The genetic transformation of plastids

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Abstract

Biolistic delivery of DNA initiated plastid transformation research and still is the most widely used approach to generate transplastomic lines in both algae and higher plants. The principal design of transformation vectors is similar in both phylogenetic groups. Although important additions to the list of species transformed in their plastomes have been made in algae and in higher plants, the key organisms in the area are still the two species, in which stable plastid transformation was initially successful, i.e., *Chlamydomonas reinhardtii* and tobacco. Basic research into organelle biology has substantially benefited from the homologous recombination-based capability to precisely insert at predetermined loci, delete, disrupt, or exchange plastid genome sequences. Successful expression of recombinant proteins, including pharmaceutical proteins, has been demonstrated in *Chlamydomonas* as well as in higher plants, where some interesting agronomic traits were also engineered through plastid transformation.

1 Introduction

Plants are defined as the organisms containing plastids. Plant cells are operating and functioning through the integrated expression networks of nuclear, mitochondrial, and plastid genes. The capability of using genetic transformation for changing components of the integrated networks allows – in basic research – to study the interplay between the different genomes. In applied research, genetic transformation can optimize plants for their performance in natural or artificial environments and can introduce new functions such as the production of recombinant proteins or novel metabolites. Stable genetic transformation of plastids was first introduced for *Chlamydomonas* almost 20 years ago (Boynton et al. 1988; Blowers et al. 1989), and was successfully applied to the higher plant *Nicotiana tabacum* L. (tobacco) soon afterwards (Svab et al. 1990). In both species transformation involves a single or very few plastid DNA molecules initially, which leads to cells or organisms containing genetically different plastomes. These are termed "heteroplasmic" (Fig. 1). Distribution of plastid DNA molecules (and, in higher plants, plastids) among the daughter cells originating from mitosis is a statistical process. As a consequence, segregation of different plastid DNA molecules occurs. Under appropriate selection this process leads to cells (organisms) containing only transformed plastomes, which are called "homoplasmic" (Fig. 1). Several

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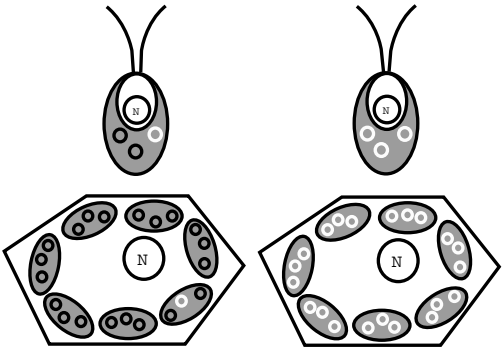

Fig. 1. Schematic representation of heteroplasmic (left) and homoplasmic (right) cells of *Chlamydomonas* (top) and a higher plant (bottom). N: nucleus; plastids are in grey; plastid DNA molecules are depicted as black (wild type) or white (transformed) circles. Note that drawings are not to scale and numbers of DNA molecules and (in higher plants) plastids are far too low.

hundred original scientific reports and numerous reviews (Table 1) on research in the area have been published in the meantime. This review attempts at serving as a reference article and at providing an actual update on this research.

1.1 Plastid biology in *Chlamydomonas* and tobacco

The basic technology of genetic transformation of plastids was developed for *Chlamydomonas reinhardtii*, and most of the technical features, like transfer of DNA via particle bombardment and some of the selection markers used, were directly applicable for higher plants. One could argue therefore that *Chlamydomonas* can serve as a model for plastid transformation in higher plants. This view is attractive since, due to the presence of only one plastid per cell and the much shorter generation time, in *Chlamydomonas* it takes only four to six weeks to reach homoplasmy, where in tobacco four to six months are necessary. As pointed out by Maliga (1993), there are however some important differences in plastid biology between single-celled algae and higher plants, which need to be kept in mind when trying to directly transfer techniques between the different groups of organisms. These differences include, e.g., the size of the genome and the genes present, the number of genome copies per cell, and the number of nucleoids per plastid. The morphology and intracellular location of plastids, and the occurrence of tissue specific plastid forms in higher plants are further differences. Furthermore, the fate of plastids before and after fertilization, the option to switch between photoautotrophic, mixotrophic and heterotrophic growth conditions in *Chlamydomonas,* whereas the target tissues for plastid transformation in higher plants always are heterotrophic in vitro cultures, and, finally, the shorter time to reach homoplasmy (Fig. 1) in the single-celled alga as compared to higher plants (Table 2) are also factors different in the two model systems.

Table 1. Reviews on genetic transformation of plastids.

Author(s)	Year	Title
Howe CJ	1988	Organelle transformation.
Butow RA, Fox TD	1990	Organelle transformation: shoot first, ask questions later.
Maliga P	1993	Towards plastid transformation in flowering plants.
Maliga P et al.	1993	Plastid engineering in land plants: a conservative genome is open to change.
Dix PJ, Kavanagh TA	1995	Transforming the plastome: genetic markers and DNA delivery systems.
Rochaix JD	1995	*Chlamydomonas reinhardtii* as the photosynthetic yeast
Rochaix JD	1997	Chloroplast reverse genetics: new insights into the function of plastid genes.
Bock R	1998	Analysis of RNA editing in plastids.
Kofer W et al.	1998a	PEG-mediated plastid transformation in higher plants.
Bock R	2000	Sense from nonsense: how the genetic information of chloroplasts is altered by RNA editing.
Bogorad L	2000	Engineering chloroplasts: an alternative site for foreign genes, proteins, reactions and products.
Daniell H	2000	Genetically modified food crops: current concerns and solutions for next generation crops.
Hager M, Bock R	2000	Enslaved bacteria as new hope for plant biotechnologists.
Heifetz PB	2000	Genetic engineering of the chloroplast.
Nickelsen J, Kück U	2000	The unicellular green alga *Chlamydomonas reinhardtii* as an experimental system to study chloroplast RNA metabolism.
Bock R	2001	Transgenic plastids in basic research and plant biotechnology.
Daniell H et al.	2001a	Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants.
Heifetz PB, Tuttle AM	2001	Protein expression in plastids.
van Bel AJ et al.	2001	Novel approach in plastid transformation.
Daniell H	2002	Molecular strategies for gene containment in transgenic crops.
Daniell H, Dhingra A	2002	Multigene engineering: dawn of an exciting new era in biotechnology.
Daniell H et al.	2002	Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology.
Maliga P	2002	Engineering the plastid genome of higher plants.
Staub JM	2002	Expression of recombinant proteins via the plastid genome.
Maliga P	2003	Progress towards commercialization of plastid transformation technology.

Author(s)	Year	Title
Walmsley AM, Arntzen CJ	2003	Plant cell factories and mucosal vaccines.
Bock R	2004	Studying RNA editing in transgenic chloroplasts of higher plants.
Bock R, Khan MS	2004	Taming plastids for a green future.
Franklin SE, Mayfield SP	2004	Prospects for molecular farming in the green alga *Chlamydomonas reinhardtii*.
Lorence A, Verpoorte R	2004	Gene transfer and expression in plants.
Maliga P	2004	Plastid transformation in higher plants.
Ramesh VM, Bingham SE, Webber AN	2004	A simple method for chloroplast transformation in *Chlamydomonas reinhardtii*.
Tregoning J et al.	2004	New advances in the production of edible plant vaccines: chloroplast expression of a tetanus vaccine antigen, TetC.
Xiong L, Sayre RT	2004	Engineering the chloroplast encoded proteins of *Chlamydomonas*.
Daniell H et al.	2005a	Chloroplast-derived vaccine antigens and other therapeutic proteins.
Daniell H et al.	2005b	Breakthrough in chloroplast genetic engineering of agronomically important crops.
Daniell H et al.	2005c	Chloroplast genetic engineering to improve agronomic traits.
Khan MS et al.	2005	Phage phiC31 integrase: a new tool in plastid genome engineering.
Ma JK et al.	2005	Molecular farming for new drugs and vaccines. Current perspectives on the production of pharmaceuticals in transgenic plants.
Maliga P	2005	New vectors and marker excision systems mark progress in engineering the plastid genome of higher plants.
Mayfield SP, Franklin SE	2005	Expression of human antibodies in eukaryotic micro-algae.
Nugent JM, Joyce SM	2005	Producing human therapeutic proteins in plastids.
Chase CD	2006	Genetically engineered cytoplasmic male sterility.
Daniell H	2006	Production of biopharmaceuticals and vaccines in plants via the chloroplast genome.
Dhingra A, Daniell H	2006	Chloroplast genetic engineering via organogenesis or somatic embryogenesis.
Lu XM et al.	2006	Chloroplast transformation.
Lutz KA et al.	2006a	Construction of marker-free transplastomic tobacco using the Cre-loxP site-specific recombination system.
Bock R	2006	Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering and molecular farming.

Table 2. Features of *Chlamydomonas reinhardtii* and *Nicotiana tabacum* as model species for plastid transformation[a].

Feature		*Chlamydomonas reinhardtii*	*Nicotiana tabacum*
features of the organism			
organization		single-celled	multicellular, highly differenti-ated
time to reach homoplasmy[b]		three to four weeks	three to four months
ploidy level	haploid	+	(-)
	diploid	+	+
culture conditions	autotrophic	+	-
	mixotrophic	+	-
	heterotrophic	+	+
transformable	nucleus	+	+
	mitochondrion	+	-
	plastid	+	+
genome sequenced	nucleus	+	-
	mitochondrion	+	-
	plastid	+	+
features of the organelle			
plastid morphology		cup-shaped	lentiform
plastid size (µm diameter)		eight to ten	five to ten
plastid fusion after fertilization		+	-
plastids per cell[c]		1	100
plastid type	proplastid	-	+
	etioplast	-	+
	chloroplast	+	+
	leucoplast	-	+
	chromoplast	-	+
	gerontoplast	-	+
eyespot		+	-
pyrenoid		+	-
features of the plastome			
nucleoids per plastid		10	10-50
plastome copies per cell[c]		80	500-10000
plastome size		203.395 bp	155.943 bp
size of inverted repeat		21,2 kbp	26,4 kbp
protein genes		69	101
RNA genes		40	45
GC content		37%	34%
coding sequences		38%	49%
short dispersed repeats		20%	-

[a] Compiled from GenBank entries BK000554 (*Chlamydomonas*) and Z00044 (tobacco), respectively, and from Grossman et al. (2003), Maliga (1993), Maul et al. (2002), Rochaix (1995), and Yukawa et al. (2005).

[b] See Fig. 1

[c] In the case of tobacco the term "cell" refers to fully developed mesophyll cells

2 General procedures

Differences in the structure of the organism between (single-celled) algae and multicellular and highly-differentiated higher plants primarily have an influence on the selection process in plastid transformation, while the basic processes of introduction of DNA into the organelle, of integration of sequences into the plastid DNA and of gene expression control are similar. Therefore, we will first describe procedures, which have been used irrespective of the species in question and will then address algae and higher plants separately.

2.1 Gene transfer methods

Stably transformed lines have primarily been generated by using two methods to deliver transforming DNA into plastids, the particle gun-mediated biolistic process and treatment of isolated protoplasts with polyethylene glycol (PEG) [for a detailed description of the particle bombardment process see e.g. Boynton et al. 1988; Lutz et al. 2006a; for PEG treatment, see Kofer et al. 1998a)]. The mechanism of entry of the transforming DNA is assumed to be by mechanical impact: microprojectiles supposedly, after passing the cell wall, penetrate the organelle's envelope, thus, carrying the DNA inside. It is not known whether or how a chloroplast envelope would reseal after penetration. The mechanism of DNA entry after PEG-treatment is even less clear. The assumption is that PEG produces transient 'holes', in the plasma membrane through which DNA can enter into the cell (Paszkowski et al. 1984). This would lead to deposition of plasmids into the cytosol, although it remains completely unknown how the DNA could subsequently reach the inside of the plastids. If, however, there is transfer of DNA from the cytosol into the plastids, then it is conceivable that also with particle bombardment plasmids are primarily delivered into the cytosol and enter the organelle afterwards. Particle bombardment (Boynton et al. 1988) is the method primarily used for the genetic transformation of plastids in algae as well as in higher plants. PEG-treatment of protoplasts (Golds et al. 1993) was successfully used in a number of higher plant species (see Table 9). In tobacco, plastid transformation is highly efficient irrespective of the methods used for DNA delivery. Another, but less efficient, technique is vortexing of cell-wall deficient algal cells with glass beads (Kindle et al. 1991). A femtosyringe-based microinjection procedure was used to deliver a GFP gene into plastids (Knoblauch et al. 1999; van Bel et al. 2001), and transient expression was clearly achieved, stable transformants were, however, not described. Earlier reports on *Agrobacterium*-mediated plastid transformation were never subsequently confirmed (de Block et al. 1985; Venkateswarlu and Nazar 1991).

2.2 Transformation vectors

Naturally, the vector design depends on the purpose of a specific experiment. In contrast to stable transformation, for transient expression a plasmid carrying a functional expression cassette would suffice, and no sequences necessary for stable integration are required.

2.2.1 Transient expression

Relatively few reports have been published on transient expression in plastids in vivo. Note, that gene products detected in experiments analysing transient expression might at least in part be due to transcription from sequences integrated via co-integrate formation (Klaus et al. 2004), if vectors containing extended plastome sequences were used. Daniell et al. (1990) reported expression of chloramphenicol acetyl transferase in bombarded tobacco suspension cells, but no expression was found after electroporation of suspension cell-derived protoplasts. The expression was assigned to plastids (supposedly leucoplasts and not chloroplasts). Transient expression of GUS following particle bombardment in tobacco (Ye et al. 1990; Daniell et al. 1991; Seki et al. 1995) or PEG-treatment of leaf protoplasts in *Nicotiana plumbaginifolia* (Spörlein et al. 1991) was also reported. In the protoplasts, the GUS protein, which was co-purified with plastids, was proteinase stable, in contrast to protein derived from a nucleo/cytosolic reporter construct. GFP served as a reporter for transient expression after particle bombardment (Hibberd et al. 1998) and after femtosyringe-mediated microinjection (Knoblauch et al. 1999). Expression is quite cumbersome to detect and difficult to quantify after particle bombardment or microinjection. Thus, a versatile, reliable and easy to use system for quantitative transient expression studies is still missing.

2.2.2 Stable transformation

The organelle's recombination system requires sequences on the transformation vector with sufficient homology to the target plastome to allow for homologous recombination. Such 'homologous flanks' are generally about 1 kbp in length. Shorter flanks would presumably reduce recombination efficiency, while significantly longer flanks cause technical problems with vector construction. Expression cassettes in plastid transformation vectors require regulatory elements, such as promoters, 5' UTRs, ribosome binding sites and 3' UTRs, which are compatible with the plastid gene expression machinery. A heterologous transcription system can also be used, consisting of a foreign RNA polymerase and an expression cassette, which is equipped with a suitable promoter (McBride et al. 1994). Further modifications consist of a "downstream box" for enhanced translation efficiency (Kuroda and Maliga 2001a; Herz et al. 2005), fusion and/or purification tags for enhanced protein stability and facilitation of protein extraction (Leelavathi and Reddy 2003), and protease cleavage sites, if authentic starting amino acids are required for a desired protein end product (Staub et al. 2000). Artificial operons may

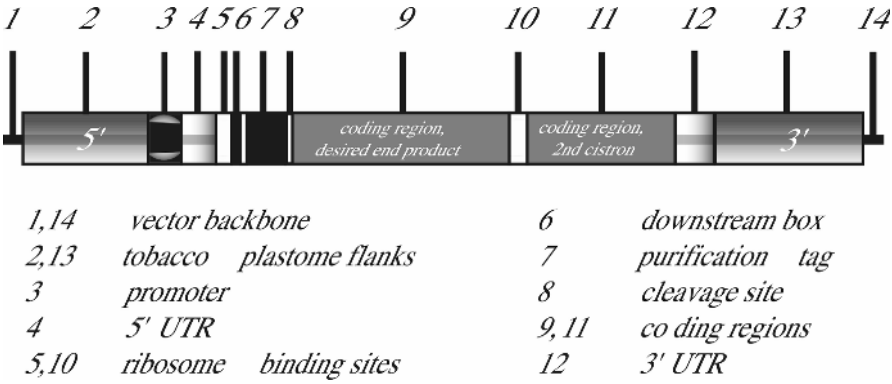

Fig. 2. Elements of a dicistronic plastid transformation vector.

contain one or more cistrons (Staub and Maliga 1995a; Lössl et al. 2003; Arai et al. 2004; Quesada-Vargas et al. 2005; Herz et al. 2005). A plastid transformation vector for the insertion of a dicistronic operon is depicted in Fig. 2. A selection marker gene is certainly also required for the transformation process. If the second cistron is used for this purpose, it is safe to assume that the protein encoded by the first cistron is also transcribed in the selected lines, due to read-through transcription. Alternatively, a selection marker cassette could be positioned elsewhere on the same transformation vector or on a different transformation vector and used in a co-transformation approach, which works efficiently in plastid transformation (Carrer and Maliga 1995; Herz et al. 2005). Up to four genes combined in a single operon were successfully introduced into the tobacco plastome (Nakashita et al. 2001; Lössl et al. 2003; Arai et al. 2004; Quesada-Vargas et al. 2005), and it might be possible to co-introduce and co-express even higher numbers of cistrons.

Not all elements given in Fig. 2 are absolutely required. Separate promoters are not necessary, if transcription is mediated by endogenous transcription start signals (Staub and Maliga 1995a). Such "operon extension vectors" are described in detail by Herz et al. (2005). Interestingly, the level of expression may even be higher if transcription is controlled by an endogenous rather than a separate promoter. It is also possible to use incomplete expression cassettes on co-transforming, separate transformation vectors, since complete and functional expression cassettes can be assembled from such "split vectors" by homologous recombination inside the plant after transformation (Herz et al. 2005).

Lutz et al. (2004) demonstrated that the integrase of phage phiC31 can be used for integrating foreign DNA into the tobacco plastome, if in a preceding transformation step suitable recognition sequences (attB) had been introduced into the target plastome. The real advantage of this approach remains to be demonstrated. Integration of attB elements relies on the endogenous recombination system – which was speculated to be rate-limiting – and the integrase has to be supplied either via another stable transformation or transiently.

2.2.3 Episomal maintenance of foreign sequences

Considerable effort was invested in studying the possibility of introducing constructs that are not integrating into the plastid chromosome and are maintained as episomes. In *Chlamydomonas*, Kindle et al. (1994) found highly amplified plasmid copies that were capable of correcting a photosynthetic growth defect. Suzuki et al. (1997) analyzed the transformed lines further and found characteristic rearrangements in both copies of the inverted repeat. Attempts to generate plastid transformants using vectors without any homology to the recipient plastome failed. Re-transformation of the lines that contained amplified plasmid copies with standard-type vectors surprisingly led to loss of the amplified plasmids. Thus, the mechanisms leading to establishment of plasmids apparently capable of autonomously replicating in plastids are presently not understood. Interestingly, plastid transformation in *Euglena gracilis* (Doetsch et al. 2001) also involved episomal elements. In tobacco, a potentially autonomously replicating element, NICE 1, was described (Staub and Maliga 1994, 1995b). However, as correctly stated by the authors, replication of this element could have also occurred while integrated into the plastid chromosome (see also: Klaus et al. 2004). Interestingly, Mühlbauer et al. (2002) did not find any influence on plastid replication activity after inactivation or deletion of the NICE 1 sequences from the tobacco plastome.

2.3 Marker gene removal

Marker removal approaches are useful, when the number of available selection markers is limited and multiple consecutive transformation steps are required for generating a desired end product. Furthermore, expression of marker genes constitutes an unnecessary metabolic burden on transplastomic plants. In addition, public concern requests removal of antibiotic resistance marker genes from transgenic plants intended for human consumption or animal feed. Three different strategies are available for transplastomic lines: direct repeat-mediated loop-out recombination, segregation of different plastomes after use of separate transformation vectors for selection marker and gene of interest, and marker excision using site-specific recombinases.

2.3.1 Direct repeat-mediated loop-out recombination

The highly active recombination system of plastids leads to loop-out recombination of introduced sequences, if transformation generates direct repeats in the plastome. This needs to be considered when designing transformation vectors, since undesired loss of sequences might follow otherwise (Maliga et al. 1993; Zou et al. 2003). On the other hand, direct repeat-mediated loop-out recombination can also be used for marker removal as initially shown for *Chlamydomonas* by Fischer et al. (1996) and later for higher plants by Iamtham and Day (2000) and Durfourmantel et al. (2006). The marker is maintained as long as a selective pressure is present. After removal of selection, marker gene sequences are excised; however,

transformants cannot be distinguished phenotypically from wild type lines. Therefore, this system benefits from the availability of a secondary selection system, e.g., herbicide resistance (Iamtham and Day 2000). Interestingly, the same approach can be applied for targeted gene inactivation in chloroplasts (Kode et al. 2005, 2006). In a different approach, Klaus et al. (2004) used transformation vectors with a different architecture. They positioned the selection marker cassette outside the homologous flanks, such that the marker can never become stably integrated. This approach was stimulated by their observation that recombination via a single flank occurs routinely, leading to the formation of vector co-integrates, which are later resolved through secondary recombination events. Secondary recombination within co-integrate structures automatically results either in plastomes identical to those of the acceptor lines or marker-free plastomes containing the gene of interest. Klaus et al. (2004) applied phenotypical selection using pigmentation. Alternatively, use of a secondary selection marker, PCR screening or other visible markers could also be conceived as a means to assist in detecting the regenerates containing the gene of interest. A difference in using vectors with the marker gene in the vector backbone lies in the fact that loop-out recombination can only occur after integration of vector sequences into the plastome, whereas in the process described by Fischer et al. (1996) and Iamtham and Day (2000) the marker gene might be lost even prior to the transformation event itself. Whether this might reduce transformation efficiency, is not known.

2.3.2 Co-transformation and segregation

Co-transformation was first shown to be possible in tobacco plastids by Carrer and Maliga (1995). In *Chlamydomonas*, Fischer et al. (1996) inserted the resistance marker into an essential gene. Thus insertion of the marker, i.e., disruption of the essential gene, could not be driven to homoplasmy and segregation allowed for the recovery of lines containing the gene of interest but not the marker gene. Ye et al. (2003) used two different vectors in tobacco and a scheme, which was initially based on spectinomycin as the selective inhibitor and subsequently on an herbicide. The rational behind this scheme is that initial selection with herbicides is not possible in plastid transformation, whereas after enrichment for transplastomes, the level of herbicide tolerance might be sufficient to, in a heteroplasmic situation, allow for segregation of lines, which carry the herbicide but not the antibiotic resistance genes. Indeed, 20% of the recovered lines fulfilled this criterion.

2.3.3 Use of site-specific recombinases

CRE recombinase-mediated marker removal from transplastomic tobacco was independently reported from two different groups (Hajdukiewicz et al. 2001; Corneille et al. 2001). CRE recombinase, derived from the P1 bacteriophage, mediates insertion or excision of sequences, provided that recognition elements, *loxP* sites, are present on the recombination substrate molecules. Marker gene removal, thus, requires directly repeated *loxP* elements flanking the marker gene in the plastome. CRE recombinase can be expressed from a nuclear expression cassette,

translated in the cytosol and then introduced into the plastid through the organelle's import machinery. When executing this approach, additional plastome rearrangements were found that were not necessarily only due to 'cryptic' *lox* sites in the plastome (Corneille et al. 2003) but were either based on short direct repeats or on recombination 'hot spots'. CRE recombinase seems to generally increase recombination activity in the plastome. Introduction of the recombinase into the transplastomic lines can either be established by *Agrobacterium*-mediated stable or transient (Lutz et al. 2006a, 2006b) nuclear transformation or by crossing a transplastomic line with a suitable nuclear transformant. Marker removal through *Agroinfiltration*-based transient expression (Lutz et al. 2006a, 2006b) is efficient and clearly preferable since removal of stably integrated expression cassettes from the nuclear genome is not necessary. In addition, plastome rearrangements no longer occur once the CRE recombinase is absent.

3 Plastid transformation in algae

3.1 Expression control elements

In *Chlamydomonas reinhardtii*, extensive efforts have been made to identify the crucial cis-acting determinants that regulate chloroplast gene expression by systematic site-directed mutagenesis of plastid 5′ and 3′ regions after co-integration or co-transformation of selectable marker genes (see 3.2). As a consequence, several elements that affect RNA stability and translational activities have been mapped especially in the 5′ UTRs of various chloroplast mRNAs (see Herrin and Nickelsen 2004; and chapters by Stern and Danon in this issue).

Expression of foreign genes in algae was performed with only a limited set of 5′ and 3′ regions as listed in Table 4. However, the analysis of reporter gene expression after systematic testing of various combinations of these 5′ and 3′ regulatory elements revealed that the *atpA* and *psbD* 5′ regions including the respective promoters and 5′ UTRs confer the highest expression rates for both the *uidA* and *gfp* reporter genes (Ishikura et al. 1999; Kasai et al. 2003; Barnes et al. 2005). In contrast, the *rbcL* and *psbA* 5′ regions produce less mRNA and protein while the nature of the 3′ UTR had only a small impact on reporter gene expression. Overall, a direct correlation of mRNA and protein levels was observed with some notable exceptions (Barnes et al. 2005; Kato et al. 2006).

To date, it remains to be clarified whether similar to the situation in vascular plants (see 4.1), viral, or artificial cis-regulatory elements work also in *C. reinhardtii*. However, it was demonstrated that neither the *psbA* 5′ region from wheat nor the spinach *psbB* 5′ region consisting of the promoter and 5′ UTR each were capable of producing stable *aadA* reporter gene mRNA in *C. reinhardtii* chloroplasts despite the fact that the genes were efficiently transcribed (Nickelsen 1999). This suggests that the post-transcriptional principles of chloroplast gene expression in algae and plants differ to some extent.

3.2 Resistance marker genes

Three principle strategies have been used for selecting chloroplast transformants of *Chlamydomonas reinhardtii*. The initial selection scheme was based on the wide range of chloroplast mutants with photosynthetic defects, which had been isolated during several decades of classical genetic work. These mutants were complemented by the respective intact wild type genes resulting in restored photoautotrophy. For instance, Boynton et al. (1988) used in their pioneer work a *C. reinhardtii atpB* deletion mutant, which they transformed with an *atpB* gene fragment. Another example is represented by the *tscA* gene that enabled the restoration of photosystem I activity in the chloroplast mutant *H13* (Goldschmidt-Clermont et al. 1991).

A second strategy involved the use of mutations within rRNA genes that confer resistances to antibiotics like spectinomycin, streptomycin, or erythromycin (for an overview see Goldschmidt-Clermont 1998). Moreover, mutations in the *psbA* gene conferring resistance to herbicides like metribuzin or DCMU were used for selection of transformants (Przibilla et al. 1991; Newman et al. 1992) and in the red alga *Porphyridium sp*., a mutant form of the chloroplast-encoded acetohydroxyacid synthase (AHAS) gene allowed the selection of chloroplast transformants using the herbicide sulfometuron methyl (Lapidot et al. 2002).

Finally, a third - and nowadays commonly applied - strategy is based on the expression of bacterial genes whose gene products inactivate antibiotics. The *aadA* gene from *Escherichia coli* conferring resistance to spectinomycin and streptomycin is widely used in *C. reinhardtii* (Goldschmidt-Clermont 1991) and, more recently, the *aphA-6* gene from *Acinetobacter baumannii* has also been shown to be suitable for selecting chloroplast transformants on kanamycin- or amikacin-containing media (Bateman and Purton 2000).

3.3 Targeted inactivation

Although the long-standing isolation of chloroplast mutants of *Chlamydomonas reinhardtii* had already enabled one to assign distinct functions to several chloroplast genes, the establishment of the chloroplast transformation system by Boynton et al. (1988) immediately opened the door for the systematic inactivation of chloroplast genes of unknown function. The first targeted gene disruption affected PsaC, a subunit of PS I, which was shown to be essential for PS I activity (Takahashi et al. 1991). At the same time and as mentioned above (3.2), the chloroplast *tscA* locus was mapped by biolistic complementation of the mutant strain *H13* and shown to encode a small RNA which is required for the trans-splicing process generating mature *psaA* mRNA and, thus, active PS I (Goldschmidt-Clermont et al. 1991). In the meantime, 36 genes of the *C. reinhardtii* genome have been inactivated, which are listed in Table 3, representing an updated version of the one published by Grossman et al. (2003). Only six genes turned out to be essential, i.e., could not be brought to homoplasmy. These include three genes for subunits of the chloroplast-encoded RNA polymerase, a ribosomal protein gene, the *clpP*

Table 3. Inactivated chloroplast genes in *Chlamydomonas reinhardtii*.

Gene	Inactivation status	Reference
RNA-polymerase		
rpoB1	heteroplasmic	Fischer et al. 1996
rpoB2	heteroplasmic	Fischer et al. 1996
rpoC2	heteroplasmic	Fischer et al. 1996
photosystems		
psaA	homoplasmic	Redding et al. 1999
psaB	homoplasmic	Redding et al. 1999
psaC	homoplasmic	Takahashi et al. 1991
psaJ	homoplasmic	Fischer et al. 1999
tscA	homoplasmic	Goldschmidt-Clermont et al. 1991
ycf3	homoplasmic	Boudreau et al. 1997a
ycf4	homoplasmic	Boudreau et al. 1997a
psbA	homoplasmic	Bennoun et al. 1986
psbC	homoplasmic	Rochaix et al. 1989
psbD	homoplasmic	Erickson et al. 1986
psbE	homoplasmic	Morais et al. 1998
psbH	homoplasmic	Summer et al. 1997; O´Connor et al. 1998
psbI	homoplasmic	Kunstner et al. 1995
psbK	homoplasmic	Takahashi et al. 1994
psbT	homoplasmic	Ohnishi and Takahashi 2001
psbZ	homoplasmic	Swiatek et al. 2001
petA	homoplasmic	Kuras and Wollman 1994
petB	homoplasmic	Kuras and Wollman 1994
petD	homoplasmic	Kuras and Wollman 1994
petG	homoplasmic	Berthold et al. 1995
petL	homoplasmic	Takahashi et al. 1996
atpA	homoplasmic	Drapier et al. 1998
atpB	homoplasmic	Shepherd et al. 1979
atpE	homoplasmic	Robertson et al. 1990
RUBISCO		
rbcL	homoplasmic	Spreitzer et al. 1985
ribosomal proteins		
rps3	heteroplasmic	Liu et al. 1993
protease		
clpP	heteroplasmic	Huang et al. 1994; Majeran et al. 2000
chlorophyll synthesis		
chlB	homoplasmic	Li et al. 1993
chlL	homoplasmic	Suzuki and Bauer 1992
chlN	homoplasmic	Choquet et al. 1992
others		
cemA	homoplasmic	Rolland et al. 1997
ccsA	homoplasmic	Xie and Merchant 1996
ORF1995	heteroplasmic	Boudreau et al. 1997b

gene and ORF1995. Recently a procedure was described which allows the analysis of the function of such essential genes by reducing the gene product levels. This strategy, named translational attenuation, is based on the finding that reduced

Table 4. Chloroplast expression of foreign genes in *Chlamydomonas reinhardtii*.

Protein	Expression	Insertion site	Expression construct	Reference
reporter proteins				
ß-glucuronidase (*Escherichia coli*)	0.08%	*rbcL-psaB*	*PatpA 5'atpA uidA 3'atpA*	Ishikura et al. 1999
ß-glucuronidase (*Escherichia coli*)	0.009%	*rbcL-psaB*	*PrbcL 5'rbcL uidA 3'rbcL*	Ishikura et al. 1999
ß-glucuronidase (*Escherichia coli*)	34.4 nmol/h mg	*atpB-IR*	*PpetD 5'petD uidA 3'rbcL*	Sakamoto et al. 1993
luciferase (*Renilla reniformis*)	n.a	*tscA-chlN*	*PatpA 5´atpA rluc 3´atpA*	Minko et al. 1999
luciferase (*Vibrio harveyi*, codon adapted)	450 U/µg	*psbA-5SrRNA*	*PpsbA 5´psbA luxCt 3´rbcL*	Mayfield and Schultz 2004
luciferase (*Photinus pyralis*, codon adapted)	variable	*psbN-psbT*	*PpsbD 5´psbD lucCP 3´atpB*	Matsuo et al. 2006
luciferase (*Photinus pyralis*, codon adapted)	variable	*ORF2971-psbD*	*PtufA 5´tufA lucCP 3´atpB*	Matsuo et al. 2006
GFP (*Aequorea aequorea*)	0.006%	*psbA-5SrRNA*	*PrbcL 5´rbcL GFPncb 3´rbcL*	Franklin et al. 2002
GFP (*Aequorea aequorea*, codon adapted)	0.5%	*psbA-5SrRNA*	*PrbcL 5´rbcL GFPct 3´rbcL*	Franklin et al. 2002
other proteins				
RecA (*Escherichia coli*)	n.a	*atpB-IR*	*PatpA 5´atpA recA 3´rbcL*	Cerrutti et al. 1995
fusion of VP1 and cholera toxin B (FMDV and *Vibrio cholerae*)	3%	*chlL*	*PatpA 5´atpA CTBVP1 3´rbcL*	Sun et al. 2003
large single-chain antibody (*Homo sapiens*)	n.a	*psbA-5SrRNA*	*PrbcL 5´rbcL HSV8-lsc 3´rbcL*	Mayfield et al. 2003
large single-chain antibody (*Homo sapiens*)	n.a	*psbA-5SrRNA*	*PatpA 5´rbcL HSV8-lsc 3´rbcL*	Mayfield et al. 2003
allophycocyanin (*Spirulina maxima*)	2%	*chlL*	*PatpA 5´rbcL apcAapcB 3´rbcL*	Su et al. 2005

protein synthesis rates which are obtained after alteration of the AUG start codon can already cause severe phenotypes (Chen et al. 1993). Correspondingly, after mutation of the *clpP* initiation codon to AUU the degradation of the cytochrome b_6f complex was affected suggesting that ClpP is involved in quality control of this photosynthetic complex (Majeran et al. 2000). Most inactivated genes encode photosynthetic functions and, thus, are not essential for cell viability on acetate-containing medium (Table 3).

Several site-directed mutants for distinct amino acids in diverse photosynthetic subunits were generated which provides a very detailed view on the structure/function relationships in photosynthesis (for a review see: Xiong and Sayre 2004; Marin-Navarro and Moreno 2006).

3.4 Introduced genes, expressed proteins

Despite the extraordinary significance of the chloroplast transformation system in *Chlamydomonas reinhardtii* for elucidating scientific aspects, biotechnological applications were considered only relatively recently. Nevertheless, as compiled in Table 4, several foreign genes have now successfully been expressed in the algal chloroplast. Besides reporter genes like β-glucuronidase, luciferase, and green fluorescent protein (GFP), high-yield expression (3% of total soluble protein) of a fusion protein consisting of VP1 protein from the foot-and-mouth disease virus and cholera toxin B subunit has been achieved. Antigenicity was demonstrated suggesting that transplastomic *C. reinhardtii* cells might be a source for mucosal vaccines (Sun et al. 2003). In addition, a fully active human antibody directed against glycoprotein D of the herpes simplex virus was expressed in the alga (Mayfield et al. 2003) verifying that pharmaceutical proteins can be synthesized in *C. reinhardtii* chloroplasts. An enhancement of gene expression was observed after adaptation of codon-usage of foreign genes to the plastid codon usage. This appears to reflect an important aspect for future algal biotechnological applications (Franklin and Mayfield 2004).

3.5 Transformed species

Although recent years have seen substantial improvements in genetic engineering of the nuclear genomes of a variety of algae including several multicellular seaweeds like *Porphyra*, *Gracilaria*, *Ulva*, and *Laminaria* (Qin et al. 2005), to date, only three chloroplast genomes from algae have successfully been transformed. Besides *C. reinhardtii*, the chloroplasts of *Euglena gracilis* were transformed with an *aadA* cassette which contained *E. gracilis* expression control elements and shown to be resistant to spectinomycin (Doetsch et al. 2001). However, despite the presence of suitably-sized homologous flanking chloroplast DNA sequences, the transforming DNA was not stably integrated into the chloroplast genome but, instead, was inherited as an episomal element during continuous selection on antibiotics (Doetsch et al. 2001). Further work is required to elucidate the potential of this transformation system, which represents the first one for an alga containing complex chloroplasts, a feature that developed during secondary endosymbiosis (Delwiche 1999). Moreover, this system might pave the way for the genetic engineering of complex plastids from other algae of higher oecological and/or economical importance like diatoms or brown algae.

In contrast to *E. gracilis*, the unicellular red alga *Porphyridium spec*. containing primary chloroplasts can be stably transformed after integration of the transform-

ing DNA into the chloroplast genome (Lapidot et al. 2002). Single crossover events have been observed after homologous recombination-mediated integration of a mutant AHAS gene conferring resistance to the herbicide SMM (see 3.2) into the chloroplast genome. However, homoplasmy was not reached under the applied experimental conditions leaving the question open whether transformants can be maintained under non-selective conditions. Interestingly, transformation rates were shown to significantly increase after synchronization of cell cultures in light/dark regimes and particle bombardment immediately after the dark phase (Lapidot et al. 2002). This procedure might be valuable also for other algal species, which have so far not been accessible to chloroplast transformation.

4 Plastid transformation in higher plants

4.1 Expression control elements

Quite a number of different regulatory elements have been tested for heterologous gene expression in plastids of higher plants (Table 5). Only very few of the elements are routinely used in plastid expression vectors (see also Table 8): the strong constitutive plastid 16S rRNA promoter in combination with the viral T7G10-5'-UTR (Staub et al. 2000; Kuroda and Maliga 2001b) or alternatively with a synthetic ribosomal binding site (rbs) consisting of the terminal 18 bp of the *rbcL*-5'-UTR (Svab and Maliga 1993). The light-regulated *psbA* control elements (promoter, 5'-UTR and 3'-UTR) are also frequently used (Staub and Maliga 1993; Fernandez-San Millan et al. 2003). These control elements have been shown to generally generate superior expression levels. Very high expression levels could also be obtained with the T7-system (promoter and 5'-UTR) relying on nuclear expressed and plastid imported T7-polymerase (McBride et al. 1994) or with operon extension vectors under the control of strong endogenous promoters (Staub and Maliga 1995a; Herz et al. 2005). Sometimes a T7-terminator was introduced in addition to a plastid 3'-UTR to ensure termination, when T7-polymerase was used to transcribe transplastomic genes (Magee et al. 2004b; Lössl et al. 2005).

As expression in plastids is predominantly controlled at the post-transcriptional level (Stern et al. 1997), the 5'-UTR is an important determinant of the expression level (Eibl et al. 1999). Another important feature is the N-terminal sequence of the gene of interest, which can be modified by fusion tags (Kuroda and Maliga 2001a; Herz et al. 2005).

A potential problem using control elements homologous to endogenous control elements is the risk of undesired recombination events (Svab and Maliga 1993). One such example was recently described for the *psbA*-3'-UTR (Rogalski et al. 2006). To avoid this potential problem some groups used plastid control elements from different species (Reddy et al. 2002; Zhou et al. 2006). However, homologous elements have frequently been used without reported recombination problems.

In some cases the 5'-UTR of the gene of interest was used as a ribosomal binding site and no extra 5'-UTR was included, especially when polycistronic operons have been introduced into the plastome (e.g. De Cosa et al. 2001; Madoka et al. 2002; Lössl et al. 2003).

Most 3'-UTRs do not terminate transcription, rather they merely act as processing and stabilising elements (Stern and Gruissem 1987). No substantial differences in the suitability of different 3'-UTRs for expression vectors have been reported (Eibl et al. 1999), so the 3'-UTR seems to be only of minor importance compared to promoter and 5'-UTR.

Table 5. Regulatory elements used in higher plant plastid transformants[a].

Regulatory element	Reference
promoters	
16S rRNA	Svab and Maliga 1993
psbA	Staub and Maliga 1993
T7G10[b,c]	McBride et al. 1994
clpP	Sriraman et al. 1998
trc[b]	Newell et al. 2003
rbcL	Herz et al. 2005
PHS[b,d]	Buhot et al. 2006
atpI	Wurbs et al. 2007
5'-untranslated regions	
rbcL (rbs)	Svab and Maliga 1993
psbA	Staub and Maliga 1993
T7G10[b]	Staub et al. 2000
atpB	Kuroda and Maliga 2002
clpP	Kuroda and Maliga 2002
rpl22	Herz et al. 2005
psbC	Herz et al. 2005
psaB	Herz et al. 2005
IREScp148[b]	Herz et al. 2005
atpI	Wurbs et al. 2007
3'-untranslated regions	
psbA	Staub and Maliga 1993
rps16	Zoubenko et al. 1994
rbcL	Eibl et al. 1999
rpl32	Eibl et al. 1999
rrnB	Newell et al. 2003
Ta[b]	Buhot et al. 2006

[a] Expression control elements were used in various combinations.
[b] Regulatory elements not of plastid origin: *trc* (*E. coli),* PHS (*E. coli groE* heat shock promoter), T7G10 (phage T7 gene 10 promoter), IREScp148 (internal ribosome entry site of the coat protein of a crucifer-infecting tobamovirus), *Ta* (*E. coli* threonine attenuator).
[c] T7-RNA polymerase needed.
[d] Chimeric transcription factor needed.

4.2 Inducible gene expression

A number of reasons make inducible gene expression in plastids highly desirable. If an economically feasible pre- or post-harvest induction were available, metabolic drain during growth and development could be avoided. Furthermore, negative effects of gene product(s) or metabolic changes caused by novel gene products might be a problem, if expression were constitutive (Lössl et al. 2003; Herz et al. 2005; Chakrabarti et al. 2006). Finally, it would be very valuable for basic research, if plastid gene expression could be switched on and off at will and at desired time-points.

Expression of plastid genes is not primarily controlled at the transcriptional level through regulated promoters that supply differential gene expression in response to physiological, developmental, or tissue specificity parameters. Therefore, inducible expression in plastids cannot be achieved using endogenous plastid control elements. External control was first described using a plastid transgene under control of the phage T7 promoter in combination with T7 polymerase encoded by a nuclear transgene and imported into the organelle (McBride et al. 1994). Controlled expression is achieved to a certain extent (Magee et al. 2004a), and negative effects observed during constitutive expression of genes of interest (Lössl et al. 2003) were avoided, when the same genes were transcribed by an ethanol induced T7 polymerase (Lössl et al. 2005). The system is, however, not optimal. The T7 promoter is recognized in in vitro experiments by the nucleus encoded plastid RNA polymerase (Lerbs-Mache 1993). This would, if true also in vivo, lead to background expression in the non-induced state. Furthermore, expression of some plastid genes is altered in the presence of T7 polymerase even if the genes do not contain a T7 promoter (Magee and Kavanagh 2002), and the low level of expression typical for most nuclear inducible promoters in the absence of an inducer may be sufficient to cause an undesirable phenotype (Magee et al. 2004a, 2007). Buhot et al. (2006) reported using the eubacterial *E. coli* groE heat shock promoter, which is not recognized by the plastid transcription machineries. Controlled expression was achieved through transient expression from a nuclear expression cassette of a chimeric sigma factor that mediates the interaction of the plastid encoded plastid RNA polymerase (PEP) and the eubacterial promoter. It remains to be seen how the system performs if combined with an inducible nuclear promoter.

Yet another approach towards inducible gene expression in plastids is based on CRE recombinase-mediated excision of the selection marker gene leaving its AUG translation start codon behind (Tungsuchat et al. 2006). Thus, a gene of interest lacking an own start codon is brought into contact with the non-excised start codon of the excised marker gene. The advantage of the system lies in the fact that it is not sensitive to read-through transcription. Control is executed by generating a translatable open reading frame and GFP was used as the reporter protein. Prior to excision there is no detectable GFP, while accumulation of GFP is found to constitute up to 0.3% of the total cellular protein after excision. Again, a transgene expressed from the nucleus is required to trigger plastid expression: primary transplastomic lines harbouring an inactive gene of interest were transformed in a

second step in their nuclear genome using *Agrobacterium*-mediated gene transfer. Once the activation has occurred it cannot be reversed, and it remains to be seen, how the approach can be adapted for practical purposes.

A direct induction system, which is independent of nuclear gene expression, is based on constitutive repression of a plastid transgene by the lac repressor and induction with isopropyl-ß-D-galactopyranoside (IPTG) (Mühlbauer and Koop 2005). Increase of the level of reporter protein (GFP) was about 20-fold. This system is also not optimal, since there is low-level expression in the non-induced state. It is, however, attractive, since post-harvest induction is possible (Mühlbauer and Koop 2005), avoiding spraying of IPTG in the open field, which might be ecologically undesirable.

All the approaches towards inducible plastid gene expression developed so far are useful for basic research and for lab-scale expression studies. Inducible expression for production-scale application remains a prominent challenge in plastid transformation technology.

4.3 Resistance marker genes and selection schemes

In comparison to nuclear transformation protocols the number of selection genes successfully used for plastid transformation is relatively small (Table 6). With one exception all the direct selection markers provide resistance to the aminoglycoside antibiotics spectinomycin, streptomycin, and kanamycin. These compounds inhibit protein synthesis by specifically binding to the organelle's prokaryotic 70S ribosomes. Pioneering work with tobacco transformation was achieved using plastid marker genes isolated from plants that were resistant to streptomycin and spectinomycin (Svab et al. 1990; Staub and Maliga 1992). Two specific point mutations in the *rrn16* gene (Spc^+ and Str^+) and one mutation in the *rps12* gene (Str^+) alter ribosome structure and prevent antibiotic binding. Similar gene sequences, cloned from the *Solanum nigrum* plastome, have been successfully used for transformation of tobacco (Kavanagh et al. 1999) and more recently tomato (Nugent et al. 2005). However, much higher efficiencies of transformation have been reported using dominant chimeric antibiotic resistance genes. The most universally used marker is the *aadA* gene, which detoxifies spectinomycin and streptomycin (Goldschmidt-Clermont 1991; Svab and Maliga 1993). Translational fusions between *aadA* and *gfp* (FLARE-S) have also been used to generate bifunctional proteins that can be used for visual tracking of the transformation process (Khan and Maliga 1999). Marker genes giving resistance to kanamycin, *nptII* (Carrer et al. 1993) and *aphA-6* (Huang et al. 2002) have also been described. A novel approach for cotton plastid transformation involved the simultaneous use of *nptII* and *aphA-6* to detoxify kanamycin. The double gene/single selection strategy was shown to be more efficient than using the *aphA-6* gene alone (Kumar et al. 2004b).

To date only one non-antibiotic resistance marker has been described for direct selection of plastid transformants, the *badh* gene from spinach (Daniell et al. 2001b). In tobacco, extraordinarily high transformation efficiencies were claimed using this gene in combination with the selection agent betaine aldehyde, which is

Table 6. Selection genes for higher plant plastid transformation.

Selection agent	Gene	Mutation, Enzyme	Reference[a]
direct selection			
spectinomycin	*rrn16*	point mutation in 16S rRNA	Svab et al. 1990
streptomycin	*rrn16*	point mutation in 16S rRNA	Svab et al. 1990
streptomycin	*rps12*	point mutation in rps12	Staub and Maliga 1992
spectinomycin and streptomycin	*aadA*	aminoglycoside 3' adenyltransferase	Svab and Maliga 1993
spectinomycin and streptomycin	*gfp* + *aadA*	green fluorescent protein fused with aminoglycoside 3' adenyltransferase (FLARE-S)	Khan and Maliga 1999
kanamycin	*nptII*	neomycin phosphotransferase II	Carrer et al. 1993
kanamycin	*aphA-6*	aminoglycoside phosphotransferase	Huang et al. 2002
betaine aldehyde	*badh*	betaine aldehyde dehydrogenase	Daniell et al. 2001b
secondary selection			
phosphinothricin (glyphosinate ammonium)	*bar*	phosphinothricin acetyltransferase	Iamtham and Day 2000
glyphosate	*epsps*	resistant form of 5-enolpyruvylshikimate-3-phosphate synthase	Ye et al. 2003
isoxaflutole	*hppd*	4-hydroxyphenylpyruvate dioxygenase	Dufourmantel et al. 2007
negative selection			
5-fluorocytosine	*codA*	cytosine deaminase	Serino and Maliga 1997

[a] Only the first publication on each marker is cited.

inactivated to non-toxic glycine betaine. It should be noted, however, that no further reports verifying the system have been published.

Secondary selection genes, while not suitable for direct selection, can be used to confer a selective advantage where a dominant population of transformed plastid chromosomes has first been established using antibiotic selection. Such markers are particularly useful for counter-selection strategies, which result in the removal of antibiotic resistance markers from transformed plants. Genes conferring resistance to the herbicides phosphinothricin/glyphosinate ammonium (Iamtham and Day 2000; Ye et al. 2003), glyphosate (Ye et al. 2003) or isoxaflutole (Dufourmantel et al. 2007) have all been used successfully in this way.

Bacterial cytosine deaminase (*codA*) has been shown to be a suitable negative selection marker for tobacco plastid transformation. Cytosine deaminase converts the selection agent 5-fluorocytosine to a toxic metabolite 5-fluorouracil and leads to cell death (Serino and Maliga 1997). Cells that do not express the enzyme grow normally when plated on 5-fluorocytosine. Corneille et al. (2001) later demonstrated the functionality of the negative selection system for monitoring the excision of *codA* using the CRE-*lox* recombination system.

Higher plant plastid transformation necessitates the development of selection systems to meet highly demanding criteria. Selective advantage must be generated on two levels, that of the plastid and that of the individual cell. A typical tobacco mesophyll cell contains as many as 100 plastids each with up to 100 plastome copies (see Table 2 for an overview of plastid biology). Although the precise mechanism of plastid transformation is unknown, it can be speculated that it is a rare event, perhaps initially only occurring as one transformed molecule within a single plastid. Appropriate selection conditions must be chosen to amplify the transformed molecules such that they become the dominant plastome type. The removal of all wild type plastomes can prove difficult and sometimes very time consuming. Conventionally this has been performed by making cycles of repeated regeneration from leaf explants on selection medium, such that cell division and organelle segregation ultimately lead to stable homoplasmic tissues (Svab and Maliga 1993). Dix and Kavanagh (1995) have described the possible benefit of using plastid genes carrying point mutations to speed up the process of selecting for homoplasmic transformants. Recessive-type markers as opposed to dominant selectable markers such as *aadA* do not cause localized detoxification of the selection agent, which could conceivably maintain heteroplastomy. However, much lower transformation frequencies are generally obtained using genes carrying point mutations compared to the dominant selection markers. A novel selection system was described by Klaus et al. (2003) to improve selection of transformants and also accelerate segregation towards homoplasmy. Firstly, homoplastomic pigment-deficient mutants were produced following site-specific deletion of photosynthesis-related genes using the *aadA* gene and spectinomycin selection (see section 4.4). These acceptor lines were propagated in vitro and used as an alternative to wild type plants for re-transformation using reconstitution vectors carrying *aphA-6* together with foreign sequences of interest. Transformants recovered after kanamycin selection had a wild type appearance due to complementation of the previously deleted plastome sequences and these regenerants could clearly be distinguished from untransfomed tissues. Surprisingly, PCR showed that the primary regenerants were already homoplasmic, suggesting that green tissues have a strong selective advantage over pigment deficient ones.

4.4 Targeted inactivation

Reverse genetic analysis is quite straightforward in tobacco due to the precise recombination system active within plastids. To date, 38 genes of the tobacco plastome have been inactivated to analyse or confirm their function (Table 7). Inactivation or deletion of plastid genes has been achieved by site-specific integration of a dominant marker (e.g. Burrows et al. 1998), replacement with a frame-shifted mutant (Horvath et al. 2000), or CRE/*lox* mediated excision (Kuroda and Maliga 2003). Recently, a deletion method based on the insertion of a direct repeat,

Table 7. Inactivated chloroplast genes in *Nicotiana tabacum*.

Gene	Inactivation status	Reference
RNA-polymerase		
rpoA	homoplasmic	Serino and Maliga 1998; De Santis-Maciossek et al. 1999; Klaus et al. 2003
rpoB	homoplasmic	Allison et al. 1996; De Santis-Maciossek et al. 1999
rpoC1	homoplasmic	Serino and Maliga 1998; De Santis-Maciossek et al. 1999
rpoC2	homoplasmic	Serino and Maliga 1998
tRNA		
$trnV_{GAC}$	homoplasmic	Corneille et al. 2001; Hajdukiewicz et al. 2001
photosystems		
psaJ	homoplasmic	Schöttler et al. 2007a
psbA	homoplasmic	Baena-Gonzales et al. 2003
psbE	homoplasmic	Swiatek et al. 2003a
psbF	homoplasmic	Swiatek et al. 2003a
psbI	homoplasmic	Schwenkert et al. 2006
psbJ	homoplasmic	Hager et al. 2002; Swiatek et al. 2003a
psbL	homoplasmic	Swiatek et al. 2003a
petA	homoplasmic	Monde et al. 2000; Klaus et al. 2003
petB	homoplasmic	Monde et al. 2000
petD	heteroplasmic	Monde et al. 2000
petL	homoplasmic	Fiebig et al. 2004; Schöttler et al. 2007b
ycf3	homoplasmic	Ruf et al. 1997; Klaus et al. 2003
ycf6 (petN)	homoplasmic	Hager et al. 1999
ycf9 (lhbA, psbZ)	heteroplasmic, homoplasmic	Mäenpää et al. 2000; Ruf et al. 2000; Baena-Gonzales et al. 2001; Swiatek et al. 2001
RUBISCO		
rbcL	homoplasmic	Kanevski and Maliga 1994; Kode et al. 2006
acetyl-CoA-carboxylase		
accD	heteroplasmic	Kode et al. 2005
NDH complex		
ndhA	heteroplasmic	Kofer et al. 1998b
ndhB	homoplasmic	Shikanai et al. 1998; Horvath et al. 2000
ndhC	homoplasmic, heteroplasmic	Burrows et al. 1998; Kofer et al. 1998b
ndhH	heteroplasmic	Kofer et al. 1998b
ndhF	homoplasmic	Martin et al. 2004
ndhI	heteroplasmic	Kofer et al. 1998b
ndhJ	homoplasmic	Burrows et al. 1998
ndhK	homoplasmic, heteroplasmic	Burrows et al. 1998; Kofer et al. 1998b
DNA replication		
oriA	homoplasmic	Mühlbauer et al. 2002
oriB	heteroplasmic	Mühlbauer et al. 2002

Gene	Inactivation status	Reference
RNA binding		
sprA	homoplasmic	Sugita et al. 1997
ribosomal proteins		
rps14	heteroplasmic	Ahlert et al. 2003
rps18	heteroplasmic	Rogalski et al. 2006
protease		
clpP1	heteroplasmic	Shikanai et al. 2001; Kuroda and Maliga 2003
hypothetical chloroplast open reading frames		
ycf1	heteroplasmic	Drescher et al. 2000
ycf2	heteroplasmic	Drescher et al. 2000
ycf10 (cemA)	homoplasmic	Swiatek et al. 2003b

Note: alternative gene names are given in brackets.

flanking the gene to be deleted and the selection marker was described (Kode et al. 2006). A subsequent loop-out recombination then eliminates the desired gene together with the selection marker.

Homoplasmic plant lines could be obtained, for most inactivated genes, allowing clear assignment of an observed phenotype. Although many of these mutants were defective or impaired in photosynthesis, the lines could be grown readily on sugar-containing media. However, in a few cases only heteroplasmic inactivation could be obtained suggesting an essential role of the gene even under heterotrophic conditions. These genes comprise *ycf1* and *ycf2* whose function is not yet clear (Drescher et al. 2000), the protease subunit gene *clpP1*, which is essential for shoot development (Kuroda and Maliga 2003) and the ß-carboxyl transferase subunit encoded by *accD*, which is required for fatty acid synthesis (Kode et al. 2005). Plastid ribosomal proteins (e.g. S14 and S18) seem to be essential for cell survival in tobacco, but not necessarily in all higher plants (Rogalski et al. 2006; Ahlert et al. 2003). The genes coding for plastidic NAD(P)H dehydrogenase seem to be dispensable under optimal growth conditions (Burrows et al. 1998; Kofer et al. 1998b; Horvath et al. 2000).

4.5 Introduced genes, expressed proteins

To date, a large number of heterologous genes have been expressed in plastids of higher plants including reporter proteins to monitor efficiency of regulatory elements, modified endogenous proteins, agronomic traits like herbicide resistance, insect resistance, pathogen resistance, output traits such as pharmaceutical proteins, vaccines or bioplastics, and a diverse group of heterologous enzymes (Table 8). The absence of a glycosylation system and the prokaryotic nature of the plastid expression system make the plastid compartment an unsuitable system for some proteins, whereas many others have been successfully expressed. The reported expression levels range from 0.001 to over 40% of the total soluble protein (TSP).

Very high expression levels (> 10% TSP) seem in some cases to delay plant development or result in a chlorotic phenotype (Tregoning et al. 2003; Chakrabarti et al. 2006). Given the differences in methods of quantification, the reported levels of expression need to be interpreted with some care. Most of the reported expression levels are maximum values, which were obtained under optimal conditions. Stable proteins such as GUS accumulate *in planta* such that the highest levels are found in mature plants (Herz et al. 2005), whereas proteins more susceptible to degradation like interferon (Leelavathi and Reddy 2003) or VP6 (Birch-Machin et al. 2004) occur at higher levels in young leaves. Depending on the regulatory elements, light conditions also influence the expression level (Fernandez-San Millan et al. 2003; Watson et al. 2004; Herz et al. 2005; Wirth et al. 2006). In general, the expression level in plastids is higher than with conventional nuclear expression in plants, but lower than the levels obtained with recent transient expression technology (Gleba et al. 2005). However, it should be clear that no expression system is universally suitable for every protein. The characteristics of the protein of interest have to fit with the chosen expression system. Unfortunately, this cannot be predicted in advance, and needs to be tested experimentally. As such there are also examples for proteins, which could not be expressed in plastids like haemoglobin (Magee et al. 2004b), ß-zein (Bellucci et al. 2005), or haemagglutinin (Lelivelt et al. 2005).

Almost all proteins were expressed in tobacco plastids except GUS (tobacco and petunia), neomycin phosphotransferase (tobacco, cotton), GFP (tobacco, potato, lettuce, poplar and rice), AAD-GFP (tobacco, rice and *Lesquerella*), HPPD (tobacco and soybean), Bt-toxin (tobacco, oilseed rape and soybean), BADH (tobacco and carrot), lycopene-ß-cyclase (tobacco and tomato), and haemagglutinin (lettuce). See Table 9 for additional information.

Whereas most expression studies in plastids rely on the endogenous PEP/NEP polymerases, there is also the possibility to use an orthologous polymerase such as the T7-polymerase to achieve transcription in plastids. Expression of a plastid-localised *uidA* gene by the aid of a nuclear expressed and plastid-targeted T7-polymerase resulted in very high transcript and protein levels (McBride et al. 1994). High transcript levels do, however, not necessarily result in high levels of translated protein (Magee et al. 2004a, 2004b). There is growing evidence that correct folding and proteolytic stability of the target protein are more important determinants of the expression level than transcription and translation efficiency (Birch-Machin et al. 2004). When GUS was fused to the N-terminus of interferon-γ the expression level increased from 0.1 to 6% and the half-life of the fusion protein increased from 6 to 48 hours compared to the unmodified interferon-γ although both versions were under the control of identical regulatory elements (Leelavathi and Reddy 2003). Similar results were obtained with recombinant epidermal growth factor (Wirth et al. 2006).

Unlike in many other expression systems, codon usage plays only a minor role in the plastid expression system of *N. tabacum*, probably because of the relatively balanced codon frequency (Maliga 2003). Nevertheless, heterologous gene expression was modestly increased (up to 2.5-fold), if the codon usage was adjusted to the relatively AT-rich plastid genome of tobacco (Ye et al. 2001; Tregoning et

al. 2003). On the other hand, at least in vitro translation efficiencies do not always correlate with codon usage (Nakamura and Sugiura 2007). Although mRNA editing occurs in resident plastome genes, no editing of heterologous genes has ever been observed.

Staub and co-workers (2000) established an elegant expression system for mature somatotropin in plastids by fusing the mature somatotropin domain to an ubiquitin domain, which is only processed to mature protein by endogenous cytosolic ubiquitin-protease during the extraction procedure but not in the intact plastid. However, one additional amino acid was removed from the N-terminus in most of the processed somatotropin. This could arise from incorrect processing by cytosolic ubiquitin-protease or from a secondary protease activity. In fact, most endogenous proteins expressed in plastids are processed post-translationally by methionine-aminopeptidase and/or peptide-deformylase (Giglione and Meinnel 2001). In the case of the RUBISCO large subunit even two N-terminal amino acids are removed post-translationally (Houtz et al. 1989). Currently, little is known about post-translational modifications of recombinant proteins in plastids. Analysis of recombinant hydroxyphenyl-pyruvate dioxygenase (HPPD) in plastids showed that the starting methionine was cleaved off, but no further modifications were detected (Dufourmantel et al. 2007). However, when tetanus toxin (TetC) was expressed in tobacco plastids the initiator methionine was not removed post-translationally, but around half of the TetC was expressed as a slightly larger, modified protein (Tregoning et al. 2003). Comparative analysis of mature amino-terminal sequences of twelve recombinant proteins expressed in chloroplasts suggests that recombinant proteins comply with the N-terminal processing rules proposed for endogenous plastid proteins (Fernandez-San Millan et al. 2007).

Recently lipidation and functional activity of a recombinant bacterial lipoprotein expressed in tobacco chloroplasts was reported (Glenz et al. 2006). The protein was only lipidated when the appropriate signal sequence was present. This is also a prerequisite for lipidation in bacteria and cyanobacteria. The main fraction of the protein was lipidated but unlipidated protein and lipoprotein variants were also present. Another important aspect is the correct formation of disulfide bonds, which can be achieved in the cytosol of prokaryotic hosts like *E. coli* only in specially modified strains (Bessette et al. 1999). It was shown that all disulfide bonds of somatotropin where formed correctly inside plastids (Staub et al. 2000), making it a suitable host for disulfide-containing proteins.

To date most recombinant proteins have been extracted from green leaves, but in some plant species other organs like seeds, fruits, or tubers present attractive sources for protein extraction, because of advantages in transportation and storage. However, expression in chloroplasts seems to be much higher compared to other plastid types, such as amyloplasts or chromoplasts. Expression of an AAD-GFP fusion protein (FLARE-S) was detected in non-green tissues including petals and roots of transplastomic tobacco (Khan and Maliga 1999). However, the expression level of GFP in potato tubers was only 0.05% TSP compared to 5% TSP in green tissues (Sidorov et al. 1999). Kumar et al. (2004a), on the other hand, report only a minor decrease of BADH-expression in carrot roots compared to carrot leaves. In transplastomic tomato fruits the expression level of the *aadA* selection marker un-

der control of the constitutive 16S-promoter was half as high as in the green leaves (Ruf et al. 2001). High expression of recombinant HPPD under control of the light-regulated *psbA* promoter and 5'-UTR was reported in transplastomic tobacco leaves, but also at a lower level in seeds and petals, whereas no expression was detectable in roots (Dufourmantel et al. 2007). In soybean expression of *Bt*-toxin was detected in leaves, stems and seeds but not in root tissue (Dufourmantel et al. 2005).

Recombinant HPPD (4-hydroxyphenylpyruvate dioxygenase) in transplastomic tobacco and soybean provided improved tolerance to the herbicide isoxaflutole compared to nuclear transgenic plants (Dufourmantel et al. 2007). But in the case of EPSPS (5-enolpyrovylshikimate-3-phosphate synthase) expression, transplastomic lines showed no higher resistance to the herbicide glyphosate than nuclear transformants, despite much lower expression levels of EPSPS in the nuclear transformants (Ye et al. 2001). The reason for the different resistance levels might be the alternative mode of action of glyphosate (inhibitor of aromatic amino acid biosynthesis) and isoxaflutole (inhibitor of tocopherol- and plastoquinone-biosynthesis). Glyphosate is toxic for all cell types whereas isoxaflutole is only toxic to photosynthetic cells. Thus, plastid expression of HPPD is particularly well suited since only expression in the chloroplast is needed whereas plastid expression of recombinant proteins in non-green tissues is generally much lower, limiting the efficiency of EPSPS in these cells (Dufourmantel et al. 2007). Plastidic expression of PAT (phosphinothricin acetyltransferase) resulted in high tolerance to the herbicide phosphinothricin, an inhibitor of glutamine biosynthesis (Lutz et al. 2001; Kang et al. 2003b).

Besides herbicide resistance, another promising area for transplastomic plants is metabolic engineering. The expression of chorismate pyruvate lyase in plastids yields p-hydroxybenzoic acid, which is a precursor for liquid crystal polymers (Viitanen et al. 2004). Recently, the ß-carotene level in transplastomic tomato fruits was shown to be increased by expression of bacterial lycopene-ß-cyclase, which converts lycopene into ß-carotene (Wurbs et al. 2007). Lycopene-ß-cyclase from the fungus *Phycomyces blakesleeanus* could not be expressed successfully due to mRNA instability (Wurbs et al. 2007).

Plastid-localised expression of the *phb*-operon from *Ralstonia eutropha* has also been described (Lössl et al. 2003; Arai et al. 2004; Lössl et al. 2005). The *phb*-operon encodes ß-ketothiolase, acetyl-CoA reductase and PHB synthase. These enzymes catalyse the synthesis of polyhydroxybutyrate, which is a biodegradable plastic, from the plastidic precursor acetyl-coenzyme A. The expression of functional polycistronic operons is a major advantage of plastid transformation over other transformation methods in plants. However, change of metabolic flux or product toxicity may enforce regulation of the genes or pathways that are introduced (Lössl et al. 2005).

The expression of the bacterial *cry*-operon comprising ORF1, ORF2, and *cry2Aa2* is another example for the expression of a large polycistronic operon in plastids (De Cosa et al. 2001). ORF2 supports crystallisation of the *Bt*-toxin leading to the formation of Bt-crystals within the plastids. However, the quoted expression level of 46% total soluble protein is somewhat misleading as extracts

Table 8. Proteins expressed in plastids of higher plants.

Protein	Expression	Insertion site	Expression construct	Reference
reporter proteins				
ß-glucuronidase	2.5%	*trnV-16S*	*PpsbA 5'psbA uidA 3'psbA*	Staub and Maliga 1993
ß-glucuronidase	0.5%	*trnN-trnR*	*Prrn 5'T7G10 uidA aadA 3' rpl32*	Herz et al. 2005
ß-glucuronidase	3,7%	*trnN-trnR*	*Prrn 5'T7G10 5AAsyn-uidA aadA 3' rpl32*	Herz et al. 2005
ß-glucuronidase	1.5%	*trnS-orf74*	*Prrn 5'T7G10 5AA-uidA aadA 3' rpl32*	Herz et al. 2005
ß-glucuronidase	3.8%	*rps12-orf131*	*Prrn 5'T7G10 5AAsyn-uidA aadA 3'rpl32*	Herz et al. 2005
ß-glucuronidase	10.8%	*psbA-trnH*	*OpsbA 5'T7G10 5AAsyn-uidA aadA 3'rpl32*	Herz et al. 2005
ß-glucuronidase	20-30%	*rps12-trnV*	*PT7G10 5'T7G10 uidA 3'psbA*	McBride et al. 1994
neomycin phos. transf.	1.0%	*rbcL-accD*	*Prrn 5'rbcL 5AArbcl-neo 3'psbA*	Carrer et al. 1993
neomycin phos. transf.	0.3%	*rps12-trnV*	*Prrn 5'clpP neo 3'rbcL*	Kuroda and Maliga 2002
neomycin phos. transf.	0.8%	*rps12-trnV*	*Prrn 5'atpB neo 3'rbcL*	Kuroda and Maliga 2002
neomycin phos. transf.	7%	*rps12-trnV*	*Prrn 5'atpB 14AAatpB-neo 3'rbcL*	Kuroda and Maliga 2001a
neomycin phos. transf.	10.8%	*rps12-trnV*	*Prrn 5'rbcL 14AArbcl-neo 3'rbcL*	Kuroda and Maliga 2001a
neomycin phos. transf.	0.16%	*rps12-trnV*	*Prrn 5'T7G10 10AApts-neo 3'rbcL*	Kuroda and Maliga 2001b
neomycin phos. transf.	16.4%	*rps12-trnV*	*Prrn 5'T7G10 10AAT7G10-neo 3'rbcL*	Kuroda and Maliga 2001b
neomycin phos. transf.	23%	*rps12-trnV*	*Prrn 5'T7G10 3AAsyn-neo 3'rbcL*	Kuroda and Maliga 2001b
GFP	5%	*rps12-trnV* potato	*Prrn 5'rbs gfp 3'rps16*	Sidorov et al. 1999
GFP	5.5%	*rbcL-accD*	*Prrn 5'rbs gfp 3'rrnB*	Newell et al. 2003
GFP	36%	*rbcL-accD* lettuce	*PpsbA 5'psbA gfp 3'rps16*	Kanamoto et al. 2006
GFP	n.a.[c]	*rbcL-accD* poplar	*PpsbA 5'psbA gfp 3'rps16*	Okumura et al. 2006

Protein	Expression	Insertion site	Expression construct	Reference
AAD-GFP fusion protein (FLARE-S)	8%	*rps12-trnV*	*Prrn 5'atpB 14AAatpB-aadA-gfp 3'psbA*	Khan and Maliga 1999
AAD-GFP fusion protein (FLARE-S)	18%	*rps12-trnV*	*Prrn 5'rbcL 14AArbcl-aadA-gfp 3'psbA*	Khan and Maliga 1999
CTB-GFP fusion protein	21%	*trnI-trnA*	*Prrn 5'rbs aadA 5'psbA ctb-gfp 3'psbA*	Limaye et al. 2006
eYFP	n.a.[c]	*rps12-trnV*	*Pphs 5'rbs eyfp 3'ta*	Buhot et al. 2006
plastid proteins				
acetyl-CoA carboxylase	17-63 pmol / min mg	*accD*	*Prrn 5'accD accD 3'accD*	Madoka et al. 2002
RUBISCO (large subunit)	wild type level	*rbcL-replacement*	*PrbcL 5'rbcL rbcL-histag 3'rbcL*	Rumeau et al. 2004
RUBISCO (small subunit)	wild type level	*trnI-trnA*	*PpsbA 5'psbA rbcS 3'psbA*	Dhingra et al. 2004
RUBISCO (bacterial[a])	1/3 wild type level	*rbcL-replacement*	*PrbcL 5'rbcL rbcM aadA 3'rps16*	Whitney and Andrews 2001
herbicide resistance				
EPSPS	n.a.[c]	*rbcL-accD*	*Prrn 5'rbs aadA epsps 3'psbA*	Daniell et al. 1998
EPSPS	0.001%	*rps12-trnV*	*Prrn 5'rbcL CP4bact 3'rps16*	Ye et al. 2001
EPSPS	0.002%	*rps12-trnV*	*Prrn 5'rbcL CP4syn 3'rps16*	Ye et al. 2001
EPSPS	0.2%	*rps12-trnV*	*Prrn 5'T7G10 CP4bact 3'rps16*	Ye et al. 2001
EPSPS	0.3%	*rps12-trnV*	*Prrn 5'T7G10 CP4syn 3'rps16*	Ye et al. 2001
EPSPS	10%	*rps12-trnV*	*Prrn 5'T7G10 14AAgfp-CP4syn 3'rps16*	Ye et al. 2001
PAT	7%	*rps12-trnV*	*Prrn 5'atpB 14AAatpB-bar 3'rbcL*	Lutz et al. 2001
PAT	n.a.[c]	*trnI-trnA*	*Prrn 5'rbs aadA bar 3'psbA*	Kang et al. 2003b
HPPD	n.a.[c]	*rps12-orf131*	*Prrn 5'rbs hpd 3'rbcL*	Falk et al. 2005
HPPD	5%	*rbcL-accD*	*PpsbA 5'psbA hppd 3'rbcL*	Dufourmantel et al. 2007
HPPD	5%	*rps12-trnV* soybean	*Prrn 5'T7G10 hppd 3'rbcL*	Dufourmantel et al. 2007

Protein	Expression	Insertion site	Expression construct	Reference
insect resistance				
Bt toxin	3%	*rbcL-accD*	*PpsbA 5'psbA(rice) cry1Ia5 3'psbA(rice)*	Reddy et al. 2002
Bt toxin	n.a.[c]	*rps12-trnV* soybean	*Prrn 5'T7G10 cry1Ab 3'rbcL*	Dufourmantel et al. 2005
Bt toxin	n.a.[c]	*rps7-ndhB* oilseed rape	*Prrn 5'rbs cry1Aa10 3'psbA(rice)*	Hou et al. 2003
Bt toxin	3-5%	*rps12-trnV*	*Prrn 5'rbcL cry1Ac 3'rps16*	McBride et al. 1995
Bt toxin	2-3%	*rbcL-accD*	*Prrn 5'rbs aadA cry2Aa2 3'psbA*	Kota et al. 1999
Bt toxin	46.1%[b]	*trnI-trnA*	*Prrn 5'rbs aadA ORF1 ORF2 cry2Aa2 3'psbA*	De Cosa et al. 2001
Bt toxin	10%	*trnI-trnA*	*Orrn 5'cry cry9Aa2 3'rbcL*	Chakrabarti et al. 2006
pathogen resistance				
MSI-99	n.a.[c]	*rps12-trnV*	*Prrn 5'rbs msi99 aadA 3'psbA*	DeGray et al. 2001
pharmaceutical proteins				
somatotropin	0.2%	*rps12-trnV*	*PpsbA 5'psbA hgh 3'rps16*	Staub et al. 2000
somatotropin	1%	*rps12-trnV*	*PpsbA 5'psbA ubq-hgh 3'rps16*	Staub et al. 2000
somatotropin	7%	*rps12-trnV*	*Prrn 5'T7G10 ubq-hgh 3'rps16*	Staub et al. 2000
HSA	0.02%[b]	*trnI-trnA*	*Prrn 5'rbs aadA hsa 3'psbA*	Fernandez-San Millan et al. 2003
HSA	11.1%[b]	*trnI-trnA*	*PpsbA 5'psbA hsa 3'psbA*	Fernandez-San Millan et al. 2003
insulin like growth factor	33%[d]	*trnI-trnA*	*PpsbA 5'psbA igf 3'psbA*	Daniell et al. 2005a
interferon α5	n.a.	*trnI-trnA*	*PpsbA 5'psbA ifnA5 3'psbA*	Daniell et al. 2005a
interferon α2b	18%[d]	*trnI-trnA*	*PpsbA 5'psbA ifnA2b 3'psbA*	Daniell et al. 2005a
interferon-γ	0.1%	*rbcL-accD*	*PpsbA 5'psbA ifnG 3'psbA*	Leelavathi and Reddy 2003
interferon-γ	6%	*rbcL-accD*	*PpsbA 5'psbA histag-uidA-ifnG 3'psbA*	Leelavathi and Reddy 2003
haemoglobin	n.d.	*rbcL-accD*	*PT7G10 5'T7G10 hba hbb 3'rps16-T7G10*	Magee et al. 2004b

Protein	Expression	Insertion site	Expression construct	Reference
Guy's 13 antibody	n.a.	*trnI-trnA*	*Prrn 5'rbs igA-G 3'psbA*	Daniell et al. 2005a
single-chain camel antibody fragment	low level	*rps12-trnV*	*PT7G10 5'T7G10 abl 3'rps16-T7G10*	Magee et al. 2004a
epidermal growth factor	n.d.	*16S-trnI*	*PpsbA 5'psbA hegf 3'rps16*	Wirth et al. 2006
epidermal growth factor	low level	*16S-trnI*	*PpsbA 5'psbA 186AAuidA-hegf 3'rps16*	Wirth et al. 2006
vaccines				
TetC (tetanus)	10%	*rps12-trnV*	*Prrn 5'atpB tetC(bact) 3'rbcL*	Tregoning et al. 2003
TetC (tetanus)	25%	*rps12-trnV*	*Prrn 5'T7G10 tetC(bact) 3'rbcL*	Tregoning et al. 2003
TetC (tetanus)	10%	*rps12-trnV*	*Prrn 5'T7G10 tetC(syn) 3'rbcL*	Tregoning et al. 2003
LT-B (enterotoxigenic *E. coli*)	2.5%	*trnI-trnA*	*Prrn 5'rbs aadA ltb 3'psbA*	Kang et al. 2003a
LTK63 (enterotoxigenic *E. coli*)	3.7%	*trnI-trnA*	*Prrn 5'rbs aadA ltk63 3'psbA*	Kang et al. 2004
CT-B (cholera)	4.1%	*trnI-trnA*	*Prrn 5'rbs aadA ctb 3'psbA*	Daniell et al. 2001c
VP6 (rotavirus)	3%	*rbcL-accD*	*Prrn 5'rbs vp6 3'rrnB*	Birch-Machin et al. 2004
VP6 (rotavirus)	0.6%	*rbcL-accD*	*PpsbA 5'psbA vp6 3'rrnB*	Birch-Machin et al. 2004
2L21 peptide (virulent canine parvovirus)	31%	*trnI-trnA*	*PpsbA 5'psbA ctb-2l21 3'psbA*	Molina et al. 2004
2L21 peptide (virulent canine parvovirus)	23%	*trnI-trnA*	*PpsbA 5'psbA gfp-2l21 3'psbA*	Molina et al. 2004
PA (anthrax)	18%	*trnI-trnA*	*PpsbA 5'psbA pag 3'psbA*	Watson et al. 2004
F1-V (plague)	14.8%	*trnI-trnA*	*PpsbA 5'psbA caF1-lcrV 3'psbA*	Daniell et al. 2005a
Haemagglutinin (influenza)	n.d.	*trnI-trnA lettuce*	*Prrn(lettuce) 5'rbs aadA ha 3'psbA(lettuce)*	Lelivelt et al. 2005
VP1 (foot and mouth disease)	2-3%	*trnK-psbA*	*Prrn 5'rbs vp1 3'psbA*	Li et al. 2006a
lipoprotein A (lyme disease)	1%	*rbcL-accD*	*PpsbA 5'psbA ospA-histag 3'psbA*	Glenz et al. 2006
lipoprotein A (lyme disease)	10%	*rbcL-accD*	*PpsbA 5'psbA ospA-histag (without signal sequence) 3'psbA*	Glenz et al. 2006

Protein	Expression	Insertion site	Expression construct	Reference
NS3 (hepatitis C)	2%[d]	*n.a.*	*PpsbA 5'psbA ns3 3'psbA*	Daniell 2006
ORF2 fragment (hepatitis E)	0.1%	*trnM-trnG*	*PpsbA(rice) 5'psbA (rice) e2 3'psbA(rice)*	Zhou et al. 2006
VCA (Epstein-Barr virus)	0.004%	*rbcL-accD*	*PpsbA(rice) 5'psbA (rice) vca 3'psbA (rice)*	Lee et al. 2006a
spike protein subunit (SARS)	0.2%	*rbcL-accD*	*PpsbA 5'psbA histag-s1 3'psbA*	Li et al. 2006b
LecA surface antigen (amebiasis)	7%[d]	*n.a.*	*PpsbA 5'psbA lecA 3'psbA*	Daniell 2006
enzymes				
mercuric ion reductase; organomercurial lyase	n.a.[c]	*trnI-trnA*	*Prrn 5'rbs aadA merA merB 3'psbA*	Ruiz et al. 2003
xylanase	6%	*rbcL-accD*	*PpsbA 5'psbA(rice) xynA 3'psbA(rice)*	Leelavathi et al. 2003
chorismate pyruvate lyase (CPL)	35%	*trnI-trnA*	*PpsbA 5'psbA ubiC 3'psbA*	Viitanen et al. 2004
betaine aldehyde dehydrogenase	9 nmol/min mg	*trnI-trnA*	*Prrn 5'rbs aadA badh 3'psbA*	Daniell et al. 2001b
betaine aldehyde dehydrogenase	10-13 nmol/min mg	*trnI-trnA carrot*	*Prrn 5'rbs aadA 5'T7G10 badh 3'rps16*	Kumar et al 2004a
ß-ketothiolase	14.7 units/mg	*trnI-trnA*	*PpsbA 5'psbA phaA 3'psbA*	Ruiz and Daniell 2005
trehalose-6-phosphate synthase	5 µmol / min mg	*trnI-trnA*	*Prrn 5'rbs aadA tps1 3'psbA*	Lee et al. 2003
anthranilate synthase (α-subunit)	n.a.[c]	*rpl32-trnL*	*Prrn 5'rbs asa2 3'rpl32*	Zhang et al. 2001a
lycopene-ß-cyclase	n.a.[c]	*trnfM-trnG* (tomato and tobacco)	*PatpI 5'atpI crtY 3'rps16*	Wurbs et al. 2007
lycopene-ß-cyclase	n.d.	*trnfM-trnG* (tomato and tobacco)	*PatpI 5'atpI carRA 3'rps16*	Wurbs et al. 2007
bio-plastics				
PBP (GVGVP)	n.a.	*trnI-trnA rbcL-accD*	*Prrn 5'rbs aadA eg121 3'psbA*	Guda et al. 2000
PHB operon	10-160 ppm PHB	*rbcL-accD*	*Prrn 5'rbs aadA phbC phbA phbB 3'psbA*	Arai et al. 2004
PHB operon	1383 ppm PHB	*trnN-trnR*	*PT7G10 5'T7G10 phbC phbA phbB 3'phbB-rbcL-T7*	Lössl et al. 2005

Protein	Expression	Insertion site	Expression construct	Reference
storage protein				
ß-zein	n.d.	*trnM-trnG*	*Prrn 5'rbs g2 3'rbcL*	Bellucci et al. 2005

Note: Not all expression construct variants could be included. The expression data refers to percentage of total soluble protein (TSP). When this data was not available, enzyme activity or amount of end product (in ppm) is shown, n.a. data not available, n.d. no expression detectable.
The insertion-site shows the endogenous genes between which the insertion of the expression-cassette takes place. If not stated otherwise, all genes were inserted into the tobacco plastome. The expression cassette designates the promoter (P) or operon extension (O); 5'-UTR (5') (rbs, synthetic ribosomal binding site derived from the *rbcL* 5'-UTR); gene(s) present in the cistron, components of fusion-proteins are connected by hyphens, if truncated versions were used the number of amino acids (AA) is indicated (syn, synthetic sequence; pts, synthetic plastid downstream sequence; or name of the ORF); and 3'-UTR (3') at the end of the cistron. If not stated otherwise, all control elements are from tobacco.
[a] Homodimeric rbcM from *R. rubrum*, for an overview of additional RUBISCO-variants expressed in tobacco plastids see Andrews and Whitney (2003); [b] Quantification is based on protein solubilised in 50 mM NaOH; [c] Only biological activity determined; [d] No details given.

solubilised in 50 mM NaOH were used for quantification. The precise recombination mechanism in plastids allows exact modification of endogenous proteins. Replacement of endogenous RUBISCO (large subunit) by a RUBISCO-protein containing a C-terminal HisTag did not alter RUBISCO expression levels, which is the most abundant plant protein with 30-65% TSP. But the transplastomic plants accumulated high amounts of zinc, due to the presence of the HisTag (Rumeau et al. 2004). RUBISCO was also the target of more intensive modifications, e.g., replacement of plant *rbcL* by bacterial *rbcM* (Whitney and Andrews 2001). Description of the various modifications would exceed the scope of this article and they are excellently reviewed in Andrews and Whitney (2003).

Many of the enzymes listed in Table 8 are associated with beneficial agronomic traits: trehalose-6-phosphate synthase conferring drought tolerance (Lee et al. 2003), ß-ketothiolase conferring male sterility (Ruiz and Daniell 2005), betaine-aldehyde dehydrogenase (BADH) conferring salt tolerance (Kumar et al. 2004a), mercuric ion reductase resp. organomercurial lyase enabling phytoremediation (Ruiz et al. 2003).

Vaccines are the most prevalent class of pharmaceutical proteins expressed in plastids of higher plants. To date 14 different vaccines have been expressed in tobacco plastids and all extracted and analysed proteins have shown immune response in animals. It has often been proposed to use plant-made vaccines directly as edible vaccines, taking advantage of cheap production cost and easy application (Tregoning et al. 2004; Daniell et al. 2005a; Daniell 2006). However, edible vaccines would have to face the high standards of pharmaceutical production and potential risk of amalgamation with food plants (Fox 2006). It is, therefore, more likely that for human vaccines, the proteins would be extracted, purified and for-

mulated as with existing production procedures. Nevertheless with an increasing world population the need for cheap vaccine production also increases, making plastid expression systems an attractive alternative.

4.6 Transformed species

Plastid transformation technology for tobacco was first described over 15 years ago (Svab et al. 1990). However, despite numerous additional publications describing improvements in the efficiency of tobacco transformation the transfer of the technology to other plants has proven relatively difficult. Table 9 summarizes the current status of higher plant plastid transformation. It should be stressed that in addition to tobacco, fertile homoplasmic plants have only been described for *N. plumbaginifolia*, tomato, soybean, *Lesquerella*, cotton, petunia, and lettuce. Furthermore, with four exceptions (potato, tomato, soybean, and lettuce) the remaining species are all documented as single publications only. As such there is no great depth of knowledge in the field regarding reproducibility and potential for improvement. The favoured method for transformation has been particle bombardment using explants as target tissue (e.g. leaves, callus, or suspension cells). Various efficiencies have been observed using this approach, as many as 40 leaf bombardments were needed to obtain a single plastid transformant from *Arabidopsis* (Sikdar et al. 1998), whereas in soybean (Dufourmantel et al. 2004) and, recently, in tomato (Wurbs et al. 2007) one transformant per shot or better have been described.

Less widely used is PEG-mediated plastid transformation of protoplasts. Some success has been reported in *N. plumbaginifolia* (O'Neill et al. 1993), tomato (Nugent et al. 2005), lettuce (Lelivelt et al. 2005), cauliflower (Nugent et al. 2006), and the moss *Physcomitrella* (Sugiura and Sugita 2004). The difficulties in isolating and culturing protoplasts and obtaining good plating efficiencies after treatment with PEG, are most probably a major restricting factor using this approach.

Direct comparison of transformation efficiencies between species is inappropriate, as vector constructs are rarely identical and different selection systems were utilized. However, it is readily apparent that even the best efficiencies reported are generally much lower than those typically obtained in tobacco, where bombardment can yield one to fourteen transformants per shot with leaves (Svab and Maliga 1993; Daniell et al. 2001c), four or more events per plate of bombarded cell suspension cells (Langbecker et al. 2004), and three to 47 transformants can be obtained for every million protoplasts treated with PEG (Koop et al. 1996). There are several reasons given in the literature for the lower transformation efficiencies observed in non-tobacco species, including, reduced activity of plastid homologous recombination (Sikdar et al. 1998), a focus on green tissues containing fully developed chloroplasts (Bogorad 2000), and use of heterologous elements for vector construction (Skarjinskaia et al. 2003). While the influence of these factors cannot be excluded there is no collective evidence that any are limiting progress in the field. The critical components for success are more likely rapid transformation protocols allowing for the efficient treatment of large numbers of cells or explants,

Table 9. Development of plastid transformation systems for higher plants.

Species[a]	System[b]	Genes[c]	Efficiency[d]	Status	Reference
Nicotiana tabacum (tobacco)	PG, leaves	*Nicotiana tabacum* rrn16 (Spc^+/Str^+)	3 lines from 148 shots	homoplasmic T_0 plants and T_1 progeny	Svab et al. 1990
	PG, leaves	aadA	84 lines from 79 shots	homoplasmic T_0 plants and T_1 progeny	Svab and Maliga 1993
	PEG, ppts	*Nicotiana tabacum* rrn16 (Spc^+/Str^+)	5 lines from 1.0 x 10^6 treated ppts	homoplasmic T_0 plants, T_1 not described	Golds et al. 1993
	PEG, ppts	aadA	118 lines from 6.0 x 10^6 treated ppts (best 47 from 1.0 x 10^6)	homoplasmic T_0 plants and T_1 progeny	Koop et al. 1996
	PG, cell suspension	aadA, gfp	best > 4 per shot[e]	homoplasmic T_0	Langbecker et al. 2004
Nicotiana plumbaginifolia (tex mex tobacco)	PEG, ppts	*Nicotiana tabacum* 16S rRNA (Spc^+/Str^+)	2 lines from 10^6 treated ppts	homoplasmic T_0 plant and T_1 progeny	O'Neill et al. 1993
Arabidopsis thaliana (mouse ear cress)	PG, leaves	aadA	2 lines from 201 shots (best 1 from 40)	homoplasmic T_0 plants but not fertile	Sikdar et al. 1998
Solanum tuberosum (potato)	PG, leaves	aadA, gfp	6 lines from 150 shots (best 2 from 12)	homoplasmic T_0 plants. no seed, tubers	Sidorov et al. 1999
	PG, leaves	aadA, gfp	14 lines from 282 shots	homoplasmic T_0 plants. no seed, tubers	Nguyen et al. 2005
Oryza sativa (rice)	PG, cell suspension	FLARE-S (aadA + gfp)	12 lines from 25 shots	heteroplastomic T_0 plants, no T_1 progeny	Khan and Maliga 1999
	PG, callus	aadA, gfp	2 lines from 120 shots	heteroplastomic T_0 and T_1 plants	Lee et al. 2006b
Glycine max (soybean)	PG, cell suspension	aadA	1 line from 984 shots	heteroplastomic callus, no plants regenerated	Zhang et al. 2001b
	PG, callus	aadA	18 lines	homoplasmic	Dufourman-

Species[a]	System[b]	Genes[c]	Efficiency[d]	Status	Reference
			from 8 shots	T_0 plants and T_1 progeny	tel et al. 2004
	PG, callus	aadA, cry1Ab	1 line from 11 shots	homoplasmic T_0 plant and T_1 progeny resistant to larval damage.	Dufourmantel et al. 2005
	PG, callus	aadA, hppd	1 line from 14 shots	homoplasmic T_0 plant and T_1 progeny, resistant to herbicide	Dufourmantel et al. 2007
Lycopersicon esculentum (tomato)	PG, leaves	aadA	6 lines from 60 shots (best 3 from 20)	homoplasmic T_0 plants and T_1 progeny	Ruf et al. 2001
	PEG, ppts	*Nicotiana tabacum* rrn16 (Spc^+,Str^+) *S. nigrum*, rrn16 (Spc^+) and rps12 (Str^+)	1 line from every 1.5 x 10^6 ppts treated	homoplasmic T_0 plants and T_1 progeny	Nugent et al. 2005
	PG, leaves	aadA, crtY, carRA	1-2 lines per shot	homoplasmic T_0 and T_1 progeny	Wurbs et al. 2007
Lesquerella fendleri (bladder pod)	PG, leaves	FLARE-S (aadA + gfp)	2 lines from 51 shots	segregating T_1 progeny from a grafted shoot	Skarjinskaia et al. 2003
Brassica napus (oilseed rape)	PG, cotyledon petioles	aadA, cry1Aa10	4 lines from 1000 explants (number of shots not described)	T_0 plants heteroplasto-mic, resistant to larval damage, T_1 progeny not described	Hou et al. 2003
Physcomitrella patens (spreading earth-moss)[f]	PEG, ppts	aadA	14 lines from 1.4 x 10^6 treated ppts	heteroplasmic and homoplasmic lines	Sugiura and Sugita 2004
Gossypium hirsutum (cotton)	PG, callus	aphA-6, nptII	30 lines from 199 shots (best 13 from 31 shots)	homoplasmic T_0 plants and T_1 progeny	Kumar et al. 2004b
Daucus carota	PG, callus	aadA, badh	9 lines	homoplasmic	Kumar et al.

Species[a]	System[b]	Genes[c]	Efficiency[d]	Status	Reference
(carrot)			from 284 shots (best 4 from 30 shots)	T_0 plants with increased salt tolerance, T_1 progeny not described	2004a
Petunia hybrida (petunia)	PG, leaves	aadA, gus	3 lines from 31 shots	homoplasmic T_0 plants and T_1 progeny	Zubko et al. 2004
Solanum rickii (wild nightshade)	PG, internode sections	aadA	2 lines from 1 shot	T_0 plants no T_1 progeny described	Matveena et al. 2005
Lactuca sativa (lettuce)	PEG, ppts	aadA, gfp, HA	9 lines from 5.6 x 10^6 treated ppts	homoplasmic T_0 plants and T_1 progeny, no expression of HA	Lelivelt et al. 2005
	PG, leaves	aadA, gfp	6 lines from 10 shots	homoplasmic T_0 plants and T_1 progeny	Kanamoto et al. 2006
Brassica oleracea (cauliflower)	PEG, ppts	aadA	1 line from 3.0 x 10^6 treated ppts	homoplasmic T_0 plant, no progeny	Nugent et al. 2006
Populus alba (poplar)	PG, leaves	aadA, gfp	10 lines from 30 shots	homoplasmic T_0 plants (5-10 years required for sexual maturity)	Okumura et al. 2006
Marchantia polymorpha (liverwort)[f]	cell suspension	aadA	30 lines from 10 shots (best 24 from 5 shots)	homoplasmic callus lines	Chiyoda et al. 2007

[a] For tobacco (*N. tabacum*) only representative papers are given. Other species are listed in the order in which they were first published together with subsequent additional reports.
[b] PG (particle gun), PEG (polyethylene glycol), ppts (protoplasts).
[c] *aadA* (aminoglycoside 3'-adenyltransferase), *nptII* (neomycin phosphotransferase), *aphA-6* (aminoglycoside phosphotransferase), *gfp* (green fluorescent protein), *gus* (ß-glucuronidase), *cry1Ab/cry1Aa10 (Bt* crystal toxin proteins), *hppd* (4-hydroxyphenylpyruvate dioxygenase), *crtY* (lycopene ß-cyclase from *Erwinia herbicola*), *carRA* (lycopene ß-cyclase from *Phycomyces blakesleeanus*), *HA* (haemagglutinin).
[d] Average efficiency for published work, direct comparison of results is difficult since different transformation and selection regimes were employed and in some cases putative transformants were not all analyzed in detail. Where appropriate optimal transformation results are given.
[e] A range of bombardment parameters tested.
[f] Moss species are listed together with higher plants.

construction of species specific transformation vectors, a suitable selection marker and use of tissues with a high regeneration capacity such that fertile plants can be recovered. Of particular merit is the recent report describing the extension of plastid transformation technology from herbaceous plants to the woody tree species poplar (Okumura et al. 2006). In contrast, limited success has been achieved with monocotyledonous plants. To date, there are only two reports on rice, both of which describe integration of foreign sequences into the plastome but no homoplasmic plants were recovered (Khan and Maliga 1999; Lee et al. 2006b).

When species, closely related to tobacco, prove difficult to transform in their plastome, an interesting approach can be used exploiting the fact that plastids in tobacco can be transformed. Kuchuk et al. (2006) transformed the plastomes of five different recalcitrant solanaceous species after transferring their plastids into tobacco; thus, generating cytoplasmic hybrids with tobacco supplying the nuclear genome and the other species donating the cytoplasmic genomes.

5 Perspectives

Plastid transformation offers a basic tool for the study of plastid gene function and regulation but has also opened up the possibility to use the technology for commercial applications. The very high expression levels observed for recombinant proteins make the system ideal for applications involving plant-made-pharmaceuticals. Tobacco has received the most attention, since it is easily transformed and is a non-food crop. To date, over 50 different recombinant proteins have been expressed in tobacco. A major class of these proteins includes vaccine-related antigens. Considerable progress has also been made in the last few years for plastid-based expression in edible crop species. While it is highly unlikely that edible vaccines will meet with regulatory approval for humans such approaches could conceivably be useful for animal vaccination or serve as an alternative to tobacco as a production platform. The ongoing challenge will be to demonstrate that a plant-based production system offers an effective alternative to conventional fermenter production.

Plastid transformants offer an additional advantage compared to nuclear transformants for genetic safety, since transgenes are maternally inherited in most crops. Improved safety coupled with high expression and the ease of selectable marker elimination may lead to a new generation of transgenic crops expressing useful agricultural traits.

The most striking limitation of plastid transformation is the lack of substantial progress with monocotyledonous species, which include the agriculturally important cereal crops. However, the outlook is encouraging; a combination of improved transformation technologies coupled with an increase in the number of groups working in the field should deliver reproducible systems for these crops in the coming years.

In algae, challenges for genetic engineering of chloroplasts include the further optimization of foreign gene expression. This will initially involve the model sys-

tem *Chlamydomonas reinhardtii*. The development of transformation protocols for complex plastids of ecologically or economically relevant groups like diatoms and brown algae will provide important tools for basic as well as applied studies.

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