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Cover illustration: Fruit-bearing shoots of the Brazilian Atlantic Forest understorey tree Psychotria brachyceras (Rubiaceae). This species accumulates an unusual monoterpine indole alkaloid, brachycerine, which is induced in response to wounding, UV-exposure, jasmonate application, drought and osmotic stress. Its function is probably related to its capacity to mitigate reactive oxygen species, acting both as antioxidant and antimutagenic agent.

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Preface

As sessile organisms, plants have evolved an amazing array of metabolic pathways leading to molecules capable of responding promptly and effectively to stress situations imposed by biotic and abiotic factors. These pathways, often recruited during biological evolution from essential primary metabolism pathways upon initial gene duplication, combine carbon, hydrogen, oxygen, nitrogen, and sulfur into molecules that are capable of herbivore deterrence, tri-trophic signaling (i.e., attracting herbivore predators and parasites), pathogen inhibition, integrating defense responses, UV absorption, quenching of reactive oxygen species, allelopathic activity, attracting pollinators, or seed dispersing animals, heat dissipation, among other activities. Throughout the kingdom Plantae, there are tens of thousands of molecules, frequently restricted to specific taxonomic groups, which play this major role of plant interaction with the environment and are extremely important for plant fitness; these are historically referred to as secondary metabolites, also known as natural products.

In spite of our detailed knowledge of only a fraction of plant secondary metabolic pathways, particularly when considering tropical and subtropical phytodiversity, some major patterns have emerged, revealing a complex regulation of this metabolism. Both constitutive and inducible secondary metabolism activity can be found in different plant species, and frequently an intermediate profile of metabolic activity between these two strategies can be found. A strict spatial and temporal control of gene expression ensures the correct accumulation pattern of various secondary products. Organ-specific, tissue-specific, and cell type-specific expression of different portions of a single metabolic pathway are not uncommon in the biosynthesis of alkaloids and terpenes, for example. The required transport of metabolic intermediates constitutes an additional level of regulation. Within cells, different compartments participate in biosynthetic steps, including chloroplasts, cytoplasm, endoplasmic reticulum, vacuoles, nucleus, and special structures, such as alkaloid vesicles. The induction of secondary metabolism gene expression by wounding, herbivore-derived molecules, pathogen elicitors, and oxidative stress caused by heat, drought, flooding, UV light, or temperature extremes is often mediated by integrating signaling molecules such as salicylic acid, jasmonic acid, and their derivatives. Ontogeny and circadian clock controlled expression are also important features of plant secondary metabolism. At the level of gene expression, the presence of master regulatory transcription factors is a common theme in the metabolism of phenolics, alkaloids, and glucosinolates. These regulators are considered some of the best targets for engineering secondary metabolic pathways.

As can be easily realized, effective engineering of plant secondary metabolism pathways is not a trivial task, mostly due to the various levels of regulation and the often observed close dependence of subcellular, tissue, and organ differentiation for effective expression of metabolic steps. Nonetheless, examples of successful engineering of plants, plant cell cultures, and heterologous expression of plant secondary metabolism genes in microbes are available. The purpose of this book is to provide a source of detailed practical information on some of the most important methods employed in the engineering
of plant secondary metabolism pathways and in the acquisition of essential knowledge in performing this activity. Some examples in which the application of these methods has allowed significant advances in the major goal of tailoring plants or microorganisms to become factories of relevant plant secondary metabolites are highlighted. Some emerging strategies for metabolic engineering, still incipiently used in secondary metabolism, are also described, pointing to future directions in the field. The book is intended to be a resourceful reference tool for researchers (molecular biologists, plant physiologists, biochemists, chemical engineers, agronomists) engaged in the challenging task of modifying some of the most intricate products of plant evolution. Hopefully, it will assist scientists’ efforts in the goal of sustainably supplying, in a fast changing planet, the ever growing needs of humankind for natural chemicals, such as pharmaceuticals, nutraceuticals, agrochemicals, food and chemical additives, biofuels, and biomass.

Arthur Germano Fett-Neto
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Chapter 1

Plant Secondary Metabolism and Challenges in Modifying Its Operation: An Overview

Naíla Cannes do Nascimento and Arthur Germano Fett-Neto

Abstract

Plants have metabolic pathways leading to tens of thousands of secondary products capable of effectively responding to stress situations imposed by biotic and abiotic factors. These pathways, often recruited from essential primary metabolism pathways upon initial gene duplication, are frequently restricted to specific taxonomic groups and play a major role in the plant × environment interaction. A strict spatial and temporal control of gene expression ensures the correct accumulation pattern of various secondary products. The required transport of metabolic intermediates constitutes an additional level of regulation. The induction of secondary metabolism gene expression by wounding, herbivore-derived molecules, pathogen elicitors, and oxidative stress caused by heat, drought, flooding, UV light, or temperature extremes is often mediated by integrating signaling molecules such as jasmonate, salicylic acid, and their derivatives. Ontogeny and circadian clock-controlled gene expression are also important features of plant secondary metabolism, as are master regulatory transcription factors. These regulators are attractive targets for engineering secondary metabolic pathways. In spite of the complexity of secondary metabolism, important advances have been achieved, leading to success stories in engineering this diverse reservoir of useful molecules.

Key words: Plant secondary metabolism, environmental stress, development, elicitors, metabolic engineering, transcription factors, microbial expression, plant transformation.

1. Introduction

Plant secondary metabolites, often referred to as natural products, comprise a wide array of non-protein molecules, often of relatively low molecular weight, that occur in certain taxa and are not essential for basic growth and development, but are frequently involved in environmental adaptation to both biotic and abiotic stresses. Secondary metabolites also have important roles in pollination and seed dispersion, as well as in the interaction of plants with mutualist species of bacteria and fungi. In addition, chemical
interactions with other plants (e.g., allelopathy), host recognition by herbivores, and indirect defense based on the attraction of parasites and predators of herbivores (tri-trophic interactions) often involve secondary metabolites (1). Therefore, the adaptive and evolutionary significance of plant secondary metabolites is undisputed.

From the human point of view, many of these thousands of molecules have interesting applications in pharmacology, chemical industry, novel materials, agriculture, and forestry. Even with a sparse and fragmentary knowledge of plant secondary metabolites in plants, mostly centered in a few medicinal, crop, and forestry species, this type of natural chemicals has made a huge impact in human life. Take the example of quinine as an antimalarial. Without this remedy, it is quite probable that the colonization of the Americas would have had a different outcome. The discovery and development of secondary metabolism-derived drugs, such as the antitumoral taxol from yew and the antileukemic vincristine from vinca, have been making possible the continuity of many human lives (2). Because of their structural complexity, often including several chiral centers, plant secondary metabolites are very difficult to synthesize chemically in adequate yields, making plants or cells and tissues derived thereof the only commercially viable sources of these molecules (3).

Detailed knowledge of plant secondary pathways is restricted to a few plant species. The chemical profile of the vast majority of the 300,000 estimated plant species is unknown, particularly considering the wealth of tropical and subtropical species, which are either understudied or unknown to science. The picture that has emerged from the few cases in which plant secondary metabolism has been well described indicates that there is a large variation in the biochemical pathways involved and in their regulation. This observation points to the need of intensive investigations to unveil the “big picture” of this metabolic diversity and paves the way to uncover and explore its myriad of applications.

The pathways leading to the biosynthesis of natural products in plants have been the object of significant research efforts to better understand their operation and to optimize production of useful metabolites (3). Nonetheless, effective metabolic engineering of numerous plant natural product pathways remains a challenge. Among the hurdles on the way are (a) lack of sustainable source of biomass, (b) poor effectiveness of transformation and regeneration systems, (c) need to evaluate accumulation profile throughout developmental stages and upon environmental changes, (d) difficult synthesis and labeling of biosynthetic intermediates, (e) problems in the establishment of axenic cultures to separate genuine plant metabolism from endophyte or plant–endophyte interaction metabolism, and (f) limited knowledge of transport systems for metabolites in organelles and between cells.
In this chapter, an outline of the main features of plant secondary metabolic pathways will be presented, with emphasis on the bottlenecks associated with the improvement of biosynthetic flow to metabolites of interest. This will be a preamble to the ingenious techniques described in this volume of *Methods in Molecular Biology* which aims at assisting researchers in manipulating this dazzling complex, dynamic, and relevant part of plant metabolism. Some of the regulatory points in plant secondary metabolism and potential strategies to engineer them are shown in Fig. 1.1.

One of the most impressive aspects of plant secondary metabolites is their diversity. Whereas a few hundred metabolites can be assigned to the core of primary metabolism (photosynthesis, respiration, and metabolism of carbohydrates, fatty acids, and nitrogen), estimates indicate that 200,000 metabolites take part in secondary metabolism (terpenes, polyketides, phenolics, alkaloids, cyanogenic glycosides, glucosinolates, and non-protein amino acids) (3). The biochemical and evolutionary origin of secondary metabolites can be traced to primary compounds and genes of primary metabolism, respectively. Secondary metabolites often arise from amino acids, nitrogenous bases, and intermediates of photosynthetic, respiratory, carbohydrate, or fatty acid metabolism. From an evolutionary point of view, most secondary metabolism genes appear to have arisen from the recruitment of primary metabolism genes by events of duplication, followed by accumulation of mutations and functional diversification (1).

The impressive numbers of secondary metabolites are the product of relatively small modifications in core compounds from which a large number of derivatives arise. This provides plants with a “natural combinatorial chemistry” approach to select ecologically relevant compounds for various adaptive functions in the course of evolution. The catalysts of secondary metabolism can be roughly divided into parent compound enzymes and modifying enzymes. The former catalyze reactions that provide the basic skeletons of compound series, whereas the latter change parent compound structure (monooxygenases, methyltransferases, glucosyltransferases, hydroxilases, etc.). In terpene metabolism, some terpene cyclases have been found to convert one substrate into many different products, significantly adding to chemical diversity (4). The predominant products formed by these enzymes are likely controlled by cofactor availability and other aspects of enzyme subcellular environment.
Fig. 1.1. (a) Outline of main regulatory points involved in secondary metabolite production. Numbers with asterisks indicate potential strategies for modifying carbon flux through secondary pathways or for insertion of new pathways. Organelles from left to right: chloroplast, nucleus, and vacuole. Lower case letters represent genes, upper case letters represent metabolites, and boxed upper case letters represent proteins. TRP, protein transport; TRM, metabolite transport; and TF, transcription factor. Dashed lines represent nuclear entry of transcription factors. Solid dark lines are catalytic steps. (b) Alternatives of heterologous systems for partial or total expression of plant secondary pathways (left) and main regulatory inputs that must be considered and can be manipulated in pathway engineering (right).
Diversity of secondary metabolites also results from mixed origin compounds. It is quite common to find compounds that arise from the combination of intermediates derived from different metabolic pathways. Typical examples are the flavonoids, which are assembled from intermediates of the acetate–malonate and shikimate pathways, and taxol, a diterpene amide that derives from isopentenyl biphosphate (terpene pathway), and phenylalanine (phenolic pathway) (5). The involvement of two or more metabolic pathways in the biosynthesis of some secondary metabolites contributes to a high degree of complexity in the regulation of metabolic flux leading to these compounds.

3. Signal Transduction and Environmental Responsiveness

One of the hallmarks of plant secondary metabolism is its capacity to respond to environmental signals. As sessile organisms, plants acquired a complex metabolic machinery to produce chemicals that allow them to cope with various types of stresses, both biotic and abiotic. In that regard, phytohormones and other signal molecules often play a key role in the transduction of the environmental information to trigger a metabolic response that is adaptive to the particular circumstances. The activation of secondary pathways is often triggered by molecules, such as jasmonate, ethylene, abscisic acid, salicylic acid, and nitric oxide, which are produced upon herbivore or pathogen attack. Ethylene and abscisic acid are also induced by a variety of abiotic stresses, including flooding, drought, salinity, UV-B, and extreme temperatures (6).

Most stresses result in the transient accumulation of reactive oxygen species (ROS), which are important in signaling, but become toxic if accumulated (7). Several secondary metabolites are induced by ROS. These chemical species may not only act as signaling molecules to regulate secondary metabolism genes but also be the target of the metabolites derived from the catalytic activity of the respective enzymes. Various natural products can quench and mitigate reactive species, contributing to regulate the antioxidant defenses and protect nucleic acids, proteins, and membranes. The shielding and antioxidant capacity of flavonoids to protect against UV and its ROS-driven effects on plant cells is well documented (8), and increasing evidence points to a similar role for some monoterpene indole alkaloids (9).

The signaling pathways connecting environmental status to changes in gene expression converge on four main regulation levels: transcriptional control, mRNA stability, post-translational protein modification, and selective proteolysis (10). The activation, release from membranes, inhibition, or proteolysis of
transcription factors (TF) or TF-sequestering proteins, affecting their concentration, nuclear mobility, or DNA-binding activity, often determine the production of enzymes that are responsible for the phenotypic metabolic profiles (11, 12). Another very important level of regulation that is just starting to be disclosed for plant secondary metabolism is RNA stability, orchestrated by a wide array of small RNA molecules. Effective RNA interference techniques have been applied to modify the profile of secondary metabolites by changing the expression of regulatory proteins or to knock down biosynthetic steps (13).

4. Secondary Metabolism and Defense Strategies: Phytoanticipins, Phytoalexins, and Everything in Between

Defense strategies based on secondary metabolism can vary between plant species and with interactions involving specific types of aggressors, both herbivores and pathogens. In general, plant defense metabolites can be pre-existent or constitutive (phytoanticipins) or their biosynthesis may be induced upon challenge (phytoalexins) (14). A special case of constitutive defense corresponds to the accumulation of precursor compounds often stored in vacuoles; upon damage, these metabolites are released and modified by enzymes which are either co-activated in these circumstances or made available by de-compartmentalization (e.g., glucosinolates) (1).

Numerous species, however, have intermediate profiles in which defense compounds can be accumulated in basal levels and further induced upon specific challenges. Examples of this can be found in the production of monoterpene indole alkaloids of Psychotria sp. (9). Although the strategies outlined above have been classified based on responses to biotic stresses, they could easily be extended to describe accumulation profiles of secondary metabolites in response to abiotic stresses.

5. Organ, Tissue, Cell, and Subcellular-Specific Metabolism: Developmental Controls Organize Structure and Function

In many cases, the expression of plant secondary pathways is strongly controlled in both time and space. Typical examples include the production of volatile terpenes and flavonoid pigments in floral tissues, the accumulation of flavonoids in the epidermis of UV-treated leaves, and the production of monoterpene glandular trichomes of peppermint (2). The circadian pattern of expression of terpene biosynthetic genes, enzyme
activities, and specific metabolite profiles also illustrates the coupling of secondary metabolism with time cues (15).

Studies on alkaloids have shown that secondary metabolic pathways can be distributed in different tissues and cell types, each one of them expressing a portion of the pathway leading to a final product. A major site for the biosynthesis of monoterpenoid alkaloids of *Catharanthus roseus* is the leaf epidermis, although further steps of their biosynthesis have been shown to take place in other tissues, such as palisade idioblasts, laticifers, and possibly phloem parenchyma cells. The biosynthesis of the tropane alkaloid scopo-lamine in *Atropa belladonna* and *Hyoscyamus muticus* takes place in roots and involves different tissues. An early and a late enzyme of the alkaloid biosynthesis (putrescine N-methyl transferase and hyoscyamine 6β-hydroxylase) localize to the pericycle, whereas an intermediate enzyme in the pathway (tropinone reductase I) resides in the endodermis and inner cortical cells (16).

In spite of a relatively limited number of case studies, the distribution of portions of some pathways of secondary metabolism in various organs and tissues of plants implies the transport of intermediates between cell types. However, little is known on the mechanisms of transport, specificity characteristics, or nature of the transporters. The involvement of ABC-type transporter proteins has been shown for alkaloids and isoprenoids (17). This is certainly a field that demands much investigation to allow better metabolic engineering, since it represents a different checkpoint for regulation of carbon flux through secondary metabolic pathways.

Several subcellular compartments can be involved in the biosynthesis of secondary metabolites. In the production of monoterpane indole alkaloids, for example, the cytosol, the chloroplast, the endoplasmic reticulum, and the vacuole host enzymes that take part in assembling these metabolites (16). In the production of glucosinolates and cyanogenic glucosides, the vacuole can act as a storage compartment for final products, which are released upon wounding to generate isothiocyanates or cyanide, both having herbivore deterrence properties (1). The enzymes chalcone synthase and chalcone isomerase, from the flavonoid biosynthesis pathway, have been localized to the nucleus of *Arabidopsis thaliana* cells, where accumulation of some flavonoids was also observed, in agreement with reports on other plant species (8).

The adaptive value of subcellular compartmentalization of secondary metabolic pathways must be a driving force for its establishment in various unrelated species and molecular backbones. Reduced competition for common substrates, optimized control of pathway inhibition by end products, self-protection (safe storage of potentially harmful metabolites), better enzyme activity in subcellular niches, and better flux control are among
the possible advantages of this biochemical architecture. Once again, transporter systems for certain metabolites and enzyme sorting systems between compartments become a requirement and a potential regulatory level. The cellular and subcellular complexities associated with the expression of secondary pathways help to explain why in many cases undifferentiated cell cultures fail to form bioactive or industrially relevant metabolites (9).

6. Channeling, Regulatory Proteins, and Metabolic Flux

Metabolic channeling is one of the reasons that explain why some secondary pathways are capable of quickly producing relatively large amounts of required metabolites upon cell perception of specific signals. Co-localization of related enzymes to specific compartments or membranes and the establishment of multienzyme complexes provide prompt transfer of metabolic intermediates between catalysts as well as prevent the accumulation of potentially toxic compounds. The latter can be found in the metabolism of phenylpropanoids (18). The availability and production of co-factors may also be favored by these spatial arrangements.

The control of metabolic flux in biochemical pathways has been recognized as the result of the combined inputs of all enzymes in the pathway. Enzymes and metabolites behave as a system in such a way that changes in one of its components will often affect others (19). This is the general concept behind metabolic flux analysis. Every enzyme in a pathway contributes to flux control of metabolites, although some may exert a larger effect. Unlike constants of enzyme kinetics, the flux control coefficient (contribution of an enzyme to the flux control in a metabolic pathway) is not a fixed value, varying with factors such as developmental stage or physiological status.

Taking this systemic feature of metabolic pathways into account, it is not hard to understand why increasing the activity of a single enzyme often falls short of improving final metabolite yields (9). Whenever possible, strategies to engineer metabolism should take into account steady-state concentrations of intermediates, maintenance of minimum pools to avoid major unbalance in pathway operation, enzyme cofactor availability and regeneration capacity, product sequestration in vacuoles or other compartments to avoid pathway inhibition, transport of metabolites across membranes and cell types, and the role of transcription factors (19) and/or small RNAs in regulating the expression of functionally related biosynthetic genes (13). Some of these aspects are
Transcription factors (TFs), both positive and negative, are interesting targets for metabolic engineering, since they can regulate the expression of several biosynthetic enzyme genes in an integrated and coordinated fashion (19). However, as is the case of flavonoid metabolism, more than one class of transcription factors has to be modulated to affect the expression of target genes that receive mixed inputs from different transcriptional regulators (20). The existence of transcriptional networks characterized by complex interactions, such as TFs sequentially regulating the transcription of other counterparts, auto-regulation of TF gene transcription, and transactivating activity depending on TFs interactions at the protein level, is observed in plants (21). This reflects the systemic feature of transcriptional networks that control enzyme genes, just as is observed with the flux of metabolites at the biochemical level. In theory, the modular nature of these transcriptional regulation proteins, with DNA sequence-specific binding and protein transactivating domains, allows the design of tailor-made proteins to regulate metabolic pathways.

Metabolic engineering has a long history in microorganisms, but is much younger in plants. However, large-scale culture systems available for microorganisms are very attractive as platforms for producing metabolites of interest. In spite of the compartmentalization and tissue differentiation requirements for the correct function of plant secondary metabolism, some pathways can be entirely or at least partially introduced and expressed in bacteria and yeast (2) (Fig. 1.1b). Plant cell cultures can also be effective ways to produce secondary products, even without need for genetic modification, using environmental control and elicitors to improve production, reaching commercial scale viable yields (5).

Bacteria are versatile organisms to express plant secondary metabolism steps or even short pathways. In particular, *Escherichia coli* is widely used with this purpose. Fast growth rates, ease of transformation, simplicity of culture media, large variety of strains and mutants (also allowing codon bias modifications), high expression rates for foreign proteins, and plasmid-based expression systems are just some of the advantages of using this species to produce plant secondary metabolites and enzymes (22). Similar features can be listed for yeast, namely *Saccharomyces cerevisiae* and *Pichia pastoris*, with the additional advantage of “built-in” eukaryotic post-translation modification
capacity. Genetic manipulations using *P. pastoris* are similar to those of *S. cerevisiae*. However, because of the strong preference of *P. pastoris* for respiratory metabolism, extremely high cell densities can be achieved without the accumulation of high ethanol concentrations as is often the case in high-density cultures of *S. cerevisiae* (23). In baker’s yeast, short pathways have been effectively expressed, and the organism has been used to produce much needed and hard to obtain labeled intermediate and parental compounds for biosynthetic studies of plant secondary metabolism, as exemplified by taxadiene (22).

Plant cell cultures resemble some of the features of microorganism cultures, although growth is much slower (increasing contamination chance), cells are less resistant to shearing stress in bioreactors, culture media are more complex, and subpopulations are often out of synchrony during culture (24). However, some plant cell cultures are capable of producing secondary metabolites of interest in large scale, using slightly modified bioreactors to accommodate for their specific features (e.g., impeller design, airlift technology, and aeration levels) (24). One of the advantages of cell cultures is the easy application of elicitors, such as jasmonate, and feeding of limiting precursors to boost metabolic flow toward products of interest. Two-step culture media systems, with a nutrient-rich growth media and nutrient-limited elicitor-containing production media, can be applied to cell cultures. A continuous two-phase organic solvent extraction layer (physically separated from cells) can assist in metabolite extraction and in reducing feedback inhibition of pathways. Cell sorting methods based on metabolite expression via fluorescent detection systems can afford more homogeneous cell populations for enhanced production of target compounds (25).

Cell immobilization techniques can also be used to promote the accumulation of secondary metabolites; the establishment of cell layers or “pseudotissues” creates an environment for different cell–cell signaling and metabolite gradients that may favor secondary metabolism pathway expression (24). Plant cell cultures can also be transformed via *Agrobacterium* or biobalistic strategies to improve yields. When metabolite release to culture medium is limited and preservation of cell biomass is desired, small concentrations of cell permeabilization agents (e.g., dimethyl sulfoxide) may be employed.

Organ cultures can be an alternative to produce secondary metabolites in bioreactor setups. Roots are especially useful for this purpose, since they have a tendency to exude various metabolites, facilitating recovery. Gas-phase trickle-bed reactors, in which medium is re-circulated from a reservoir, sprayed over the root mass, flowing down through the root surface, have been designed for root cultures. Transformation of plant explants with wild or engineered strains of *Agrobacterium rhizogenes* generally results
8. Using Bacterial Genes and Plastid Engineering to Improve Plant Secondary Metabolism

Bacteria are also a rich source of secondary metabolites, particularly carotenoids, and bacterial genes can be used to improve the profile of human health-promoting terpenes in plants. One of the most important examples of this strategy is the development of golden rice, expressing the pathway to the vitamin A precursor β-carotene, using both bacterial and plant genes under the control of an endosperm-specific promoter (27).

A similar successful strategy was used to insert a phytoene synthase gene (crtB) derived from a soil bacterium Pantoea ananatis into flax plants, yielding transgenic seeds with about 20-fold increase in total carotenoid content (28). Although these are examples of nuclear genome engineering, the expression of bacterial genes in plastid genomes can also be used, taking advantage of the prokaryotic nature of plastids stemming from their endosymbiotic origin. The introduction of nuclear genes in plastid genomes is also feasible with some further modifications.

Plastids have many useful features for secondary metabolism engineering, including operon arrangement, no need for transit peptides, high genome copy number, and level of protein expression (29).

9. Transfer of Specific Secondary Pathways to Host Plant Species

The transfer of secondary metabolic pathways between plant species is of high interest in agriculture, pharmaceutical sciences, and forestry. Target metabolite accumulation and valuable traits conferred by secondary metabolites for plant defense against herbivores and pathogens, nutraceutical content, and abiotic stress resistance are in high demand in both non-cultivated species of interest and crops. Some limitations for the effective metabolic profile modification are related to difficulties in multigenic pathway transfer, transformation and regeneration of modified plants, and development of specific cell types involved in production and storage (e.g., glandular trichomes).

In spite of these potential difficulties, entire plant-derived metabolic pathways have been effectively transferred from one species to another (30). The availability of simpler and more
efficient cloning techniques, exemplified by the ligation-free cloning technique USER, allowing the one-step assembly of complex DNA constructs, has been the key element in some of these achievements (31).

The conversion of the model plant *A. thaliana* into a cyanogenic plant through the transfer of the complete pathway leading to the cyanogenic glycoside dhurrin, using *Sorghum bicolor* genes, was carried out (32). The transgenic plants showed improved resistance to herbivores. The final portion of the glucosinolate biosynthetic pathway (C–S lyase, glycosyltransferase, and sulfotransferase) from *Arabidopsis* has been introduced in tobacco. To achieve this important step toward the goal of engineering glucosinolates into non-cruciferous plant species, an expression construct made up of a single polycistronic open reading frame was used, allowing the control of the three coding sequences by a single promoter (31). Although still significantly based on model plant species, these examples show that wider engineering of crop species with secondary metabolic pathways is on its way.

10. Conclusion

The handful of plant species which have had their secondary metabolism investigated to significant detail, at the level of metabolites, proteins, and genes, provide us with a sketchy picture of the architecture and functioning of secondary metabolism and its diversity. There are various tools to engineer plant secondary metabolism, and several of them have been shown to be effective. However, to fully realize the benefits of manipulating the inner chemical factories of plants and their refined responses to the environment, there is a long way to go. This is a very fortunate time to work with plant secondary metabolism.

References


Suppression Subtractive Hybridization as a Tool to Identify Anthocyanin Metabolism-Related Genes in Apple Skin

Yusuke Ban and Takaya Moriguchi

Abstract

The pigmentation of anthocyanins is one of the important determinants for consumer preference and marketability in horticultural crops such as fruits and flowers. To elucidate the mechanisms underlying the physiological process leading to the pigmentation of anthocyanins, identification of the genes differentially expressed in response to anthocyanin accumulation is a useful strategy. Currently, microarrays have been widely used to isolate differentially expressed genes. However, the use of microarrays is limited by its high cost of special apparatus and materials. Therefore, availability of microarrays is limited and does not come into common use at present. Suppression subtractive hybridization (SSH) is an alternative tool that has been widely used to identify differentially expressed genes due to its easy handling and relatively low cost. This chapter describes the procedures for SSH, including RNA extraction from polysaccharides and polyphenol-rich samples, poly(A)+ RNA purification, evaluation of subtraction efficiency, and differential screening using reverse northern in apple skin.

Key words: Anthocyanin, apple (*Malus domestica*) skin, differential screening, gene expression, poly(A)+ RNA purification, RNA extraction, suppression subtractive hybridization (SSH).

1. Introduction

Anthocyanins, which impart red coloration in apple skin, belong to the diverse group of ubiquitous secondary metabolites known as flavonoids. The pigmentation of anthocyanins is an important determinant of consumer preference and marketability as well as an attractive factor to pollinators. Anthocyanin biosynthesis has been well characterized in the flowers of petunia (*Petunia hybrida*) and snapdragon (*Antirrhinum majus*) and in the kernels of maize (*Zea mays*) (1). As such, the biosynthetic pathway is one of the well-known pathways in plants (2). It has been known that anthocyanin metabolism is induced by various environmental
factors (3) and controlled through the coordinated expression of biosynthetic genes in many plants (1). For example, in apple, both ultraviolet (UV)-B (280–320 nm) irradiation and low temperatures stimulate the production of anthocyanins (4, 5). The mRNA levels of *chalcone synthase* (*CHS*), *flavanone 3-hydroxylase* (*F3H*), *dihydroflavonol 4-reductase* (*DFR*), *anthocyanidin synthase* (*ANS*), and *UDP-glucose: flavonoid 3-O-glucosyltransferase* (*UFGT*) were up-regulated by both low temperature and UV-B irradiation (6). In addition, the expression of *MdMYBA*, a key transcription factor for anthocyanin accumulation, was also induced by low temperature and UV-B irradiation (7). Therefore, it can be considered that most of the genes involved in anthocyanin biosynthesis appear to participate in the development of pigmentation under the regulation of certain environmental factors. In this sense, the identification of genes whose expression is affected by the relevant environmental factors is a useful strategy for elucidating the mechanisms underlying the physiological process leading to pigmentation.

In order to isolate differentially expressed genes, microarrays have been widely used in plants. However, this technique needs special apparatus and is highly expensive. Thus, availability of microarrays is often limited and not commonly used. In addition, availability of commercial microarrays is restricted to model plants, although anthocyanins are widely distributed in numerous plant species. In apple, although about 180,000 ESTs have been deposited in public databases (8, 9), no commercial microarrays for the species have become available so far. Therefore, it is difficult to utilize microarray technology in many studies involving anthocyanins. Suppression subtractive hybridization (SSH) is an alternative tool that has been widely used to identify differentially expressed genes due to its easy handling and relatively low cost (10). SSH is a powerful technique to compare two populations of mRNA and obtain clones of genes that are differentially expressed. Although there are many steps in the method of SSH, the basic theory is simple. First, cDNA is synthesized from 2 μg of poly(A)+ RNA generated from the two types of tissues being compared: we refer to the cDNA that contains specific (differentially expressed) transcripts as tester and the reference cDNA as driver. The tester and driver cDNAs are then digested with *RsaI*. The digested tester cDNA is subdivided into two portions, and each is ligated with a different cDNA adaptor. The driver cDNA has no adaptors. Tester and driver cDNAs are then hybridized. The hybridized sample is subjected to PCR that permits exponential amplification of cDNAs which differ in abundance, whereas amplifications of sequences of identical abundance in the two populations are suppressed. The amplified cDNAs can be directly inserted into a T/A cloning vector. This cloning allows identification of differentially expressed RNAs by sequence and/or hybridization analysis.
This chapter describes the procedures for SSH including the steps from RNA extraction to differential screening, based on our previous study (11), in which we performed SSH with the apple skin irradiated with UV-B to isolate other genes participating in anthocyanin biosynthesis apart from those located in the flavonoid biosynthetic pathway and MYB. The protocols described in this chapter can be utilized for polysaccharide- and polyphenol-rich samples such as fruits and flowers.

2. Materials

2.1. Total RNA Extraction

1. Miracloth® (Calbiochem, San Diego, CA).
2. RNase-free water, made by adding 0.001% (v/v) diethylpyrocarbonate (DEPC), stirring overnight, and then autoclaving.
3. Extraction buffer (see Note 1): 200 mM sodium borate (pH 9.0), 30 mM ethylene glycol tetraacetic acid (EGTA), and 1% (w/v) sodium dodecyl sulfate (SDS). Use RNase-free water and store at room temperature. The extraction buffer is supplemented with 10 mM dithiothreitol (DTT), 2% (w/v) polyvinylpyrrolidone (PVP, MW 40,000), and 1% (v/v) Nonidet P-40 (NP-40) just prior to use.
4. Proteinase K (10 mg/mL). Use RNase-free water and store at −20°C.
5. 2 M potassium chloride (KCl). Use RNase-free water, autoclave, and store at 4°C.
6. 10 and 2 M lithium chloride (LiCl). Use RNase-free water, autoclave, and store at 4°C.
7. 10 mM Tris–HCl (pH 7.5). Use RNase-free water, autoclave, and store at 4°C.
8. 2 M K-acetate (pH 5.5). Use RNase-free water, autoclave, and store at 4°C.
9. Absolute ethanol.
10. 70% ethanol. Use RNase-free water and store at 4°C.

2.2. Poly(A)+ RNA Purification

1. RNase-free water.
2. Oligotex™-dT30 <Super> (Takara Bio Inc., Shiga, Japan) (mRNA purification kit).
3. SUPREC™-01 (Takara Bio Inc.) (DNA purification kit).
4. Washing buffer: 10 mM Tris–HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA, pH 7.5), 0.5 M sodium chloride (NaCl), and 0.1% (w/v) SDS. Use RNase-free water and store at room temperature.
5. 5 M NaCl. Use RNase-free water, autoclave, and store at room temperature.

6. 3 M Na-acetate (pH 5.2). Use RNase-free water, autoclave, and store at room temperature.

7. Dr. GenTLE™ precipitation carrier (Takara Bio Inc.).

8. Absolute ethanol.

9. 70% ethanol. Use RNase-free water and store at 4°C.

2.3. Suppression Subtractive Hybridization (SSH)

1. PCR-Select subtractive hybridization kit (Clontech, Palo Alto, CA): the kit includes AMV reverse transcriptase, cDNA synthesis primer, first-strand buffer, second-strand enzyme cocktail, second-strand buffer, T4 DNA polymerase, RsaI, RsaI restriction buffer, T4 DNA ligase, adaptors 1 and 2R, DNA ligation buffer, dilution buffer, hybridization buffer, PCR primer 1, nested PCR primers 1 and 2R, dNTP mix, EDTA/glycogen mix, ammonium acetate, and sterile water.

2. Advantage 2 polymerase mix (Clontech).

3. Absolute ethanol.

4. 70% ethanol. Use RNase-free water and store at 4°C.


2.4. Evaluation of Subtraction Efficiency

1. Primer set for the reference gene which is not differentially expressed such as ubiquitin and actin (see Note 2).

2. AmpliTaq Gold and GeneAmp PCR buffer II (Applied Biosystems, Foster City, CA).

3. Tris-acetate-EDTA (TAE) buffer: 40 mM Tris-acetate and 1 mM EDTA.

4. Agarose (electrophoresis grade).

2.5. Differential Screening Using Reverse Northern

2.5.1. Preparation of Membrane

1. TA cloning kit (Invitrogen, San Diego, CA).

2. Luria–Bertani (LB) medium, ampicillin, isopropyl-beta-thio galactopyranoside (IPTG), and 5-bromo-4-chloro-indoly-β-D-galactoside (X-gal).

3. 10×12 cm nylon membrane (Hybond N+, Amersham Biosciences, Piscataway, NJ).

4. Nested PCR primers 1 and 2R (Clontech) (see Note 3).

5. TaKaRa Ex Taq (Takara Bio Inc.).

6. 0.6 N NaOH; prepare just prior to use.

7. 0.25% (w/v) bromophenol blue (BPB).

8. 0.5 M Tris–HCl (pH 7.5)
2.5.2. Labeling Probes

1. SuperScript first-strand synthesis system II for RT-PCR (Invitrogen): the kit includes RNase-free water, oligo(dT) primer, 10× RT buffer, MgCl₂, DTT, RNase OUT™ Superscript II, and RNase H.

2. DIG DNA labeling mix (Roche Diagnostics, Mannheim, Germany).

2.5.3. Hybridization, Washing, and Detection

1. 20× saline sodium citrate (SSC): 750 mM NaCl and 75 mM tri-sodium citrate (pH 7.0), autoclave, and store at room temperature.

2. Maleic acid buffer: 0.1 M maleic acid and 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5, autoclave, and store at room temperature.

3. 10× blocking stock solution: dissolve blocking reagent (Roche Diagnostics) 10% (w/v) in maleic acid buffer under constant heat in a microwave oven, autoclave, and store at −20°C.

4. 1× blocking solution: dilute 10× solution 1:10 with maleic acid buffer. Prepare just prior to use.

5. 10% (w/v) SDS.

6. High SDS hybridization buffer: 50% formamide, 5× SSC, 2× blocking solution, 0.1% lauroylsarcosine and 7% SDS.

7. CSPD solution: dilute CSPD (Roche Diagnostics) (chemiluminescent substrate for alkaline phosphatase) 1:100 in detection buffer, and store at 4°C with dark condition.

8. Washing buffer: maleic acid buffer, 0.3% (v/v) Tween 20.

9. Detection buffer: 0.1 M Tris–HCl and 0.1 M NaCl; adjust to pH 9.5, autoclave, and store at room temperature.

10. Antibody solution: centrifuge anti-digoxigenin-AP, Fab fragments (Roche Diagnostics) at 9,500×g for 5 min at 4°C in the original vial prior to use, and pipette the necessary amount carefully from the surface. Dilute anti-digoxigenin-AP 1:5,000 (150 mU/mL) in blocking solution.

11. X-ray film.

3. Methods

3.1. Total RNA Extraction (see Note 4)

1. Pipette 35 mL of extraction buffer into a 50-mL tube. Warm in a water bath to 80°C.

2. Grind 10 g of tissue to a fine powder in liquid nitrogen using mortar and pestle. Transfer the ground tissue into an empty 50-mL tube.
3. Add the heated buffer to the ground tissue by decanting. Vortex well until the powder is fully dispersed and thawed in the buffer.

4. Add 2.5 mL of proteinase K, mix gently, and incubate with mild agitation (∼100 rpm) on a shaker at 42°C for 1.5 h.

5. Add 3.6 mL of 2 M KCl, mix gently, and incubate on ice for 1 h.

6. Centrifuge at 11,900×g for 20 min at 4°C and filter the supernatant through Miracloth into a fresh 50-mL tube.

7. Add 0.25 volume of 10 M LiCl (final concentration of 2 M LiCl), mix gently, and incubate on ice at 4°C overnight.

8. Centrifuge at 11,900×g for 20 min at 4°C. Discard the supernatant by gentle decanting without dislodging the pellet.

9. Suspend the pellet with 2.5 mL of ice-cold 2 M LiCl. Separate into two aliquots and transfer to fresh 2.0-mL tubes.

10. Centrifuge at 16,100×g for 20 min at 4°C. Discard the supernatant by gentle decanting.

11. Repeat steps 9 and 10 once.

12. Suspend the pellet with 750 μL of 10 mM Tris–HCl (pH 7.5) for each one of the separate tubes (see Note 5).

13. Centrifuge at 16,100×g for 10 min at 4°C. Transfer the supernatant to a fresh 1.5-mL tube by pipetting.

14. Add 75 μL of 2 M K-acetate (pH 5.5) to each one of the separated tubes, mix gently, and incubate on ice for 15 min.

15. Centrifuge at 16,100×g for 10 min at 4°C. Transfer the supernatant to a fresh 2.0-mL tube by pipetting.

16. Add 2.5 volume of absolute ethanol. Incubate at −80°C for 1 h.

17. Centrifuge at 16,100×g for 30 min at 4°C. Discard the supernatant by gentle decanting without dislodging the pellet.

18. Add 1.5 mL of 70% ethanol and centrifuge at 16,100×g for 5 min at 4°C.

19. Discard the supernatant by gentle decanting and dry up the pellet.

20. Dissolve the pellet with appropriate volume of RNase-free water.

21. RNA quantification is performed spectrophotometrically at wavelengths of 230, 260, and 280 nm. To confirm the RNA quality, the RNA is electrophoresed on a 1.2% agarose gel containing formaldehyde. Intact total RNA typically exhibits two bright bands corresponding to ribosomal 28S and 18S RNA with a ratio of intensities of ∼1.5–2.5:1.
3.2. Poly(A)$^+$ RNA Purification

1. Prepare 600 μL of total RNA solution (the amount of total RNA should be more than 350 μg) with RNase-free water.
2. Add 150 μL of Oligotex$^{TM}$-dT30 <Super> and mix well. Incubate at 70°C for 10 min.
3. Cool the mixture immediately on ice with absolute ethanol (−20°C).
4. Add 75 μL of 5 M NaCl and mix well. Incubate at 37°C for 10 min.
5. Centrifuge at 13,800×g for 5 min at room temperature. Discard the supernatant by gentle pipetting.
6. Add 500 μL of wash buffer and mix well.
7. Centrifuge at 13,800×g for 5 min at room temperature. Discard the supernatant by gentle pipetting.
8. Add 100 μL of RNase-free water and mix well. Incubate at 65°C for 5 min.
9. Apply the mixture to the SUPREC$^{TM}$-01.
10. Centrifuge at 3,900×g for 10 min at room temperature and collect the flowthrough.
11. Add 100 μL of RNase-free water (pre-heated to 65°C) to mix by pipetting.
12. Centrifuge at 3,900×g for 10 min at room temperature and collect the flowthrough.
13. Add 400 μL of RNase-free water to collected flowthrough.
14. Repeat steps 2–12 once.
15. Add 0.1 volume of 3 M Na-acetate (pH 5.2) to the collected samples and vortex.
16. Add 4 μL of Dr. GenTLE$^{TM}$ precipitation carrier and vortex.
17. Add 2.5 volume of ethanol and vortex.
18. Centrifuge at 16,100×g for 15 min at 4°C and discard the supernatant by gentle decanting without dislodging the pellet.
19. Add 1 mL of 70% ethanol and centrifuge at 16,100×g for 5 min at 4°C.
20. Discard the supernatant by gentle decanting and dry up the pellet.
21. Dissolve the pellet with 5 μL of RNase-free water.
22. Poly(A)$^+$ RNA quantification is performed spectrophotometrically at wavelengths of 230, 260, and 280 using 0.5 μL of poly(A)$^+$ RNA solution. To confirm the quality, 0.5 μL of poly(A)$^+$ RNA solution is electrophoresed on a
1.2% agarose gel containing formaldehyde. Poly(A)+ RNA appears as a smear with no ribosomal RNA bands present (see Note 6).

### 3.3. Suppression Subtractive Hybridization (SSH)

The protocols in this section are excerpted from PCR-Select™ cDNA subtraction kit user manual (Clontech). For the detailed protocols, it is strongly recommended to refer to the appropriate Clontech manuals. In this section, cDNA that contains specific (differentially expressed) transcripts and the control cDNA are referred to as tester and driver, respectively. For example, if the researcher wants to isolate UV-B-inducible genes, refer to the cDNA from UV-B-irradiated sample as tester and the cDNA from control sample as driver.

#### 3.3.1. First-Strand cDNA Synthesis

1. For tester and driver, combine the following components in a sterile 0.5-mL microtube (do not use a polystyrene tube): poly(A)+ RNA (2 μg) 4 μL and cDNA synthesis primer (10 μM) 1 μL. Mix contents and spin briefly in a centrifuge.
2. Incubate at 70°C for 2 min in a thermal cycler.
3. Cool on ice for 2 min and briefly centrifuge.
4. Add the following to each reaction: 5× first-strand buffer 2 μL, dNTP mix (10 mM each) 1 μL, sterile water 1 μL, and AMV reverse transcriptase (20 U/μL) 1 μL.
5. Gently vortex and briefly centrifuge the tubes.
6. Incubate the tubes at 42°C for 1.5 h in an air incubator.
7. Place on ice to terminate first-strand cDNA synthesis and immediately proceed to next section.

#### 3.3.2. Second-Strand cDNA Synthesis

1. Add the following components to the first-strand synthesis reaction tubes (containing 10 μL): sterile water 48.4 μL, 5× second-strand buffer 16 μL, dNTP mix (10 mM) 1.6 μL, and 20× second-strand enzyme cocktail 4 μL.
2. Mix contents and briefly spin. The final volume should be 80 μL.
3. Incubate at 16°C for 2 h in water bath or in thermal cycler.
4. Add 2 μL of T4 DNA polymerase. Mix contents well.
5. Incubate at 16°C for 30 min in a water bath or in a thermal cycler.
6. Add 4 μL of 20× EDTA/glycogen mix to terminate second-strand synthesis.
8. Vortex thoroughly and centrifuge at 14,000 rpm for 10 min at room temperature to separate phases.
9. Carefully collect the top aqueous layer and place in a fresh 0.5-mL microcentrifuge tube.
10. Add 100 μL of chloroform:isoamyl alcohol (24:1).
11. Repeat steps 8 and 9.
12. Add 40 μL of 4 M NH₄OAc and 300 μL of 95% ethanol. Proceed to next step immediately.
13. Vortex thoroughly and centrifuge at 14,000 rpm for 20 min at room temperature.
14. Carefully collect the supernatant.
15. Overlay the pellet with 500 μL of 80% ethanol.
16. Centrifuge at 14,000 rpm for 10 min.
17. Remove the supernatant.
18. Air-dry the pellet for about 10 min to evaporate residual ethanol.
19. Dissolve precipitate in 50 μL of sterile water.

### 3.3.3. Rsal Digestion

1. Add the following reagents: dscDNA (double-stranded cDNA; from Section 3.3.2 step 19) 43.5 μL, 10× Rsal restriction buffer 5 μL, and Rsal (10 U/μL) 1.5 μL.
2. Mix by vortexing and briefly centrifuge.
3. Incubate at 37°C for 1.5 h.
4. Add 2.5 μL of 20× EDTA/glycogen mix to 45 μL of the digest mixture.
7. Vortex thoroughly and centrifuge at 14,000 rpm for 10 min at room temperature to separate phases.
8. Carefully collect the top aqueous layer and place in a fresh 0.5-mL tube.
10. Repeat steps 7 and 8.
11. Add 25 μL of 4 M NH₄OAc and 187.5 μL of 95% ethanol. Proceed to next step immediately.
12. Repeat step 7.
13. Remove the supernatant.
14. Gently overlay the pellets with 200 μL of 80% ethanol.
15. Centrifuge at 14,000 rpm for 5 min.
16. Carefully remove the supernatant.
17. Air-dry the pellets for 5–10 min.
18. Dissolve the pellet in 5.5 μL of sterile water and store at −20°C.
3.3.4. Adaptor Ligation

1. Dilute 1 μL of \textit{RsaI}-digested tester cDNA (from \textbf{Section 3.3.3} step 18) with 5 μL of sterile water.

2. Prepare a ligation master mix by combining the following reagents in a 0.5-mL microcentrifuge tube: sterile water 3 μL, 5× ligation buffer 2 μL, and T4 DNA ligase (400 U/μL) 1 μL. Tubes containing the master mix should be prepared in a set of two.

3. Ligate the two kinds of adaptors (adaptors 1 and 2R) separately as follows. The reaction mixtures containing adaptors 1 and 2R are designated as tester 1-1 and tester 1-2, respectively. Combine the following reagents in a 0.5-mL microcentrifuge tube in the order shown: tester 1-1: diluted tester cDNA 2 μL, adaptor 1 (10 μM) 2 μL, and master mix 6 μL (from step 2); tester 1-2: diluted tester cDNA 2 μL, adaptor 2R (10 μM) 2 μL, and master mix 6 μL. Pipette mixture up and down to mix thoroughly.

4. In a fresh microcentrifuge tube, mix 2 μL of tester 1-1 and 2 μL of tester 1-2. After ligation is complete, this will be unsubtracted tester control.

5. Centrifuge briefly the three kinds of tubes (tester 1-1, tester1-2, and unsubtracted tester control) and incubate at 16°C overnight.

6. Add 1 μL of EDTA/glycogen mix to stop ligation reaction.

7. Heat samples at 72°C for 5 min to inactivate the ligase.

8. Briefly centrifuge the tubes. Adaptor-ligated tester cDNAs and unsubtracted tester control are now complete.

9. Remove 1 μL from unsubtracted tester control and dilute into 1 mL of sterile water. This sample will be used for PCR (\textbf{Section 3.3.7}).

10. Store samples at –20°C.

3.3.5. First Hybridization

1. Adaptor 1-ligated tester 1-1 (from \textbf{Section 3.3.4} step 10) and adaptor 2R-ligated tester 1-2 (from \textbf{Section 3.3.4} step 10) are separately hybridized with \textit{RsaI}-digested driver cDNA (from \textbf{Section 3.3.3} step 18). Combine the following reagents in a 0.5-mL microcentrifuge tube in the order shown: hybridization sample 1: \textit{RsaI}-digested driver cDNA (from \textbf{Section 3.3.3} step 18) 1.5 μL, adaptor 1-ligated tester 1-1 (from \textbf{Section 3.3.4} step 10) 1.5 μL, and 4× hybridization buffer 1 μL; hybridization sample 2: \textit{RsaI}-digested driver cDNA (from \textbf{Section 3.3.3} step 18) 1.5 μL, adaptor 2R-ligated tester 1-2 (from \textbf{Section 3.3.4} step 10) 1.5 μL, and 4× hybridization buffer 1 μL.
2. Overlay samples with one drop of mineral oil and centrifuge briefly.
3. Incubate samples at 98°C for 1.5 min in a thermal cycler.
4. Incubate samples at 68°C for 8 h. Samples may hybridize for 6–12 h. Do not let the incubation exceed 12 h.

3.3.6. Second Hybridization (see Note 8)

1. The two samples from the first hybridization (hybridization samples 1 and 2 from Section 3.3.5 step 4) are mixed together, and fresh denatured driver cDNA (from Section 3.3.3 step 18) is added for further enrichment of differentially expressed sequences. Add the following reagents into a sterile tube: Rsal-digested driver cDNA (from Section 3.3.3 step 18) 1 μL, 4× hybridization buffer 1 μL, and sterile water 2 μL.

2. Place 1 μL of this mixture in a 0.5-mL microcentrifuge tube and overlay it with one drop of mineral oil.
3. Incubate at 98°C for 1.5 min in a thermal cycler.
4. Remove the tube of freshly denatured driver from the thermal cycler. Use the following procedure to simultaneously mix the driver with hybridization samples 1 and 2 (prepared in Section 3.3.5 step 4).
   a. Set a micropipettor at 15 μL.
   b. Gently touch the pipette tip to the mineral oil/sample interface of the tube containing hybridization sample 2.
   c. Carefully draw the entire sample partially into the pipette tip. Do not be concerned if a small amount of mineral oil is transferred with the sample.
   d. Remove the pipette tip from the tube and draw a small amount of air into the tip, creating a slight air space below the droplet of sample.
   e. Repeat steps b–d with the tube containing the freshly denatured driver. The pipette tip should now contain both samples (hybridization sample 2 and denatured driver) separated by a small air pocket.
   f. Transfer the entire mixture to the tube containing hybridization sample 1.
   g. Mix by pipetting up and down.
5. Briefly centrifuge if necessary.
6. Incubate reaction at 68°C overnight.
7. Add 200 μL of dilution buffer and mix by pipetting.
8. Heat at 68°C for 7 min in a thermal cycler.
9. Store at –20°C.
3.3.7. PCR Amplification
(see Note 9)

1. The first PCR reaction is performed in a total volume of 25 μL comprising each diluted cDNA (i.e., subtracted sample from Section 3.3.6 step 9 and the diluted unsubtracted tester control from Section 3.3.4 step 9) 1 μL, sterile water 19.5 μL, 10× PCR reaction buffer 2.5 μL, dNTP mix (10 mM) 0.5 μL, PCR primer 1 (10 μM) 1 μL, and 50× Advantage cDNA polymerase mix 0.5 μL.

2. Mix well by vortexing and briefly centrifuge the tube.

3. Incubate the reaction mix at 75°C for 5 min in a thermal cycler to extend the adaptors. (Do not remove the samples from the thermal cycler.)

4. Immediately commence thermal cycling. After pre-PCR heating at 94°C for 25 s, a reaction cycle of 94°C for 10 s, 66°C for 30 s, and 72°C for 1.5 min is repeated 27 times (see Note 10).

5. Dilute 3 μL of each primary PCR mixture in 27 μL of sterile water.

6. The second PCR reaction is performed in a total volume of 25 μL comprising each diluted primary PCR product mixture (from step 5) 1 μL, sterile water 18.5 μL, 10× PCR reaction buffer 2.5 μL, dNTP mix (10 mM) 0.5 μL, nested PCR primer 1 (10 μM) 1 μL, nested PCR primer 2R (10 μM) 1 μL, and 50× Advantage cDNA polymerase mix 0.5 μL.

7. Mix well by vortexing and briefly centrifuge.

8. Immediately commence thermal cycling. A reaction cycle of 94°C for 10 s, 68°C for 30 s, and 72°C for 1.5 min is repeated 10–12 times (see Note 10).

9. Store reaction products at −20°C.

3.4. Evaluation of Subtraction Efficiency

1. Dilute the subtracted and unsubtracted secondary PCR products (from Section 3.3.7 step 9) about 10-fold with sterile water. The concentration of subtracted and unsubtracted products should be roughly equal (see Note 11).

2. The PCR reaction is performed in a total volume of 30 μL comprising 1 μL of each diluted secondary PCR product (from step 1), 200 μM dNTPs, 150 μM MgCl₂, 0.5 μM of non-differentially expressed gene primers, 0.75 U of AmpliTaq Gold and GeneAmp PCR buffer II.

3. Immediately commence thermal cycling. After pre-PCR heating at 95°C for 12 min, a reaction cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 1.5 min is repeated 38 times.
4. 5 μL of PCR reaction mixture is removed after 18, 23, 28, 33, and 38 cycles and placed in clean tubes. These samples are analyzed on a 2% agarose gel.

5. Example of the results using apple ubiquitin (Ub) is shown in Fig. 2.1. For the unsubtracted cDNA, the apple Ub was amplified after 18 cycles. However, for the subtracted cDNA, the Ub product was only detected after 38 cycles. The reduction of apple Ub abundance in the subtracted cDNA ensures that SSH was successfully achieved (see Note 12).

![Fig. 2.1. Confirmation of SSH efficiency using apple ubiquitin (Ub). PCR was performed on the subtracted (lanes 1–5) and unsubtracted (lanes 6–10) cDNA with the Ub primers. Numbers of PCR cycles are indicated above the panel. Lane M indicates 100 bp ladder marker.](image)

3.5. Differential Screening Using Reverse Northern (see Note 13)

3.5.1. Preparation of Membrane

1. Subclone the subtracted cDNAs (from Section 3.3.7 step 9) into the pCR2.1 vector and transform to *Escherichia coli* using a TA cloning kit. Spread the transformed *E. coli* to LB plate with ampicillin/IPTG/X-gal and incubate at 37°C overnight.

2. Pick up and culture white clones in 100 μL of LB medium with ampicillin using 96-well cell culture plate. Incubate at 37°C overnight with continuous shaking at 150 rpm.

3. Inserts of cDNA clones are amplified by PCR using cultured LB medium as templates in 96-well PCR plate. The PCR reaction is performed in a total volume of 20 μL comprising 1 μL of cultured LB medium, 80 μM dNTPs, 150 μM MgCl₂, 0.5 μM of nested PCR primers 1 and 2R, 0.5 U of TaKaRa Ex Taq and 1× TaKaRa Ex Taq buffer. After pre-PCR heating at 94°C for 30 s, a reaction cycle of 95°C for 10 s and 68°C for 3 min is repeated 30 times. The remaining culture medium is stored as glycerol stocks at −80°C for further analysis (e.g., sequencing and expression analysis).

4. Dispense 5 μL of 0.6 N NaOH and 2 μL of 0.25% BPB and transfer 5 μL of the PCR products to 96-well microtiter plate. Mix gently by pipetting.
Fig. 2.2. Selection of cDNAs up-regulated in UV-B-treated apple skin by reverse northern. Two identical cDNA blots of PCR-amplified inserts derived from the subtracted cDNA library were hybridized with the probes from + UV-B-treated (tester) and – UV-B-treated (driver) skin. The arrows indicate the spots which show higher intensity in the tester sample.

5. Spot 1.2 μL of the mixtures onto a 10×12 cm nylon membrane in 96-well format (Fig. 2.2). To perform experimental replication, at least four membranes should be prepared.

6. Soak the membranes in 0.5 M Tris–HCl (pH 7.5) for 3 min and rinse with distilled water for 1 min.

7. Fix the PCR product on a nylon membrane using UV cross-linker (e.g., Stratalinker) at energy of 120,000 μJ and dry up the membrane at 65°C.

8. Put the prepared membranes into a plastic hybridization bag. The membranes can be stored at −20°C.

3.5.2. Labeling Probes

1. Mix 7.5 μg of each total RNA (adjust to 7 μL with equipped RNase-free water to the kit) from tester and driver samples with 1 μL of an oligo(dT) primer and 2 μL DIG DNA labeling mix.

2. Incubate the mixture at 65°C for 5 min and chill on ice for 1 min.

3. Add 2 μL of a 10× RT buffer, 4 μL of 25 mM MgCl₂, 2 μL of 0.1 M DTT, and 1 μL of RNase OUT™ and incubate at 42°C for 2 min.

4. Add 1 μL of Superscript II, incubate at 42°C for 50 min and then at 72°C for 15 min.

5. Add 1 μL RNase H and incubate at 37°C for 20 min. Total volume of labeled probe should be 21 μL.

3.5.3. Hybridization, Washing, and Detection

1. Add 10 mL of a high SDS hybridization buffer to plastic hybridization bags (from Section 3.5.1) and incubate at 42°C for 1 h.

2. Add 100 μL of high SDS hybridization buffer to 21 μL of each probe solution prepared from tester and driver samples (from Section 3.5.2).

3. Boil the probe mixture for 5 min and chill on ice for 1 min.
4. Discard a high SDS hybridization buffer from the plastic hybridization bag.

5. Add 5 mL of a fresh high SDS hybridization buffer and each probe mixture to the plastic hybridization bag. Incubate overnight at 42°C.

6. Wash the membranes twice with 2× SSC, 0.1% SDS for 15 min at room temperature.

7. Wash twice with 0.1× SSC, 0.1% SDS at 68°C for 15 min.

8. Rinse in washing buffer at room temperature for 3 min.

9. Incubate in 100 mL blocking solution at room temperature for 30 min.

10. Incubate in 20 mL antibody solution at room temperature for 30 min.

11. Wash twice in 100 mL washing buffer at room temperature for 5 min.

12. Equilibrate in 20 mL detection buffer at room temperature for 5 min.

13. Incubate in 20 mL freshly prepared CSPD solution in the dark for 5 min.

14. Wrap the hybridized membranes with plastic films and expose these membranes simultaneously to X-ray film for 1 h.

15. Examples of the signals for reverse northern are shown in Fig. 2.2. Select the spots which show higher intensity in the tester sample (indicated with arrows). Reverse northern should be conducted with at least two replicates. Proceed with the reproducible spots for further analysis (e.g., sequencing and expression analysis).

4. Notes

1. Sodium borate decahydrate may be hard to dissolve. In that case, dissolve sodium borate decahydrate with continuous heating at 65°C.

2. The sequence of PCR product should not contain RsaI site. The example of apple Ub primers are as follows: 5′-TCGCTGGAAAAGCAGCTTTGAAGA-3′ and 5′-GCTTTCCGGCAAAGAATCAGACG-3′.

3. Nested PCR primer 1 (5′-TCGAGCGGCCGCCCAGGT-3′) and nested PCR primer 2R (5′-AGCGTGGTCGCGGCCAGGT-3′) are available through usual
oligonucleotide ordering companies. Desalted purification grade is enough for this analysis.

4. This protocol was developed based on the hot borate method (12) and can be applied to polysaccharide- and polyphenol-rich samples such as fruit and flowers.

5. If the pellet could not be well suspended, increase the volume of 10 mM Tris–HCl. Subsequently, change the volume of solutions used in the later steps at the same ratio.

6. The ratio of poly(A)⁺ RNA contained in total RNA varies with species and tissues. For example, 0.25% poly(A)⁺ RNA was contained in total RNA from mature apple skin.

7. Adaptors will not be ligated to the driver cDNA in this section.

8. Do not denature the primary hybridization samples at this stage. Also, do not remove the hybridization samples from the thermal cycler for longer than is necessary to add fresh driver.

9. All cycling parameters were optimized on a GeneAmp PCR Systems 9700 (Applied Biosystems). Cycling parameters for other thermal cycler machines may require optimization.

10. To prevent the amplification of undesired sequences, PCR cycles should be optimized (13). In many cases, reduction of the PCR cycles gives favorable results. In our previous study (11), the first and second PCR cycles were determined as 25 and 8 cycles, respectively.

11. To check the concentration of the subtracted and unsubtracted secondary PCR products, analyze 8 µL of each secondary PCR product on a 2% agarose gel run in 1× TAE buffer.

12. If you do not observe a decrease in abundance of PCR product in the subtracted sample, repeat the PCR amplification (Section 3.3.7) with reduced PCR cycles (see Note 10).

13. Alternatively, PCR-Select differential screening kit (Clontech) can be used for differential screening. For details, please see the PCR-Select differential screening kit user manual.

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References


Identification of Regulatory Protein Genes Involved in Alkaloid Biosynthesis Using a Transient RNAi System

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Abstract

RNAi (RNA interference, RNA silencing) is a powerful tool in functional genomics. We report here the use of transient RNAi to isolate regulatory factor genes involved in isoquinoline alkaloid biosynthesis in Coptis japonica protoplasts. Double-stranded (ds) RNAs prepared against candidate regulatory factors, which were predicted from an EST library, were introduced into C. japonica protoplasts by polyethylene glycol (PEG)-mediated transformation, and their effects were monitored by real-time reverse transcription (RT)-polymerase chain reaction (PCR). The potential of this transient RNAi system to characterize the functions of regulatory factor genes in alkaloid research is discussed.

Key words: RNA interference (RNAi), RNA silencing, transcription factor, transient RNAi.

Abbreviations

MES 2-morpholinoethanesulfonic acid;
PEG polyethylene glycol;
RNAi RNA interference;
RT reverse transcription;
TF transcription factor.

1. Introduction

Higher plants produce a wide range of secondary metabolites, which have been used as dyes, fragrances, and pharmaceuticals. Despite their economic importance, the biosynthetic pathways of many secondary metabolites have not been fully characterized.
due to the complexity of the products and their relatively low biosynthetic enzyme activity. While the transcriptional activation of whole biosynthetic enzymes by a master regulator would be useful for characterizing biosynthetic pathways, only a few transcriptional factors in alkaloid biosynthesis have been isolated; e.g., the ORCA transcriptional factor for the strictosidine synthase gene in the terpenoid indole alkaloid biosynthetic pathway (1, 2). Recently, we identified the WRKY gene as a general regulator using the transient RNAi method (3). We discuss here the potential of RNAi for the isolation of regulatory factors.

In general, the activation tagging method is a powerful tool for isolating master genes in the regulation of gene expression (4). However, it is not always easy to isolate the true target gene, especially when multiple transcription factors are involved. For example, ORCA3 in terpenoid indole alkaloid biosynthesis controls only some of the biosynthetic genes (1). Considerable improvements in methodology would be needed to elucidate a complicated transcriptional network. Whereas T-DNA tagging to knockout gene functions is generally used in Arabidopsis, most medicinal plants are not suitable for this approach due to the low efficiency of stable transformation, low regeneration, and large genome size. Thus, another approach using the overexpression of a candidate transcription factor in host cells has been examined with partial success (5, 6). Again, this simple approach using the overexpression of transcription factors would not be sufficient for identifying the function of transcription factors, since biosynthetic genes in secondary metabolism are controlled by multiple transcriptional regulators, as has been described for terpenoid indole alkaloid biosynthesis (1, 7).

Thus, we examined another approach to isolate transcription regulator(s) using high berberine-producing C. japonica cells as a model material with a novel transient RNAi method that was recently developed for C. japonica cells (Fig. 3.1; 8). This newly developed RNAi system using artificially prepared double-strand RNAs degrades target mRNA very effectively in a sequence-specific manner and is useful for isolating a possible transcriptional regulator (a WRKY protein gene; CjWRKY1) from an EST library of high berberine-producing C. japonica cells. While this RNAi method is more effective than antisense RNA technology, which is commonly used to reduce gene expression in plant cells, the construction of stable expression vectors for RNAi is tricky due to the nature of the inverted repeat structure (9, 10). Since research on gene function involved in biosynthetic pathways does not require stable transformation, we used RNAi coupled with rapid transient assays (8). We also discuss various perspectives of transient RNAi.
Identification of Regulatory Protein Genes in Alkaloid Biosynthesis

2. Materials

1. dsRNA synthesis (see Note 1): T7 RiboMAX™ express RNAi system (Promega), RNase-free water, isopropanol, 70% ethanol, and gene-specific PCR primers containing the 28-nucleotide minimal T7 promoter sequence (5’-TAATACGACTCACATAGGGGAGACCAC-3’) at the 5’ terminal followed by a 21-nucleotide gene-specific region.

2. DNA template preparation (see Note 1): thermal cycler for PCR (e.g., PE 9700, Perkin-Elmer), Taq DNA polymerase for PCR (e.g., GoTaq DNA polymerase, Promega), and Wizard SV gel and PCR clean-up system (Promega).

3. Protoplast preparation reagents (see Note 2): cell wall digestion solution (e.g., for cultured Coptis japonica cells, digestion solution contains 0.4% Onozuka cellulase R10 (Yakult Pharmaceutical), 0.2% macerozyme R10 (Yakult Pharmaceutical), 0.01% pectolyase Y23 (Seishin Pharmaceutical), 0.6 M sorbitol, 20 mM MES at pH 5.5, and 5 mM MgCl₂, which was pre-treated at 50°C for 30 min before filter sterilization with Millex GP (pore size 0.22 μm, Millipore)).
4. Plant materials (see Note 2): cultured *C. japonica* cell line 156-1, which was originally selected by small aggregate cloning of *C. japonica* Makino var. *dissecta* (Yatabe) Nakai (11), was further transformed with extra scoulerine 9-O-methyltransferase expression vector, and then maintained in liquid Linsmaier–Skoog medium containing 3% sucrose, 10 μM naphthalene acetic acid, and 0.01 μM 6-benzyladenine (12).

5. Protoplast transformation reagents (13) (see Note 3): W5 solution contains 154 mM NaCl, 125 mM CaCl₂·2H₂O, 5 mM KCl, and 5 mM glucose, pH 5.8. MaCaM solution contains 0.4 M and 20 mM CaCl₂·2H₂O. These solutions are autoclaved and the pH is then adjusted to 5.8 with 5 mM sterilized MES. Polyethylene glycol (PEG) solution contains 40% PEG6000 (Nacalai, Japan) dissolved in 0.4 M mannitol and 0.1 M Ca(NO₃)₂·4H₂O and is adjusted to pH 10. All reagents are sterilized in an autoclave.

6. Protoplast culture vessel – six-well plate (IWAKI, Chiba, Japan).

7. Dual-luciferase assay system (Promega) contains passive lysis buffer (Promega), LUC assay reagent II, and Stop & Glo reagent.

8. Detector: luminometer (e.g., Berthold Lumat 9507 (Berthold)), fluorescent microscopy (e.g., Nikon), UV-spectrophotometer for RNA quantification (e.g., Shimadzu BioSpec-mini).

9. RNA preparation (see Note 1): RNeasy kit (Qiagen), RNaseOUT™ recombinant RNase inhibitor (Invitrogen), RNase-free DNase (Promega), and Superscript III reverse transcriptase first-strand synthesis system (Invitrogen).

10. Quantification of transcripts: real-time PCR machine (e.g., DNA Engine Opticon™ system, Bio-Rad). DYNAmo™ HS SYBR® Green qPCR kit (New England BioLabs) contains 2× master mix, DYNAmo hot start DNA polymerase, SYBR Green I, optimized PCR buffer, and deoxynucleotide triphosphates (dNTPs). Sense and antisense gene-specific primers.

### 3. Methods

#### 3.1. ds (double-stranded) RNA Preparation

In vitro preparation of dsRNA was performed using the protocol described by Carthew and Kennerdell (14). In brief, dsRNA was prepared by in vitro transcription with PCR products harboring T7 RNA polymerase promoters at both ends.
1. DNA templates for the preparation of dsRNA are produced from 10 to 100 ng plasmid containing target cDNA by PCR using 0.5 μM primers containing the minimal T7 promoter sequence (5’-TAATACGACTCACTATAGGGAGACCAC-3’) at the 5′ terminal followed by a 21-nucleotide gene-specific region and 0.2 mM dNTPs with 2.5 U GoTaq DNA polymerase in 100 μl of reaction mixture. A typical reaction cycle is as follows: denaturation at 94°C for 5 min, (denaturation at 94°C for 30 s, annealing at 40°C for 30 s, and extension at 72°C for 30 s) × 10 cycles, then (denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s) × 25 cycles, extension at 72°C for 5 min, and maintenance at 4°C. (Primer design: see Note 1.)

2. PCR product is cleaned up with Wizard SV gel and a PCR clean-up system.

3. dsRNAs are synthesized in vitro from dsDNA template using the T7 RiboMAX express kit according to the manufacturer’s protocol. dsRNA yield is 1–7 μg/μl. dsRNA preparation is confirmed by denatured agarose gel electrophoresis.

4. Residual DNA template is removed by treatment with RNase-free DNase.

5. The dsRNA is purified by isopropanol extraction, precipitated with 70% ethanol, dissolved in RNase-free water, and quantified by UV spectrophotometry. (see Note 1).

3.2. Protoplast Transformation

dsRNA is introduced into protoplasts essentially as described by Chiu et al. (15)

1. C. japonica protoplasts are prepared from 1- to 3-week-old cultured 156-SMT cells in cell wall digestion solution (see Section 2 and Note 2).

2. About 1 g of cells is incubated in 10 ml of solution overnight at 28°C and 30 rpm and about 1–5 million protoplasts are obtained.

3. Protoplasts are separated from undigested cell debris by filtration through 60- to 80-μm nylon mesh and then precipitated by centrifugation at 40 × g for 5 min.

4. After the protoplast preparation is washed three times in 10 ml of W5 solution, the protoplasts are suspended in MaCaM solution at 2 × 10^6 protoplasts/ml.

5. dsRNA of the candidate gene at different concentrations is added to the protoplast suspension with/without cauliflower mosaic virus 35S promoter-Renilla LUC or GFP expression plasmid as an internal control. Typically, a solution of 50 μg of dsRNA in RNase-free water is added to 1.0 × 10^6 protoplasts in 500 μl.
6. The expression plasmids and dsRNA are gently mixed with the protoplasts in MaCaM solution.

7. An equal volume of polyethylene glycol (PEG) solution (e.g., 550 μl) is gradually added, gently mixed by spinning five times, and incubated at 28°C and 30 rpm for 30 min (see Note 3).

8. Protoplasts are spun down by centrifugation at 40×g for 5 min and then re-suspended in 4 ml of W5 solution.

9. Finally, 10^6 protoplasts are suspended in 2 ml of W5 solution and cultured in a six-well plate at 28°C for 24–72 h with gentle shaking at 30 rpm in the dark.

10. The viability of protoplasts after culture is evaluated by microscopic observation (see Note 2).

11. After incubation for 24–72 h, protoplasts are harvested by centrifugation at 40×g for 5 min for quantitative real-time PCR to measure the transcript levels of RNAi target gene (transcription factor (TF) candidate) and biosynthetic genes (target of TFs) (Figs. 3.2–3.4; see Note 4).

3.3. Quantitation of Luciferase (LUC) Activity

LUC activity is measured using a dual-luciferase assay system to monitor the RNA silencing efficiency of dsRNA in the system (see Note 5).

1. C. japonica cells are harvested by centrifugation and homogenized in 100 μl passive lysis buffer.

2. 10 μl of the extracts is mixed with 50 μl LUC assay reagent II, and apparent luminescence is measured in a luminometer.

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Fig. 3.2. Lasting effects of dsRNA. Effect of transient RNAi of the CjWRKY1 gene was analyzed by real-time PCR analysis. The results shown are mean values ± SD of four measurements. The relative transcript level was calculated using the arabinogalactan protein gene as an internal control.
3. After *Photinus* LUC activity is measured, 50 μl of Stop & Glo reagent is added and the resultant *Renilla* activity is measured.
4. Data are expressed as a ratio of luminescence from *Photinus* divided by the luminescence from *Renilla*.

Total RNAs are prepared from protoplasts and treated with RNase-free DNase to avoid contamination by genomic and/or plasmid DNA from transformation. Expression levels of transcripts can be evaluated using either real-time PCR or semi-quantitative RT-PCR (Figs. 3.2–3.4; see Note 6).

1. Protoplasts transformed with dsRNAs are precipitated by centrifugation at 40×g for 5 min and then stored at −80°C until analysis.

2. About 2–7 μg of total RNA is extracted from 3 to 4 × 10⁵ protoplasts of *C. japonica* with an RNasy kit according to the protocol recommended by the manufacturer.

3. After treatment with RNase-free DNAse I, 10-μl aliquots (ca. total RNA 100–1000 ng) are subjected to reverse transcription with Superscript III reverse transcriptase according to the protocol recommended by the manufacturer. In brief, total RNA is incubated at 50°C for 60 min in the presence of 1X first-strand buffer, 5 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate, 2.5 μmol of oligo dT₂₀ primer, 40 U of RNaseOUT™ recombinant RNase inhibitor, and 200 U of SuperScript™ III reverse transcriptase in a total volume of 20 μl. The sequence of the oligo dT primer is 5′-GCTGTCAACGATACGCTACGT AACGGCAGCAGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
7. The final PCR products are checked for homogeneity by a melting curve analysis (72–95°C) according to the manufacturer’s instructions. Each quantitative PCR is performed at least in duplicate.

8. The final result is expressed as a relative fold by comparing the target gene transcripts to the internal control (actin or ATPase), which is determined by the equation

\[ 2^{(C(t)_{\text{target}} - C(t)_{\text{internal control}})} \] (see Note 8).

3.5. Perspectives

It is now clear that many dsRNAs effectively down-regulate their own target genes (e.g., CjWRKY1-3 by dsRNA of CjWRKY1-3), although there are a few exceptions, such as CjWRKY4 (Figs. 3.2–3.4). This highly efficient gene silencing enables the screening of candidate genes involved in the regulation of downstream biosynthetic gene expression. Thus, CjWRKY1 was identified as the first general transcription factor in isoquinoline alkaloid biosynthesis (3). This screening is very simple; just induce RNAi in a candidate transcription factor gene and monitor the transcript level of downstream biosynthetic genes. The usefulness of this method is also evident since dsRNAs of other WRKY homologues did not interfere with the expression of CjWRKY1 (Fig. 3.4). While we cannot avoid the effect of being off-target due to sequence similarity, the high silencing efficiency even with short dsRNA fragments of 37 bp facilitates the rapid and efficient screening of candidates (16). We should emphasize that secondary metabolism, especially the one involved in defense mechanisms, would be an important target for this analysis, since biosynthetic genes are often induced by wounding signals generated during protoplast isolation and cultivation. This analytical system would also be suitable for gene expression, which can be induced by some signals such as jasmonate. On the other hand, it might be difficult to characterize the biosynthetic genes when the target gene products such as enzymes and metabolites accumulate, since the effect of gene silencing of a target biosynthetic gene is masked by the presence of pre-existing products. However, Zhai et al. (17) recently reported that transient RNAi might also be suitable for the functional analysis of biosynthetic genes such as γ-glutamylcysteine synthase, a key enzyme in glutathione biosynthesis. While glutathione would be involved in defense responses, this result suggests the broad potential of this method. Another limitation of this technology is the difficulty of protoplast isolation and manipulation. When it is difficult to obtain good materials for preparing active protoplasts, a transient assay with microbombardment using a particle gun system might be a suitable substitute for protoplast isolation. The potential of transient RNAi is still growing.
4. Notes

1. While many kits are mentioned in this protocol, users may choose other products, which are reliable and may be available locally. In addition, users may follow a more conventional protocol, such as molecular cloning (18) or another protocol (e.g., 19). For example, RNase-free water can be prepared as shown in the literature (18, 19).

PCR primers are designed using the Primer3 program (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). To facilitate RT-PCR measurement of the transcripts under standard reaction conditions, a stringent set of criteria are used for primer design. These include a predicted melting temperature of 60±2°C, primer lengths of 20–24 nucleotides, guanine–cytosine (GC) contents of 45–55%, and a PCR amplicon length of 60–200 base pairs (bp).

The various dsRNA fragments are prepared from different parts of the nucleotide sequence to evaluate the effect of sequence length and position in the coding sequence on the silencing efficiency. Longer fragments are better for silencing, but an off-target effect may induce the silencing of other genes and hinder characterization. Thus, primers for the dsRNA template should be selected to give a length of 200–400 base pairs.

2. For the transient assay using protoplasts, the viability of protoplasts is critical. For the preparation of viable protoplasts, the conditions of the plant/cell materials and protoplast preparation, such as the cell wall digestion enzyme composition, are crucial. The protoplast isolation efficiency and viability must be checked before transformation using microscopy. Viable protoplasts are smooth and efficiently stained with neutral red or fluorescent diacetate. Dead cells show irregular shapes and are stained with Evans Blue. Since the number of protoplasts isolated from cell materials varies based on the plant variety, tissue types, and tissue/cell culture conditions, the standard efficiency of isolation should be recorded (e.g., 5 × 10^6 protoplast/g fresh weight of cultured Coptis japonica cells) and preparation conditions with low isolation efficiency should not be used for transformation. The cell wall composition also varies considerably among materials. Thus, it is essential that the cell wall digestion enzyme solution is optimized. The enzyme solution is heat labile and should be sterilized using a filter. However, commercial enzyme products often contain additives such as starch. Thus, the additives in the enzyme preparation might be dissolved at 50°C or removed by centrifugation.
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3. W5 solution developed for transient assays of *Arabidopsis* protoplasts (13) is effective for minimizing the damage to *C. japonica* protoplasts compared to standard plant media (20, 21) with various levels (0–25%) of sucrose or glucose (8).

For protoplast transformation, PEG-mediated transformation is cost-effective and efficient, whereas electroporation would also be suitable. In PEG treatment, the selection of PEG is important: PEG6000 would be preferable to PEG4000. PEG also contains some additives. Thus, different products can give different transformation efficiencies.

Additionally, protoplasts are fragile, but a high concentration of PEG is very viscous. Thus, PEG should be added gradually drop by drop and mixed slowly. Optimization of the transformation conditions and protoplast preparation with a transformation efficiency of more than 90%, which can be monitored using *GFP* expression, is recommended, although a transformation system of even 30–50% would still be useful.

4. An RNA silencing effect is evident at 24 h after dsRNA treatment and lasts for 72 h (see Fig. 3.2). The silencing efficiency also depends on the materials and dsRNA sequences used (Fig. 3.4; 3).

5. For the optimization of RNAi experiments, the luciferase gene contained 1 µg of CaMV35S promoter-driven *Photinus LUC* expression plasmid, and CaMV35S promoter-driven *Renilla LUC* expression plasmid, and 0.5, 1, 5, and 10 µg of full-length *Photinus LUC* dsRNA can be introduced for every 10⁵ protoplasts.

6. Semi-quantitative PCR would be more convenient, since it can be done using GoTaq DNA polymerase (Promega) in a PE 9700 (Perkin-Elmer). PCR is basically carried out with the following thermal cycle: a denaturation step at 95°C for 2 min followed by 25–30 cycles of 95°C for 15 s, 45°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. This method has been used to successfully amplify up to four independent gene targets with different amplification cycle requirements in a single PCR reaction tube. The following primer sets were used for the detection of transcripts of isoquinoline alkaloid biosynthesis genes in *Coptis japonica*: for *SMT* – 5'-GGATTTCTTTTGTA TGCTGG-3' and 5'-TCTCTATCCGTCTCCCAATC-3'; for *4OMT* – 5'-ATGTTCGAGAATAAAAGCCTC-3' and 5'-ACCACATAAAAGCCTC-3'; for *6OMT* – 5'-GCAGTGCAACTTGA TCT AGCC-3' and 5'-AGCTGG TTTTCTCAGGGTG-3'; for *CNMT* – 5'-GCAACAG...
AGTTGAGACCTTG-3' and 5'-GCTGCTAGTCCTTT
CTGGATG-3'; and for arabinogalactan protein – 5'-GC
GTGCAAGGTGGTTTTATGC-3' and 5'-AGAACA
CTCTGCATGTTGTGA-3'. These primers are designed
to produce specific amplicons and the addition of dsRNA
does not to interfere with specific amplification of the target
transcript by PCR. The levels of transcripts of monitored
genes are evaluated using densitometry after agarose gel
electrophoresis.

7. The fluorescence signal from each real-time quantitative
PCR reaction is collected as a normalized value plotted
versus the cycle number. Reactions are characterized by
comparing threshold cycle ($C_t$) values. $C(t)$ is the frac-
tional cycle number at which the sample fluorescence signal
passes a fixed threshold above the baseline. Quantitative val-
ues are obtained from the $C(t)$ values at which the increase in
signal associated with the exponential growth of PCR prod-
uct starts to be detected according to the manufacturer’s
instructions (Bio-Rad, Hercules, CA).

8. The actin, ATPase, or arabinogalactan protein genes were
used as internal controls to normalize the target biosynthetic
gene expression. However, the expression levels of these
internal standard genes also fluctuate. Thus, it is important
to select a suitable internal standard gene.

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Chapter 4

Site-Directed Mutagenesis and Saturation Mutagenesis for the Functional Study of Transcription Factors Involved in Plant Secondary Metabolite Biosynthesis

Sitakanta Pattanaik, Joshua R. Werkman, Que Kong, and Ling Yuan

Abstract

Regulation of gene expression is largely coordinated by a complex network of interactions between transcription factors (TFs), co-factors, and their cognate cis-regulatory elements in the genome. TFs are multidomain proteins that arise evolutionarily through protein domain shuffling. The modular nature of TFs has led to the idea that specific modules of TFs can be re-designed to regulate desired gene(s) through protein engineering. Utilization of designer TFs for the control of metabolic pathways has emerged as an effective approach for metabolic engineering. We are interested in engineering the basic helix-loop-helix (bHLH, Myc-type) transcription factors. Using site-directed and saturation mutagenesis, in combination with efficient and high-throughput screening systems, we have identified and characterized several amino acid residues critical for higher transactivation activity of a Myc-like bHLH transcription factor involved in anthocyanin biosynthetic pathway in plants. Site-directed and saturation mutagenesis should be generally applicable to engineering of all TFs.

Key words: Transcription factors, site-directed mutagenesis, saturation mutagenesis, yeast one-hybrid, transient protoplast expression.

1. Introduction

Coordination of transcriptional control of biosynthetic genes has emerged as a major mechanism dictating the production of secondary metabolites in plant cells. Such regulation of biosynthetic pathways can be achieved by specific transcription factors (TFs). TFs have been isolated and characterized for a number
of plant metabolic pathways. Transgenic expression for some of these TFs has resulted in regulation of the biosynthesis of flavonoids and terpenoid indole alkaloids (1). TFs are typically multidomain proteins that arise evolutionarily through protein domain shuffling. The modular nature of TFs has led to the idea that specific modules of TFs can be re-designed to regulate desired gene(s) through protein engineering. The ability to generate designer TFs has the potential to aid in our understanding of gene regulation and is of biotechnological interest. In the past few years, impressive advances have been achieved in the engineering of TF-like proteins (2–4). Engineered TFs have been shown to be capable of regulating endogenous gene expression.

We are interested in engineering the basic helix-loop-helix (bHLH, Myc-type) transcription factors. The Myc-type bHLH proteins are relatively well characterized and are involved in several well-known plant pathways. Here, we describe two approaches, site-directed mutagenesis and saturation mutagenesis, for functional study of TFs involved in plant secondary metabolite biosynthesis. Site-directed mutagenesis is an invaluable tool used to modify DNA sequences at a defined site in a pre-determined way, which is widely used for studying protein structure–function relationships. Saturation mutagenesis allows the substitution of one or more specific sites against all possible mutations in a gene sequence.

Efficient screening methods are also essential for analyzing the property of the individual mutants. We have used two efficient screening methods to characterize the TF mutants: yeast transactivation assay and transient plant protoplast expression assay. Yeast transactivation assay is a commonly used method to test the transactivation potential of TFs in yeast cell. Transient expression in plant protoplasts is a powerful tool to evaluate the transactivation activities of the mutants in vivo, especially in an environment mimicking the inside of a plant cell. Compared to the stable transformation, transient expression has the advantages of speed, convenience, and flexibility (5). By random, site-directed, and saturation mutagenesis in combination with efficient screening, we have successfully improved the transactivation properties of a bHLH TF involved in plant anthocyanin biosynthesis (4, 6).

2. Materials

2.1. Plasmids and Primers

1. Plasmids for yeast expression: pYES2-1 (Clontech, USA). This vector contains the GAL4 DNA-binding domain.
(amino acids 1–147), ADH1 promoter, and ADH1 transcription termination signal. The vector generates a fusion of the GAL4 DNA-binding domain and a protein of interest cloned into the multicloning site (MCS) in correct orientation and reading frame.

2. Reporter and effector plasmids for transient protoplast expression: (a) reporter plasmid, a modified pKYLX80 plasmid (7) containing five tandem repeats of GAL responsive elements, minimal CaMV 35S promoter (−46 to +8 relative to transcription start site), a fire-fly luciferase gene, and rbcs terminator and (b) effector plasmid, a modified pBlueScript plasmid containing the transcription factor under the control of mirabilis mosaic virus (MMV) full-length transcript promoter, GAL4 DNA-binding domain (amino acids 1–147), and rbcs terminator (6, 7, 8). The effector plasmid generates a fusion of GAL4 DNA-binding domain and protein of interest cloned into the MCS in correct orientation.

3. All synthetic oligonucleotides used were procured from Integrated DNA Technologies (Coralville, IA, USA).

2.2. Polymerase Chain Reaction (PCR), Agarose Gel Electrophoresis and Gel Purification, Escherichia coli Transformation, Plasmid Isolation, and Sequencing

1. Taq DNA polymerase and dNTPs: PfuUltra high-fidelity Taq DNA polymerase (Stratagene, USA) and 100 mM dNTPs (USB Affymetrix, USA). Prepare a 10 mM stock solution of dNTPs in sterile milli-Q water for regular use. Store all PCR reagents at −20°C.

2. Agarose and buffer used in gel electrophoresis: ultrapure low melting agarose (Invitrogen, USA). 50X Tris-acetate-EDTA (TAE) buffer (242 g Tris base, 100 ml of 0.5 M EDTA (pH 8.0), and 57.1 ml glacial acetic acid per 1 l solution). Autoclave and store at room temperature. Prepare a 1X TAE in milli-Q water for regular use. Owl separation system (Thermo Scientific, USA) is used for gel electrophoresis.

3. Gel purification of PCR products: Wizard SV gel and PCR clean-up system (Promega, USA).

4. E. coli cell for transformation: K12 TB1 cells (F– ara Δ(lac-proAB) [Φ80dlac Δ(lacZ)M15] rpsL(StrR) thi bsdR; New England BioLabs, USA).

5. Luria–Bertani (LB) medium: sodium chloride (NaCl, 10 g/l), Bacto tryptone (10 g/l), and Bacto yeast extract (5 g/l). Add 15 g/l Bacto agar to LB medium to make LB agar plate. Dissolve in water and autoclave.

6. Plasmid isolation from E. coli cells: Wizard Plus SV minipreps (Promega, USA).
7. Reagents and instrument for DNA sequencing: GenomeLab DTCS quick start kit, GenomeLab separation gel, GenomeLab separation buffer (Beckman Coulter, USA), and Beckman Coulter CEQ-8000 sequencer. Store the DTCS quick start kit at \(-20^\circ\text{C}\), and the separation buffer and gel are stored at \(4^\circ\text{C}\).

2.3. Yeast Strain, Yeast Culture, and Transformation Medium

1. Yeast strain: Y187 (Saccharomyces cerevisiae Y187: \(\text{MAT}\alpha, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4\Delta, met-, gal80\Delta, MEL1,\) and \(\text{URA3::GAL1 UAS-Gal1TATA-lacZ; Clontech, USA}\)).

2. YPDA medium: Bacto yeast extract (10 g/l), Bacto peptone (20 g/l), dextrose (20 g/l) and adenine hemisulfate salt (15 ml of 0.2% solution per liter), and Difco agar (15 g/l). Prepare a 40% dextrose solution in water, filter sterilize and add to the medium (50 ml/l) after autoclaving.

3. Reagents for yeast transformation: 50% polyethylene glycol (PEG 3350; Sigma), 1 M lithium acetate (LiAc), and salmon sperm (SS) DNA (2 mg/ml). Dissolve 50 g of PEG in 30 ml sterile water on a stirring plate and adjust the volume to 100 ml. To make a 1 M solution, dissolve 5.1 g of LiAc in 50 ml water. Filter sterilize the solutions and store at \(4^\circ\text{C}\). Dissolve SS DNA in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), aliquot in small tubes, and store at \(-20^\circ\text{C}\).

4. Synthetic dropout (SD) medium: Difco yeast nitrogen base without amino acids (6.7 g/l; BD Biosciences, USA), dextrose (20 g/l), and tryptophan dropout supplement (740 mg/l; Clontech). Dissolve all the components in water and adjust medium pH to 5.8 before autoclaving.

5. Plasmid isolation from yeast cells: Zymoprep yeast plasmid isolation kit (Zymo Research, USA).

2.4. Buffers and Reagents Used for \(\beta\)-Galactosidase Assay

1. Buffer Z: sodium phosphate dibasic (\(\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}; 16.1\) g/l), sodium phosphate monobasic (\(\text{NaH}_2\text{PO}_4 \cdot 8\text{H}_2\text{O}; 5.5\) g/l), potassium chloride (KCl; 0.75 g/l), and magnesium sulfate (\(\text{MgSO}_4 \cdot 7\text{H}_2\text{O}; 0.246\) g/l). Adjust the pH to 7.0, autoclave, and store at room temperature.

2. Buffer Z with X-gal: dissolve 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside (X-gal) in \(N, N\)-dimethylformamide (DMF; Sigma) at a concentration of 20 mg/ml and store at \(-20^\circ\text{C}\). Solution for filter paper assay is made with 100 ml buffer Z, 270 \(\mu\text{l}\) \(\beta\)-mercaptoethanol (\(\beta\)-ME; Sigma), and 1.67 ml X-gal solution.
3. Buffer Z with ONPG: prepare fresh 5-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) solution in buffer Z at a concentration of 4 mg/ml. Solution for liquid assay is made fresh with 100 ml buffer Z, 270 μl of β-ME, and 160 μl of ONPG solution.


1. Cell suspension culture medium: Murashige and Skoog’s medium with minimal organics (Sigma, USA), potassium phosphate (KH₂PO₄; 204 mg/l), pyridoxine HCL (0.5 mg/l), nicotinic acid (0.5 mg/l), thiamine HCL (0.5 mg/l), 2,4-dichlophenoxyacetic acid (2,4-D; 0.2 mg/l), and kinetin (0.1 mg/l). Adjust medium pH to 5.8 before autoclaving. Culture medium, vitamins, and phytohormones are procured from Sigma, USA.

2. MMC solution: mannitol (91.1 g/l), 2-morpholinoethane sulfonic acid (MES, 1.95 g/l), and calcium chloride (CaCl₂·2H₂O, 1.47 g/l).

3. Enzyme solution: cellulase Onozuka RS (0.75%; Yakult Honsa, Japan) and pectinase (0.075%; Sigma). Dissolve the enzymes in MMC solution on a stirring plate and filter sterilize using Millipore filter (0.22-μm size; Millipore, USA) (see Note 1).

4. Sucrose solution: sucrose (25 g/100 ml), MES (1.95 g/l), and CaCl₂·2H₂O (1.47 g/l).

5. Electroporation buffer: mannitol (91.1 g/l), potassium chloride (KCl, 5.21 g/l), and MES (975 mg/l).

6. Protoplast culture medium: mannitol (91.1 g/l), MES (1.95 g/l), CaCl₂ 2H₂O (1.47 g/l), KH₂PO₄ (27 mg/l), KNO₃ (101 mg/l), MgSO₄ (120 mg/l), KI (2 mg/l), and sucrose (30 g/l).

7. Mannitol agarose solution: dissolve 9 g of mannitol and 1 g of low melting agarose in 100 ml milli-Q water and autoclave. 1 ml of this solution is used to coat a 25-mm sterile Petri dish.

8. Electroporator: Gene Pulser II apparatus with the capacitance extender II (model 165–2107; Bio-Rad, USA).

9. Reagents and instrument for luciferase assay: luciferase assay system (Promega, USA). Luminometer (model no. TD2020; Turner Designs, USA).
3. Methods

3.1. Primer Design for Site-Directed and Site-Saturation Mutagenesis

Site-directed and site-saturation mutagenesis are carried out using one-step method (9). Complementary primers with corresponding mutations are synthesized and used in PCR amplification of the template plasmid. Here, we use the yeast plasmid, pAS2-1 (Clontech, USA) containing the TF cDNA. The primers in the pair must complement each other at the 5′-terminus instead of the 3′-terminus to avoid primer self-extension. For saturation mutagenesis, the codon of the targeted amino acid is randomized to NNK in forward primer and MNN in reverse primer (where N = A or T or G or C; K = G or T; and M = A or C). We routinely use primers that are 40–45 bp long with $T_m$ values ranging from 75 to 83°C. A few considerations are important for designing primers for site-directed and saturation mutagenesis: first, the targeted mutation should be introduced in both primers; second, at least eight non-overlapping bases should be at the 3′-end of each primer; third, at least one G or C should be placed at the end of each terminus; fourth, the desired mutation is in the middle of the primer with 10–15 bases of correct sequences on both sides.

3.2. PCR, Gel Purification of the PCR Products, DpnI Digestion, and Transformation into E. coli Cells

1. PCR amplifications are carried out in a total volume of 50 μl containing 50 ng plasmid DNA, 0.4 μM primer pair, 200 μM dNTPs, and 2 U of PfuUltra high-fidelity DNA polymerase. PCR is performed on a Peltier thermal cycler (MJ Research, USA) using the following program: pre-heat 94°C for 2 min; 16 cycles of 94°C for 30 s, 55°C for 1 min, and 68°C for 10 min (1 kb/min); followed by incubation at 68°C for 1 h.

2. Prepare 1% agarose gel in 1X TAE buffer and pour the gel in a gel tray. The gel should polymerize in 30 min. Once the gel has set, carefully remove the comb, add running buffer (1X TAE) to the unit, load the PCR product in a well, load a 1 kb DNA ladder in another well, connect it to the power supply, and run at 95 V until bands are well separated.

3. The PCR products are purified using Wizard SV Gel and PCR clean-up system and then treated with DpnI (New England Biolabs, USA) for 1 h to remove parental plasmid. The enzyme is heat inactivated at 75°C for 15 min.

4. For transformation, 10 μl of the digested PCR product is added to 100 μl of chemically competent TB1 cells, incubated on ice for 30 min, heat shocked for 2 min in a 37°C water bath, and kept for 2 min on ice. 1 ml of LB medium is added to the tube and incubated at 37°C with continuous
shaking (200 rpm). After 1 h the cells are pelleted in a microcentrifuge at 4000 rpm for 5 min, plated on LB plate containing 100 μg/ml ampicillin, and placed in a 37°C incubator overnight.

3.3. Plasmid Isolation and Sequencing of the Mutated Plasmids

1. The transformants are grown in 2 ml of LB medium containing 100 μg/ml ampicillin at 37°C overnight. Plasmids are isolated from the overnight cultures using Wizard Plus SV minipreps and eluted in water.

2. Sequencing PCR is performed in a total volume of 20 μl containing 300 ng of plasmid, 1 μl of primer (10 μM solution), and 8 μl of reaction mix (GenomeLab DTCS quick start kit). The PCR products are purified and dissolved in sample loading solution as per the manufacturer’s instructions. Sequencing to verify the mutation is performed in Beckman Coulter sequencer CEQ-8000. The sequencing data are analyzed and mutant plasmids are selected for functional characterization.

3.4. Transformation of the Mutated Plasmids into Yeast

1. The mutant plasmids are transformed into yeast strain Y187 using the lithium acetate/single-stranded carrier DNA/polyethylene glycol protocol (10) with minor modifications. A single colony of Y187 is inoculated in 2 ml YPDA medium and grown overnight at 30°C with shaking (250 rpm). The overnight culture is diluted in 50 ml YPDA medium and the OD$_{600}$ of the culture is adjusted to 0.2. The culture is grown for an additional 3–4 h at 30°C with shaking. The OD$_{600}$ of the culture at harvest should be 0.4–0.6.

2. The cells are harvested in a 50-ml sterile tube by centrifugation at 4000 rpm for 5 min in a Sorvall Legend RT Plus. The medium is discarded, cells are resuspended in 20 ml of sterile water, and centrifuged for 5 min at 4000 rpm. The water is discarded, cells are resuspended in 1 ml of 100 mM LiAc, centrifuged for 30 s, and the supernatant is carefully removed.

3. The cells are finally resuspended in 500 μl of 100 mM LiAc. 100 μl of the cell suspension is transferred to a fresh 1.5-ml tube. The reagents are added to the tube in the following order: 240 μl of 50% PEG, 36 μl of 1 M LiAc, 25 μl of SS DNA, and 0.5–1.0 μg of plasmid DNA in 50 μl water. The tube is vortexed for few seconds, incubated at 30°C for 30 min followed by 20 min at 42°C. Cells are centrifuged for 30 s at 7000 rpm in a microcentrifuge, supernatant is removed, and the pellet is resuspended in 500 μl of sterile water. 100 μl is plated on SD medium and incubated at 30°C for 3 days.
3.5. Colony-Lift, Filter Paper β-Galactosidase Assay

This is a quick and sensitive method to screen a large number of mutants within a short time. The filter paper assay is performed using the protocol described in yeast protocol handbook (Clontech).

1. A sterile filter paper (7.5 cm; VWR, USA) is presoaked in 2 ml of buffer Z with X-gal solution in a 95-mm sterile Petri dish.

2. Another dry, sterile filter paper is carefully placed over the surface of the plate of colonies using a forceps and gently rubbed with the sides of the forceps to help the colonies cling to the filter paper.

3. The filter paper is carefully lifted off the plate of colonies using a forceps and dipped in liquid nitrogen for 10–15 s and then allowed to thaw at room temperature for few seconds.

4. The thawed filter paper is then carefully placed, with colony side up, on the presoaked filter paper, incubated at room temperature, and checked periodically for appearance of blue colonies.

5. The yeast colonies with high β-galactosidase activity produce a strong blue color. The stronger blue color colonies most likely contain TF mutants with higher transactivation activity compared to the wild type. These colonies are selected for liquid β-galactosidase assay. The liquid culture assay is carried out to quantify the transactivation activity of the mutant TFs.

3.6. Liquid β-Galactosidase Assay

1. Transformed yeast colonies are grown in 2 ml of SD-Trp medium at 30°C overnight. One ml of overnight culture is transferred to 5 ml of YPDA medium and incubated at 30°C for 3–4 h with shaking (250 rpm/min) until the cells are in mid-log phase (OD600 of 1 ml = 0.5–0.8).

2. In a microcentrifuge, 1.5 ml of the culture is centrifuged at 13,000 rpm for 1 min. The supernatant is carefully removed, cells are resuspended 1 ml buffer Z, centrifuged again at 13,000 rpm for 1 min, and the supernatant is again removed. The cells are finally resuspended in 300 μl buffer Z (the concentration factor is 1.5/0.3 = 5), and 100 μl of which is used for assay.

3. To a fresh 1.5 ml tube, 100 μl of the cell suspension is transferred. The tube is placed in liquid nitrogen for 1 min and then moved to a 37°C water bath for 1 min. The freeze/thaw cycle is repeated for two more times to ensure that the cells are broken open. To this, 700 μl buffer Z plus β-ME and 160 μl of ONPG are added and incubated at 30°C.

4. After the yellow color develops, 400 μl of 1 M sodium carbonate solution is added to stop the reaction. The tubes are
centrifuged at 13,000 rpm for 10 min. The supernatant is carefully removed to a fresh tube and absorbance is measured in a spectrophotometer at 420 nm.

5. The β-gal units are calculated as described in yeast protocol handbook (Clontech):

$$\beta$$-galactosidase units = \(1000 \times \frac{OD_{420}}{t \times V \times OD_{600}}\)

where \(t\) = time of incubation (in min); \(V\) = volume of the cell (0.1 ml) X concentration factor from step 2; and \(OD_{600}\) = absorbance of 1 ml culture at 600 nm. One unit of β-gal activity is defined as the hydrolysis of 1 μmol of ONPG to o-nitrophenol and D-galactose per min per cell.

3.7. Plasmid Isolation from Yeast and Cloning of the Mutant Transcription Factors into a Plant Expression Vector

1. Yeast colonies with higher β-gal activity are grown in 2 ml of SD-Trp medium at 30ºC overnight. Plasmid is isolated from the culture using the Zymoprep yeast plasmid isolation kit.

2. The yeast plasmid containing the mutant transcription factors is digested with appropriate restriction enzymes. Digested products are run on a gel and gel purified using methods described above. Gel purified fragments are ligated to the protoplast expression vector (effector plasmid) digested with same restriction enzymes.

3. The ligated products are transformed into TB1 cells and grown overnight at 37ºC. Overnight minipreps from selected colonies are made and plasmid is digested with appropriate enzymes to check for appropriate clones by running on an agarose gel.

4. The positive clones are chosen and used for protoplast electroporation.

3.8. Isolation of Tobacco Cell Suspension Protoplasts, Electroporation of Plasmids and Luciferase Assay

Tobacco cell suspension cultures (cultivar Xanthi) are maintained by subculturing to fresh medium at 4-day intervals (5 ml of culture is transferred to 50 ml medium). Three-day-old cell culture is used for protoplast isolation (see Note 2). The cells are harvested in a 50-ml tube by centrifugation at 1100 rpm for 4 min in a Sorvall Legend RT Plus. The culture medium is replaced with 30 ml enzyme solution and the cell suspension is transferred to a 250 ml flask, incubated at 26ºC in dark with slow shaking (50 rpm). After 2 h, the cell suspension is transferred to a 50-ml tube, centrifuged at 1100 rpm for 3 min, and enzyme solution is carefully removed (see Note 3). The protoplasts are washed once with 20 ml of MMC solution and then resuspended in 10 ml of MMC solution. Protoplasts are subsequently layered carefully on 25% sucrose solution and centrifuged for 4 min at 1100 rpm. Upon settling, protoplasts form a ring at the interface of sucrose and MMC solution, which is carefully recovered. They are transferred to a fresh tube and resuspended in electroporation buffer.
An aliquot of 750 μl, containing approximately $2 \times 10^6$ protoplasts in an electroporation cuvette (0.4-cm gap; 200 V and 950 μF; Bio-Rad, USA), is electroporated with 5–10 μg of the reporter and effector plasmids. Protoplasts are transferred to a new tube, centrifuged at 1100 rpm for 3 min, and electroporation buffer is carefully removed. The protoplasts are resuspended in 1 ml of culture medium and plated onto a 25-mm plate coated with agarose. After incubation at 26°C for 20–22 h, the protoplasts are harvested to measure luciferase activity.

Luciferase activity in transfected protoplasts is measured using a luciferase assay system (Promega, USA) following manufacturer’s instructions. The protoplasts are harvested in 1.5 ml tubes by centrifugation at 3000 rpm for 3 min in a microcentrifuge and the culture medium is carefully removed. To each tube, 100 μl of 1X lysis buffer is added, vortexed for 30 s to break the cells, microcentrifuged for 2 min at maximum speed, and the supernatant is carefully transferred to a fresh tube. The cell lysate (10–20 μl) is added to luciferase assay reagent (50–100 μl) and the luminescence is measured in a luminometer.

4. Notes

1. Use a freshly prepared enzyme solution for protoplast isolation.
2. A 3-day-old tobacco cell suspension is ideal for getting good protoplasts.
3. Protoplasts are delicate and must be handled carefully. Centrifugation at high speeds will damage the protoplasts.

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References


Isolation of Proteins Binding to Promoter Elements of Alkaloid Metabolism-Related Genes Using Yeast One-Hybrid

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Abstract

Controlled transcription of biosynthetic genes is one major mechanism regulating alkaloid production in plant cells. This regulation of biosynthetic pathways is achieved by specific transcription factors. Sequence-specific DNA-binding proteins interact with the promoter regions of target genes, modulating the rate of initiation of mRNA synthesis by RNA polymerase II. Gene transcription is regulated depending on tissue type and/or in response to internal signals like hormones or external signals such as microbial elicitors or UV light. Promoter elements are identified based on their ability to keep the wild-type response to these signals. Transcription factors involved in biosynthetic regulation can be isolated based on their ability to bind these specific promoter elements using yeast one-hybrid screening. Several transcription factors involved in the regulation of alkaloid metabolism-related genes have been isolated by this method. The aim of this chapter is to describe the yeast one-hybrid system for screening DNA-binding proteins potentially involved in transcriptional regulation.

Key words: Yeast one-hybrid, transcription factor, alkaloid metabolism, DNA-binding protein, promoter element, library screening.

1. Introduction

In plants, transcriptional regulation of alkaloid biosynthetic genes depends on the interaction of trans-acting protein factors with cis-acting DNA elements in a gene promoter. Once one has isolated and characterized a target cis-acting element, the next step would be the fusion of the promoter fragment to the reporter
HIS3 gene and integration of the construction into yeast chromosomal DNA. This yeast strain carrying the reporter HIS3 gene driven by the new artificial promoter of interest, usually composed of multimers of the promoter element and an yeast TATA box region, will then be used to screen a cDNA expression library cloned as a translational fusion with the heterologous GAL4 activation domain (Fig. 5.1). In this screening, yeast cells are selected for the HIS3 reporter gene activation, which is conferred by the binding of the cDNA library protein to the promoter element. This binding brings the GAL4 activation domain close to the HIS3 reporter gene, activating its expression, therefore conferring to the yeast cell the ability to grow on minimal medium without the amino acid histidine. Since the resulting clones encode fusion proteins consisting of the cDNA-encoded protein and the GAL4 activation domain, cDNAs are solely selected for binding of the corresponding proteins to the promoter element of interest. This system has been used successfully to clone several trans-acting regulators from alkaloid metabolism-related genes (1–4).

Fig. 5.1. Schematic representation of the yeast one-hybrid transcription factor screening experiment. Yeast reporter strains carrying multimers of the promoter element fused to the HIS3 gene are cotransformed with the ds-cDNAs and the pGADT7-Rec2 AD vector (Clontech) and selected for transformation and activation of the HIS3 selection gene on medium lacking leucine and histidine. Cloning of the ds-cDNAs takes place in vivo via homologous recombination with the pGADT7-Rec2 vector.
2. Materials

2.1. Complementary DNA (cDNA) Library Construction

1. Total RNA or purified mRNA (poly A+ RNA), store at −70°C.
2. 10 μM oligo(dT) primer (or CDS III primer, 5′-ATT CTA GAG GCC GAG GCG GCC GAC ATG-d(T)30 VN-3′ N = A, G, C, or T; V = A, G, or C, provided by Clontech), store at −70°C.
3. 10 μM SMART III oligonucleotide (5′-AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ATG GCC GGG-3′), provided by Clontech, store at −70°C.
4. 20 U/μL Moloney murine leukemia virus (MMLV) reverse transcriptase (RT, Clontech) or Superscript III reverse transcriptase (Invitrogen), store at −20°C.
5. 2 U/μL RNase H, store at −20°C.
6. 5X first-strand buffer: 250 mM Tris–HCl (pH 8.3), 30 mM MgCl2, 375 mM KCl, store at −20°C.
7. 20 mM dithiothreitol (DTT), store at −20°C.
8. dNTP mix: dATP, dCTP, dGTP, dTTP, 10 mM each, store at −20°C.
9. Autoclaved ultrapure (Mili-Q) water.
10. 10 μM 5′-PCR primer (5′-TTC CAC CCA AGC A GT GGT ATC AAC GCA GAG TGG-3′), store at −70°C.
11. 10 μM 3′-PCR primer (5′-GTA TCG ATG CCC ACC CTC TAG AGG CCG AGG CCG ACA-3′), store at −70°C.
12. 10X PCR buffer for long distance (LD)-PCR (Advantage 2 PCR buffer is provided by Clontech along with 10X GC-melt solution also recommended for this protocol).
13. Thermostable DNA polymerase for LD-PCR (50X Advantage 2 polymerase mix is provided by Clontech and recommended in the present protocol).
14. CHROMA SPIN TE-400 columns (Clontech) or GFX DNA purification kit (GE Healthcare).
15. 3 M NaAc (sodium acetate), pH 4.8.
16. Ethanol, store at −20°C.
17. 500 ng/μL activation-domain (AD) vector: SmaI-linearized pGADT7-Rec2 AD cloning vector (Clontech, 5).
2.2. Integration of Target Promoter Elements into Yeast Genome

1. pIC Vector (6).
2. pHIS3NB or pHIS3NX and pINT1 vectors (7) (Fig. 5.2).
3. Yeast strain Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met-, gal80Δ, MEL1, and URA3::GAL1UAS-GAL1TATA-lacZ, Clontech).
4. Materials for yeast transformation (see Section 2.3).
5. 150 mg/mL G418 solution in water. Filter sterilize and store at −20°C.

2.3. Yeast Transformation

1. YPD medium: 20 g/L Bacto-Peptone (Difco), 10 g/L yeast extract, 20 g/L glucose, adjust pH to 5.8 with HCl. Add 18 g/L agar to prepare plates.
2. YAPD medium: same as YPD medium containing 20 mg/L adenine sulfate.
3. SD medium: 6.7 g/L yeast nitrogen base without amino acids (Difco), 10 mL/L of 100X adenine, uracil or amino acids dropout stocks, 20 g/L glucose, adjust pH to 5.8 with KOH. Add 20 g/L agar to prepare plates.
4. 100X dropout stocks:
   - Adenine sulfate 2 g/L
   - L-Histidine-HCl 2 g/L
   - L-Leucine 3 g/L
   - L-Methionine 2 g/L
   - L-Tryptophan 2 g/L
   - Uracil 2 g/L.
5. Carrier DNA: 10 mg/mL ss-sonicated Salmon Sperm DNA or Herring Testes DNA, alternatively it can be bought from Clontech.
6. 10X LiAc: 1 M lithium acetate, adjusted to pH 7.5 with acetic acid.
7. 10X TE: 100 mM Tris–HCl pH 7.5/10 mM EDTA.
8. Transformation mixture (for 1 mL): 800 μL 50% PEG 4,000, 100 μL 10X LiAc, 100 μL 10X TE, prepare fresh for each transformation event.
9. Autoclaved ultrapure (Mili-Q) water.

2.4. Library Screening

1. Materials for yeast transformation.
2. DMSO.
3. Parental, circularized pGADT7-Rec2 AD cloning vector (Clontech).
Fig. 5.2. Maps of pHIS3 reporter gene cassettes and pINT1 integration vector. (a) The pHIS3NB vector region containing the cloning sites and the HIS3 reporter gene is shown. NotI, XbaI, SpeI, Smal, and EcoRI are unique sites for cloning the promoter element of interest. The HIS3 reporter gene cassette can be transferred as a NotI-BamHI fragment to the NotI-BclI sites of the pINT integration vector. (b) The pHIS3NX vector region containing the cloning sites and the HIS3 reporter gene is shown. NotI, SpeI, BamHI, Smal, and EcoRI are unique sites for cloning the promoter element of interest. The HIS3 reporter gene cassette can be transferred as a NotI-XbaI fragment to the NotI-BclI sites of the pINT integration vector. (c) The pINT1 integration vector. The APT1 gene confers resistance to G418 antibiotic. SacI, Ncol, NotI, XbaI, and BclI are unique sites. pINT1-derived vectors may be linearized at SacI and/or Ncol for homologous recombination at the PDC6 locus.
4. 3-Aminotriazole (3-AT, Sigma). Prepare 1 M solution in water, filter sterilize and store at 4°C.

5. 5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal, Clontech). Prepare X-α-Gal at a concentration of 2 mg/mL in dimethylformamide (DMF). Store in glass or polypropylene bottles at −20°C in the dark.

2.5. Isolation of Plasmid DNA from Positives

1. Acid-washed glass beads (425–600 μm, Sigma).

2. Lyticase lyophilized powder (200 U/mg solid from Sigma). Store at −20°C.

3. Yeast resuspension buffer (YRB): 0.9 M sorbitol, 50 mM EDTA pH 8.0, 4 mg/mL lyticase.

4. Yeast lysis buffer (YLB): 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA.

5. 3 M NaAc pH 5.2.


7. TE.

8. *Escherichia coli* XL-1 competent cells.

9. Materials for *E. coli* plasmid minipreps.

2.6. Analysis of *E. coli* Plasmid DNA

1. Materials for yeast transformation.

2. Original yeast strain (Y187) and control strains containing other DNA fragments fused to the HIS3 gene, if possible.

3. 5′- and 3′-PCR primers for sequencing cDNA inserts (see above).

3. Methods

3.1. Construction of cDNA Libraries

1. One first fundamental step before constructing a cDNA library for yeast one-hybrid screening of putative transcription factors is the preparation of high-quality total RNA (and mRNA) from alkaloid-producing plant cells, tissues, or organs like roots, stems, leaves or flowers (8; see Note 1).

2. Messenger RNA transcripts are then efficiently copied into ds-cDNA using RNA-dependent DNA-polymerases (reverse transcriptases) and an oligo(dT) primer (see Note 2).

3. Combine the following reagents in a sterile 0.25-mL microcentrifuge:

   1–2 μL RNA sample (0.025–1 μg poly A+ or 0.1–2 μg total RNA)

   1 μL CDS III primer (10 μM or an oligo(dT) primer of your preference)
1–2 μL autoclaved ultrapure (Mili-Q) water to bring volume up to 4 μL.

4. Mix contents and spin briefly.
5. Incubate at 72°C for 2 min.
6. Cool on ice for 2 min.
7. Spin briefly and add the following to the reaction tube:
   2 μL 5X first-strand buffer
   1 μL DTT (20 mM)
   1 μL dNTP mix (10 mM)
   1 μL MMLV-RT (20 U/μL).
8. Mix gently by tapping.
10. Incubate at 42°C for 10 min.
11. Add 1 μL SMART III oligonucleotide (10 μM).
12. Incubate at 42°C for 1 h in an air incubator or hot-lid thermal cycler (see Note 3).
13. Place the tube at 75°C for 10 min to terminate first-strand synthesis.
14. Cool the tube to room temperature, then add 1 μL RNase H (2 U/μL).
15. Incubate at 37°C for 20 min.
16. Transfer the reaction tube to ice (see Note 4).
17. Second-strand synthesis followed by long distance-PCR (LD-PCR). To do so, the number of thermal cycles should be optimized based on the amount of RNA used in the first-strand synthesis (Table 5.1). Fewer cycles generally mean fewer nonspecific PCR products.

Table 5.1
Relationship between amount of RNA and optimal number of thermal cycles. The optimal cycling parameters were determined and recommended by Clontech using a control poly A+ human placenta RNA. These parameters may vary with different templates and thermal cyclers.

<table>
<thead>
<tr>
<th>Total RNA (μg)</th>
<th>Poly A+ RNA (μg)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0–2.0</td>
<td>0.5–1.0</td>
<td>15–20</td>
</tr>
<tr>
<td>0.5–1.0</td>
<td>0.25–0.5</td>
<td>20–22</td>
</tr>
<tr>
<td>0.25–0.5</td>
<td>0.125–0.25</td>
<td>22–24</td>
</tr>
<tr>
<td>0.05–0.25</td>
<td>0.025–0.125</td>
<td>24–26</td>
</tr>
</tbody>
</table>
18. Preheat the PCR thermal cycler to 95°C.

19. To prepare sufficient ds-cDNA for transformation, set up two 100-μL PCRs for each experimental sample. Set up one reaction for the control sample. In each reaction tube, combine the following components:

- 2 μL first-strand cDNA
- 70 μL autoclaved ultrapure water
- 10 μL 10X Advantage 2 PCR buffer
- 2 μL dNTP mix (10 mM)
- 2 μL 5′-PCR primer (10 μM)
- 2 μL 3′-PCR primer (10 μM)
- 10 μL 10X GC-melt solution
- 2 μL 50X Advantage 2 polymerase mix.

20. Mix gently by flicking the tube. Centrifuge briefly.

21. Cap the tube and place it in a preheated (95°C) thermal cycler (see Note 3). The following program is recommended when employing a hot-lid PCR thermal cycler: 95°C for 30 s.

- x cycles according to Table 5.1:
  - 95°C 10 s
  - 68°C for 6 min (see Note 5)
  - 68°C for 5 min
  - 4°C until transferring the reaction tube to ice.

22. When PCR is complete, electrophoretically run a 5- to 10-μL aliquot of the PCR product from each sample alongside a DNA size marker on a 1% agarose/EtBr gel. Typical results obtained with grapevine berry poly A+ RNA are shown in Fig. 5.3. Proceed to next step or store ds-cDNA at −20°C until use.

Fig. 5.3. Double-stranded cDNAs synthesized from *Vitis vinifera* (grapevine) berry poly A+ RNA. 1 μL (1.0 μg) of poly A+ RNA was used as the template for first-strand cDNA synthesis with the CDS III primer. Next, 2 μL of the ss-cDNA was amplified by LD-PCR with 15, 18, 21, and 24 cycles as indicated. Lane M was loaded with 500 ng of EcoRI/HindIII-cut lambda DNA size marker.
23. Purify ds-cDNA with a CHROMA SPIN TE-400 column (Clontech, see Note 6). Remove the CHROMA SPIN column from the protective plastic bag and invert it several times to resuspend the gel matrix completely. Use one column for each ~95-μL cDNA sample.

24. Holding the CHROMA SPIN column upright, grasp the break-away end between your thumb and index finger and snap it off.

25. Place the end of the spin column into one of the 2-mL microcentrifuge (collection) tubes and lift off the top cap. Save the top cap and the white-end cap.

26. Centrifuge at 700 × g for 5 min. After centrifugation, the column matrix will appear semidry. This step purges the equilibration buffer from the column and re-establishes the matrix bed.

27. Remove the spin column and collection tube from the centrifuge rotor and discard the collection tube and column equilibration buffer.

28. Place the spin column into the second 2-mL microcentrifuge tube. Carefully and slowly apply your cDNA sample (~95 μL) to the center of the gel bed’s flat surface. Do not allow any sample to flow along the inner wall of the column.

29. Centrifuge at 700 × g for 5 min.

30. Remove the spin column and collection tube from the rotor and detach them from each other. The purified sample is at the bottom of the collection tube.

31. Combine duplicate experimental samples in a single tube.

32. Add the following reagents:
   - one-tenth volume of 3 M NaAc (pH 4.8)
   - 2.5 volume of 95% ethanol (~20°C).

33. Mix gently by rocking the tube back and forth.

34. Place the tube in a −20°C freezer or on a dry-ice/ethanol bath for 1 h. (Optional: You may incubate at −20°C overnight, which may result in better recovery.)

35. Centrifuge the tube at 14,000 rpm for 20 min at room temperature.

36. Carefully remove the supernatant with a pipette. Do not disturb the pellet.

37. Briefly centrifuge the tube to bring all remaining liquid to the bottom.

38. Carefully remove all liquid and allow the pellet to air-dry for ~10 min.
39. Resuspend the pellet in 20 μL of autoclaved ultrapure water and mix gently. The cDNA is now ready for ligation into an activation-domain (AD) vector or in vivo recombination with pGADT7-Rec2 directly in yeast cells (see Note 7). It may alternatively be stored at −20°C.

40. Go to Section 3.4.

3.2. Integration of Target Promoter Elements into the Yeast Genome

1. Tetramerization of the promoter fragment of interest is the first step to prepare the promoter element before integration into the yeast genome (see Note 8). Multimerization of DNA sequences can be achieved using the polylinker sequences of the pIC vector series (6, 9). Clone the isolated promoter fragment of interest as a BamHI/BglII fragment in pIC vector. Excise the monomer fragment with PstI/BglII and reintroduce between the PstI and BamHI sites of the monomer containing plasmid. The resulting plasmid will contain a head-to-tail dimer. Repeat this procedure once with the dimer-containing plasmid.

2. Introduce the tetramerized promoter fragment in the HIS3-containing vector, pHIS3NB or pHIS3NX (7) (Fig. 5.2).

3. Introduce promoter-HIS3 fusion in the yeast integration vector pINT1 (Fig. 5.2, see Note 9).

4. Integrate pINT1 carrying the promoter-HIS3 fusion into chromosomal PDC6 locus of yeast strain Y187 (Clontech) using the G418 antibiotic for selection.

5. Digest 1 μg of pINT derivative with Neol or SacI for single cross-over integrations or both enzymes for double cross-over integrations.

6. Transform yeast strain Y187 (see procedure below) with the digestion mixture and select recombinants on YAPD plates containing 150 μg/mL G418 after 3–4 days.

7. Re-streak colonies on YAPD plates containing 150 μg/mL G418 at least once.

8. Check one to three colonies for integration with Southern hybridization of BamHI-digested chromosomal DNA and the 1 kb PDC6 locus fragment isolated from pINT1 digested with Neol and XbaI as a probe. Use negative (Y187) and, if possible, positive controls.

3.3. Yeast Transformation

1. Make sure all tubes and solutions are sterile and work in flow cabinet to minimize contamination.

2. Grow the yeast strain overnight at 30°C in 10 mL YPD medium.

3. Dilute 10-fold in 50 mL YPD to OD₆₀₀ = 0.2 (~10⁷ cells/mL) and grow to OD₆₀₀ = 0.4; this will take...
approximately 4 h. 50 mL of yeast culture is necessary for 10 transformations with 1 μg of plasmid DNA.

4. Harvest cells by centrifugation for 5 min at 3,000 rpm at room temperature and resuspend cells in sterile water.

5. Pellet cells at 3,000 rpm for 5 min and resuspend in 500 μL 1X TE/1X LiAc.

6. Mix, per transformation:
   1 μg plasmid DNA
   2.5 μL Herring Testes ss-carrier DNA (freshly denatured for optimal efficiency)
   50 μL yeast suspension in 1X TE/1X LiAc.

7. Add 300 μL of transformation mixture (prepared fresh), mix gently by swirling, and shake for 30 min at 30°C.

8. Heat shock for 15 min at 42°C and immediately chill on ice.

9. Pellet cells at 2,500 rpm for 5 min, resuspend in 1 mL YPD, and shake at 30°C for 1 h. Wash once with sterile water.

10. Pellet cells at 2,500 rpm for 5 min and resuspend in 100 μL TE.

11. Plate cells on the appropriate SD medium and incubate at 30°C until colonies appear. This must take 3–5 days.

12. The transformation efficiency of this method should be around 1–5 × 10^4 colonies/μg DNA.

3.4. Library Screening

1. Depending on the promoter fragment fused to the *HIS3* gene, some background growth can occur on SD medium lacking histidine. In this case use 3-AT to suppress background (see Note 10). To determine the amount of 3-AT needed to suppress background growth, library screening conditions are imitated (see Note 11). Therefore, the yeast strain is transformed with the pGADT7-Rec2 and plated at high transformation density.

2. A 3-AT titration is made by plating pGADT7-Rec2 (parental, empty plasmid) transformants at high density on a series of SD plates without histidine and leucine containing increasing amounts of 3-AT at 15, 20, 30, 40, and 50 mM. Control plate contains histidine and no 3-AT.

3. Check after 7–10 days. The optimal 3-AT concentration is the lowest amount that inhibits growth except for very small background colonies (Often no 3-AT is needed).

4. For library screening, cotransform yeast strain Y187 carrying the promoter-*HIS3* fusion with the ds-cDNA and the *SmaI*-linearized pGADT7-Rec2 vector. It will be necessary to use an upscaled standard protocol as follows.
5. Repeat steps 1–5 of the yeast transformation protocol above (Section 3.3) with the yeast strain Y187 carrying the promoter HIS3.

6. In a sterile 15-mL tube, mix the following for transformation:
   - 20 μL ds-cDNA (Section 3.1, step 39)
   - 6 μL pGADT7-Rec2 (0.5 μg/μL)
   - 20 μL Herring Testes ss-carrier DNA, denatured
   - 500 μL yeast competent cells.

7. Add 2.5 mL of transformation mixture (prepared fresh), mix gently by swirling, and shake for 30 min at 30°C.

8. Add 160 μL DMSO, mix, and then place the tube in a 42°C water bath for 20 min and immediately chill on ice.

9. Pellet cells at 2,500 rpm for 5 min, resuspend in 3 mL YPD, and shake at 30°C for 1.5 h. Wash once with sterile water.

10. Pellet cells at 2,500 rpm for 5 min and resuspend in 6-mL TE.

11. Plate 150 μL portions on 150-mm plates with SD−histidine−leucine medium (minimal SD medium plus adenine, methionine, tryptophan, and uracil, but lacking histidine and leucine).

12. To determine the transformation efficiency and to calculate the number of clones screened, spread 100 μL of a 1:10, 1:100, and 1:1,000 dilution onto 100-mm plates with SD-leucine.

13. Grow plates for 10–15 days at 30°C.

14. Screen as many transformants as necessary to get a reasonable representation of the library (see Note 12).

15. Streak putative positive colonies on the same selection medium used for library screening. Keep these plates numbered at 4°C.

16. Re-streak them on the same selection medium containing X-α-gal. (see Note 13). False positives will turn blue.

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3.5. Isolation of Plasmid DNA from Positives

1. Plasmid preparations from yeast do not yield much, so you must prepare plasmid from the yeast positive clone and transform them into E. coli to get a reasonable amount of DNA for analysis.

2. To isolate plasmid from yeast positives, grow them in 5 mL histidine-containing SD medium selective solely for the plasmid (−leucine) for 3 days at 30°C.

3. Harvest cells by centrifugation at 6,000 rpm for 5 min.

4. Resuspend cell pellet in 200 μL of freshly prepared YRB.
5. Shake for 60 min at 30°C and centrifuge cells at 4,000 rpm for 5 min at room temperature.

6. Remove supernatant and resuspend pellet in 200 μL YLB by vortexing.

7. Add 200 μL (a spatula tip) of acid-washed glass beads and 200 μL of phenol:chloroform (1:1) and vortex at maximum speed to disrupt cells.

8. Centrifuge at 13,000 rpm for 10 min at room temperature and transfer the upper phase to a clean 1.5-mL tube.

9. Precipitate plasmid DNA by adding 0.1 volume of 3 M NaAc pH 5.2 and 2.5 volume of ethanol.

10. Centrifuge again at 13,000 rpm for 10 min. Wash the pellet with 70% ethanol and dry it under vacuum.

11. Finally resuspend in 20 μL TE.

12. Use 10 μL of this yeast plasmid preparation to transform E. coli competent cells (see Note 14).

13. After recovering recombinant E. coli colonies, miniprep plasmids to get sequencing quality DNA.

### 3.6. Analysis of E. coli Plasmid DNA

1. Digest plasmids to excise cDNA inserts. Analyze digestions on agarose gels by electrophoresis. You must visualize the same vector band for all positives and different sized insert fragments (see Note 15).

2. Retransform the yeast strain used in the screening (Y187 containing the promoter of interest) with each positive. Use the original yeast strain (Y187) and Y187 strains containing other DNA fragments fused to the HIS3 gene as negative control (see Note 16).

3. Determine sequence of inserts and search databases to detect possible homologies with described DNA-binding proteins.

4. To confirm DNA-protein interaction in vitro, clone the cDNA into an expression vector such as pET (Novagen) or pGEX (GE Healthcare), producing His-tagged or GST fusion proteins, which can be used in electrophoretic mobility shift assays (EMSA, protocols described elsewhere).

### 4. Notes

1. Several manufacturers yield very good reagents and kits for total RNA purification from different plant tissues. In
our laboratories best results were achieved with Trizol and PureLink reagents from Invitrogen and Qiagen Oligotex kits for total RNA and mRNA purification. Macherey-Nagel, GE Healthcare, Promega and Eppendorf have also quite good products and protocols for RNA isolation. We advise testing plant tissues of interest with different products and protocols since the final RNA quality will be strongly affected by the polysaccharide, fiber and extract contents of the original plant tissue.

2. The protocol here described is based on Clontech’s SMART (switching mechanism at 5′-end of RNA transcript) technology (10), which traditionally provided excellent protocols for yeast one- and two-hybrid screenings. The cDNA first-strand synthesis is followed by a PCR amplification that is particularly suited for one-hybrid construction because it consistently delivers high yields of cDNA while maintaining sequence representation. In the first-strand cDNA synthesis step, MMLV-RT is used to transcribe RNA into DNA. To prime RNA for cDNA synthesis in the present protocol, a modified oligo(dT) primer (named CDS III primer) is employed. The CDS III primer hybridizes to the 3′-end of poly A+ RNA and tends to yield full-length cDNA sequences. Other protocols, established by Clontech and other manufacturers, employ general oligo(dT) or random short (hexamer) primers, and results concerning library representation are significantly different.

3. If you use a water bath or non-hot-lid thermal cycler for this incubation, cover the reaction mixture with one drop of mineral oil before you close the tube. This will prevent loss of volume due to evaporation.

4. Any first-strand reaction mixture that is not used right away should be placed at −20°C. First-strand cDNA can be stored at −20°C for up to 3 months.

5. Program the cycler to increase the extension time by 5 s with each successive cycle. For example, in the second cycle, the extension should last 6 min and 5 s; in the third, 6 min and 10 s, and so on. These cycling parameters may not be optimal for non-hot-lid thermal cyclers.

6. We have successfully performed this DNA purification step with the GE Healthcare’s GFX Kit as well, essentially following manufacturer’s instructions. Clontech’s CHROMA SPIN columns are packed with resins that fractionate molecules based on size. Molecules larger than the pore size are excluded from the resin. These molecules quickly
move through the gel bed when the column is centrifuged, while molecules smaller than the pore size are held back. In the present protocol, a CHROMA SPIN TE-400 column is used to select for DNA molecules >200 bp. It is recommended to centrifuge CHROMA SPIN columns in a swinging bucket or horizontal rotor. Fixed-angle rotors can also be used, but there is a risk that a portion of the sample will slide down the inner side of the column instead of passing through the gel matrix, resulting in reduced or inconsistent purification.

7. A series of phage and plasmid vectors containing the GAL4 AD are available for cDNA ligation. Clontech’s vectors pACT, pACT2, pGAD-GH, and pGADT7 were and are popular ones, along with λ.ACT and λ.ACT2 bacteriophage vectors that allow direct plasmid excision/conversion via cre/lox site-specific recombination. Nevertheless the most recent pGADT7-Rec2 vector from Clontech has important advantages for yeast one-hybrid and it is recommended in the present protocol. Vector pGADT7-Rec2 is a low-copy plasmid that contains, besides the autonomous replication sequence (ARS element), a centromeric sequence (CEN element) to ensure stable segregation of the plasmid during yeast mitosis and meiosis. Low-copy plasmids such as pGADT7-Rec2 are preferred for one-hybrid analysis because they generate fewer false positives. A GAL4 AD fusion library with the pGADT7-Rec2 vector is produced by cotransforming yeast with SMART ds-cDNA and SmaI-linearized pGADT7-Rec2. The SMART ds-cDNA will recombine with the AD cloning vector in vivo to yield a complete GAL4 AD expression vector. The resulting construct will express the cDNA insert as a GAL4 AD fusion protein.

8. Alternatively, you may also use monomers or dimers, especially if the promoter element is large. Multimerization of small promoter elements is believed to enhance the chance of interaction with DNA-binding proteins.

9. Reporter gene constructions in pHIS3 vectors (7) can be transferred to the pINT1 integration vector (7) which is designed for integration of the reporter gene construction at the nonessential yeast PDC6 locus.

10. Background growth on SD medium lacking histidine may result from activation of the reporter gene by an endogenous yeast factor. Growth can be inhibited by low concentrations of 3-AT, which is a competitive inhibitor of the yeast HIS3 protein. This does not interfere with the identification of DNA-binding library proteins in a one-hybrid
screening. Nevertheless, you should not discard the possibility of isolating false positives that do not bind DNA, but enhance the activity or abundance of the endogenous yeast factor.

11. The optimal 3-AT concentration is somewhat dependent on plating density.

12. Repeat the library screening protocol as many times as necessary. Usually only about 10% of positive colonies express specific DNA-binding proteins.

13. The Y187 strain contains the MEL1 reporter gene under control of Gal4p binding site. The MEL1 gene encodes α-galactosidase, which is secreted from the yeast cell. Its activity can be easily detected by direct addition of X-α-Gal substrate to the media. Activation of this reporter gene may indicate that the cDNA library clone encodes a nonspecific DNA-binding protein.

14. Use high efficiency competent cells (at least $10^7$ cfu/μg DNA).

15. If a large number of positives has been obtained it is useful to narrow down the number of clones that need to be further analyzed. Identical or overlapping clones can be identified on the basis of restriction and by Southern cross-hybridization.

16. Confirming DNA-binding activity is essential to certify that you are not working with a false positive.

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References


Modulation of Carotenoid Accumulation in Transgenic Potato by Inducing Chromoplast Formation with Enhanced Sink Strength

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Abstract

An increasing interest in carotenoids as nutritional sources of provitamin A and health-promoting compounds has prompted a significant effort in metabolic engineering of carotenoid content and composition in food crops. The strategy commonly used in plants is to increase the biosynthetic capacity by altering the carotenogenic enzyme activities. The recent isolation of the Or gene from a cauliflower orange mutant has brought a new endeavor for carotenoid enhancement by increasing the sink strength to sequester and store the synthesized carotenoids. Potato as one of the major staple crops usually accumulates low levels of carotenoids. In this chapter, we describe a detailed protocol for metabolic engineering of carotenoids in potato plants with the Or gene and the analysis of the Or transformants.

Key words: Cauliflower Or gene, carotenoids, chromoplasts, transgenic potato, sink strength.

1. Introduction

Carotenoids are widely distributed in nature, but can only be de novo synthesized in plants (including algae) as well as in some fungi and photosynthetic bacteria. Carotenoids possess numerous health benefits to humans. They have been implicated in reducing the incidence of certain diseases and are the primary dietary sources of provitamin A (1). Vitamin A deficiency represents a major global health problem. The deficiency affects 140–250 million preschool children worldwide and causes one-quarter to a half million of them to become blind every year with half of them dying within 12 months of losing their sight.
Thus, development of food crops rich in carotenoids could help alleviate vitamin A deficiency and maximize the health benefits of carotenoids to a large number of population in the world. Significant progress has been made in quantitative and qualitative manipulation of carotenoids in food crops (3–5). The strategy commonly employed is to alter the expression of a rate-limiting enzyme or multiple enzymes in the carotenoid biosynthetic pathway in a tissue-specific manner. One of the most successful examples is Golden Rice, which has increased production of β-carotene in rice endosperm from almost nothing to 31 μg/g dry weight, a level adequate to provide the recommended dietary allowance of provitamin A for small children (6).

In 1970s, a natural mutant of cauliflower with high β-carotene accumulation in the curds was found in Canada, and subsequent genetic work showed that this was the result of a single gene (Or for Orange) mutation (7, 8). Later studies revealed that the massive β-carotene accumulation correlated with biogenesis of chromoplasts (9). Rather than directly controlling carotenoid biosynthesis, the Or gene was found to function in triggering the differentiation of proplastids and/or non-colored plastids into chromoplasts (10, 11). Such structures provide deposition sinks to effectively sequester and store large quantities of synthesized carotenoids (12).

Transformation of this cauliflower mutant Or gene into white cauliflower leads to high levels of β-carotene accumulation with the formation of chromoplasts in the transgenic curd cells (10). Remarkably, introduction of the Or gene under a tuber-specific promoter into potato tubers also conferred carotenoid accumulation in newly formed chromoplasts in a heterologous system (11). Thus, manipulation of chromoplast formation with enhanced sink strength proves as being an alternative and complementary approach for carotenoid enrichment in food crops. In this chapter, we provide a detailed, improved protocol on metabolic engineering of carotenoid accumulation with the Or gene in potato tubers.

2. Materials

2.1. Gene, Promoter, and Vectors

1. Cauliflower Or gene (GenBank accession # DQ482460).
2. Potato granule-bound starch synthase (GBSS) promoter (GenBank accession #A23740).
3. pCR2.1 TA cloning vector (K2020-20, Invitrogen) and pBluescript KS(-) vector (Stratagene) for making
Modulation of Carotenoids in Transgenic Potato Tuber

intermediate constructs. Binary vector pBI101 (Clontech) with nptII gene that confers a kanamycin resistance for vector-only control and for constructing the pBI-GBSS-Or transformation construct.

4. PfuUltra DNA polymerase (Stratagene).

2.2. Agrobacterium tumefaciens and Culture Media

1. Strains and selectable markers: the transformation protocol supports a number of variables including the potato lines (see Note 1), Agrobacterium tumefaciens strains (see Note 2), and the selectable markers (see Note 3). The binary plasmids containing plant expression cassettes are electroporated into Agrobacteria.

2. Luria–Bertani (LB) medium: 10 g/L Bacto-tryptone, 5 g/L yeast extract, and 10 g/L NaCl. Autoclave, then cool to 55°C before adding the appropriate selective agent (dependent upon the vector). Store at 4°C.

3. YM medium: 400 mg/L yeast extract, 10 g/L mannitol, 100 mg/L NaCl, and 200 mg/L MgSO₄·7H₂O. Autoclave, then cool to 55°C before adding the appropriate selective agent (dependent upon the vector). Store at 4°C.

4. Selective agent: prepare the appropriate agent according to concentration and solubility (see Note 3). Filter sterilize. Store aliquots at −20°C.

2.3. Tissue Culture and Transformation

1. Benzyladenine (BA): 1 mg/mL stock. Dissolve the powder in a few drops of 1 N HCl and then add deionized water to volume. Filter sterilize the stock and store in 1-mL aliquots in sterile 1.5-mL capacity Eppendorf tubes at 4°C.

2. Zeatin riboside (ZR): 1 mg/mL stock. Dissolve the powder in a few drops of 1 N HCl and then add deionized water to volume. Filter sterilize the stock and store in 1-mL aliquots in sterile 1.5-mL capacity Eppendorf tubes at 4°C.

3. Indole-3-acetic acid (IAA): 1 mg/mL stock. Dissolve the powder in a few drops of 1 N KOH and then add deionized water to volume. Filter sterilize the stock and store in 1-mL aliquots in sterile 1.5-mL capacity Eppendorf tubes at 4°C.

4. Naphthaleneacetic acid (NAA): 1 mg/mL stock. Dissolve the powder in a few drops of 1 N KOH and then add deionized water to volume. Filter sterilize the stock and store in 1-mL aliquots in sterile 1.5-mL capacity Eppendorf tubes at 4°C.

5. Selection agent: as appropriate for the selectable marker found in the gene construct (see Note 3).
6. Carbenicillin: 100 mg/mL stock. Dissolve the powder in deionized water and then filter sterilize. Store as 2.5-mL aliquots at −20°C (see Note 4).

7. CM medium: 4.3 g/L Murashige and Skoog (MS) salts (Caisson Laboratories, Rexburg, ID), 0.4 mg/L thiamine, 0.1 mg/L myo-inositol, 20 g/L sucrose, and 8 g/L agar. Adjust pH to 5.7 with 1 N KOH and HCl.

8. MS liquid medium: 4.3 g/L MS salts, 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.4 mg/L thiamine-HCl, 0.25 mg/L folic acid, 0.05 mg/L D-biotin, and 30 g/L sucrose. Adjust pH to 5.6 with 1 N KOH and HCl. Autoclave and store at room temperature for up to 1 month.

9. CIM medium: 4.3 g/L MS salts, 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, 0.4 mg/L thiamine, 0.25 mg/L folic acid, 0.05 mg/L D-biotin, 100 mg/L myo-inositol, 30 g/L sucrose, 1 mg/L BA, 2 mg/L NAA (added after autoclaving), and 6 g/L agar. Adjust pH to 5.6 with 1 N KOH and HCl. Autoclave, then cool to 55°C before adding NAA. Pour into approximately 40 Petri-dishes (100 × 20 mm) per liter of medium.

10. 3C5ZR selective medium: 4.3 g/L MS salts, 1 mg/L thiamine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, 100 mg/L myo-inositol, 30 g/L sucrose, 0.1 mg/L IAA (added after autoclaving), 3.4 mg/L ZR (added after autoclaving), 500 mg/L carbenicillin (added after autoclaving), appropriate selection agent (added after autoclaving), and 8 g/L agar. Adjust pH to 5.6 with 1 N KOH and HCl. Autoclave, then cool to 55°C before adding zeatin, IAA, carbenicillin, and the appropriate selection agent. Pour into approximately 40 Petri dishes (100 × 20 mm) per liter of medium.

11. CM selective medium: CM medium containing 500 mg/L carbenicillin (added after autoclaving) and the appropriate selection agent (added after autoclaving). Autoclave, then cool to 55°C before adding the carbenicillin and selection agent. Allot approximately 50 mL of medium per Magenta® GA7 box ([7.7 cm (l) × 7.7 cm (w) × 9.7 cm (h)], PhytoTechnology Laboratories).

12. Glass test tubes [2.5 cm (w) × 15 cm (l), VWR, West Chester, PA].

13. Clear plastic test tube caps.

14. Micropore tape [0.5 inch (1.27 cm); 3 M HealthCare, St. Paul, MN).

15. Sterile paper towels or filter paper.
16. Transfer to soil: Jiffy 7s (42 mm, Griffin Greenhouse Supply, Auburn, NY), 3-gallon pots (Griffin Greenhouse Supply) containing Metro Mix 360 (Griffin Greenhouse Supply), trays without holes, trays with holes, and transparent plastic tray covers (Griffin Greenhouse Supply) or clear plastic bags.

2.4. Extraction of DNA from Potato Leaves

1. DNA extraction buffer: 0.1 M Tris–HCl (pH 8.0), 0.05 M EDTA (pH 8.0), 0.5 M NaCl, 1% sodium dodecyl sulfate (SDS), and 1% β-mercaptoethanol (added just prior to use).
2. 5 M potassium acetate.
3. Isopropanol (Sigma).
4. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
5. 70% and 100% ethanol (Pharmco-AAPER).
6. 10 mg/mL RNase A solution. Store in 1-mL aliquots at −20°C.
8. FisherBrand 2.0-mL conical screw cap tube.

2.5. Southern Blot Analysis of Transgenic Potato Plants

1. 20X standard saline citrate (SSC): dissolve 175.3 g NaCl and 88.2 g sodium citrate in dH2O, adjust pH to 7.0. Add deionized water to 1 L.
2. 10% SDS.
4. DECAprime II kit (Ambion).
5. α-32P dCTP (Perkin-Elmer).
6. ULTRAhyb™ ultrasensitive hybridization buffer (Ambion).

2.6. RNA Extraction, qRT-PCR, and Northern Blot Analysis of Transgenic Potato Plants

1. Trizol reagent (Invitrogen) for extraction of total RNA from leaf tissue.
2. RNA extraction buffer for extraction of total RNA from potato tubers: 1.0 M Tris–HCl (pH 9.0), 1 M NaCl, 0.2 M EDTA, 2% CTAB, and 1% β-mercaptoethanol (added immediately prior to extraction). Prepare RNA extraction buffer in DEPC-treated water.
3. Phenol/chloroform (1:1). Prepare by mixing phenol and chloroform (Sigma).
5. 70% (prepared with DEPC-treated water) and 100% ethanol.
6. 4 M LiCl.
7. DEPC-treated water: Add 0.1% (v/v) DEPC (Sigma) to deionized water. Stir for 1 h. Autoclave after overnight treatment.
8. DNase I (Ambion Inc.).
9. Oligo dT(15–18) and SuperScript III reverse transcriptase (Invitrogen).
10. SYBR Green PCR master mix (Applied Biosystems, Foster City, CA).
11. 384-well clear optical reaction plate (Applied Biosystems).
13. 10X MOPS: Add 41.8 g MOPS, 6.8 g sodium acetate, and 20 mL EDTA (0.5 M, pH 8.0) into 1 L deionized H₂O, adjust pH to 7.0. Autoclave and keep in the dark.
14. Formaldehyde (ACS grade, VWR).
15. Formamide (for molecular biology, Sigma).

### 2.7. Analysis of Carotenoid Levels in Transgenic Potato Tubers

1. 80% acetone.
2. Ethyl acetate (HPLC grade, Mallinckrodt).
3. Triethylamine (Reagent grade, Fisher).
4. Acetonitrile (HPLC grade, EMD).
6. HPLC solvent A: 81% acetonitrile, 9% deionized H₂O, 10% ethyl acetate, and 0.1% triethylamine.
7. HPLC solvent B: 80% ethyl acetate, 18% acetonitrile, 2% H₂O, and 0.02% triethylamine.
8. Spherisorb ODS2 C₁₈ reverse phase column (5-μm particle size) (Waters, Milford, MA).
9. Waters HPLC system with a diode array detector (Waters).

### 2.8. Light Microscopic Study of Chromoplasts

1. Plastid isotonic buffer: 0.33 M sorbitol, 0.1 M Tris–HCl (pH 8.0), 5 mM MgCl₂, 10 mM NaCl, and 1X plant protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).
2. Olympus BX60 microscope equipped with a Sony CCD color camera.

### 3. Methods

#### 3.1. Making Or Transformation Construct

The Or construct contains the Or genomic DNA under the control of potato granule-bound starch synthase (GBSS) promoter (see Note 5).
1. Cloning GBSS promoter sequence from potato. Potato DNA is used as template to amplify the GBSS promoter sequence using PfuUltra DNA polymerase with a forward primer (5'-GATCTGACAAATTGCGATTTGG AAG-3') and a reverse primer containing an Neol site (5'-TACCAATGGTATGGTGCTACAAAAGGGGA ATC-3'). The amplified product is cloned into the TA cloning vector pCR2.1 to produce pTA-GBSS and sequenced.

2. Making intermediate construct of pBS-GBSS. pTA-GBSS is digested with HindIII and Neol. GBSS fragment is ligated into pBluescript KS(-) to produce pBS-GBSS.

3. Cloning cauliflower Or gene. The Or genomic DNA starting from the ATG start codon is amplified using PfuUltra DNA polymerase with a forward primer containing an Neol site (5'-TACCATGGGATCTTTGTTGGTAGATCTTG-3') and a reverse primer containing a SacI site (5'-TGAGAGCTCAGACAACCGCTGCGAGAA TG-3'). The 7.5 kb amplified product is cloned into the TA cloning vector pCR2.1 to produce pTA-Or construct and digested with HindIII to check the characteristic digestion pattern (see Note 6).

4. Making intermediate construct of pBS-GBSS-Or. pBS-GBSS and pTA-Or plasmids are digested with Neol and SacI, respectively. The Or fragment is ligated downstream of the GBSS promoter to produce pBS-GBSS-Or.

5. Generating the transformation construct of pBI-GBSS-OR. The GBSS-Or fragment is digested out of pBS-GBSS-Or plasmid with SalI and SacI and cloned into the binary vector pBI101 to generate the final construct of pBI-GBSS-OR for transformation. The nucleotide sequences of cloning sites are confirmed by DNA sequencing.

### 3.2. Potato Transformation

1. In vitro stock plants are maintained in test tubes containing 10 mL of CM medium as a source of internode segments for transformation (see Note 7).

2. Day 1, streak the Agrobacterium strain containing the binary plasmid of interest onto a plate of LB medium containing the appropriate selection agent. Incubate at 28–30°C for 48 h.

3. Day 3, select four well-formed Agrobacterium colonies to initiate a 50 mL culture in YM selective medium. Maintain at 28–30°C in a shaking incubator at 250 rpm.

4. Day 4, check the OD_{600} of the liquid culture. The OD_{600} needs to be at 0.4–0.6. Should the culture overgrow an
OD$_{600}$ of 0.6, dilute the culture with YM medium until the reading is below 0.5, and then grow about 1 h before rechecking the OD$_{600}$. Centrifuge the culture at 8,000 rpm for 10 min at room temperature before re-suspending the pellet in 50 mL MS liquid medium.

5. For each experiment or gene construct, cut approximately 100 stem internode segments of 0.5–1 cm in length from 6-week-old in vitro stock plants. Place internode segments on CIM medium plate during cutting.

6. Incubate approximately 30 internode segments in 50 mL of prepared *Agrobacterium* inoculum for 10 min, agitating occasionally (see Note 8).

7. Pipette off the *Agrobacterium* inoculum and transfer the internode segments to sterile filter paper or paper towels to remove excess inoculum.

8. Place infected internode segments back onto CIM medium plate and incubate at 19°C in the dark for 48 h (see Note 9).

9. Day 6, transfer to 3C5ZR selective medium containing 500 mg/L carbenicillin in Petri dishes (see Note 4). Seal the plates with micropore tape and incubate at 24°C ± 1°C under 16 h light/8 h dark at 45 μmol photons/m$^2$s. Transfer weekly for 1 month to fresh 3C5ZR selective medium. After 1 month, transfer every 10–14 days.

10. After approximately 8 weeks (depends upon the potato cultivar), excise regenerated shoots (1.5 cm long) and transfer into CM selective medium in Magenta® GA7 boxes (see Note 10). Cut only one transformant from each end of a stem segment (see Note 11). Culture five shoots per box.

11. After 1 month, propagate only the well-rooted plantlets by cutting a nodal segment (section containing an internode) and transferring again to CM selective medium in Magenta® GA7 boxes (see Note 12).

12. Use PCR, Southern blot or technique of choice to verify that the rooted plants contain the transgene. Confirmed transformants should be maintained on CM medium in test tubes and propagated by nodal segments every 6–8 weeks.

13. If needed, well-rooted 3- to 4-week-old in vitro plants can be transferred into soil. At least 2 h before transferring, place a tray with holes inside a tray without holes, then place the number of Jiffy 7s needed in the tray. Fill the tray with water and allow the Jiffy 7s to absorb the water. When the Jiffy 7s are completely expanded and water soaked, they are ready to use.
14. Drain the excess water from the tray and orient the Jiffy 7s, so that the holes face upward. Enlarge the hole slightly at the top of Jiffy 7 to make it easier to place the plant.

15. Remove a plant from a test tube. Be careful to not break off the roots (see Note 13). Wash all medium from the roots in tepid water by gently rubbing the roots (see Note 14).

16. Transfer the plant to a Jiffy 7. Make certain that all the roots are covered. Transfer the plant back to the tray with a label. Continue until all the plants are transferred. Cover with plastic dome (see Note 15).

17. Add water to a depth of approximately 1.5 cm. Cover with transparent plastic dome.

18. Place in a shaded area of a greenhouse (see Note 16).

19. Plants are acclimated over a 2-week period in trays covered with transparent, plastic domes. They remain completely covered for the first week and then the lids are gradually lifted over the course of the second week and completely removed at the end of that week. The tray of plants is removed from the tray without holes, and the plants are watered as needed. When the roots extend to approximately 1–2 cm beyond the Jiffy 7s, the plants are transferred to 3-gallon pots containing Metro Mix 360 and grown at 20–22°C in a greenhouse.

20. Potato tubers are harvested in approximately 3 months. The Or transgenic tubers show orange-yellow flesh color (Fig. 6.1).

Fig. 6.1. Cross sections of potato tubers. The flesh color is associated with carotenoid accumulation. WT, wild type; VC, vector-only control; L29, L55, and L88, individual Or transgenic lines.

3.3. Extraction of Genomic DNA from Transgenic Potato Plants

Many protocols for plant genomic DNA extraction are available. The procedure described here is a protocol based on the method of Dellaporta et al. (13) and produces good-quality DNA for most of the downstream applications. A larger quantity of DNA can be obtained by proportional increase of starting plant materials and volumes of reagents.
1. Harvest young potato leaves (~100 mg) into a 2.0-mL conical screw cap tube. Freeze in liquid nitrogen.

2. Quickly include one ceramic sphere (Qbiogene) per tube and crush leaves into powder for 10 s in a FastPrep instrument (see Note 17).

3. Remove ceramic sphere and add 750 μL of the DNA extraction buffer.

4. Incubate at 65°C for 10 min.

5. Add 150 μL of 5 M potassium acetate, vortex to mix, and incubate on ice for 15 min.

6. Centrifuge at 12,000 rpm for 10 min.

7. Label new set of microcentrifuge tubes and add 850 μL of isopropanol to each tube.

8. Transfer 850 μL supernatant to each new tube, mix well, and centrifuge at 12,000 rpm for 10 min to pellet DNA.

9. Pour off supernatant and add 1 mL 70% ethanol to each tube.

10. Mix and centrifuge at 12,000 rpm for 5 min.

11. Pour off the supernatant and air-dry the DNA samples on the bench for about 30 min (see Note 18).

12. Dissolve DNA samples in 40 μL TE buffer containing RNase A (20 μg/mL).

13. Incubate at 37°C for 30 min to digest RNA.

14. Use 2 μL DNA sample to measure DNA concentration at OD260.

3.4. Analysis of Or Transgenic Potato Plants by PCR and Southern Blot Analysis

3.4.1. PCR Analysis

Positive transformants are identified by PCR amplification of the Or transgene using primers of BoOrF1 (5'-ATCTC GAGGGATCAAGGAAGGGG-3') and BoOrR1 (5'-CGACG AAAACAGATCTTTCTCGTGC-3') as well as of the selective marker, nptII gene, using primers nptIIIF1 (5'-GGGTGGAGAGGCT ATT-3') and nptIIR1 (5'-GAAGGCGATAGAAGGGCG-3').

1. Digest 10 μg of genomic DNA with a proper restriction enzyme in a 50 μL reaction mixture at 37°C overnight.

2. Separate the digested DNA on a 0.8% agarose gel and transfer DNA onto a Hybond-N+ membrane following the manufacturer’s instruction.

3. Radiolabel DNA probe with α-32P dCTP using DECAprime II kit according to the manufacturer’s instruction (see Note 19).
4. Prehybridize the membrane in ULTRAhyb™ ultrasensitive hybridization buffer at 42°C for 1 h and then hybridize overnight after adding the radiolabeled probes.

5. Wash the membrane twice with 2X SSC plus 0.5% SDS at 42°C for 20 min and then twice with 0.2X SSC plus 0.5% SDS at 42°C for 20 min.

6. Detect the signals by autoradiography or PhosphorImager.

The Or transgene is driven by the tuber-specific promoter GBSS. It is essential to examine Or expression in transgenic tubers. Since potato tubers are starch and phenolics-rich storage tissues, it is difficult to extract RNA without contamination of polysaccharides and other phenolic compounds. Here we describe a modified phenol/chloroform/LiCl method to extract good-quality total RNA from potato tubers (14) (see Note 20).

1. Homogenize 2 g of potato tuber in liquid nitrogen, add 5 mL of the RNA extraction buffer, and grind for a few more minutes.

2. Transfer the homogenate into 50-mL centrifuge tube. Incubate at 65°C for 15 min.

3. Add 5 mL phenol/chloroform (1:1) and vortex for 30 s. Centrifuge for 10 min at 12,000 rpm.

4. Transfer the upper phase into a new tube and add equal amounts of chloroform: isoamyl alcohol (24:1). Vortex to mix.

5. Centrifuge at 12,000 rpm for 10 min.

6. Repeat steps 4 and 5.

7. Transfer the upper phase into a new tube, add 3 mL isopropanol, and incubate at room temperature for 10 min.

8. Centrifuge at 12,000 rpm for 15 min at 4°C to precipitate RNA.

9. Rinse the pellet with 3 mL of 70% ethanol, and air-dry at room temperature.

10. Re-suspend the pellet in 1 mL of DEPC-treated H$_2$O.

11. Add an equal volume of 4 M LiCl and incubate on ice over 3 h.

12. Recover RNA by centrifugation at 12,000 rpm for 15 min at 4°C (see Note 21).

13. Rinse the RNA pellet with 1 mL of 70% EtOH, air-dry, and dissolve in 100–200 μL of DEPC-treated H$_2$O.

14. Measure RNA concentration at OD$_{260}$ and determine RNA quality using the ratio of OD$_{260}$ over OD$_{280}$ (see Note 22).
3.5.2. qRT-PCR Analysis

1. Total RNA (5 μg) is treated with DNase I and reverse transcribed using oligo dT\(_{15-18}\) as primer and SuperScript III reverse transcriptase to generate the first-strand cDNA following the manufacturer’s instruction (Invitrogen).

2. Dilute the synthesized cDNA to 100 μL.

3. Normalize cDNA concentration in different samples based on the amplification of Actin using primers: actinF (5'-GCTTCCGGATGGTCAAGTCA-3') and actinR (5'-GGATTCAGCTGCTTCCATC-3').

4. Add 2 μL cDNA, 5 μL SYBR Green PCR master mix, 0.5 μL each of 10 μM gene-specific primers, and H2O to total 10 μL of reaction volume in a 384-well qRT-PCR plate.

5. Reaction mixtures are amplified in an Applied Biosystems 7900HT fast real-time PCR system. Thermal cycling conditions consist of a first step of denaturation at 95°C for 10 min, followed by 40 cycles of denaturation for 15 s at 95°C, and annealing/extension for 1 min at 60°C.

6. Relative expression levels were calculated using the \(\Delta\Delta C_T\) method (http://www.appliedbiosystems.com) as described by Lyi et al.(15).

3.5.3. Northern Blot Analysis

1. Total RNA (10 μg) is separated on a 1.2% agarose gel containing formaldehyde and transferred onto a Hybond-N+ membrane.

2. Preparation of probe, hybridization, washing, and signal detection are conducted as described above for Southern blot analysis. Figure 6.2 shows the expression of Or transgene in transgenic potato tubers (see Note 23).

3.6. Analysis of Carotenoid Content in Transgenic Potato Tubers by HPLC

1. Fine grind 500 mg of potato tubers in 800 μL 80% acetone with a mortar and pestle (see Note 24). Transfer homogenate into a 2.2-mL capacity centrifuge tube (see Note 25).

2. Add 500 μL ethyl acetate and vigorously vortex for 30 s to extract carotenoids.
3. Partition the extract by adding 400 μL water and vortex again.
4. Centrifuge at 12,000 rpm for 5 min.
5. Carefully transfer the upper phase into a new tube and dry under stream of nitrogen gas.
6. Re-suspend the dried samples in 200 μL ethyl acetate (see Note 26).
7. Centrifuge at 12,000 rpm for 10 min and transfer 100 μL sample into a sample vial for HPLC analysis.
8. Sample (40 μL) is injected automatically and separated on a Spherisorb ODS2 C₁₈ reverse phase column using a Waters HPLC system with a diode array detector.
9. Pigments are separated by a linear gradient between solvents A and B over 35 min at a flow rate of 1.0 mL/min. Elution program is 0 min at 100% A and 0% B; 1–25 min with a linear gradient to 0% A and 100% B; 25–30 min at 0% A and 100% B; and 30–35 min at 100% A and 0% B. The detector records absorbance spectra from 280 to 530 nm.

3.7. Light Microscopic Analysis of Chromoplasts

While it is possible to observe chromoplasts from freshly prepared hand sections of cauliflower orange mutant tissues with a light microscope (9), it is difficult to observe them directly from hand sections of fresh transgenic potato tubers due to the presence of a large number of amyloplasts. Therefore, fresh tissue (see Note 27) of potato tubers is gently homogenized first to remove large amyloplasts.

1. Fresh tissue of transgenic potato tubers is cut with a razor blade into small pieces and gently homogenized in a plastid isotonic buffer.
2. Centrifuge at 100 g for 2 min.
3. Upper suspension is dropped on a microscope slide under a cover slip and examined with an Olympus BX60 microscope equipped with a Sony CCD color camera. Images are recorded.
4. Large numbers of carotenoid-sequestering structures from broken chromoplasts can be observed at the edge of the cover slip.

4. Notes

1. Potato lines: Desiree, Russet Burbank, Yema de Huevo, and Shepody.
2. Two Agrobacterium strains we have used are LBA4404 and EHA105. We do not see any significant difference in the
number of transgenic plants recovered using these bacteria, but each has advantages and disadvantages. The strain LBA4404 is our preferred strain in spite that it is \textit{recA} positive, and constructs must be checked regularly for recombination events. The EHA105 strain is \textit{recA} negative and more virulent than LBA4404. As a result, it is often difficult to eradicate. This can lead to overgrowth of the bacteria on the explants and kill the explants.

3. Selective agents used include kanamycin (PhytoTechnology Laboratories) and bialaphos (PhytoTechnology Laboratories). The optimal concentration for each selective agent is dependent on the potato line. We have used kanamycin at 50 and 75 mg/L and bialaphos at 2 and 3 mg/L. No significant difference has been seen in transformation efficiencies between these selection agents; however, regeneration of putative transformants is slower on bialaphos than on kanamycin.

4. Substitute 300 mg/L timentin (PhytoTechnology Laboratories) if carbenicillin cannot be acquired.

5. Other tuber-specific promoters can also be used such as patatin B33 promoter.

6. Digestion of the \textit{Or} genomic DNA in a plasmid from ATG start codon to \textit{Sac}I site with \textit{Hind}III generates DNA fragments of 1.8, 1.4, 1.0, 0.9, 0.7, and 0.3 kb, and the size of vector.

7. We have found that it is important to use test tubes with clear caps for maintenance of individual potato plants as a source of internode segments for transformation. Individual plants maintained in test tubes are more vigorous than multiple plants that are maintained in Magenta® GA7 boxes, which produce plants with thin stems. We have noticed that internode segments from plants with the thickest stems result in the highest transformation efficiency (50–60%).

8. A longer period of incubation may cause water soaking damage to cells.

9. Lower co-cultivation temperatures have been shown to increase the transformation efficiency for some crops (16, 17). This has not been reported for potato; however, we have seen increased recovery of transformed lines at low temperature following expression of a gene that exhibits a negative effect on transformation efficiency.

10. We have found that the transformation efficiency (percent of infected stem segments that give rise to transformants) varies with different cultivars, selectable markers, and introduced genes. Expression of some transgenes decreases the
number of transformed lines recovered. A vector-only con-
struct (no gene of interest, only the vector with a selectable
marker) should be included as a transformation control to
determine if the introduced gene has such an effect.

11. We do not take multiple transformants from each end of a
stem segment because these could be sister clones instead
of independent transformation events. After harvesting a
shoot, we then cut off the end of the stem segment
to prevent regeneration of shoots from that end, which
helps to keep track of the regions where shoots have been
taken.

12. When selecting shoots to propagate to a second round of
selective rooting medium, be certain that plantlets have
roots growing from the cut region of the stem in the
medium but not growing from an area of a stem above
the medium. We have found that plants with roots coming
from the stem above the medium are escapes (not transfor-
mants).

13. We gently tap the bottom of the test tube to dislodge the
medium. To remove the plant, we grasp the bottom with
forceps and slowly pull the plant from the test tube.

14. Washing the medium away from the roots reduces the
chance of adverse bacterial and fungal growth that may kill
the plantlet once it is placed in soil.

15. It is important to cover the plants immediately after transfer
to Jiffy 7 to prevent wilting.

16. If new transfers are placed in direct sunlight, heat will build
up under the cover and kill the plants.

17. Work fast to make sure leaf material remains frozen during
the crushing process. Return sample tubes back to liquid
nitrogen container right after taking them out from Fast-
Prep instrument.

18. To reduce drying time, liquid remaining in the centrifuge
tube can be removed gently with a micropipette.

19. The fragment corresponding to 265–904 nt of cauliflower
Or mRNA (DQ482459) is amplified by PCR with
the primers 5′-CGACGAAACAAGATCTTCTTG and
5′-GGAATCAAAAGGAAGGA. PCR product is purified
with QIAquick PCR purification kit (QIAGEN) and then
100 ng DNA is used for probe labeling.

20. A number of other RNA isolation methods can also be fol-
lowed to extract good-quality RNA from potato tubers,
such as a method by Kumar et al. (18) and the RNeasy
plant mini kit (QIAGEN). Total RNA from leaves is
extracted using Trizol reagent.
21. Steps 10–12 can be repeated to obtain high-quality RNA.

22. The ratio of OD$_{260}$ over OD$_{280}$ should be from 1.8 to 2.0, which indicates good-quality RNA.

23. Equal loading can be verified by ethidium bromide-staining rRNA or by hybridization to an Actin probe.

24. Extraction should be performed under dim light to minimize degradation and isomerization of carotenoids.

25. It is very important to grind the tissue well in order to fully extract pigments. We usually first grind 500 mg of potato tubers with 500 μL 80% acetone, transfer the homogenate into a 2.2-mL centrifuge tube, and then use another 500 μL 80% acetone to rinse the mortar to ensure full transfer of the sample into a tube. The final extract volume should be around 800 μL due to evaporation of acetone during homogenization.

26. If saponification is needed, the dried sample is re-suspended in 200 μL 10% KOH in MeOH and saponified overnight at 4°C. Re-extract the sample with equal volume of ethyl acetate and then dry it down under a stream of nitrogen gas.

27. It is easier to observe intact chromoplasts from fresh tissues with a light microscope. Although long-term storage results in increased levels of total carotenoids (11), most of the chromoplasts are not intact.

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References


Over-expression of Rate-Limiting Enzymes to Improve Alkaloid Productivity

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Abstract

Benzylisoquinoline alkaloids are one of the most important groups of secondary metabolites and include the economically important analgesic morphine and the antimicrobial agent berberine. To improve the productivity of these alkaloids, we investigated the effects of putative rate-limiting step enzymes in alkaloid biosynthesis. We constructed several over-expression vectors for biosynthetic enzymes and introduced them into cultured California poppy, a model isoquinoline alkaloid-producing plant. HPLC/LC–MS analysis of transgenic cells revealed that these enzymes varied in their ability to increase alkaloid production. We describe the use of a rate-limiting step gene to improve alkaloid productivity.

Key words: Agrobacterium, alkaloid, co-suppression, metabolic engineering, rate-limiting enzyme, transgenic plant.

1. Introduction

Higher plants produce a wide range of chemicals, which have been used as dyes, fragrances, and pharmaceuticals. Despite their economic importance, these useful secondary metabolites are still extracted from harvested plants, mainly in the wild (1). However, the productivity of natural harvests is not sufficient and often unstable due to environmental factors, such as climate change, pathogen attack. To satisfy the demand for these compounds, alternative production systems, such as cell culture systems where cells can be grown under controlled and/or optimized conditions, have been studied (2). However, only a few selected plant
cells can produce sufficient quantities of metabolites for industrial application. We report here our attempts to improve the production of useful benzylisoquinoline alkaloids in California poppy (*Eschscholzia californica*), a model isoquinoline alkaloid-producing plant, using molecular engineering techniques.

Benzylisoquinoline alkaloids include many important pharmaceuticals, such as the analgesics morphine and codeine (*Papaver somniferum*) and the antibacterial agents sanguinarine (*P. somniferum, E. californica*) and berberine (*Coptis japonica*). While high berberine-producing cultured *C. japonica* cells have been selected (3), this productivity is still not sufficient for industrial application. In the case of other plant species, the productivity is much lower, and thus a considerable improvement of productivity is needed. While the general enhancement of biosynthetic pathways using master regulator genes is a promising approach for improving productivity, the availability of regulatory factors involved in comprehensive gene regulation in secondary metabolism is very limited (4, also see chapter 3 in this book). Thus, the ectopic expression of biosynthetic enzymes has been empirically examined to overcome the rate-limiting step to achieve increased productivity. Isoquinoline alkaloid biosynthesis serves as a good model for such investigations because it offers several advantages; different classes of benzylisoquinoline alkaloids, such as morphinan, protoberberine, and benzophenanthridine, share the same early steps in their biosynthetic pathways and many of those biosynthetic genes have been isolated. It is well established that dopamine and 4-hydroxyphenyl acetoaldehyde are condensed by norcoclarine synthase (NCS), *O*-methylated by norcoclarine 6-*O*-methyltransferase (6OMT), *N*-methylated by coclarine *N*-methyltransferase (CNMT), hydroxylated by CYP80B, and *O*-methylated by 3′-*O*-hydroxy *N*-methylcoclarine 4′-*O*-methyltransferase (4′OMT), to produce the central intermediate (S)-reticuline in isoquinoline biosynthesis (Fig. 7.1). (S)-Reticuline is further converted to protoberberine alkaloids (e.g., berberine), or morphinan alkaloids (e.g., morphine), and benzophenanthridine alkaloids (e.g., sanguinarine) (5, references therein). Since the early biosynthetic pathway is a common pathway for benzylisoquinoline alkaloid biosynthesis and all of the genes (cDNAs) mentioned above in reticuline biosynthesis have been isolated, use of the ectopic expression of biosynthetic genes to improve alkaloid productivity has been intensively studied (6). Here, we describe the ectopic expression of biosynthetic enzymes in California poppy (e.g., endogenous methylene-dioxy bridge-forming enzyme genes such as stylopine synthase (CYP719A2/A3) and cheilanthifoline synthase (CYP719A5) in benzophenanthridine-type isoquinoline alkaloid biosynthesis (7, 8). The effects of homologous gene-silencing due to the high expression of endogenous genes are also discussed.
Fig. 7.1. Isoquinoline alkaloid biosynthetic pathway in California poppy. Biosynthetic pathway from tyrosine to reticuline is a common pathway in isoquinoline alkaloid biosynthesis and cDNAs of biosynthetic enzymes for N-methylcoclaurine 3′-hydroxylase (CYP80B1), 3′-OH N-methylcoclaurine 4′-O-methyltransferase (4′OMT), berberine bridge enzyme (BBE), cheilanthifoline synthase (CYP719A5), and stylopine synthase (CYP719A2/A3) have been cloned from California poppy.

2. Materials

1. Expression plasmids: pBHE vector (9) or its equivalent has been used for the over-expression of enzymes in plants. However, Gateway-based binary vectors (e.g., pGBW2 vectors; gift from Dr. Nakagawa, Shimane University) have recently become more popular due to the ease of cloning (10–12) (see Note 1).

2. Gateway cloning system (Invitrogen): pENTR/D-TOPO cloning kit (Invitrogen) (see Note 1).

3. Bacterial strains: *Agrobacterium tumefaciens* strain LBA4404 is commonly used for plant transformation. *Escherichia coli* DH5α and TOP10 are used for the general construction of expression vector and Gateway cloning, respectively (see Note 2).

4. Plant materials: *E. californica* seeds (see Note 3).

5. Bacterial culture medium: e.g., 25 g of Luria–Bertani (LB) broth (Difco) per liter. As a substitute, 10 g of Bacto Tryptone (Difco), 5 g of yeast extract (Difco), and 5 g of NaCl.
per liter can be used. If needed, the pH can be adjusted to 7.0 with NaOH. Autoclave for 15 min at 121°C.

6. Plant germination medium: half-strength Murashige–Skoog basal medium (e.g., Sigma-Aldrich) containing 3% (w/v) sucrose. As a substitute, 1.65 g NH₄NO₃, 1.9 g KNO₃, 0.44 g CaCl₂ 2H₂O, 0.37 g MgSO₄ 7H₂O, 0.17 g KH₂PO₄, 37.3 mg Na₂-EDTA, 27.8 mg FeSO₄ 7H₂O, 6.2 mg H₃BO₃, 22.3 mg MnSO₄ 4H₂O, 8.6 mg ZnSO₄ 7H₂O, 0.83 mg KI, 0.25 mg Na₂MoO₄ 2H₂O, 0.025 mg CuSO₄ 5H₂O, and 0.025 mg CoCl₂ 6H₂O per liter can be used.

7. Co-culture medium: Murashige–Skoog basal medium containing 3% sucrose 10 μM, α-naphthalene acetic acid, 1 μM benzylaminopurine, 100 μM acetylsyringone, pH 5.2. Autoclave the medium without acetylsyringone for 15 min at 121°C. Acetylsyringone (196.2 mg dissolved in 1 ml DMSO; 1 M) is added after autoclaving.

8. Plant transformant selection medium: Murashige–Skoog basal medium containing 3% sucrose, 10 μM α-naphthalene acetic acid, 1 μM benzylaminopurine, 20 μg/ml hygromycin, and 200μg/ml cefotaxime, pH 5.7. Autoclave the medium without antibiotics for 15 min at 121°C, and then add filter sterilized antibiotics (e.g., hygromycin, kanamycin, or cefotaxime) (see Note 4).

9. Sterilization agents: 1% (w/v) benzalkonium chloride solution, 70% ethanol, and 1% sodium hypochlorite solution (effective Cl₂ concentration of 1%) (see Note 5).

10. DNA extraction reagents (from gel): Wizard SV gel and PCR clean-up system (Promega) (see Note 6).

11. Plasmid preparation regents: Wizard Plus SV minipreps DNA purification system (Promega) (see Note 6).

12. DNA amplification by PCR: PCR machine (e.g., TAKARA PCR machine), PCR quality gene-specific primers, DNA polymerase (e.g., KOD plus DNA polymerase (TOYO-BO)) (see Note 6).

13. DNA extraction reagents from plant: 2X CTAB buffer (100 mM Tris–HCl, 20 mM EDTA, 1.4 M NaCl, pH 8.0), TE (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA).

14. RNA extraction and characterization reagents: RNeasy plant mini kit (Qiagen), DNase I amplification grade (Invitrogen), SuperScript™ III reverse transcriptase (Invitrogen) (see Note 6).

15. Transformation of bacterium: electroporation machine (e.g., Gene Pulser™ system (Bio-Rad)) and cuvette.
3. Methods

3.1. Construction of Plant Expression Vector

Most popular plant expression vectors consist of cauliflower mosaic virus 35S (CaMV35S) promoter with a duplicated enhancer (El2), while a more cell-specific or developmental stage-specific promoter might be used for specific regulation. In common binary vectors, such as pBHE vector (9), expression vectors are constructed by a conventional method; target genes are cloned using restriction enzymes and ligation. Recently, however, Gateway-based vector systems such as pGWB2 vector have become more popular for use in constructing the expression vector. Gateway cloning systems which use a pENTR/D-TOPO cloning kit are more convenient and efficient for construction (10–12). In this chapter, the expression vector for alkaloid biosynthetic genes is constructed using pGWB2 vector (Fig. 7.2).

1. Full-length cDNA of an alkaloid biosynthetic gene is amplified from template plasmid or cDNA prepared by PCR using KOD plus DNA polymerase with the 5′-sense (Fw) primer, which contains a TOPO cloning site (CACC) (see Note 7). The 5′-sense primer usually contains the initiation codon and the 3′-antisense (Rv) primer contains the stop codon (e.g., CYP719A2 Fw:5′-CACCATGGAGGAGATGAAGATCCTTAG-3′, CYP719A2Rv: 5′-TTAATTACGACGGATATTGAGC-3′, CYP719A3 Fw:5′-CACCATGGAGGAGATGAAGATCCTTAG-3′, CYP719A3 Rv:5′-TTAATTACGACGGATATTGAGC-3′). The PCR product is analyzed in 1% agarose gel and the DNA fragment with the desired molecular size is then recovered and purified using Wizard SV gel and a PCR clean-up system.

2. The PCR product is integrated into TOPO vector using a pENTR/D-TOP cloning kit by incubation at room temperature for 5 min according to the manufacturer’s manual.

3. The resulting entry vector is introduced into E. coli DH5α using the heat-shock method (see Note 1). Transforms are confirmed with colony PCR with forward M13 primer GTAAAACGACGGCCAGT and a reverse primer designed for the cDNA insert.
Fig. 7.2. Construction of over-expression vector using the Gateway system. (1) Ligate a linear fragment of pENTR plasmid with a GTGG extrusion end and the PCR product of target full-length cDNA (ORF) with CACC at the 5’-end of the sense strand to produce an ENTRY cassette (attL1-ORF-att2). (2) Construct expression vector (35S-attB1-ORF-attB2) by the LR clonase reaction of the pGWB2 plasmid and ENTRY cassette.

(e.g., CYP719A2Rv, CYP719A3Rv, and CYP719A5Rv). Several bacterial colonies are picked up with toothpick to make master plate on LB medium, and each colony is suspended in a tube containing PCR reaction mixture. A typical reaction cycle is as follows: denaturation at 98°C for 5 min (denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min) × 30 cycles and then final extension at 72°C for 4 min.
5. A single colony with the desired construct is picked up from the master plate, which is grown at 37°C for 16 h, inoculated in 3 ml LB liquid medium, and incubated at 200 rpm, 37°C for 16 h. The entry plasmid is recovered with a Wizard Plus SV minipreps DNA purification system.

6. For construction of a plant expression vector, the binary vector pGWB2 is made linear with XhoI before recombination with the Gateway cloning system. After 1 h of incubation at 37°C, XhoI is inactivated at 65°C for 20 min.

7. A linear fragment of pGWB2 is mixed with TOPO entry vector and incubated with LR clonase at 25°C for 1 h, and LR clonase is then inactivated by incubation with proteinase K at 37°C for 10 min.

8. The resulting expression vector is introduced into *E. coli* (TOP10) by heat-shock treatment and cultured on an LB plate (see Note 1). Transformed *E. coli* is confirmed by colony PCR, and a single colony is selected and cultured in LB liquid medium containing 50 μg/ml each of kanamycin and hygromycin at 200 rpm, 37°C for 16 h.

9. Expression plasmid is extracted with a Wizard Plus SV minipreps DNA purification system and dissolved in sterilized water.

10. Transformation of *A. tumefaciens* LBA4404 is achieved with electroporation (13) (see Note 1). Competent *A. tumefaciens* cells (100 μl) frozen at −80°C are thawed on ice, and the expression plasmid (ca. 0.5 μg/1 μl) is then added and transformed using a Gene Pulser™ system at 1.8 V, 25 μF.

11. After electroporation, ice-cold LB medium (800 μl) is immediately added, and the mixture is incubated at 25°C for 30 min.

12. Transformed *Agrobacterium* is selected on LB agar medium containing 50 μg/ml kanamycin at 25°C for 2 days in the dark.

13. Transformant (*Agrobacterium* with expression vector) is confirmed using colony PCR as described above.

14. 5 ml of LB liquid medium (containing 50 μg/ml kanamycin) is inoculated with single colonies of *Agrobacterium* cells with expression plasmid and cultured at 200 rpm, 25°C for 2 days in the dark for plant transformation.

### 3.2. Transformation of Plant Cells

The generation of stable transformant with over-expression vector is a powerful tool for determining the rate-limiting step in metabolite biosynthesis and for creating novel metabolite-
producing plants. For the creation of novel metabolite-producing plant/cells, we can use different strategies: introduction of a novel branch in the pathway, shutting down the pathway to create/activate a new branch, or their combination. Over-expression of the rate-limiting step is used to increase productivity rather than to improve quality. While metabolite productivity is limited by the lowest activity in the pathway, it is not always easy to identify the rate-limiting step, since many steps are not rate limiting and the limiting step might be different among plant species and might vary according to the environmental conditions. While we reported that 6OMT is rate limiting and 4′OMT is not in California poppy (6), others have reported that the over-expression of N-methylcoclaurine 3′-hydroxylase (CYP80B3) enhanced alkaloid productivity in opium poppy (14). In fact, transformation itself enhances the expression of some biosynthetic genes, probably infection-responsive genes such as jasmonate-responsive genes, including those in isoquinoline alkaloid biosynthesis (6–8), and somaclonal variation during culture may enhance modification of the rate-limiting step. However, ectopic expression would still be useful for elucidating the biosynthetic network and flow. Below is our outline of a protocol for the transformation of California poppy. The actual method of plant transformation will vary based on the plant species and material conditions.

1. California poppy seeds are sterilized by soaking for 1 min each in 70% ethanol and 1% benzalkonium chloride, then soaking for 10 min in sodium hypochlorite solution. Sterilized seeds are then intensively washed three times with a sufficient volume of sterilized water for 10 min (see Note 8).

2. Sterilized seeds are inoculated on 1% agar medium containing Murashige–Skoog inorganic salts and cultivated under continuous light (100 µE/m²/s) at 25 ± 1°C for an adequate period, e.g., 3 weeks (see Note 8).

3. Seedlings are cut to a length of 5–10 mm and transferred to 12 ml of co-culture medium containing Agrobacterium (OD₆₀₀=0.6–1.0) (see Note 9).

4. After incubation for 10 min at room temperature, excess Agrobacterium suspension is removed from plant segments with blotting on a paper towel.

5. Plant segments are cultured on co-culture medium for 2 days at 25°C in the dark.

6. After 2 days of co-culture, segments are transferred to selection medium, pH 5.7. After 2 days of culture on selection medium, infected segments are transferred to fresh selection medium every 2–3 weeks.
7. Calluses are generally formed after 2 months (about three successive selection cultures). After additional selection culture on fresh selection medium for 3–4 months, calluses are transferred to liquid medium. The integration and expression of transgene are examined with cultured cells, or plants are regenerated from calluses (see Note 10).

3.3. Characterization of Transformants (DNA)

Integration of the expression vector is a pre-requisite for high expression of the introduced gene. While integration of the gene is not sufficient for high expression, it is needed to analyze DNA integration when expression levels are low. While the over-expression vector is usually integrated into the genome without modification, some specific vectors, such as RNAi vector with an inverted sequence, might lose part of its sequence during integration (see Note 1).

1. To confirm integration of the transgene, DNA is extracted from transformants. About 50–100 mg calluses are made to a fine powder in liquid nitrogen with a mortar and pestle.
2. 0.5 ml of 2X CTAB buffer warmed to 60°C is then added, and the mixture is incubated at 60°C for 30 min and transferred to a 1.5-ml tube.
3. 0.5 ml chloroform is added and the solution is gently mixed for 15 min.
4. The sample is centrifuged at 15,000×g at room temperature for 5 min to separate the water layer. 300 μl isopropanol is added to 450 μl supernatant, and the solution is mixed by vortex and then centrifuged at 15,000×g for 10 min at room temperature to sediment DNA.
5. Supernatant is removed and 500 μl of 70% ethanol is added, and DNA is sedimented by centrifugation at 15,000×g for 2 min.
6. DNA is dried under vacuum with aspiration for about 15 min, and the dried DNA is then dissolved in 50 μl TE.
7. Transgene is detected with genomic PCR using Cauliflower mosaic virus 35S forward primer (5′-GATATCTCCACTGACGTAAAGG-3′) and NOS terminator reverse primer (5′-CCCATCTCATAAATAACGTC-3′).

3.4. Characterization of Transformants (RNA)

When we evaluate the effect of over-expression of a candidate gene for the rate-limiting step, it is essential that we confirm the high expression of the introduced gene. Sometimes, the introduced gene is not expressed due to loss of the gene, improper integration (position effect) and gene-silencing due to sequence homology to an endogenous gene or the multiple integration of introduced genes. Figure 7.3 shows some results for the integration of CYP719A5 in California poppy.
Fig. 7.3. Analysis of transgenic California poppy cells transformed with CYP719A5 over-expression vector. (a) Expression of CYP719A5 and CYP719A2 genes in transgenic California poppy cells with an over-expression vector of EcCYP719A5. (b) Metabolite profiles of transformants. About 2 g of wild-type and transgenic cells with the CYP719A5 over-expression vector was cultured in 30 ml medium for 2 weeks and harvested for analysis by LC–MS. Peaks 1–7 correspond to protopine, allocryptopine, sanguinarine, 10-hydroxychelerythrine, chelerythrine, chelirubine, and macarpine, respectively. I.S. is internal standard (quinine). As the chromatogram shows, over-expression of a rate-limiting enzyme may increase the production of some metabolites and their relative composition. Compound numbers also correspond to the chemical structures shown in Fig. 7.1 (see Note 13; Todokoro et al., unpublished data).

While CYP719A5, which is homologous to CYP719A2/A3, was successfully over-expressed, over-expression of the endogenous gene (e.g., CYP719A2/A3 in our case) often induces the co-suppression of gene-expression (15, Todokoro et al., data not shown).

1. For the characterization of transgene expression, RNA is extracted from transformant. About 100–200 mg of 2-week-old calluses is frozen in liquid nitrogen immediately after harvest and stored at −80°C until use. They are then made into a fine powder with a mortar and pestle when RNA is extracted.

2. Total RNA is extracted from this fine powder of calluses with an RNeasy plant mini kit (Qiagen) in a 1.5-ml microtube according to the manufacturer’s protocol.

3. RNA is dissolved in RNase-free water, and the RNA concentration is then measured using OD at 260 nm (see Note 1).
4. Total RNA (1 μg) is treated with amplification-grade DNase I (Invitrogen) to remove DNA contaminants, and cDNA is reverse transcribed using SuperScript™ III for RT-PCR (Invitrogen) with oligo dT primer.

5. cDNA of transgene is detected using PCR with adequate forward and reverse primer sets for transgenes (see Note 11).

3.5. Characterization of Transformants (Metabolites)

Another important step to confirm the high expression of an introduced gene is to measure the amount and/or activity of the introduced gene product. When specific antibodies and a sensitive method for detecting target enzymes are not available, the direct measurement of metabolites is a useful alternative (see Note 12).

1. Cells are harvested after an adequate culture period, usually at the time of subculture, e.g., 2–3 weeks of culture. Culture medium can be recovered separately.

2. Fresh weight of cells is determined.

3. 100 mg cells are soaked overnight in 90% methanol solution (1 ml) and then sonicated three times for 1 min.

4. The cell debris is sedimented and the extract is filtered for LC–MS analysis.

5. For analysis of the medium, the cell debris in the medium is sedimented by centrifugation at 10,000×g for 10 min, and supernatant is concentrated with Sep-Pak Plus C18. The Sep-Pak Plus C18 is first activated with methanol and washed with water, and 50 ml medium is then loaded to concentrate the lipophilic metabolites. After being washed with 3 ml water, adsorbed alkaloid is eluted with 3 ml methanol.

6. Cell extracts or medium concentrates are analyzed by LC–MS (see Note 13).

4. Notes

1. While the Gateway system is convenient, conventional cloning methods using restriction enzyme digestion and ligation are also useful alternatives. See Molecular Cloning (16) or other relevant molecular biology protocols (17) when conventional methods, such as heat-shock transformation, restriction enzyme digestion, reagent preparation (e.g., RNase-free water), and competent bacterial cell preparation, are mentioned in the text.

pGWB vector is available from Dr. Tsuyoshi Nakagawa, Research Institute of Molecular Genetics, Shimane University, Matsue 690, Japan (tnakagaw@life.shimane-u.ac.jp).
A more detailed protocol for Gateway cloning with the pGWB series is also available at http://www.versailles.inra.fr/urgv/pub/pGWB_manual.pdf.

2. Other *Agrobacterium* strains might be useful when plant materials for which transformation is difficult need to be used, e.g., *Agrobacterium* with greater virulence, such as EHA101 (18).

3. Transformation efficiency, regeneration/callus formation efficiency, and secondary metabolite productivity can vary considerably when a novel plant variety is used. Several seed varieties should be compared in advance, if available (e.g., see 19).

4. Some other antibiotic, i.e., 150 μg/ml kanamycin, might be used when the NPTII gene is used as a selection marker gene. However, a suitable selection marker will vary due to differences in plant sensitivity and thus expression vectors must be selected carefully, while pGWB vector contains both hygromycin and kanamycin resistance genes. In general, hygromycin is more effective than kanamycin. In addition, bacterial cells have different sensitivities than plant cells. The concentration of antibiotics should be modified based on the literature (e.g., 17).

5. Dilute bleach solution can be substituted for 1% sodium hypochlorite solution. While this solution should ideally be prepared with sterilized water, distilled water is usually sufficient for preparation.

6. Several molecular biological reagents and kits are available on the market. The most reliable of these should be selected from among locally available materials. As an alternative, conventional methods can be followed as mentioned in Note 1. For DNA amplification for the construction of expression vector, high-fidelity DNA polymerase is critical to avoid the unexpected introduction of base modification, and the sequence integrity should be confirmed by nucleotide sequencing.

7. While full-length cDNAs are often used without a 5′- or 3′-untranslated region (UTR), some UTRs have positive effects on expression. Codon usage is also critical for gene expression in heterologous plant species. When the expression level is not sufficient, the structure of the vector (UTR requirement, codon usage, sequence preference around the start codon, etc.) as well as the promoter should be modified. Codon modification is especially important when gene silencing due to over-expression of an endogenous gene is evident.
8. Strong sterilization treatment can damage seeds. Generally, longer washing is better for seed germination, and the sterilization conditions (concentration and duration) should be optimized for the materials used. In addition, the culture conditions should be modified based on the plant species, varieties, and seed conditions. In some cases, incubation in the dark during germination might be useful for preparing more embryogenic cells (Okamoto, unpublished data). Juvenile tissues such as shoot meristem and immature seed are often preferable materials for the establishment of an embryogenic culture. A light condition of 10–30 μE/m²/s is provided under two to three 40 W fluorescent bulbs at a distance of about 30 cm.

9. Agrobacterium density and the duration of co-culture are important factors for efficient transformation. The addition of acetosyringone is another important factor when using materials that are not very transformable.

10. At the early stage of selection culture, a more frequent transfer of plant materials might be preferable to avoid the toxic effects of metabolites released from dead cells. In the later stage of selection, the concentrations of antibiotics may be reduced, e.g., 50 μg/ml kanamycin and 50 μg/ml cefotaxime after 3 months.

11. Whereas actin is used as a reference gene, it is not necessarily the most suitable marker. Since its expression also varies, some preliminary characterization is needed.

12. Liquid medium would be useful for a preliminary metabolite evaluation, when there are not enough cultured cells for the analysis. Since metabolites are often excreted from cells when metabolism is modified, the culture medium should be measured with cell extract. To prepare cell extract, fresh cells can be freeze dried or heat dried in an oven, although freeze drying is preferable to avoid chemical modification, since heat might induce chemical changes. Ethanol might be substituted for methanol. For alkaloid extraction, the addition of a weak acid would be recommended. We also recommend adding a certain amount of internal control compound to measure the metabolite content quantitatively. For example, 1 μM of quinine is included as an internal standard in isoquinoline alkaloid analysis in California poppy or C. japonica cells. While repeated extraction is recommended for quantitative measurement, the simple extraction described here would be useful for a qualitative analysis. Note that some metabolites might pass through Sep-Pak and thus not be concentrated.
13. The analytical equipment and conditions should be adjusted based on the target compounds. Our LC–MS consists of an LC–MS2010A (SHIMADZU) with lab solutions software. Column: TSK-gel ODS-80Ts 4.6 mm, I.D. × 25 cm (TOSOH); solvent condition: acetonitrile:water 65:35 with 0.05% trifluoroacetic acid. Scan mode (m/z=100–800); column temperature, 40°C. For quantification, the peak intensity is monitored with the SIM (single ion monitoring) mode.

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References


Microbial Expression of Alkaloid Biosynthetic Enzymes for Characterization of Their Properties

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Abstract

A wide variety of secondary metabolites are produced in higher plants. These metabolites are synthesized in specific organs/cells at certain developmental stages and/or under specific environmental conditions. Since these biosynthetic activities are rather restricted and difficult to detect, the biochemical characterization of biosynthetic enzymes involved in secondary metabolism has been limited compared to those involved in primary metabolism. Recently, however, progress in tissue culture and molecular biology has made it easier to study biosynthetic enzymes. Here we describe protocols for expressing some biosynthetic enzymes in Escherichia coli expression systems, since this system is both efficient and cost-effective. First, we describe a standard system for expressing biosynthetic enzymes as a soluble protein under the T7 promoter of the pET expression system in E. coli. In addition, the successful expression of cytochrome P450 in E. coli in an active soluble form with N-terminal modification is discussed, since P450 is the critical enzyme in secondary metabolite biosynthesis.

Key words: Alkaloid, microbial expression system, Escherichia coli, pET system, cytochrome P450, bioconversion.

1. Introduction

Higher plants produce divergent chemicals, such as alkaloids, terpenoids, and phenolic compounds, in secondary metabolism. Among these chemicals, alkaloids are very important in medicine due to their high biological activities. Alkaloids are low molecular weight, nitrogen-containing compounds that are found in about 20% of plant species. Most alkaloids are derived from amines produced by the decarboxylation of amino acids, such as histidine, lysine, ornithine, tryptophan, and tyrosine. Due to the
high interest in their potential for medicinal use, plant metabolic engineering has often been attempted to increase the amount of desired metabolites, and plant cells are selected to produce sufficient quantities of metabolites for industrial application (1 and references therein). In these approaches, the characterization of biosynthetic enzymes is essential. As more tools become available for working with biosynthetic enzymes, synthetic biological approaches have made it possible to reconstruct entire plant biosynthetic processes in microbial systems (2–5). In all of these attempts, it is important to express alkaloid biosynthetic enzymes in an active form in microbial systems. Whereas various microbial expression systems have been examined to express alkaloid biosynthetic enzymes easily and efficiently, practical tips are needed for the successful expression of biosynthetic enzymes.

Among biosynthetic enzymes, cytochrome P450s (P450s) play the key role in the oxidation reaction in plant secondary metabolism. P450s are microsomal (membrane-bound) enzymes, and thus expression systems based on the yeast *Saccharomyces cerevisiae* have often been used to express P450s in an active form. Although an expression system based on *E. coli* has been unsuitable for the expression of eukaryotic membrane-bound enzymes, such as cytochrome P450s, the successful expression of P450s in *E. coli* cells with modification of the N-terminal region has recently been reported. Thus, we discuss strategies for the expression of P450s in *E. coli* cells. In addition, we describe a method to characterize alkaloid biosynthetic enzymes by using a bioconversion reaction in microbial expression systems. While we focus on alkaloid biosynthetic enzymes in this chapter, the described protocol is not necessarily specific to metabolites of this type.

2. Materials

1. The expression plasmid pET-41a(+) vector (Novagen) is used for alkaloid biosynthetic enzymes. pCWori+ vector (6, 7) is used for cytochrome P450 expression.

2. Bacterial strains: *E. coli* DH5α is used for cytochrome P450 expression and for all manipulations on cloning. *E. coli* BL21(DE3) is used for the pET expression system.

3. Luria–Bertani (LB) broth: 25 g of LB broth (Difco) per liter. As a substitute, 10 g of Bacto Tryptone (Difco), 5 g of yeast extract (Difco), and 5 g of NaCl per liter can be used. If needed, the pH can be adjusted to 7.0 with NaOH. Autoclave for 15 min at 121°C.

4. Terrific Broth (TB): 47.6 g of TB modified (Sigma-Aldrich) and 8 ml of glycerol per liter. As a substitute, 12 g of Bacto
Tryptone (Difco), 24 g of yeast extract (Difco), 4 ml of glycerol, 2.2 g of KH2PO4, and 9.4 g of K2HPO4 per liter can be used. Autoclave for 15 min at 121°C.

5. Extraction buffer: 50 mM Tris–HCl, pH 7.5, containing 10% glycerol and 5 mM 2-mercaptoethanol (see Note 1).

6. Pretreatment column: Sep-Pak Plus C18 cartridge (Waters).

7. Liquid chromatography–mass spectrometry (LC–MS)

3. Methods

3.1. Construction of E. coli Expression Vector for Alkaloid Biosynthetic Genes

One of the most popular E. coli expression vectors is the pET vector system. In pET vectors, target genes are cloned under the control of strong bacteriophage T7 transcription and translation signals, and expression is induced by providing a source of T7 RNA polymerase in the host cell. pET vector systems are suitable as the first choice of E. coli expression vector for alkaloid biosynthetic genes, since many alkaloid biosynthetic genes have been successfully expressed by pET vector systems. In this chapter, the expression vector for alkaloid biosynthetic genes is constructed by using pET-41a(+) vector (see Note 2).

1. cDNA of an alkaloid biosynthetic gene is amplified by PCR where the 5′-sense primer introduces an NdeI restriction site (CATATG) that contains the initiation codon and the 3′-antisense primer introduces a unique cloning site of the expression vector that is coincident with or near the stop codon.

2. The PCR product is ligated into the NdeI site and a unique cloning site of pET-41a(+) vector to generate the E. coli expression vector.

3. The expression vector is introduced into E. coli BL21(DE3) (see Note 3).

3.2. Expression and Extraction of Alkaloid Biosynthetic Enzymes in E. coli Cells

1. Inoculate 5 ml LB broth (containing 30 mg/l kanamycin) with single colonies of E. coli cells containing expression vector. Incubate at 37°C overnight with shaking at 100–150 rpm.

2. Transfer 1 ml of overnight culture into 100 ml LB broth (containing 30 mg/l kanamycin). Incubate at 37°C with shaking at 100–150 rpm.

3. IPTG (1 mM final concentration) is added at OD600=0.5–0.8 to induce expression of the alkaloid biosynthetic gene, and the culture is incubated at 37°C at 100–150 rpm for 4 h (see Note 4).
4. *E. coli* cells are harvested by centrifugation at $8,000 \times g$ for 10 min at $4^\circ C$ and washed with ice-cold extraction buffer. Cells are re-harvested by centrifugation and stored at $-80^\circ C$.

5. Harvested cells are re-suspended in 10 ml extraction buffer and disrupted by ultrasonication.

6. After centrifugation at $12,000 \times g$ for 15 min at $4^\circ C$, the supernatant is used as a crude enzyme solution. This crude enzyme solution is stored at $-80^\circ C$ until further use.

The result of CjNCS1 expression in *E. coli* cells is shown in Fig. 8.1 as an example of the expression of an alkaloid biosynthetic enzyme by the pET vector system. CjNCS1 catalyzes the condensation of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) to produce (S)-norococlaurine in benzylisoquinoline alkaloid biosynthesis.

![Fig. 8.1. SDS-PAGE analysis of recombinant CjNCS1 expressed in E. coli cells. Lane 1: E. coli harboring empty vector; lane 2: E. coli expressing CjNCS1; and lane 3: purified CjNCS1. In E. coli expression systems, CjNCS1 tends to be expressed in inclusion bodies. We can improve the proportion of soluble CjNCS1 by performing the culture at a lower temperature (18°C).](image)

### 3.3. Construction of E. coli Expression Vectors for Alkaloid Biosynthetic Cytochrome P450s

Plant cytochrome P450s have a highly hydrophobic N-terminal region that serves as a membrane anchor region for co-translational insertion of the protein into the endoplasmic reticulum. In the *E. coli* expression system, plant cytochrome
P450s could be poorly expressed in an active form due to this membrane anchor region. Therefore, various protocols for the N-terminal modification of cytochrome P450s have been reported, especially for mammalian cytochrome P450s (10, 11). Compared to mammalian cytochrome P450s, there have been few reports on the expression of plant cytochrome P450s in *E. coli*. Among these various modifications, our selected strategies for the expression of plant cytochrome P450s in *E. coli* cells are provided in this section. We illustrate four modifications of a plant cytochrome P450, CYP80G2, by the example in Fig. 8.2. CYP80G2 catalyzes an intramolecular C–C phenol coupling of (S)-reticuline in magnoflorine biosynthesis (12).

The N-terminal amino acids of *Coptis japonica* CYP80G2

\[
\text{MDLQIALFSLIPVILVFILLLK-PPGPHPWPLIGN}\]

**membrane anchor region** **basic region** **proline conserved region**

1) \[
\text{MDLQIALFSLIPVILVFILLLK-PPGPHPWPLIGN...}
\]

**MA** \[
\text{PKYKNPGLPGPHPWPLIGN...}
\]

2) \[
\text{MDLQIALFSLIPVILVFILLLKPKYKNPGLPGPHPWPLIGN...}
\]

**MALLAVF** \[
\text{SLIPVILVFILLLKPKYKNPGLPGPHPWPLIGN...}
\]

3) \[
\text{MDLQIALFSLIPVILVFILLLKPKYKNPGLPGPHPWPLIGN...}
\]

**MALLAVF** \[
\text{PKYKNPGLPGPHPWPLIGN...}
\]

4) \[
\text{atgatctccaatgcaactgtttttccctta}
\]

**MA** \[
\text{LQIALFSL}
\]

\[
\text{atgctttcaaatgcaactgtttttccctta}
\]

**MAL** \[
\text{LQIALF}
\]

Fig. 8.2. Modification of the N-terminal amino acid/nucleotide sequences of CYP80G2 for plant cytochrome P450 expression in *E. coli* cells. (1) The second codon is changed from GAT (Asp) to GCT (Ala), which is preferred for expressing the lacZ gene (14), and the membrane anchor region (corresponding to the respective amino acid residues) is deleted. (2) The eight N-terminal amino acids are changed to a MALLAVFL motif (nucleotide sequences: ATGGCTGTGTTATAGCCAGTTTT), which correspond to the first eight amino acids of modified bovine P45017α (see Note 10; Ref. 15). (3) The 22 N-terminal amino acids (membrane anchor region) are changed to a MALLAVFL motif. (4) The second codon is changed from GAT (Asp) to GCT (Ala), and the N-terminal nucleotide sequences (corresponding to amino acid residues) are modified to enrich with AT nucleotides without changing the amino acids (15). The nucleotide sequence of CYP80G2 has been reported in GenBank™ with accession number AB288053.
1. cDNA of the cytochrome P450 gene is amplified by PCR where the 5′-sense primer introduces an Ndel restriction site (CATATG) that contains the initiation codon and the 3′-antisense primer introduces a unique cloning site of expression vector that is coincedent with or near the stop codon. The 5′-sense primers used in the N-terminal modification are as follows (Ndel restriction site is underlined):

(1) 5′-GGGCATATGGCTCTCATAATACAAAGAACCCT-3′,

(2) 5′-CCCCCATATGGCTCTGTTATTAGCAGTTTTTTTCCTTAATCCCTGTCATC-3′,

(3) 5′-CCCCCATATGGCTCTGTTATTAGCAGTTTTTTCCCTTAATACAAAGAACCCTCCCA-3′,

(4) 5′-GGGCATATGGCTCTCATAATACAAAGAACCCTGGCTT-3′.

2. The PCR product is ligated into the Ndel site and a unique cloning site of pCWori+ vector to generate the E. coli expression vector (see Note 5).

3. The expression vector is introduced into E. coli DH5α.

3.4. Expression and Extraction of Alkaloid Biosynthetic Cytochrome P450s in E. coli Cells

1. Inoculate 5 ml of LB broth (containing 100 mg/l ampicillin) with single colonies of E. coli cells containing expression vector. Incubate at 37°C overnight with shaking at 100–150 rpm.

2. Transfer 1 ml of overnight culture into 100 ml TB (containing 100 mg/l ampicillin). Incubate at 37°C with shaking at 100–150 rpm.

3. IPTG (0.5 mM final concentration) and δ-aminolevulinic acid (0.5 mM final concentration) are added at OD600 = 0.5–0.8 nm to induce expression of the cytochrome P450 gene (see Note 6), and the culture is incubated at 30°C with shaking at 100–200 rpm for 40 h (see Note 7).

4. Cells are harvested by centrifugation at 8,000×g for 10 min at 4°C and washed with ice-cold extraction buffer. Cells are re-harvested by centrifugation and stored at –80°C.

5. Harvested cells are re-suspended in 10 ml extraction buffer and disrupted by ultrasonication.

6. After centrifugation at 12,000×g for 15 min at 4°C, the supernatant is used as a cytochrome P450 crude enzyme solution. This crude enzyme solution is stored at –80°C until further use.

3.5. Alkaloid Biosynthetic Enzyme Reactions in Microbes

One of the effective methods for the characterization of alkaloid biosynthetic enzymes is bioconversion of the substrate to its corresponding product using the biosynthetic enzyme-expressing E. coli cells in vivo. Although this enzymatic conversion often
requires expensive cofactors, recombinant *E. coli* cells can supply the cofactors for bioconversion. Thus, bioconversion does not require the addition of cofactors in the reaction. This method is also useful in the case of low activity or unstable enzymes in vitro, since in vivo activity can be maintained during bioconversion.

1. Inoculate 5 ml LB broth (containing antibiotics) with single colonies of *E. coli* cells containing expression vector. Incubate at 37°C overnight with shaking at 100–150 rpm.
2. Transfer 1 ml of overnight culture into 100 ml LB (containing antibiotics). Incubate at 37°C with shaking at 100–150 rpm.
3. IPTG (0.5 mM final concentration) and the substrate are added at OD$_{600}=0.5–0.8$, and the culture is incubated at 30°C with shaking at 100–150 rpm for 40 h (*see Note 8*).
4. Culture medium is recovered, and the supernatant after protein precipitation with a one-tenth volume of 20% trichloroacetic acid is used to measure alkaloid production by HPLC or LC–MS (*see Note 9*). LC–MS analysis of the bioconversion by *E. coli*-expressing alkaloid biosynthetic enzymes is shown in Fig. 8.3.

![Fig. 8.3. LC–MS analysis of bioconversion in *E. coli* expressing alkaloid biosynthetic enzymes. Reticuline, which is an important intermediate in benzylisoquinoline alkaloid biosynthesis, is produced from dopamine by using transgenic *E. coli* expressing reticuline biosynthetic genes (4). Transgenic *E. coli* cells expressing biosynthetic genes are cultured with 2 mM dopamine in the medium. This culture produces mainly reticuline at a yield of 2 mg/l of medium within 28 h. An advantage of this system is that reticuline is produced without the addition of a methyl group donor, S-adenosyl-L-methionine (SAM), since the regeneration of SAM in microbial cells is known to maintain in vivo methylation activity during bioconversion (16). Alkaloid production is measured by LC–MS (API 3200™, Applied Biosystems Japan Ltd.) with an Agilent™ HPLC system: column, ODS-80Ts (4.6×250 mm; Tosoh Inc.); solvent system, 20% acetonitrile containing 0.1% acetic acid; flow rate, 0.5 ml/min at 40°C. Selected ion monitoring (SIM) parameters: $m/z = 153$ (3,4-DHPAA), 154 (dopamine), 288 (norlaudanosoline), 302 (3′-hydroxycoclaurine), 316 (3′-hydroxy-N-methylcoclaurine), 330 (reticuline).]
4. Notes

1. The extraction buffer is not limited to Tris–HCl buffer. Different types of buffers can be used for different purposes. Protease inhibitor cocktails are helpful for preventing recombinant proteins from unwanted degradation during their characterization.

2. The vector used here is a typical expression plasmid. Various pET vectors can be prepared for any purpose. For example, pET-16b is used for the quick and easy detection of expressed proteins with an anti-His tag antibody and purification of the fusion protein using a nickel-chelating column. Fusion protein could improve the solubility and expression level of the target enzyme.

3. Other *E. coli* strains can be used. When the vector does not itself carry a *lacI* gene, strains that express *lac* repressor from a *lacI* gene present on a compatible plasmid or carried on the chromosome should be used to reduce the basal expression of the target gene. BL21-CodonPlus(DE3)(Stratagene) or Rosetta, (Novagen), can be used to resolve the codon bias problem that reduces the expression level of eukaryotic protein in *E. coli* cells.

4. Optimization may be required to express the target enzyme in an active form. Critical factors for optimized expression are (1) IPTG concentration: 10 μM–1 mM, (2) IPTG induction point: OD_{600}=0.4–1.5, (3) induction time: 1–48 h, and (4) induction temperature: 16–37°C. When the recombinant protein is expressed in inclusion bodies, reduction of the IPTG concentration or a lower induction temperature helps to improve the proportion of soluble enzyme.

5. pKK223-3 vector (Pharmacia) or pCWori+ vector has often been used to express eukaryotic cytochrome P450s in *E. coli* cells. Therefore, we use pCWori+ vector, which contains two Tac promoter cassettes upstream of an *NdeI* restriction cloning site, which coincides with the initiation codon. pCWori+ vector is not available commercially and must be obtained from cytochrome P450 research groups.

6. In some cases, the addition of δ-aminolevulinic acid, which is the heme precursor, can improve the expression level of cytochrome P450 (13).

7. The induction temperature should be 25–30°C. An induction temperature above 30°C results in the accumulation of recombinant protein in inclusion bodies, and an induction
temperature below 25°C results in a decrease in the expression level of recombinant protein. The shaking speed and induction time also affect the optimal expression level of recombinant protein and therefore must be optimized. Note that the cap is also critical. Aluminum foil is not suitable for achieving high aeration. A silicone sponge plug or cotton plug is recommended.

8. Optimization may be required to convert the substrate into the corresponding product (see Note 4). Variation of the pH in the culture broth may interfere with uptake of the substrate by *E. coli* cells. If the transformation efficiency is low, buffer that corresponds to the pKₐ of the substrate should be used in place of LB. *E. coli* cells expressing the alkaloid biosynthetic enzyme are transferred from LB to the buffer and cultured with the substrate.

9. LC–MS is the optimum instrument for the analysis of alkaloids. Alkaloids can be analyzed with high sensitivity by using LC–MS in the positive mode, since alkaloids are low molecular weight and nitrogen-containing compounds. If the alkaloid concentration is low, it can be concentrated from the supernatant using a Sep-Pak Plus C18 cartridge before measurement.

10. Various mammalian cytochrome P450s have been successfully expressed using the first eight amino acids of modified bovine P45017α. N-terminal modification with this motif is a recommended strategy for expression in *E. coli*. The best type of N-terminal modification varies according to the cytochrome P450 examined.

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**References**


Producing a Recombinant Flavin-Containing Monooxygenase from *Coffea arabica* in *Escherichia coli* for Screening of Potential Natural Substrates

Igor Cesarino and Paulo Mazzafera

Abstract

Only few biological functions have been related with flavin-containing monooxygenases (FMOs) in plants, such as specific roles in auxin biosynthesis, pathogen defense, and metabolism of glucosinolates. Biochemical characterization using recombinant proteins is a promising approach to determine the precise specificity of plant FMOs for potential natural substrates. FMOs may be very difficult to express in a soluble form due to their highly hydrophobic nature and this can be improved by fusing them to solubility-enhancing proteins, such as maltose-binding protein (MBP) and N-utilization substance A (NusA). Here we describe the expression of a recombinant FMO from *Coffea arabica* as a maltose-binding protein fusion in *Escherichia coli* and its purification by affinity chromatography, producing a ready-to-use protein for enzymatic activity assays.

**Key words:** Flavin-containing monooxygenase, maltose-binding protein, *Coffea arabica*.

1. Introduction

A large number of genes coding flavin-containing monooxygenases (FMOs) are found in plant genomes, but only few biological functions have been related with these enzymes in plants. In animals, specially mammals, it is believed that FMOs evolved to protect from an attack of lipophilic nucleophilic chemicals (e.g., secondary metabolites) in the early environmental conditions (1). Indeed, a FMO was identified as responsible for the detoxification of host plant-acquired pyrrolizidine alkaloids in the alkaloid-defended arctiid moth *Tyria jacobaeae* (2), showing that
FMOs can be related with metabolism of secondary metabolites, at least in animals. Recent researches revealed specific functions of plant FMOs, such as participation in auxin biosynthesis pathway (3), pathogen defense (4), and biosynthesis of glucosinolates (5), amino acid-derived secondary metabolites present in the order Brassicales. Biochemical characterization using recombinant proteins is a promising approach to determine the specificity of plant FMOs for potential natural substrates, including secondary metabolites.

Many members of biochemically interesting families of proteins, including kinases, phosphatases, membrane-associated proteins, and many other enzymes, are extremely difficult to produce as recombinant soluble proteins in *Escherichia coli* (6). This also seems to be the case of FMOs, due to their often highly hydrophobic nature, with possible multiple internal sites of membrane association. Over the years, much effort has been put into optimizing *E. coli* as an expression host for eukaryotic proteins. This strategy has generated a wide arsenal of tools, which can be used to increase the yield of proteins in a soluble form (7). However, the number of targets purified is much smaller than the number of targets cloned. In terms of full-length proteins, only 10% of proteins from Eukarya can be expressed in *E. coli* as soluble proteins (http://targetdb.pdb.org) (7). Producing soluble proteins for purification has continued to be a major bottleneck in the field, despite the improvement in protein purification techniques during the past decade (6). Thus, this chapter does not emphasize cloning procedures, usually based on following manufacturer’s instructions, although we provide a list of the molecular biology kits that could be used. Here, we intend to provide an optimized approach for expression and purification of an FMO from *Coffea arabica*.

2. Materials

2.1. Molecular Biology Kits for Cloning Procedures

1. RNA isolation and treatment with DNase: TRIzol® (Invitrogen, Carlsbad, CA) and “TURBO DNA-free” (Ambion, Austin, TX).

2. cDNA synthesis: “Superscript First-Strand Synthesis System for RT-PCR” (Invitrogen).

3. Production and purification of PCR products: “Platinum PCR High-Fidelity Supermix” (Invitrogen) and “illustra GFX PCR DNA and Gel Band Purification Kit” (GE Healthcare, Chalfont St. Giles, UK).
4. Cloning: “pGEM T-easy Vector System” (Promega, Madison, WI); Restriction enzyme EcoRI (Promega); Competent E. coli strain: “One Shot® Mach1™-T1R Competent Cells” (Invitrogen).


6. Expression Vector: pETMBP_1a (EMBL, Heidelberg); Restriction enzymes: FastDigest NcoI and FastDigest XhoI (Fermentas, Glen Burnie, MD); E. coli expression strain: “Rosetta™ (DE3) Competent Cells” (Novagen, Darmstadt) (see Note 1).

2.2. Protein Expression and Preparation of Bacterial Lysate

1. Luria-Bertani (LB) Medium: for 1 L dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl, plus 2 g glucose (see Note 2) in 950 mL deionized water. Adjust the pH to 7.0 with NaOH and bring the volume up to 1 L. Autoclave on liquid cycle for 20 min. Store at 4°C or room temperature.

2. Kanamycin (PhytoTechnologies, Lenexa, KS) is dissolved at 100 mg/mL in water, filter-sterilized, and stored at –20°C. Chloramphenicol (USB Corporation, Cleveland, OH) is dissolved at 34 mg/mL in methanol and stored at –20°C.

3. Isopropyl β-D-thiogalactoside (IPTG; Fermentas) is dissolved at 1 M in water, filter-sterilized, and stored in aliquots at –20°C.

4. Lysis buffer: 100 mM potassium phosphate, pH 8.0, 500 mM NaCl. Filter-sterilize and store at 4°C (see Note 3).

5. Lysozyme (Roche, Basel, Switzerland) is dissolved in water at 0.5 mg/mL and phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO) is dissolved in isopropanol at 0.5 mM. PMSF is a highly toxic protease inhibitor and must be handled with care.

2.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)


3. 30% acrylamide/bis solution (37.5:1) (care should be taken due to neurotoxic effects of unpolymerized acrylamide/bis solution), N,N,N′,N′-tetramethyl-ethylenediamine (TEMED, Merck/EMD, Darmstadt) and autoclaved water.

4. Ammonium persulfate: prepare 10% (w/v) solution of ammonium persulfate in water and immediately freeze in single use aliquots at –20°C. Sodium dodecyl sulfate
(SDS): prepare 10% (w/v) stock solution in water and store at room temperature.

5. Isopropanol.

6. Sample buffer (2X): 100 mM Tris–HCl, pH 6.8, 20% (w/v) glycerol, 400 mM β-mercaptoethanol, 0.1% (w/v) bromophenol blue, 4% (w/v) SDS. Store in aliquots at –20°C.

7. Running buffer (5X): 125 mM Tris, 960 mM glycine, 0.5% (w/v) SDS. Store at room temperature. Dilute 100 mL of the 5X running buffer with 400 mL of water.


9. Staining solution: 54% (v/v) methanol, 13.5% (v/v) acetic acid, 0.27% Coomassie Brilliant Blue R250 (Sigma). Maintain solution under agitation for 2 h and then filter through Whatman #1 paper (Whatman, Maidstone, UK). Store at room temperature. Solution should be prepared in a fume hood to minimize exposure to methanol and acetic acid vapors.

10. Destain solution A: 30% methanol (v/v), 10% acetic acid (v/v), 60% distilled water. Destain solution B: 5% methanol (v/v), 7% acetic acid (v/v), 88% water. Prepare both solutions in a fume hood.

2.4. Purification by Affinity Chromatography

1. Poly-Prep Chromatography Columns (BioRad, Hercules, CA).

2. Amylose Resin (New England Biolabs) stored in 20% ethanol at 4°C.

3. Elution buffer: 100 mM potassium phosphate, 500 mM NaCl, 10 mM maltose (Merck). Adjust pH to 8.0 and store at 4°C.

3. Methods

Perhaps the most important consideration for recombinant protein expression and purification is the experimental purpose for which the protein will be used. The production of soluble and functionally active proteins is often important in the case of biochemical and structural studies (12). Methods described below are meant to produce an active FMO for enzymatic activity assays and functional studies. FMOs usually have a hydrophobic nature due to internal sites of membrane association, result-
Producing a Recombinant Flavin-Containing Monoxygenase 125

In insoluble aggregates that lack functional activity. The solubility of recombinant proteins can be improved by fusing them to solubility-enhancing proteins, such as maltose-binding protein (MBP) and N-utilization substance A (NusA) (8). We used such strategy to produce a recombinant FMO from *C. arabica*, whose nucleotide sequence was obtained from the EST Coffee Genome Project (9). A contig was identified by homology as an *FMO* gene, coding for a putative 41.3 kDa FMO with high similarity to the *Arabidopsis* protein YUCCA10, which seems to be involved in the tryptophan-dependent auxin biosynthesis pathway. We heterologously expressed that putative coffee protein to investigate whether it presents FMO-like activity. Total RNA was isolated from leaves and first-strand cDNA was synthesized for the amplification of the full-length open reading frame by RT-PCR, using specific primers. The complete coding sequence was cloned into pETMBP_1a and the expression construct was transformed into the *E. coli* strain Rosetta™ (DE3). Using vector pETMBP_1a (EMBL, Heidelberg – may be obtained under request at http://www.pepcore.embl.de/index.html), a target protein is fused in-frame with an N-terminal tag comprised of two affinity tags, an oligohistidine (6xHis) and MBP, which can be used for both affinity purification and solubility enhancement. All these steps were carried out using kits cited above and following the manufacturer’s instructions. Then the tagged protein was purified and subjected to a battery of substrate tests to confirm FMO activity.

Even though a solubility-enhancing tag may improve soluble expression of recombinant FMO, it still can be sequestered into insoluble inclusion bodies. Avoiding the formation of these aggregates can be accomplished through adjusting expression and purification parameters. Due to the variety of factors that may contribute to difficulties encountered in the expression of an FMO in *E. coli*, each optimized procedure will be discussed as soon as it appears.

### 3.1. Culture Growth and Protein Extraction

1. Inoculate 4 mL LB medium supplemented with glucose (2 g/L) and containing 50 μg/mL kanamycin and 34 μg/mL chloramphenicol in a 15 mL flask. Grow the cultures overnight at 37°C with shaking (200–300 rpm). To help evaluate the results, we recommend including a suitable positive expression control, which consists in bacteria transformed with empty pETMBP-1a (in this case, the recombinant protein produced will be green fluorescent protein – GFP, as a maltose-binding protein fusion).

2. Inoculate 500 mL of media (supplemented with glucose and antibiotics) pre-warmed at room temperature with 2 mL of the overnight cultures and grow at 37°C with
shaking (200–300 rpm) until the OD$_{600}$ is 0.5–0.7 (approximately 2 h). The cells should be in mid- to late log phase of the growth curve to ensure maximal yield while avoiding the problems with cells going into stationary phase, such as induction of proteases.

3. Induce expression by adding IPTG to a final concentration of 0.3 mM (see Note 4).

4. Incubate the culture at 25°C with shaking (250 rpm) for 5 h (see Note 5).

5. Harvest the cells by centrifugation at 5,000 rpm for 10 min at 4°C. Proceed with purification or store the cells overnight at –20°C (or –80°C for future use).

6. Thaw the cell pellet for 15 min on ice and resuspend the cells in 10 mL lysis buffer. The volume of the lysis buffer required depends on the expression level of the recombinant protein. In this case, we used 1 mL lysis buffer for each 50 mL culture media.

7. Add lysozyme at 0.5 μg/mL and PMSF at 0.5 μM and incubate on ice for 30 min.

8. Disrupt the cells using a sonicator equipped with a microtip, dividing total volume in aliquots of 1 mL. If the lysate is very viscous, try sonication without the microtip using total volume of lysate (10 mL), but intervals between pulses should increase (see Note 6).

9. Centrifuge lysate at 15,000 rpm for 20 min at 4°C to pellet cellular debris. Recover the soluble phase (supernatant) and maintain it on ice until the purification procedures. For monitoring the efficiency of cell disruption and the level of soluble recombinant protein, a supernatant sample and a sample of the insoluble phase (resuspended cellular debris pellet) should be electrophoresed in denaturing conditions (see Section 3) (Fig. 9.1).

10. Even if inclusion bodies are formed and apparently no soluble recombinant FMO is detected with SDS-PAGE, some MBP-tagged FMO may often remain soluble. Thus, we recommend proceeding with the purification protocol described below.

3.2. Purification of MBP-Tagged FMO by Affinity Chromatography

1. Load 1 mL amylose resin into a column with the bottom outlet capped. Start packing procedure by removing bottom cap, allowing the resin to settle. Do not allow resin to dry. If this occurs, resuspend resin in lysis buffer and repack the column (see Note 7).

2. Equilibrate column with 10 column volumes (10 mL) of lysis buffer.
Fig. 9.1. Solubility determination of MBP-tagged FMO. Cells were harvested immediately before induction (0 h) and after 5 h of induction with 0.3 mM IPTG. Samples of the soluble and insoluble fractions were prepared as described in Section 3 and analyzed by SDS-PAGE. Solid arrow points to the recombinant MBP-tagged FMO, which presents 84.4 kDa. Dotted arrow indicates the presence of recombinant protein in soluble fraction. Lane M: molecular weight marker.

3. Apply clarified lysate to column and collect the flow-through on ice. Save flow-through for SDS-PAGE analysis (see Note 8).

4. Wash column with 20 column volumes (20 mL) of lysis buffer and save washing fraction for SDS-PAGE analysis.

5. Elution is carried out in three steps with different volumes of elution buffer. Start with 1 column volume (1 mL) of elution buffer, followed by 1.2 column volume (1.2 mL) and 0.8 column volume (0.8 mL), collecting separately each fraction for further SDS-PAGE analysis. Maintain purified fractions on ice. The last fraction should contain little of the recombinant protein, but depending on the expression level of the recombinant protein, these volumes may not be enough for total elution of the target protein. Scale-up elution volumes according to expression level (see Note 9) (Fig. 9.2).

1. These instructions assume the use of Hoefer® miniVE minigel system (Amersham Biosciences, GE Healthcare), but are easily adaptable to other systems. Prepare a 10% resolving gel by mixing 2.5 mL of 4X resolving buffer with
Fig. 2. Purification of MBP-tagged FMO by affinity chromatography in amylose resin. Solid arrow indicates recombinant FMO and its respective molecular weight (84.4 kDa). Lane M: molecular weight marker; lane P: resuspended cellular debris pellet; lane FT: flow-through; lane W: washing; lanes E1, E2, and E3: elution 1, 2, and 3, respectively.

3.9 mL water, 3.4 mL acrylamide/bis solution, 100 μL 10% SDS, 100 μL 10% ammonium persulfate solution, and 30 μL TEMED. Pour the gel (6–7 mL) into Hoefer apparatus, leaving space for a stacking gel and overlay with isopropanol. The unused resolving gel solution (3–4 mL) will indicate when gel is well polymerized (about 15 min). Pour off the isopropanol and carefully dry the gel top with filter paper.

2. Prepare the stacking gel by mixing 0.63 mL of 4X stacking buffer with 3.4 mL water, 0.9 mL acrylamide/bis solution, 50 μL 10% SDS, 50 μL 10% ammonium persulfate solution, and 20 μL TEMED. Pour the gel (about 2 mL) on the polymerized resolving gel and insert the comb. The stacking gel should polymerize in about 15 min. Once the stacking gel has set, pour the running buffer into the Hoefer apparatus and carefully remove the comb.

3. Prepare samples by mixing 10 μL of 2X sample buffer with 10 μL of each fraction. Close the tube and then boil samples for 5–10 min. Cool samples to room temperature. Load
10 μL of each sample in a well, reserving external wells for the molecular weight marker.

4. Complete the assembly of the gel unit and connect to a power supply. Run the gel at 100 V until samples have entered and migrated through the stacking gel and then at 150 V for protein separation in the resolving gel, which will take about 3 h, when the migration dye (bromophenol blue) reaches the end of the gel.

5. At the end of the run, the gel unit is disconnected from the power supply and disassembled. The stacking gel is discarded and the resolving gel is submerged in enough staining solution that the gel floats freely in the tray. Shake for 30 min and slowly, to minimize gel cracking.

6. Rinse gel in distilled water and replace staining solution with destain solution A. Shake slowly for 30–60 min. Remove destain solution A and replace with destain solution B. Change destain solution B periodically until the gel background is clear. Use caution, however, because excessive destaining may lead to loss of band intensity. Store the gel in destaining solution B at room temperature for further documentation.

4. Notes

1. For expression of eukaryotic proteins, it is often important to use *E. coli* strains supplemented with additional tRNAs to overcome the effects of codon bias, such as low expression levels, frame shifts, truncation, and early termination. These effects may occur especially when two or more adjacent rare codons are presented in a gene sequence. Exposito-Rodriguez and colleagues (10) reported the production of a truncated tomato FMO due to two adjacent arginine codons that are used in *E. coli* at very low frequency. We recommend previous determination of rare codons in your DNA sequence, using specific programs (e.g. “Rare Codon Caltor”, http://www.doe-mbi.ucla.edu/~sumchan/caltor.html) and the use of Rosetta™ (DE3) (Novagen), BL21 derivative carrying additional tRNAs.

2. Amylose resins are affected by amylase activity in crude cell lysates. Adding glucose to bacterial growth media helps to suppress amylase expression and alleviates the problem. Still, amylose resin can be regenerated and reused only a few times (8–10) to avoid a significant drop in the yield. The packed resin may be regenerated as follows: water – three column
volumes, 0.1% SDS – three column volumes, water – one column volume, 20% ethanol – five column volumes.

3. We have tried Tris–HCl and potassium phosphate buffers, at pH’s from 8.0 to 8.5 with identical results. However, we noticed an increase in the yield of soluble protein using high ionic strength (500 mM NaCl), which maintains solubility and stability of the widest variety of proteins. Non-ionic detergents such as Triton X-100 and Tween 20 (both at 0.1%) may avoid hydrophobic or ionic interactions between proteins, acting as solubilization agents, although some fusion proteins may not bind efficiently on amylose resins in the presence of Triton or Tween.

4. IPTG is a highly stable synthetic analog of lactose and is used to induce the expression of cloned genes, which are under control of the lac operon. While lactose is consumed during induction, IPTG cannot be broken down and cells remain at high inducing level. Despite metabolic differences, protein expression can usually be induced by both IPTG and lactose. However, in the case of pETMBP_1a, induction with lactose can result in low expression levels and, consequently, reduced yield. We recommend using IPTG for induction of FMO expression.

5. Two factors that might contribute to difficulties encountered when expressing foreign proteins in E. coli are the rate of translation and the rate of protein folding, which are almost an order of magnitude faster in E. coli as compared with eukaryotic systems (6). Frequently, the result of a high concentration of incorrectly folded recombinant protein is the formation of inclusion bodies. An alternative to overcome these problems is to adjust expression conditions such that protein production occurs in slower rates, allowing newly transcribed recombinant proteins enough time to fold properly. Lower temperatures and reduced IPTG concentrations will reduce expression leading to a higher amount of soluble protein. We recommend expressing at 25°C with 0.3 mM of IPTG as default.

6. Because FMO-like proteins show a considerable thermal lability (11), sonication pulses should be short (10–15 s separated by 1 min intervals) and the extract should be maintained on ice all the time. In fact, cells and protein solutions, in addition to buffers and related solutions, should be kept at 0–4°C at all times.

7. Due to insertion of two different affinity tags, 6xHis and MBP, protein expression using pETMBP_1a allows purification by both Ni-NTA (QIAGen, Düsseldorf, Germany) and amylose resins. Moreover, two different affinity purification
regimes could be applied sequentially, obtaining a highly purified protein. If the recombinant FMO in the lysate is present at a very low concentration or 6xHis tag is not fully accessible, batch procedure may be a solution. The batch procedure entails binding the protein to the resin in solution and then packing the protein-resin complex into a column for the washing and elution steps (12). If that is the case, add 1 mL of the resin (amylose or Ni-NTA) to 5 mL cleared lysate and mix gently by shaking (200 rpm) at 4°C for 60 min. Then, load the mixture into a column and proceed with the same steps described for column purification.

8. If the lysate is very viscous, flow rate may decrease and the collection of flow-through fraction will take a long time. If this is the case, do not forget to keep the system at 0–4°C, due to FMO thermal lability.

9. Despite the effort of washing contaminant proteins with lysis buffer, some endogenous proteins may interact with amylose resin, which requires more stringent washing steps. If desired, a step gradient of elution buffer in wash buffer may be used, increasing maltose concentration up to 10 mM. However, our experience suggests the utilization of different volumes of 10 mM maltose elution buffer, applying a smaller volume in the beginning to wash contaminant proteins.

References


Efficient Production of Active Form Recombinant Cassava Hydroxynitrile Lyase Using Escherichia coli in Low-Temperature Culture

Hisashi Semba, Eita Ichige, Tadayuki Imanaka, Haruyuki Atomi, and Hideki Aoyagi

Abstract

Hydroxynitrile lyase (MeHNL, EC 4.1.2.39) is a useful enzyme for production of optically active cyanohydrin compounds. Production of MeHNL can be increased by substituting rare codons of the natural sequence of cassava (Manihot esculenta) MeHNL. However, most of the MeHNL produced by this method was in an insoluble form in Escherichia coli expression system. In order to increase the productivity of active form of MeHNL, the effects of cultivation temperature were investigated. When the cultivation temperature was reduced, the cell yield and the ratio of soluble MeHNL increased significantly. The enzyme activity and yield at low-temperature cultures (17°C) were 850 times higher than those obtained at the optimum growth temperature of 37°C. The rate of MeHNL production in the present study was calculated as 3,000 U/h. Low-temperature cultivation is very effective in improving the productivity of the active form of MeHNL and has more potential for large-scale production of MeHNL for optically active cyanohydrin production.

Key words: Hydroxynitrile lyase, HNL, recombinant enzyme, high-density culture, low-temperature cultivation, cyanohydrins.

1. Introduction

Optically active cyanohydrins are useful intermediate compounds that are used in various chiral pharmaceuticals and pyrethroid-type insecticides. Hydroxynitrile lyases (HNLs), which exist only in plants, are now receiving much attention as enzyme catalysts, which catalyze the synthesis of cyanohydrin from HCN and
carbonyl compounds. HNL is present in *Euphorbiaceae* plants such as cassava (*M. esculenta*) and rubber tree (*Hevea brasiliensis*). HNL from cassava is called MeHNL (EC 4.1.2.39) and its genes have been cloned from cassava leaves (1). Furthermore, MeHNL production by recombinant *E. coli* has also been studied (2, 3). However, the productivity was not high enough for industrial application. Hasslacher et al. (4) reported overexpression of HNL from *H. brasiliensis* using *Pichia pastoris* expression system. Methanol-utilizing yeast incorporating *Pichia* expression system resulted in higher productivity than that of the *E. coli* system. However, high cell density cultivation of yeast requires very long cultivation time, and recombinant enzyme recovery by disruption of cells is also difficult because of its tough cell wall.

*E. coli* is widely used as a host for recombinant protein production. However, efficient production of the active form of the recombinant enzyme depends on various culture conditions. Furthermore, the proteins produced in many cases are accumulated in the host cell as an inclusion body. Application of enzyme reaction to chemical industries requires drastic reduction in the cost of the enzymes. It is therefore necessary to develop simple and efficient systems for production of biocatalysts. Although various techniques have been reported for refolding of inclusion bodies, most of these methods are difficult to operate in large-scale production systems (5). Co-expression of chaperon protein has also been reported but this is not a promising method (6). Temperature shift cultivation method has also been reported (7, 8). In this method, the cell is cultivated at the optimum growth temperature (the cell growth phase) and subsequently the cultivation temperature is reduced for protein expression (the protein production phase). Although this method is relatively simpler than other methods, our study revealed that the temperature shift cultivation did not result in efficient productivity of active form of MeHNL (9).

In this chapter, we describe the construction of a MeHNL overexpression version derived by codon preference substitution from plant to that of *E. coli* for efficient production of soluble active MeHNL under low-temperature cultivation system.

### 2. Materials

#### 2.1. Strain and Vectors

1. Host strain, *E. coli* BL21(DE3) and vector, pET-21a were purchased from Novagen® (Merk, Darmstadt, Germany).
2. cDNA of modified MeHNL gene was inserted between *Nde*I and *BamHI* restriction site of the expression vector.
2.2. Reagents and Equipments

1. PCR reagents: 10X PCR buffer (attached to the DNA polymerase kit), dNTP mix, 25 mM MgCl₂, forward primer (40 pmol), reverse primer (40 pmol), and KOD DNA polymerase (Toyobo, Osaka, Japan).

2. Cultivation media (NS-2 medium): 40 g/L glycerol, 10 g/L (NH₄)₂SO₄, 2 g/L KH₂PO₄, 6 g/L K₂HPO₄, 40 g/L yeast extract (Yeast P2G, Asahi Breweries, Tokyo, Japan), 1 g/L MgSO₄·7H₂O, and 1 g/L antifoam agent (Adekanol LG-109, Asahidenka, Tokyo, Japan), pH 6, supplemented with 0.1 g/L ampicillin. MeHNL production medium is NS-2 medium containing 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and ampicillin. Both ampicillin and IPTG are sterilized by filtration and added after autoclaving of NS-2 medium.

3. 2-L jar-fermentor with a working volume of 1.2 L (B.E. Marubishi, Tokyo, Japan).

4. Spectrophotometer (UV–Vis) with cuvette temperature control.

5. Na-citrate buffer (0.2 M, pH 5.5).

6. Ultrasonic disruptor (Tomy, Tokyo, Japan).

7. D, L-mandelonitrile (Sigma-Aldrich, St-Louis, MO).

8. BCA protein assay reagent kit (Pierce, IL).

3. Methods

3.1. Design of Codon-Substituted MeHNL Sequence for Overexpression

Codon substitution of native MeHNL sequence can be carried out by referring to the codon usage of genome of *E. coli* K-12 MG1655 (Xana Genome, Microbial genome database) and aspartase of *E. coli* (10). Comparison of codon usage between natural MeHNL and *E. coli* genes is shown in Table 10.1 (see Notes 1–3).

1. Codon substitution step 1: lower usage codons of arginine (AGA and AGG) are substituted to high-frequency codon (CGU).

2. Codon substitution step 2: relatively lower frequency codons are substituted: valine (GUA to GUU), histidine (CAU to CAC), phenylalanine (UUU to UUC), tyrosine (UAU to UAC), and stop codon (UGA to UAA).

3. Construction of finally modified sequence: ModSHNL-3, with replacing of 98/259 codons of native MeHNL sequence (shown in Fig. 10.1; see Note 4).
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<tr>
<td>GCC</td>
<td>0.27</td>
<td>A</td>
<td>0.19</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>GCA</td>
<td>0.20</td>
<td>A</td>
<td>0.31</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>GCG</td>
<td>0.36</td>
<td>A</td>
<td>0.17</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

Table 10.1
Comparison of the codon usage of *E. coli* genome, aspartase sequence from *E. coli*, and MeHNL sequence from cassava

<table>
<thead>
<tr>
<th>Codon</th>
<th>Frequency</th>
<th>AA</th>
<th><em>E. coli</em> genome</th>
<th>Aspartase</th>
<th>MeHNL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAU</td>
<td>0.57</td>
<td>Y</td>
<td>0.31</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>UAC</td>
<td>0.43</td>
<td>Y</td>
<td>0.69</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>UAA</td>
<td>*</td>
<td></td>
<td>0.63</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>UAG</td>
<td>*</td>
<td></td>
<td>0.07</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>CAU</td>
<td>H</td>
<td></td>
<td>0.57</td>
<td>0.13</td>
<td>0.78</td>
</tr>
<tr>
<td>CAC</td>
<td>H</td>
<td></td>
<td>0.43</td>
<td>0.88</td>
<td>0.22</td>
</tr>
<tr>
<td>CAA</td>
<td>Q</td>
<td></td>
<td>0.35</td>
<td>0.16</td>
<td>0.67</td>
</tr>
<tr>
<td>CAG</td>
<td>Q</td>
<td></td>
<td>0.65</td>
<td>0.84</td>
<td>0.33</td>
</tr>
<tr>
<td>AAU</td>
<td>N</td>
<td></td>
<td>0.45</td>
<td>0.08</td>
<td>0.86</td>
</tr>
<tr>
<td>AAC</td>
<td>N</td>
<td></td>
<td>0.55</td>
<td>0.92</td>
<td>0.14</td>
</tr>
<tr>
<td>AAA</td>
<td>K</td>
<td></td>
<td>0.77</td>
<td>0.89</td>
<td>0.55</td>
</tr>
<tr>
<td>AAG</td>
<td>K</td>
<td></td>
<td>0.23</td>
<td>0.11</td>
<td>0.45</td>
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<tr>
<td>AAG</td>
<td>K</td>
<td></td>
<td>0.23</td>
<td>0.11</td>
<td>0.45</td>
</tr>
<tr>
<td>UAU</td>
<td>H</td>
<td></td>
<td>0.57</td>
<td>0.13</td>
<td>0.78</td>
</tr>
<tr>
<td>UUG</td>
<td>L</td>
<td></td>
<td>0.13</td>
<td>0.00</td>
<td>0.17</td>
</tr>
<tr>
<td>UUC</td>
<td>L</td>
<td></td>
<td>0.00</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>UUA</td>
<td>L</td>
<td></td>
<td>0.00</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>UUG</td>
<td>L</td>
<td></td>
<td>0.00</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>UUA</td>
<td>L</td>
<td></td>
<td>0.00</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>UUG</td>
<td>L</td>
<td></td>
<td>0.00</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>UUG</td>
<td>L</td>
<td></td>
<td>0.00</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>UUG</td>
<td>L</td>
<td></td>
<td>0.00</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>UUG</td>
<td>L</td>
<td></td>
<td>0.00</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

* asterisk represents termination codon
Fig. 10.1. Codon substitution of MeHNL gene. Upper: codon-substituted MeHNL sequence (modSHNL-3). Lower: Natural MeHNL sequence (Genbank accession no. Z29091). Identical base is indicated by an asterisk. The native MeHNL sequence contained rare codons for expression in *E. coli*.
3.2. Preparation of Substituted MeHNL Sequence by Self-Priming PCR

1. Synthesis of oligo-DNA fragments as primers (80–100 bp including 40 bp of complemented region; see Note 5).

2. First-step PCR: using initial primers (in the middle region of MeHNL sequence), without template (illustrated in Fig. 10.2; see Note 6).

3. PCR reaction mixture: 5 μL of 10X PCR buffer (attached to the DNA polymerase kit), 5 μL of dNTP mix, 2 μL of 25 mM MgCl₂, 1 μL of forward primer (40 pmol), 1 μL of reverse primer (40 pmol), 35 μL of water, and 1 μL of KOD DNA polymerase (Toyobo, Osaka, Japan).

4. PCR reaction: 1 cycle – 98°C × 10 min and 25 cycles – 98°C × 1 min, 67°C (this temperature depends on annealing temperature of complementary region of primers used) × 20 s, and 74°C × 20 s.

5. Second- to final-step PCR: using elongating primers with the former step PCR product as the template.

6. PCR reaction mixture from the second step: 5 μL of 10X PCR buffer (attached to the DNA polymerase kit), 5 μL of dNTP mix, 2 μL of 25 mM MgCl₂, 1 μL of forward primer (40 pmol), 1 μL of reverse primer (40 pmol), 1 μL of template DNA (PCR product from the previous step), 34 μL of water, and 1 μL of KOD DNA polymerase.

7. Sequence confirmed final-length DNA is prepared as an inserting DNA fragment for expression. Using 5′-end and 3′-end primers link suitable restriction enzyme site and insert fragment into expression vector (pET21a, BamHI, and NdeI digested). Transform plasmid into the host cell, BL21(DE3) (see Notes 7 and 8).
3.3. Cultivation

Media and Low-Temperature Cultivation Methods

Codon substitution increases the overall production of MeHNL (Table 10.2). However, the ratio of soluble form (active) to the insoluble form (inactive) of recombinant MeHNL may be quite low (Fig. 10.3; see Note 9). Most of the produced MeHNL exists as an inclusion body in the transformants (Figs. 10.4 and 10.5). This suggests that the productivity of the active MeHNL increases due to increase in the ratio of the soluble to insoluble forms of MeHNL.

Table 10.2

Effect of codon substitution on the active MeHNL production

<table>
<thead>
<tr>
<th>Modified MeHNL gene</th>
<th>Codon substitution sites</th>
<th>Relative specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeHNL10</td>
<td>Native sequence (Z29091)</td>
<td>100</td>
</tr>
<tr>
<td>modSHNL-1</td>
<td>R; AGA → CGU (28, 92, 129, 187), AGG → CGU (46, 155, 175)</td>
<td>101</td>
</tr>
<tr>
<td>modSHNL-2</td>
<td>R; modSHNL-1, V; GUA → GUU (2, 253), H; CAU → CAC (5, 10, 14, 20), F; UUU → UUC (6), Y; UAU → UAC (157), stop; UGA → UAA (259)</td>
<td>127</td>
</tr>
<tr>
<td>modSHNL-3</td>
<td>R, V, H, F, Y, stop; modSHNL-2, others; illustrated in Fig. 10.1</td>
<td>145</td>
</tr>
</tbody>
</table>

The numbers in parentheses represent the codon substitution site. MeHNL activity increased 1.45 times higher than that of the native sequence.

Fig. 10.3. SDS-PAGE of transformants. Lanes: 1 – soluble fraction of native MeHNL, 2 – insoluble fraction of native MeHNL, 3 – soluble fraction of modSHNL-1, 4 – insoluble fraction of modSHNL-1, 5 – soluble fraction of modSHNL-2, 6 – insoluble fraction of modSHNL-2, 7 – soluble fraction of modSHNL-3, 8 – insoluble fraction of modSHNL-3. Arrow represents recombinant MeHNL (approximately 30 kDa).
Fig. 10.4. Relationship between cultivation temperature and soluble MeHNL production. S, soluble fraction; I, insoluble fraction. Arrow represents the recombinant MeHNL (approximately 30 kDa). Lowering the cultivation temperature significantly enhanced the productivity of soluble MeHNL.

Fig. 10.5. Effect of cultivation temperature on MeHNL production. (a) Effect of cultivation temperature on cell yield and soluble MeHNL activity. Closed columns: cell growth; open columns: specific MeHNL activity. (b) Effect of cultivation temperature on productivity of active form of MeHNL. A transformant (modSHNL-3) was cultivated in shake flasks at 17, 19, 22, and 37°C using the NS-2 medium. The cell concentration and enzyme productivity were improved by lowering the temperature at the beginning of the cultivation. When the cultivation was done at 17°C, the cell yield increased five times while the specific activity of MeHNL increased 170 times (A). Therefore, the soluble MeHNL yield increased 850 times compared to the result obtained at 37°C (B).
1. Cultivate host bacteria in NS-2 medium (Section 2.2, step 4) (see Note 10).

2. Seed cultivation: one loopful of freeze stock of transformant is inoculated into 100 mL of NS-2 medium supplemented with 0.1 g/L ampicillin in shake flask and cultivated at 17°C for 48 h (see Note 11).

3. Setting up of jar-fermentor: 2-L jar-fermentor with a working volume of 1.2 L (B.E. Marubishi, Tokyo, Japan). Agitation and aeration rates are 680 rpm and 1 vvm (volume/volume/min), respectively. The temperature is adjusted at 17°C.

4. Inoculation: 24 mL of full-grown seed is inoculated into the jar-fermentor containing 1.2 L of production medium (NS-2 medium supplemented with 0.1 g/L ampicillin and 1 mM of IPTG) and the cultivation is initiated. The pH of the cultivation is maintained at pH 6.0 using 28% ammonia solution.

5. Monitoring of the growth: cell growth is measured as OD value at 660 nm (see Note 12).

6. Cultivation continues until growth reaches stationary phase. In this condition, the DO (dissolved oxygen concentration)
is maintained above 0 ppm during the cultivation period (Fig. 10.6; see Notes 13 and 14).

### 3.4. MeHNL Activity Assay

1. The culture broth is centrifuged at $15,000 \times g$ for 5 min, and the cell pellet is suspended in Na-citrate buffer (0.2 M, pH 5.5).

2. The cell suspension is sonicated by an ultrasonic disruptor under the following conditions: output, 2; time, 5 min; and duty cycle, 50 (Tomy, Tokyo, Japan).

3. The homogenate is centrifuged ($15,000 \times g$ for 10 min), and the supernatant is used for MeHNL activity assay.

4. MeHNL activity measurement is carried out by the production of benzaldehyde from D,L-mandelonitrile as the substrate (11).

5. The wavelength of spectrophotometer is adjusted to 249.6 nm, and the temperature of cuvette holder sets at 20°C.

6. 7 mL of 0.2 M Na-citrate buffer (pH 5.5) is dispensed in a screw-cap vial and incubated at 20°C.

7. 0.93 μL of D,L-mandelonitrile (Sigma-Aldrich, St-Louis, MO) is added to the vial and mixed in vortex.

8. 3 mL of substrate solution is dispensed to each cuvette (sample and reference).

9. 10 μL of enzyme solution is added and the absorbance measured every 10 s.

10. HNL activity is calculated from the following equation. One unit of MeHNL activity is defined as the amount of enzyme that decomposes 1 μmol of D,L-mandelonitrile in 1 min. 

    Volume activity (U/mL) = \[ \frac{[\text{total volume (mL)} \times \text{absorbance inclination (mABS/min)}]}{12.35 \times \text{sample volume (mL)} \times 1,000}] \]

11. Protein concentration can be measured using a BCA protein assay reagent kit (Pierce, IL) with bovine serum albumin as a standard according to the manufacturer’s instructions.

### 4. Notes

1. Cloning of the native MeHNL gene can be carried out using a previously described procedure (3).
2. The cloned sequence can be confirmed by comparison with published MeHNL sequence [MeHNL10 (EMBL accession no. Z29091)].

3. General DNA manipulations can be performed in standard fashion as described by Sambrook et al. (12).

4. It was found that aspartase gene from *E. coli* showed very high expression in our previous study. Therefore, we based this sequence to construct substituted sequence for high expression.

5. Design of several restriction enzyme recognition sites in the artificial sequence may be useful for later processes.

6. Purchase oligo-DNA fragments from a reliable producer. Especially, longer fragment (>100 bp) may include mistakes.

7. In this method, fidelity of DNA polymerase is quite important. KOD DNA polymerase (Toyobo) or other high-fidelity polymerase should be used. Confirming of sequence of PCR products should be carried out in each step.

8. A few errors may be found in the finally prepared DNA sequence, in such case, site-direct mutagenesis is available for error correction. Several kits are available (e.g., QuickChange® site-directed mutagenesis kit, Stratagne, La Jolla, CA).

9. For high-density cultivation, defined NS·2 medium is used. In the low-temperature culture, IPTG is added at the beginning of culture and the temperature maintained at 17°C throughout the cultivation period.

10. Seed cultivation at 37°C may cause longer lag period in main fermentation. Therefore, we recommend that both seed culture and enzyme production culture should be done at the same temperature.

11. Under optimum growth temperature (37°C), recombinant protein synthesis in pET system (which includes a strong T7 promoter) would be too rapid for protein expression, and most of the produced proteins would be in the form of insoluble inclusion body (Fig. 10.3).

12. The maximum specific growth rate (μ_{max}) declined from 1.27 to 0.12 h^{-1}. A temperature of 17°C is therefore recommended for practical production of MeHNL by this strain.

13. The results of our study revealed that addition of an inducer at the beginning of the culture and maintaining low temperature throughout the culture period are effective in increasing the productivity of MeHNL. At 37°C,
limitation of dissolved oxygen concentration (DO) was observed when the cell density increased. On the other hand, in the low-temperature culture (at 17° C), the rate of growth and the rate of nutrient consumption decreased. This implies that the oxygen consumption rate would also decrease, leading to maintenance of aerobic condition and thus increase in the final cell concentration.

14. The MeHNL production rate in this procedure was calculated to be 3,000 U/h. This production process can be successfully scaled up to 2-m^3 scale.

References


Introduction of the Early Pathway to Taxol Biosynthesis in Yeast by Means of Biosynthetic Gene Cluster Construction Using SOE-PCR and Homologous Recombination

Pia Dahm and Stefan Jennewein

Abstract

Metabolic engineering of plant natural product pathways in heterologous systems requires the highly concerted action of several biosynthetic genes. Besides the functional heterologous expression of the genes encoding the natural product biosynthetic pathway, often additional extensive modifications in the host primary metabolism are also needed, in order to obtain efficient supply of the required biosynthetic building blocks to support the engineered natural product biosynthesis. Selection markers in heterologous expression systems, like baker’s yeast (*Saccharomyces cerevisiae*), are often limited and the chromosomal insertion prevents later modifications of engineered pathway, e.g. exchange of gene promoters, or the introduction of additional genetic regulatory elements in a timely manner. Thus the construction of biosynthetic gene clusters on episomal expression vectors seems a logical solution for this dilemma. Although manipulation of long DNA fragments still represents a challenge, by using PCR and in vitro homologous recombination, we assembled a biosynthetic gene cluster for the concerted heterologous expression of three important genes for the metabolic engineering of taxoid biosynthesis in yeast.

**Key words:** Biosynthetic gene clusters, assembling of DNA fragments, engineering of taxoid biosynthesis in yeast.

1. Introduction

Since the beginning of civilization natural products have been used in matters of human health and disease. Hence, extensive screening campaigns for natural products were carried out leading to the identification of an enormous number of natural
products in plants and micro-organisms. Today more than 30,000 different terpenoids and 12,000 alkaloids have been defined (1). Several of these isolated compounds possess valuable biological activities. However, due to highly complex chemical structures with numerous stereocentres, synthesis by chemical means remains challenging. In addition, many of the natural producers are not amenable for established large-scale cultivation by reason of slow growth, low extraction yields or special cultivation requirements (2).

Establishing easy to culture microbial fermentation strains for the production of natural products, too difficult to be synthesized by chemical means or obtained by extraction from plant material, may offer an attractive alternative mean of supply. Taxol® and other clinical useful taxoids are still derived by extraction of plant material, either collected or obtained from plant cell culture (3–6). The metabolic engineering of \textit{S. cerevisiae} for the total biosynthesis of Taxol® from a simple carbon source such as glucose offers therefore an attractive alternative for the production of the widely used anticancer drug.

Metabolic engineering of complex natural product pathways is, however, still in its infancy today. Major obstacles in the metabolic engineering are found in the limited availability of efficient tools for the coordinated heterologous expression of multiple biosynthetic genes. Many natural product biosynthetic pathways require the coordinated expression of an array of genes responsible for distinct enzymatic reactions, not only of the natural product pathway itself, but also of the underlying primary metabolism, responsible for the delivery of the required metabolic building blocks. Taxol biosynthesis involves approximately 20 enzymatic steps starting from the universal diterpenoid building block geranylgeranyl diphosphate. Recombinant production of diterpenoid natural products in \textit{S. cerevisiae} is severely hampered by insufficient supply of geranylgeranyl diphosphate from the yeast primary metabolism (7, 8). Thus, genetic modifications of the primary metabolism were necessary to establish metabolic flux towards taxoid biosynthesis (Fig. 11.1).

Due to the limited availability of suitable selection markers in \textit{S. cerevisiae}, in particular, but also true for most other host organisms in general, efficient methodologies for the construction of biosynthetic gene clusters are needed. A limited number of technologies for the fusion of DNA fragments already exist (see Section 3), but they all bear certain advantages and disadvantages. Here we report on a new approach, which was successfully used to fuse three genes, as well as their individual promoters and terminators for optimized production of taxadiene in yeast.
Fig. 11.1. Schematic construction of engineered taxadiene biosynthetic pathway. Precursors IPP and DMAPP are formed via yeast endogenous mevalonate pathway and connected by head-to-tail condensation to form GGPP by the archaean geranylgeranyldiphosphate synthase (GGPPSsa) from *S. acidocaldarius*. Cyclization reaction to generate taxadiene is catalyzed by codon optimized taxadiene synthase (TDSco) in committing step of taxol biosynthesis. The limiting step of mevalonate pathway is bypassed expressing a truncated version of *HMG-CoA*-reductase *thIMGR* missing regulation domain.

2. Materials

2.1. Performing SOE-PCR

1. Template (in case of genomic DNA 100 ng, plasmid DNA 5–30 ng)
2. *Proofreading* DNA polymerase creating blunt-ended PCR products
3. dNTP’s 25 mM each
4. Primers 50 μM (synthesized by Invitrogen (Karlsruhe, Germany)) adding homologous overlaps to fragment (*see Section 3.1*)
5. Sterile ddH₂O
6. Agarose gel for analysis of PCR products [0.8–1.0% (w/v)] and equipment for electrophoresis (chamber, power supply)
7. PCR purification kit or in case of unspecific or smeary products agarose gel extraction kit
2.2. PCR Amplification of Fragments and Preparation for StarGate® Cloning

1. Template (5–30 ng)
2. *Proofreading* DNA polymerase which creates blunt-ended PCR products
3. dNTP’s 25 mM each
4. 5’-Phosphorylated primers 50 μM (synthesized by Invitrogen)
5. Sterile ddH₂O
6. Agarose gel for analysis of PCR products [0.8–1.0% (w/v)] and equipment for electrophoresis (chamber, power supply)
7. PCR purification kit or in case of unspecific or smeary products agarose gel extraction kit

2.3. Creating StarGate® Donor vectors

1. Standard entry cloning kit [IBA BioTAGnology (Göttingen, Germany)]
2. Purified PCR product 4–16 nM
3. 2YT medium (16 g/L peptone, 5 g/L NaCl, 10 g/L yeast extract)
4. 2YT agar plates (2YT medium supplied with 18 g/L agar–agar, 50 mg/L kanamycin, 50 mg/L X-Gal) can be used up to 3 months, when stored at 4°C
5. Restriction endonucleases *Xba*I and *Hind*III (NEB) (*see Note 9*)

2.4. Transfer into StarGate® Fusion Vectors

1. StarGate® Fusion Cloning Set (IBA BioTAGnology). Make sure to dilute F3 with the appropriate diluent before use
2. Donor vectors with inserts generated in Section 2.3
3. 2YT medium (16 g/L peptone, 5 g/L NaCl, 10 g/L yeast extract)
4. 2YT agar plates (2YT medium supplied with 18 g/L agar–agar, 100 mg/L ampicillin, 50 mg/L X-Gal) can be used for 3 months, when stored at 4°C
5. Restriction endonuclease *Bgl*II (NEB) (*see Note 9*)

2.5. Assembling of Genes

1. StarGate® Fusion Cloning Set (IBA BioTAGnology). Make sure to dilute F6 with the appropriate diluent before use
2. Fusion vectors generated in Section 2.4
3. 2YT medium (16 g/L peptone, 5 g/L NaCl, 10 g/L yeast extract)
4. 2YT agar plates (2YT medium supplied with 18 g/L agar–agar, 50 mg/L kanamycin, 50 mg/L X-Gal) can be used for 3 months, when stored at 4°C
5. Restriction endonucleases *XbaI* and *HindIII* (NEB) (see Note 9)

### 2.6. Further Fusion Steps

1. StarGate® Fusion Cloning Set (IBA BioTAGnology)
2. Donor vector with integrated fusion construct generated in Section 2.5
3. Further fusion vectors with integrated fragments to be fused to initial fusion construct Section 2.4. The choice of fusion vector thereby is depending on desired position of the particular fragment in relation to the first-round construct (if planned to be positioned at 5′-end of fusion product, choose upstream vector for recombination with first-round construct, if fragment 3 is to be fused at the 3′-end, a downstream vector has to be used)
4. 2YT medium (16 g/L peptone, 5 g/L NaCl, 10 g/L yeast extract)
5. 2YT agar plates (2YT medium supplied with 18 g/L agar–agar, 100 mg/mL X-Gal and 50 mg/mL ampicillin or 100 mg/L kanamycin)
6. Restriction endonucleases *BglII*, *XbaI* and *HindIII* (NEB)

### 2.7. Creating Expression Vector

1. Gateway® Cloning components (Invitrogen): donor vector pDONR221, BP™ and LR™ Clonase II
2. 2YT medium (16 g/L peptone, 5 g/L NaCl, 10 g/L yeast extract)
3. 2YT agar plates (2YT medium supplied with 18 g/L agar–agar, 50 mg/L kanamycin) can be used for 12 weeks, when stored at 4°C
4. 2YT medium supplied with 50 mg/L kanamycin
5. 2YT agar plates (2YT medium supplied with 18 g/L agar–agar, and 100 mg/mL ampicillin)

### 3. Methods

Several methods for fusion of DNA fragments are reported in the literature with restriction- and ligation-based assembly (15) representing probably the most applied ones. It embodies a brilliant invention for fusion of genes, but becomes more and more unpractical with growing construct length, since it depends on unique restriction sites. Development of PCR-based methods to assemble DNA fragments was therefore considered to disburden the problems of relying on suitable restriction enzyme sites
present in the DNA constructs. The procedure of SOE-PCR (Splicing by overlap extension PCR) invented by Horton et al. in 1989 (10) resembles the first of several approaches based on fusion through PCR techniques (11, 12). However, these methods are dependent on the efficient cross-annealing of intermediate PCR products, which can be challenging on thermodynamic grounds. In addition to the widely used PCR-based methods, numerous in vivo recombination approaches have been described employing recombination systems in their natural hosts.

One very recently reported method, making use of a bacterial recombination mechanism, represents the so-called domino method – an in vivo homologous recombination technology between overlapping sequences in Bacillus subtilis (13). This method was successfully used for the assembly of DNA fragments to a total length of up to 134 kb. Nevertheless, each DNA piece has to be subcloned into a constructed vector that allows accommodating large DNA fragments stably as part of the B. subtilis genome. Thus the assembling procedure becomes labour and time consuming. The presumably most astonishing achievement regarding in vivo recombination was made by Gibson et al., who performed the chemical synthesis and assembling of the complete Mycoplasma genitalium genome (14). Seventy-two kilobytes large DNA fragments were assembled into bacterial artificial chromosomes in Escherichia coli. When molecules became too large to be stably propagated in bacteria, the complete synthetic genome was fused by transformation-associated recombination cloning in yeast.

The most recent way of successful gene fusion in an eukaryotic system via in vivo recombination is certainly represented by the DNA assembler (15), a one-step construction of large biochemical pathways by in vivo homologous recombination in S. cerevisiae. However, this certainly up-to-date technology unfortunately lacks the possibility of easily exchangeable single components for flexible gene expression. Besides exploiting recombination skills in vivo, recombinatorial tools have been extracted for use in vitro as well, such as the Cre/lox system originating from bacteriophage P1. It has been successfully used to eliminate or activate gene expression, reduce the number of copies of integrated genes and to direct the site-specific insertions of transgenes (16).

Another prominent in vitro recombination system first described in 1975 (17) is now commercially available by Invitrogen. The MultiSite Gateway® technology is lambda phage based and mimics in vivo integration of viral DNA into host DNA, whereas insertion occurs by genetic recombination between phage attachment site attP and bacterial attachment site attB. The provided kit can be used for simultaneously assembling of
up to four DNA fragments into one vector. Although Invitrogen offers a broad set of Gateway® technology-compatible expression vectors, unfortunately the system is limited in the number of fragments that can be fused, which makes it not convenient for the assembly of large parts of entire biosynthetic pathways.

Besides the mentioned ones, other in vitro systems have been published like SLIC (sequence and ligation-independent cloning) (18), which is based on in vitro homologous recombination and single strand annealing for assembly of multiple DNA fragments, or the InFusion technology (19, 20) now available from Clontech. The latter is based on in vitro recombination of an insert with a linearized vector. Since the DNA fragments are added sequentially by linearizing the generated fusion construct at the intended integration site after each round, the demand of unique restriction sites when fusing large and numerous DNA fragments remains.

A completely different mode of action features the USER™ (Uracil-specific excision reagent) technology (21), whereby a single deoxyuridine (dU) residue is placed 6–10 nt away from the 5′-end of each primer. After amplification of the fragments to be fused with these primers the dU is excised by a USER mix to generate 3′ single-stranded DNA extensions. By designing the primers with complementary ends to each other, they can be assembled through the generated single-stranded DNA overlaps. A drawback of this system is the need of dU-containing primers plus a specialized DNA polymerase needed for performing PCR amplification using dU-containing primers.

The shortest recombination sites required for successful in vitro recombination are clearly offered by the StarGate® cloning technology from IBA BioTAGnology, which is based on recombinatorial sites consisting of only four nucleotides that have to be attached to the DNA fragment. The fragments to be fused and the intermediate fusion constructs are separately cloned into vectors by recombination before fusion, which maintains a high degree of flexibility by facilitating the exchange of certain fragments for order variations. Since the DNA pieces are cloned into the vectors by recombination, the procedure is not dependent on any restriction or ligation-based steps, making it suitable for numerous and large DNA fragments. The success rate of vector construction and fusion reaches almost 100%, so that this method is not time or labour intensive in spite of sequential assembling steps. Still, it is not suitable for assembly of DNA segments which have to be fused seamlessly, since it inserts an intergenic sequence between the fused fragments.

Apparently, multitudinous techniques have been developed for fusion of DNA fragments, all holding benefits and drawbacks, so that the ideal method has not been found yet. One solu-
tion therefore seems to be the combination of several systems to counterbalance the shortcomings of the individual methods currently available. By combining three of the mentioned methods, construction of one plasmid harbouring three fused genes [geranylgeranyldiphosphate synthase from *Sulfolobus acidocaldar-\textit{ius} GGPP\textit{Sa}, \textit{tHMG-CoA}-reductase \textit{tHMG\textit{R}}, codon-optimized taxadiene synthase (\textit{TDSco})] and associated regulation units (promoters: \textit{gap1\textit{p}} and \textit{adh1\textit{p}}; terminators: \textit{pho5\textit{tt}} and \textit{gcn4\textit{tt}}) was successfully performed. To maintain subsequent replacement of regulation units for balancing expression levels, the genes were fused to the respective promoters and terminators by SOE-PCR first, before being assembled with adjacent genes using StarGate\textsuperscript{®} technology. The design of intended construct therefore was divided in the following parts (Fig. 11.2).

3.1. Primer Design and Performing SOE-PCR

1. Choice of DNA polymerase: it is important to use a proof-reading DNA polymerase to reduce the risk of mutations. Furthermore, you should take into consideration that blunt ends are needed for SOE-PCR and the following recombination reaction. Although every polymerase fulfilling these conditions is suitable, the Herculase\textsuperscript{®} II fusion DNA polymerase from Stratagene was used featuring extremely short elongation times combined with high product yield and reliability.

2. Since the genes have been expressed separately before (8), the respective expression vectors were used as template for amplification. The promoters \textit{gap1} and \textit{adh1} as well as the terminators \textit{gcn4\textit{tt}} and \textit{pho5\textit{tt}} were amplified using genomic DNA extracted from *S. cerevisiae*. Primers for amplification of SOE fragments should have a melting temperature of 60–70°C and add an overlap homologous to the adjacent fragment. An example of the SOE primers for fusion of \textit{adh1} promoter with \textit{tHMG\textit{R}} gene is given below (Fig. 11.3). For performing PCR reaction using Herculase\textsuperscript{®} II fusion DNA polymerase, the following components should be mixed in a 250 μL PCR tube:

- Template in case of gDNA 100 ng
- In case of plasmid DNA 5–20 ng
- Herculase buffer 5X 10 μL
- dNTP mix (25 mM each) 0.5 μL
- Herculase II fusion DNA polymerase 0.5 μL
- Primers (50 μM) 1 μL each
- ddH\textsubscript{2}O add 50 μL

PCR programme:
- 95°C 2 min (initial denaturation step)
Fig. 11.2. Design of intended construct consisting of three subsequently performed technologies. **a** Fusion of genes with regulation units using SOE-PCR. TDSco, codon-optimized Taxadiene synthase gene; pho5tt, phosphatase terminator; gap1p, glyceraldehyde-3-phosphate dehydrogenase promoter; GGPPSsa, archean geranylgeranyl diphosphate synthase gene from *S. acidocaldarius*; gcn4tt, transcriptional activator protein terminator; adh1p, alcohol dehydrogenase promoter; thMGR, truncated HMG-CoA reductase gene lacking regulation domain. **b** Fusion of SOE-products via StarGate® technology. **c** Final expression plasmid constructed using Gateway® recombination.

PCR products were checked via agarose gel electrophoresis and purified using the PCR purification kit from Macherey/Nagel (Düren, Germany) or – in case of additional
Designed primers for amplification of *adh1p* and *tHMGR* for further fusion via SOE-PCR. Overlapping regions are marked in bold letters. *Adh1p*, alcohol dehydrogenase promoter; *tHMGR*, truncated *HMG-CoA reductase* gene; SOE *tHMGR* for, 5′-primer used for amplification of *tHMGR* gene adding overlaps for fusion with *adh1* promoter using SOE-PCR; SOE *adh1p* rev, 3′-primer for amplification of *adh1* promoter adding overlaps for fusion with *tHMGR* gene using SOE-PCR.

unspecific PCR products – via extraction kit (see Note 3) to use for SOE-PCR.

3. Amplified DNA fragments were fused by SOE-PCR to generate constructs as described above. Determination of used template concentration depends on fragment size and should result in an equal molecular ratio (see PCR mix below). An example of PCR mix and programme is given below:

<table>
<thead>
<tr>
<th>DNA fragment</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>adh1p</em> (700 bp)</td>
<td>12 ng</td>
</tr>
<tr>
<td><em>tHMGR</em> (1600 kb)</td>
<td>5 ng</td>
</tr>
</tbody>
</table>

Herculase buffer 5X

dNTP mix (25 mM each) 0.5 μL

Herculase II fusion DNA

polymerase

ddH$_2$O add 48 μL

PCR programme for generating fusion construct *adh1p-tHMGR*

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>2 min</td>
<td>(initial denaturation step)</td>
</tr>
<tr>
<td>95°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>50°C</td>
<td>30 s</td>
<td>10X</td>
</tr>
<tr>
<td>72°C</td>
<td>30 s/kb</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>3 min</td>
<td>(final elongation step)</td>
</tr>
</tbody>
</table>

Add terminal primers (1 μL each): in this case ADH1-for and tHMGR-rev

For amplification of fused PCR product use the following PCR programme:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>2 min</td>
<td>(initial denaturation step)</td>
</tr>
<tr>
<td>95°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>60–70°C</td>
<td>30 s</td>
<td>20X</td>
</tr>
<tr>
<td>72°C</td>
<td>30 s/kb</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>3 min</td>
<td>(final elongation step)</td>
</tr>
</tbody>
</table>
PCR products were checked via agarose gel electrophoresis, fragments of right length were purified using a DNA extraction kit from Machery/Nagel and then used for StarGate® amplification (see Section 3.2). Be sure to elute with water to avoid high salt concentrations.

3.2. Primer Design and Preparation of Inserts for StarGate Cloning

1. The primers for amplification of DNA fragments to be fused by StarGate® technology must be phosphorylated at the 5′-end and show a melting temperature of 60–65°C (see Note 1). To determine the required temperature you can use the following formula (see Note 2):

\[ T_m = [(A + T) \times 2 + (C + G) \times 4] - 4 = 60 - 65°C \]

2. For successful integration into acceptor vector recombination sites have to be attached to both ends (5′-end: AATG; 3′-end: GGGA) (Fig. 11.4).

---

**StarGate TDSeo rev**

NNN-AAGTAGTGTTAGTAGTTACCT-5′

5′-ATGGATGATATTCCAGATT-NNN-TTCATCAACATTACATTAA-3′

3′-TACCTATATAAGTGTAA-NNN-AAGTAGTGTTAGTAGTTACCT-5′

In case of the TDSeo gene, the start codon was completely replaced by the 5′-end recombinatorial site AATG. The recombinatorial site at the 3′-end of the tHMGR gene was attached to the gene sequence including the yeast own stop codon, since it varies from the one included in the recombination site. In case of fragments flanked by non-coding DNA regions (gap1p-GGPPSsa-gcn4tt, TDSeo-pho5tt, and adh1p-tHMGR) recombination sites were added without cutting off the initial ends (see Note 2).

For performing the PCR reaction using Herculase® II fusion DNA polymerase, components should be mixed in a 250 μL PCR tube as described below.

The success of PCR can be checked via agarose gel electrophoresis and the needed products can be purified using the PCR purification kit from Machery/Nagel or – in case of additional unspecific PCR products – via DNA extraction kit (see Note 3). The DNA must be eluted with water instead of...
Template 5–20 ng
Herculase buffer 5X 10 μL
dNTP mix (25 mM each) 0.5 μL
Herculase II fusion DNA polymerase 0.5 μL
Primers (50 μM) 1 μL each
ddH2O add 50 μL
PCR programme:

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Time</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>2 min</td>
<td>(initial denaturation step)</td>
</tr>
<tr>
<td>95</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>60–65</td>
<td>30 s</td>
<td>30X</td>
</tr>
<tr>
<td>72</td>
<td>30 s/kb</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>3 min</td>
<td>(final elongation step)</td>
</tr>
</tbody>
</table>

supplied elution buffer to minimize inhibiting salt concentrations and quantified by UV spectroscopy at 260 nm.

3.3. Creating the StarGate Donor Vector

1. All fragments to be fused have to be cloned into a donor vector separately (see Note 10).
2. Purified PCR products are diluted with water to a concentration of 4–16 nM. This concentration is dependent on fragment size and corresponds to the following values:

- 0.5 kb fragment → 1.4–5.6 ng/μL
- 1 kb fragment → 2.8–11.2 ng/μL
- 1.5 kb fragment → 4.2–16.8 ng/μL
- 2 kb fragment → 5.6–22.4 ng/μL
- 2.5 kb fragment → 7–28 ng/μL.

3. For recombination 14 μL of each diluted PCR product and 1 μL of supplied StarSolution E are added to one tube of pENTRY-IBA10 (10 μL aliquots supplied by Entry cloning kit), respectively (see Note 4).
4. Mix the samples gently by pipetting up and down and centrifuge briefly to settle down the reaction components.
5. Incubate reactions for at least 1 h at 22°C (see Note 5).
6. One vial of supplied competent E. coli cells for each reaction is thawed on ice and 10–15 μL of reaction mixture added, respectively. Mix samples carefully by pipetting and incubate on ice for at least 30 min (see Note 6).
7. The heat shock is performed for 5 min at 37°C, followed by gentle mixing via tapping the tube and cooling on ice for 5 min.
8. Add 600–900 μL of 2YT medium and incubate at 37°C for 45 min to generate the kanamycin resistance (see Note 7).

9. Plate 100–500 μL of mix on selection plates (50 mg/L of kanamycin, 50 mg/L X-Gal) and incubate at 37°C overnight.

10. Pick three to five white colonies into selection medium and isolate plasmid DNA via Nucleospin Plasmid kit (Macherey/Nagel) (see Note 3).

11. Positive plasmids can be detected by performing restriction analysis using restriction endonucleases XhoI and HindIII as described by the manufacturer (see Note 9). Restriction analysis is checked via gel electrophoresis. Positive samples should result in a 1.7 kb fragment for the vector backbone plus a fragment of the size of insert (take into account potential XbaI and HindIII restriction sites within the insert (see Fig. 11.5).

Fig. 11.5. Restriction analysis of generated donor vectors. A 1 kb ladder (NEB); B–C XbaI/HindIII restriction analysis of generated pDONORs (Insert 2.2 kb); E Negative control.

3.4. Transfer into StarGate® Fusion Vectors

1. All fragments to be fused have to be cloned into a donor vector before.

2. Depending on intended position within the final fusion construct, the DNA fragments have to be cloned in different fusion vectors. The DNA fragment to be positioned at the 5′-end has to be transferred into an upstream fusion vector (here: TDSco-pho5tt), the fragment intended at the 3′-end into a downstream fusion vector (here: gap1p – GGPPSsa – gcn4tt). There are several types of upstream vectors (pNFUSE-IBA derivatives) differing in their intergenic region, which after fusion is placed between the two fragments. A list of all commercially available regions
can be found on the StarGate® homepage (see Note 11). Since the fusion construct was intended to be expressed in yeast the pNFUSE-IBA-SD1 vector was used for cloning of \emph{TDSco-pho5tt}. For subsequent fusion, upstream fusion vectors are combined with the same type of downstream fusion vector (pCFUSE-IBA).

3. Add 12 μL of the diluted donor vectors pDONOR: \emph{TDSco-pho5tt} and pDONOR: \emph{gap1p-GGPPSsa-gcn4tt} (2 ng/μL, dilute with water) and 1 μL of StarSolutions F1-3, respectively, to the supplied aliquot of upstream or downstream fusion vector (see Note 4).

4. Mix gently by pipetting up and down and centrifuge briefly to settle down the reaction components.

5. Incubate the reaction mixture for at least 1 h at 30°C (see Note 5).

6. Thaw a vial of supplied competent \emph{E. coli} cells on ice and add 10–15 μL of reaction mixture. Mix carefully by pipetting and incubate on ice for 30 min (see Note 6).

7. Heat shock for 5 min at 37°C (see Note 6), mix gently by tapping the tube and cool on ice for 5 min.

8. Add 600–900 μL of 2YT medium (see Note 7) and incubate at 37°C for 30 min to generate the ampicillin resistance.

9. Plate 100–500 μL of mix on selection plates (100 mg/L of ampicillin, 50 mg/L X-Gal) and incubate at 37°C overnight.

10. Pick three to five white colonies into selection medium and isolate plasmid DNA via Nucleospin Plasmid kit (Macherey/Nagel) (see Note 3).

11. Detect positive plasmids (pNFUSE-IBA-SD1::\emph{TDSco-pho5tt} and pCFUSE::\emph{gap1p-GGPPSsa-gcn4tt}) performing restriction analysis using restriction endonuclease \emph{BciVI} as described by the manufacturer. Check restriction analysis via gel electrophoresis. Positive samples should result in 1.48 kb and 350 bp fragments for the vector backbone plus a fragment of the size of insert (take into account potential restriction sites within the insert).

3.5. Assembling of \emph{TDSco-PHO5tt} with \emph{gap1p-GGPPSsa-gcn4tt}

1. Fusion of two DNA fragments depends on connection by intergenic region derived from chosen upstream vector and is achieved by recombination of an upstream vector (pNFUSE-IBA SD1::\emph{TDSco-pho5tt}) with a downstream vector (pCFUSE::\emph{gap1p-GGPPSsa-gcn4tt}). In the course of that
reaction, the two fragments are assembled in a new donor vector pDONOR::\textit{TDSco-pho5tt-gap1p-GGPPSsa-gcn4tt}.

2. Add 6 μL of each fusion vector containing one of the DNA fragments (dilute with water to a concentration of 4 ng/μL) and 1 μL of StarSolutions F4–F6, respectively, to a 10 μL -aliquot of donor vector pENTRY-IBA20 (see Note 4).

3. Centrifuge briefly to collect the reaction components and incubate the reaction mixture for at least 1 h at 22°C (see Note 5).

4. Thaw a vial of supplied competent \textit{E. coli} cells on ice and add 10–15 μL of reaction mixture. Mix carefully by pipetting and incubate on ice for 30 min (see Note 6).

5. Heat shock for 5 min at 37°C (see Note 5), mix gently by tapping the tube and cool on ice for 5 min.

6. Add 600–900 μL of 2YT medium (see Note 7) and incubate at 37°C for 45 min to generate the kanamycin resistance.

7. Plate 100-500 μL of mix on selection plates (50 mg/L of kanamycin, 50 mg/L X-Gal) and incubate at 37°C overnight.

8. Pick 3-5 white colonies into selection-medium and isolate plasmid DNA via Nucleospin Plasmid kit (Macherey/Nagel).

9. Detect positive plasmids performing restriction analysis using restriction endonucleases \textit{XbaI} and \textit{HindIII} as described by provider (see Note 9). Check restriction analysis via gel electrophoresis. Positive samples should result in a 1.7 kb fragment for the vector backbone plus a fragment of the size of insert (take into account potential restriction sites within the insert).

### 3.6. Multiple Fusions

For adding a further fragment (\textit{adh1p-tHMGR}) to the generated fusion construct \textit{TDSco-pho5tt-gap1p-GGPPSsa-gcn4tt}, it had to be transferred into a fusion vector again according to Section 3.4. The resulting upstream fusion vector (pNFUSE-IBA SD1::\textit{TDSco-pho5tt-gap1p-GGPPSsa-gcn4tt}) could then be recombined with its counterpart pCFUSE::\textit{adh1p-tHMGR} (always upstream with downstream vector) containing the fragment to be fused to the first-round fusion construct as described in Section 3.5. This results in generation of a new donor vector pDONOR::\textit{TDSco-pho5tt-gap1p-GGPPSsa-gcn4tt-adh1p-tHMGR} containing fused fragments 1–3. The number of assembled DNA fragments seems not to be limited by the StarGate® technology, although incubation times get longer with growing of the construct (see Note 5).
3.7. Creating a Destination Vector

There are a set of destination vectors offered by IBA BioTAGnology for expression in different host organisms ranging from bacteria and yeast to mammals. Nevertheless, the choice is limited regarding promoter strength and induction of transgene expression. To further broaden the options, the StarGate® system was combined with an additional technology. Since the Gateway technology offers a vast variety of expression vectors differing in selection markers, copy number and promoter induction, it resembles a sophisticated method for proceeding after successful fusion via StarGate®. There are two ways of sample preparation: (i) the final fusion construct can be amplified with primers adding Gateway® sites for subsequent recombination into one of the numerous Gateway® destination vectors available, which possibly would mean the risk of mutations when the template gets too large. (ii) Gateway® sites are added to the outside located DNA fragments of intended construct by directly including in primers for amplification of StarGate® insert. The sequence of combined Gateway®–StarGate® primers is illustrated below.

Gateway® primer forward (StarGate sites are typed in bold letters): 5’-AACGGTGACCACAAGTTTGTACAAAAAAGCAGGCTAAAA-gene specific nucleotides-3’

Gateway® primer reverse (StarGate® sites typed in bold letters): 5’-TCCCGGGGACCACCTTTGTACAAAGAAAGCTGGGTC-gene specific nucleotides-3’

4. Notes

1. Primers used for amplification of StarGate® inserts should be phosphorylated. Alternatively the insert can be phosphorylated by T4-polynucleotide kinase after PCR amplification. However, this often results in a less effective approach and should be avoided.

2. If you follow the instructions of how to design the primers, amplification should work out trouble free. Nevertheless, the matter of creating the right primers can be simplified by using the “StarPrimer D’signer 2.0” programme provided on the IBA-TAGnology website.

3. There are numerous companies offering kits for extraction and purification, generally all applying the same technique. In case of single PCR product without any byproducts, you can perform PEG precipitation to get rid of enzymes, primers and inhibiting salts.
3.1 Add 150 μL of TE-buffer (10 mM Tris–HCl, 1 mM EDTA, pH8.0) to a 50 μL amplification reaction containing your PCR product.

3.2 Add 100 μL of 30% PEG 8000, 30 mM MgCl₂ (store at −20°C). Vortex and centrifuge immediately at 16,000×g (13,200 rpm in a conventional benchtop microcentrifuge) for 30–45 min at room temperature.

3.3 Note that in most cases longer centrifugation time may increase the amount of recovered DNA.

3.4 Remove the supernatant carefully immediately after centrifugation. Keep in mind that the pellet will be clear and not visible.

3.5 Dissolve the pellet in 20–50 μL TE buffer.

4. All solutions supplied by the StarGate® kits have to be stored at −80°C. Centrifuge briefly before use to collect fluid that might stick to the cover. Special attention should be paid to the guidelines for dilution of F3 and F6.

5. Incubation time can vary depending on insert size. When inserted or fused DNA fragments exceed 4 kb, incubation should be performed overnight. Keep the rest of reaction mixture for backup, in case you stop the reaction too soon.

6. *E. coli* cells supplied by the StarGate® kits are highly competent and should be used. In case of running out of stocks, you can use any competent cells (no matter of electro- or chemically competent) that feature ability of blue–white screening. Irrespective of the transformation procedure, cells should be stored at −80°C and be thawed on ice right before use. In case of chemically competent cells, heat shock can be performed alternatively for 45 s at 42°C.

7. Although cells grow faster in 2YT, inoculation medium is interchangeable with SOC or LB medium, which can all be stored at room temperature after sterilization and are stable for several months.

SOC medium:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>0.5%</td>
</tr>
<tr>
<td>Tryptone</td>
<td>2%</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>10 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 mM</td>
</tr>
</tbody>
</table>

Glucose should be passed through a 0.2 μm filter and added after autoclaving the solution with the remaining ingredients.
8. Usually kanamycin is dissolved in water (50 mg/mL) and therefore should be passed through a 0.2 \( \mu \text{m} \) filter before adding to medium. Ampicillin is dissolved in ethanol (100 mg/mL) and needs no sterilization. Antibiotic stocks can be used for years when stored at –20°C.

9. BciVI is an isoschizomer of BfuI. Both restriction endonucleases and XbaI and HindIII are available from different companies, should be stored at –20°C and used as described by the manufacturer. Be sure not to add more than 1/10 volume of endonuclease in all to avoid inhibition of restriction by the glycerol in which enzymes are stored in.

10. There are reagents to perform control reactions supplied by StarGate® kits. However, since the StarGate® technique was successful in almost 100% of cases, using kit ingredients like enzymes and cells seems to be wasting valuable resources and time.

11. IBA BioTAGnology offers a set of upstream vectors for performing StarGate® fusion technique each integrating a different intergenic sequence by which DNA fragments are linked to each other after fusion. They should be chosen depending on selected expression system. The exact sequence can be found in the StarGate® manual (www.stargate-cloning.com).

References


Biocatalytic Synthesis of Tritium (³H)-Labelled Taxa-4(5),11(12)-diene, the Pathway Committing Precursor of the Taxoid Diterpenoids

Hans Schmeer and Stefan Jennewein

Abstract

Availability of isotope-labelled metabolites often proves to be an essential prerequisite for the successful identification of a biosynthetic pathway. Also for the metabolic engineering isotope-labelled, either radioactive or non-radioactive, biosynthetic intermediates represent valuable tools for the assessment of metabolic flux, identification of unexpected biosynthetic side reactions, or for the confirmation of the functionality of the engineered reaction step. Most often the required compounds are neither available commercially nor prepared by chemical means within an acceptable time span and effort. Biocatalytic synthesis of early pathway intermediates may offer an attractive solution for this problem. For the metabolic engineering of taxol biosynthesis in a heterologous host, like yeast, isotope-labelled taxanes represent useful tools for the establishment and functional assessment of the introduced biosynthetic steps. Using Taxus chinensis taxadiene synthase expressed in the heterologous organism Pichia pastoris, we describe a method for the biocatalytic synthesis of tritium (³H)-labelled taxa-4(5),11(12)-diene, which represents the pathway committing biosynthetic precursor for all taxoid diterpenoids.

Key words: Terpenoids, taxol biosynthesis, taxadiene, radioactive probes, metabolic flux analysis, metabolic engineering.

1. Introduction

With more than 55,000 structurally diverse members reported, the terpenoids comprise the largest group of natural products (1, 2). All terpenoids are derived from the isoprenoid building blocks dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), which are either derived via the mevalonic acid or...
1-deoxy-D-xylulose-5-phosphate pathway. DMAPP and IPP serve then as substrates for a group of enzymes known as prenyltransferases for the synthesis of the linear prenyl diphosphates such as geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), yielding the universal mono- (C\textsubscript{10}), sesqui- (C\textsubscript{15}) and diterpenoid (C\textsubscript{20}) precursors, respectively. The biosynthetic pathway committing step leading to the individual mono-, sesqui- and diterpenes is then catalysed by specific terpene cyclases using these universal prenyl diphosphates.

In contrast to other natural product biosynthetic pathways, in terpene biosynthesis the entire carbon skeleton core structure is established by the action of a single enzymatic reaction. The obtained core carbon skeleton is then in most cases further modified by specific monoxygenases, acyltransferases, etc., yielding the individual terpenoids. Whereas some terpenoids, like the ones belonging to the family of essential oils, can be obtained from nature in large quantities, many complex terpenoids are only encountered in low abundance naturally. Often these high-value compounds are too complex to be synthesized economically by chemical means. Metabolic pathway engineering either of the native host (whole plant or plant cell culture) or the establishment of recombinant microbial production strains (based on \textit{Escherichia coli} or yeast) offers promising alternatives for the production of some high-value terpenoids.

Over the last three decades the highly oxygenated diterpenoid natural product taxol, derived from yew (\textit{Taxus} spp.), has emerged as one of the most efficient drugs in the treatment of a multitude of cancers. Since the first isolation in 1969 and structural determination of taxol (3), supply of this valuable compound is a major challenge. Biotechnological approaches, such as the fermentative production of higher taxanes via \textit{Taxus} cell culture (4) or the metabolic engineering of yeast (\textit{Saccharomyces cerevisiae}) (5–7), are promising undertakings to overcome the limitations of supply.

For the efficient metabolic engineering, in addition to the genes coding for the individual biosynthetic steps, access to the biosynthetic intermediates is also needed. In particular radioactive-labelled biosynthetic precursors are of great benefit, since they provide an excellent tool for the identification of unexpected metabolic side reactions in the engineered host, in addition to the highly sensitive and quantitative detection of established biosynthetic steps. The universal biosynthetic precursor of taxol biosynthesis is represented by the tricyclic [9.3.1.0\textsuperscript{3,8}] pentadecane hydrocarbon taxa-4(5),11(12)-diene, which is formed by cyclization of GGPP, catalyzed by the taxadiene synthase (8) (\textbf{Fig. 12.1}). Since there are only a few syntheses of terpenoid precursor molecules reported, most of which are extensive procedures with minor yields, the biocatalytic conversion of the
isoprenyl diphosphates GPP, FPP and GGPP utilizing a terpene synthase appears as a promising alternative for the synthesis of any terpenoid precursor. This is especially for the synthesis of radioactive-labelled terpenoids, since the isoprenyl diphosphate building blocks GPP, FPP and GGPP are commercially available as tritium (³H)-labelled derivatives.

In comparison to the majority of other terpenoid precursors, a chemical synthesis for taxa-4(5),11(12)-diene is reported, but due to the fact that this is an extensive 12 step procedure, with a low overall yield of less than 0.6% (9), a biocatalytic conversion of [³H]-GGPP seems to be the method of choice for the synthesis of [³H]-taxa-4(5),11(12)-diene. Here we report the optimized heterologous expression of taxadiene synthase, a typical terpene synthase, using a shuttle expression vector system, suitable for the expression in E. coli and Pichia pastoris. While E. coli produces predominantly irreversible misfolded inclusion bodies and only traces of soluble enzyme, the expression in P. pastoris results in a good yield of active taxadiene synthase, sufficient for the subsequent conversion of the isoprenyl diphosphate [³H]-GGPP to the key biosynthetic precursor of the taxol biosynthesis [³H]-taxa-4(5),11(12)-diene.

2. Materials

2.1. Cloning of the Taxus chinensis Taxadiene Synthase into P. pastoris Expression Vector

1. As a template for the PCR reaction, any plasmid carrying cDNA of the T. chinensis taxadiene synthase gene or any other terpene synthase can be applied.

2. PCR mix: Herculase® II fusion DNA polymerase, 5X Herculase® II fusion DNA polymerase buffer (Agilent Technologies, Böblingen, Germany), deoxy ribonucleotide triphosphate mix (25 mM each dNTP).

3. The oligonucleotides illustrated in Fig. 12.2 were synthesized by Invitrogen (Karlsruhe, Germany).
Fig. 12.2. Oligonucleotides for the PCR amplification of the *T. chinensis* taxadiene synthase cDNA. The area shaded in grey represents the nucleotides complementary to the terpene synthase gene (in this case taxadiene synthase).

4. Standard polynucleotide purification kit for the purification of 0.1–10 kb large polynucleotides, e.g. QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

5. Standard agarose gel electrophoresis equipment.

6. DNA size marker covering DNA sizes between 0.5 and 10 kb, e.g. 1 kb DNA-ladder (Carl-Roth, Karlsruhe, Germany).

7. Standard plasmid isolation kit for the isolation of plasmid DNA from *E. coli*, e.g. Plasmid Mini Kit (Qiagen, Hilden, Germany).

8. Restriction endonucleases *Eco*RI and *Not*I, the corresponding buffer 10X NEB3 and 10X BSA solution (New England Biolabs, Frankfurt (M), Germany).

9. T4 DNA Ligase (HC), 10X Reaction Buffer: 300 mM Tris–HCl, pH 7.8, 100 mM MgCl₂, 100 mM DTT, 10 mM ATP (Promega, Mannheim, Germany).

10. *P. pastoris* and *E. coli* shuttle expression vector pPICHOLI-1 (Mobitec, Göttingen, Germany).

11. Chemical or electro competent *E. coli* cells, applicable for plasmid isolation, such as *Mach1*™ (Invitrogen, Karlsruhe, Germany) with a competency of at least $10^7$.

12. The antibiotic phleomycin D1 (*Zeocin™*, Invitrogen, Karlsruhe, Germany), effective against both, bacteria and eukaryotes, e.g. *P. pastoris* (see **Note 1**).
13. LBZ medium: 1% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride in ddH2O, sterilized by autoclaving. Zeocin 50 μg/ml is added, when the temperature of the autoclaved medium is chilled below 50°C, LBZ plates containing additional 1.5% (w/v) agar–agar.

14. T7 promoter and terminator standard primers for sequencing.

2.2 Transformation of P. pastoris with pPICHOLI-1:TDS

1. YPD medium: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose in ddH2O, sterilized by autoclaving.

2. YPDZ plates: YPD medium containing additional 1.5% (w/v) agar–agar and 100 μg/ml Zeocin, added below 50°C after autoclaving.

3. P. pastoris wild-type strain X33 (Invitrogen, Karlsruhe, Germany).

4. Resuspension buffer: 10 mM Tris–HCl, pH 7.5, 100 mM lithium acetate, 10 mM dithiotreitol, 600 mM sorbitol.

5. 1 M sorbitol solution.

6. pPICHOLI-1:TDS expression vector.

7. Standard equipment for electroporation of prokaryotes, such as 2 mm Ø electro cuvettes and a electroporation device, e.g. Multiporator (Eppendorf, Wesseling-Berzdorf, Germany).

8. A shaking incubator, applicable at 160 rpm and 28–31°C.

2.3 Heterologous Expression of T. chinensis Taxadiene Synthase in P. pastoris

1. YPGZ medium: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glycerol in ddH2O, sterilized by autoclaving, 100 μg/ml Zeocin, added below 50°C after autoclaving.

2. YNB solution: 10% (w/v) ammonium sulphate, 3.4% (w/v) yeast nitrogen base without ammonium sulphate and amino acids in ddH2O.

3. Biotin solution: 0.02% (w/v) biotin in ddH2O.

4. BMMMYZ medium: 1% (w/v) yeast extract, 2% (w/v) peptone, 14 mM K2HPO4, 86 mM KH2PO4, pH 6.0 in 900 ml ddH2O, after autoclaving the medium is cooled to 50°C and 100 μg/ml Zeocin, 0.5% (w/v) methanol, 100 ml YNB solution and 2 ml biotin solution are added by sterile filtration.

5. Glass beads, with a diameter of 0.25–0.50 mm, e.g. Glasperlen (Carl-Roth, Karlsruhe, Germany).

6. Cell disruption buffer: 25 mM HEPES, pH 6.8, 5 mM dithiotreitol, 5 mM sodium metabisulphite, 5 mM sodium ascorbate, 10 mM MgCl2.
2.4. Expression Analysis of Taxadiene Synthase Using Histidine Tag-Directed Western Blot

1. 5X SDS sample buffer: 225 mM Tris–HCl, pH 6.8, 50% (v/v) glycerol, 5% (w/v) SDS, 0.05% (w/v) bromophenol blue, 250 mM dithiotreitol.

2. Standard SDS-PAGE gel electrophoresis and western blot equipment.

3. Protein ladder, covering protein sizes between 15 and 150 kDa, e.g. prestained page ruler (Fermentas, St. Leon-Rot, Germany).

4. Nitrocellulose transfer membranes, e.g. Protran (Whatman, Dassel, Germany).

5. PBS Tween buffer: 137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM di-sodium hydrogenphosphate, 1.5 mM potassium di-hydrogenphosphate, 0.05% (v/v) Tween 20 in ddH2O, should be pH 7.4 without adjusting.

6. Blocking buffer: PBS Tween buffer containing 3% (w/v) BSA.

7. Antibody 1: specifically binding to 6X histidine tag, we used the rabbit anti HIS-tag antibody (Rockland Immunochemicals, Gilbertsville, USA).

8. Antibody 2: specifically binding to the constant domain of the Antibody 1, conjugated to alkaline phosphatase; we used the goat anti rabbit AP antibody (Jackson ImmunoResearch, Newmarket, Suffolk, UK).

9. AP buffer: 100 mM Tris–HCl, pH 9.5, 100 mM sodium chloride, 5 mM magnesium chloride.

10. NBT solution: 5% (w/v) nitro blue tetrazolium in 70% dimethylformamide.

11. BCIP solution: 5% (w/v) 5-bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide.

12. AP substrate mix: 132 μl NBT solution, 66 μl BCIP solution in 20 ml AP buffer (prepare fresh immediately before alkaline phosphatase reaction).

2.5. GC/MS Verified [1-3H]-GGPP-Based Activity Test of Taxadiene Synthase

1. Reaction buffer: 25 mM HEPES, pH 8.5, 5 mM dithiotreitol, 5 mM sodium metabisulphite, 5 mM sodium ascorbate, 10 mM MgCl2, 10% glycerol.

2. Running solvent for thin-layer chromatography (TLC): cyclohexane/ethylacetate (9/1), prepare freshly before each run.

3. Radio-TLC reader (RITA Star, Raytest, Straubenhardt, Germany) or liquid scintillation counter (LS6000, Beckman Coulter, Krefeld, Germany) for the detection of tritium radiation.
4. Non-radioactive geranylgeranyl diphosphate (Sigma-Aldrich, Munich, Germany).
5. [1-3H]-labelled all trans geranylgeranyl diphosphate, 1 mCi/ml, 0.05–0.06 mCi/mmol (Biotrend, Cologne, Germany).
6. N-Pentane for the extraction of taxa-(4)5,11(12)-diene.
7. Concentrated sodium chloride solution (brine).
8. TLC silica plate: TLC Plate Silica gel 60 (Merck, Darmstadt, Germany).
10. GC column for the separation of taxa-4(5),11(12)-diene (Rxi™ – 5 ms (30 m, 0.25 mm ID), Restek, Bad Homburg, Germany).

2.6. Purification of the N-Terminal Truncated C-Terminal Histidine-Tagged Taxadiene Synthase

1. Equilibration buffer: 25 mM HEPES, pH 8.0, 500 mM sodium chloride, 10 mM imidazol, 5 mM dithiotreitol, 10 mM magnesium chloride, 1 mM PMSF.
2. Washing buffer: 25 mM HEPES, pH 8.0, 500 mM sodium chloride, 20 mM imidazol, 5 mM dithiotreitol, 10 mM magnesium chloride, 1 mM PMSF.
3. Elution buffer: 25 mM HEPES, pH 8.0, 500 mM sodium chloride, 150 mM imidazol, 5 mM dithiotreitol, 10 mM magnesium chloride, 1 mM PMSF.
4. Storage buffer: 25 mM HEPES, pH 6.8, 5 mM dithiotreitol, 5 mM sodium metabisulphite, 5 mM sodium ascorbate, 5 mM magnesium chloride, 1 mM PMSF.
5. Nickel-NTA Agarose (Qiagen, Hilden, Germany).
6. Any column suitable for gravity flow chromatography, e.g. Econo-Pac columns (Bio-Rad, Munich, Germany).
7. Dialysis tubing with a MWCO <50.000 Da, e.g. Spectra/Por 4 (Carl-Roth, Karlsruhe, Germany).

3. Methods

Since it has been reported that many terpene synthases yield predominantly inclusion bodies by heterologous overexpression in *E. coli* (10–12), as it is also the case for taxadiene synthase, we decided to produce the needed taxadiene synthase protein in the well-established expression system *P. pastoris*. For the heterologous expression of the N-terminal truncated (lacking the plastidic
targeting sequence) *T. chinensis* taxadiene synthase, we use the shuttle vector system pPICHOLI-1. As illustrated in Fig. 12.3, pPICHOLI-1 possesses features, which allow replication in *E. coli* (ColE1 Ori) and *P. pastoris* (PARS1). Thus, in contrast to other *P. pastoris* expression systems, the use of the autosome replicating pPICHOLI-1 expression vector does not require the genomic integration of the expression construct associated with the cumbersome selection for good transgene expression clones.

Transgene expression of pPICHOLI-1 in *P. pastoris* is under the control of the strong methanol-inducible AOX1 promoter. For selection pPICHOLI-1 possesses a gene coding for the bleomycin-resistance protein from *Streptoalloteichus hindustanus* (ShBle), also mediating resistance towards phleomycin D1 (Zeocin), an antibiotic effective against both bacteria and eukaryotes. The primers for the PCR amplification and cloning of the terpene synthase cDNA are illustrated in Fig. 12.2, while the variable part, representing the terpene synthase gene-specific oligonucleotides, is shaded in grey. The constant part of the primers provides the essential features, such as the C-terminal 6X histidine tag (R-Primer) and the 5′ non-transcribed region (5′UTR) with the Shine–Dalgarno and Kozak sequence.
Biocatalytic Synthesis of Tritium (³H)-Labelled Taxa-4(5),11(12)-diene (F-Primer) for the ribosomal binding in *E. coli* and *P. pastoris*, respectively.

In this protocol, the variable part of the primers is designed for the amplification of a “pseudomature” variant of the *T. chinensis* taxadiene synthase, which is N-terminal truncated by 61 amino acids, what is estimated to be the size of the N-terminal plastidic transit peptide, which is proteolytically cleaved off in the 79 kDa mature protein, purified from *Taxus* plants (8). The truncation of the enzyme is essential, since it is known that the heterologous expression of the full size cDNA results in the formation of a 98 kDa protein with almost no enzymatic activity.

### 3.1. Cloning of the *T. chinensis* Taxadiene Synthase into *pPICHOLI-1* Vector

1. In Table 12.1 the 50 µl reaction mix, which is prepared on ice in a 200 µl PCR tube, and the applied PCR reaction protocol are described.

#### Table 12.1
PCR reaction for the amplification of the taxadiene synthase gene

<table>
<thead>
<tr>
<th>PCR reaction mix</th>
<th>PCR reaction protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µl <em>Tc-tds</em> cDNA template</td>
<td>3 min 95°C</td>
</tr>
<tr>
<td>1 µl Primer-F</td>
<td></td>
</tr>
<tr>
<td>1 µl Primer-R</td>
<td>30 s 95°C</td>
</tr>
<tr>
<td>10 µl 5x Herculase buffer</td>
<td>30 s 55°C</td>
</tr>
<tr>
<td>36 µl ddH₂O</td>
<td>90 s 72°C</td>
</tr>
<tr>
<td>0.5 µl dNTP mix</td>
<td>10 min 72°C</td>
</tr>
<tr>
<td>0.5 µl Herculase</td>
<td>Forever 16°C</td>
</tr>
</tbody>
</table>

2. Following the PCR reaction, 5 µl of the product is analysed by separation together with a DNA size marker, covering DNA sizes between 0.5 and 5 kb by standard DNA gel electrophoresis techniques, applied at 120 V for 1 h, using a 0.8% agarose gel.

3. If the desired PCR product appears at the size of 2.4 kb, without any other DNA impurities, as for example primer dimers, purify the PCR product by the use of a standard nucleotide purification kit, otherwise optimise PCR reaction or extract band from gel.

4. In the next step the purified PCR product and *pPICHOLI-1* expression plasmid are digested by the simultaneous use of the two restriction endonucleases *Eco*RI and *Not*I, which efficiently digest the *pPICHOLI-1* plasmid and PCR product, amplified with Primer-F and Primer-R in a double
digestion mix. After determination of the DNA concentration, two reaction mixes are setup in a total volume of 20 μl each (adjusted with ddH₂O), with 2 μl 10X BSA solution, 2 μl 10X NEB3 buffer, 1 μl of each endonuclease (10 units μl⁻¹) and 2 μg of either the pPICHOLI-1 plasmid or PCR product. After incubation for 5 h at 37°C the digested pPICHOLI-1 plasmid and PCR product are purified by the use of a standard polynucleotide purification kit.

5. After determination of the DNA concentration, the digested pPICHOLI-1 plasmid and PCR product are applied in a 20 μl ligation mix (adjusted with ddH₂O), using 2 μl of 10X reaction buffer, 1 μl T4 Ligase (HC) (10 units μl⁻¹) and the pPICHOL-1 plasmid and PCR product in a molar ratio of 1:5, with 70 ng pPICHOL-1 plasmid and 240 ng PCR product.

6. After incubation of the ligation reaction for 10–24 h, 2–10 μl of it is directly applied to transform any competent E. coli strain, with a competency of at least 10⁷ and suitable for subsequent plasmid isolation. We transformed the electro competent E. coli strain Mach1™ in 2 mm Ø electro cuvettes by application of the Multiporator apparatus from Eppendorf with the settings for prokaryotes at 2.5 kV. Using any other electroporation device, recommendable values for the electroporation are 2.5 kV, 25 μF and 600 Ω. Positively transformed cells are selected by the incubation at 37°C on LBZ plates.

7. After incubation for 12–24 h, depending on the efficiency of the ligation reaction and the competency of the cells, 100 or more colonies should appear, from which 10 are picked to inoculate 3 ml LBZ medium.

8. When the culture reaches an OD₆₀₀ ≈ 2 (6–8 h), cells are collected by centrifugation in an Eppendorf tube, and plasmids are isolated by the use of a standard plasmid isolation kit, suitable for E. coli.

9. The isolated plasmids are analysed for successful integration of the PCR product by EcoRI/NotI restriction digest, as described before, digesting 1 instead of 2 μg isolated plasmid DNA at 37°C for 3 h.

10. Analysed by gel electrophoresis, two single bands at ~3.6 kb, representing the pPICHOLI-1 plasmid, and at ~2.4 kb, representing the truncated taxadiene synthase gene, should be detectable (Fig. 12.4).

11. If the two described DNA bands are detectable, it is recommendable to sequence the plasmid by the use of T7 promoter and terminator standard sequencing primers. The
Biocatalytic Synthesis of Tritium ($^3$H)-Labelled Taxa-4(5),11(12)-diene

3.2. Transformation of P. pastoris with pPICHOLI-1:TDS Vector DNA

1. For the preparation of electro-competent *P. pastoris* cells, a 5 ml YPD preculture of *P. pastoris* strain X33, which was incubated overnight, shaking at 160 rpm/28°C is used to inoculate 100 ml YPD with an OD$_{600}$ = 0.2 (*see Note 2*).

2. The culture is kept shaking at 160 rpm/28°C up to an OD$_{600}$ = 1.5.

3. Then, after chilling for 5 min on ice, the culture is centrifuged for 10 min at 3000×$g$ (4°C), the supernatant is discarded and the cell pellet resuspended in 8 ml resuspension buffer.

4. After 30 min of incubation at RT (without shaking), the cells are centrifuged for 10 min at 3000×$g$ (4°C).

5. The supernatant is discarded and the cells are resuspended in 1.5 ml ice-cold 1 M sorbitol by gently pipetting up and down and then transferred into an Eppendorf tube, which is centrifuged for 1 min at 8000×$g$ (4°C) in a tabletop microcentrifuge (it is recommended to keep the Eppendorf tube on ice in between each centrifugation step).

6. After five cycles of washing with ice-cold 1 M sorbitol, the cells are resuspended in a final volume of 350 μl ice-cold 1 M sorbitol, which leads to a final cell density of about $1^{10}$ cells/ml.

7. The electro-competent cells are transferred into eight ice-cold Eppendorf tubes, 60 μl cells suspension each. At this stage, the electro-competent cells can either be stored at −80°C, remaining sufficiently competent for at least 3
months, or they are used directly in the electroporation procedure described below.

8. At the beginning of the electroporation procedure, two 60 μl aliquots of electro-competent X33 cells are thawed on ice and then transferred into two sterile 2 mm Ø electro cuvettes by gentle pipetting. Electroporation cuvettes should be pre-chilled on ice for at least 5 min.

9. 0.5–3 μg of the expression plasmid pPICHOLI-1:TDS DNA and the same amount of empty vector pPICHOLI-1 plasmid DNA as a negative control are added to the electro-competent cells, after 5 min of incubation on ice a 5 ms voltage pulse of 1.5 kV is applied at 25 μF and 186 Ω by the use of any electroporation device suitable for the electroporation of prokaryotes. We used the Multiporator apparatus from Eppendorf with the settings for prokaryotes at 1.5 kV.

10. Immediately after the voltage pulse (see Note 3), 800 μl of ice-cold 1 M sorbitol is added to the electro-cuvettes and the cells are transferred into a 15 ml Falcon tube and incubated for 1 h at 28°C (without shaking).

11. 50, 150 and 600 μl of the cell suspension is plated on 28°C pre-warmed YPDZ plates and incubated for 2–3 d in the dark at 28°C, until the first colonies appear. By using 1 μg of plasmid DNA, approximately 100–500 white colonies can be expected.

3.3. Heterologous Expression of T. chinensis Taxadiene Synthase in P. pastoris

1. Two colonies, one derived from the transformation with pPICHOLI-1:TDS plasmid DNA and the other from the empty vector control, are picked from the YPDZ plates and used to inoculate 5 ml YPGZ preculture (see Note 4). Usually all colonies on the YPDZ plates should carry the expression plasmid, therefore a screening for expression is not necessary, and it is sufficient to pick a single colony (see Note 5).

2. After incubation overnight, shaking at 160 rpm (28°C), a 500 ml BMMYZ culture is inoculated with the preculture up to an OD_{600} = 1 and incubated for 36–48 h, shaking at 160 rpm (28°C), adding 0.5% methanol each 12 h.

3. The cells are centrifuged for 10 min at 3000×g (4°C), the supernatant is discarded and the cells are resuspended in 10 ml cell disruption buffer and transferred into a 50 ml Falcon tube.

4. Glass beads are added until a ca. 3 mm overlay of cell disruption buffer remains and the Falcon tube is chilled on ice for
5 min, before the cells are disrupted by five repeats of 1 min vigorously shaking followed by 1 min chilling on ice.

5. After the cell disruption, 5 ml of ice-cold cell disruption buffer are added to the Falcon tube, which is subsequently shaken for 30 s and centrifuged for 10 min at $9000 \times g$ ($4^\circ C$). The supernatant, containing the cellular proteins, is transferred to an ice-chilled 50 ml Falcon tube. This protein extraction procedure is repeated two further times, ending up with about 15 ml of crude extract.

6. The protein concentration, which is determined by Bradford analysis, is usually found between 5 and 10 mg/ml.

7. The success of expression is analysed by histidine tag-directed western blot and by enzyme activity assay as described in the two following sections.

8. If active enzyme is detectable and the crude extract is not further purified, 50% glycerol is added and it is stored at $–20^\circ C$, being stable for at least 4 months.

3.4. Expression Analysis of Taxadiene Synthase by Histidine Tag-Directed Western Blot

1. 80 $\mu$l of the crude extract or the purification fractions (described in Section 3.6) is mixed with 20 $\mu$l 5X SDS sample buffer and boiled for 5 min at $>95^\circ C$.

2. An adequate volume of the protein ladder and the protein samples from both the negative control and the pPICHOLI-1:TDS expression are loaded in same amount, 10–40 $\mu$g of total protein, to a 10% SDS gel.

3. The proteins are separated by applying 170 V for 45 min.

4. The separated proteins are transferred from the SDS gel to a nitrocellulose membrane by standard western blot techniques. In this specific case, transferring the proteins by tank blotting for 90 min at 120 V can lead to a more efficient transfer of the $\sim 85$ kDa histidine-tagged taxadiene synthase to the membrane, compared to semi-dry blotting.

5. After western blotting, the membrane is incubated for 1 h in blocking buffer and next is washed twice in PBS Tween buffer.

6. The antibody 1 is properly diluted in PBS Tween buffer (in this case 1:10000) and then incubated together with the membrane for 5–24 h at RT.

7. After three times washing in PBS Tween buffer for 10 min each step, the antibody 2, properly diluted in PBS Tween buffer (in this case 1:5000), is incubated together with the membrane for 1–3 h at RT.

8. Followed by three further 10 min washing steps in PBS Tween buffer, and 15 min equilibration in AP buffer, the AP
substrate mix is finally added and incubated together with the membrane for about 5–15 min. If taxadiene synthase is expressed successfully, and the blotting procedure worked well, dark blue signals of proper size should become visible at a size of about \( \sim 85 \) kDa.

1. The reaction mix of the activity test is listed in Table 12.2.
2. The crude extract from the blank pPICHOLI-1 plasmid expression serves as a negative control.

### Table 12.2

**Reaction mix for the activity assay**

<table>
<thead>
<tr>
<th>Activity test</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>166 ( \mu l ) reaction buffer</td>
<td></td>
</tr>
<tr>
<td>30 ( \mu l ) enzyme fraction</td>
<td></td>
</tr>
<tr>
<td>3 ( \mu l ) non-radioactive GGPP (6.66 nmol)</td>
<td></td>
</tr>
<tr>
<td>1 ( \mu l ) [1-3H]-GGPP (0.05 nmol)</td>
<td></td>
</tr>
</tbody>
</table>

3. In each 200 \( \mu l \) test reaction, a total of 6.71 nmol geranylgeranyl diphosphate (6.66 nmol non-radioactive and 0.05 nmol [1-\(^3\)H]-labelled GGPP) is applied. This is consistent with a molar concentration [total GGPP] \( \approx 34 \) \( \mu M \), which is about twofold the value of the \( K_M \) of the truncated, histidine-tagged *T. chinensis* taxadiene synthase, that was determined to be 14.8 ± 0.9 \( \mu M \).

4. The total protein concentration in each test reaction is determined by Bradford analysis of the protein solution (crude extract or purification fractions).

5. The reaction is initiated by the addition of a mix of the non-radioactive and [1-\(^3\)H]-GGPP to the pre-warmed (31°C) reaction mix.

6. After incubation for 2 h at 31°C, 50 \( \mu l \) of the 200 \( \mu l \) reaction is loaded stepwise on a TLC silica plate and analysed by thin-layer chromatography, using cyclohexane/ethylacetate (9/1) as a running solvent.

7. The TLC plate is analysed using a Radio-TLC reader. A typical chromatogram of a reaction carried out with a freshly prepared crude extract is illustrated in Fig. 12.5. Alternatively, the TLC silica plate can be scraped off and the single fractions of the silica gel can be analysed in a liquid scintillation counter.

8. The strongly hydrophilic substrate GGPP does not move significantly in the running solvent and is localized nearby the starting point, whereas the strongly hydrophobic
product taxa-4(5),11(12)-diene has a high affinity for the eluent and is expected to be localized near the running solvent front, where a distinctive peak is detectable in the chromatogram.

9. To further confirmation the identity of the peak as taxa-4(5),11(12)-diene, the reaction is analysed by GC/MS.

10. Therefore the silica gel in the region of the peak is scraped off with a sharp scalpel, transferred into a 5 ml glass tube and three times extracted with pentane.

11. After evaporation of the pentane to a total volume of 50 μl, the sample is analysed applying the described GC column and GC/MS device by running the following GC oven protocol: 200°C (3 min), 200–270°C (18 min), 270°C (3 min).

12. As shown in Fig. 12.6, two peaks are detectable, which exhibit the typical mass spectra of taxa-4(5),11(12)-diene (8.2 min) and its isomer taxa-4(20),11(12)-diene (7.6 min). Consequently, the peak localized close to the running solvent front of the chromatogram represents the product of the taxadiene synthase, a mixture of taxa-4(5),11(12)-diene (~95%) and taxa-4(20),11(12)-diene (~5%), indicating that the procedure described is a suitable method for the determination of taxadiene synthase activity.

13. Beside the peaks of taxadiene and GGPP, there is a third, smaller peak visible, running a little further than GGPP. This peak has the same retention factor as a tritium-labelled reference of geranylgeraniol. It is probable that parts of the
Fig 12.6. GC/MS analysis of taxadiene. Typical MS spectra of the two taxadiene isomers taxa-4(5),11(12)-diene (a) and taxa-4(20),11(12)-diene (b).
Biocatalytic Synthesis of Tritium (³H)-Labelled Taxa-4(5),11(12)-diene

GGPP are dephosphorylated by the action of *P. pastoris* phosphatases, forming geranylgeraniol. This peak is not detectable in activity tests carried out with nickel-purified enzyme fractions only by using the *P. pastoris* crude extract.

14. The synthesis yield of [³H]-taxa-4(5),11(12)-diene is consistent with approximately 68% of all integrated peaks, subtracted from the background. Since radioactive [1-³H]-GGPP is converted by the same rate, as the non-radioactive substrate, the total turnover of the reaction is 68%. With further knowledge of the total amount of GGPP and enzyme, applied in the reaction, the specific activity of the enzyme fraction can be determined (Formula 12.1). A typical specific activity in a freshly prepared crude extract lies between 1 and 2 nmol/h/mg.

\[
\text{Specific activity (nmol h}^{-1}\text{mg}^{-1}) = \frac{0.01 \times \text{[turnover (%)} 3\text{H-taxadiene]} \times \text{[total amount GGPP substrate]} (\text{nmol})}{\text{[total amount of enzyme]} (\text{mg}) \times \text{[reaction duration]} (\text{h})}
\]

(Formula 12.1)

Formula for the calculation of the specific activity

3.6. Purification of the N-Terminal Truncated C-Terminal Histidine-Tagged Taxadiene Synthase

1. The heterologously expressed N-terminal truncated C-terminally histidine-tagged *T. chinensis* taxadiene synthase quickly loses activity in the *P. pastoris* crude extract. Therefore care should be taken that the enzyme is kept on ice during the entire purification procedure and that the purification is carried out as quick as possible in a 4°C room.

2. The cells are disrupted by the use of glass beads as described before, using equilibration buffer instead of cell disruption buffer.

3. The crude extract is cleared of any insoluble particles by centrifugation for 30 min at 75000×*g* (10°C) and subsequent filtration through a 0.22 μm Ø filter membrane.

4. In parallel 4 ml nickel-NTA agarose stored in 10% ethanol is transferred to a gravity flow column, the nickel-NTA agarose is allowed to settle down and ethanol is discarded by gravity flow through the column, ending up with approximately 2 ml moist nickel-NTA agarose. Take care that there is always a small overlay of supernatant remaining so that the column is never running dry.

5. After the equilibration of the nickel column with five column volumes (CV) of equilibration buffer, the cleared supernatant is loaded onto the column and allowed to pass it by gravity flow, keeping 1 ml crude extract load and the flow-through fraction for further analysis via SDS-PAGE and western blot.
6. The column is washed with five CV washing buffer; keep the washing fraction for further analysis via SDS-PAGE and western blot.

7. Finally the nickel-bound proteins are eluted with three CV elution buffer.

8. The elution fraction is transferred into a dialysis tubing and dialysed for 4–8 h at 4°C in storage buffer.

9. After addition of 50% glycerol the enzyme can be stored at 20°C, stable for at least 4 months.

10. Flow-through, washing and elution fraction are analysed by SDS-PAGE, western blot (Fig. 12.7) and activity assay.

3.7. Scaled-Up Synthesis and Purification of \(^{3}\text{H}\)-taxa-4(5),11(12)-diene

1. After determination that cloning, expression and purification leads to an enzyme fraction with sufficient activity, a scaled-up reaction is set up, to convert \([1-^{3}\text{H}]\)-GGPP to \(^{3}\text{H}\)-taxadiene, as described in Table 12.3.

2. First, 100 μl of the \([1-^{3}\text{H}]\)-GGPP dissolved in ethanol is transferred into a 5 ml glass tube and the ethanol is quantitatively (see Note 6) evaporated with a N\(_2\) gas stream under a fume hood.

| Table 12.3 |
| Reaction mix for the scaled-up production of \(^{3}\text{H}\)-taxa-4(5),11(12)-diene |
| Large scale reaction |
| 170 μl reaction buffer |
| 30 μl enzyme fraction |
| 100 μl \(^{3}\text{H}\)-geranylgeranyl diphosphate (5 nmol) |
3. Then 170 μl of the reaction buffer and subsequently 30 μl of the enzyme fraction is added and the reaction is incubated for 5 h at 31°C. Every 5 h an additional 10 μl dose of the enzyme fraction is added to the reaction. The reaction is stopped after 30–40 h by addition of one volume of concentrated sodium chloride solution and two volumes of pentane.

4. The 5 ml tube is vortexed for 1 min and then centrifuged for 5 min at 5000 × g in a tabletop centrifuge. The upper pentane layer is transferred into a fresh 5 ml glass tube and the extraction is repeated twice by the addition of two volumes of pentane each time.

5. The pooled pentane phases are evaporated to a final volume of 20–30 μl and quantitatively loaded on a TLC silica plate and then separated by thin-layer chromatography as described in Section 3.5.

6. After analysis via Radio-TLC reader the area of [^3]H-taxadiene is marked on the TLC silica plate. The silica gel is scraped off using a sharp scalpel and transferred to a 5 ml glass tube.

7. The silica gel material is extracted three times with two volumes of pentane and the pooled organic phases are analysed via Radio-TLC reader, as described above. A single [^3]H-taxadiene peak with the typical retention factor of 0.93 should appear, representing the purified [^3]H-taxadiene.

4. Notes

1. The antibiotic phleomycin is unstable at high temperatures and intense light exposure. Therefore, particular care must be taken that the antibiotic is never added to any medium at temperatures >50°C and that cultures containing phleomycin are incubated in the dark.

2. P. pastoris needs very high levels of oxygen for proper growth. Therefore, it is advisable to use baffled flasks and a high shaking frequency, optimally >200 rpm.

3. The ice-cold 1 M sorbitol solution should be added immediately after the voltage pulse since P. pastoris is very sensitive to electroporation procedures and the 1 M sorbitol helps the stressed cells to recover from the electroporation pulse, leading to a significant increase in survival rate.
4. It is important to use a medium free of glucose, such as YPGZ, since glucose inhibits the AOX1 promoter expression.

5. Compared to other expression cassettes, like for example the pPICZ vectors (Invitrogen), which integrate into the genome of *P. pastoris* by homologous recombination, the autosomal pPICHOLI-1 vector does not integrate into the *P. pastoris* genome. While screening for a positive clone, sufficiently expressing the gene of interest, is often a time consuming procedure, in the case of genomic integrating systems, this is not a problem at all for the expression vector pPICHOLI-1. Normally all positively selected *P. pastoris* clones carry equal amounts of the pPICHOLI-1 expression vector and produce equal amounts of the heterologous protein.

6. It is necessary to remove the ethanol quantitatively, since traces of ethanol will inhibit the reaction.

References

USER Cloning and USER Fusion: The Ideal Cloning Techniques for Small and Big Laboratories

Hussam H. Nour-Eldin, Fernando Geu-Flores, and Barbara A. Halkier

Abstract

The explosive development of the field of molecular biology has led to the need for simpler and more efficient cloning techniques. These requirements are elegantly met by the ligation-free cloning technique called USER cloning. USER cloning is suitable not only for everyday and high-throughput cloning but also for the one-step construction of complex DNA constructs, which can be achieved in a variant called USER fusion. In this chapter, we present a general protocol for converting any vector into a USER-compatible vector, together with protocols for both USER cloning and USER fusion.

Key words: Ligation-free cloning, high-throughput cloning, seamless DNA fusion, multiple PCR fragment assembly, uracil excision-based cloning, artificial microRNA, homologous recombination, Gateway cloning.

1. Introduction

Together with the invention of PCR, the exploitation of restriction enzymes/ligase for cloning DNA fragments gave birth to the field of molecular biology. More than 20 years later, restriction digests/ligation remain being the standard cloning technique in most laboratories. However, the field has developed immensely and the need for simpler and more efficient cloning techniques has become evident. For example, functional characterization of genes from newly sequenced genomes requires the construction of gene libraries for which high-throughput cloning techniques are desired. Moreover, the assembly of complex DNA constructs requires the fusion of several DNA fragments, which is...
cumbersome with conventional cloning and therefore avoided in many research projects.

Uracil excision-based cloning was conceived in the early 1990s as a ligation-independent cloning technique that could rival conventional cloning (1). In the following years, the concept was further developed (2, 3) and in 2003, New England Bio-labs (NEB) launched the USER (uracil-specific excision reagent) Friendly Cloning Kit (4). Although simple and efficient, NEB’s version of USER cloning was published as not being compatible with proofreading polymerases, which made the technique unattractive. Shortly after, our lab faced the challenge of cloning a library of 240 genes into an expression vector. Having recognized the hidden-jewel status of USER cloning, we learned that the basis of the incompatibility had already been elucidated (5), and that a compatible proofreading polymerase, Pfu Turbo® Cx Hotstart DNA polymerase, had just been launched to the market by Stratagene, but for a completely different purpose (6). With this knowledge and after having optimized NEB’s design of USER-compatible vectors, we made a USER-compatible expression vector and USER cloned our 240 fragments in a total time of 3 weeks and having an efficiency close to 95% (7, 8).

Another big challenge our lab faced at that time was the creation of complex DNA constructs made of up to four large fragments (~1 kb each) that had to be assembled seamlessly. For this purpose, overlapping PCR was the technique commonly used. However, we tested whether we could use the USER-cloning principle to fuse the different PCR products prior to their conventional cloning as a fused fragment. The success of this idea (9) led us to develop a method for the simultaneous fusion and cloning of multiple PCR products (without prior ligation) into a USER-compatible vector, which turned out to be almost as efficient as cloning a single fragment. We dubbed this technique USER fusion (10).

USER cloning and USER fusion are nowadays routinely used in our lab, and the number of fellow labs that have adopted them is continuously growing, not only because of their simplicity and efficiency, but also because of the open-source nature, allowing researchers to adapt the technique to suit their specific needs (7, 10–15).

2. Materials

2.1. Construction of USER-Compatible Vectors

Destination vector with multiple cloning site (MCS). Appropriate restriction enzymes and buffers for digestion of destination vector. USER cassette (ordered as two 5′-phosphorylated
oligonucleotides from an oligonucleotide synthesis company). T4-DNA ligase enzyme and buffer. Competent *Escherichia coli* cells.

### 2.2. Digestion of USER-Compatible Vectors for Cloning

PCR purification kit, appropriate restriction enzymes, and buffers for digestion of the USER-compatible vector. As an example, if a USER cassette is designed and inserted as described in Section 3.1 and Note 1 the following restriction and nicking enzyme are required: *PacI* (NEB), *Nt.BbvCI* (NEB).

### 2.3. USER Cloning and USER Fusion

PfuTurbo® Cx Hotstart DNA polymerase (Stratagene), USER reagent (NEB), CaCl₂ competent *E. coli* cells (>10⁶), Taq polymerase, *DpnI* (NEB).

### 3. Methods

The power of USER cloning lies in the ability to generate long, complementary overhangs in both PCR product and destination vector. These overhangs can anneal to each other to form a stable hybridization product that can be used to transform *E. coli* without prior ligation. Most importantly, the overhangs on the PCR fragments are custom-made and their generation is not dependent on the introduction of restriction sites.

For the generation of overhangs in the PCR product, a short (∼8 nt) sequence that ends in a single deoxyuridine residue is included as an upstream extension in each primer used to amplify the target DNA. Subsequently, the resulting PCR product is treated briefly with a commercial mix of uracil DNA glycosylase (16) and DNA glycosylase-lyase Endo VIII (17, 18). These enzymes, which are included in the USER™ enzyme mix (NEB), remove the two single deoxyuridine residues and enable the dissociation of the short, single-stranded fragments lying upstream from the cleavage sites (Fig. 13.1). For the generation of overhangs in the destination vector, a short cassette is inserted into it (Section 3.1), so that digestion with a restriction and a nicking enzyme creates the desired overhangs (Section 3.2). As mentioned, the long overhangs on a treated PCR product and a digested vector form a stable hybridization product that can be used to transform *E. coli* without prior ligation (Section 3.3, USER cloning). The fact that long, custom-made overhangs can be generated on PCR products can be exploited to generate a series of PCR products with complementary overhangs. This enables the generation of a hybridization product consisting of a vector and multiple PCR products, which can be USER cloned into a compatible vector almost as easily as a single PCR product (Section 3.4, USER fusion).
3.1. Construction of a USER-Compatible Vector

1. Any vector with a multiple cloning site can be made USER compatible by inserting a small (≤30 nt) ‘USER cassette’. The overall structure of a USER cassette consists of a single restriction recognition site flanked by a nicking recognition site to each side. In between the restriction and the respective nicking sites, a single variable deoxynucleotide (nt) is placed.

2. Design the USER cassette by starting from the restriction site in the middle and build outward. This restriction site should not occur in the target vector and the cleavage should occur after a [T] in both strands (Note 1). Place a random, but non-complementary nt to each side. These ‘variable nts’ will be responsible for the directionality of the cloning
(Note 2). In each side, the variable nt is then followed by a nicking site. The nicking sites are placed in opposite orientations to each other so that two different 3’-overhangs are obtained in the vector backbone after digestion with both the restriction and the nicking enzyme (Note 3). The length of the designed overhangs should be 7–15 nt (Note 4). An example of a USER cassette is given in Fig. 13.1 (Note 5).

3. Choose two restriction sites in the multiple cloning site of the destination vector into which the cassette can be cloned. Add extra nts to the USER cassette described so far that would mimic the overhangs that would be generated on the cassette after digestion if it had had the full restriction sites at each end. Order two 5’-phosphorylated oligonucleotides which when annealed to each other, create the desired USER cassette with the extra overhangs. An example of such oligonucleotides is provided in Fig. 13.2 (Note 5).

4. Dissolve or dilute the phosphorylated oligonucleotides to give a stock concentration of 100 μM. Mix the solutions in a 1:1 ratio and dilute 10 times to give a 5 μM solution of assembled USER cassette.

5. Double digest the destination vector with the restriction enzymes selected in the multiple cloning site using the conditions recommended by the manufacturer, preferably overnight. Purify the digested vector using a spin column-based purification kit (Note 6).

6. Prepare four ligation reactions by mixing 0, 1, 4, and 15 μl of 5 μM USER cassette with 1 μl of digested vector, 2 μl of 10X T4 DNA ligase buffer, and 2 μl of T4 DNA ligase in a 20 μl ligation reaction. Incubate overnight at 16°C.
7. Transform competent *E. coli* by heat shock or electroporation. If the plasmid confers ampicillin resistance, plate onto ampicillin-containing plates directly. Otherwise, incubate the cells for 1 h at 37°C in a small volume of non-selective liquid media (shaking not necessary) and plate on selective media afterward. Incubate the plates overnight at 37°C.

8. Grow overnight cultures from several colonies appearing on the plates. Isolate plasmid DNA and verify the insertion by restriction digestion of the plasmids. In the analysis, include the unique restriction enzyme in the middle of the USER cassette. Send a plasmid preparation from a positive colony for sequencing.

3.2. Preparing USER-Compatible Vectors for Cloning

1. Inoculate five overnight cultures of at least 2 ml each with *E. coli* containing the USER-compatible vector. Purify plasmid DNA using a spin column-based commercial kit. Use one spin column per 2–3 ml overnight culture, eluting each in 60 μl of water. Pool the eluates (Note 7).

2. Digest ~20 μg of the purified plasmid with 20 units of the restriction enzyme at the appropriate temperature and in a total volume of 200 μl. Incubate overnight (Note 8).

3. The following day, add additional 10 units of restriction enzyme together with 30 units of nicking enzyme and incubate for further 2 h at the appropriate temperature.

4. Analyze 2 μl of the digestion by gel electrophoresis to verify complete linearization. A single band of the expected molecular weight should be seen.

5. Purify the digested vector using a spin column-based purification kit. Use a single spin column and elute in 100 μl TE buffer (Note 6).

6. Perform cloning of a test PCR fragment (Note 9) using 1 μL of the purified digested vector and Section 3.3. Based on the number of colonies obtained, dilute the vector preparation as to obtain ~200 colonies when repeating the cloning with 1 μl of diluted vector preparation (Note 10). Ideally, under these conditions, no colonies should grow on the negative control. Aliquot and store the diluted vector preparation (for example, in ~20 μl aliquots) at –20°C to avoid repeated freezing and thawing.

3.3. USER Cloning of Single PCR Fragments

1. Design the template-annealing part of your PCR primers as usual and add 5’ extensions that complement the overhangs of the digested USER vector, except that the 3’-end [T] in these extensions is replaced with a [U] (Note 11). As an example, the 5’ extensions for any vector containing the
USER cassette shown in Fig. 13.1 would be GGCTTAAU for the forward primer and GGTTHAAU for the reverse primer. Order the primers from your usual oligonucleotide provider (Note 12).

2. Perform PCR using the [U]-containing primers and PfuTurbo\textsuperscript{TM} C\textsubscript{5} Hotstart DNA polymerase following the manufacturer’s instructions (Note 13). Verify a successful PCR reaction by analyzing 1 \( \mu \)l of the product by gel electrophoresis.

2. If the template for the PCR reaction is a plasmid with the same selection marker as the USER-compatible target vector, selective degradation of the plasmid template will be necessary before USER treatment and cloning. For this purpose, add 20 units of \( DpnI \) per 50 \( \mu \)l of PCR reaction and incubate at 37\(^\circ\)C for 1 h (Note 14). Heat-inactivate the enzyme at 80\(^\circ\)C for 20 min before proceeding to step 4. Alternatively, the PCR product may be purified from an agarose gel after electrophoresis (eluting in water), which is also recommended if the PCR reaction results in multiple fragments.

3. Mix 10 \( \mu \)l of the raw (or the \( DpnI \)-treated PCR product) with 0.75 units USER\textsuperscript{TM} enzyme mix (NEB) and 1 \( \mu \)l linearized vector (Note 15). Prepare a negative control reaction with 10 \( \mu \)l of water instead of PCR product. Incubate at 37\(^\circ\)C for 15 min followed by 15 min at 25\(^\circ\)C.

4. Add 50–100 \( \mu \)l chemically competent (CaCl\(_2\) \( E.\ coli \) cells (>10\(^6\)) to the entire reaction and transform \( E.\ coli \) by heat shock (Notes 16 and 17). If the plasmid confers ampicillin resistance, plate onto ampicillin-containing plates directly. Otherwise, incubate the cells for 1 h at 37\(^\circ\)C in a small volume of non-selective liquid media (rotation is not necessary) and plate on selective media afterward. Incubate the plates overnight at 37\(^\circ\)C.

5. Many more colonies should have been obtained from the USER reaction with PCR product than from the negative control reaction (Note 18). Verify the correct insertion of the PCR product in three of these colonies by colony PCR or by growing overnight cultures, isolating plasmid DNA, and analyzing the plasmid by either PCR or restriction analysis (Note 19). It is advisable to use combinations of vector-specific and insert-specific primers when verifying insertion via PCR as this will give the least false-positive results. Send a plasmid preparation from a positive colony for sequencing. For general troubleshooting tips, see Table 13.1.
Table 13.1
General troubleshooting tips for USER cloning and USER fusion

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause and solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsuccessful PCR amplification</td>
<td>Try the following three troubleshooting tips, separately or simultaneously. Increase polymerase amounts (use 5.0–7.5 units instead of 2.5). Include 5% DMSO. Increase dNTP concentration from 1 to 2 mM while increasing PCR buffer concentration to 1.5X. If all these fail, try different annealing temperatures by running a gradient PCR. If no band can be seen from the gradient experiment, order new PCR primers with extended template-annealing regions</td>
</tr>
<tr>
<td>Low number of colonies</td>
<td>The amount of colonies can be limited by one of these factors: cell competency, vector concentration, and PCR product concentration. Improve any of these to get more colonies</td>
</tr>
<tr>
<td>Low efficiency (high number of colonies, but many negative ones)</td>
<td>Check whether the negative control (with no PCR product) has yielded a high number of colonies (&gt;1000). If this is the case, the USER-compatible vector might not have been fully digested. Prepare a new stock of digested vector (Section 3.2), this time increasing the amount of restriction enzyme and/or incubation times in steps 2 and 3. If the reaction with PCR product yielded more colonies than the negative control, a common cause for low efficiency is contamination from a PCR template plasmid that carries the same marker gene as the USER-compatible vector. Proceed as described in Section 3.3 (step 3) and Note 14. On top of this, in the case of USER fusion, make sure that approximately equimolar amounts of PCR products are used, as stated in Section 3.4 (step 7)</td>
</tr>
<tr>
<td>Unsuccessful cloning (all colonies negative or no colonies at all)</td>
<td>Verify that electroshek has not been used for the transformation (Note 16). Ensure that the correct version of the nicking enzyme has been used when digesting the USER-compatible vector. There are two versions of the BbvCI nicking enzyme. Nt.BbvCI is the correct version to use for the USER cassette shown in Figs. 13.1 and 13.2 (and not Nb.BbvCI). Repeat the cloning, this time with the test PCR product from Section 3.2 (step 6) and Note 9 (as positive control) in parallel with the PCR product of interest. If plenty of colonies are obtained from the positive control, but none with the PCR product of interest, order new primers and perform a new PCR. Occasionally, oligonucleotides carry fatal errors, like absence of deoxyuridines or errors in the 5′ extension. If only a few or no colonies are obtained from this positive control, make sure that the stock of digested vector used for the cloning has not been frozen and thawed repeatedly. If this is the case, increase the volume of vector used (up to 5 μl per reaction) or, alternatively, prepare a fresh stock of digested vector using Section 3.2. Do not forget to store the fresh stock of digested vector in independent aliquots.</td>
</tr>
</tbody>
</table>
3.4. USER Fusion of Multiple PCR Fragments

1. Design an in silico version of your final fusion construct using your preferred sequence analysis software (Note 20). Design the construct exactly as you want it to look like. PCR fragments can be fused completely seamlessly or with a small (<100 nt) custom-made linker in between (Note 21) (Fig. 13.3a).

2. In the region surrounding each junction site (where two PCR products are to be joined), identify a region in which an [A] and a [T] are spaced 6–17 nt apart, with the [T] coming downstream from the [A]. These regions will constitute the overlap between the respective PCR fragments and will become overhangs during the fusion process. In the case of seamless fusions, these [A]-to-[T] regions ideally span the junctions themselves (Fig. 13.3b). In the case of long linkers, these regions are ideally located in the linker itself. However, these requirements are not strict and the location of the [A]-to-[T] overlap regions in relation to the junction sites or eventual linkers is very flexible (Fig. 13.3c, d). If possible, choose [A]-to-[T] overlap regions of different lengths at each junction and of lengths different than the overhangs on the digested destination vector. As mentioned, the overlap regions will become overhangs during the fusion process, and having different overhang lengths will minimize the risk of incorrect annealing.

3. Mark the selected overlap regions clearly on your sequence on the computer screen as this will greatly facilitate the ensuing primer design (Fig. 13.3b–d).

4. For the first PCR fragment, the forward primer will consist of the standard 5′ extension (for the forward primer) for cloning into the USER-compatible vector (including the [U]; see Section 3.3, step 1) followed downstream by the template-annealing sequence (~20 nt). For the first fragment the reverse primer is designed over four steps. Step 1: From the sequence on the computer screen select ~20 nts on the top strand lying upstream of the previously defined overlap region (connecting the first and second fragment) and which are all part of fragment 1. If the overlap region is not spanning the junction, e.g., situated on fragment 2 or part of a linker, include the eventual sequence lying between the end of fragment 1 and the overlap region in the selection (Fig. 13.3c, d). Step 2: Add the overlap region itself to the selection (including the [A] and [T]). Step 3: Make the reverse complement for this sequence. Going from the 5′- to 3′-end the primer should now consist of the overlap region ending in a [T] (which is complementary to the [A] in the top strand), followed by any eventual sequence lying
Fig. 13.3. Primer design for USER fusion. a Representation of a final-fusion construct composed of four different fragments. In order to design primers, the construct is first assembled in silico (Section 3.4, step 2). In the construct shown, fragments 1 and 2 as well as fragments 2 and 3 are to be joined seamlessly, while fragments 3 and 4 are to be joined with a small linker in between. b A hypothetical sequence surrounding the first junction site, with a selected overlap region ([A]-to-[T] region) spanning the junction site (Section 3.4, step 3). Based on this overlap region, primers 1 (reverse primer for amplifying the first fragment) and 2 (forward primer for amplifying the second fragment) are designed (Section 3.4, steps 4 and 5). c A hypothetical sequence surrounding the second junction site. Because of the particular sequence shown for fragment 2, an overlap region that spans the junction site cannot be identified. Therefore, a suitable overlap region in fragment 3 (but close to the junction site) has been selected. Based on this overlap region, primers 3 and 4 are designed. d A hypothetical sequence surrounding the third junction site, including an 18-bp linker. Ideally, the overlap region would be located on the linker itself. However, because of the particular sequence shown for fragment 3 and the linker, such situation is not possible. The selected overlap region spans the junction between the linker and fragment 4. Primers 5 and 6 are designed accordingly. e Sequences of primers 1–6, stated from the 5’- to the 3’-end.
between the overlap region and fragment 1, and ending with the ~20 nts selected in step 1. Step 4: Substitute the [T] at the end of the overlap region with a [U] (Fig. 13.3b–e).

5. For the second PCR fragment, the forward primer is designed over two steps. Step 1: On the top strand select the previously defined overlap region (connecting the first and second fragment) ending in the [T]. If the overlap region is not spanning the junction, e.g., situated in fragment 1 or a linker, include the eventual sequence lying between the overlap region and the beginning of fragment 2 in the selection. Step 2: Add ~20 nts of template-annealing sequence. Step 3: Substitute the [T] at the end of the overlap region with a [U] (Fig. 13.3b–e).

6. Continue in the same fashion along the consecutive series of junctions between PCR fragments. For the last PCR fragment, the reverse primer will consist of the specific 5′ extension (for the reverse primer) for cloning into the USER-compatible vector (see Section 3.3, step 1) upstream of the template-annealing sequence.

7. Carry out the PCR amplification, USER treatment, and cloning by following steps 2–6 from Section 3.3. For step 4, instead of adding 10 μl of a single PCR product, add 10–20 μl of an approximately equimolar mixture of each of the PCR products to be fused (Note 22). Using this protocol, a large number of PCR products can be joined together seamlessly within a short period of time and with a minimum of effort (Note 23). For general troubleshooting tips, see Table 13.1.

4. Notes

1. Ideally, choose restriction enzymes with 8 bp restriction sites in order to maximize the possibility that other vectors, especially large ones, can be made USER compatible using the same cassette. From the 8 bp cutters currently available from NEB, only four cleave right after a [T]: AsiSI, PaeI, PmeI, and SwaI.

2. For the last 3 years, we have worked with USER-compatible vectors carrying single ‘variable nts’ between the restriction and nicking sites. After cloning more than 1000 PCR products, we have yet to observe a fragment that is inserted in the wrong orientation. This indicates that a single base pair difference between the 8 nt vector overhangs is sufficient to ensure directional cloning. However,
the number of nts lying between the restriction and nicking site may be increased and/or varied, which might be beneficial when having more than one USER cassette in a single vector and attempting simultaneous PCR product insertion into the different sites (13). In any case, we do not recommend a high number of variable nts that would lead to vector overhangs of more than 15 nt (Note 4).

3. Any nicking enzyme can be used, but we recommend nicking enzymes with long recognition sites like \textit{Nt.BbvCI} (7-bp site). In addition, full activity in the recommended buffer for the restriction enzyme is preferable. In principle, there is no requirement for the nicking enzyme not to cut other places in the destination vector. However, we recommend avoiding an enzyme that would nick the destination vector in two adjacent sites \(<100 \text{ bp} (4))\). This may lead to the dissociation of a small (single-stranded) oligonucleotide if the sites are equally oriented or to a virtual double strand break if the sites are oppositely oriented.

4. We do not recommend designing vector overhangs longer than 15 nt, since this might prevent the small complementary single-stranded fragments that remain in the mixture after enzyme digestion from dissociating efficiently.

5. We have chosen to insert the same USER cassette (depicted in \textbf{Fig. 13.1}) into all our destination vectors. This simplifies primer design and enables repeated use of PCR fragments for cloning into any of our USER vectors. Initially, we had designed a USER cassette with two \textit{PacI} sites at the center (7); however, we have found a single \textit{PacI} site to be sufficient, as depicted in \textbf{Fig. 13.1}.

6. For very large vectors, purification by alcohol precipitation may give better yields.

7. For routine vector preparation, we do not measure the DNA concentration in our plasmid purification. We do, however, make sure that an aliquot (typically 3 µl) of the purified plasmid gives a band of similar intensity to the strongest band of our preferred DNA ladder (typically 3 µl of a 1 µg/µl stock, strongest band having 0.1 µg/µl).

8. The amount of enzymes used in this protocol was chosen based on standard unit definitions (1 unit = 1 µg of DNA digested in 1 h in a 50 µl reaction). Under the mentioned conditions, overnight digestion by \textit{PacI} (which is stable for >8 h) should lead to complete digestion. However, for combinations of restriction and nicking enzymes other than \textit{PacI} and \textit{Nt.BbvCI}, we recommend aliquots to be taken out at various time points and analyzed for linearization by
Cloning and USER Fusion

1. Gel electrophoresis. Adjust amounts of restriction enzyme and length of digestion time accordingly.

9. Any PCR fragment with appropriate [U]-containing 5’ extensions will be suitable as a test fragment. We recommend using a ~1000 bp fragment and making a large PCR reaction (~500 μl) to keep as a positive control stock.

10. We prefer this method of normalizing the concentration of digested vector, since different vectors of similar size might perform differently based on their intrinsic sequences or on the efficiency of the nicking/restriction digestion. Routine preparation of digested pCAMBIA2300-35Su (7) by Section 3.2 gives enough material for 500–1000 cloning reactions.

11. When working with very long overhangs (>14 nt), we recommend replacing one or more of the [T]s in the remaining part of the 5’ extension by a [U]. This will increase the efficiency of overhang formation by deoxyuridine excision.

12. Most oligonucleotide providers do offer synthesizing deoxyuridine-containing primers, although at a higher cost than conventional primers. However, significant price reduction may be obtained.

13. Currently, this is the only commercially available proof-reading DNA polymerase that is compatible with [U]-containing primers. It carries a single-point mutation that abolishes the ‘read ahead’ function that otherwise makes proofreading polymerases stall when encountering a [U] in the template strand (5). For the majority of applications, the commercial Pfu Cx polymerase performs well. However, for some applications such as amplifying genomic plant DNA it is necessary to use increased amounts of polymerase (5–7.5 units per 50 μl) to obtain a PCR fragment. An improved version of Pfu Cx (with respect to yield and performance on difficult targets) may become available (Morten H. H. Nørholm, personal communication, see Note 24). Alternatively, mixtures of non-proofreading and proofreading polymerases can be used.

14. DpnI is a 4-bp cutter that only acts when its recognition site is methylated. Plasmid DNA isolated from bacteria is methylated, while the synthetic PCR product is not. The PCR product does not need to be purified before treatment with DpnI, since, from our experience, the enzyme is fully active in PfuTurbo® Cx buffer. After DpnI treatment, the PCR product does not require purification before treatment with USER™ enzyme mix and heat inactivation is sufficient.
15. When USER cloning a purified PCR fragment eluted in water the reaction must be buffered. According to the specifications for the USER\textsuperscript{TM} enzyme mix, the enzyme is fully active in all tested DNA polymerase buffers. A comprehensive list of compatible buffers can be found online (4). We prefer to perform the USER treatment in either the PfuTurbo\textsuperscript{®} C\textsubscript{x} Hotstart DNA polymerase buffer or standard Taq buffer.

16. Transformation should not be done by electroporation, as the electroshock causes the circular hybridization product (USER-treated PCR product + digested vector) to dissociate and no positive colonies will be obtained.

17. Different protocols for heat shock transformation of *E. coli* cells exist, some of them very long. In our experience, 2 min initial incubation on ice, 1\(\frac{1}{2}\) min heat shock at 42°C, and 2 min final incubation on ice are sufficient for an efficient transformation.

18. Ideally, the negative control reaction should not yield colonies. However, this is rarely the case. Sometimes, less colonies are obtained from the USER reaction with PCR product than from the negative control reaction. Surprisingly, we have often found that, in these cases, the fewer colonies from the reaction with PCR product are almost all positive. Therefore, colonies from the reaction with PCR product should always be tested.

19. We use PCR on colonies when performing the cloning of numerous PCR products, which avoids growing numerous overnight cultures from which to prepare plasmids for analysis. However, when cloning less than 10 different PCR products, we skip the PCR on colonies and only analyze the purified plasmids from overnight cultures. Typically, the three selected colonies are positive, and we then send one colony for sequencing.

20. The design can also be done using pencil and paper, but we recommend using a sequence analysis software, as automatic display of the complementary DNA strand (in the desired fusion sequence) makes primer design much easier.

21. Since the linkers are to be included as part of the PCR primers, the length of the eventual linkers is limited by how long synthesized primers can be before they start carrying sequence errors. In our experience primers larger than 80 nt usually carry errors that are only discovered after the whole USER fusion has been performed. However, the quality of DNA synthesis continues to improve.
22. It is sufficient to estimate the relative molar concentration of the PCR products by analyzing aliquots (1–5 μl) by gel electrophoresis, which is routinely done to check the success of the PCR reactions. Remember that the intensity of the staining is proportional to the mass of stained DNA and not to the number of moles. For example, if two PCR products, one twice the size of the other, are stained equally on an agarose gel, then the bigger PCR product is present in half the concentration of the other one (in terms of number of moles/unit of volume). Then, if these two fragments are to be fused, one should add ~7.3 μl of the biggest product and ~3.7 μl of the smallest one to the USER reaction.

23. So far, we have not reached the limit of USER fusion with regard to number of fragments and length of the fusion product. The largest fusion construct we have made consisted of four fragments of 2 kb, 3 kb, 700 bp, and 300 bp into a 10-kb vector. Success rate was approximately 50% (50% of the analyzed colonies were positive), so we expect that fusing a larger number of fragments will still be possible.

24. The [U] compatible PfuX7 polymerase has now been described as a V93Q version of Pfu which has been fused to the small DNA-binding protein Sso7d from the thermophilic archaea Sulfolobus solfataricus (19). The polymerase has been tested in a number of laboratories with outstanding and superior performance on complex templates such as plant genomic DNA or very large templates (>10kb). Plasmids carrying the improved polymerase for production and His-tag purification can be obtained from Dr. Morten H. H. Nørholm or from our lab.

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References

Chapter 14

Enrichment of Carotenoids in Flaxseed by Introducing a Bacterial Phytoene Synthase Gene

Masaki Fujisawa and Norihiko Misawa

Abstract

Carotenoids are well-known natural pigments, typically ranging from yellow to red. Carotenoids are industrially utilized as functional materials due to their strong antioxidant properties. Phytoene synthesis is known to be a rate-determining step in the entire carotenoid biosynthetic pathway in plants. We show methods of pathway engineering for the enrichment of carotenoids in flaxseed (linseed; Linum usitatissimum L.), which is an industrially important oleaginous crop. A phytoene synthase gene (crtB) derived from a soil bacterium Pantoea ananatis (formerly called Erwinia uredovora) strain 20D3 was introduced into L. usitatissimum WARD cultivar. The resulting transgenic flax plants formed orange seeds, which contained phytoene, α-carotene, β-carotene, and lutein. The total carotenoid amount in the transgenic seeds was 156 μg/g fresh weight at the maximum, corresponding to 18.6-fold increase compared with that of untransformed controls.

Key words: Carotenoids, linseed, flax, phytoene synthase, provitamin A.

1. Introduction

Carotenoids are C_40 isoprenoids (tetraterpenes) and well-known natural pigments, typically ranging from yellow to red, which are produced in all plants and algae. They are biosynthesized in the plastids (chloroplasts) from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) that are generated through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (also called non-mevalonate pathway) (1) (Fig. 14.1). Carotenoids are essential to plants as components of the light-harvesting antenna complex in photosynthesis and as a precursor of a plant hormone, abscisic acid (ABA). The pigments are also indispensable
in humans as a precursor of vitamin A (retinol), since vitamin A (retinol) deficiency (VAD) causes serious symptoms such as irreversible blindness and corneal drying (2). Moreover, carotenoids are expected to protect human body from oxidative stresses by removing free radicals, which are considered as a main cause of a number of chronic diseases such as cancer and cardiovascular diseases (3, 4). For these reasons, carotenoids have received worldwide attention as functional ingredients in food.

Linseed flax (*Linum usitatissimum* L.) is an industrially important oleaginous crop. Flaxseed is an excellent source of seed oil and dietary fiber. Linseed oil abundantly includes α-linolenic acid (18:3), which is a type of ω-3 fatty acid and essential for human health, and lignan that is one of phytoestrogens and acts as an antioxidant (4). If carotenoids are enriched in flaxseed in addition to these nutrients, it would be expected to enhance its utility for human health. We have performed genetic manipulation of the carotenoid biosynthetic pathway in flaxseed through *Agrobacterium*-mediated transformation with the
bacterial phytoene synthase gene \((crtB)\), which was derived from a soil bacterium \(Pantoea ananatis\) (formerly called \(Erwinia uredovora\)) strain 20D3. Since phytoene synthesis from geranylgeranyl diphosphate (GGPP) is known to be a main rate-determining step in the entire carotenoid biosynthetic pathway (5–8), the introduction of \(crtB\) for its overexpression is an effective way to reinforce this step and lead to elevation of the total carotenoid amount in flaxseed. Here, we describe methods for transformation of flax plants using the \(crtB\) gene and for carotenoid analysis of resultant transgenic flaxseed using high-performance liquid chromatography with photodiode array detector (HPLC–PDA). The resulting transgenic flax plants generate orange seeds (embryos), in which the total carotenoid amounts are significantly increased, while untransformed flax plants form light-yellow seeds.

2. Materials

2.1. Plant Materials and Agrobacterium-Mediated Transformation

All of media and tools should be pre-sterilized by the supplier or sterilized by autoclave (at 121°C for 15 min) or dry heat (at 180°C for 3 h) prior to use.

1. Flaxseed [elite line WARD cultivar (accession PI523082)] is distributed from the North Central Regional Plant Introduction Stations (NCRPIS), part of the United States National Plant Germplasm System. Store at 4°C.

2. \(Agrobacterium tumefaciens\) strain GV3101 (pMP90) harboring plasmid pBIScrtB or pBIFcrtB (see Fig. 14.2 and Note 1): the glycerol (15–25%) stocks are stored at –80°C.

![Fig. 14.2. Structure of plasmids pBIScrtB and pBIFcrtB that are used in this protocol. Abbreviations: LB and RB, left and right borders of T-DNA; P nos and T nos, promoter and terminator of nopaline synthase gene \((nos)\); \(nptII\), neomycin phosphotransferase II gene; P CaMV 35S, cauliflower mosaic virus (CaMV) 35S constitutive promoter; P AtFAE1, fatty acid elongase 1 gene \((FAE1)\) seed-specific promoter from \(A. thaliana\); tp, transit peptide sequence from pea ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) small subunit (SSU).]
3. MS liquid medium: Murashige and Skoog (MS) medium (pH 5.6–5.8) (9) composed of MS basal salts mixture (Wako, Osaka, Japan), MS vitamin (Sigma-Aldrich, St. Louis, MO), and 3% sucrose. Store at 4°C.

4. MS agar medium: MS liquid medium with 0.8% Bacto Agar (BD, Franklin Lakes, NJ). Store at 4°C.

5. Germination medium: 30 mL of MS agar medium is dispensed in a plant box (CUL-JAR300; Iwaki Lab. Ware, Chiba, Japan). Store at 4°C.

6. Inoculation medium: 25 mL of MS agar medium is dispensed in a plastic Petri dish (Φ 90 × 15 mm, Terumo Corporation, Tokyo, Japan). Store at 4°C.

7. Shoot regeneration medium: 25 mL of MS agar medium containing 200 mg/L cefotaxime sodium (Claforan, Duchefa Biochemie BV, Haarlem, the Netherlands), 50 mg/L kanamycin, 0.1 mg/L 1-naphthaleneacetic acid (NAA), and 1 mg/L benzyladenine (BA) is dispensed in a plastic Petri dish (Φ 90 × 20 mm, Terumo). Store at 4°C.

8. Root regeneration medium: 25 mL of MS agar medium containing 1 mg/L NAA and 100 mg/L Claforan is dispensed in a plant box (Iwaki). Store at 4°C.

9. YEB liquid medium (1.0 g/L yeast extract, 5.0 g/L beef extract, 5.0 g/L polypeptone, 0.1 g/L MgSO₄ · 7H₂O in deionized water) (10) containing 50 mg/L kanamycin and 25 mg/L rifampicilin. Store at 4°C.

10. 70% (v/v) ethanol. Store at room temperature.

11. 2% (v/v) sodium hypochlorite. Prepare just before use.

12. 50 ml clear polypropylene (PP) centrifuge tube.

13. Distilled water.

14. Plant box (Iwaki) filled halfway with vermiculite is prepared for acclimation.

15. Growth chamber is set with the following conditions: 14 h at 20°C under continuous light and 10 h at 18°C in darkness.


17. Pot (18–24 cm diameter, 2.2–5.1 L) filled with common culture soil is prepared for cultivation.

18. Filter paper (No. 2, 300 mm diameter, Advantec Toyo, Tokyo, Japan).

19. Liquid fertilizer: dilute a stock solution of common liquid fertilizer with water to appropriate concentration. We use
Enrichment of Carotenoids in Flaxseed

2.2. Carotenoid Analysis

1. Methanol (HPLC grade).
2. Tris/NaCl solution: 50 mM Tris–HCl (pH 7.5) and 1 M NaCl. Store at room temperature.
3. Chloroform (HPLC grade).
4. Ethyl acetate (HPLC grade).
5. HPLC: a 2695 separation module (Waters, Milford, MA, USA) equipped with a 2996 photodiode array (PDA) detector (Waters).
6. Nova-pak C18 reverse phase column (3.9 × 300 mm, 60 Å pore size, and 6 μm particle size, Waters) is attached to the separation module.
7. Separation solvent (mobile phase): acetonitrile/methanol/2-propanol (90/6/4). All reagents should be HPLC grade.
8. 12 mm × 32 mm polypropylene screw neck vial (300 μL) with LectraBond cap and PTFE/Silicone septa (Waters).
9. 15 mL clear polypropylene (PP) centrifuge tube.
10. Mortar and pestle.
11. Centrifugal evaporator.

3. Methods

3.1. Preparation of Flax Hypocotyls

1. Put 100 flaxseeds into a 50 mL centrifuge tube. Pour 30 mL of 70% (v/v) ethanol into the tube and wash the seeds for 1 min.
2. Remove the ethanol solution and rinse the seeds twice with 30 mL of distilled water. Remove the water and pour 30 mL of 2% (v/v) sodium hypochlorite and incubate for 20 min with gently shaking for sterilization on the surface of the seeds.
3. Remove the sodium hypochlorite solution and rinse the seeds more than five times with 30 mL of distilled water.
4. Put the seeds onto germination medium in plant boxes (25 seeds per box) and incubate them in a growth chamber until the generation of hypocotyls with a small true leaf (approximately for 10 d; see Note 2).
3.2. Inoculation of Agrobacterium to Flax Explants

1. *A. tumefaciens* GV3101 (pMP90) strain harboring plasmid pBIScrB or pBIFcrB are cultured in two 50 mL disposable tubes containing total 25 mL of YEB liquid medium at 28°C in dark with shaking for 2 d before inoculation in the flax hypocotyls.

2. Collect the *A. tumefaciens* cells by centrifugation (7,000 rpm, 10 min, 4°C).

3. Remove the supernatant and suspend the cells with 25 mL of MS liquid medium per tube. Transfer the suspension into a plastic Petri dish.

4. Remove cotyledons and roots from the flax seedlings using scalpel and forceps on a plate (e.g., a sterilized square plastic Petri dish) and cut the hypocotyls (2–3 cm long) to form 1 cm long explants.

5. Move quickly the explants into the *Agrobacterium* suspension culture to avoid dryness and immerse them for 20–40 min.

6. Collect the explants and put them onto filter paper to remove an excess of the suspension culture.

7. Plate the explants onto inoculation medium, seal the dishes with parafilm, and incubate them in growth chamber for 3 d.

3.3. Removal of Agrobacterium from Flax Explants and Regeneration of Transformed Flax Shoots

1. Prepare MS liquid medium containing 0.5 g/L Claroan into a new plastic Petri dish and immerse the explants in the above medium to remove the *Agrobacterium* cells.

2. Put the explants onto a filter paper to remove an excess of the medium and move them onto shoot regeneration medium (almost 100 explants per dish).

3. Seal the dish with parafilm and incubate in growth chamber for 3 weeks. The explants and the generated calli are transferred onto fresh shoot regeneration medium every 3 weeks. An example of the results is shown in Fig. 14.3. If *Agrobacterium* colonies appear around the explant or callus during incubation, move the other explants without *Agrobacterium* onto a new medium and continue incubation.

4. After second subculture (45 d after the *Agrobacterium* inoculation), a shoot begins to be regenerated from the callus. Cut out the callus with the shoot from the explant and culture it independently on shoot regeneration medium until the shoot grows to reach the lid of the dish (at least approximately 3 cm long). An example of the results is shown in Fig. 14.3 (see Note 2).
3.4. Root Formation of the Regenerated Flax Shoots and Cultivation of the Transgenic Flax Plants

1. Separate the 3 cm long shoot from the callus and place the shoot in root generation medium.

2. After root is generated and elongated enough, put out the transformed plantlet from the medium and carefully remove the medium from the roots.

3. Plant the plantlet into vermiculite in a plant box and pour liquid fertilizer. Cover the box (e.g., with a plastic bag) to
maintain humidity and incubate it in growth chamber for a week. Make small holes in the bag and gradually widen them for acclimation of the plantlet.

4. Remove the bag and incubate it in growth chamber for another week.

5. Put the plantlet together with vermiculite into cultivation soil in a pot and cultivate it in a physically closed greenhouse. Water the plant not to dry and pour liquid fertilizer every week. When the transformed plant grows to some extent, a support could be needed to avoid it falling down. Flowers are generated in a month, and then mature seeds are formed in another month. For self-pollination, it is recommended to handle pollen with a cotton swab. Mature seeds are collected, air dried, and stored at 4°C (see Note 3).

3.5. Extraction of Carotenoids

We have extracted total carotenoids from flaxseed, based on the method described by Fraser et al. (11) and also by Fujisawa et al. (12) as follows. Whenever possible, all manipulations should be carried out on ice and shielded from strong light.

1. Weigh each flaxseed and grind it using mortar and pestle.
2. Add 1.5 mL of methanol and put the suspension into a 15 mL centrifuge tube. Mix it by inversion for 5 min at 4°C.
3. Add 1.5 mL of Tris/NaCl solution in the tube, mix it, and incubate for 10 min on ice.
4. Add 1 mL of chloroform in the tube, mix it, and incubate for 10 min on ice.
5. Centrifuge at 3,500 rpm (3000 × g) for 5 min at 4°C.
6. Collect lower phase (chloroform phase) into a 1.5 mL tube. If the aqueous phase remains colored, add another 1 mL of chloroform to the phase and extract again.
7. The chloroform extracts are dried by centrifugal evaporation (or under a stream of nitrogen). A dried residue is stored under an atmosphere of nitrogen at −20°C prior to HPLC.
8. Dissolve the dried residue with 100 μL of ethyl acetate and transfer it into a 300 μL vial for HPLC (see Note 4).

3.6. Carotenoid Analysis Using HPLC–PDA

We have analyzed carotenoids in flaxseed in accordance with the method described by Misawa et al. (13).

1. Set the vial on a rack and inject 10–20 μL of the sample for HPLC–PDA analysis. Carotenoids are analyzed using the separation solvent with a flow ratio of 1 mL/min at 25°C. Carotenoids are detected at wavelengths of 460 nm for lutein, α-carotene, and β-carotene or 287 nm for phytoene.
2. Carotenoids are identified and quantified by comparing retention time, spectra, and peak area with those of the authentic standards. An example of the results is shown in Fig. 14.4 (see Note 5).

4. Notes

1. The gene encoding phytoene synthase (crtB) from soil bacterium *P. ananatis* strain (ATCC 19321) (14) was fused with the transit peptide (tp) sequence from the pea ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) small subunit to import the crtB gene product (CrtB) to plastids (5, 15–17). For the expression of crtB, we adopted the cauliflower mosaic virus (CaMV) 35S constitutive promoter and the *Arabidopsis thaliana* FAE1 seed-specific promoter (18). These promoter-tp-crtB constructs were inserted into the T-DNA region of the binary vector pBI121 (Clontech, Mountain View, CA) by replacing its CaMV 35S promoter-GUS construct.

2. The growth rate of the seedlings depends on the condition of the seeds, temperature, and/or light condition in the growth chamber.

3. During subculture (especially earlier stage), shoots that lack the *nptII* gene as well as *crtB* occasionally are regenerated. To distinguish transformed shoots from these false-positive
(escape) ones, all regenerated shoots are screened by PCR analysis using the primer pairs for the *crtB* (12).

4. If debris is found, the sample should be filtered prior to HPLC–PDA analysis.

5. In this case, two transgenic flax, S10-1 (pBIScrtB) and F28-5 (pBIFcrtB) lines, formed orange seeds (embryos) which contained 20.4 and 24.0 μg/g fresh weight (FW) of lutein, 26.8 and 12.5 μg/g FW of α-carotene, 58.2 and 49.4 μg/g FW of β-carotene, and 23.9 and 14.4 μg/g FW of phytoene, respectively, while untransformed seeds contained 8.4 μg/g FW of lutein, which corresponded to the total carotenoid amount. Total carotenoid amounts in these seeds were 156.3 and 100.2 μg/g FW (corresponding to 18.6- and 11.9-fold increase), respectively, compared with those of untransformed controls (12). These results suggest that the flux of phytoene synthesis from GGPP was first promoted by the expressed *crtB* gene product (CrтB) and then phytoene was consecutively metabolized to the downstream metabolites α-carotene, β-carotene, and lutein, as catalyzed by endogenous carotenoid biosynthetic enzymes in seeds. For more details, see Fujisawa et al. (12)

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Chapter 15

Metabolic Engineering by Plastid Transformation as a Strategy to Modulate Isoprenoid Yield in Plants

Tomohisa Hasunuma, Akihiko Kondo, and Chikahiro Miyake

Abstract

Plants synthesize a large number of isoprenoid compounds that have diverse structures and functions. All isoprenoids are synthesized through consecutive condensation of five-carbon precursors, isopentenyl diphosphate (IPP) and its allyl isomer dimethylallyl diphosphate (DMAPP). With recent success in the cloning of genes that encode the enzymes of isoprenoid biosynthesis, genetic engineering strategies for the improvement of plant isoprenoid metabolism have emerged. Plastid transformation technology offers attractive features in plant genetic engineering. It has many advantages over nuclear genome transformation: high-level foreign protein expression, no need for a transit peptide, absence of gene silencing, and convenient transgene stacking in operons. We demonstrated that this technology is a remarkable tool for the production of isoprenoids in plants through metabolic engineering. The expression of bacterial genes encoding CrtW (β-carotene ketolase) and CrtZ (β-carotene hydroxylase) or cyanobacterial genes encoding DXR (1-deoxy-D-xylulose-5-phosphate reductoisomerase) in the plastid genome leads to alteration in isoprenoid content of tobacco leaves.

Key words: Plastid transformation, metabolic engineering, isoprenoid, carotenoid, astaxanthin.

1. Introduction

The isoprenoids, which constitute the most diverse group of natural products, serve numerous biochemical functions in plants (1). They play essential roles as photosynthetic pigments (carotenoids, the side chain of chlorophylls), as quinones in electron carriers, components of membranes (sterols), in subcellular targeting and regulation (prenylation of proteins), and as hormones (gibberellins, brassinosteroids, abscisic acid, cytokinins). With recent success in the cloning of genes that encode the enzymes of isoprenoid biosynthesis (Fig. 15.1), genetic engineering strate-
Fig. 15.1. Biosynthetic pathway of isoprenoids (a) and carotenoids (b). Enzymes are indicated by their gene assignment symbols: DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; CrtW, β-carotene ketolase; CrtZ, β-carotene hydroxylase; GAP, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; HMBPP, hydroxymethylbutenyl-4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; Phytyl-PP, phytyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.
gies for the improvement of plant isoprenoid metabolism have emerged (2, 3).

Plastids of higher plants have their own genome that can be genetically engineered by insertion of foreign genes. Plastid transformation offers several advantages over nuclear transformation and appears to be the method of choice to engineer metabolic pathways that are localized in the plastid, although the technology is presently applicable to only a few crops (4, 5). The expression of a transgene in plastids eliminates the need for a transit peptide required for the import of proteins synthesized in the cytosol into plastids (6). Moreover, following translocation across the plastid membranes, the transit peptide needs to be proteolytically removed to yield a mature functional protein. However, nuclear transformants can accumulate unprocessed proteins that are not functional and that can interfere with the expected results. These problems can be circumvented by using plastid transformation. In addition, because the plastid genome is highly polyploid (500–10,000 genome copies per cell) (7), the expression level of a transgene is likely to be high because it is present in high copy number and thus has the potential to confer higher levels of enzyme protein accumulation compared to nuclear transgene expression (8). Recently, the expression of a cyanobacterial 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) gene under the control of the tobacco \textit{psbA} promoter caused 350-fold higher DXR activity, which led to increased levels of various isoprenoids such as chlorophyll \textit{a}, \textit{b}-carotene, lutein, solanesol, and \textit{b}-sitosterol (9). Furthermore, we succeeded in producing more than 0.5% (dry weight) high-value carotenoid, astaxanthin (more than 70% of total carotenoids), in tobacco leaves, which turns the leaves reddish brown, by expressing both genes encoding CrtW (\textit{b}-carotene ketolase) and CrtZ (\textit{b}-carotene hydroxylase) from the marine bacterium \textit{Brevundimonas} sp. in the chloroplasts (10). In the transplastomic tobacco, total carotenoid content was 2.1-fold higher than that in wild-type tobacco.

The procedure for plastid transformation is technically demanding, and the construction of a plantlet regeneration system from either leaf explants or callus that will be damaged by the gene transfer manipulation (e.g., particle bombardment and chemical treatment) is required. Tissue growth conditions including the concentration of selection agents in cultures have great influence on the efficiency of transformant regeneration. Until recently, the application of plastid transformation to metabolic pathway engineering was restricted to the model species such as tobacco, which can be transformed relatively easily. In this chapter, we introduce the methods for generating transplastomic tobacco plants, and describe the procedures for analyzing transgene expression and the level of metabolites in the transplastomic plants.
2. Materials

2.1. Production and Growth of Transplastomic Plants

1. Murashige–Skoog (MS) medium (11) (adjusted to pH 5.7 with KOH) supplemented with MS salts (Wako Pure Chemical Industries, Ltd., Osaka, Japan), MS organics (1 mg l⁻¹ thiamine, 5 mg l⁻¹ pyridoxine, 5 mg l⁻¹ nicotinic acid, 2 mg l⁻¹ glycine, 100 mg l⁻¹ inositol), 30 g l⁻¹ sucrose, and 2 g l⁻¹ gellan gum (Wako).

2. Agripot AP-2 containers (φ 78 mm × 110 mm; Wako).

3. Biolistic PDS-1000/He delivery system (Bio-Rad Laboratories, Inc., Hercules, CA) and consumables: rupture disks (900 psi), macrocarriers, and stopping screens.

4. RMOP medium consisting of MS salts, MS organics, 1 mg l⁻¹ 6-benzyladenine, 0.1 mg l⁻¹ α-naphthaleneacetic acid (see Note 1), and 30 g l⁻¹ sucrose at pH 5.7 plus 2 g l⁻¹ gellan gum.

5. Soil: Metro-Mix 350 (Sun Gro Horticulture, Vancouver, Canada).


2.2. Southern Blot Analysis

1. DNA isolation kit: DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany).

2. DNA purification kit: MinElute PCR Purification Kit (Qiagen).

3. 0.8% agarose gel prepared by melting Seakem GTG agarose (Takara Bio, Shiga, Japan) in TAE (40 mM Tris base, 20 mM acetate, 1 mM EDTA, pH 8.0).


5. 20 × SSC: 3 M NaCl and 0.3 M sodium citrate (adjusted to pH 7.0 with NaOH).

6. Hybridization bag: Hybri-Bag Hard (Cosmo Bio, Tokyo, Japan).

7. AlkPhos Direct Labeling and Detection System (GE Healthcare) containing labeling reagents, detection reagents, and hybridization buffer.

8. Primary wash buffer: 2 M urea, 0.1% SDS, 50 mM sodium phosphate (pH 7.0), 150 mM NaCl, 1 mM MgCl₂, and blocking reagent (GE Healthcare).

9. Secondary wash buffer: 50 mM Tris base, 100 mM NaCl, and 2 mM MgCl₂.
2.3. Protein Analysis

1. Medium I: 20 mM Tricine-KOH (pH 7.6), 5 mM EGTA (pH 8.0), 5 mM EDTA (pH 8.0), 10 mM NaHCO₃, 0.33 M sorbitol, 0.4 mg ml⁻¹ ascorbate, and 1 mg ml⁻¹ BSA.

2. Medium II: 20 mM HEPES-KOH (pH 7.6), 2.5 mM EDTA (pH 8.0), 5 mM MgCl₂, and 0.33 M sorbitol.

3. Percoll solution: 1 ml of 70% Percoll (GE Healthcare) in Medium II is slowly injected under 40% Percoll in Medium II in a 15 ml centrifuge tube to prepare a Percoll double layer.

4. High-speed homogenizer: Polytron (Kinematica AG, Littau, Switzerland).

5. Filter: Miracloth (Cosmo Bio).

6. Assay buffer: 150 mM Tris–HCl (pH 7.2), 5 mM MgCl₂, 5 mM β-mercaptoethanol, and 1 mM thiamine diphosphate.

7. 1-Deoxy-D-xylulose-5-phosphate (DXP), available from Echelon (Salt Lake City, UT).


9. 2× sample buffer: 125 mM Tris–HCl (pH 6.8), 0.002% (w/v) bromophenol blue, 4% (w/v) SDS (sodium dodecyl sulfate), 10% (v/v) β-mercaptoethanol, and 40% (w/v) glycerol.

10. Blotting buffer: 50 mM Tris-glycine (pH 8.3) and 20% (v/v) methanol.

11. Electro-blotting machinery: Trans-Blot SD cell (Bio-Rad).

12. Washing buffer (TBST): Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20.

13. ECL Plus Western Blotting Detection System (GE Healthcare) containing ECL blocking agent, anti-rabbit IgG [HRP (horseradish peroxidase)-linked whole antibody], and detection reagents.

2.4. Measurement of Metabolites in Transplastomic Plants

1. Leaf punch available from Fujiwara Scientific Company (Tokyo, Japan).

2. Ball mill: Mixer mill MM301 (Retsch GmbH, Haan, Germany) and zirconia ball (φ 5 mm).

3. High performance liquid chromatograph (HPLC) for pigment analysis: ACQUITY UPLC system (Waters, Milford, MA).
4. HPLC column for pigment analysis: ACQUITY BEH Shield RP18 column (2.1 × 150 mm, 1.7 μm particle size) (Waters).

5. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄ (adjusted to pH 7.4 with HCl).

6. Tocol and undecaprenol available, respectively, from Wako and from Larodan Fine Chemicals AB (Malmo, Sweden).

7. Derivatization reagent: N-trimethylsilylimidazole (TMSI)-H (GL Sciences, Tokyo, Japan).

3. Methods

In transplastomic plants, transgenes are inserted into the plastid genome by homologous recombination. The authors chose the *rbcL* and *accD* genes to use as the anchoring region to initiate site-specific integration into the plastid genome (Fig. 15.2), although other genes have been used as an anchoring region (4). In plastid transformation, polycistrionic multigene expression is achieved under the control of a single promoter (10) since plastids possess a prokaryotic gene expression system. This enables modulating sequential metabolic reactions in a single transformation procedure.

![Fig. 15.2. Chloroplast genome structure around the integration site in transformants and in wild-type plants. Bars indicate the position of probes used in Southern blot analysis. The *aadA* gene confers spectinomycin resistance for the selection of transformed shoots. *Prm*, 16S rRNA promoter; *TpsbA*, *psbA* terminator; *Trps16*, *rps16* terminator.](image)

The integration of transgenes is verified by Southern blot analysis. The accumulation level and activity of foreign protein is determined by immunoblot analysis and enzyme assay, respectively. The procedures for analyzing the levels of isoprenoids produced in plant leaves such as chlorophylls, carotenoids, tocopherols, solanesol (nonaprenol), phytol, and β-sitosterol are also described.
3.1. Production and Growth of Transplastomic Tobacco Plants

1. Sterile wild-type tobacco (*Nicotiana tabacum* L. cv Xanthi) plants are grown on agar (gellan gum)-solidified MS medium in an Agripot.

2. Young leaves (leaf width 2–4 cm) are placed (see Note 2) adaxial side up on RMOP medium in a Petri dish (ϕ 90 mm) to cover an area 4–5 cm in diameter, and incubated overnight under low light conditions (5 μmol photons m⁻² s⁻¹).

3. 0.6 μm gold particles (see Note 3) are prepared for transformation by mixing 20 μl of a suspension of 2.5 mg gold in 230 μl of water, 250 μl of 2.5 M CaCl₂, 25 μl of plasmid DNA (1 mg ml⁻¹ in water), and 50 μl of 0.1 M spermidine (see Note 4). The manipulation is carried out on ice.

4. The particle/DNA mixture is incubated on ice for 10 min (see Note 5) and centrifuged for 1 min at 13,000×g. After removal of the supernatant, the particles are rinsed with 500–600 μl of ethanol by centrifugation (1 min, 13,000×g). The particles are dissolved in 60 μl of ethanol and used for particle bombardment (5.4 μl per bombardment).

5. The gold particles coated with transforming DNA, are introduced into the precultured leaves by the PDS-1000/He biolistic device.

6. The bombarded leaves are placed under low-light conditions (5 μmol photons m⁻² s⁻¹) for 2 d, followed by cutting of the leaves into sections (3 mm × 3 mm) and placement of the sections on RMOP medium containing 500 μg ml⁻¹ spectinomycin dihydrochloride.

7. Green calli that form on the bleached leaves are subcultured onto the same selective medium. Regenerated shoots are rooted on MS medium containing 500 μg ml⁻¹ spectinomycin dihydrochloride. Homoplastomic transgenic shoots are obtained by repeated shoot regeneration on the RMOP selective medium, and are then rooted on the MS selective medium to obtain T₀ plants.

8. T₀ plants are transplanted into soil in pots (700 ml plant⁻¹) and are cultured in a growth chamber at 25°C under a 16 h light/8 h dark regime at a photon flux density of 200–400 μmol m⁻² s⁻¹. Plants are fertilized three times a week.

3.2. Southern Blot Analysis

1. Total plant DNA is extracted using a DNA isolation kit.

2. 2 μg of DNA is digested with *Eco*RV (see Note 6), purified by a DNA purification kit, and fractionated on a 0.8% agarose gel (130 mm × 130 mm) for 12 h at 50 V. DNA size standards are run in the same gel.
3. After the DNA in the gel is stained by ethidium bromide, the DNA size standards are measured with a ruler to determine their migration distance.

4. The gel is sequentially immersed and shaken in (1) 0.25 M HCl, (2) 0.5 M NaOH containing 1.5 M NaCl, and (3) 0.1 M Tris–HCl (pH 7.2) containing 1.5 M NaCl.

5. DNA fragmented in the gel is transferred onto a nylon membrane by capillary blotting in 20× SSC overnight and then cross-linked by ultraviolet light.

6. The nylon membrane is dried for 30 min at room temperature and incubated with 50 ml of pre-warmed hybridization buffer at 56°C for 30 min in a hybridization bag.

7. DNA probes for hybridization are prepared; 500–1000 bp DNA fragments produced by PCR (see Note 7) are purified by a DNA purification kit and directly labeled with alkaline phosphatase by the AlkPhos System.

8. The probe (20–40 ng) is added to the hybridization bag and the hybridization mixture is incubated at 56°C overnight.

9. Hybridized membrane is washed sequentially with primary wash buffer at 56°C for 10 min (two times) and with secondary wash buffer at room temperature for 5 min (two times).

10. Hybridized probes are detected with chemiluminescent reagents and an image analyzer.

### 3.3. Protein Analysis

#### 3.3.1. Extraction of Total Stroma Protein

1. 1–2 g of tobacco leaf is cut into 1 cm² sections, put into a pre-cooled centrifuge tube (50 ml) filled with 25 ml Medium I, and shredded with a Polytron homogenizer at 4°C.

2. The homogenate is filtered through Miracloth and centrifuged at 3000×g at 4°C for 5 min.

3. The resultant green pellet is gently, quickly, and fully suspended in Medium II by pipetting with a wide-mouthed pipette tip (see Note 8).

4. The suspension is put on pre-cooled double-layered Percoll solution and centrifuged in a swinging rotor at 3500×g at 4°C for 10 min.

5. Intact chloroplasts present at the interface between 40 and 70% Percoll solutions are transferred into a new centrifuge tube and suspended in 4 ml Medium II.

6. The suspension is centrifuged at 2000×g at 4°C for 3 min. The resultant pellet (intact chloroplast) is resuspended in Medium II and the mixture is then centrifuged again.
3.3.2. DXR Activity Measurement

1. DXR activity is determined in 1 ml of assay buffer containing 1 mM DXP, 1 mM MnCl$_2$, 0.125 mM NADPH, and stroma fraction. The reaction is initiated by adding NADPH to a final concentration of 0.125 mM in the assay buffer.

2. The oxidation of NADPH is monitored with a spectrophotometer at 340 nm for 10 min at 25°C; an absorption coefficient of 6220 M$^{-1}$ cm$^{-1}$ is assumed. One unit of enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADPH per minute. Controls are performed in the absence of cell-free extracts.

3.3.3. Immunoblot Analysis

1. Protein concentrations in the stroma fraction are determined by Bradford assay. Diluted sample solution (200 μl) is mixed well with Bradford reagent (800 μl) and incubated for 5 min at room temperature. After the determination of $A_{595}$ by a spectrophotometer, the amount of stroma protein is estimated from a calibration curve made using BSA (1–10 μg).

2. 250 ng of stroma proteins are mixed with 2× sample buffer, denatured by heating at 95°C for 3 min, and placed on ice for 5 min. Proteins in the stroma fraction and unstained protein size standards are separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12.5% gel (120 × 100 mm) at 20 mA.

3. The electrophoretic gel is shaken in blotting buffer for 30 min. A polyvinylidene fluoride membrane is immersed in methanol for 10 s and slowly shaken in blotting buffer for 30 min.

4. Separated stroma proteins are electro-blotted onto the membrane at 15 V for 30 min.

5. The membrane is immersed in blocking solution (50 mg ml$^{-1}$ ECL blocking reagent in TBST) in a hybridization bag and shaken overnight at 4°C (see Note 9).

6. The membrane is washed for 15 min in TBST (three times) and shaken in TBST containing polyclonal IgG antibody prepared against the target protein.

7. The membrane is washed for 15 min in TBST (three times) and shaken in TBST containing anti-rabbit IgG linked peroxidase diluted 1:1000.

8. The membrane is washed for 15 min in TBST (three times), then 1 ml of ECL Plus Western Blotting Detection Reagent is applied onto the membrane.
9. Blotted proteins are detected by a chemiluminescent image analyzer. The accumulation level of foreign protein is determined by the intensity of the chemiluminescent signal, which is converted to protein concentration by comparison with known concentrations of purified foreign protein (Fig. 15.3). The level of foreign protein is estimated as percentage of total stroma protein using the amount of stroma protein applied to the gel as the value for total protein.

<table>
<thead>
<tr>
<th>DXR standard [ng]</th>
<th>Transformant</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>#3</td>
<td>#5</td>
</tr>
<tr>
<td>2</td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td></td>
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<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 15.3. Immunoblot analysis to study the accumulation level of Synechosystis DXR protein in leaves of transplastomic tobacco plants. Total stroma protein from leaves (250 ng lane$^{-1}$) was probed with an antibody raised against recombinant DXR derived from Synechosystis sp. Purified DXR standard (1, 2, 5, 10, 20, 50 ng lane$^{-1}$) was included for quantification.

3.4. Measurements of Metabolites in Transplastomic Plants

3.4.1. Pigment Analysis

1. A 15 mm leaf disc is punched from a tobacco leaf, put into a 2 ml centrifuge tube and immediately frozen in liquid nitrogen. The disc is ground to fine powder with a ball mill (see Note 10).

2. 200 μl methanol is added to the tube along with the internal standard (e.g., lycopene, 4 μg). The suspension is mixed by inversion for 5 min at 4°C.

3. 50 mM Tris–HCl (pH 7.5) containing 1 M NaCl is added (200 μl) and a further incubation at 4°C for 10 min is carried out.

4. 800 μl chloroform is added to the mixture, which is incubated on ice for 10 min.

5. A clear partition is formed by centrifugation at 3000×g for 5 min at 4°C. The hypophase is removed with a Pasteur pipette and the aqueous phase is re-extracted with chloroform (800 μl).

6. The pooled chloroform extracts are dried under a stream of nitrogen. The dried residue (see Note 11) is dissolved in acetonitrile and applied to HPLC.

7. Pigments in samples are analyzed by reverse-phase HPLC (see Note 12). Pigments are separated using an ACQUITY BEH Shield RP18 column operating at 35°C and eluted using 30% solvent A (methanol:water [50:50, v/v]) for the first 3.5 min followed by a 4.5 min linear gradient to 100% solvent B (acetonitrile) that continues isocratically until the end of the 11 min separation. A conditioning phase (11–15 min) is then used to return the column to the initial
concentrations of A and B. A flow rate of 0.6 ml min\(^{-1}\) is used throughout chromatography, and the eluate is monitored continuously from 200 to 500 nm with a photodiode array detector.

8. Pigments are identified by their retention time and absorption spectra (Table 15.1) and quantified by integrating peak areas at \(A_{445}\). The peak areas are converted to molar concentrations by comparison with authentic standards (see Note 13).

### Table 15.1
**Pigments separated on reverse-phase HPLC system and their spectral characteristics (in the eluting solvent) used in identification based on photodiode array detection**

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Retention time (min)</th>
<th>Spectral characteristics (nm at (\lambda_{\text{max}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotenoids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adonirubin</td>
<td>2.95</td>
<td>480</td>
</tr>
<tr>
<td>Adonixanthin</td>
<td>2.61</td>
<td>464</td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>2.42</td>
<td>423, 448, 476</td>
</tr>
<tr>
<td>Astaxanthin</td>
<td>1.99</td>
<td>480</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>4.40</td>
<td>474</td>
</tr>
<tr>
<td>(\alpha)-Carotene</td>
<td>9.14</td>
<td>424, 447, 475</td>
</tr>
<tr>
<td>(\beta)-Carotene</td>
<td>9.26</td>
<td>453, 480</td>
</tr>
<tr>
<td>(\beta)-Cryptoxanthin</td>
<td>7.33</td>
<td>427, 452, 480</td>
</tr>
<tr>
<td>Echinenone</td>
<td>7.52</td>
<td>459</td>
</tr>
<tr>
<td>Fritschiellaxanthin</td>
<td>2.53</td>
<td>457, 474</td>
</tr>
<tr>
<td>3-Hydroxyechinenone</td>
<td>3.78</td>
<td>467</td>
</tr>
<tr>
<td>4-Ketoantheraxanthin</td>
<td>1.86</td>
<td>457, 474</td>
</tr>
<tr>
<td>Lutein</td>
<td>3.32</td>
<td>423, 448, 476</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>1.40</td>
<td>413, 438, 466</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>1.74</td>
<td>418, 441, 470</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>3.44</td>
<td>427, 453, 479</td>
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<tr>
<td>Chlorophylls</td>
<td></td>
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</tr>
<tr>
<td>Chlorophyll (a)</td>
<td>8.53</td>
<td>430</td>
</tr>
<tr>
<td>Chlorophyll (b)</td>
<td>7.46</td>
<td>458</td>
</tr>
</tbody>
</table>

1. Two pieces of a \(\phi\) 15 mm leaf disc are punched from a tobacco leaf, put into a 2 ml centrifuge tube, and immediately frozen in liquid nitrogen. The disc pieces are ground to fine powder with a ball mill.
2. 200 μl PBS, 500 μl methanol, and 250 μl chloroform are added to the tube. The suspension is mixed for 2 min.

3. After a 10 min incubation at room temperature, 250 μl chloroform is added to the tube along with internal standard (e.g., 0.25 μg of tocol, 2 μg of undecaprenol). The mixture is mixed well with a vortex mixer.

4. 200 μl water is added to the mixture, which is mixed well with the vortex mixer.

5. A clear partition is formed by centrifugation at 16,000×g for 3 min at 4°C. The hypophase is removed with a Pasteur pipette and the aqueous phase is re-extracted with 400 μl chloroform. This extraction is repeated one more time.

6. The pooled organic phase is dried under a stream of nitrogen. The dried residue is dissolved in 2-propanol/n-hexane (50:50, v/v) and subjected to conventional HPLC (see Note 14).

7. Prenyl lipids are separated using an ODS column (see Note 14) operating at 40°C, using 95% solvent C (methanol/2-propanol/water [60:40:5, v/v]) for the first 15 min followed by a 20 min gradient to 100% solvent D (2-propanol/n-hexane [30:70, v/v]). A flow rate of 1 ml min⁻¹ is used. A conditioning phase (35–45 min) is then used to return the column to the initial concentrations of solvents C and D.

8. Solanesol (nonaisoprenol) and undecaprenol are detected at 210 nm.

9. α- and γ-tocopherol and tocol are detected with a fluorescence detector with excitation and emission wavelengths 290 and 330 nm, respectively.

10. Peaks are identified by their retention time and by absorption spectra using a photodiode array detector and are quantified by integrating peak areas. The peak areas are converted to molar concentrations by comparison with authentic standards.

3.4.3. Measurement of Phytol and β-Sitosterols

1. Four pieces of a φ 15 mm leaf disc are punched from a tobacco leaf, put into a 2 ml centrifuge tube, and immediately frozen in liquid nitrogen. The disc pieces are ground to a fine powder with a ball mill.

2. 500 μl 2 M ethanolic KOH along with internal standard (100 μg dihydrocholesterol) is added to the tube. The suspension is incubated at 80°C for 2.5 h for alkaline hydrolysis.

3. 200 μl saturated KCl and 500 μl n-hexane are added to the suspension.
4. A clear partition is formed by centrifugation at 16,000×g for 30 min at 4°C. The hypophase is removed with a Pasteur pipette and the aqueous phase is re-extracted with 500 μl n-hexane. This extraction is repeated three more times.

5. The pooled organic phase is dried under a stream of nitrogen. The dried residue is dissolved in n-hexane, trimethylsilylated at 60°C for 30 min using a derivatization reagent, and subjected to gas chromatography–mass spectrometry (GC–MS) (see Note 15).

6. The GC is programmed at an initial temperature of 200°C for 3 min, with a ramp of 15°C min⁻¹ to 320°C, and a final time of 7.5 min, using helium as the carrier gas at 1 ml min⁻¹ with a split ratio of 25:1. The injector port, interface, and MS source temperature were 230°C, 250°C, and 250°C, respectively. The electron multiplier is set at 1393 V.

7. Phytol and β-sitosterol are identified by their retention time and fragmentation pattern and quantified by integrating peak areas. The peak areas are converted to molar concentrations by comparison with authentic standards.

---

4. Notes

1. 100 mg of 6-benzyladenine and 10 mg of α-naphthale-necetic acid are, respectively, dissolved in 500 μl of 1 M NaOH, diluted with 1000 ml water, dispensed, and stored at –30°C.

2. Major veins of leaves are removed with a sterile scalpel before placing them on the Petri dish.

3. Before using, 2.5 mg of the gold particles are rinsed with ethanol and water by centrifugation (13,000×g for 1 min) and stored in 200 μl of water at –30°C.

4. The suspension is mixed after each addition of solutions.

5. The particle/DNA mixture is mixed with a vortex mixer for 10 s each minute 10 times.

6. The EcoRV site is located both upstream of rbcL and in accD, which is a homologous recombination region useful for tobacco plastid transformation (Fig. 15.2).

7. PCR is performed to amplify target sequence (transgene or homologous recombination region) (Fig. 15.2) using a gene-specific primer set.

8. It is necessary to avoid suspending the white starch pellets present around the green pellets because the yield of chloroplast is decreased due to incorporation of starch.
9. It is necessary to immerse the membrane quickly into the hybridization bag to prevent the blotted membrane from drying out.

10. All subsequent manipulations are carried out on ice and the sample is shielded from strong light whenever possible.

11. Dried residues are stored under an atmosphere of nitrogen at \(-20^\circ C\) prior to HPLC.

12. The ACQUITY UPLC system is suitable for the measurement of pigments such as chlorophylls and carotenoids, because an ACQUITY BEH Shield RP18 column achieves complete separation of pigment compounds.

13. Authentic standards are available from Wako and Caroten-Nature GmbH (Lupssingen, Switzerland).

14. The authors use an LC-10 series HPLC (Shimadzu, Kyoto, Japan) and an Inertsil ODS-3 column (4.6 \( \times \) 250 mm, 5 \( \mu \)m particle size) (GL Sciences) for the analysis of prenyl lipids.

15. The authors use a TRACE DSQ GC-MS (Thermo Electron Corporation, Waltham, MA) equipped with a CP-Sil 8CB low bleed/MS column (30 m \( \times \) 0.25 mm i.d., 0.25 \( \mu \)m film thickness, Varian, Inc., Palo Alto, CA) for the analysis of phytol and sterols.

Acknowledgments

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References


Chapter 16

Engineering High Yields of Secondary Metabolites in Rubia Cell Cultures Through Transformation with Rol Genes

Victor P. Bulgakov, Yuri N. Shkryl, and Galina N. Veremeichik

Abstract

Among the different methods currently used to improve yields of secondary metabolites in cultured plant cells, the method involving transformation by rol genes represents an example of relatively new technology. These genes, isolated from plasmids of the plant pathogen Agrobacterium rhizogenes, are potential activators of secondary metabolism in transformed cells from the Solanaceae, Araliaceae, Rubiaceae, Vitaceae, and Rosaceae families. In some cases, the activator effect of individual rol genes was sufficient to overcome the inability of cultured plant cells to produce large amounts of secondary metabolites. Stimulation of production characteristics of cultured plant cells mediated by the rol genes was shown to be remarkably stable over long-term cultivation. In this chapter, we describe transformation of Rubia cordifolia L. cells with the rol genes as an example of metabolic engineering of secondary metabolites.

Key words: Agrobacterium rhizogenes, rol genes, isochorismate synthase, anthraquinones, secondary metabolism.

1. Introduction

Hairy roots are known to be produced as a response to integration of the wild-type T-DNA of A. rhizogenes into the plant genome (1, 2). The interest in rol genes stems from the well-known fact that hairy root cultures, derived from various plants species, stably produce high amounts of secondary metabolites (3, 4). Among T-DNA genes, three rol genes, rolA, rolB, and rolC or a combination (rolABC), seem to be most efficient at inducing production of secondary metabolites (5). Although it is known that the rol genes act via transcriptional activation of
defense genes, the mechanism of activation is unclear. Evidence indicates that the rol genes mediate uncommon signal transduction pathways in plants. They act on phytoalexin production independently of plant defense hormones and the calcium-dependent NADPH oxidase pathway (6). Likewise, the production of secondary metabolites in rolC-transformed cells is not dependent on an oxidative burst (7). The extent of secondary metabolism activation varies between studied plant species, from 2- to 300-fold depending on the group of secondary metabolites and the plant species (6). In some cases, the transformation with the rol genes provoked a biphasic effect with initial suppression and subsequent activation of biosynthesis for particular groups of secondary metabolites (6, 8). Transformation with the rol genes is especially useful in those cases where different methods commonly used to increase secondary metabolite production (cell selection, elicitor treatments, and addition of a biosynthetic precursor) only slightly enhance cell productivity.

The rolB and rolC genes are most interesting candidates for plant biochemical engineering. A high expression of the rolB gene in transformed R. cordifolia cells dramatically increases accumulation of anthraquinones and the transcription of a key biosynthetic gene (ICS) encoding isochorismate synthase (5). However, excessive expression of the rolB gene inhibits cell growth. In comparison to the rolB gene, the rolC gene activates anthraquinone biosynthesis to a lesser extent. However, this gene possesses an interesting ability to increase cell growth. When combined, rolA, B, and C genes increase anthraquinone content and do not suppress biomass accumulation (5). Evidence indicates that each of the rol genes has its own role in plant metabolic processes (6).

2. Materials

2.1. Agrobacterium Strains

1. For transformation, we use A. tumefaciens strain GV3101 (see Note 1), which is a disarmed derivative of the nopaline C58 strain, harboring the pMP90RK vir-helper plasmid (9). Expression cassettes contain rol genes derived from the wild-type A. rhizogenes A4 strain (11), including pPCV002-A (rolA under the control of its own native promoter), pPCV002-ABC (rolA, rolB, and rolC under the control of their own native promoters), pPCV002-CaMVBT (rolB under the control of 35S CaMV promoter), and pPCV002-CaMVC (rolC under the control of 35S CaMV promoter) (10). The T-DNA of the pPCV002 vector includes the chimeric npt-II gene, which renders resistance to kanamycin.
2. Luria–Bertani medium (LB), 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, adjusted to pH 6.5 with 5 N KOH.

3. Kanamycin and carbenicillin should be dissolved aseptically in water and ethanol respectively, at 100 mg/mL and stored at –20°C. GV3101-derived strains are grown in the LB medium supplemented with appropriate antibiotics which help to maintain plasmids. pMP90RK is maintained with 50 mg/L kanamycin sulfate, and pPCV002 is maintained with 100 mg/L carbenicillin (pMP90RK contains a marker for kanamycin resistance and pPCV002 contains a marker for carbenicillin resistance).

4. Taq-polymerase, 10X Taq-Buffer, and dNTPs (Syntol, Russia, Moscow or Invitrogen, Carlsbad, California, USA).

5. Primer pairs for Agrobacterium verification can be seen in Table 16.1.

6. Polymerase chain reaction instrument, iQ5 thermal cycler (Bio-Rad Laboratories, Inc., USA) or equivalent.

7. Big dye terminator cycle sequencing kit (Applied Biosystems, Forster City, USA) or equivalent.

8. ABI 3130 genetic analyzer (Applied Biosystems) or equivalent.

9. 100 mM acetosyringone solution in DMSO, stored at –20°C.

10. The WB/A medium. WB/A is a modification of Murashige and Skoog medium (12) in which the concentration of ammonium nitrate is decreased (see Note 2). Macronutrients include 400 mg/L NH₄NO₃, 440 mg/L CaCl₂·2H₂O, 370 mg/L MgSO₄·7H₂O, 1900 mg/L

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direct and reverse primer sequences</th>
<th>Size of PCR product, bp</th>
</tr>
</thead>
</table>
| rolA  | 5'-ATGGAAATTAGCCGGAGCTA-3'  
      | 5'-TAAATCCGGTAGTTTGTG-3'     | 303                     |
| rolB  | 5'-ATGGATCATCCAATTGCTA-3'  
      | 5'-TAAAGCTTCTTCTTCAGCTG-3'  | 780                     |
| rolC  | 5'-ATGGCCTGAAGACGACCTG-3'  
      | 5'-TTAGCCGATTGCAAACCTTG-3'  | 543                     |
| virD2 | 5'-AGCCCGAGATTTGTTTGTCAGG-3'  
      | 5'-TGTTGGCCCGAGGCATGTCC-3'  | 417                     |
KNO₃, and 170 mg/L KH₂PO₄. Microelements include 8.6 mg/L ZnSO₄·7H₂O, 6.2 mg/L H₃BO₃, 22.3 mg/L MnSO₄·4H₂O, 0.025 mg/L CuSO₄·5H₂O, 0.83 mg/L KI, 0.25 mg/L Na₂MoO₄·2H₂O, 0.025 mg/L CoCl₂·2H₂O, 37.3 mg/L Na₂EDTA, and 27.8 mg/L FeSO₄·7H₂O. Organics include 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxin-HCl, 0.2 mg/L thiamin-HCl, 0.2 mg/L 6-benzylaminopurine, 2 mg/L α-naphthalacetic acid, and 25,000 mg/L sucrose. Solid WBA medium also contains 6000 mg/L agar. The medium is adjusted to pH 5.6 with 5 N KOH before autoclaving. The medium is sterilized at 120°C for 20 min.

11. An incubator with an orbital shaker to grow *Agrobacterium tumefaciens* at 28°C.

### 2.2. Transformation of Plant Explants

1. Sterile plantlets, cultivated at 25°C with a 16/8 h light/dark photoperiod in hormone-free agarized WBA medium, which contains half-strength macroelements.

2. Antibiotic-free agarized WBA medium, 30 mL in 100 mL Erlenmeyer flasks.

3. Selection medium: agarized WBA medium supplemented with cefotaxime (250 mg/L) or with cefotaxime (250 mg/L) and kanamycin (10 mg/L) (see Note 3).

4. A solution (0.1% w/v) of Diocide (contains a detergent, N-cetylpyridinium bromide, and disinfectant, ethanolmercuric chloride, in the proportion 2:1), Novokuznetsk Chem-Pharm Company, Russia, or an equivalent disinfectant.

5. Cefotaxime (or Claforan, Hoechst, Germany), 100 mg/mL sterile stock solution in water, stored at –20°C.

6. Forceps and scalpels.

7. A tissue culture room, at 25°C, with a 16 h day/8 h night photoperiod.

8. A tissue culture room, maintained at 25°C in the dark.

9. General tissue culture and plant culture materials.

### 2.3. Transformation of Suspension Cultures

1. *R. cordifolia* suspension-cultured cells (growing in WBA medium, in the dark, at 25°C, on an orbital shaker at 125 rpm).

2. Liquid WBA medium, 50 mL in 250-mL Erlenmeyer flasks.

3. Selection medium. Solid WBA medium supplemented with cefotaxime (250 mg/L) and kanamycin (10–50 mg/L), 30 mL in 100-mL Erlenmeyer flasks.

4. 100 mM acetosyringone solution in DMSO, stored at –20°C.
5. Sterile polypropylene filters (35-μm pore size).
6. A tissue culture room, maintained at 25°C in the dark.
7. An orbital shaker.
8. General tissue culture and plant culture materials.

2.4. Quantitative Analysis of Gene Expression

1. RNA extraction buffer: 100 mM Tris–HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% CTAB, 2% polyvinylpyrrolidone, and 2% β-mercaptoethanol added just prior to use (see Note 4).
2. Water bath.
3. 10 M LiCl water solution (see Note 5), stored at +4°C.
4. 96% ethanol.
5. Chloroform.
6. UV–Vis spectrophotometer.
7. M-MLV reverse transcriptase with 5X buffer, dNTP mixture, and oligo(dT) primer.
8. Real-time PCR instrument (iQ5 thermal cycler, Bio-Rad Laboratories, Inc., USA) supplied with Optical system software v. 2.0 or equivalent.
9. Primer pairs for real-time PCR (see Table 16.2).
10. Real-time PCR kit (2.5× mixture) with SYBR Green I and ROX (as a reference dye) (Syntol, Russia).
11. General molecular biology materials and equipment.

<table>
<thead>
<tr>
<th>Table 16.2</th>
<th>Primers used for studying expression of the rol genes, ICS gene (anthraquinone biosynthesis), and actin gene (constitutive expression reference) by real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Direct and reverse primer sequences</td>
</tr>
<tr>
<td>rolA</td>
<td>5'-GCCGGACGCTTCCGGAGTTATAT-3'</td>
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<tr>
<td></td>
<td>5'-CCAAAGGAGTGGTGCAGT-3'</td>
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<tr>
<td>rolB</td>
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<td></td>
<td>5'-CCGAGAGTCGCAGGGTAG-3'</td>
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<tr>
<td>Rc-ICS</td>
<td>5'-TATCATCCCTTCCATCCAACCTCC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCGCCAAGAAAACCCACAG-3'</td>
</tr>
<tr>
<td>Rc-actin</td>
<td>5'-CAATCCACAAGGTCTATCTGTA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ATGGCAGGGGTGTGAATGT-3'</td>
</tr>
</tbody>
</table>
2.5. Determination of Cell Growth

2. Foam plastic rack with a 2-cm diameter slot.
3. 100-mL Erlenmeyer flasks containing 30 mL agarized WBA medium (35 flasks per one culture).

2.6. Estimation of Anthraquinone Content

1. Hot air source (60°C).
2. 5 N HCl solution.
3. 96% ethanol.
4. Spectrophotometer.

3. Methods

Two approaches may be used to transform cells of *R. cordifolia* with the *rol* genes. The first approach includes transformation of leaf explants or sterile, clonally cultivated plantlets. The second approach includes the transformation of cell suspension cultures. As a rule, it is easier to transform plant explants than cell suspension cultures. However, cell suspension cultures have an obvious advantage over plant explants, such as in cases where homogeneous starting material should be used to compare effects of individual genes.

3.1. Agrobacterium Preparation

1. Vector plasmids are electroporated into competent *Agrobacterium* cells prior to use. Alternatively, transformed GV3101 strains can be stored in agar columns or in 20% glycerol solution (see Note 6). GV3101 strains containing pPCV002/pMP90RK binary vector should be grown in the LB medium, supplemented with 50 mg/L kanamycin sulfate and 100 mg/L carbenicillin, at 28°C in the dark.

2. Dip a sterile loop into the agar column, or a frozen stock culture in glycerol, and streak onto selective LB agar plates using the four-quadrant method. Incubate at 28°C for 2 days for single colony appearance (see Note 7).

3. Prepare PCR reactions with primer sets listed in Table 16.1. Pick up six to eight colonies with 1–10 μL tips into individual PCR tubes and perform the reaction with 25–30 cycles. Check the results of the PCR with 1% (w/v) agarose gel with ethidium bromide (final concentration 0.5 μg/mL). To purify PCR products corresponding to positive colonies, precipitate them with 80% ethanol and clean twice with 70% ethanol. Sequence PCR products with the same primers and compare the results with GeneBank sequences: *rolA*
4. Streak the chosen Agrobacterium colony onto selective LB agar plates supplemented with 200 μM acetylsyringone and incubate the colony overnight at 28°C.

5. Incubate selected Agrobacterium overnight in 5 mL of LB medium containing 50 mg/L kanamycin sulfate and 100 mg/L carbenicillin at 28°C and 250 rpm shaking.

6. Inoculate 100 μL of the bacterial culture in 1 mL of LB containing 200 μM of acetylsyringone and incubate for 5 h at 28°C using 250 rpm shaking to a final OD$_{600}$ 0.4–0.6 units. Use two tubes for each construct to obtain a large enough quantity of bacterial cells.

7. Precipitate A. tumefaciens by centrifugation (2000 × g, 5 min) and then resuspend bacterial cells in 1 mL of liquid medium WBA (see Section 2.1, step 10). This Agrobacterium suspension is used for transformation.

3.2. Transformation of Plant Explants

1. Wild-growing and greenhouse plants, as well as sterile clonally cultivated plantlets, can be used as a source of explants for transformation (see Note 8). Young leaves and stems of R. cordifolia are the best source for the induction of transgenic calli. Cut off the sufficient number of explants with a blade and put them in a beaker (leaves may be placed in water and kept in a refrigerator at +4°C for several hours). Put explants in disinfectant solution for 5 min and then rinse them thoroughly with distilled water. Under sterile conditions, decant the solution and rinse explants several times with sterile distilled water.

2. Inoculation of explants with Agrobacterium. We describe here two equally effective methods. 
   (A) Pass a scalpel over the streak of the 1-day-old Agrobacterium culture on LB plate (see Section 3.1, step 4) to obtain visible traces of cells. Cut leaflets into the pieces of 0.5 × 0.5 cm size with the scalpel containing Agrobacterium.

   (B) Cut leaflets into the pieces of 0.5 × 0.5 cm size with a scalpel and dip them into the Agrobacterium containing liquid WBA medium (see Section 3.1, step 7) for 10 min.

3. Transfer the inoculated explants onto solid WBA medium. Incubate explants with Agrobacterium for 2–4 days (see Note 9).

4. Transfer explants to the solid WBA selection medium, supplemented with cefotaxim (250 mg/L) to eliminate
Agrobacterium and with kanamycin (10 mg/L) for selection of transgenic calli. Kanamycin can be added to the selection medium after induction of primary calli (see Note 10).

5. After calli induction (4–5 weeks), they must be separated from the rest of the explants and placed in direct contact with fresh selection medium. Increase kanamycin concentration up to 25 mg/L for the second subculture and to 50 mg/L for the third and subsequent subcultures. Use cefotaxim (250 mg/L) for three to four subcultures to eliminate Agrobacterium cells. Cultivate the calli with 4-to 5-week intervals.

6. Watch closely for morphological differentiation of primary calli and select different phenotypes of transformed calli. This is the best time to obtain cultures with different expression of a target gene, growth rate, and biosynthetic activity. Select, for this purpose, small 1- to 2-mm homogeneous aggregates. As the rol genes often induce root formation, one can select root-forming aggregates that can be used for establishment of transformed root cultures. To obtain a homogeneous non-differentiated, transformed callus culture, select non-root-forming aggregates in two to three subsequent subcultures.

7. Long-term cultivation of transformed cultures:
   (A) Cultivate callus cultures using liquid or solid, antibiotic-free 
       WBA medium.
   (B) Cultivate transformed root cultures in liquid, antibiotic-
       free WBA medium in which 6-benzylaminopurine and 
       \( \alpha \)-naphthylacetic acid are replaced by 3-indolebutyric 
       acid (1 mg/L).

3.3. Transformation of Callus/Suspension Cells

1. Suspension cultures are best suited for transformation at the 
   exponential stage of growth (4–6 days for R. cordifolia sus-
   pension culture). Under sterile conditions, supplement the 
   suspensions with acetosyringone to a final concentration of 
   100 \( \mu \)M.

2. Add 100 \( \mu \)L of Agrobacterium from inoculation medium to 
   50 mL of plant cell suspension and co-cultivate for 2–3 days 
   at 25\(^\circ\)C.

3. Add cefotaxim aseptically to a final concentration of 
   250 mg/L directly to the suspension culture and cultivate 
   for 10 days.

4. The suspension-cultured cells are filtered using a sterile 
   polypropylene filter (35 \( \mu \)m pore size). Cells and cell aggreg-
   rates are plated homogeneously on agarized WBA medium 
   in 100-mL Erlenmeyer flasks supplemented with cefotaxim 
   (250 mg/L) and kanamycin (50 mg/L).
5. During 4–5 weeks of the first passage of the subculture, small growing cell aggregates will appear against brown necrosis cell clusters. Surviving and growing cell clusters should be selected and subcultured three times with a month period on the same medium.


3.4. Quantitative Analysis of Gene Expression

1. RNA isolation.
   1.1. Vigorously grind 200 mg of a tissue in 1 mL pre-warmed (65°C) extraction buffer for 2–3 min using a mortar and pestle (see Note 11).
   1.2. The mixture is transferred to 2-mL tubes and subsequently incubated in a 65°C water bath for 5 min. Add an equal volume of chloroform and store for 10 min on ice (mix thoroughly every 2 min), then centrifuge at 15,000×g for 10 min at 4°C (see Note 12).
   1.3. To selectively precipitate RNA, add 0.25 volumes of 10 M LiCl and store the sample overnight at 4°C (see Note 13).
   1.4. Precipitate RNA by centrifugation for 20 min at 4°C, and wash with ice-cold 70% EtOH, dry in air, and dissolve in 100 μL DEPC-treated water.
   1.5. Determine RNA concentration and 260/280 nm ratios using a spectrophotometer. Run 1% agarose gel to visualize the integrity of the RNA (see Note 14). An example of RNA quality determination is presented in Fig. 16.1.

2. cDNA synthesis. A solution containing 2.5 μg of mRNA and 2.5 μM oligo d(T)_{16} is preheated for 5 min at 72°C and cooled on ice. The reverse transcription should be performed in 50 μL containing 1X M-MLV buffer, 0.2 mM of each dNTP, and 4 U M-MLV reverse transcriptase. Reaction is carried out for 1 h at 36°C, followed by 10 min at 72°C.

3. Quantitative, real-time PCR of rol genes and ICS gene.
   3.1. The gene-specific primer pairs are listed in Table 16.2. The suitability of the primers’ sequences, in terms of efficiency of annealing, is evaluated using the Primer Premier 5.0 program.
   3.2. Amplify the cDNA using a premixed real-time PCR kit with an iQ5 thermal cycler. The reaction mixture (25 μL) should contain 0.25 μM of both direct and reverse primers. The PCR conditions are as follows: 95°C for 3 min, 35 cycles of 95°C for 15 s, and 60°C for 30 s. An example of a real-time PCR result is shown in Fig. 16.2.
Fig. 16.1. Analysis of the quality of all RNA samples isolated from *R. cordifolia*-transformed cultures. Samples were run on an Experion™ Automated Electrophoresis Station with an RNA StdSens LabChip® kit. L, RNA ladder; 1–12, RNA samples. A box (right panel) indicates fluorescence of the RNA sample under analysis. This probe is presented in the left panel. Main peaks reflect relative abundance of 18S and 28S RNAs. The ratio 28S/18S reflects quality of the sample. The best ratio is 2. A sample of RNA cannot be used if the ratio is less than 0.4.

![RNA Quality Analysis](image1.png)

<table>
<thead>
<tr>
<th>Fragment Number</th>
<th>Fragment Name</th>
<th>Start Time</th>
<th>End Time</th>
<th>Area</th>
<th>% of Total Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18S</td>
<td>40.25</td>
<td>42.50</td>
<td>65.16</td>
<td>17.04</td>
</tr>
<tr>
<td>2</td>
<td>28S</td>
<td>46.55</td>
<td>47.25</td>
<td>89.66</td>
<td>23.45</td>
</tr>
</tbody>
</table>

RNA Area: 382.41
RNA Concentration: 187.03 ng/μl
Ratio [28S/18S]: 1.39

Fig. 16.2. Expression of the ICS gene and rol genes in *R. cordifolia* calli transformed with *rolA*, *rolB*, *rolC*, and *rolABC* genes. Real-time PCR data are presented. RFU is relative fluorescent units.

![Gene Expression](image2.png)
3.5. Determination of Cell Growth Dynamics

1. Prepare twenty-five 100-mL Erlenmeyer flasks. Under sterile conditions, zero the weight of a flask with the medium and put 200 mg of callus inoculum.

2. Take five flasks every 5 days, harvest callus biomass, weigh electronically, and dry under hot air flow (60°C) for 5–6 h.

3. Store samples at room temperature before anthraquinone determination.

3.6. Estimation of Anthraquinone Content

1. Grind the samples to powder using a mortar and pestle.

2. Impregnate 100 mg of the dried, powdered calli with 0.3 mL 5 N HCl for 30 min and then extract with 3 mL of ethanol at room temperature for 2–4 h using a shaker (see Note 15).

3. Clear the supernatant by centrifugation at 10,000 × g for 2 min.

4. Determine anthraquinone content photometrically with a UV–Vis spectrophotometer. The purpurin content of ethanol extract is determined by absorption at 515 nm. The content of munjistin is quantified by absorption at 421 nm, subtracting the absorption due to purpurin at this wavelength. Purpurin and munjistin are predominant anthraquinones in rol gene-transformed *R. cordifolia* callus cultures and represent 90% of anthraquinones. The effect of the transformation on anthraquinone content is presented in Table 16.3.

<table>
<thead>
<tr>
<th>Table 16.3</th>
<th>Amount of anthraquinones (percent dry wt.) in <em>R. cordifolia</em> calli non-transformed and transformed by the rol genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-transformed</td>
</tr>
<tr>
<td>Total anthraquinones*</td>
<td>0.4–0.5</td>
</tr>
</tbody>
</table>

*Note that Table 16.3 shows the percentage of anthraquinones in non-transformed and transformed *R. cordifolia* calli.

4. Notes

1. GV3101/pMP90RK is a convenient and powerful transformation system. In our laboratory, this vector was successfully used for transformation of numerous plant species, including species that have been regarded as recalcitrant...
to transformation (woody plants, leguminous plants, etc.). The transformation usually proceeds during a single experiment. *A. tumefaciens* EHA105/pTiBo542 (13), a disarmed derivative of the succinamopine C58 strain, is also an appropriate vector system.

2. Such an ammonium concentration stimulates production of most groups of secondary metabolites and ensures a high rate of cell growth.

3. Antibiotics are aseptically added to the medium after autoclaving.

4. We usually prepare fresh extraction buffer before RNA isolation using sterile stock solutions of 1 M Tris–HCl (pH 8.0), 500 mM EDTA, 10 M NaCl, 10% (w/v) CTAB, 10% (w/v) polyvinylpyrrolidone, and sterile water. The extraction buffer can be prepared in a large volume, sterilized by autoclaving, and stored at +4°C in hermetic flasks.

5. High molarities of LiCl solutions are hazardous if in contact with skin.

6. Strains of *A. tumefaciens* can be typically stored for half a year and up to 1 year in an agar column. Use autoclaved 2-mL tubes, two-thirds full of medium. Inoculate each tube with a loop containing enough bacteria biomass. Seal the vial tightly and store at +4°C. Alternatively, *A. tumefaciens* can be stored for 1–2 years in a glycerol solution (20% w/v) at –20°C. Bacteria can be stored also at –70°C for many years without loss of viability. Autoclave 20% glycerol solution (15 min, 121°C), cool, and store it in a refrigerator. Transfer bacterial cultures grown overnight into a tube with glycerol. Mix and store at –20°C or at –70°C. For any storage method, to revive a stored strain, streak a single colony onto a LB plate containing appropriate antibiotics.

7. We recommend verifying sequences of constructs that will be used for genetic transformation. Sequences of *rol* genes should be checked before transformation. It is useful to periodically test for the presence of *virD2* gene, to be sure that you are working with *Agrobacterium* vector, but not with the *E. coli* vector.

8. It is convenient to use sterile, clonally cultivated plantlets, because there is no need to expose explants to aggressive disinfectant sterilization procedures. Ten plantlets are enough to perform any transformation procedure. As a rule, one 30-day-old plantlet carries 5–10 leaflets.
9. A critical issue for successful transformation is the choice of co-cultivation time. This time should be long enough to ensure gene transfer. However, a long period of co-cultivation leads to excessive agrobacterial growth and total inhibition of plant cells. A good choice for recalcitrant plants is a long (3–4 days) co-cultivation at relatively low temperatures (18–20°C). In any case, transfer inoculated explants to a medium containing cefotaxim at the first appearance of visible *Agrobacterium* infection.

10. We seldom use the recommendation, by many guides, to add antibiotics for bacterium inhibition (cefotaxim) and a plant selective marker (kanamycin) simultaneously. The triple stress (*Agrobacterium* infection, cefotaxim, and kanamycin action) often leads to rapid death of plant cells. It is better to use kanamycin selection after 1 month of cultivation and gradually increase kanamycin concentrations, from 10 to 50–100 mg/L, for three to four subcultures. In particular, a delay of kanamycin selection improves induction of transgenic calli in *R. cordifolia* explants.

11. In most cases, there is no need to use liquid nitrogen for plant material disruption. Instead, use hot extraction buffer (65–68°C), grind plant material vigorously for 1–2 min, use adequate material-buffer proportion (100–200 mg/mL for plant material with high water content and 50–100 mg/mL for dry material), and autoclaved (or sterile) plastics and solutions. Latex gloves are also recommended.

12. An additional step of nucleic acid precipitation, with 2.5 volume of ethanol, enhances efficiency of RNA isolation.

13. To reduce the time it takes for the RNA isolation procedure, add 1 volume of 12 M LiCl to precipitate RNA and store it at –20°C for 30 min. However, this method of quick RNA isolation reduces the amount of RNA.

14. To improve assessment of RNA quality, run the RNA samples on an Experion™ Automated Electrophoresis Station with an RNA StdSens LabChip® kit. This procedure allows rapid and accurate determination of RNA quality. Additionally, mRNA concentration can be also determined by subtraction of rRNA concentration from the total RNA concentration.

15. Anthraquinones can precipitate when stored in ethanol extract for a long time.
Acknowledgments

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References


Abstract

Plant cell cultures provide an important method for production and supply of a variety of natural products, where conditions can be easily controlled, manipulated, and optimized. Development and optimization of plant cell culture processes require both bioprocess engineering and metabolic engineering approaches. Cultures are generally highly heterogeneous, with significant variability amongst cells in terms of growth, metabolism, and productivity of key metabolites. *Taxus* cultures produce the important anti-cancer agent Taxol® (i.e., paclitaxel) and have demonstrated significant variability amongst cell populations in culture with regard to paclitaxel accumulation, cell cycle participation, and protein synthesis. To fully understand the link between cellular metabolism and culture behavior and to enable targeted metabolic engineering approaches, cultures need to be studied at a single cell level. This chapter describes the application of plant cell flow cytometric techniques to investigate culture heterogeneity at the single cell level, in order to optimize culture performance through targeted metabolic engineering. Flow cytometric analytical methods are described to study *Taxus* single cells, protoplasts, and nuclei suspensions with respect to secondary metabolite accumulation, DNA content, cell size, and complexity. Reproducible methods to isolate these single particle suspensions from aggregated *Taxus* cultures are discussed. Methods to stain both fixed and live cells for a variety of biological markers are provided to enable characterization of cell phenotypes. Fluorescence-activated cell sorting (FACS) methods are also presented to facilitate isolation of certain plant cell culture populations for both analysis and propagation of superior cell lines for use in bioprocesses.

**Key words:** Plant cell culture, flow cytometry, cell sorting, FACS, *Taxus*, paclitaxel, culture heterogeneity, secondary metabolite, DNA content.

1. Introduction

Plant cell cultures provide an important method for production of various natural products (e.g., pharmaceuticals, colors, specialty chemicals) in which environmental conditions can be easily
controlled, manipulated, and optimized in order to obtain high quantities of these valuable compounds (1). Development of an optimal plant cell culture process requires a successful combination of bioprocess engineering and metabolic engineering approaches. Adapting existing strategies, which are often developed using well-studied cell types (e.g., mammalian, yeast, and bacteria), does not necessarily translate into productive and viable plant cell culture processes because of the inherent challenges associated with plant cells that ultimately lead to low and variable product yields. While a majority of research on plant cell culture optimization has been focused on system characterization (e.g., reactor design, medium optimization, and nutrient utilization), there have been few studies emphasizing the link between cellular metabolism and culture heterogeneity (2–6).

Cell–cell heterogeneity in culture results in unpredictable shifts in product accumulation over time and within cultures cultivated at the same time. Cell aggregation creates distinct microenvironments owing to differences in oxygen and nutrient availabilities, resulting in alterations in gene expression and metabolic function, which may be observed as phenotypic changes in culture behavior. These changes include variations in metabolic pathway participation and metabolite accumulation as well as cell subpopulation variability where certain populations of cells do not participate in growth or secondary metabolite accumulation (7). Most studies concerning metabolite production via cell culture technology rely on culture-average parameters. These measurements involve averages of culture properties over a group of cells, and are insufficient to describe culture heterogeneity as they neglect variations at the single cell level. Analyzing cell populations with different properties, such as varying levels of metabolite accumulation, at the single cell level can significantly contribute to the understanding of the inherent molecular and metabolic differences amongst cells in culture and lead to new approaches for controlling culture heterogeneity and production variability. Techniques for investigating single cell properties, most notably flow cytometry, can provide insight into the nature of culture heterogeneity and when adapted with a sorting functionality allow for recovery of distinct sub-populations for further analyses (e.g., gene expression profiling, metabolite profiling, DNA content, and ploidy measurements).

Flow cytometry is a powerful tool to count, analyze, and sort single particles (e.g., cells, protoplasts, nuclei, chromosomes, beads) in suspension. Flow cytometry allows for simultaneous multiparameter analysis of cells and/or cellular molecules, based on their light scatter and fluorescence properties, within heterogeneous populations (8). The quantitative evaluation of cellular characteristics on a per-cell basis across the entire population set facilitates rapid, accurate, and sensitive analyses of culture
Flow Cytometry in Plant Metabolic Engineering

Table 17.1
Examples of some important flow cytometric-measurable parameters and measurement techniques and/or fluorescent probes

<table>
<thead>
<tr>
<th>Measurable variables</th>
<th>Measurement techniques and/or associated probe(s)</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell count and size</td>
<td>Scatter dot plots</td>
<td>Nicotiana tabacum (9)</td>
</tr>
<tr>
<td>DNA, ploidy, cell cycle</td>
<td>DAPI, mithramycin, propidium iodide</td>
<td>Dioscorea (10)</td>
</tr>
<tr>
<td>Chlorophyll content</td>
<td>Autofluorescence</td>
<td>Nicotiana tabacum (11)</td>
</tr>
<tr>
<td>Cell viability</td>
<td>PI and fluorescein diacetate (FDA)</td>
<td>Brassica napus (12)</td>
</tr>
<tr>
<td>Cell-wall components</td>
<td>Specific antibodies, Calcofluor white</td>
<td>B. napus (13)</td>
</tr>
<tr>
<td>Apoptosis and related studies</td>
<td>Annexin-V</td>
<td>Nicotiana plumbaginifolia (14)</td>
</tr>
<tr>
<td>Membrane fluidity</td>
<td>Diphenylhexatriene (DPH)</td>
<td>Lupinus albus L. (15)</td>
</tr>
<tr>
<td>Membrane potential</td>
<td>Rhodamine 123</td>
<td>Solanum tuberosum L. (16)</td>
</tr>
<tr>
<td>Intracellular Ca^{2+}</td>
<td>Indo-1, fura-2</td>
<td>Barley aleurone (17)</td>
</tr>
<tr>
<td>Gene expression</td>
<td>GFP or other reporter genes</td>
<td>Arabidopsis (18)</td>
</tr>
<tr>
<td>Intracellular molecules</td>
<td>Specific dyes, antibodies tagged with fluorescence after cell fixation and permeabilization</td>
<td>Coptis japonica (19)</td>
</tr>
</tbody>
</table>

Flow cytometry has been used extensively by scientists for the study of mammalian and microbial cell populations for biotechnological applications (20). Plant cell flow cytometry is, however, still an emerging field where many researchers adapt existing techniques developed for mammalian and microbial cells. Sorting and analysis of various plant particles/organelles (e.g., nuclei, protoplasts, chromosomes, and pollen grains) have been performed, with applications ranging from production of pharmaceuticals and specialty chemicals to crop breeding. A genomic chromosome library has been constructed through flow cytometric sorting of banana (*Musa balbisiana*) nuclei (21). Specific green fluorescent protein (GFP)-positive protoplasts of *Arabidopsis thaliana* have been isolated using fluorescence-activated cell sorting (FACS) and analyzed for gene expression (22). Chromosomes differing in DNA content have been sorted and isolated from suspension cells of *Haplopappus gracilis* using flow cytometry (23). Transcriptional profiling of *Arabidopsis* tissues was performed by obtaining highly purified pollen grains through flow cytometric
sorting (24). However, because of the large size of some plant protoplasts and non-uniformity of intact single cells (i.e., those cells that contain an intact cell wall), it is challenging to use conventional methods and flow cytometers for analysis and sorting (25). Plant cells are relatively large in size (ca. 20–100 μm), limiting the use of a typical nozzle in the cytometer. The dimensions of the particles to be sorted, including intact single cells, should be compatible with the flow nozzle. It is generally assumed that for a smooth and clog-free run, the flow nozzle/orifice should be at least four times the particle size, therefore demanding special size instrument nozzles when sorting larger plant cells (26).

Intact single cells, as opposed to protoplasts, are relatively non-isotropic, which adds complexity in the application of flow cytometric methods. Moreover, large sized particles create instabilities in the flow stream, which mandates that the system flow rate and sheath pressure be maintained at low values, necessitating longer runs. Researchers have studied the physics of the cytometric sorting process to establish correlations which explain and can ultimately predict flow and droplet formation in a sorter. For instance, there have been reports of inter-dependence between parameters such as sorting efficiency, particle diameter, flow cell nozzle diameter, sheath pressure, and drive frequency (26). An extensive optimization of these parameters can lead to stable hydrodynamic flow conditions, resulting in efficient droplet formation and successful sorting of plant cells. Vacuoles, which are largely comprised of water and may constitute up to 90% of a plant cell, render plant cells fragile and shear-sensitive, thus affecting cell health and viability during sterile operations such as live cell staining and sorting. Special care (e.g., low centrifugation speeds and reduced agitation rates) must be taken during sample preparation and instrument operation to avoid any potential detrimental cellular effects. Plant cells, unlike animal and microbial cells, are non-uniform in shape, creating problems with signal detection that lead to incorrect optical measurements. To overcome this issue, a significant number of cells (~10,000 or more) should be analyzed in the cytometer, to ensure an accurate representation of the entire population set.

Another crucial difference between plant cells and other systems that limits the application of flow cytometry is the tendency of plant cells to aggregate in suspension. The first step in isolating a single particle suspension from aggregated plant cell suspensions is to induce single cell generation using enzymatic digestion to weaken the middle lamella that cements adjoining cells in an aggregate (3). Following single particle isolation, cells tend to sediment in suspension, which can complicate cytometer operation. Xanthan gum, a relatively inert material, has been used to keep large biological particles suspended during flow cytometric analysis and sorting (27).
This chapter describes the application of different flow cytometric techniques to analyze a variety of plant particles, including intact single cells. A comprehensive population analysis can be used to both optimize bioprocess conditions for growth and productivity and to identify targets for metabolic engineering through focusing on productive cells. Here we describe methods developed in *Taxus* plant suspensions; however, all techniques can be easily adapted to other plant culture systems. Flow cytometric methods are described to investigate heterogeneity in *Taxus* single cells, protoplasts, and nuclei suspensions with respect to secondary metabolite accumulation, DNA content, cell size, and complexity. A variety of reliable methods to isolate these single particle suspensions (i.e., single cells, protoplasts, nuclei) from aggregated *Taxus* suspensions are discussed. Techniques to stain cells for different types of biological molecules, that provide information about the metabolic, genomic, and phenotypic state of these cultures, are also detailed. Finally, sorting of plant protoplasts and intact single cells for the isolation of distinct populations is described.

### 2. Materials

#### 2.1. Cell Culture

**Maintenance and Methyl Jasmonate Elicitation**

1. Gamborg’s B-5 basal medium with minimal organics (Sigma-Aldrich, St. Louis, MO) (28) is dissolved in nanopure water at 3.2 g/L (see Note 1).

2. Sucrose, grade I (plant cell culture tested; Sigma-Aldrich) is added to the basal medium solution at 20 g/L.

3. Stock solutions of α-naphthalene acetic acid (NAA) and benzyladenine (BA) (Sigma-Aldrich) are made in nanopure water, and stored at 2–8°C for up to 6 months.

4. Supplemental anti-oxidants and nutrients: ascorbic acid (150 mg/L), L-glutamine (non-animal source) (6 mM) and citric acid (150 mg/L) (Sigma-Aldrich) are dissolved in nanopure water, filter sterilized through a 0.22 μm Millex® GP filter unit (Millipore) and added to the autoclaved medium. This solution is made fresh for each culture transfer (see Notes 2 and 3).

5. Pyrex glass shake flasks (125 ml) and Bellco semi-permeable foam closures (Vineland, NJ).

6. A working solution of methyl jasmonate (MJ) (95%; Sigma-Aldrich) is prepared by adding 50 μL of MJ to 450 μL of 95% (v/v) ethyl alcohol and 500 μL of nanopure water. The solution is then vortexed and filtered through a 0.2 μm
Gelman acrodisc PVDF filter (Sigma-Aldrich) into a sterile container prior to culture addition.

2.2. Isolation of Single Cells and Protoplasts from Aggregated Cultures

1. Working solution of osmoticum is prepared by dissolving 0.5 M D-mannitol and 0.3% (w/v) dextran sulfate sodium salt (Sigma-Aldrich) in nanopure water. The pH is adjusted to 5.5 and the osmoticum is then autoclaved at 121°C for 15 min and stored at room temperature for up to 48 h (see Note 4).

2. Cellulase from *Trichoderma viride* (Sigma-Aldrich) and Pectolyase Y-23 (MP Biomedicals, LLC, Solon, OH).

3. Miracloth® (Calbiochem, San Diego, CA) (cut into squares of 4 × 4 in.), 80 μm nylon mesh (Sefar American, Depew, NY) (cut into squares of 3 × 3 in.), aluminum foil, vacuum flask, funnel, spatula, and 25 mL Erlenmeyer flasks are sterilized via autoclaving.

4. Phosphate-buffered saline (PBS) at 0.01 M prepared in nanopure water.

5. Sucrose solution at 0.5 M prepared in nanopure water.

6. Paraformaldehyde (1% (w/v)) (Sigma-Aldrich).

7. Centrifuge tubes (15 and 50 mL).

8. BD Falcon 5 mL polystyrene round-bottom tube (BD Biosciences, Bedford, MA).

9. BD 22G 1½ Precision Glide® needle and 1 mL syringe (BD & Co., Franklin Lakes, NJ).

10. Hemacytometry – V167014 light microscope (VWR), Leica Zoom 2000 microscope (Leica Microsystems, Buffalo, NY), or the like hemacytometer glass slides and cover slips (VWR).

2.3. Immunofluorescent Staining for Paclitaxel

1. Working solutions of primary mouse monoclonal anti-paclitaxel antibody (Cardax Pharmaceuticals, Aiea, HI) and secondary goat anti-mouse phycoerythrin (PE)-conjugated antibody (Southern Biotech, Birmingham, AL) are prepared in BPT [0.25% (w/v) bovine serum albumin, 0.05% (v/v) Tween-20, 0.02% (w/v) sodium azide in 10 mM PBS]. These solutions are stored at 2–8°C until use.

2. Solution of TBS-T (50 mM Tris–HCl, 0.15 M sodium chloride, 0.05% (v/v) Tween-20, 0.02% (w/v) sodium azide) stored at 28°C.

2.4. Flow Cytometric Analysis and Sorting

1. BD LSR II analytical flow cytometer (Becton Dickinson, San Jose, CA) with light source suitable for excitation of the fluorochromes used in the study (PE, PI, and FITC) (e.g., an Argon laser tuned to 488 nm) and BD LSR II User’s Guide.
The cytometer should be capable of evaluating multiparameter cellular properties (e.g., fluorescence and light scatter).

2. BD FACS Vantage SE™ cell sorter (Becton Dickinson, San Jose, CA) configured with 488 nm air-cooled lasers and BD FACS Vantage User’s Guide. The sorter should be equipped with a MacroSort™ option and a large nozzle (200 μm) for sorting of large plant particles.

3. Software for the analysis of flow cytometric data (e.g., BD FACSDiva™ (Becton Dickinson, San Jose, CA) or FlowJo (Tree Star, Inc., Ashland, OR)).

4. Calibration beads for BD LSR II analytical flow cytometer – Sphero™ rainbow fluorescent particles, 3.0–3.4 μm (BD Biosciences, San Diego, CA).

5. Calibration beads for BD FACS Vantage cell sorter – AlignFlow™ flow cytometry alignment beads, 2.5 μm for 488 nm excitation; and AlignFlow™ flow cytometry alignment beads, 2.5 μm for 633 nm excitation (Molecular Probes, Inc., Eugene, OR).

6. Megabead NIST Traceable Particle Size Standard, 25.0 μm (Polysciences, Inc., Warrington, PA).

7. Sheath buffer (sterile PBS or 0.9% sodium chloride).

8. Cytometer sterilization kit (10% bleach, 70% ethanol, and distilled water) (can be prepared at the cytometer facility; follow the manufacturer’s manual).

2.5. Preparation of Nuclei Suspensions from Aggregated Cultures

1. Nuclei isolation buffer is prepared by dissolving MgCl₂ (45 mM), 4-morpholinepropanesulfonic acid (MOPS) ≥ 99.5% (titration) (20 mM), sodium citrate (30 mM), and Triton X-100 [0.3% (v/v)] (Sigma-Aldrich) in nanopure water and adjusting the pH to 7. The isolation buffer is filtered through a 0.2 μm Gelman acrodisc PVDF filter and stored at –20°C in aliquots of 10 mL. This buffer should not be refreezed once thawed.


3. Polystyrene (100 × 15 mm) Petri dishes.

2.6. Staining of Nuclear DNA

1. Propidium iodide (PI) stock solution of 1 mg/mL is made in nanopure water, filtered through a 0.2 μm Gelman acrodisc PVDF filter, and stored at –20°C in 1 mL aliquots, for single use only.

2. Ribonuclease A (activity > 97.9 units/mg) (Fisher Scientific, Hampton, NH) stock solution of 1 mg/mL is made in nanopure water and heated for 15 min at 90°C to render any DNases inactive. The solution is stored at –20°C in aliquots of 1 mL, for single use only.
3. Methods

As a first step in analyzing plant cells using flow cytometry it is crucial to develop reliable methods for isolation of plant-derived particles (i.e., nuclei, protoplasts, and intact single cells). Plant cells aggregate via a middle lamella layer that cements neighboring cell walls together. While cellulose is the primary component of the plant cell wall, the middle lamella is largely composed of pectin. Enzymes such as cellulase and pectolyase Y-23 are used, at appropriately evaluated concentrations, to prepare single cells and protoplasts from aggregated cultures. Since paclitaxel is primarily stored in the cell wall, as is the case with many hydrophobic secondary metabolites (29, 30), intact isolated single cells must be used to characterize populations with regard to paclitaxel accumulation. An indirect immunofluorescent procedure is employed to identify paclitaxel-accumulating populations through flow cytometry. Protoplasts, isolated using enzymatic digestion, are useful in the study of size, complexity, and intracellular protein content. Isolated nuclei can be stained for DNA and protein content using PI and FITC, respectively, and are useful in the study of nuclear protein synthesis, ploidy levels, and cell cycle participation. Here we present detailed methods for the isolation of nuclei, protoplasts, and intact single cells from aggregated plant cultures, along with appropriate staining techniques, to enable population analyses via flow cytometry.

3.1. Cell Culture Maintenance and Methyl Jasmonate Elicitation

1. The cell lines P991, P93AF, PO93X (*Taxus cuspidata*), and CO93D (*Taxus canadensis*), obtained from US Plant Soil and Nutrition Laboratory (Ithaca, NY), are maintained by sub-culturing every 2 weeks into fresh medium consisting of Gamborg’s B-5 basal medium (3.2 g/L) and sucrose (20 g/L). The medium is supplemented with 2.7 μM NAA and 0.1 μM BA and is adjusted to a pH of 5.5. Media is transferred into 125 mL shake flasks (40 mL media per flask), capped with Bellco foam closures, and sterilized via autoclaving at 121°C for 15 min. Media is then brought to room temperature before supplemental anti-oxidants and nutrients are added. Ten milliliters of cell culture suspension from a 14-day-old culture, with 2–3 mL of packed cell
volume per 10 mL, are transferred to each of the 125 mL flasks. All cultures are maintained in gyratory shakers (125 rpm) at 23°C in the dark.

2. Elicitation of the *Taxus* cell cultures to up-regulate the genes involved in secondary metabolite accumulation is performed with MJ 7 d post-transfer at 200 μM (31). A working stock solution of MJ is added to the culture flasks under sterile conditions.

3. Isolation of Single Cells and Protoplasts from Aggregated Cultures

3.2. Isolation of Single Cells

1. For isolation of single cells, an enzyme solution is prepared by adding 0.04% (w/v) cellulase and 0.5% (w/v) pectolyase Y-23 in osmoticum (see Notes 5 and 6). The mixture is filter sterilized through a 0.22 μm Millex® GP filter unit (Millipore) and then transferred to sterile containers (e.g., 25 mL flasks, six-well plates). The volumes of the enzyme mixture and isolated single cell suspension can be varied according to the required application (see Note 7).

2. Cells from late exponential phase of the culture period are vacuum-filtered using Miracloth® and added to the enzyme mixture. The ratio of cells added to the enzyme mixture is kept at a value of 1 g cells/5 mL solution. Enzymatic digestion is carried out at typical culture conditions (see Section 3.1.1) for 4 h.

3. Post-digestion, the single cell suspension is centrifuged at $1000 \times g$ for 5 min (see Note 8). After centrifugation, the supernatant is carefully removed with a syringe and needle (see Note 9).

4. PBS is added to the cells to increase the total volume to the required working volume. Single cells are then purified by filtering twice through 80 μm nylon mesh into new centrifuge tubes. Filtration is performed by pouring/transferring (using a cut 1 mL pipette tip) the cell suspension through the nylon mesh (held over an open centrifuge tube) and collecting the filtrate in the tubes.

5. Cell density is then measured using hemacytometry. A suitable working range of cell density for flow cytometry and sorting is $(0.5–1.0) \times 10^6$ cells/mL. The cell suspension should be diluted, if necessary, with PBS to achieve this cell density. Care should be taken to account for the loss of cells during cell counting and subsequent staining and filtering steps, especially when using small volumes (i.e., less than 2 mL) of solution (see Notes 9 and 10).

6. The isolated single cells can be utilized for several types of analysis. Cells may be fixed and stained for paclitaxel accumulation (see Section 3.3) and analyzed in the BD LSR II flow cytometer (see Section 3.4.1) or used for cell
sorting through the BD FACS\textsuperscript{V}antage cell sorter (see Section 3.4.2). In order to avoid any loss of important metabolic information in the isolated single cells, samples should be analyzed and/or sorted in the cytometer immediately following preparation (see Note 11).

### 3.2.2. Isolation of Protoplasts

1. For formation of protoplasts, the enzyme solution is prepared at a different concentration than that required for single cell generation. One percentage (w/v) cellulase and 0.1\% (w/v) pectolyase Y-23 is dissolved in osmoticum (see Note 5). The mixture is filter sterilized through a 0.22 μm Millipore\textsuperscript{G}P filter unit (Millipore) into a sterile container.

2. Cells are added to the enzyme mixture and digestion is carried out as described in Section 3.2.1, step 2.

3. Post-digestion, the suspension is centrifuged at 300×\textit{g} for 5 min (see Note 9). The supernatant is carefully removed using a syringe and needle (see Note 9).

4. Enzyme-free osmoticum is added to the sample to increase the volume to the required working volume. The sample is then filtered through 80 μm nylon mesh into sterile centrifuge tubes (see Section 3.2.1, step 4).

5. Approximately one-third volume of 0.5 M sucrose is added to the sample and mixed by inversion. The sample is allowed to sit for 20–30 min, during which time the sucrose and osmoticum separate due to density differences and the protoplasts settle in a noticeable ring at the liquid–liquid interface. The protoplasts are then carefully removed using a 1 mL cut pipette tip.

### 3.3. Immunofluorescent Staining for Paclitaxel

1. The isolated single cells (as described in Section 3.2.1, step 6) are fixed with 1\% (w/v) paraformaldehyde at 4°C for 1 h. The cells are then washed two times with an equal volume of PBS to remove any remaining fixative. Washing includes the following steps: (i) centrifuge at 400×\textit{g} for 1 min; (ii) remove the supernatant using a syringe and needle (see Note 9); (iii) add PBS; and (iv) repeat starting at step (i).

2. The fixed cells are then divided into two equal volumes and transferred to suitable containers. The first sample serves as the fluorescence control and is treated only with the secondary antibody. The second sample is incubated with both primary and secondary antibody to stain specifically for paclitaxel.

3. To stain for paclitaxel, the sample is incubated with the primary antibody at a dilution ratio of 1:500 (see Notes 12 and 13) at typical culture conditions (125 rpm, 23°C, and dark) for 1 h. After incubation, the sample is washed two
times with one-half volume of TBS-T, in a similar manner as described in step 1 above. Both the stained sample and the control sample are then incubated with secondary antibody at a dilution ratio of 1:500 for 1 h at typical culture conditions (see Notes 12 and 13). Thereafter, each sample is washed two times with TBS-T as described above.

4. Both the control and stained samples are filtered through 80 μm mesh (see Section 3.2.1, step 4), re-suspended in equal volume of PBS, and transferred to 5 mL round-bottom tubes for flow cytometric analysis (see Note 14).

3.4. Flow Cytometric Analysis and Sorting

3.4.1. Flow Cytometric Analysis of Paclitaxel Accumulation

1. The BD LSR II is used for the analysis of paclitaxel accumulation in stained single cells. The cytometer setup is performed according to the instructions in the BD LSR II User’s Guide. Typical steps in the setup procedure include starting the computer and the cytometer, setting up the optical filters and mirrors, preparing sheath and waste containers, and configuring the fluidics system. A cytometer quality control (QC) procedure is performed prior to sample analysis. To optimize laser and fluidics performance, QC calibration beads (Spherotech rainbow fluorescent particles) are run first, results recorded, and setup parameters are adjusted based on bead performance. Refer to the BD LSR II User’s Guide for more details.

2. Once the cytometer setup is complete, the settings for the sample type and fluorochromes used are selected (i.e., selection of filter sets to be used to collect data) before running a sample. The BD LSR II is generally equipped with 488 and 633 nm fixed alignment lasers. The primary blue 488 nm laser can detect five fluorochromes (including the PE fluorochrome conjugated to the antibody used in the immunostaining for paclitaxel), while the red 633 nm laser can detect three fluorochromes.

3. The control cell sample (see Section 3.3.3) is run first through the BD LSR II cytometer (see Note 15). Typically, data collected for immunofluorescent analysis include forward scatter (FSC), side scatter (SSC), and PE-fluorescence. Data are visualized real-time using scatter dot plots (FSC and SSC) and histograms (PE). Voltage settings on the photomultiplier tubes (PMT) for each detector must be adjusted so that measurements are scaled properly to spread across the dot plot; otherwise data points may be grouped at the maximum or minimum of the relevant axis. Additionally, the PE detector voltage should be set so that the control histogram falls at the low end of the fluorescent range, since positively stained samples will have a higher fluorescence and must not reach the maximum of the range. Once voltage settings are adjusted, they remain unchanged for analysis of subsequent
samples so that different samples can be directly compared to each other (see Note 16).

4. Subsequently, data for 10,000 control cells are recorded. If necessary, the FSC threshold can be adjusted and/or a manual gate drawn to exclude debris from the population of interest and defined as population P1 (Fig. 17.1a).

5. The stained cell sample is then run on the flow cytometer and data for 10,000 cells are recorded. The stained sample histogram is analyzed and the non-overlapping part, using the same relative fluorescence cutoff, is obtained. The cells in this region are the PE-positive cells and represent the cells in the culture that are positively stained for paclitaxel (Fig. 17.1b, c). Cells having fluorescence which exceeds 99.5% of that of the control cells are defined as positively stained cells (8).

**Fig. 17.1.** a Flow cytometric scatter dot plot (FSC vs. SSC) of *T. cuspidata* P991 single cells stained for paclitaxel (see Section 3.3). The figure shows a method for gating the population of interest while recording the fluorescent data. A manual polygon-shaped gate, excluding the debris and aggregates, is drawn in the dot plot before recording the data. Debris, which is characterized by low FSC and low SSC values in the dot plot, is largely comprised of cellular fragments and undesirable sub-cellular particles. While these are often counted as individual events by the cytometer, they are not intact single cells and do not accurately represent the correct metabolic information pertaining to the stained cells. Aggregates and larger particles, which are formed by fusion of two or more cells, are also not included in the analysis, owing to their incorrect optical measurements. b and c Flow cytometric histograms of control (secondary antibody only) and stained (primary and secondary antibody) samples of *T. cuspidata* P991 single cells: b cells were not elicited with methyl jasmonate (-MJ), i.e., “low/nonpaclitaxel-accumulating” cells; c cells were elicited with methyl jasmonate (+MJ), i.e., “paclitaxel-accumulating cells.” Both elicited and nonelicited cells were stained under identical conditions (see Section 3.3) and can thus be compared on the basis of their fluorescent histograms. Percentage positive is defined as the percentage of stained cells above the threshold set by 99.5% of control cells (8). In this experiment, nonelicited cells were 2% positive, while elicited cells were 89% positive, indicating that significantly more elicited cells accumulate paclitaxel. The relative fluorescent intensity, defined as the difference between the mean fluorescence of stained and unstained cells, can also be used to compare samples. In this experiment, the relative fluorescent intensities of nonelicited and elicited cells were 882 and 2895, respectively, also indicating that elicited cells accumulate more paclitaxel than nonelicited cells. These data indicate cell–cell heterogeneity with regard to secondary metabolite accumulation, as demonstrated by the distribution in paclitaxel-related fluorescence in the samples. This type of analysis can be used to understand secondary metabolite heterogeneity in cell culture populations to ultimately suggest strategies for optimizing accumulation and stability.
3.4.2. Sorting of Single Cells and Protoplasts

1. A BD FACS Vantage, a special order system equipped with a 200 μm nozzle and a MacroSort™ option, is used for sorting of single cells and protoplasts. The sorter setup is performed according to the instructions in the BD FACS Vantage User’s Guide, which includes, starting up the instrument, instrument optimization and quality control, and sample processing. QC calibration beads (AlignFlow™ flow cytometry alignment beads, 2.5 μm for 488 nm excitation) are run and the data are used to align the 488 nm laser. The signal from this primary laser is optimized for consistency by adjusting controls such as nozzle alignment, objective lens, FSC obscuration bar, beam focus, fluorescence focus, etc. If using a two-color sorting, the signals from the secondary laser (633 nm) should also be optimized using the AlignFlow™ flow cytometry alignment beads, 2.5 μm for 633 nm excitation. Refer to the BD FACS Vantage User’s Guide for more details.

2. Before recording data, instrument settings (FSC and SSC voltages, FSC threshold, and fluorescence PMT voltages) are optimized by running large beads (Megabead NIST Traceable Particle Size Standard, 25.0 μm), which represent an average plant cell size. These beads are sorted based on scatter properties and the configuration settings corresponding to high sort purity (>90%) are recorded. The instrument is subsequently kept at the same settings to sort plant cells and protoplasts (see Note 17). Refer to the BD FACS Vantage User’s Guide for more details.

3. Isolated single cells (see Section 3.2.1) and protoplasts (see Section 3.2.2) are then separately run through the cell sorter. The data are acquired on a FSC vs. SSC scatter dot plot for a minimum of 10,000 events. The events, corresponding to the cells to be sorted, are selected from the dot plot based on high FSC and SSC properties, and gated as P1 (Fig. 17.2a, c). These cells/protoplasts are then sorted in the FACS Vantage and collected in tubes containing sterile 50:50 medium (see Note 11).

4. The sorted cells/protoplasts are then analyzed for sort purity by running the recovered populations through the sorter. The scatter data corresponding to the sorted cells/protoplasts are recorded and the number of events lying inside the gated P1 region is determined (Fig. 17.2b, d). Sort purity is defined as the percentage of events in the post-sorting plot that fall within the P1 region.

3.5. Preparation of Nuclei Suspensions from Aggregated Cultures

1. For preparation of nuclei suspensions, cells from day 12 of the culture (note: here cells were day 12, but cells could be isolated from any culture time) are vacuum-filtered through
Fig. 17.2. Flow cytometric scatter dot plots of sorting of single cells (a and b) and protoplasts (c and d) by size: a) single cells before sorting, 52% (P1) of the entire parent events were selected for sorting based on high FSC and SSC values; b) single cells after sorting, 91% of the selected events fall within P1 in the sorted cells plot; c) protoplasts before sorting, 62% (P1) of the entire parent events were selected for sorting based on high FSC and SSC values; and d) protoplasts after sorting, 93% of the selected events fall within P1 in the sorted protoplasts plot. Single cells and protoplasts were isolated from T. cuspidata cell line P991 (see Section 3.2) and were run through a BD FACSVantage (see Section 3.4.2). High sort purities (91% for single cells and 93% for protoplasts) were obtained for sorting based on size. These results demonstrate the feasibility of sorting both intact Taxus cells and protoplasts. These distinct populations differing in size and complexity can be isolated and further analyzed to investigate culture heterogeneity.

Miraclenth® and approximately 0.5 g of cells are transferred to the center of a plastic Petri dish.

2. The frozen nuclei isolation buffer is thawed at room temperature (approximately 30 min) and allowed to liquefy completely. Approximately 1–3 mL of the cold nuclei isolation buffer is added to the Petri dish (32).

3. Cells are immersed in the buffer and chopped immediately with the mini-glass scraper for 5 min (32) (see Note 18). The resultant sample is mixed properly with the buffer several times by tilting the Petri dish to ensure uniform chopping of the entire sample. If necessary, additional buffer can be added to facilitate chopping (Fig. 17.3a).
**Fig. 17.3.**

(a) Preparation of *T. cuspidata* P991 nuclei suspensions: cells were placed in a Petri dish with the isolation buffer and chopped with a mini-glass scrapper (see Section 3.5); and (b) histogram of PI fluorescence intensity as a measurement of relative 2C nuclear DNA content obtained after PI staining and subsequent flow cytometric analysis of the isolated nuclei suspension (see Sections 3.6 and 3.7). Note that there are two peaks in the histogram. A nucleus in the first peak has two copies of the unreplicated genome and has a relative DNA content of 2C. Similarly, a nucleus in the second peak has 4C relative DNA content. The two peaks can be gated and their mean fluorescence intensities determined which can be used to estimate the absolute DNA content using known standards. This method can be used to explore culture variability by determining the DNA content/genome size variation across different cell lines and under different culture conditions.

4. The chopped sample is then transferred to a 15 mL centrifuge tube and PBS is added to make up the volume to 10 mL.

5. The nuclei suspension is then filtered through 80 μm mesh (see Section 3.2.1, step 4) and transferred into sterile 15 mL centrifuge tubes for staining of DNA.

### 3.6. Staining of Nuclear DNA

1. For staining of DNA, both PI and Ribonuclease A are added to the nuclei suspension (isolated from nonelicited cultures as described above) at final concentrations of 50 μg/mL.
each (see Note 19). The sample is mixed by inverting the tubes several times. The sample is then incubated on ice for 15 min.

2. The PI-stained sample is filtered through 80 μm mesh and transferred to 5 mL round-bottom tubes for flow cytometric analysis.

3.7. Flow Cytometric Analysis of Stained Nuclei

1. The BD LSR II analytical flow cytometer is used to measure the fluorescence of the stained nuclei suspensions. The fluorochrome used is PI, which can be detected using the 488 nm laser under the PI detector. Refer to Section 3.4.1 for cytometry setup and other related procedures.

2. The PI-stained sample is run in the flow cytometer and data for 5,000 events are acquired in a histogram chart containing PI fluorescence intensity as a measure of relative nuclear DNA content. The scatter dot plot is gated manually to eliminate background noise arising from debris or aggregates (see Notes 20 and 21) (Fig. 17.3b).

3. Minimize interruptions during operation of the flow cytometer (see Notes 22 and 23).

4. Notes

1. Nanopure water used to prepare solutions should have a resistivity greater than 18 MΩ cm.

2. All culture medium preparation and cultivation steps should be carried out in a laminar flow hood under sterile conditions.

3. Supplemental plant anti-oxidants and nutrients should be made fresh and added to the autoclaved media under sterile conditions. These compounds are labile and degrade upon autoclaving.

4. Osmoticum should be prepared a day before single cell and protoplast isolation to enable potential equilibrium and stored at room temperature.

5. Cell wall digesting enzymes are labile and will denature upon heating, so ensure that enzyme solutions are not heated to facilitate dissolution. Also, do not vortex enzymes, as proteins are more readily denatured at air–water interfaces, particularly at high speeds. Dissolve the enzymes by adding small amounts in succession with intermittent mixing/shaking through inversion by hand.

6. Specific enzyme concentrations to efficiently dissociate aggregates into single cells and protoplasts must be
determined for different plant species and cell types. The single cell yield (SCY), which represents the percentage of cell clusters that contain only a single cell, serves as an indicator of effective disaggregation and can be obtained using hemacytometry (3). Working with flow cytometry necessitates the use of single cell suspension and, therefore, obtaining a high SCY is critical to ensure accurate representation of aggregated cultures.

7. The amounts of isolated single cells and protoplasts depend on the relevant application. For instance, relatively greater volumes will be required for a cell sorting application as compared to analytical flow cytometric evaluation. Typical reaction containers used are 15 mL flasks, six-well plates, and 1.5 mL centrifuge tubes.

8. Isolated single cells and protoplasts should be handled with special care to avoid any breakage. Do not centrifuge for either longer periods or at higher speeds than indicated.

9. Minimize sample/cell loss during washing and other steps by removing the supernatant carefully with a syringe and needle, instead of either decanting or using a pipette tip.

10. When diluting the isolated cell suspension to the required level, always consider to account for cell loss during subsequent steps (e.g., staining, washing). It is advisable to maintain a cell density slightly above the recommended levels to account for any potential loss during manipulation.

11. It is advisable to analyze/sort samples in the flow cytometer immediately after preparation. However, if required, isolated single cells can be stored overnight in a tailor-made 50:50 medium (50% fresh medium + 50% conditioned medium from day 12 of the culture period) without affecting viability or SCY.

12. In order to avoid any fluorescence loss, protect the cells from light while incubating with the fluorophore-attached secondary antibody by wrapping containers with metal foil or keeping the containers inside a larger opaque container.

13. Optimal antibody concentrations must be determined in a titration experiment in which increasing concentrations of antibodies are used until fluorescence saturation is reached. In the case of abundant molecules present in high concentrations, for which saturation is not possible, a constant antibody concentration may be used, in which case relative fluorescence shifts can be compared between different samples.

14. Single cells can be filtered a final time through 80 μm mesh prior to flow cytometric analysis, if any aggregation is observed in the sample. This is particularly important for
cell sorting applications as aggregates can complicate the creation of a stable hydrodynamic flow in the instrument.

15. Gently invert the round-bottom tubes containing cells to be analyzed several times to properly suspend cells and then run enough cells (∼2,000, provided sufficient cells are available) in the BD LSR II cytometer before recording data. This will help stabilize the sample cell counting rate.

16. Once the appropriate PMT voltage settings for the control are obtained, do not change them. Both the control and the stained sample should be run at the same voltage settings. Since the control sample is used to set the level of background fluorescence, it is advisable to check for any possible non-specific binding of the secondary antibody. Ensure that the background fluorescence (i.e., control) is on the order of endogenous fluorescence associated with unstained (i.e., no secondary antibody) cells.

17. Sorting of single cells and protoplasts should be performed at optimized flow and sorting conditions for the instrument (26). Parameters may vary with the type of instrument and sample; therefore, an extensive optimization to obtain a suitable working range for the following parameters should be carried out – sheath pressure, drop-drive frequency, break-off point, drop-delay range, and sample differential.

18. The amount of cells and buffer as well as chopping time should be determined according to the species to enable effective nuclei isolation. Note that some cells may be more resistant to breakage. The razor should be sharp and used only once. Do not chop vigorously or significant cell damage will occur.

19. As PI also binds to dsRNA, add RNase simultaneously with PI to the nuclei suspension. Also, heat the RNase solution at 90°C for 15 min to deactivate any DNase activity.

20. In order to obtain PI peaks without undesirable low channel signals, choose an appropriate PMT voltage threshold to minimize off signals coming from cell debris and autofluorescent compounds. The scatter dot plots may be gated to exclude any debris and aggregates as described previously.

21. In case of significant debris and aggregates in the isolated nuclei suspension, the concentration of the nonionic detergent (i.e., Triton X-100) can be increased in the isolation buffer. Increasing concentrations aid in the release and cleaning of nuclei solutions, by decreasing the aggregation affinity of nuclei and debris without affecting the fluorescence properties of the dye molecule (33). Besides,
there are several possible reasons (e.g., improper staining protocol, vigorous chopping, blunt razor blade, incorrect instrument operation and data acquisition, negative cytotoxic effects, recalcitrant tissues) for broad peaks and large amount of debris background that should be appropriately addressed (34).

22. Avoid air bubbles in the flow cytometer system by minimizing sample mixing and tube changes during operation.

23. If there is a clog in the flow chamber or line tubing, or the sheath pressure and/or the event rate drops unexpectedly, pause the sorting, clean the flow chamber, tubing, in-line filters, and inspect for leaks. Cleaning is performed using the cytometer sterilization kit (10% bleach, 70% ethanol, and distilled water) as mentioned in the User’s Guide for the instrument.

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References


Phenylpropanoid Biosynthesis in Leaves and Glandular Trichomes of Basil (Ocimum basilicum L.)

Cícero Deschamps and James E. Simon

Abstract

Basil (Ocimum basilicum L.) essential oil phenylpropanes are synthesized and accumulate in peltate glandular trichomes and their content and composition depend on plant developmental stage. Studies on gene expression and enzymatic activity indicate that the phenylpropene biosynthetic genes are developmentally regulated. In this study, the methylchavicol accumulation in basil leaves and the enzyme activities and gene expression of both chavicol O-methyltransferase (CVOMT) and eugenol O-methyltransferase (EOMT) were investigated in all leaves at four plant developmental stages. Methylchavicol accumulation decreased over time as leaves matured. There was a significant correlation between methylchavicol accumulation and CVOMT ($r^2 = 0.88$) enzyme activity, suggesting that the levels of biosynthetic enzymes control the essential oil content. CVOMT and EOMT transcript expression levels, which decreased with leaf age, followed the same pattern in both whole leaves and isolated glandular trichomes, providing evidence that CVOMT transcript levels are developmentally regulated in basil glandular trichomes themselves and that differences in CVOMT expression observed in whole leaves are not solely the result of differences in glandular trichome density.

Key words: Ocimum basilicum, essential oil, biosynthesis, trichomes.

1. Introduction

Basil (Ocimum basilicum L.) leaves contain terpenes and aromatic phenylpropanes in the essential oil fraction. Initial developmental studies on essential oil accumulation in Ocimum species demonstrated differences in composition between young and mature leaves (1, 2). Total content and composition of essential oils change with leaf development, although most constituents are
present at all leaf developmental stages. The total essential oil content decreases at leaf maturity.

Peltate and capitate glandular trichomes (glands), distinguished by size and number of head cells, are present on the surface of basil leaves (2), and the essential oil phenylpropanes, including methylchavicol, appear to be produced exclusively in peltate glands (3). Because the phenylpropanes are specifically synthesized in peltate glands, differences in production and accumulation of these compounds should be due to variations in activity of enzymes present in peltate glands. Consequently, monitoring whole leaves (or leaf parts) for essential oil content and composition, mRNA levels, or enzymes specific to the phenylpropene pathway actually monitors these factors in peltate glands diluted by components from the rest of the leaf. In addition, because of the variation of gland density, differences measured in leaves for mRNA level, enzyme activity, and phenylpropene content may be due not only to differences in gene expression but also to differences in gland density.

Initial investigations of enzyme activities in the phenylpropene pathway in basil (4, 5) suggested that this pathway may be developmentally regulated. The young developing tissues appear to be the primary sites of essential oil biosynthesis, as young leaves display much higher levels of enzyme activity than more developed leaves, where the activity is negligible. Likewise, the basal region possesses higher enzymatic activity than middle and apical regions of the leaf.

Studies on gene expression in basil (3, 5) indicated that leaf age and leaf part (basal, middle, and apical) affect the expression level of specific genes. For example, young leaf tissues have higher levels of transcripts for genes involved in the phenylpropene pathway than mature tissues, and basal regions of both young and mature tissues contained higher transcript levels than apical regions (5). These results from basil are similar to the results from work on peppermint (Mentha × piperita), which showed that the developmental regulation of monoterpene biosynthesis and accumulation in glandular trichomes resides at the level of gene expression (6).

The previous studies in basil (3, 5), however, did not investigate the level of gene expression, enzyme activity, and phenylpropene accumulation throughout plant development. In addition, no systematic studies have investigated processes that occur in basil glands through plant development. The optimization of a gland isolation technique for this plant species (3, 7) now allows us to investigate in situ the developmental regulation of gene expression, enzyme activity, and metabolite accumulation in the phenylpropene pathway in basil peltate glands. The availability of basil breeding lines rich in one volatile phenylpropene, such as methylchavicol (in chemotype EMX-1), permits the study
of regulation and expression of specific genes and correlation with accumulated phenylpropenes. We describe the methods used for a systematic analysis of the factors that affect phenylpropene accumulation in basil leaves throughout plant development, highlighting the importance of considering developmental stages, organ- and cell-specific expression in the study of secondary metabolism.

2. Materials

2.1. Plant Material and Growth Conditions

1. *O. basilicum* L. seeds (EMX-1 chemotype methylchavicol) from Newe Ya’ar Research Center, Agricultural Research Organization Israel (see Note 1).
2. Redi-Earth coir mix potting soil.
4. Liquid nitrogen.

2.2. Essential Oil Extraction and Volatile Oil Analysis

1. 200 mg of dried (37°C) leaf samples.
2. MTBE (*tert*-butyl methyl ether, Fisher, Pittsburgh, PA).
3. Safrole (internal standard).
4. Pasteur pipette.
5. Anhydrous Na₂SO₄.
6. Silicagel 60 (230–400 mesh, Merck, Darmstadt, Germany).
7. 0.2 μm syringe filter (Millipore, Fisher, Pittsburgh, PA).
8. Agilent GC system 6890 Series coupled to a mass selective detector.
10. HP5-MS (30 m, 0.25 ID, 0.25 mm) column (Agilent, Palo Alto, CA).

2.3. Enzyme Assays

1. 100 mg of frozen leaves
2. Protein extraction buffer (10:1, w/v), consisting of 50 mM BisTris [2-[bis(hydroxyethyl)amino]-2-(hydroxymethyl)-1-propane-1,3-diol]HCl, pH 8.0, 14 mM β-mercaptoethanol, and 10% (w/v) glycerol.
3. S-[methyl-¹⁴C] adenosyl-l-Met (25 μCi ml⁻¹, Amersham Pharmacia Biotech, UK) buffer A (same as extraction buffer but pH 7.5).
4. 2N HCl.
2.4. Total RNA Extraction, RNA Blot Analysis, and RT-PCR Analysis

1. 200 mg of quick frozen whole leaves (see Note 2).
2. Trizol reagent (Invitrogen, Carlsbad, CA).
3. 100 mg of each peltate and capitate glands (quick frozen).
4. RNeasy kit (Qiagen, Valencia, CA).
5. 32P-labeled OMT probe.
6. CVOMT1 primers (5′-TTTCCCAATTACTCAAGGCC-3′ and 5′-CCCTCCAACGCCAACCTGC-3′).
7. Specific primers for CVOMT to distinguish from EOMT.
8. RT-PCR Introductory System (Promega, Wisconsin, MA).
9. AMV reverse transcriptase (Promega, Madison, WI).
10. 1.0% agarose gel.
12. 32P-labeled OMT.
13. 18S basil DNA probes.

2.5. Glandular Trichomes Isolation

1. 15 g of fresh young (less than 2 cm in length) leaf tissue.
2. 14 mM β-mercaptoethanol.
4. 50 g of glass beads (0.5 mm in diameter, Biospec Products, Inc.).
5. 50 mM Tris-HCl.
6. 200 mM d-sorbitol.
7. 20 mM sucrose.
8. 10 mM KCl.
9. 5 mM MgCl2.
10. 0.5 mM K2PO4.
11. 5 mM succinic acid.
12. 1 mM EGTA.
13. 0.6% [w/v] methylcellulose
14. 1% [w/v] polyvinilpirroldone, 360,000 Mr.
15. 350, 105, 41 and 21-μm mesh cloth.

3. Methods

3.1. Plant Material and Growth Conditions

Seeds are germinated in 4 cm deep cells in trays containing potting soil. After emergence, one seedling is left in each cell and grown under controlled conditions in the greenhouse (24°C/21°C day/night temperature cycle and 13 h natural light
photoperiod). Plants should be harvested at four developmental stages (stage B, when plants have two pairs of true leaves; stage D, four pairs of true leaves; stage F, six pairs of true leaves; and stage H, eight pairs of true leaves), when the newest pair of leaves is 10 days old (approximately 1 cm long). The numbering of leaf pairs is done purposefully in consecutive order from the first pair of true leaves which are coded as stage A through the stage of plant growth where basil exhibits eight pairs of true leaves or stage H. For essential oil extraction, three replicates using 10 plants per replicate are used (30 plants total). For total protein and RNA extraction, 10 plants are harvested for sample collection. Separate leaves from each internode, freeze them in liquid nitrogen, and store at −70°C for later extractions.

### 3.2. Essential Oil Extraction and Volatile Oil Analysis

Plants are harvested manually and leaves are physically separated from the rest of the plant parts and dried at 37°C until a constant weight is achieved. Essential oil extraction is carried out using 200 mg of dried samples by gentle shaking overnight at room temperature with 5 ml MTBE (tert-butyl methyl ether) containing 0.1 μl ml⁻¹ safrole as an internal standard. The extract is cleaned by passing it through a small column (Pasteur pipette) containing equal parts of anhydrous Na₂SO₄ and Silicagel 60 (230–400 mesh). The sample is then filtered through a 0.2 μm syringe filter.

The volatile components are analyzed using a GC system with an HP5-MS (30 m, 0.25 ID, 0.25 mm) column for gas chromatography (GC) coupled to mass spectrometric (MS) and flame ionization (FID) detection. Oils are injected using an autosampler. The inlet temperature is set at 200°C and the column temperature program includes 60°C for 1 min, a ramp of 4°C min⁻¹ to 200°C, followed by a 15 min hold at 200°C. The helium flow rate is 1 ml min⁻¹. Individual compound identifications can be achieved by matching Kovats index values (8) to the literature (9) and by comparison of mass spectra to a mass spectral library (Wiley 275.L). Example of methylchavicol levels in leaves and cotyledons at different developmental stages is shown in Table 18.1.

### 3.3. Enzyme Assays

Assays are performed from 100 mg of frozen leaves after grinding the tissues in a chilled mortar with a pestle in the presence of liquid nitrogen. Protein extraction and assays are performed as described (3). Frozen powdered leaves are mixed with ice-cold protein extraction buffer (10:1, w/v), consisting of 50 mM BisTris [2-[bis(hydroxyethyl)amino]-2-(hydroxymethyl)-1-propane-1,3-diol]HCl, pH 8.0, 14 mM β-mercaptoethanol, and 10% (w/v) glycerol, followed by incubation on ice for 30 min. Next the protein extract is obtained by centrifuging the mixture at 14,000×g for 20 min at 4°C. The clarified
Table 18.1
Methylchavicol accumulation (µg g\(^{-1}\) DW) and chavicol O-methyltransferase (CVOMT) specific activity (pkat mg\(^{-1}\) of protein) in cotyledons and leaves of basil in developmental stages B (two pairs of leaves), D (four pairs of leaves), F (six pairs of leaves), and H (eight pairs of leaves)\(^1\)

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Plant tissue</th>
<th>B</th>
<th>D</th>
<th>F</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methychavicol accumulation</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cotyledons</td>
<td>61.18 Ca</td>
<td>66.15 Da</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Leaf–1st pair</td>
<td>478.45 Ba</td>
<td>245.65 Cb</td>
<td>255.31 Cb</td>
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<tr>
<td>Leaf–2nd pair</td>
<td>1751.58 Aa</td>
<td>569.79 Bb</td>
<td>383.62 Bc</td>
<td>429.08 Cbc</td>
<td></td>
</tr>
<tr>
<td>Leaf–3rd pair</td>
<td>572.66 Ba</td>
<td>491.31 Ba</td>
<td>433.36 Ca</td>
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</tr>
<tr>
<td>Leaf–4th pair</td>
<td>1252.69 Aa</td>
<td>496.84 Bb</td>
<td>667.92 Bb</td>
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<td></td>
</tr>
<tr>
<td>Leaf–5th pair</td>
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<td>738.48 Bb</td>
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<tr>
<td>Leaf–6th pair</td>
<td>1009.10 Aa</td>
<td>992.25 Aa</td>
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<td></td>
<td></td>
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<tr>
<td>Leaf–7th pair</td>
<td>1285.73 A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf–8th pair</td>
<td>914.65 AB</td>
<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>CVOMT activity</th>
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<tbody>
<tr>
<td>Cotyledons</td>
<td>0.43 Ba</td>
<td>0.21 Cb</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Leaf–1st pair</td>
<td>1.01 Aa</td>
<td>0.83 Bab</td>
<td>0.57 Db</td>
<td>0.59 Cb</td>
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<tr>
<td>Leaf–2nd pair</td>
<td>1.27 Aa</td>
<td>1.06 Bab</td>
<td>0.79 Cbc</td>
<td>0.55 Cc</td>
<td></td>
</tr>
<tr>
<td>Leaf–3rd pair</td>
<td>1.11 Ba</td>
<td>0.88 Cab</td>
<td>0.78 Cb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf–4th pair</td>
<td>1.85 Aa</td>
<td>1.29 Bb</td>
<td>1.25 Bb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf–5th pair</td>
<td>1.52 ABa</td>
<td>1.11 Bb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf–6th pair</td>
<td>1.37 Aa</td>
<td>1.72 Aa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf–7th pair</td>
<td>1.84 A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf–8th pair</td>
<td>1.97 A</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

\(^1\)Means followed by the same capital letter within the columns and small letter within the rows are not significantly different by Tukey’s test at \(P<0.05\). Re-drawn from (16).
NA = not assayed

Supernatant was transferred to a new tube and used immediately for enzyme assays. CVOMT and EOMT activities are determined by measuring the formation of radiolabeled methylchavicol from chavicol and methyleugenol from eugenol, respectively, in presence of S-\([methyl-^{14}\text{C}]\) adenosyl-\(\text{L-Met}\) (25 \(\mu\)Ci ml\(^{-1}\), Amersham Pharmacia Biotech, UK) and protein extracts (~5 \(\mu\)g of total protein). The radiochemical assay mixture consists of 35 \(\mu\)l of buffer A (same as extraction buffer but pH 7.5), 5 \(\mu\)l of protein extract, 5 \(\mu\)l of substrate solution (in ethanol, final concentration of substrates of 0.1 mM for CVOMT and EOMT), and \(^{14}\text{C-SAM}\) (50,000 counts per min per reaction), in a total volume of 50 \(\mu\)l. After incubation of 30°C for 0.5 h, the reactions are
stopped by addition of 6 μl of 2 N HCl. Radiolabeled products are then extracted by addition of 100 μl ethyl acetate followed by vortexing and centrifugation at 14,000×g for 4 min. After phase separation, 50 μl of the upper (ethyl acetate) phase are removed for scintillation counting. Verification of assay product identity is achieved as described (3). Examples of obtained results for CVOMT activity are shown in Table 18.1.

3.4. Total RNA Extraction, RNA Blot Analysis, and RT-PCR Analysis

Total RNA extraction is performed from 200 mg of whole leaves using the Trizol reagent (Invitrogen, Carlsbad, CA) and 100 mg of each peltate and capitate glands, using the RNeasy kit (Qiagen, Valencia, CA), following the instructions of the manufacturers. For RNA blot analysis, a 32P-labeled OMT probe is constructed from a gel-purified PCR product using CVOMT1 primers (5′-TTTCCCAAATTACTCAAGGCC-3′ and 5′-CCCTCCAACGCCAAGCCC-3′). Examples of RNA levels in leaves and glandular trichomes of basil are shown in Figs. 18.1 and 18.2.

To evaluate the level of transcription according to leaf age and plant development of CVOMT, specific primers that distinguish CVOMT from EOMT are used for reverse transcription-PCR (RT-PCR) procedure. First strand synthesis reaction is carried out using the RT-PCR Introductory System (Promega, Wisconsin, MA), with some modifications in the protocol. A 20 μl reaction containing all reagents and 1 μg of total RNA

![Fig. 18.1](image-url)

Fig. 18.1. (a) RNA blot analysis showing changes in steady-state mRNA levels for O-methyltransferase (OMT) enzymes in EMX-1 basil whole leaves at developmental stage F (six pairs of leaves). From left to right: (lane 1), second (lane 2), third (lane 3), fourth (lane 4), fifth (lane 5), and sixth (lane 6) pairs of leaves. Five micrograms of total RNA was loaded in each lane. (b) Blot rehybridization with a basil 18S rDNA probe to standardize sample results. (c) RT-PCR analysis of chavicol O-methyltransferase (CVOMT) expression in EMX-1 basil top whole leaves as described for (a). One microgram of total RNA was used for each reverse transcriptase reaction. (d) Ribosomal bands visualized with ethidium bromide that were used to verify equal loading of total RNA for reverse transcriptase reactions. RT-PCR reactions were run in triplicate and 10 μl of each reaction was loaded on gel and transferred to membrane. Re-drawn from (16).
Fig. 18.2.  

(a) RNA blot analysis showing changes in steady-state mRNA levels for O-methyltransferase (OMT) enzymes in 10-day-old (lanes 1, 3, 5, and 7) and 30-day-old (lanes 2, 4, 6, and 8) EMX-1 basil whole leaves. Leaves were collected from the second (lanes 1 and 2), fourth (lanes 3 and 4), sixth (lanes 5 and 6), and eighth (lanes 7 and 8) leaf positions. Five micrograms of total RNA was loaded in each lane. 

(b) Blot rehybridization with a basil 18S rDNA probe to standardize sample results. 

(c) RT-PCR analysis of chavicol O-methyltransferase (CVOMT) expression in EMX-1 basil whole leaves of different ages, as described for (a). One microgram of total RNA was used for each reverse transcriptase reaction. 

(d) Ribosomal bands visualized with ethidium bromide that were used to verify equal loading of total RNA for reverse transcriptase reactions. RT-PCR reactions were run in triplicate and 10 μl of each reaction was loaded on gel and transferred to membrane. 

(e) RT-PCR analysis of chavicol O-methyltransferase (CVOMT) expression in peltate glands, as described for (a). One microgram of total RNA was used for each reverse transcriptase reaction. 

(f) Ribosomal bands visualized with ethidium bromide that were used to verify equal loading of total RNA for reverse transcriptase reactions. RT-PCR reactions were run in triplicate and 10 μl of each reaction was loaded on gel and transferred to membrane. Re-drawn from (16).

from EMX leaves is used for reverse transcription. The reaction is carried out at 42°C for 1 h using the AMV reverse transcriptase (Promega, Madison, WI). The PCR amplifications are run in triplicate and 5 μl of the 2× diluted first reaction product is used for amplification. A total of 25 amplification cycles is optimal for the subsequent PCR reactions, resulting in detectable bands for CVOMT. After PCR, the amplified products are separated on a 1.0% agarose gel and transferred to a nitrocellulose membrane. The DNA gel blottings are performed in triplicates with 10 μl of each RT-PCR reaction. RNA blot analysis of total RNA and DNA blot analysis of the PCR products is performed by hybridization at 42°C with 32P-labeled OMT and 18S basil DNA probes under standard conditions (10). Examples of CVOMT levels obtained by RT-PCR analysis of whole leaves are shown in Figs. 18.1c and 18.2c.
3.5. Glandular Trichomes Isolation

For gland separation, we follow the method originally developed (11), modified for use in basil (7), and later optimized (3) (see Note 3). A total of 15 g of tissue are placed in a beaker (kept on ice) and soaked in ice-cold deionized water in the presence of 14 mM of β-mercaptoethanol for 0.5–1.0 h to facilitate the swelling and gland removal. The water is decanted and the leaves transferred to the 300-ml beveled flask supplied with a Bead Beater model 1107900 (Biospec Products, Inc., Bartlesville, OK) along with 40–50 g of glass beads (0.5 mm in diameter, Biospec Products, Inc.) and approximately 250 ml of ice-cold gland isolation buffer (50 mM Tris–HCl, 200 mM D-sorbitol, 20 mM sucrose, 14 mM β-mercaptoethanol, 10 mM KCl, 5 mM MgCl₂, 0.5 mM K₂PO₄, 5 mM succinic acid, 1 mM EGTA, 0.6% [w/v] methylcellulose, and 1% [w/v] polyvinilpirrolidone, 360,000 Mᵣ). Glands are removed from basil leaves with three pulses of 1 min at 30 V, with 1 min rest between pulses. The glands are then separated from leaf material by passing the resulting mixture consecutively through a 350 μm mesh to separate out leaves and glass beads and a 105 μm mesh cloth to remove leaf debris. The peltate glands with an average diameter of 80 μm are collected in 41 mesh, which allows the capitate glands (average diameter of 30 μm) to pass through and then be collected on a 21 μm mesh (see Note 4). The collected glands are next washed at least three times on the mesh cloth with ice-cold gland storage buffer, transferred to a 1.5 ml microcentrifuge tube, placed on ice, and centrifuged at 10,000×g for 1 min at 4°C. Total RNA is then extracted as previously described (3) (see Note 5). Example of CVOMT expression found in glandular trichomes is shown in Fig. 18.2e.

3.6. Perspectives

Glandular tissues are considered a target for bioengineering in aromatic plants. For some of these species, like basil and mint, Agrobacterium tumefaciens transformation systems have been developed and can be used to obtain transgenic plants with altered or novel composition of essential oils (12, 13). The isolation of trichome-specific promoters will allow controlled expression of genes of interest in this differentiated cell type and modification of the contents of essential oil constituents of economic importance (14–16).

4. Notes

1. Although the development of techniques to isolate glandular trichomes allows the production of highly enriched protein
extracts and of highly enriched mRNA levels to construct cDNA libraries, the gene expression evaluations should be carried out using plant chemotypes with high concentration of the target chemical constituents in the essential oil. This will make it possible to get enriched tissues to monitor specific gene expression and/or to detect the specific activity of regulatory enzymes.

2. Transcript expression may be affected by gland density (more glands per unit area on young leaves) or of differences in expression within the glands themselves as the leaves age. For basil and other aromatic plants which accumulate essential oil in glands, young leaves which present greatest trichome density at young stages of morphological maturation should be used to study the factors that regulate essential oil biosynthesis.

3. To isolate the glands, all buffers must be cold and low temperature must be kept during all the isolation processes. The recommended rest between pulses must also be carefully observed.

4. To monitor the success of isolation and the gland integrity at the end of the protocol, gland samples should be collected and visualized under optical microscope.

5. Glands should be next sonicated on ice before nucleic acids and protein extraction assays, with rest intervals for cooling down, until gland cells are completely lysed.

Acknowledgments

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References


Chapter 19

Fusion with Fluorescent Proteins for Subcellular Localization of Enzymes Involved in Plant Alkaloid Biosynthesis

Patrícia Duarte, Johan Memelink, and Mariana Sottomayor

Abstract

To establish the role in alkaloid metabolism of candidate genes identified in silico or by Omics approaches, it may be essential to determine the subcellular localization of the encoded proteins. The fusion with fluorescent proteins (FP) may now be used as a quite effective and reliable tool to investigate this question. The methodology involves the choice of the FP, the design and production of the appropriate FP fusions, and the use of a transient or stable transformation protocol applied to a homologous or heterologous plant system. This chapter describes the application of this methodology to an enzyme involved in indole alkaloid biosynthesis, with general considerations on the development of the approach.

Key words: Fluorescent proteins, GFP, Catharanthus roseus, Arabidopsis, subcellular localization, vacuole, confocal microscopy.

1. Introduction

Alkaloids are a large and diverse group of secondary metabolites including some of the most remarkable pharmacological products of plant origin, like the muscle relaxants (+)-tubocurarine and papaverine, the anticancer agents vinblastine and vincristine, or the still number one painkiller, morphine (1, 2). In plants, alkaloids are thought to play a role in defense against herbivores, which would explain their strong physiological activity in animals. In plant cells, alkaloids and many other secondary metabolites accumulate in the vacuole, separated from much of the cell physiological activities with which they could interfere. Likewise, a few
alkaloid biosynthetic enzymes have been shown to be located in the vacuole (1–4).

Due to the complexity of alkaloid biosynthetic pathways and the low concentrations of alkaloid biosynthetic enzymes in plant tissues, a thorough knowledge of alkaloid biosynthetic pathways is still lacking in many cases (2, 5). However, we can now use new research strategies involving the characterization of recombinant proteins produced from candidate genes selected by homology-based screening or from differential transcriptomic/proteomic analyses (5). In this context, determining the subcellular localization of the candidate proteins may be essential to firmly establish their role in cells. In order to approach this question, fusions with fluorescent proteins (FPs) may be used as a quite effective and reliable tool, provided proper care is taken in the design of the fusions, the choice of the FP, and/or the conditions needed to obtain fluorescence in the case of vacuolar or apoplast localization.

1.1. Outline of the Experimental Procedure

We describe the methodology used to determine the vacuolar localization of the main leaf class III peroxidase (CrPrx1) of the medicinal plant *Catharanthus roseus*, which has been implicated in the biosynthesis of the anticancer alkaloids vinblastine and vincristine (6). The procedure employed involved the following steps:

1. Choice of the fluorescent reporter to be used as the sGFP (S65T) (7, 8).
2. Design and construction of the CPrx1-GFP fusions needed to evaluate the subcellular sorting of CrPrx1.
3. Transient and stable transformation of *C. roseus* cells by particle bombardment with the different constructs.
4. Polyethylene glycol-mediated transfection of protoplasts from *Arabidopsis* cell cultures with the different constructs.
5. Analysis of transformed cells for the subcellular localization of GFP fluorescence using confocal microscopy.

Some theoretical and practical considerations for the choice of the FP and of the transformation system and the design of fusions are discussed below.

The use of FPs in plant biology has become a powerful and widespread tool and a number of excellent papers reviewing the use and applications of FPs are available (9–13). GFP, as the first FP to be isolated, characterized, and applied, is still among the most used FPs in plant studies, although YFP and the red variants mRFP and mCherry are gaining preference due to higher brightness and stability, particularly under the acidic pH of the vacuole and the apoplast (10, 14). The two main GFP variants
used in plant cells are the mGFP5 developed by Jim Haseloff and colleagues (http://www.plantsci.cam.ac.uk/Haseloff/imaging/GFP.htm), (15, 16) and the sGFP(S65T), similar to EGFP, developed for human cells (17) and first applied to plants by Chiu, Niwa, and colleagues (7, 8).

In spite of the reported difficulty on observing GFP fluorescence in the vacuole (10, 18), both mGFP5 and sGFP(S65T) were used successfully in vacuoles (4, 6, 18–23), with no special difficulty mentioned in those reports. However, according to Tamura and colleagues (18) and to our own experience (6), at least sGFP(S65T) shows fluorescence in the vacuole only if the transgenic plants or cells are maintained in the dark for 48 h prior to observation. Tamura and colleagues (18) show that this is due to a light-induced conformational change of GFP that makes it susceptible to degradation by vacuolar papain-type cysteine protease(s) under acidic pH. Our experience with sGFP(S65T) fusions showed us that vacuolar fluorescence was only observed in either C. roseus cells or Arabidopsis protoplasts if the cells were previously incubated in the dark. It was also quite evident that the fluorescence observed in the vacuoles of C. roseus cells was much fainter than in vacuoles of Arabidopsis protoplasts for the same constructs. Although this may result from differences in the expression levels in the two systems, it is also possible that this is due to different proteolytic or pH properties of the vacuoles from the two cell types. In fact, it has been recently reported that similar mGFP5-derived fusions presented high vacuolar fluorescence in Arabidopsis transgenic plants, but very low vacuolar fluorescence in Nicotiana tabacum or Nicotiana benthamiana, a fact that was attributed to differences in the vacuolar accumulation of light-dependent proteases (21).

As stated above, mRFP has been suggested to be more stable in the vacuole (10, 14) and has also been successfully used as a vacuolar marker (14). Moreover, the red variant mCherry seems to have superior photostability, rendering mRFP obsolete (see Note 1) (10, 11). The problem with the RFPs is that, although their fluorescence is easily distinguished from the red autofluorescence of chloroplasts during computer-mediated observation at the confocal microscope, this is not the case when using an epifluorescence microscope. In any case, for the purpose of investigating the subcellular localization of uncharacterized proteins, the use of sGFP (S65T) seems to us perfectly suitable, provided a 48 h dark incubation is applied before observation.

1.3. Choice of the Transformation System

The ideal transformation system to use when investigating the subcellular localization of uncharacterized proteins by fusion with FPs is not only the homologous species, but even the tissue/cell type where the protein is usually expressed, since differences in
subcellular accumulation have been reported for the same FP fusions in different tissues or cells of the same plant (21, 23). The easiest way to do this is probably through a transient transformation protocol using either Agrobacterium infiltration, particle bombardment, or polyethylene glycol-mediated transfection of protoplasts. Such protocols are available for a number of plant species and can be optimized for the plant species of interest. In parallel, transient expression in an heterologous system such as Arabidopsis can be performed using the protocol below.

These transient transformation protocols may not always yield sufficient material for biochemical characterization of the fusion proteins, namely for the characterization of their size using GFP antibodies, in order to determine if a putative or confirmed sorting signal is excised and constitutes a propeptide. For such characterization, stable transformed cells or plants may be necessary, as described below for the transformation of C. roseus cells.

1.4. Design of Fusions

The position of the protein under investigation in relation to the FP is crucial in determining the correct final destination of the fusion protein, since N-terminal or C-terminal sorting signals will not be recognized if they are sandwiched between the two sequences of the fusion. Therefore, the rules of thumb for the design of GFP fusions capable of indicating reliably the subcellular localization of the protein of interest are the following: (i) perform an exhaustive in silico analysis of the full-length deduced protein sequence in order to determine the putative presence of subcellular sorting signals or propeptides and (ii) design carefully the fusions, locating correctly the putative sorting/propeptide sequences and the putative mature protein sequence at the N-terminus or C-terminus of GFP (or both in case of doubt).

In our case, to investigate the subcellular sorting of CrPrx1 and the respective sorting signal, we considered the presence in the full-length protein sequence of a signal peptide directing the nascent polypeptide to the ER (SP), and the presence of a C-terminal extension (CTE), compared to all the mature Prx proteins sequenced. Accordingly, we designed fusion constructs with GFP, attaching the SP to its N-terminus and the CTE to its C-terminus (Fig. 19.1). We also designed constructs including the mature protein sequence with and without the CTE, attached to the C-terminus of SP-GFP.

2. Materials

2.1. Plasmids, Constructs, and Cloning

1. Plasmid pTH-2 corresponding to pUC18 carrying the 35SΩ-sGFP(S65T)-nos construct and an ampicillin/carbenicillin-resistance marker (7, 8). May
be requested from Yasuo Niwa (niwa@fns1.u-shizuoka-ken.ac.jp).

2. Plasmid pTH-2BN lacks the stop codon and is used for fusions at the GFP C-terminus (24). May be requested from Johan Memelink (j.memelink@biology.leidenuniv.nl).

3. Plasmid pGL2 harbouring the hygromycin B-resistance gene (25). May be requested from Jerzy Paszkowski (jerzy.paszkowski@bioveg.unige.ch).

4. Primers used for amplification of CrPrx1 sequences are shown in Table 19.1.

5. Luria-Bertani (LB) medium: dissolve 10 g of bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl in water and adjust the volume to 1 L. Sterilize by autoclaving. Solid
Table 19.1
Primers used for the amplification of CrPrx1 sequences

<table>
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<tr>
<th>Amplification product</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>SP</td>
<td>SPF-ACGCGTCCAGACAAATGGCT TTTTCTTTTCAACTTCTCTGC</td>
<td>SPR-CATGCCATGCGCCGCAGGTT GTTTGAAGCATAATAGG</td>
</tr>
<tr>
<td>mCrPrx1-CTE</td>
<td>MPF-GAAGATCTTTACCACCAACAGTGAGTGACTTTTC</td>
<td>CTER-CCGCTCGAGTTAAACAT AGACAAGCGAAATTCAGC</td>
</tr>
<tr>
<td>mCrPrx1</td>
<td>MPF-GAAGATCTTTACCACCAACAGTGAGTGACTTTTC</td>
<td>MPR-CCGCTCGAGTTTTTCG AAGTGAACATCTGGATC</td>
</tr>
<tr>
<td>CTE</td>
<td>CTEF-GAAGATCTTTAGCTGCTG CCATGGGAGGTTCTTC</td>
<td>CTER-CCGCTCGAGTTAAACAT AGACAAGCGAAATTTCAGC</td>
</tr>
</tbody>
</table>

SP, signal peptide; mCrPrx1, mature CrPrx1 protein; CTE, C-terminal extension. Bold, primer designation; italic, oligonucleotide extension introducing the new restriction site; underlined, start codon and stop codon. See position of primers in Fig. 19.1a

medium is supplemented with 15 g/L of Bacto-agar (Becton Dickinson).


7. Carbenicillin (Duchefa): 200 mg/mL in water (toxic, avoid contact with skin, or breathing dust). Filter-sterilize through a 0.22 μm membrane (Millipore, Bedford, MA, USA). Store at –20°C and add to the LB medium, prior to use, to the concentration of 200 μg/mL.

8. Qiagen CompactPrep Plasmid Midi Kit (Qiagen GmbH, Düsseldorf, Germany).

2.2. *C. roseus* Cell Cultures

1. MP183L cell line (26). May be requested from Johan Memelink (J.Memelink@biology.leidenuniv.nl).

2. Linsmaier-Skoog-13 (LS-13) medium: standard LS including vitamins (27) (Duchefa) with 30 g/L sucrose, 2 mg/L 1-naphthalene acetic acid (NAA), and 0.2 mg/L kinetin (Kin). Adjust pH to 5.8 with KOH, autoclave 20 min at 120°C, and store at 4°C (stable for several months).

3. Kin and NAA (Duchefa): 100x stock solutions with a concentration of 20 and 200 mg/L, respectively. For a stock of 100 mL, dissolve the powder in 1 mL of KOH 1 M, add water to the final volume, and store at 4°C (stable for several months).

2.3. Preparation of *C. roseus* Cells for Particle Bombardment

1. Solid LS-13 medium: LS-13 supplemented with 0.7% of plant tissue culture agar (Imperial laboratories). Autoclave 20 min at 120°C and store at 4°C (stable for several months).
2. Whatman filter paper No. 4, 42.5 mm diameter (Schleicher & Schuell).

3. Sterile porcelain Büchner funnel maximum content 25 mL, for 40 mm diameter filters (Haldenwanger 127C/0 Technische Keramik GmbH, Berlin, Germany).

4. Small Petri dishes (60 × 15 mm), vacuum pump, and Erlenmeyer vacuum flask.

2.4. Pre-treatment and Coating of Tungsten Particles

1. Tungsten particles Pioneer Hi-Bred, 1.8 μm.

2. 0.1 M HNO₃.

3. Ethanol.

4. Sonicator 1: VibraCell VCX130 equipped with a SM1004 microtip (Sonics and Materials, Newtown, USA).

5. Sonicator 2: VibraCell VC300 equipped with a cup horn model 07-88 (Sonics and Materials, Newtown, USA).

2.5. Coating of Tungsten Particles

1. 2.5 M CaCl₂: dissolve 3.67 g of CaCl₂·2H₂O in 10 mL of sterile water. Filter-sterilize with a 0.22 μm membrane (Millipore, Bedford, MA, USA) and store at –20°C indefinitely.

2. Spermidine (free base): 0.1 M in sterile water. Filter-sterilize as above and store at –20°C. This solution must be prepared fresh every month.

2.6. Bombardment of Plant Cells with a Helium-Powered Particle Gun

1. Home-built particle gun according to (28).

2. Support screens 13 mm SS (Poretics Corporation, Livermore, CA, USA).

3. Dispersal screens: stainless steel screens, mesh 100 μm (Poretics Corporation, Livermore, CA, USA).

4. Rings: Teflon gasket 13 mm (Poretics Corporation, Livermore, CA, USA).

5. Sonicator: VibraCell VC300 equipped with a cup-horn model 07-88 (Sonics and Materials, Danbury, Newtown, USA).

6. LS-13 medium supplemented with 50 μg/mL hygromycin B.

7. Hygromycin B (concentrated solution from Calbiochem-Novabiochem): dilute in water to 50 mg/mL and adjust pH to 7 (very toxic, avoid contact with skin or eyes, causes severe burns). Filter-sterilize as above and store at 4°C for several months. Add to the liquefied solid culture medium prior to plating.

2.7. Subculturing of Resistant Calli

1. Solid selection medium: solid LS-13 medium supplemented with 50 μg/mL hygromycin B.

2.8. Conversion of Calli into Cell Suspensions

1. Selection medium: LS-13 medium supplemented with 50 μg/mL hygromycin B.
2. Orbital shaker.

2.9. Fluorescence or Confocal Laser Scanning Microscopy

1. Filter set for an excitation wavelength of 488 nm and an emission wavelength of 522 nm ± 16 nm.

2.10. Arabidopsis Cell Cultures

2. Culture medium: 30 g/L sucrose, 3.2 g/L Gamborg’s B5 basal medium with mineral organics (Sigma G5893), and 0.2 mg/L NAA. Adjust pH to 5.8 with KOH and autoclave 20 min at 120°C.

2.11. Preparation of Arabidopsis Protoplasts

1. Medium A: 0.4 % Macerozyme R-10 (Yakult), 2 % Cellulase “Onozuka” R-10 (Yakult), 12% sorbitol. Dissolve sorbitol first, add enzymes, heat in microwave for 15–20 s to 50–60°C, stir for 1 h, adjust to pH 5.8 with HCl. Filter-sterilize and store at ~20°C in 20 mL aliquots.
2. Plastic disposable 70 μm cell sieves (BD Biosciences #352350).
3. Medium B: culture medium in which sucrose is replaced by 0.1 M glucose plus 0.25 M mannitol. Adjust pH to 5.8 with KOH or HCl and autoclave 20 min at 120°C.
4. Counting chamber: Bürcker, depth 0.1 mm.

2.12. Transformation of Arabidopsis Protoplasts by Polyethylene glycol-Mediated Transfection

1. PEG solution: 25% PEG 4000 (Fluka), 0.2 M mannitol, and 0.1 M CaCl2.2H2O. Filter-sterilize and store at ~20°C indefinitely.
2. Sterile 24-well cell culture plates.

3. Methods

3.1. Constructs, Cloning, and Plasmid Midi-Preps

Steps 1–3 must be customized according to the objectives of each researcher. Here, in Steps 1–3, we describe the procedure used for CrPrx1.

1. The constructs designed to study the subcellular sorting of CrPrx1 are shown in Fig. 19.1. The CrPrx1 sequences used
in fusions were amplified by PCR with primers extended to include the adequate restriction sites, using the CrPrx1 cDNA as template (Table 19.1).

2. The amplified CrPrx1 signal peptide (SP) was cloned in frame in the plasmid pTH-2 using a SalI/NcoI ligation to generate the construct 35S-SP-GFP.

3. The same was done for the plasmid pTH2BN, resulting in a 35S-SP-GFP construct without stop codon, which was then used to further insert the CTE or the sequence of mature CrPrx1 with and without the CTE at the C-terminus of GFP. A BglII/XhoI ligation generated the constructs 35S-SP-GFP-CTE, 35S-SP-GFP-mCrPrx1, and 35S-SP-GFP-mCrPrx1-CTE (Fig. 19.1).

4. Use all construct plasmids to transform E. coli XL1-blue competent cells as follows: mix 1 μg of plasmid DNA with 100 μL of cells on ice, heat shock at 42°C for 30 s, put the tubes back on ice.

5. Plate 5–10 μL on LB solid medium supplemented with carbenicillin 200 μg/mL.

6. Obtain pure plasmid preps using 50 mL of overnight E. coli cultures and the Qiagen CompactPrep Plasmid Midi Kit according to the manufacturer’s instructions.

### 3.2. C. roseus Cell Cultures

1. Sub-culture C. roseus cell cultures weekly by pipetting 7.5 mL of culture into 50 mL of fresh LS-13 medium, in 250 mL Erlenmeyer flasks with foam stoppers.

2. Grow in an orbital shaker at 125 rpm, under a 16 h light photoperiod, at 25°C.

### 3.3. Preparation of C. roseus Cells for Particle Bombardmentss(30)

Perform all the steps in a laminar flow hood.

1. Prepare small Petri dishes with 6–8 mL of solid LS-13 medium.

2. Dilute 4-day-old cell suspension cultures of C. roseus 1:1 with fresh LS-13 medium.

3. Resuspend the cells by swirling the flask, pipette 3–8 mL, pour onto a pre-wetted sterile filter paper in a sterile Büchner funnel, and apply vacuum (see Note 2). Cells must form an even thin layer and should not be too wet (see Note 3).

4. Place the filter with the cells onto the solid medium and put the lid on the Petri dish until bombardment.

### 3.4. Pre-treatment of Tungsten Particles

1. Pre-treat the tungsten particles by adding 2 mL of 0.1 M HNO₃ to 375 mg of tungsten particles in a sterile 10 mL tube.
2. Sonicate with the sonicator 1 microtip for 20 min in an ice/water bath at maximum amplitude.

3. Centrifuge for 1 min at 2000 rpm, remove the HNO₃, resuspend the particles in 2 mL of sterile water, and repeat centrifugation.

4. Remove the water, add 2 mL of ethanol, and sonicate 2 s in a sonication cup (sonicator 2) using the settings duty cycle 50% and output control 10.

5. Centrifuge as above and remove the ethanol.

6. Add again 2 mL of ethanol, sonicate as in Step 4, and aliquot the particle suspension in 25 μL portions in sterile 1.5 mL tubes, vortexing vigorously each time before taking an aliquot.

7. Leave the tubes opened in a sterile flow cabinet overnight, allowing the particles to air dry.

8. Close the tubes and store at room temperature indefinitely.

3.5. Coating of Tungsten Particles

Perform all the steps in a laminar flow hood.

1. For transient transformation, add 10 μg of plasmid DNA (obtained in Section 3.1, step 6), in a total volume of 10 μL of sterile water to the dry particles and mix well by flicking the tube.

2. For stable transformation (see Note 4), mix 8 μg of test plasmid DNA with 2 μg of plasmid pGL2 (hygromycin B resistance) in a final volume of 10 μL of sterile water, add to the dry particles, and mix well by flicking the tube.

3. Add 25 μL of 2.5 M CaCl₂ and mix by flicking.

4. Add 10 μL of 0.1 M spermidine and mix by flicking.

5. Leave the particles to sediment for at least 15 min and remove 15 μL of supernatant.

3.6. Bombardment of Plant Cells with a Helium-Powered Particle Gun

1. Mount the particle gun inside a laminar flow hood and clean with ethanol prior to use.

2. Sterilize by submersion in ethanol all the equipment to be used during the bombardment procedure, namely loaders, screens, rings, and Petri dish base, and allow to air dry inside the laminar flow hood.

3. Sterilize with ethanol and under flame the tweezers needed to handle and assemble the bombardment apparatus.

4. Sonicate the coated particles for 2 s in a sonication cup using duty cycle 50% and output control 10, and immediately pipette 2 μL of the particle suspension onto the loader screen. You may wish to avoid sonication at this step, which may lead to DNA fragmentation. Instead you may sonicate the dry particles in a small volume of sterile water in
Section 3.5, step 1, previous to the addition of DNA. And just vortex at this stage.

5. Place the loader and dispersal screens into the gun and place an opened Petri dish with the cells 15 cm below.

6. Bombard the cells using 2.5 Bars of helium pressure under partial vacuum ($\leq 50$ mBar) (see Note 5).

7. Release vacuum, cover the Petri dish, and seal with two rounds of parafilm.

8. For each construct, bombard three to five Petri dishes.

9. If transient expression is the goal, incubate cells at 25°C, in the dark, for 1–2 d prior to observation under the confocal laser scanning microscope (go directly to Section 3.9) (see Note 6).

10. If the goal is to obtain stable transgenic cell lines, incubate cells for 24 h at 25°C, under a 16 h light photoperiod, covering the Petri dishes with a piece of filter paper to lower the light level.

11. After 24 h, transfer the filters into new Petri dishes containing solid LS-13 medium supplemented with 50 μg/mL hygromycin (see Note 7) and incubate at 25°C under a 16 h light photoperiod until calli appears – usually it takes about 4–5 weeks (see Note 8).

3.7. Subculturing of Resistant Calli

1. In order to avoid chimeric transformants, transfer single hygromycin-resistant calli of 1–2 mm diameter to solid selection medium and allow to grow for several weeks. Transfer about 30 calli for each construct (see Note 9).

2. After 2–4 weeks, transfer a small piece of each callus to fresh solid selection medium to increase the probability that homogeneous clones are obtained.

3. Analyse calli for the presence and expression of the construct of interest (GFP fluorescence or PCR and RT-PCR). Analyse positive lines under the confocal microscope (Section 3.9). Calli can be converted into cell suspensions for further studies.

3.8. Conversion of Calli into Cell Suspensions

In order to obtain a stable cell suspension culture a critical cell density needs to be maintained. Suspension cells can be easily observed in the confocal microscope, or can be used for protoplast or vacuole isolation for characterization of the fusion proteins using anti-GFP antibodies.

1. For each callus line, transfer an area of ca. 2–4 cm$^2$ of callus tissue (see Note 10) to 5 mL of liquid selection medium in a 100 mL Erlenmeyer flask. Disperse cell clumps with forceps, this will immediately create a cell suspension like mixture.

2. Incubate the flasks at 25°C, under a 16 h light photoperiod, on an orbital shaker at 125 rpm.
3. Keep adding 5 mL of fresh selection medium whenever cell density becomes high, so that gradually the culture volume is increased up to 20 mL (see Note 11).

4. When a volume of 20 mL of dense cell suspension is reached (after about 2–3 weeks), transfer the whole suspension into a 250 mL Erlenmeyer flask containing 50 mL of selection medium.

5. Sub-culture weekly, starting with 20 mL of inoculum in the first week, reducing gradually to 10 mL over the weeks, as the cells start to grow as a regular cell suspension. This takes about 4–5 weeks.

3.9. Fluorescence or Confocal Laser Scanning Microscopy

1. Place cells in a drop of water on a glass microscope slide.

2. Visualize using an excitation wavelength of 488 nm and an emission wavelength of 522 nm ± 16 nm (see Notes 12 and 13). In our case, we used an Axioplan upright microscope (Zeiss) equipped with a Bio-Rad MRC1024ES scan-head with a krypton/argon laser.

3.10. Arabidopsis Cell Cultures

1. Sub-culture Arabidopsis cell cultures weekly by pipetting 5 mL of culture into 50 mL of fresh medium in 250 mL Erlenmeyer flasks with foam stoppers.

2. Grow on an orbital shaker at 125 rpm, under a 16 h light photoperiod, at 25°C.

3.11. Preparation of Arabidopsis Protoplasts(31)

1. The day before protoplast preparation, transfer 10 mL of a 5-day-old culture into 40 mL of fresh medium and maintain at the normal growth conditions.

2. Pour the cells into one 50 mL Falcon tube, centrifuge for 5 min at 80×g without brake, and remove all the supernatant with a pipette, removing the last drops with a P1000 micropipette with the tip touching the bottom of the tube.

3. Resuspend the cells in 20 mL of medium A (enzyme mixture) and incubate the tube at 28°C, in the dark, for 2–3 h with gentle shaking (e.g. 60 rpm).

4. Separate protoplasts from undigested cells by filtration through a plastic disposable 70 μm cell sieve and add 30 mL of medium B.

5. Pellet protoplasts at 80×g for 5 min without brake, remove most of the supernatant by aspiration with a pipette and resuspend carefully the protoplasts in 50 mL of medium B.

6. Pellet protoplasts again as before, remove the supernatant, and resuspend carefully the protoplasts in 15 mL of medium B.
7. Determine the number of protoplasts per mL using a counting chamber.

8. Dilute again to 50 mL with medium B, pellet as above, and resuspend the protoplasts to a concentration of $4 \times 10^6$ per mL.

3.12. Transformation of Arabidopsis Protoplasts by Polyethylene glycol-Mediated Transfection (31)

1. Before preparing the protoplasts, aliquot the DNAs to be transfected into 2 mL sterile tubes. The ideal amount of plasmid DNA may vary for each construct and needs to be optimized. A good starting point is 10 μg.

2. Prepare the plate wells putting 4.5 mL of medium B in the wells to be used.

3. Add 250 μL of protoplast suspension ($10^6$ cells) to each of the 2 mL tubes containing the DNA (pipette cells using a 1 mL plastic tip).

4. Add one volume of PEG solution, drop by drop, and flick the tube every five drops until the solution is mixed. Leave the tubes standing for 15 min at RT.

5. Transfer the cells to the plate wells containing medium B and incubate for 48 h at 25°C, without agitation, in the dark. Analyse under the confocal microscope.

4. Notes

1. FP mCherry can be obtained for example at http://www.bio.purdue.edu/people/faculty/gelvin/nsf/protocols_vectors.htm. With this FP the infiltration of tobacco leaf epidermis with *Agrobacterium* (10, 32) can be used as a transformation system, since mCherry shows high fluorescence even in this biological material (33).

2. The cell suspension has to evenly and completely cover the filter. For transient expression purposes a thin layer of cells on the filter is required corresponding to ca. 4 mL of an average grown diluted culture. Conversely, when the goal is to obtain stably transformed cells, a thicker layer of cells on the filter paper is required corresponding to ca. 8 mL of an average grown diluted culture.

3. Cells should become lighter coloured/whitish.

4. In stable transformation, one important control experiment is the bombardment of cells with particles coated with pGL2 plus an empty vector and with one of the GFP plasmids alone. Transformation with the first will yield control calli. Transformation with the latter will confirm that the
conditions used are selective for calli transformed with the hygromycin B resistance gene.

5. To increase the transformation efficiency or the transient expression levels, cells can be bombarded two or three times.

6. A 24–48 h dark treatment is essential for observation of GFP fluorescence in the vacuole (Section 1.2) (18).

7. The Petri dishes with medium are always prepared fresh and must not have any condensation. The plates should not be turned upside down.

8. Routinely 20–50 hygromycin B-resistant calli are obtained per bombarded Petri dish.

9. The standard co-transformation frequency is 50–75%, about 10–20 hygromycin-resistant calli should be obtained containing the construct of interest and expressing it to some level. High-expression calli appear at about 5–10%.

10. A portion of each callus must be transferred onto a separate plate with solid medium as a back-up. This back-up callus culture should be sub-cultured onto fresh medium every 4 weeks.

11. A relatively high cell density is important for rapid cell division. Therefore, at this stage, each cell suspension should be evaluated independently for dilution rate.

12. Under the confocal microscope, GFP-labelled cells appear as bright green. Yellowish fluorescence may occur and it does not reflect GFP expression.

13. When the transformation efficiency is low, a pre-scan of the filters with the bombarded cells may be performed using a low-magnification epifluorescence microscope, in order to spot the fluorescent cells and use these cells to prepare the microscope slide.

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Immunohistochemical Localisation of a Putative Flavonoid Transporter in Grape Berries

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Abstract

Flavonoids are a class of secondary metabolites present in large amounts in grapevine (Vitis vinifera L.), which are involved in several aspects of its physiology (e.g. protection against biotic and abiotic stress). Even if the biosynthetic pathways of flavonoid sub-classes have been largely characterised, the mechanisms of their transport and accumulation to the final target sites are still not completely understood. Unanticipated insights have been obtained by probing plant tissues with pure antibodies targeting bilitranslocase (BTL, TCDB # 2.A.65.1.1), a mammalian transporter involved in the absorption and tissue distribution of dietary flavonoids. The occurrence of a BTL homologue has also been found in grape berries, in both tegumental layers of skin and pulp vascular bundles. In the skin, the expression of this protein starts from véraison (starting of the change in colour and softening of berries) and increases up to a maximum at the harvest stage, matching the same temporal pattern of flavonoid accumulation.

Key words: Bilitranslocase, Vitis vinifera L., berry tissues, immunolocalisation, microsomes, SDS-PAGE, Western blotting.

1. Introduction

Mammalian bilitranslocase (BTL) is a membrane protein involved in bilirubin transport in the liver (1–4), which is also expressed in absorptive epithelia (5, 6). Besides this activity, BTL mediates anthocyanin uptake from the bloodstream into the liver (7). Indeed, BTL is competitively inhibited by several grape
anthocyanins, including aglycones and their mono- and di-glycosylated derivatives (8).

Since anthocyanins are plant natural products, it seemed reasonable to investigate if plants express a similar transporter. Indeed, such hypothesis has been confirmed by immunochemical identification of a plant BTL homologue in various plant tissues. The presence of the carrier, at both cellular and tissue levels, has been demonstrated not only in carnation (Dianthus caryophyllus L.) petals (9) and red grape (Vitis vinifera L.) berries (10), but also in other species (unpublished data) using immunochemical approaches. In grape berries, the BTL homologue was specifically seen in tonoplast and at the interface of plasmalemma/cell wall in both tegumental layers of skin and pulp vascular bundles. In addition, the expression of the BTL homologue was assessed in microsomes prepared from either berry pulp or berry skin by means of Western blot immunodetection at different berry maturation stages. Results show that the translocator is detectable from véraison, and that its expression pattern depends on tissue localisation, being higher in the skin with respect to the pulp. Moreover, the expression of BTL homologue exhibits a continuous increase until the harvest stage in the skin, whereas in the mesocarp it shows a bell shape pattern, peaking at the early maturation stage (10).

These results suggest a relation between the up-regulation of the transporter protein and the upcoming demand of flavonoid accumulation during late ripening of the berry. In addition, the vascular bundle localisation suggests a putative function of the BTL homologue in the long-distance transport of secondary metabolites in tissues not devoted to flavonoid accumulation. In this chapter, we describe the methods for immunohistochemical localisation of this putative flavonoid transporter in grape berries.

2. Materials

2.1. Grape Berries and Seeds

1. Berries from V. vinifera L. cv Merlot collected from grapevines grown in the “Tenuta Villanova” vineyard, Farra d’Isonzo, Italy.

2.2. Tissue Preparation for Immunolocalisation

1. Fixation solution (see Note 1): 20 μg/mL formaldehyde solution (37% v/v) (see Note 2) and 7.5 μg/mL sucrose (see Note 3) in Na-Phosphate buffer (20 mM Na₂HPO₄, 20 mM KH₂PO₄, pH 7.2). Store at 4°C.

2. Graded ethanol series: ethanol solutions (50, 70, 80, 95 and 100%, v/v); ethanol:Histoclear solutions (3:1, 1:1 and 1:3, v/v), freshly prepared.
3. Histoclear: biodegradable solvent, which substitutes for xylene (National Diagnostics, Pittsburgh, PA, USA).


5. Plastic disposable base moulds (Fisher Scientific, Pittsburgh, PA, USA) with proper tissue-embedding rings (Fisher Scientific, Pittsburgh, PA, USA) and tissue cassettes (Fisher Scientific, Pittsburgh, PA, USA).

2.3. Sectioning of Samples

1. Rotary microtome (Leica, Milan, Italy) equipped with knife blade (Edge-Rite L.P., Cleveland, OH, USA).

2. Poly-L-lysine-coated microscope slides (Superfrost plus gold, Menzel-Glaser, Braunschweig, Germany) and microscope cover slips (22 × 24 mm or 24 × 32 mm, depending on the section size) (Menzel-Glaser, Braunschweig, Germany) (see Note 4).

3. Special slide racks (Kartell, USA) to be heated in the oven.

2.4. Rehydration

1. Phosphate-buffered saline (PBS): prepare 10X stock solution with 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄ and store at room temperature. Dilute 1:9, just before use (adjust to pH 7.4 with HCl if necessary).

2. Coplin jar (Sigma-Aldrich, Milan, Italy).

3. Graded ethanol series: ethanol solutions (100, 95, 85, 70, 50 and 30%, v/v), freshly prepared.

2.5. Immunolabelling for BTL Homologue

1. Blocking buffer: 1% (w/v) nonfat dry milk in PBS, freshly prepared.

2. Wash buffer: 1% (v/v) Tween 20 in PBS. Store at 4°C if used longer than 1 week.

3. Primary and secondary antibody dilution buffer: 1% (w/v) nonfat dry milk in wash buffer, freshly prepared. Store at 4°C.

4. Primary antibody solution: 3.3 μg/mL anti-BTL antibody in primary and secondary antibody dilution buffer. This antibody is prepared by immunisation with the peptide EFTYQLTSSPTC and purified by immunoaffinity chromatography (9). Store the serum in aliquots at −20°C and dilute it just before use. Do not freeze and thaw it more than once.

5. Secondary antibody solution: monoclonal anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Milan, Italy), to be stored at 4°C and diluted at 1:2500 in primary and secondary antibody dilution buffer just before use.
6. Alternative secondary antibody solution: goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich, Milan, Italy). The antibody is stored at 4°C and diluted at 1:2500 in primary and secondary antibody dilution buffer just before use.

7. Staining solution: 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium tablet (BCIP/NBT, Sigma Fast) (Sigma-Aldrich, Milan, Italy) is dissolved in 10 mL water, freshly prepared (see Note 5).

8. Dehydrating solutions: 70 and 100% (v/v) ethanol, freshly prepared.


2.6. Microsomal Fraction Isolation from Grape Berry Skin and Pulp

1. Microsomal extraction buffer (MEB): 0.4 M sucrose, 20 mM HEPES–Tris (pH 7.6), 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithioerythritol (DTE), 1 mM phenylmethylsulfonyl fluoride (PMSF). Store at 4°C.

2. Microsomal wash and resuspension buffer (MWRB): 0.25 M sucrose, 20 mM Tris–HCl (pH 7.5). Store at 4°C.

2.7. Sample Preparation for Electrophoresis

1. Stacking gel buffer (SGB): 0.5 M Tris–HCl (pH 6.8), 0.4% (w/v) sodium dodecyl sulfate (SDS). Store at 4°C.

2. SDS solution: 10% (w/v) SDS. Store at 4°C.

3. Glycerol solution: 75% (w/v) glycerol. Store at 4°C.

4. β-mercaptoethanol.

5. Bromophenol blue solution: 0.025% (w/v) bromophenol blue, 2% (w/v) glycerol. Store at 4°C.

2.8. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Running gel buffer (RGB): 1.5 M Tris–HCl (pH 8.8), 0.4% (w/v) SDS. Store at 4°C.

2. SGB: see Section 2.7, step 1.

3. Acrylamide/bis solution for running gel (ASRG): \( T = 30\% \) (w/v) acrylamide/bis, \( C = 3\% \) (29.1:0.9) acrylamide/bis. Store at 4°C.

4. Acrylamide/bis solution for stacking gel (ASSG): \( T = 30\% \) (w/v) acrylamide/bis, \( C = 5\% \) (19:1) acrylamide/bis. Store at 4°C.

5. Glycerol solution: see Section 2.7, step 3.

6. \( N,N,N,N' \)-Tetramethylethlenediamine (TEMED), to be stored at room temperature in the dark.

7. Ammonium persulfate solution (NPS): prepare 20% (w/v) solution, aliquot in Eppendorf tubes (200 μL) and immediately freeze. Store at –20°C.
8. Isobutanol, store at room temperature.
9. Outer running buffer (ORB): 25 mM Tris, 192 mM glycine, freshly prepared.
10. Inner running buffer (IRB): 0.1% (w/v) SDS in ORB, freshly prepared.
11. ColorBurst electrophoresis marker (Sigma-Aldrich, Milan, Italy).

2.9. Gel Staining
1. Coomassie stain fixing solution: 7.5% (v/v) acetic acid, freshly prepared.
2. Coomassie staining solution: 1% (w/v) Coomassie Brilliant Blue R-250 in water, filter to avoid solid suspensions; 4.5% (v/v) of this solution is used to prepare the staining solution with 5.4% (w/v) trichloroacetic acid, 5.8% (v/v) acetic acid, 16.8% (v/v) methanol.
3. Silver stain fixing solution I: 40% (v/v) methanol and 10% (v/v) acetic acid, freshly prepared.
4. Silver stain fixing solution II: 5% (v/v) methanol and 5% (v/v) acetic acid, freshly prepared.
5. Na-Thiosulfate: 0.02% (w/v) Na-Thiosulfate, freshly prepared.
6. AgNO₃ solution: 0.1% (w/v) AgNO₃, freshly prepared (see Note 5).
7. Silver stain developing solution: 2.5% (w/v) Na₂CO₃, 0.03% (w/v) formaldehyde solution (37% v/v), freshly prepared (see Note 6).
8. Stop solution: 5% (w/v) acetic acid, freshly prepared.

2.10. Western Blotting (Electroblotting) for BTL Homologue: Semidry Method
1. Conditioning and transfer buffer (CTB): 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, freshly prepared.
2. Blotting membrane: nitrocellulose membrane 0.45 μm (Sigma-Aldrich, Milan, Italy).
3. Extra-thick cellulose paper (Whatman, Springfield Mill, United Kingdom).
4. Ponceau red staining solution: 0.2% (w/v) Ponceau red (Sigma-Aldrich, Milan, Italy) in 3% (w/v) trichloroacetic acid and 5% (v/v) acetic acid (see Note 7).
5. Tris-buffered saline (TBS): 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, freshly prepared.

2.11. Immunodetection
1. TBS: see Section 2.10, step 5.
2. TBS-Tween: 0.05% (v/v) Tween 20 in TBS, freshly prepared.
3. Methods

The BTL homologue present in berry and seed of *V. vinifera* L. cv Merlot is detected by immunochemical analysis. This analysis is accomplished throughout the fruit maturation, sampling berries at the crucial developmental stages. In this context, the choice of using both immunohistochemistry and Western blotting is useful for understanding how the putative function of this membrane protein is compartmentalised in tissues. First, Western blotting analysis, performed with membrane fractions obtained from berry skin and pulp, allows confirming the specific reaction of the antibodies with two close protein bands, the intensity of which increases upon grape berry ripening. Second, immunohistochemistry permits to study the distribution of the target BTL homologue in recalcitrant fruits such as grape berry, within tissues and cell types, otherwise difficult to isolate. The method chosen is mainly that described by Walker et al. (11), where a mild chemical fixation is used for preserving epitope display while maintaining a good resolution of the intracellular morphology. Further, artefacts are routinely monitored by using negative controls or by assessing specificity of the visualised target antigen on immunoblots of SDS-PAGE gels.

### 3.1. Harvest and Storage of Grape Berry and Seed

1. Collect berries from *V. vinifera* L. cv Merlot at different developmental stages and immediately store them at –20°C. Sampling stages are: 31 d after the calyptra dehiscence, pre-véraison, véraison, early ripening, full maturation, harvest.

2. For Western blotting analysis, dissect skin from pulp by peeling and deseeding frozen berries. Use whole berries for immunolocalisation analysis.
3.2. Tissue Preparation for Immunolocalisation

1. Fix approximately four frozen berries by incubation in 100 mL of fixation solution for 24 h at 25°C with stirring.

2. At the same time, prepare the tissue-embedding medium in a large jar or beaker filled with approximately 120 Paraplast chips and put it at 55–60°C to melt overnight.

3. Before embedding, it is necessary to dehydrate the tissue through a graded ethanol series:

<table>
<thead>
<tr>
<th>Ethanol</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% ethanol</td>
<td>30 min</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>30 min</td>
</tr>
<tr>
<td>80% ethanol</td>
<td>30 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>30 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>30 min</td>
</tr>
<tr>
<td>Ethanol:histoclear 3:1</td>
<td>30 min</td>
</tr>
<tr>
<td>Ethanol:histoclear 1:1</td>
<td>30 min</td>
</tr>
<tr>
<td>Ethanol:histoclear 1:3</td>
<td>30 min</td>
</tr>
<tr>
<td>Histoclear</td>
<td>30 min</td>
</tr>
</tbody>
</table>

4. For each step, add enough solutions to cover the berries. At the end of each incubation time, discard the solution and replace it with the next one. These steps are performed at room temperature under fume hood. After the last step, gradually substitute the Histoclear with tissue-embedding medium, which finally saturates berries. Pour the melted tissue-embedding medium (a quantity corresponding to approximately 20 chips) into the vessel containing Histoclear solution and the berries and incubate it at 55°C for 2–4 h. Then, remove the Histoclear/Paraplast mix and add fresh melted tissue-embedding medium. This procedure must be replicated for three times a day, for 2 d. Cast the embedded berry blocks in plastic disposable base moulds and store them at room temperature before sectioning.

3.3. Sectioning of Samples

1. Before processing, keep the embedded berry blocks at −20°C for 10 min (see Note 8). For sectioning, use a rotary microtome with freshly sharpened knife blade (see Note 9) and cut 10–15 μm thick sections. Mount the berry ribbons in water on poly-L-lysine-coated microscope slides, properly
labelled by using a permanent marker. Place the slides in racks into closed slide box and dry them at 42°C overnight. At this stage, dried slides can be stored inside closed box at 4°C.

3.4. Rehydration

1. De-paraffinisation and rehydration of tissue sections are performed before immunostaining of slides with the primary and secondary antibodies. Place the slides into a coplin jar, fitting five microscope slides vertically, and deparaffinise in Histoclear for 15 min twice at room temperature. This step is performed under fume hood.

2. Re-hydrate the slides through incubation in solutions with graded ethanol series

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>85% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>30% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>PBS</td>
<td>10 min</td>
</tr>
</tbody>
</table>

3. For each step, add enough solution to cover slides. At the end of each incubation time, discard the solution and replace it with the next one.

3.5. Immunolabelling for BTL Homologue

1. Saturate unspecific binding sites using 100 mL blocking buffer at 37°C for 45 min in a moist chamber. After incubation, wash the slides in PBS for 10 min. Then, incubate the sections with 10 mL primary antibody solution (alternatively, with pre-immune serum, or omitting primary antibody, as negative controls) at 37°C for 90 min. After incubation, wash the slides three times in wash buffer for 15 min each.

2. The following steps have to be performed only if the secondary antibody is conjugated with fluorescent dye. Incubate the slides with 10 mL FITC-conjugated secondary antibody solution at 37°C for 1 h in a humid chamber (see Note 10). After incubation, wash the sections three times in wash buffer and immediately analyse the sections under UV light by an epifluorescence microscope (Leitz Fluovert, Wetzlar, Germany). Images are managed by ImageJ® (open source software developed by the National Institutes of Health – USA, http://rsbweb.nih.gov/ij/) in order to avoid background signal. An example result is shown in Fig. 20.1a.
Fig. 20.1. Fluorescence and light microscopy sections of grape berries at the harvest stage. 

3. The following steps have to be performed only if an antibody conjugated with alkaline phosphatase is used. Incubate the slides with 10 mL alternative secondary antibody solution at 37°C for 1 h. After incubation, wash the sections three times in wash buffer for 5 min each and finally incubate them in the staining solution. Stop the colour reaction by transferring the slides into water. Rapidly dehydrate the slides by dipping in 70% ethanol for 30 s and then in 100% ethanol for 1 min. Add 200 μL of Aquatex to the edge of the slides and place a cover slip slowly on the slide. Then, remove the extra-mounting medium by wiping with blotting paper. Finally, place the slides into a chamber for 1 d and use the epifluorescence microscope, visualising the sections.
under visible light. Otherwise, it is also possible to analyse
sections by means of a stereomicroscope (Nikon SMZ-U,
Tokyo, Japan) for obtaining images of the whole berry (see Note 11). An example result is shown in Fig. 20.1b.

3.6. Microsomal Fraction Isolation from Grape Berry Skin and Pulp

1. Homogenise approximately 30 g of grape berry skin or
deseeded pulp by an Ultra-turrax (Ika Werk, Mölndal,
Sweden) blender in 200 mL MEB at 4°C (see Note 12).
Perform this step and the following ones at 4°C.

2. Filter the homogenate through a 100 μm nylon mesh, adjust
the filtrate pH to 6.5 with KOH and centrifuge at 4500 rpm
(2800×g) for 5 min in a centrifuge (Sorvall RC-5B, SS-34
rotor, DuPont Company, DE, USA).

3. Centrifuge the supernatant at 10,500 rpm (13,000×g) for
12 min in a Sorvall RC-5B centrifuge with SS-34 rotor.

4. Filter again the supernatant through 100 μm nylon mesh
and ultracentrifuge at 36,000 rpm (100,000×g) for 36 min
in an ultracentrifuge (Beckman L7-55, Ty 70ti rotor, Fuller-
ton, CA, USA).

5. Resuspend the pellets in 100 mL of MWRB and ultra-
centrifuge at 36,000 rpm (100,000×g) for 36 min in a Beck-
man L7-55 centrifuge with Ty 70ti rotor.

6. Resuspend the final microsomal membrane fraction in
approximately 1 mL of MWRB.

3.7. Sample Preparation for Electrophoresis

1. Add 5 μL SGB, 5 μL SDS solution, 5 μL glycerol solution,
2 μL β-mercaptoethanol, 2 μL bromophenol blue solution
to microsomes (20 μg total microsomal protein) in a final
volume of 50 μL.

2. Heat samples at 95°C for 3 min.

3.8. SDS-PAGE

1. Gel casting: the following recipe is designed for twin gels,
one for total protein staining and the other for immunoblot
analysis (see Note 13).

2. To prepare a mini gel (7 × 10 cm, 1.5 mm thick) with 12%
(w/v) final acrylamide gel concentration, pour into a beaker
5 mL RGB, 6 mL ASRG, 8.9 mL water. Add 100 μL NPS
and 10 μL TEMED, gently stir for a few seconds to obtain
a uniform solution. Pour 6 mL of this solution into the tem-
plate and completely cover the solution surface with 1 mL
isobutanol to obtain a sharp upper surface (see Note 14).
Leave at room temperature until the solution is completely
polymerised (see Note 15). Discard the isobutanol from the
casting and extensively wash the gel surface with distilled
water to remove all the isobutanol from the template. Gen-
tly dry the gel surface with blotting paper.
3. Prepare the solution for the stacking gel with 2 mL SGB, 1 mL glycerol solution, 0.8 mL ASSG, 4.2 mL water. Add 40 μL NPS, 6 μL TEMED to the solution of the stacking gel and gently stir to obtain a uniform solution. Pour 3 mL into the casting and insert the 10-well comb (see Note 16).

4. Prepare 500 mL of ORB and 130 mL of IRB.

5. When the polymerisation is completed, gently remove the comb and mount the two castings into the assembly frame. Pour the IRB into the inner container, so as to cover completely the gel lane surface, and pour the remaining ORB into the outer container.

6. Prepare the protein samples, as described in Section 3.7. With a chromatography syringe, carefully inject the samples into the bottom of the lanes, as a sharp, blue uniform band. Use the red button as reference for numbering the different sample lanes, starting from the first (or second) lane, to be loaded with 5 μL of ColorBurst electrophoresis marker.

7. To perform the electrophoretic run, close the apparatus with the lid and connect the wires to the power supply unit. The power supply unit is set at maximum voltage (1 kV) and constant current (40 mA) (see Note 17). The bromophenol blue tracks the front of the electrophoretic run, so stop the current when this dye is about 2 mm from the bottom of the gel. Disconnect the electrophoresis unit from the power supply, remove the lid and discard the ORB and IRB. Open the castings and slide the glass sandwiches, gently open them by forcing the lateral spacers. While still on the glass, cut the gel on the upper corner in correspondence to the red button of the apparatus to track gel orientation.

3.9. Gel Staining

1. For Coomassie blue staining, take one gel and slide it into 100 mL Coomassie stain fixing solution for 5–10 min with gentle shaking (see Note 18). Put the gel into 100 mL of Coomassie staining solution overnight. To remove the background, put the gel in water, under gentle shaking, and replace the water until the blue background disappears.

2. For silver gel staining (see Note 19), take one gel and slide it into 100 mL silver stain fixing solution I for 1 h with gentle shaking. Then, replace it with 100 mL silver stain fixing solution II, overnight at 4°C, with gentle shaking. Wash the gel with water three times each for 20 min. Transfer the gel into 100 mL Na-Thiosulfate for 1 min. Wash the gel twice for 2–4 min with water. Place the gel into 100 mL AgNO₃ solution under a powerful and uniform light source for 30 min. Wash twice with water for 1 min. Staining is brought about by placing the gel in approximately 50 mL of silver stain.
developing solution. Replace the solution with a fresh one until the protein bands are clearly visible and against a clear background (see Note 20). Stop staining development by transferring the gel into 100 mL of stop solution.

3. Acquire the gel image using a digital camera or a scanner (see Note 21).

3.10. Western Blotting (Electroblotting) for BTL Homologue: Semidry Method

1. Transfer of SDS-PAGE gel proteins to nitrocellulose membrane is performed by Bio-Rad® (Hercules, CA, USA) semidry apparatus. Put the unstained gel into a glass vessel with 100 mL CTB for the first washing lasting at least 30 min. In a separate glass vessel with 100 mL CTB, put both the nitrocellulose membranes, cut a few millimetres larger than the size of the gel and the extra-thick cellulose paper.

2. Put the wet extra-thick cellulose paper on the lower surface of the semidry apparatus (anode). Lay the nitrocellulose membrane on the extra-thick paper. Put on the gel taking care that its cut is at the upper right corner; lay it down, starting from one side. Cover the gel with another wet extra-thick cellulose paper (see Note 22). Assemble the apparatus with the upper electrode lid (cathode) and connect it to the power supply set to 250 mA and 15 V for a maximum transfer time of 90 min (see Note 23).

3. Disconnect the unit from the power supply and disassemble it. Remove both the paper and the running gel; cut the right corner of the nitrocellulose membrane to track the orientation of the lanes.

4. Put the nitrocellulose membrane immediately into Ponceau red staining solution. This reversibly stains the proteins. After 10 min, put the membrane into TBS and wash it until the background colour fades away and protein bands become visible. This image can be acquired by a camera or a scanner. With a soft lead pencil mark the protein lanes and, if necessary, the molecular standard bands; wash out finally all the red colour from the membrane with TBS.

3.11. Immunodetection

1. To saturate unspecific binding sites on the blotted membrane, put it into a glass vessel containing 100 mL of saturation buffer under gentle shaking for 1 h at room temperature. Then, wash the membrane three times, each one with approximately 20 mL TBS for 10 s.

2. Transfer the membrane into a small glass vessel containing 10 mL primary antibody solution (alternatively, with pre-immune serum as negative controls) under gentle shaking at 4°C overnight (see Note 24).
3. Then, discard the primary antibody solution (or the corresponding negative controls) and wash the membrane four times, each one with 50 mL TBS-Tween for 5 min.

4. Transfer the membrane into a small glass vessel containing 10 mL secondary antibody solution and gently shake for 90 min at room temperature (see Note 24).

5. Then, discard the secondary antibody solution and wash the membrane three times, each with 50 mL TBS-Tween for 5 min and twice again each with 50 mL TBS for 5 min.

6. Transfer the membrane in a glass vessel or in a Petri dish containing the staining solution and gently stir it for few seconds. When the colour stain reaches the right intensity and before the background becomes too intense, stop the reaction by dipping the membrane in water. After drying, store the nitrocellulose membrane wrapped in aluminium foil or scan it to save as an electronic image. An example result is shown in Fig. 20.2.

![Fig. 20.2](image-url)

Fig. 20.2. Immunoblotting of proteins cross-reacting with the anti-BTL antibody in grape berries at the harvest stage. Proteins obtained from microsomes isolated from skin and pulp tissues are separated by SDS-PAGE, transferred onto a nitrocellulose membrane and probed with the anti-BTL antibody. The presence of BTL homologue is shown by secondary antibody-phosphatase reaction product BCIP/NBT. Values on the left represent the molecular mass markers.

4. Notes

1. Unless stated otherwise, prepare all solutions and perform all washings in water with a resistivity of 18.2 MΩ cm.

2. Care should be taken to handle this reagent since it is toxic.

3. In substitution to the usual strong fixation FAA (formaldehyde/acetic acid/ethyl alcohol), choose a weak fixation solution to limit any damage on berry mesocarp.

4. Wear gloves.

5. Do not expose to direct light. Wear gloves because it is irritant to skin.
6. Given its toxicity, it is recommended to use a fume hood and to wear gloves when handling formaldehyde.

7. Recover the solution after use and store it at room temperature for the next stainings. The solution can be used several times.

8. Berries collected at the last maturation stages are difficult to cut without damaging the internal structure, because they are large and soft. For this reason, store the embedded berries at –20°C for easier cuts. During sectioning, additional problems could arise due to coiling of thin sections. To prevent this, use a pressed tip of a paintbrush to flatten out the section.

9. Sectioning of the seeds usually requires titanium-made razor blade, but if they are embedded inside the fruit a normal blade is sufficient.

10. It is recommended to perform incubation in the dark.

11. Collect several images from different regions of a berry under a microscope and then paste them together to assemble the view of the whole section.

12. DTE and PMSF must be added to MEB just before the homogenisation.

13. Always wear gloves, while handling gels and nitrocellulose membranes, to avoid both contact with acrylamide/bis solutions and contamination of gels and nitrocellulose membrane with skin-derived matter. Prepare solutions and gels under a fume hood.

14. The casting must stand on a horizontal flat surface.

15. To control if the gel is completely solidified, check the solution left in the beaker. Polymerisation time could be shortened by increasing the temperature, e.g. in oven set at 30°C.

16. To avoid air bubble entrapment, insert the 10-well comb inclined at one edge.

17. Great care has to be taken using the apparatus, due to the high voltage.

18. A good indication of protein fixation is when the bromophenol blue bands in the gel have changed from blue to yellow.

19. To be used when small amounts of protein have to be detected.

20. The volume of silver stain developing solution is about 500 mL. Take care to remove the gel from the staining solution before the background becomes stained.
21. Protect the gel between two transparency films.
22. At each step roll a glass tube to eliminate air bubbles trapped inside.
23. In the case of extra thin gels (i.e. 0.5 mm), a shorter transfer time (i.e. 60 min) is enough to efficiently transfer proteins.
24. The primary and secondary antibody solutions must completely cover the blotting membrane.

Acknowledgements

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References


Electrogenic Bromosulfalein Transport in Isolated Membrane Vesicles: Implementation in Both Animal and Plant Preparations for the Study of Flavonoid Transporters

Sabina Passamonti, Federica Tramer, Elisa Petrussa, Enrico Braidot, and Angelo Vianello

Abstract

Bromosulfalein is an organic anion dye used in the study of a variety of membrane carriers expressed in animal tissues and involved in transport of drugs and metabolites. The spectrophotometric assay of electrogenic bromosulfalein transport in membrane vesicles, isolated from various mammalian organs or tissues, enables to specifically measure the transport activity of bilitranslocase (TCDB 2.A.65.1.1). The latter is a bilirubin- and flavonoid-specific transporter expressed in rat liver, the organ where its function has been best characterized. The spectrophotometric assay of electrogenic bromosulfalein transport requires minimal volumes of membrane vesicles, is completed within 1 min, and, therefore, is a useful tool to screen the transporter spectrum of potential substrates, by testing them as reversible inhibitors of bromosulfalein transport kinetics. Furthermore, the assay enables to study the progress of time-dependent inactivation of bromosulfalein transport, caused by different protein-specific reagents, including specific anti-sequence antibodies. Inactivation can be retarded by the presence of substrates in a concentration-dependent manner, enabling to derive the dissociation constants of the transporter–substrate complex and thus to gain further insight into the transporter structure–function relationship. This assay, implemented in membrane vesicles isolated from plant organs, has paved the way to the discovery of homologues of bilitranslocase in plants.

Key words: Bromosulfalein, membrane transport, flavonoids, bilitranslocase, enzyme kinetics, enzyme inactivation, rat liver, rat kidney, carnation petal, grape berry.

1. Introduction

Bromosulfalein (BSP) is a synthetic dye introduced in 1925 as a clinical tool aiming at the assessment of the liver function (1). When injected intravenously, it is rapidly cleared from the blood...
by liver uptake, converted to a glutathione conjugate, and then actively transported into the bile (2). These steps are partly shared with bilirubin (3), a tetrapyrrolic compound arising from heme catabolism (4) and responsible for jaundice (yellow skin pigmentation), a clinical sign of hemolysis and/or liver dysfunction. BSP has been used in innumerable studies for the investigation of hepatic membrane transporters involved in the uptake and excretion of drugs, bile salts, and bilirubin (5).

The electrogenic BSP transport assay has been first set up in proteoliposomes made with the BSP-binding membrane protein bilitranslocase (6); subsequently, it was implemented in rat liver plasma membrane vesicles (7). Its unique feature is that the exclusive driving force for anionic BSP movement into vesicles is the membrane potential. Under these conditions, only the quinoidal, but not the phenolic tautomer of BSP, or related phthaleins (e.g., thymol blue) are transported (8).

Anthocyanins are natural compounds displaying pH indicator properties based on quinoidal–phenolic tautomerism. They are strong competitive inhibitors of electrogenic BSP transport in rat liver plasma membrane vesicles, revealing that bilitranslocase is a mammalian flavonoid transporter (9, 10).

A monoclonal antibody able to inhibit electrogenic BSP transport in rat liver plasma membrane vesicles and to identify a single membrane protein by Western blotting has been used to isolate a cDNA from a rat liver cDNA library (11, 12). This information permitted to deduce the primary structure of bilitranslocase. Anti-sequence bilitranslocase antibodies specifically inhibit electrogenic BSP transport in rat liver plasma membrane vesicles (11, 13).

Electrogenic BSP transport has also been implemented in isolated membrane vesicles (microsomes, plasma membranes, and tonoplast) from plant organs (e.g., carnation petals (13), grape berries (14), and other sources) and flavonoids have been found to be reversible inhibitors thereof. Anti-sequence bilitranslocase antibodies are also specific inhibitors of electrogenic BSP transport in plant membrane vesicles (13, 14). Thus, this assay enables to stretch the investigation of the function of bilitranslocase and related flavonoid transporters across the kingdoms of living species.

2. Materials

Unless otherwise specified, reagents are purchased from Sigma-Aldrich, Milan, Italy. Solutions are prepared with ultrapure water of resistivity $= 18.2 \text{M} \Omega \cdot \text{cm}$ (Milli-Ro and Milli Q, Millipore Co., Bedford, MA).
2.1. Plant Materials

1. Red carnation petals from *Dianthus caryophyllus* L., purchased at a local market.

2. Grape fresh berries from *Vitis vinifera* L., collected at the harvest stage (see Note 1). Dissect pulp from skin by peeling and deseeding berries.

2.2. Isolation of Microsomal Vesicles from Carnation Petals

1. Microsomal extraction buffer (MEB): 0.25 M sucrose, 20 mM HEPES–Tris, pH 7.6, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithioerythritol (DTE), 1 mM phenylmethylsulfonyl fluoride (PMSF) (see Note 2), 0.6% (w/v) polyvinylpolypyrrolidone, and 0.3% (w/v) bovine serum albumin (BSA). Store at 4°C (see Note 3).

2. Microsomal wash buffer (MWB): 0.25 M sucrose, 20 mM Tris–HCl, pH 7.5. Store at 4°C.

3. Microsomal resuspension buffer (MRB): 0.25 M sucrose, 20 mM Tris–HCl, pH 7.5, 0.1% (w/v) fatty acid free BSA. Store at 4°C.

2.3. Isolation of Tonoplast Vesicles from Carnation Petals

1. Microsomal resuspension buffer for tonoplast isolation (MRBTI): 0.25 M mannitol, 2.5 mM HEPES–BTP, pH 7.2, 0.1% BSA (w/v), 0.1 mM PMSF, 1 mM DTE (see Note 4). Store at 4°C.

2. Tonoplast wash buffer (TWB): see MWB. Store at 4°C.

3. Step gradient solution (SGS): 6% (w/v) Dextran T-500 in 10 mL of TWB. Store at 4°C.

4. Tonoplast resuspension buffer (TRB): see MRB.

2.4. Isolation of Plasma Membrane Vesicles from Carnation Petals

1. Aqueous polymer two-phase partitioning buffer (APTPB): 0.25 M sucrose, 5 mM phosphate buffer (Na₂HPO₄ /KH₂PO₄), pH 7.8, 4 mM KCl. Store at −20°C.


3. Dextran stock solution: 20% (w/v) Dextran T-500. Store at −20°C.

4. Polyethylene glycol (PEG) stock solution: 40% (w/v) PEG 3350. Store at −20°C.

5. Dextran and PEG two-phase system: two-phase system has to be prepared previously in three Corex® glass vessels. Two-phase system contains 6.5% polymer concentration (see Note 5), APTPB, and water, according to the following scheme:
The additions have to be done measuring weights on a balance; for proper centrifugation run check carefully the weight of the different vessels, because their balancing cannot be adjusted during phase separation. Mix all the components by vigorous inversion of the phases for at least 20 times. Finally, store the vessels at 4°C overnight, allowing phase separation by gravity; alternatively, obtain phase partition by centrifugation at 1500 × g for 5 min in a centrifuge (Sorvall RC-5B, swinging bucket HB-4 rotor, DuPont Company, DE, USA). Each vessel contains 14 mL of the two-phase system except the first one, to which the microsomal suspension has to be added.

6. Upper phase wash buffer (UPWB): see MWB. Store at 4°C.

7. Carnation plasmalemma resuspension buffer (CPRB): see MRB.

**2.5. Isolation of Microsomal Vesicles from Grape Berries**

1. Buffer A: 0.5 M glucose, 5 mM EDTA, 80 mM Na-pyrophosphate, 1% (w/v) polyvinylpyrrolidone at pH 8.2 with ascorbic acid, 0.2% (w/v) BSA, 1% (w/v) defatted casein, 5 mM DTE, 20 mM β-mercaptoethanol, and 1 mM PMSF. Store at 4°C (see Note 6).

2. Buffer B: 0.3 M sorbitol, 0.5% (w/v) polyvinylpyrrolidone, 1 mM Na-EDTA, 0.2 M KCl, 0.2% (w/v) BSA, 5 mM DTE, 1 mM PMSF in 25 mM BTP-MES, pH 7.0. Store at 4°C (see Note 7).

3. Buffer C: 20% (v/v) glycerol, 0.25 M sucrose, 0.025% (w/v) BSA in 20 mM HEPES–Tris, pH 7.0. Store at 4°C.

**2.6. Isolation of Plasma Membrane Vesicles from Rat Liver**

1. Female rats (*Rattus norvegicus*–Wistar strain), 2–3 months old, weighing 200–250 g, raised at appropriate animal house facilities.

2. Buffer A: 0.25 M sucrose, 0.2 mM CaCl₂, 10 mM HEPES, pH 7.4 (see Note 8). Store at 4°C.

3. Buffer B: 0.25 M sucrose, 10 mM HEPES, pH 7.4. Store at 4°C.

4. Buffer C: 39% sucrose (w/v) in 10 mM HEPES, pH 7.4. Store at 4°C.
5. Buffer D: 20% sucrose (w/v) in 10 mM HEPES, pH 7.4. Store at 4°C.
6. 0.2 M EDTA-NaOH, pH 7.4. Store at 4°C.

2.7. Transport Assay Solutions
1. BSP stock solution (see Note 9).
2. Transport assay buffer for membrane vesicles isolated from rat liver or from carnation petals: 4–40 μM BSP in 0.1 M potassium phosphate buffer, pH 8.0.
3. Transport assay buffer for membrane vesicles isolated from grape berries: 2–48 μM BSP in 0.2 M KCl in 20 mM HEPES–Tris, pH 8.0.
4. Vesicle suspension (3–5 mg protein/mL).
5. Valinomycin (5 or 20 mg/mL in methanol) (see Note 10).

2.8. Dependence on Membrane [Protein]
1. Transport assay buffer: 40 μM BSP in 0.1 M phosphate buffer, pH 8.0.
2. Five serial dilutions of vesicles in 0.25 M sucrose, 10 mM HEPES, pH 7.4 (e.g., 1–5 mg protein/mL, 10–15 μL each).
3. Valinomycin (2.5–10 mg/mL in methanol) (see Note 11).

2.9. Dependence on [Valinomycin]
1. Transport assay buffer: 40 μM BSP in 0.1 M phosphate buffer, pH 8.0.
2. Vesicle suspension (1–5 mg protein/mL).
3. Five serial dilutions of valinomycin in methanol (e.g., 1–5 mg/mL, 50 μL each).

2.10. Dependence on Extra-Vesicular [K+]  
1. Buffer A: 0.1 M potassium phosphate buffer, pH 8.0, containing BSP (e.g., 24 μM).
2. Buffer B: 0.1 M sodium phosphate buffer, pH 8.0, containing BSP (e.g., 24 μM).
3. Prepare the following serial solutions in spectrophotometric cuvettes:

<table>
<thead>
<tr>
<th>Test no.</th>
<th>Buffer A (mL)</th>
<th>Buffer B (mL)</th>
<th>K⁺ (mEq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.0</td>
<td>0.0</td>
<td>0.3780</td>
</tr>
<tr>
<td>2</td>
<td>1.6</td>
<td>0.4</td>
<td>0.3024</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>0.8</td>
<td>0.2268</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>1.2</td>
<td>0.1512</td>
</tr>
<tr>
<td>5</td>
<td>0.4</td>
<td>1.6</td>
<td>0.0756</td>
</tr>
</tbody>
</table>

4. Vesicle suspension (ca. 5 mg protein/mL).
5. Valinomycin (3–5 mg/mL in methanol).
2.11. Dependence on Extra-Vesicular Osmolarity

1. Buffer A: 0.1 M potassium phosphate, pH 8.0 (0.296 osm/L).
2. Buffer B: 40% sucrose (w/v) in Buffer A (1.464 osm/L, resulting from the sum of 1.168 osm/L sucrose plus 0.296 osm/L potassium phosphate, pH 8.0).
3. Buffer C: 30 μM BSP in Buffer A.
4. Prepare the following serial solutions in spectrophotometric cuvettes:

<table>
<thead>
<tr>
<th>Test no.</th>
<th>Buffer A (mL)</th>
<th>Buffer B (mL)</th>
<th>Buffer C (mL)</th>
<th>Osm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.50</td>
<td>0.00</td>
<td>1.50</td>
<td>0.296</td>
</tr>
<tr>
<td>2</td>
<td>0.45</td>
<td>0.05</td>
<td>1.50</td>
<td>0.325</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
<td>0.20</td>
<td>1.50</td>
<td>0.413</td>
</tr>
<tr>
<td>4</td>
<td>0.15</td>
<td>0.35</td>
<td>1.50</td>
<td>0.500</td>
</tr>
<tr>
<td>5</td>
<td>0.00</td>
<td>0.50</td>
<td>1.50</td>
<td>0.588</td>
</tr>
</tbody>
</table>

5. Vesicle suspension (ca. 3–5 mg protein/mL).
6. Valinomycin (5 mg/mL in methanol).

2.12. Dependence on Extra-Vesicular [BSP]

1. Transport assay buffer: 4–40 μM BSP in 0.1 M potassium phosphate buffer, pH 8.0 (e.g., 4, 8, 12, 16, 20, 24, 32 μM BSP).
2. Vesicle suspension (ca. 3 mg protein/mL).
3. Valinomycin (5 mg/mL in methanol).

2.13. Reversible Inhibition by Bile Pigments and Flavonoids

1. Prepare bile pigment solutions as follows (see Note 12).
2. Solution A: 5 mM bilirubin in dimethyl sulfoxide (DMSO) (e.g., 5.85 mg/2 mL).
3. Solution B: 0.5 mM bilirubin in DMSO (e.g., 100 μL solution A + 900 μL DMSO).
4. Solution C: 0.1 mM bilirubin in DMSO (e.g., 200 μL solution B + 800 μL DMSO). This is the solution to be used in the transport assays.
5. Proceed similarly with biliverdin.
6. 0.1 M flavonoid stock solutions in DMSO (see Note 13).
7. Transport assay buffer: 4–40 μM BSP in 0.1 M phosphate buffer (pH 8.0) (see Section 2.12).
8. Vesicle suspension (ca. 3 mg protein/mL).
9. Valinomycin (5 mg/mL in methanol).
2.14. Time-Dependent Inactivation of Bromosulfalein Transport

1. Transport assay buffer: 40 μM BSP in 0.1 M phosphate buffer, pH 8.0.
2. Vesicle suspension (ca. 3 mg protein/mL).
3. Valinomycin (5 mg/mL in methanol).
   Protein-specific reagents to be added to the vesicle suspension are as follows:
4. Stock solutions of cysteine-specific reagents are as follows:
   3 mM p-hydroxymercuribenzoic acid (p-HMB) (see Note 14).
   15 mM β-mercaptoethanol in 0.1 M HEPES, pH 7.4.
   0.1 M N-ethylmaleimide (NEM) in 0.1 M HEPES, pH 7.4 (see Note 15).
   5 mM dithionitrobenzoic acid (DTNB) in 0.1 M HEPES, pH 7.4 (see Note 16).
5. Stock solutions of arginine-specific reagents:
   0.2 M phenylglyoxal in ethanol:water (1:1, v/v).
   0.2 M methylglyoxal in 0.1 M HEPES, pH 7.4.
   1 M arginine in water titrated to neutral pH with HCl.
6. Stock solutions of serine-specific reagents:
   0.2 M phenylmethanesulfonyl fluoride (PMSF) in DMSO.
   Further diluted solutions (2–12 mM) can be prepared in 0.1 M HEPES, pH 7.4/DMSO (10:1, v/v).
   0.25–0.5 M pyridine 2-aldoxime methiodide (2-PAM or pralidoxime) in DMSO.

2.15. Sequence-Specific Bilitranslocase Antibodies

1. Antibody A is obtained by immunizing rabbits (Oryctolagus cuniculus, white New Zealand strain) with a multiantigen peptide (MAP)-based system (15), using the peptide EDSQGQHLSF, corresponding to segments 65–75 of the primary structure of bilitranslocase. The MAP-based system is conjugated to the antigen-carrier protein Keyhole Limpet Hemocyanin (KLH) via 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC). A kit with KLH and EDC crosslinker is manufactured by Pierce. Sera are purified by affinity chromatography, conjugating the MAP-based system to Affi-Gel 10 (BioRad, Hercules, CA, USA). Further details are given in (11) and on the web (http://www.bmb.leeds.ac.uk/phageAb/appendixg.html). The MAP-based system can also be bound to AminoLink Coupling Resin (Pierce, Rockford, IL, USA). Purified antibody fractions (in 0.1 M Tris-glycine, pH 8.0, supplemented with 0.5 mg/mL BSA) are stored at −20°C.
2. Antibody B is obtained by immunizing rabbits with the peptide EFTYQLTSSPTC, corresponding to segments 235–246 of the primary structure of bilitranslocase. Both
conjugation of the peptide to KLH and affinity purification of the antibodies can be carried out using the Imject Sulfhydryl Reactive Antibody Production and Purification Kit with mcKLH (Pierce) and following the instructions provided therein.

3. [IgG] in the fractions is quantified by the method of Bradford (16), using bovine IgG (Sigma) as a standard. Rabbit IgG is used in control tests. They are available from Sigma or can be purified from pre-immune serum by affinity chromatography through Protein G-agarose (Sigma); they can also be obtained from the unbound fraction of affinity chromatography of either antibody A or antibody B.

3. Methods

If not specified otherwise, ice-cold solutions should be used for the preparation of membrane vesicles. Protein determinations are carried out by the Bradford method (16).

3.1. Microsomal Fraction Isolation from Carnation Petals

1. Homogenize ca. 40 g of petals (claw deprived and cut into small pieces) by an Ultra-turrax (Ika Werk, Mölndal, Sweden) blender in 200 mL MEB at 4°C. Perform this step and the following ones always at 4°C.

2. Filter the homogenate through eight layers of cheesecloth and centrifuge the filtrate at 2800×g for 5 min in a centrifuge (Sorvall RC-5B, SS-34 rotor, DuPont Company, DE, USA).

3. Centrifuge the supernatant at 13,000×g for 12 min in a Sorvall RC-5B centrifuge with SS-34 rotor.

4. Filter again the supernatant through two layers of cheesecloth and ultracentrifuge at 100,000×g for 36 min in an ultracentrifuge (Beckman L7-55, Ty 70ti rotor, Fullerton, CA, USA).

5. Resuspend the pellets in 100 mL of MWB and ultracentrifuge at 100,000×g for 36 min in a Beckman L7-55 centrifuge with a Ty 70ti rotor.

6. Resuspend the final microsomal membrane fraction in ca. 1 mL of MRB at a final protein concentration of 3 mg/mL.

3.2. Tonoplast Isolation from Carnation Petal Microsomes

1. Remove microsomal pellets using a cut pipette tip and resuspend in 6 mL of MRBTI; layer the suspension onto 10 mL of SGS.

2. Ultracentrifuge tonoplast vesicles at 70,000×g for 2 h in a Beckman L7-55 centrifuge with a Beckman SW28 swinging bucket rotor.
3. Collect the sharp band of membranes at the interface (tonoplast), dilute it 10-fold in TWB, and keep at 8°C for 1 h.

4. Ultracentrifuge again this suspension for 40 min at 90,000×g. Resuspend the pellet in 1 mL of TRB.

3.3. Plasma Membrane Isolation from Carnation Petal Microsomes

1. Resuspend microsomal pellets with 3 mL of MRBPI.
2. Add the microsomal suspension to the first Corex® vessel. Mix vigorously for at least 20 times to allow best melting of the phases.
3. Separate the two phases by centrifugation at 1500×g for 5 min in a centrifuge (Sorvall RC-5B, swinging bucket HB-4 rotor, DuPont Company, DE, USA). Plasma membrane right-side out vesicles partition preferably to the upper phase, while the other membranes remain in the lower phase.
4. Repeat repartition of 6 mL of the upper phase against fresh lower phase, previously deprived of an identical volume of fresh upper phase. This one could be used to further partition the first lower phase and to recover additional purified membranes.
5. Centrifuge again at 1500×g for 5 min to obtain phase separation.
6. Repeat points 4 and 5 once for the last third Corex® vessel. All the upper phases undergo three runs over a lower phase.
7. Collect and mix all the upper phases and resuspend them with UPWB. Upper phase mixture has to be diluted at least eightfold to avoid phase polymer contamination.
8. Ultracentrifuge plasma membrane vesicles at 120,000×g for 1 h in a Beckman L7-55 centrifuge with Ty 70ti rotor.
9. Resuspend the pellet in 1 mL of CPRB to obtain a final protein content of ca. 1 mg/mL. Membrane suspension is enriched at about 90% in plasma membrane right-side out vesicles.

3.4. Microsomal Fraction Isolation from Grape Berry Pulp

1. Homogenize ca. 50 g of grape berry pulp by an Ultra-turrax blender in 150 mL Buffer A. Perform this step and the following ones at 4°C (see Note 17).
2. Filter the homogenate through a 100 μm nylon mesh and centrifuge at 8000×g for 20 min in a Sorvall RC-5B centrifuge with SS-34 rotor.
3. Collect the supernatant and ultracentrifuge at 100,000×g for 36 min in a Beckman L7-55 ultracentrifuge with Ty 70ti rotor.
4. Discard the supernatant and resuspend the pellets in 100 mL of Buffer B and ultracentrifuge again at 100,000×g for 36 min.
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5. Resuspend the pellet in a minimal volume of Buffer C (0.4 mL) (see Note 18) and store it at −20°C.

3.5. Plasma Membrane Isolation from Rat Liver

1. Fill polyethylene centrifuge tubes with Buffer C at room temperature; seal them with parafilm and cool them in ice.

2. Euthanize three rats by decapitation, excise their livers, mince them in ice-cold Buffer A, and repeatedly discard the latter until traces of blood have been removed (see Note 19).

3. Transfer aliquots (ca. 5 mL) of minced liver in a Potter-Elvehjem homogenizer (spherical bottom) containing ca. 30 mL of ice-cold Buffer A; fix the homogenizer’s Teflon pestle on the holder of an electrical rotator and allow it to turn at about 600 rpm; carefully set the rotating pestle into the homogenizer, kept in a plastic beaker containing thawing ice, and slowly move the latter upward, so that the pestle reaches the homogenizer bottom; repeat this (ca. 15 strokes) until the suspension is homogeneous (see Note 20). Repeat this with other aliquots of minced liver.

4. Dilute the homogenate to 230 mL with buffer A and supplement it with 0.1 M EDTA (see Note 21).

5. Centrifuge the homogenate at 1000 × g (Centrikon T-324, rotor A8.24, Kontron Instruments, Watford, United Kingdom) for 10 min at 4°C in polycarbonate centrifuge tubes.

6. During this time, add 15 mL of ice-cold Buffer D to the centrifuge tubes containing ice-cold 16 mL Buffer C (see step 1) (see Note 22).

7. At the end of step 5, collect both the supernatant material and the fluffy layer over the pellet.

8. Centrifuge this material in polycarbonate tubes at 20,000 × g (Centrikon T-324, rotor A 8.24, Kontron Instruments, Watford, United Kingdom) for 30 min at 4°C.

9. During this time, complete step 6.

10. At the end of step 8, discard the supernatant material and resuspend the pellet in 24 mL of ice-cold Buffer B (see Note 23). Add aliquots (4 mL) thereof onto the discontinuous sucrose density gradient (prepared at steps 6 and 9) (see Note 24) and centrifuge the gradient at 50,000 × g (Centrikon T-1055, rotor TST 28.38, Kontron Instruments, Watford, United Kingdom) for 150 min at 4°C.

11. Collect the plasma membrane vesicles at the heavy–light interface of the gradient by aspirating them into a Pasteur pipette connected with a rubber tube.
12. Dilute threefold this membrane fraction in Buffer B and centrifuge it at 70,000×g (Centrikon T-1055, rotor TFT 70.13, Kontron Instruments, Watford, United Kingdom) for 40 min at 4°C.

13. Resuspend the pellet in a minimal volume of Buffer B (0.3 ml) (see Note 25) and store it in aliquots at −80°C.


1. BSP is a pH indicator dye, featured by fast (17), proton-dependent quinoidal–phenolic tautomerism (pK = 8.5) (Fig. 21.1). The quinoidal species absorbs light (λ_{max} = 580 nm) and solutions thereof look purple; upon acidification, the achromic phenolic species prevails and solutions turn colorless.

2. The extra-vesicular medium is buffered at pH 8.0 and contains K^+.

3. The intravesicular medium is buffered at pH 7.4 and contains no K^+.

4. The plasma membrane is (relatively) impermeable to inorganic cations, in particular H^+ and K^+.

5. The plasma membrane is selectively permeable to BSP (see Note 26).

6. Given these features, it is expected that transport of BSP from the medium into plasma membrane vesicles can be recorded as a net decrease of A_{580}, because [BSP] in the medium decreases and, at the same time, any intravesicular [BSP] increase goes undetected, because BSP is essentially achromic at pH 7.4.

7. The plasma membrane can be permeabilized to K^+ by addition of valinomycin (see Note 27), thus generating a transmembrane potential that is positive inside vesicles.

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**Fig. 21.1** Three-dimensional structure of bromosulfalein tautomers. The equilibrium between quinoidal (left) and the phenolic (right) species depends on the solution pH. Both structures were drawn and optimized by ACD/ChemSketch freeware, version 10.
8. Since BSP is negatively charged at the assay pH, it is expected that its electro-chemical equilibrium across the plasma membrane can be altered by a change of the membrane potential.

The assay of BSP transport into plasma membrane vesicles is carried out in two sequential phases, both of which are continuously recorded by dual wavelength spectrophotometer.

1. Phase 1: allow BSP to move from the medium into vesicles according to its electro-chemical potential until the equilibrium is attained.

2. Phase 2: generate a K⁺ current across the membrane by adding valinomycin and allow BSP to further move from the medium into vesicles, this time being driven exclusively by the membrane potential, until the final equilibrium is attained. The initial rate of the latter phase of transport is referred to as electrogenic (synonymous with electrophoretic) BSP transport.

1. Set the spectrophotometer [Sigma ZWS-11C, Sigma Instruments GmbH, Berlin, Germany, or Agilent 1200 diode array and multiple wavelength detector, Agilent, Santa Clara, CA, USA] at two wavelengths, i.e., \( \lambda_1 = 580 \text{ nm} \) and \( \lambda_2 = 514 \text{ nm} \), the former being the absorbance peak of BSP and the latter being the reference (see Note 28).

2. Amplify the \( A_{580-514} \) signal of the Sigma ZWS-11C instrument 10- or 20-fold and feed it into a real-time recorder on paper tape advancing at 12 cm min⁻¹. The signal of the Agilent instrument is controlled by Agilent Instrument 1 software (Santa Clara, CA, USA).

3. Add 2 mL of transport assay solution (containing BSP at a concentration chosen in the range 4–40 \( \mu \text{M} \), depending on the aim of the experiment, see below) into an optical disposable cuvette (1 cm optical path) containing a magnetic bar (8 mm).

4. Set the cuvette into the optical cell holder of the spectrophotometer. Switch on the magnetic stirrer (see Note 29). Record the output signal for at least 5 s before adding vesicles to obtain the basal line (see Note 30).

1. Open the cover of the optical cell holder and add vesicles (2–20 \( \mu \text{L} \), ca. 10 \( \mu \text{g protein} \)) to the transport assay solution by means of a micropipette.

2. This causes the optical signal to go temporarily out of scale. The output returns within the recorder scale upon closure of the cell holder, a few seconds later, and a steady state is attained within 15–20 s (Fig. 21.2).
3. The signal record is now shifted down by a considerable amount both because of the sudden increase in turbidity of the solution due to vesicle addition and because of the movement of BSP from the medium into the vesicles (see Note 31).

4. The driving force for BSP transport into vesicles is provided by both the chemical and the electrical potential.

1. Deliver valinomycin (1–3 μL) by means of a routine work syringe (Exmire MS*10SL, http://www.exmire.com/pd_stnd_04.html#rout); thread its needle through a 1 mm hole on the cover to avoid interruption of the signal recording due to opening of the cover (Fig. 21.2).

2. The signal decrease, which is linear for its first part, is due to entry of BSP into vesicles (see Note 31) under the sole driving force provided by the K⁺ diffusion potential and is, therefore, referred to as electrogenic BSP transport. Only the latter is examined kinetically.

3. Calculate the initial rate of electrogenic BSP transport ($v_0 = \mu$mol/min/mg protein) either manually, by drawing a line extrapolating from the initial, linear segment of $A_{580-514}$ decrease caused by valinomycin addition and recorded on paper tape (Sigma ZWS-11C instrument), or by Agilent Instrument 1 software (Agilent 1200 diode array instrument) (see Note 32). The differential ($A_{580-514}$) extinction coefficient ($\varepsilon_{580-514}$) of BSP is $11.5 \times 10^3$ M⁻¹ cm⁻¹.
3.11. Biophysics of Electrogenic BSP Transport: Dependence on the Amount of Protein Added

The dependence of the rate of electrogenic BSP transport on the amount of vesicles (i.e., of protein) added into the assay solution should be established with any new preparation of membrane vesicles [e.g., (13)].

1. Fill cuvettes with 2 mL transport assay buffer.
2. Carry out assays by adding sequentially 2 μL of each vesicle dilution \( (n = 3) \) and 2 μL valinomycin (5 mg/mL) (see Note 33); proof of correct assay’s conditions and initial rate measurements is obtained when linear regression data analysis returns a predicted value of the intercept \( y_0 = 0 \) and \( r^2 > 0.98 \).

3.12. Biophysics of Electrogenic BSP Transport: Dependence on Valinomycin

The dependence of the rate of electrogenic BSP transport on the membrane potential can be examined by varying the amount of valinomycin added into the assay solution.

1. Fill cuvettes with 2 mL transport assay buffer.
2. Carry out assays by adding sequentially 2 μL of vesicle suspension and 2 μL of each valinomycin dilution \( (n = 3, \text{ each}) \) (see Note 34). Proof of correct assay conditions and initial rate measurements is obtained when linear regression data analysis returns a predicted value of \( y_0 = 0 \) and \( r^2 > 0.98 \) [e.g., (13)].

3.13. Biophysics of Electrogenic BSP Transport: Dependence on Extra-Vesicular \([K^+]\)

The dependence of the rate of electrogenic BSP transport on the membrane potential can be also examined by varying \([K^+]\) into the assay solution.

1. Fill cuvettes with 2 mL transport assay buffer.
2. Carry out assays by adding sequentially 2 μL of vesicle suspension and 2 μL valinomycin to the transport assay solutions containing graded amounts of \([K^+] \ (n = 3, \text{ each}) \). Proof of correct assay’s conditions and initial rate measurements is obtained when linear regression data analysis returns a predicted value of \( y_0 = 0 \) and \( r^2 > 0.98 \) (see Note 35).


The extent of electrogenic BSP transport, i.e., the intravesicular amount of BSP at steady state following valinomycin addition, linearly depends on the osmolarity of the assay solution [e.g., (13)]. Water flow out of vesicles results in a decrease of BSP-accessible intravesicular volume.

1. Fill a series of cuvettes with 2 mL transport assay buffer containing increasing [sucrose].
2. Carry out assays by adding sequentially 2 μL of vesicle suspension and 2 μL valinomycin into the transport assay solutions containing graded [sucrose].
3. The extent of BSP electrogenic transport (nmol BSP) is estimated by the amplitude of the \( A_{580-514} \) drop that occurred
in the interval started with addition of valinomycin and ended with attaining the steady state (see Note 36).

3.15. Kinetics of Electrogenic Bromosulfalein Transport: Dependence on [BSP]

The dependence of the rate of electrogenic BSP uptake in different membrane vesicles on the dye concentration in the medium is described by a simple hyperbola, in compliance with the Michaelis–Menten model (10, 13, 14, 18–20).

1. Fill a series of cuvettes with 2 mL transport assay buffer containing increasing [BSP] (e.g., 4, 8, 12, 16, 20, 24, 32, 40 μM).

2. Carry out assays by adding sequentially 2 μL of vesicle suspension and 2 μL valinomycin into each cuvette.

3. Measure the initial rate of electrogenic BSP uptake \( v_0 = \text{nmol min/mg protein} \); plot \( v_0 \) vs. [BSP] and calculate the best fitting hyperbola: \( y = ax/(b + x) \), where \( y = v_0 \), \( x = [\text{BSP}] \), \( a = V_{\text{max}} \), \( b = K_m \).


Bile pigments (i.e., both bilirubin and biliverdin) modify the substrate dependence of BSP electrogenic uptake in liver, but not in carnation petal microsomes (13), causing competitive inhibition. Flavonoids act as competitive, non-competitive, or mixed-type inhibitors of electrogenic BSP transport in membrane vesicles of various biological sources (9, 13, 20).

1. Fill a series of cuvettes with 2 mL transport assay buffer containing increasing [BSP] (e.g., 4, 8, 12, 16, 20, 24, 32, 40 μM).

2. Supplement each cuvette with a minimal volume (2 μL) of the inhibitors dissolved in DMSO; test at least two different inhibitor concentrations (see Note 37); when using flavonoids, check for their optical interference (see Note 38).

3. Carry out assays by adding sequentially 2 μL of vesicle suspension and 2 μL valinomycin into each cuvette.

4. Run the control tests supplementing the transport assay solution with the same volume of DMSO alone.

5. Measure the initial rate of electrogenic BSP uptake \( v_0 \), plot \( v_0 \) vs. [BSP] and calculate the best fitting hyperbola: \( y = ax/(b + x) \), where \( y = v_0 \), \( x = [\text{BSP}] \), \( a = V_{\text{max}} \), \( b = K_m \). Competitive inhibitors increase apparent \( K_m \) according to the equation: \( K_{mi} = K_m(1 + [I]/K_i) \), where \( K_i \) is the inhibition constant, \( I \) is the inhibitor, \( K_{mi} \) and \( K_m \) are the apparent \( K_m \) values in either the presence or the absence of the inhibitor, respectively. Non-competitive inhibitors decrease apparent \( V_{\text{max}} \) according to the equation \( 1/V_{\text{max1}} = 1/V_{\text{max}}(1 + [I]/K_i) \), where \( V_{\text{max1}} \) and \( V_{\text{max}} \) are the apparent \( V_{\text{max}} \) values either in the presence or in the absence of the inhibitor, respectively (see Note 39).
3.17. Time-Dependent Inactivation of Bromosulfalein Transport: Procedure and General Features

Electrogenic BSP uptake in rat liver plasma membrane vesicles is inactivated by various protein reagents binding to the side chains of cysteine (Section 3.22), arginine (Section 3.23), and serine (Section 3.24) and by specific anti-sequence bilirubin translocase antibodies (Section 3.25). The inactivation reaction is slow (time scale of minutes), [reagent] and temperature dependent, and irreversible. Inactivation tests are carried out in two phases.

Phase 1 consists of the pre-incubation of membranes with protein-specific reagents:

1. Prepare a thermostated (37°C) water bath with an Eppendorf tube holder.
2. Put Eppendorf tubes containing membrane vesicles in the water bath for at least 30 s to allow membranes to warm up to 37°C.
3. Start the inactivation reaction by adding protein-specific reagents (1–3 μL, at a single concentration, see Sections 3.22–3.25 for specific details) to the membrane vesicle suspension (20–50 μL) in Eppendorf tubes.
4. If it is desired, repeat the above step to test a series of [reagent].
5. Carry out controls by adding reagent-free solutions to the membrane vesicle suspension (20–50 μL) in Eppendorf tubes.
6. Withdraw samples from each pre-incubation mixture (test and control) (3–5 μL) at various times for up to 30–40 min to assay transport activity.
Phase 2 consists of the electrogenic BSP assay of pre-incubated membranes:
7. Add samples of each pre-incubation mixture to the assay cuvette containing 2 mL transport assay solution at a single [BSP] (typically 40 μM) (see Note 40) at room temperature (21°C).
8. Start electrogenic BSP transport by injecting valinomycin.
9. Measure the initial rate of electrogenic BSP uptake (see Section 3.10). Time series of both inhibited transport activity (T1) and control transport activity (T0) is thus acquired.

1. Transfer of vesicles to the assay cuvette stops the inactivation reaction because protein-specific reagents are diluted 0.4–1 × 10³-fold in the transport assay medium.
2. At the same time, the composition of the transport assay solution is virtually unchanged under either control or inactivation conditions. The pre-incubation conditions can, therefore, be varied at will, while enabling to assess T1/T0 under constant transport assay conditions.

3.18. Time-Dependent Inactivation of Bromosulfalein Transport: Implications

1. Transfer of vesicles to the assay cuvette stops the inactivation reaction because protein-specific reagents are diluted 0.4–1 × 10³-fold in the transport assay medium.
2. At the same time, the composition of the transport assay solution is virtually unchanged under either control or inactivation conditions. The pre-incubation conditions can, therefore, be varied at will, while enabling to assess T1/T0 under constant transport assay conditions.
3. Further favorable features are given by the electrogenic BSP transport assay taking place at a lower temperature than the pre-incubation (21°C vs. 37°C) and being completed within seconds, a time scale much lower than the inactivation reaction (minutes).

1. Express the extent of inactivation of each time sample as $T_1/T_0$ (relative transport activity).
2. Plot $T_1/T_0$ data vs. time (see Note 41).
3. To obtain the inactivation parameters, fit the following equation to data: $y = y_0 + ae^{-kt}$, where $y = T_1/T_0$, $y_0$ = residual $T_1/T_0$ at steady state, $a = 1 - y_0$, $e = 2.7183$, $t$ = time, and $k$ = inactivation rate constant (min$^{-1}$) (see Note 42).
4. If a series of $k$ values are obtained at various [reagent], plot $k$ vs. [reagent]. A straight line should be obtained by linear regression analysis, whose slope corresponds to the second-order rate constant of inactivation, $k_2$ (min$^{-1}$ M$^{-1}$).

The inactivation kinetics can be profoundly influenced by the presence of bilitranslocase substrates in the pre-incubation mixture, showing that the substrate–transporter complex inactivates at a slower rate than the free transporter (11, 13, 21). High-affinity substrates, such as bilirubin, can therefore be supplemented at relatively low absolute concentrations. Under these conditions, it is possible to observe quasi-complete protection from inactivation at saturating [substrate] and, therefore, to obtain an intrinsic control that the reagent did not disrupt the supra-molecular intactness of vesicles [e.g., (21)]. When using anti-sequence antibodies, such protection shows that the antibody is targeting a substrate-specific (bile pigment- or flavonoid-specific) transporter (13). Even more, a quantitative investigation of the inactivation protection as a function of [substrate] enables to derive the substrate–transporter dissociation constants.

The inactivation protection effect can be investigated either at constant [inhibitor]–variable [ligand] (22) (Fig. 21.3a) or, vice versa, at variable [inhibitor]–constant [ligand]. The latter procedure will not be described in detail, but can be worked out based on classical work (23). Inactivation tests are carried out in two phases.

Phase 1 consists of pre-incubation of membranes with protein-specific reagents and increasing [substrates]:
1. Prepare a thermostated (37°C) water bath with an Eppendorf tube holder; put Eppendorf tube(s) containing membrane vesicles in the water bath to allow membranes to be at 37°C.
2. Add bilitranslocase substrates (2–5 μL) to the membrane vesicle suspension (20–50 μL) in Eppendorf tubes at least 30 s before proceeding to the next step.
3. Start the inactivation reaction by adding protein-specific reagents (3–5 μL, at a single concentration, see Sections 3.22–3.25 for specific details) to the membrane vesicle suspension (20–50 μL) in Eppendorf tubes.

4. Repeat the inactivation reaction (step 3) at serial [substrate] (see Note 43).

5. Carry out controls by adding: (a) reagent-free solutions to the membrane vesicle suspension (20–50 μL) in Eppendorf tubes to obtain $T_0$; (b) substrate-free solutions to the membrane vesicle suspension (20–50 μL) in Eppendorf tubes to obtain $T_1$ in the absence of substrates.

6. Withdraw samples of each pre-incubation mixture (test and control) (3–5 μL) at various times for up to 30–40 min to assay transport activity and proceed to phase 2.

Phase 2 consists of the electrogenic BSP assay of pre-incubated membranes (see Section 3.17):

For the calculation of inactivation kinetics parameters, proceed as follows:

1. Express the extent of inactivation of each (time) sample as $T_1/T_0$ (relative transport activity).

2. Plot $T_1/T_0$ data vs. time (see Note 44) for each [substrate] tested (Fig. 21.3a).
3. Fit the following equation to data: \( y = y_0 + ae^{-kt} \), as described in Section 3.19 to obtain the inactivation rate constant parameters in the absence \( (k_0) \) and in the presence of substrates \( (k_{S1}, k_{S2}, \ldots k_{Sn}) \).

4. Plot the ratios of inactivation rate constants in the presence and absence of substrates \( (k_{S1}/k_0; k_{S2}/k_0; \ldots k_{Sn}/k_0) \) against \( [1 – (k_S/k_0)/[S]] \). A straight line should be obtained by linear regression analysis (Fig. 21.3b). Its slope is \( K_d \), the dissociation constant of the transporter–substrate complex, and its intercept provides a measure of the maximum protection by the substrate against inhibition, with complete protection producing a value of 0 (see Note 44).

**3.21. Recovery from Time-Dependent Inactivation of Bromosulfalein Transport**

Electrogenic BSP transport activity can be recovered in those cases in which the protein-specific adduct can be displaced by the addition of inactivation reversal reagents, which attack the protein–adduct bond and restore the protein amino acid side chain (see Sections 3.22–3.25 for specific cases). Under appropriate conditions, recovery can be complete, thus providing an intrinsic control that the inactivation reagent did not disrupt the supra-molecular intactness of vesicles. Even more, reactivation may be accelerated by the presence of substrates, again showing the specificity of the molecular target (i.e., the electrogenic BSP transporter) of the inactivation/activation cycle. The substrate-induced acceleration of reactivation can be assessed quantitatively, enabling to derive dissociation constants of the complex between substrates and the inactivated transporter (18). Inactivation tests are carried out in two phases.

Phase 1 consists of the pre-incubation of membranes with protein-specific reagents and inactivation reversal reagents added in sequence:

1. Prepare a thermostated \((37^\circ C)\) water bath with an Eppendorf tube holder; put Eppendorf tube(s) containing membrane vesicles in the water bath, to allow membranes to be at \(37^\circ C\).

2. Start the inactivation reaction by adding protein-specific reagents \((1–3 \, \mu L, \text{ at a single concentration, see Sections 3.22–3.25 for specific details})\) to the membrane vesicle suspension \((20–50 \, \mu L)\) in Eppendorf tubes.

3. Add reversal inactivation reagents \((1–5 \, \mu L, \text{ at a single concentration, see Sections 3.22–3.25 for specific details})\) without or with substrates (see Note 45).

4. Carry out controls by adding reversal reagent-free solutions, either in the absence or in the presence of substrates, to the membrane vesicle suspension \((20–50 \, \mu L)\) in Eppendorf tubes.
5. Withdraw samples of each pre-incubation mixture (test and controls) (3–5 μL) at various times for up to 20 min to assay transport activity and proceed as described above (Section 3.5.2).

Phase 2 consists of the electrogenic BSP assay of pre-incubated membranes (see Section 3.17):

For the calculation of inactivation kinetics parameters, proceed as below:

1. Express the extent of inactivation of each (time) sample as $T_I/T_0$ (relative transport activity).
2. Plot $T_I/T_0$ data vs. time.
3. Fit the following equation to data: 
   \[ y = 1 - e^{-kt}, \]
   where $y = T_I/T_0$, $e = 2.7183$, $t = $ time, and $k =$ reactivation rate constant (min$^{-1}$) (see Note 46).

### 3.22. Specific Cases:

#### Inactivation by Cysteine-Specific Reagents and Reactivation by Mercaptoethanol

**Inactivation by $p$-HMB**

1. Start the inactivation reaction by adding 1.5 μL $p$-HMB solution to 23.5 μL rat liver plasma membrane vesicles.
2. The reaction mixture may be supplemented with substrates, e.g., BSP (4–40 μM), bilirubin (2–50 nM), or any other substrate.
3. Use 0.5–2.5 mM $p$-HMB, so to attain final $[p$-HMB] = 30–150 μM.
4. Withdraw aliquots (3 μL, ca. 10 μg protein) of the suspension at the indicative time intervals of 0, 3, 6, 9, 12, 15, 20 min for the electrogenic BSP assay (see Section 3.17) (see Note 47).
5. Start the reactivation reaction by the addition of a molar excess of β-mercaptoethanol (see Note 48).

#### 3.22.1. Inactivation by DTNB

1. Start the inactivation reaction by adding 1.5 μL DTNB solution to 23.5 μL rat liver plasma membrane vesicles.
2. The reaction mixture may be supplemented with substrates, e.g., BSP (4–40 μM), bilirubin (2–50 nM), or any other substrate.
3. Use 0.25–5 mM DTNB, so to attain final [DTNB] = 15–300 μM.
4. Withdraw aliquots (3 μL, ca. 10 μg protein) of the suspension at the indicative time intervals of 0, 3, 6, 9, 12, 15, 20, 30 min for the electrogenic BSP assay (see Section 3.17) (see Note 47).
5. Start the reactivation reaction by the addition of β-mercaptoethanol (see Note 48).

#### 3.22.2. Inactivation by NEM

1. Start the inactivation reaction by adding 2.5 μL NEM solution to 22.5 μL rat liver plasma membrane vesicles.
2. The reaction mixture may be supplemented with substrates, e.g., BSP (4–40 μM), bilirubin (2–50 nM), or any other substrate.

3. Use [NEM] in the range 1–10 mM (final [NEM] = 0.1–1 mM).

4. Withdraw aliquots (3 μL, ca. 10 μg protein) of the suspension at the indicative time intervals of 0, 3, 6, 9, 12, 15, 20 min for the electrogenic BSP assay (see Section 3.17) (see Note 47).

5. Reactivation cannot be accomplished, since NEM forms a stable thioether bond with thiols.

3.23. Specific Cases: Inactivation by Arginine-Specific Reagents and Reactivation

1. Start the inactivation reaction by adding 2.5 μL methylglyoxal or phenylglyoxal to 22.5 μL rat liver plasma membrane vesicles.

2. The reaction mixture may be supplemented with substrates, e.g., BSP (4–40 μM), bilirubin (2–50 nM), or any other substrate.

3. Use reagents in the range 25–250 mM (final concentration = 2.5–25 mM).

4. Withdraw aliquots (3 μL, ca. 10 μg protein) of the suspension at the indicative time intervals of 0, 3, 6, 9, 12, 15, 20, 30 min for the electrogenic BSP assay (see Section 3.17) (see Note 47).

5. Start the reactivation reaction by the addition of a small molar excess of arginine (e.g., add 1 μL 1 M arginine to 24 μL vesicles pre-incubated with 20 mM methylglyoxal). Reactivation can also be obtained by diluting vesicles six-fold in 10 mM HEPES/0.15 M NaCl/0.25 M sucrose (pH 7.4) (see Note 49), due to the fact that the phenylglyoxal–arginine complex is unstable when the excess reagent is removed (24). To speed up reactivation, the dilution buffer can be supplemented with arginine.

3.24. Specific Cases: Inactivation by Serine-Specific Reagents and Reactivation

1. Start the inactivation reaction by adding 1.5 μL PMSF to 22.5 μL rat liver plasma membrane vesicles.

2. The reaction mixture may be supplemented with substrates, e.g., BSP (4–40 μM), bilirubin (2–50 nM), or any other substrate.

3. Use PMSF in the range 2–12 mM (final concentration = 0.125–0.75 mM).

4. Withdraw aliquots (3 μL, ca. 10 μg protein) of the suspension at every minute in the time window 0–10 min, since the reaction is fast (21).

5. Start the reactivation reaction by the addition of pyridine 2-aldoxime methiodide (2-PAM or pralidoxime), a specific
antidote of protein phosphorylation or sulfonylation (25). The reactivation rate depends on [2-PAM] and is best observed at 2-PAM/PMSF molar ratio ranging from 2 to 5. Samples should be assayed as soon as possible after addition of the oxime at 1–2 min intervals for ca. 20 min.

3.25. Specific Cases: Inactivation by Sequence-Specific Bilitranslocase Antibodies

1. Start the inactivation reaction by adding either antibody A or antibody B in the range 1–80 μg IgG/mL (final concentration = 0.2–20 μg IgG/mL) to plasma membrane vesicles (see Note 50).

2. The reaction mixture may be supplemented with substrates, e.g., BSP (4–40 μM), bilirubin (2–50 nM), or any other substrate.

3. Withdraw aliquots (3 μL, ca. 10 μg protein) of the suspension to be assayed for electrogenic BSP transport as described above at 2 min intervals for 30 min.

4. Notes

1. For transport assay, use fresh material to improve competence and integrity of membrane vesicles.

2. Care should be taken to handle this reagent since it is toxic.

3. DTE and PMSF must be added to MEB just before the homogenization.

4. DTE and PMSF must be added to MRBTI just before the homogenization.

5. Different polymer concentrations may be used depending on plant species and organ analyzed.

6. BSA, casein, DTE, β-mercaptoethanol, and PMSF must be added to Buffer A just before the homogenization.

7. DTE and PMSF must be added to Buffer B just before resuspension.

8. Solutions for membrane vesicle preparations are free of phenylmethanesulfonfonyl fluoride, a protease inhibitor reagent, since it is an irreversible inhibitor of electrogenic BSP transport in rat liver plasma membrane vesicles (1, 2).

9. Dissolve ca. 0.15 g BSP in 0.2 mL 0.1 M NaOH to obtain ca. 0.9 M BSP; dilute it with water to 10 mL to obtain ca. 18 mM BSP in 2 mM NaOH. Determine its concentration spectrophotometrically ($\varepsilon_{580} = 64 \times 10^3 \, M^{-1} \, cm^{-1}$). A 5 mM solution can also be prepared in DMSO.

10. Store valinomycin at −20°C in a tight vial to avoid methanol evaporation. This solution is stable for months.
Solutions of 5 and 20 mg/mL are typically used for rat liver plasma membrane vesicles and grape berry microsomes, respectively.

11. Consider to repeat this experiment with increasing valinomycin additions, as detailed in Section 2.9.

12. Use only bile pigment solutions prepared immediately before transport tests. Wrap tubes with aluminium foil.

13. Keep flavonoid solutions in the dark at room temperature; shelf life is a few days.

14. Dissolve 5.7 mg of \( p \)-HMB (sodium salt, m.m. 361.70) in 60 μL of 0.1 M NaOH (it does not dissolve in DMSO) and vigorously mix; add 150 μL of 5 M NaCl, vigorously mix; dilute to 5 mL with water. Diluting this solution to 0.15 mM in 10 mM HEPES, pH 7.4, causes no pH changes. Check \([p-HMB]\) spectrophotometrically (\( \varepsilon_{255} = 4.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \)) (26).

15. Check \([\text{NEM}]\) spectrophotometrically (\( \varepsilon_{305} = 0.62 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \)) (26).

16. Check \([\text{DTNB}]\) spectrophotometrically by adding an excess \( \beta \)-mercaptoethanol (\( \varepsilon_{412} = 14.15 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \), at 25°C) (27).

17. The preparation of intact and competent vesicles is a prerequisite to perform transport assay in microsomal fraction of grape berry, a recalcitrant fruit. For this purpose, the method suited for measuring the vacuolar pumps, inorganic pyrophosphatase and ATPase (28), has been chosen for the electrogenic BSP transport assay.

18. Increase the microsomal protein concentration in order to add small volumes of vesicle suspension to BSP transport assay.

19. To obtain a fairly well exsanguinated liver, hold the decapitated rat vertically by its tail and exert some pressure on its abdomen until the blood flowing out of the resected neck vessels will cease.

20. The relative movement of the pestle about the homogenizer must be slow, so as to avoid formation of vacuum bubbles.

21. EDTA is used to chelate Ca\(^{2+}\) in Buffer A. This will prevent subcellular particles to aggregate and thus to be collected in the low-speed supernatant.

22. To obtain a sharp interface between buffer C and buffer D, use a Pasteur glass pipette connected to a rubber tube and control delivery of ice-cold buffer D by mouth, so as to generate a slow laminar flow. The sharp interface is
needed to efficiently separate plasma membrane vesicles from microsomes. Prepare this discontinuous sucrose gradient immediately before use.

23. Use a Potter-Elvehjem homogenizer (spherical bottom).
24. Do it gently in order not to mix up the gradient.
25. Take care not to resuspend the tiny brownish material (contaminating sub-mitochondrial particles) at the center of the pellet.
26. The liver plasma membrane permeability to BSP is known to be due to specific membrane carriers (3, 5, 29).
27. This macrocyclic antibiotic is a K⁺-specific ionophore (30). Upon addition to the assay cuvette, it dissolves in the vesicle membrane, increasing its permeability to K⁺ and therefore generating a membrane potential.
28. No specific absorbance changes at $\lambda = 514$ nm occur during the assay (31). The optical signal resulting from $\lambda_1 - \lambda_2$ subtracts most (but not all) of the absorbance due to the turbidity of the membrane suspension, thus substantially increasing the signal to noise ratio (32).
29. Take care that the stirring rate is high enough to allow fast mixing while avoiding generation of air bubbles.
30. The signal might drift due to heating of the transport assay solution (29), thus it is essential to keep it at room temperature. Unusual signal oscillations might be caused by air bubbles formed by exceedingly fast stirring or by any other particulate object floating in the solution, thus the latter must be filtered and the cuvettes must be clean (keep them covered and rinse them if necessary). Further oscillations could be because of electrical interference caused by contiguous instruments.
31. The drops of the signal output caused by the additions of vesicles and valinomycin, respectively, indicate a decrease in concentration of the light-absorbing quinoidal species of BSP in the transport assay solution. Two explanations are possible. The pH of the transport assay solution has decreased, causing a corresponding loss of the quinoidal tautomer of BSP in favor of the phenolic, colorless one. This is however not true, because no pH change is recorded upon either addition (7), as expected, for the transport assay solution is heavily buffered (0.1 M potassium phosphate, pH 8.0). Then, the possibility left is that BSP has moved from the assay solution into the less alkaline vesicular compartment (buffered at pH 7.4), where colorless BSP prevails.
32. Due to the signal disturbance caused by valinomycin addition (an instantaneous decrease of the signal), avoid the first 1.5 s and then consider the following 5 s for calculation of the initial rate. The signal disturbance can also be quenched by supplementing valinomycin stock solution with some triethanolamine.

33. Consider repeating this experiment with increasing valinomycin additions, as detailed in Section 3.12.

34. When using grape berry microsomes, a higher range of valinomycin concentrations should be used (5–20 μg/mL, 15–60 μg).

35. If \( y_0 > 0 \) and \( r^2 > 0.98 \), repeat the assay with solutions A and B devoid of BSP. Measure the valinomycin-dependent absorbance decrease and subtract this value from the measurements obtained in the corresponding experiments carried out with BSP-containing solutions. Valinomycin-dependent \( K^+ \) diffusion into vesicles might determine osmotic water flow into and swelling of vesicles, detectable as an optical output response.

36. The extent of the \( A_{580-514} \) drop lessens with increasing [sucrose] in the transport assay solution. To extrapolate the extent of BSP electrogenic transport at infinite [sucrose] in the medium, plot BSP transport (nmol) vs. L/osm. Using a carnation petal plasmalemma preparation, BSP electrogenic transport at infinite [sucrose] was zero (13), but not so using rat liver plasma membranes (7). The physical basis of the latter effect might be that \( A_{580-514} \) drop is accounted for by both intravesicular transport and valinomycin-induced absorption on the plasma membrane. However, one should also consider that sucrose-induced shrinking of vesicles might also be accompanied by ion diffusion from the vesicles into the medium. Potassium ions might be involved. In fact, some \( K^+ \) might diffuse into vesicles before the addition of valinomycin, exploiting extant permeability pathways (\( K^+ \) channels).

37. Testing two or more inhibitor concentrations enables to obtain numerous independent sets of kinetic parameters (apparent \( K_m \) and \( V_{max} \)); the derived \( K_i \) values should match and can be averaged. This is particularly indicated with mixed-type inhibitors (9). However, this may not be possible if the activity range of the inhibitor is close to its solubility limit [e.g., bilirubin (13)].

38. Some flavonoids, especially anthocyanins, absorb at \( A_{580-514} \). To control their optical interference in the assay, they should be added to BSP-free transport assay solution (0.1 M potassium phosphate, pH 8.0); absorbance changes
at $A_{580-514}$ following the sequential addition of vesicles and valinomycin should be recorded. The flavonoids tested so far did not interfere with electrogenic BSP transport assay (9, 10, 13, 14).

39. Preliminary tests of BSP electrogenic transport inhibition by flavonoids can be done using flavonoid stock solutions (0.1 M). Competitive and non-competitive inhibitors better display their activity in the low (4–10 μM) and high (30–40 μM) [BSP] range, respectively. Based on the results obtained, more diluted flavonoid solutions can be used and tested over the entire [BSP] range.

40. The mechanism whereby both cysteine and arginine reagents inactivate electrogenic BSP transport in rat liver plasma membrane vesicles is rather unusual, since they decrease $K_m$ rather than $V_{max}$, as described in detail in (18). As a consequence, the inactivation is better detected at [BSP] < 40 μM, while it is not appreciably detected at [BSP] at which the uptake rate approaches $V_{max}$; in order to combine signal reliability (sufficient extent of valinomycin-induced decrease of $A_{580-514}$) with appreciable apparent inactivation, the transport assay solution should contain 25–40 μM BSP.

41. In membrane vesicles from either rat liver or carnation petals, $T_0$ is stable at 37°C for up to 35 min.

42. Relative transport activity ($T_I/T_0$) decays by pseudo-first-order reaction kinetics, reaching a steady state when $T_I/T_0 = 0.5–0.6$.

43. Test at least four increasing concentrations of substrate. Some preliminary trials might be needed in order to choose the correct serial [substrate].

44. The reaction of an inhibitor with an enzyme in the presence and absence of the enzyme substrate can be described by the following scheme, proposed by Scrutton and Utter (22):

$$E + S \rightleftharpoons ES \quad (21.1)$$

$$E + I \rightarrow E - I \quad (21.2)$$

$$ES + I \rightarrow ES - I \quad (21.3)$$

in which the enzyme (E) and the substrate (S) form a reversible complex (ES) (Eq. 21.1), featured by the dissociation constant $K_d = [E][S]/[ES]$. Both the free enzyme
E and the complex ES can in principle react with an irreversible inhibitor (I) to form either a binary (Eq. 21.2) or a ternary (Eq. 21.3) complex, with respective rate constants $k_1$ and $k_2$. If, under conditions in which both [S] and [I] >> [E], the equilibrium between E, S, and ES is attained much faster than the inhibition reaction steady state, then the following relationship applies:

$$\frac{k_S}{k_0} = \frac{k_2}{k_1} + K_d\frac{1 - (k_S/k_0)}{[S]} \quad (21.4)$$

where $k_S$ and $k_0$ are the pseudo-first-order inactivation rate constants in the presence and absence of the substrate, respectively.

45. Substrates of the electrogenic BSP transporter have been shown to accelerate the reactivation rate when inactivation was caused by either cysteine- or arginine-specific reagents (18, 33, 34), but not by serine-specific reagents (21, 35).

46. The fit might not be satisfying, because the onset of reactivation might be delayed (lag phase). This can be observed at sub-optimal reversal reagent/inactivation reagent molar ratio. The mechanism underlying such complex kinetics has been investigated and discussed in detail (18), where the equations best fitting to reactivation data are presented.

47. The volume of both the vesicle suspension and the inhibitor solution can be adjusted as function of [protein] and [inhibitor] of the stock solutions.

48. The progress of reactivation depends on [mercaptoethanol] and is best observed at mercaptoethanol/$p$-HMB molar ratio ranging from 2 to 5. Samples should be assayed as soon as possible after addition of mercaptoethanol at 2 min intervals for ca. 15 min. The same applies to DTNB.

49. Do not exceed sixfold dilution (i.e., from 3 to 0.5 mg protein/mL) if time-dependent reactivation tests are carried out at 37°C. If tests are carried out in ice, rat liver plasma membrane vesicles can be diluted up to 16-fold (i.e., from 3 to 0.19 mg protein/mL).

50. Appropriate controls are made by incubating vesicles in the presence of commercial rabbit IgG or IgG purified from preimmune rabbit serum or with the unbound fraction of affinity-purified immune serum. Antibodies are the sole time-dependent inhibitors of electrogenic BSP transport tested in membranes isolated from plant sources.
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