5. Expression and Regulation of Transgenes for Selection of Transformants and Modification of Traits in Cereals*

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ABSTRACT. To effectively use genetic transformation strategies to improve agricultural crops, it is vital to understand the underlying factors that control transgene expression and stability. This information will be required to generate transgenic crops with a stable, predictable and consistent performance. We will in this review discuss factors, carried or encoded by the transgene expression cassette, that affect gene expression levels and patterns in transgenic cereals.

1. Introduction

The development of genetic transformation techniques for most of the important cereals has opened up new possibilities for genetic improvement of these crops. Among the various conditions that affect the outcome of a plant transformation process are factors involved in control and stability of transgenes. Efficient regulation of gene expression is important during the entire course of a transformation procedure in order to: (i) efficiently select transformed cells or tissues, (ii) regenerate healthy transgenic plants with desired phenotype, and (iii) maintain stability and expression pattern of the inserted gene. Temporal and spatial programs of transgene expression are mainly directed by interactions between host regulatory factors and different control elements present on or encoded by the transgene. However, the behavior of foreign genes when inserted into the plant genome can be very complex and unpredictable during development and in response to environmental stimuli. The future application of molecular biology for improvement of agronomic performance in crops will depend largely on revealing the molecular mechanisms involved in different aspects of gene regulation.

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We will, in the following sections, discuss factors that are currently known to be of importance for regulation of gene expression in cereals. These factors include choice of genes, promoters, regulatory sequences, leader sequences, codon usage, introns, and polyadenylation sequences. In addition, we will also consider conditions that may affect the stability and maintenance of the transgene when it is integrated into the host genome.

2. Marker Genes

The first two steps of a genetic transformation protocol involve delivery of a gene cassette into recipient cells, followed by expression of delivered genes. The result of these events can be assessed by assays of transient expression of a reporter gene introduced into plant cell cultures or intact plant tissues. These analyses do not require insertion of the transgene into the host genome, and are frequently used to test promoter and gene functions. The ideal reporter gene encodes a protein, preferably with an enzymatic activity, that is easy to quantify within a reasonable time (1–48 hours) after DNA delivery. It is also preferable that the marker protein shows low background activity in plants, does not affect plant development, is relatively resistant to proteases, and is stable over a wide range of pH values and temperatures. Analysis of gene expression in stably transformed plants requires a marker gene system with a relatively low detection limit. When markers are monitored for changes in gene expression, it is required that the marker gene transcript and encoded protein have short half-lives. Only a few of the currently available reporter genes encode proteins that meet all of the above mentioned criteria. In the following paragraphs we will describe some of the commonly used reporter gene systems for development of cereal transformation protocols, and for expression analysis of promoter function and tissue-specificity in transgenic cereals.

The chloramphenicol acetyltransferase gene

Early studies on cereal transformation used the chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) enzyme as a scorable marker to monitor promoter function, and to optimize gene delivery parameters (for examples see Bruce et al., 1989, Callis et al., 1987, Fromm et al., 1985, Hauptmann et al., 1987, Kartha et al., 1989, Lee et al., 1989). The CAT gene is isolated from the *Escherichia coli* transposon Tn*9* and encodes conversion of the antibiotic chloramphenicol into acetylated derivatives, which all lack antibiotic activity. Both enzymatic and immuno-chemical assays are available to measure CAT activity and amount of CAT protein, respectively, as an index of CAT gene expression (Chibbar et al., 1991, Seed and Sheen 1988). These assays are sensitive, but suffer from the drawbacks of being tedious and destructive. Plants contain low amounts of endogenous CAT-like

activities, or CAT-inhibiting substances, which interfere with the CAT assay (Chibbar et al., 1991).

The β-glucuronidase gene

The *Eschericia coli* gene, *uidA* (Jefferson et al., 1986), encoding the enzyme β-glucuronidase (GUS; EC 3.2.1.31), has been the most widely used reporter gene for the analysis of function and tissue-specificity of promoters in plant systems including cereals. Protein targeting studies with cereal genes have also employed the GUS marker (Chan et al., 1994). The usefulness of the GUS protein is partly due to the ability of the enzyme to tolerate extensions at both the amino- and the carboxy-termini (Datla et al., 1991, Jefferson 1987), which facilitates construction of hybrid genes. The GUS enzyme hydrolyses β-glucuronide compounds, many of which are commercially available, and gives reaction products that can be quantified by spectrophotometric or spectrofluorometric methods (Jefferson et al., 1986, 1987). Histochemical detection of *uidA* expression in plant material is performed by staining with the indigogenic substrate 5-bromo-4-chloro-3-indolyl-1-glucuronide, which is converted into a clearly visible blue precipitate as a result of GUS activity (Jefferson et al., 1986). However, GUS assays with intact tissues or organs have been reported to be leaky, because production of the blue color is not always restricted to cells where *uidA* is expressed (Bowen 1992). This limits the use of the GUS marker for cell-type expression studies. Another complication with the GUS system is related to the high stability of the enzyme, which causes a non-linear correlation between protein accumulation and rates of transcription and translation (Hensgens et al., 1992, Jefferson et al., 1987). The initial studies performed using the GUS marker gene suggested that plants contain very little or no endogenous glucuronidase activity (Jefferson et al., 1986). However, over the years, it has become evident that most plant species have intrinsic glucuronidase-like activities, especially in reproductive tissues, that obscure measurements of low GUS activity levels (Hänsch et al., 1995, Hu et al., 1990, Terada and Shimamoto, 1990). Various procedures to eliminate these interfering activities from plant extracts have been presented, and include removal of low molecular weight fluorescent compounds by gel filtration (Chibbar et al., 1991, 1993), or specific inactivation of endogenous GUS-like activities by a 55°C heat-treatment (Hänsch et al., 1995) or incubation of the enzymatic reaction in the presence of 20% methanol (Kosugi et al., 1990). Despite the above-mentioned drawbacks with the GUS system, it has been very useful for optimizing genetic transformation parameters in order to develop stable transformation protocols for most cereals.

The anthocyanin genes

A set of marker systems used in cereals include genes that stimulate the endogenous anthocyanin accumulation that results from deposition of red and purple pigments in the vacuoles of plant tissues (Ludwig and Wessler, 1990, Marrs et al., 1995). The colored phenotype can be visualized *in vivo* and followed throughout development. This marker system needs a functional anthocyanin pathway in the host plant, and cereals such as maize and barley have so far been shown to fulfill this requirement (Klein et al., 1989, Olsen et al., 1993). At least 20 loci are involved in the anthocyanin production in maize, and among these are genes encoding *trans*-acting factors of the *myc*-like and the *myb*-like classes (Coe et al., 1988, Dooner et al., 1991). The anthocyanin biosynthetic genes of transgenic plants are induced by introduction and expression of one or more members of the regulatory genes, which will confer a distinct, tissue-specific and developmentally regulated pigmentation pattern in maize (Lloyd et al., 1992, Ludwig and Wessler, 1990, Lusardi et al., 1994). For example, production of the *C1* and the *R* gene products stimulate transcription of a UDP glucose flavonol 3-*O*-glucosyl transferase gene, denoted *Bronze-1 (Bz1)* in maize, which encodes one of the last enzymes in the anthocyanin biosynthesis pathway. The *Bz1* gene product induces production of a pigment in tissues that are normally not colored in maize (Goff et al., 1990), giving a pigmentation pattern that is determined by the specific combination of expressed *trans*-acting factors. The advantage of the anthocyanin marker gene lies with its non-destructive detection system and lack of a substrate requirement, making it a suitable marker for tissue-specific and cell fate studies during development (Ludwig and Wessler, 1990, Lusardi et al., 1994). However, the detection limit for anthocyanin production is higher than for GUS activity.

The firefly luciferase gene

The gene encoding luciferase activity (LUC; EC 1.13.12.7) in the common North American firefly, *Photinus pyralis* (de Wet et al., 1987), has been the most frequently used luciferase marker for monitoring gene expression in cereals (Callis et al., 1987, Fromm et al., 1990). The firefly luciferase catalyses adenosine triphosphate (ATP)-dependent oxidation of the substrate luciferin to give a concomitant release of photons at 560 nm (McElroy and DeLuca, 1978). Light production is quantified with high sensitivity in a luminometer or a liquid scintillation counter, upon addition of Mg^{2+}-ATP and luciferin to cell extracts. Alternatively, luciferase activity can be determined *in vivo* by non-destructive methods using a photon-counting low light video camera (Millar et al., 1992). The luciferase is normally targeted to peroxisomes, but targeting can be blocked by mutations of the carboxy-terminal three amino acids without loss of enzymatic activity (Barnes

1990). The enzyme can also be directed to other sub-cellular compartments by linkage to alternative targeting signals (Schneider et al., 1990). Variability in uptake and distribution of the luciferin substrate in different plant tissues or organelles may affect the luciferase-mediated light production (Schneider et al., 1990). Other drawbacks with the luciferase marker system include the rather expensive equipment needed to quantify light production and the relatively large variability in luciferase assays caused by the short half-life of the enzyme. Maize plants transformed with the luciferase gene driven by the cauliflower mosaic virus (CaMV) 35S promoter-*Adh1* intron 1 cassette have been generated and shown to produce luciferase activity in leaves (Fromm et al., 1990). The luciferase marker could also be used to study temporal or tissue-specific gene expression patterns, as demonstrated in tobacco and *Arabidopsis* (Schneider et al., 1990, Millar et al., 1992).

The green fluorescent protein gene

A reporter gene system employing the green-fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, has recently been developed (Chalfie et al., 1994, Sheen et al., 1995) and found to function in plants (Pang et al., 1996, Sheen et al., 1995). The bioluminescent jellyfish produces green light when GFP is energized by blue light emitted by a Ca^{2+}-aequorin complex (Shimomura et al., 1962). The GFP is highly stable for 24 hours or more in maize protoplasts (Sheen et al., 1995) and shows tolerance to temperatures up to 65°C, pH values from 2 to 11, 1% sodium dodecyl sulfate, 6*M* guanidium chloride, and is relatively resistant to most proteases (Cubitt et al., 1995).

Production of the marker protein in transgenic plants can be visualized by fluorescence microscopy as bright green fluorescence after excitation of the plant tissues with near UV or blue light. GFP absorbs light maximally at 395 nm, with a minor peak at 470 nm, and emits maximum green light at 509 nm, with a shoulder peak at 540 nm. No exogenously added substrates or co-factors are needed for detection of fluorescence, which is very stable and gives virtually no photo-bleaching. Emission of fluorescence does not interfere with cell viability and the marker gene can, therefore, be used to study gene expression *in vivo*. The active site of the GFP is composed of a Ser_{65}-Tyr_{66}-Gly_{67} tripeptide, which is cyclized and oxidized, to form a *p*-hydroxybenzylidene-imidazo-lidinone chromophore. The reaction occurs in many cellular environments and appears to be independent of enzymes or co-factors (Heim et al., 1994). A gene encoding a modified GFP with excitation maximum at 382 nm and emission maximum at 448 nm has been produced by mutating the Tyr_{66} codon to a His codon (Heim et al., 1994). Thus, two forms of GFP exist giving green or blue fluorescence, respectively, which permits simultaneous use of two GFP reporter genes.

The first transgenic plants expressing the GFP gene produced a low level of fluorescence, which limited the full exploitation of the marker for plant research. Recently, modified GFP genes have been developed and reported to be highly active in plants (Haseloff et al., 1997, Pang et al., 1996, Reichel et al., 1996, Rouwendal et al., 1997). A 150-fold improvement in GFP production was achieved by replacement of the Ser_{65} codon with a threonine or cysteine codon, and incorporation of the potato ST-LS1 second intron into the *gfp* coding region in the vicinity of a 5′ cryptic splicing site (Pang et al., 1996). Fluorescence produced by expression of the modified GFP genes in transgenic wheat, maize, tobacco, and *Arabidopsis* could readily be detected using a long-wave ultraviolet lamp (Pang et al., 1996).

To summarize, GFP has great potential as a marker for gene expression or protein transport studies in plants during plant growth and development, because the emitted fluorescence can easily be monitored. Therefore, this marker could also be useful for identification and selection of transformed cells or tissues, eliminating the need for selectable marker genes. Among all the reporter genes discussed above, the modified GFP marker gene seems to be the most useful, because it encodes most of the desired features of an ideal marker.

3. Selectable Marker Genes

Identification and selection of transformed cells and tissues can be based on two different characteristics conferred by the transformant (Bowen, 1993). These are: (i) traits that confer cell viability to transformed cells in the presence of a selective agent, and (ii) traits that confer a distinguishing phenotype to transformed cells or tissues. Both strategies require a high level of selectable marker gene expression during the early stages of the regeneration program to expeditiously select transformed cells or tissues, and to minimize the time spent in tissue culture. A high level of marker gene expression is provided by the use of constitutive promoters, which have played an important role in the development of genetic transformation protocols for plants in general, and cereals in particular. A number of selectable marker genes have been used to produce transgenic plants (Bowen, 1993), and of these, only a few have been effective in selection of transformed cereals. Some of the commonly used selectable marker genes will be discussed in the following sections.

3.1 Markers that Confer Cell Viability under Selective Conditions

Selection programs based on viability of transformed cells are generally more efficient than selection based on phenotype, because the transformed cells have a growth advantage over non-transformed cells. Two different

types of selectable marker genes can be used to confer cell viability to transformed cells: (i) genes encoding antibiotic resistance, and (ii) genes encoding herbicide resistance. Antibiotics and herbicides used in regeneration schemes cause the death of untransformed cells by interfering with basic mechanisms of cell division and growth. The selectable marker gene product provides protection of transformed cells by any of three strategies: (i) detoxification of the selective agent by enzymatic modification, (ii) production of a modified target with reduced affinity for the selective agent, or (iii) over-expression of the target. In practice, selectable markers that use the first two mechanisms to alleviate the effect of the selective agent have been more effective than markers that use the third mechanism. The usefulness of each agent for selection of transformed cells or tissues varies between different cereals. Some cereals show high levels of resistance or tolerance to certain antibiotics or herbicides, rendering these agents unsuitable for a selection-regeneration protocol. Most selective agents have, to varying extents, a negative effect on the regeneration of transformed cells into plants. Therefore, the selection system most useful for a certain cereal needs to be tested empirically.

Antibiotic resistance markers

The aminoglycoside group of antibiotics that includes kanamycin, geniticin (G418), hygromycin and paromomycin has been widely used in cereal transformation. These antibiotics bind to the 30S ribosomal subunit, thus inhibiting translation in both bacteria and plastids of eukaryotic cells. Prokaryotes carrying resistance genes for these antibiotics inactivate the drugs by acetylation of amino groups or modification of hydroxyl groups by phosphorylation or adenylation (Benveniste and Davies, 1973). Only resistance genes encoding phosphorylation of aminoglycosidic antibiotics (Beck et al., 1982, Tenover et al., 1989) have been used as selectable marker genes in the production of transgenic plants.

The neomycin phosphotransferase (APHII; EC 2.7.1.95) gene (*nptII*) of the Tn*5* transposon is frequently used as a selectable marker gene in dicot plant transformation, and has also been employed to develop transgenic cereals. Kanamycin, as a selection agent has been used in the production of transformed maize plants (D'Halluin et al., 1992, Rhodes et al., 1988). Several factors have made kanamycin less useful in selection of transformed wheat, barley and rice, such as inherent high levels of natural resistance (Dekeyser et al., 1989, Hauptmann et al., 1988), negative effects on regeneration of rice plants (Battraw and Hall, 1992, Zhang et al., 1988) and high levels of albinism in barley (Ritala et al., 1994). Because of these problems, other aminoglycosidic antibiotics inactivated by NPTII have been tested as selection agents. Geneticin was found to be effective in selection of *nptII*-transformed wheat (Nehra et al., 1994), barley (Funatsuki et al., 1995, Weir

et al., 1996), rice (Chan et al., 1993) and tritordeum (Barcelo et al., 1994). Paromomycin has successfully been used to select transgenic oat (Torbert et al., 1995) and wheat (Båga & Chibbar, unpublished) plants.

Resistance to the antibiotic hygromycin B is conferred by the *Escherichia coli hph* gene product (EC 2.7.1.119), which detoxifies the drug by 4-O-phosphorylation (Rao et al., 1983). The hygromycin selection system has been very effective in rice transformation, where plants resistant to the antibiotic have been regenerated from electroporated protoplasts (Shimamoto et al., 1989), bombarded immature zygotic embryos (Christou et al., 1991) and embryogenic callus transformed by *Agrobacterium tumefaciens* (Hiei et al., 1994). Transgenic maize (Walters et al., 1992) and wheat (Ortiz et al., 1996) have also been produced through selection on media containing hygromycin.

The use of the methotrexate/dihydrofolate reductase (MTX/DHFR) selection system in plant transformation was first demonstrated in tobacco and turnip cells (Brisson et al., 1984, De Block et al., 1984), and the grass *Panicum maximum* (Hauptmann et al., 1988). The folate analogue MTX binds to the catalytic sites of DHFR (EC 1.5.1.3.), which catalyzes the NADPH-dependent reduction of folate to tetrahydrofolate, an essential co-factor in the synthesis of glycine, purines and thymidine (Blakley, 1969). Protection from MTX action in transgenic plants is provided by expression of bacterial or animal DHFR genes. Transgenic maize resistant to MTX was obtained by transformation with a modified mouse DHFR gene (Golovkin et al., 1993).

Herbicide resistance markers

Several selection strategies based on resistance to commonly used broad-spectrum herbicides have been developed for transformation of plants. The use of herbicide resistance genes for cereal transformation may be preferred, with regard to the concerns about presence of antibiotic-resistance genes in human food products. This selection strategy also provides the regenerated plant with a useful agronomic trait for the control of weeds. However, the use of herbicide resistant genes as selectable markers is not advised for oat and sorghum (Vasil, 1994), which easily cross-fertilize with their respective wild relatives, wild oat species and Johnson grass (*Sorghum halipanse*).

Bialaphos (L-phosphinothricin; Glufosinate-ammonium) is a naturally occurring antibiotic synthesized by *Streptomyces hygroscopicus* (Ogawa et al., 1973). The drug consists of a tripeptide made up from two L-alanine molecules and a L-glutamic acid analogue called phosphinothricin (PPT). The antibiotic effect is mediated by PPT, which inhibits glutamine synthetase, a key enzyme involved in conversion of L-glutamate to L-glutamine in plant cells. Prevention of L-glutamine synthesis in plants leads to a concomitant accumulation of ammonia and cell death (Tachibana et al.,

1986). Resistance to L-PPT or bialaphos is mediated by the enzyme phosphinothricin acetyl transferase (PAT), which inactivates the PPT molecule by acetylation. The *bar* gene isolated from *Streptomyces hygroscopicus* encodes PAT, and has successfully been used as a selectable marker gene for most cereals (Vasil, 1996), including wheat (Becker et al., 1994, Nehra et al., 1994, Vasil et al., 1992, Weeks et al., 1993), rice (Christou et al., 1991), maize (Fromm et al., 1990, Gordon-Kamm et al., 1990), rye (Castillo et al., 1994), barley (Wan and Lemaux, 1994), sorghum (Casas et al., 1993), oats (Somers et al., 1992) and triticale (Zimny et al., 1995). Selection of plants resistant to herbicide application is most efficient at moderate L-PPT concentrations and under conditions that lower the metabolic rate of explants (De Block et al., 1995). This selection strategy also reduces the number of escapes or non-transformed cell lines, which may be a problem with L-PPT or Bialaphos selection (Christou et al., 1991, Dennehey et al., 1994). Selection and regeneration on L-PPT media was reported to result in a higher number of transformed maize plants as compared to using kanamycin resistance as a selection strategy (Omirulleh et al., 1993, Register et al., 1994).

Glyphosate (phosphomethylglycine) inhibits the activity of the plastid-localized enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS); EC 2.5.1.19), a key enzyme in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan. Resistance to glyphosate is obtained by modification of the target site or by inactivation of the herbicide. A gene coding for a glyphosate-tolerant form of EPSPS has been isolated from the *Agrobacterium* strain CP4 (Barry et al., 1992, Padgette et al., 1991). Inactivation of the glyphosate molecule is catalyzed by glyphosate oxido-reductase (GOX) encoded by a gene from *Achromobacter* sp. strain LBAA. GOX catalyses cleavage of the C-N bond of glyphosate to produce amino-methylphosphonic acid (AMPA) and glyoxylate (Barry et al., 1992). A synthetic GOX gene with an optimized sequence for plant expression is used as a herbicide resistance gene in plant transformation. Glyphosate tolerance in plants is achieved by targeting CP4 EPSPS and GOX to chloroplasts.

Transgenic wheat callus expressing a glyphosate-resistant EPSPS was first obtained by Vasil et al., (1991). Transformation of wheat cells with the CP4 EPSPS gene and/or the GOX gene, followed by selection on glyphosate-containing media, allows for rapid production of transgenic cell lines (Qureshi, Jordan and Chibbar, personal communication). Glyphosate has also been used as a selective agent to produce glyphosate-resistant transgenic wheat plants (Zhou et al., 1995). A limitation with the use of glyphosate as both a selectable and scorable marker is that a complex procedure is required for assay of EPSPS and GOX activities.

The sulfonylureas and imidazolinones class of herbicides inhibit acetolactate synthase (ALS; EC 4.1.3.18) activity in bacteria, yeast and plants (Chaleff and Mauvais 1984, Falco and Dumas, 1985, Ray, 1984, Shaner et al., 1984). The ALS enzyme is involved in biosynthesis of the

branched chain amino acids leucine, isoleucine and valine. Resistance to sulfonylureas in plants can be induced by overexpression of the ALS gene, or a gene encoding a sulfonylurea-resistant form of ALS. Expression of sulfonylurea-resistant ALS (Haughn and Somerville, 1986) was used to select and regenerate sulfonylurea-resistant maize (Fromm et al., 1990) and rice (Li et al., 1992).

The number of genes that can be successfully introduced into cereals is currently restricted, due to the limitation in available selectable marker genes. Therefore, to allow pyramiding of multiple traits into transgenic cereals, it would be desirable to develop transformation methods that do not require selectable markers, or methods that will allow removal of the selectable marker gene from the genome of the transgenic plant. Such techniques will also satisfy the public concern about safety and the environmental impact of antibiotic- or herbicide-resistance genes in crop plants (Flavell et al., 1992, Yoder and Goldsbrough, 1994). Production of transgenic cereals without selection is possible (Ritala et al., 1994, Zhang and Wu, 1988), but requires an intensive screening of regenerated plants. This approach is, therefore, only feasible for small-scale production of transgenic cereals, or when the transgenic and the non-transgenic phenotypes can be distinguished. Removal of transgene sequences from dicot plant genomes has been demonstrated by introduction and expression of gene recombination systems, based on the action of transposable elements (Ebinuma et al., 1997; Goldsbrough et al., 1993) or site-specific recombinases (see review by Kilby et al., 1993). Recently, Lyznik et al., (1996) demonstrated removal of an inserted NPTII gene from transgenic maize cells by the use of the yeast FLP/FRT recombination system. The FLP-mediated recombination frequency was 2–3% when the recombinase was transiently expressed in protoplasts of kanamycin-resistant callus.

Another strategy to produce marker-free transgenic cereals involves placement of the marker gene and the gene of interest on two different vectors, which are then simultaneously introduced into plants by direct DNA delivery methods. Although co-transformation frequencies of 50% or higher have been reported in cereals (Barcelo et al., 1994, Vasil et al., 1991, Wan and Lemaux, 1994), it has been difficult to separate the two introduced genes in subsequent generations (Peng et al., 1995, Spencer et al., 1992). This is likely due to insertion of introduced genes at a single locus or at closely linked loci.

Recently, Komari et al., (1996) reported a very promising approach for producing marker-free rice plants. Their strategy is based on separation of the selectable and the non-selectable marker gene by placing them on two separate T-DNA cassettes. Both gene cassettes are carried by the same super binary vector, and introduced into rice by *Agrobacterium*-mediated transformation. Transgenic rice plants showed a 47% co-transformation frequency of the two T-DNA cassettes, and the selectable marker and non-

selectable marker were separated in about half of the progeny of the co-transformants. The high frequency of marker-free transgenic rice plants obtained reinforces the notion that *Agrobacterium*-mediated transformation, in contrast to direct DNA delivery methods, does not favor insertion of transgenes as linked loci. This strategy has good potential to gain wide usage as the use of *Agrobacterium*-mediated transformation method is extended to cereals other than wheat, rice, maize, and barley (see chapter by Komari and Kubo, this volume).

3.2 Markers that Confer a Distinct Phenotype to Transformed Cells

Identification of transformed cells by a non-destructive method would be advantageous, as it would not require exposure of transformed cells to a selectable agent, which may negatively affect regeneration into plants. In addition, regenerated plants are free from antibiotic- or herbicide-resistance genes. Cornejo et al., (1993) and Omirulleh et al., (1993) have successfully recovered transgenic rice and maize, respectively, through identification of transformed cells by a gentle GUS staining. Although this approach appears promising, it suffers from very low survival frequencies (1–5%) of identified GUS positive cells (Omirulleh et al., 1993). The main problem lies with accumulation of the indigogenic dye crystals, which reach lethal levels before GUS-positive cells have been identified. The use of this selection strategy also requires that the GUS marker gene is driven by a strong promoter, which may be a disadvantage in a fully regenerated plant. A promising approach would be to use the GFP marker system instead of the GUS marker for screening of putative transformants (Haseloff et al., 1997).

4. Promoters

4.1 Constitutively Active Promoters used in Development of Transgenic Cereals

Efficient selection of transgenic cells or plants during a regeneration program requires a high level production of the marker protein to protect transformed cells from the action of the selective agent. This is ensured by using a strong and constitutively active promoter to drive transcription of the selectable marker gene. Constitutive promoters are also used to produce herbicide-, insect- or pathogen-resistant crops, which require ubiquitous expression of the transgene during all stages of plant development. The highly efficient promoters employed in animal or dicot plant systems have generally been isolated from genes involved in fundamental cell processes, or from viral genomes. This is also true for the strong promoters functional in cereal systems, exemplified by the CaMV 35S promoter (Odell et al.,

1985) and the 5′ regions of the rice actin *Act1* (McElroy et al., 1991) and the maize polyubiquitin *Ubi1* (Christensen et al., 1992) genes. DNA fragments encoding these promoter functions are frequently linked to antibiotic- or herbicide-resistance genes to construct selection vectors for cereals (Christensen et al., 1992, Christensen and Quail, 1996, Fromm et al., 1986, 1990, McElroy et al., 1990, 1991). Efficient gene expression in cereals generally requires the presence of homologous or heterologous introns positioned between the promoter and the selectable marker gene. Assays of transient gene expression in cell cultures or intact plant tissues can evaluate the usefulness of an expression vector for monocot species, before the construct is used in stable transformation experiments. These assays are often of value for predicting the promoter strength when inserted into the plant genome (Caplan et al., 1992, Li et al., 1997).

The rice Act1 promoter

The rice *Act1* 5′ region commonly used to construct chimeric genes includes the 1.3-kb 5′ upstream region, the 5′ non-coding exon 1, intron 1 and the 5′ part of the first coding exon of the *Act1* gene (McElroy et al., 1990). Removal of the first intron from the *Act1* gene eliminates reporter gene expression driven by the *Act1* promoter (McElroy et al., 1990). Localization of *Act1*-GUS gene expression in transgenic rice, wheat and maize has revealed that the *Act1* 5′ region is active in both vegetative and reproductive tissues (Nehra et al., 1994, Zhang et al., 1991), thus reflecting the universal production of actin in all types of plant cells. The *Act1* 5′ region is one of the most constitutive and active promoters identified for cereal species, and so has been used to express a number of genes conferring resistance to antibiotics and herbicides. For example, transgenic wheat (Nehra et al., 1994), barley (Funatsuki et al., 1995), rice (Zhang et al., 1991) and maize (Zhong et al., 1996) have all been obtained using the *Act1* 5′ region to drive a selectable marker gene.

The maize Ubi1 promoter

The *Ubi1* and *Ubi2* promoters are part of two different maize polyubiquitin genes, that are regulated by cell cycle and induced by stress factors such as heat (Christensen et al., 1992, Cornejo et al., 1993, Takimoto et al., 1994). The *Ubi1* 5′ region has similar structural organization to that of the rice *Act1* 5′ region, where the first coding exon is preceded by a non-coding exon and an intron. Expression constructs carrying the *Ubi1* 5′ region include the first exon and intron, although no direct evidence for their role in gene expression has been provided. *Ubi* promoters have been shown to drive high levels of transient gene expression in several cereal and grass species (Taylor et al., 1993, Schledzewski and Mendel, 1994). The *Ubi1* 5′ region

is very active in transiently transformed rice protoplasts, and drives gene expression levels that are much higher than those provided by expression cassettes carrying the rice *Act1-Act1* intron 1, the CaMV 35S-*Adh* intron 1 or the maize *Adh-Adh* intron 1 (Cornejo et al., 1993). The very strong activity from the *Ubi1* 5′ region in rapidly dividing cells and regenerable tissues of rice (Cornejo et al., 1993), made selection cassettes based on the *Ubi1* 5′ region very useful for stable transformation of rice (Cornejo et al., 1993, Li et al., 1997, Toki et al., 1992). In addition to rice, transgenic wheat (Vasil et al., 1993, Weeks et al., 1993), rye (Castillo et al., 1994), and barley (Wan and Lemaux, 1994) have also been produced using the *Ubi1* 5′ region linked to selectable marker genes. Rice plants with the *Ubi1* 5′ region linked to the GUS reporter gene show very high levels of GUS activity in young roots, but only moderate levels of marker activity in pollen, leaf vascular tissues and stomata (Cornejo et al., 1993). The promoter activity decreases dramatically when the plants mature, which may be of an advantage for development of crops where the *Ubi1* 5′ region drives expression of antibiotic-resistance genes.

The CaMV 35S promoter

Some of the early experiments performed with promoters that were known to be very effective in dicot systems demonstrated very low activity in cereals (Fromm et al., 1985, Hauptmann et al., 1987, Keith and Chua, 1986). For example, the CaMV 35S promoter was 10 times less active in electroporated protoplasts of *Triticum monococcum* as compared to electroporated dicot protoplasts (Hauptmann et al., 1987). The gene expression pattern directed by the CaMV 35S promoter was also reported to differ between monocot and dicot plants (Battraw and Hall, 1990). Expression of the GUS gene under the control of the CaMV 35S promoter was on average 10 times higher in rice leaves as compared to leaves of tobacco transformed with the same construct. In contrast, there was no difference in GUS activity levels in the roots of transgenic rice and tobacco. It was also noted that the CaMV 35S promoter, which is constitutively active in tobacco (Jefferson et al., 1987, Odell et al., 1985), was nonfunctional in certain parts of rice flowers such as the palea, stigma and anthers (Terada and Shimamoto, 1990). The different behavior of the CaMV 35S promoter in dicot and monocot systems is probably due to differences in quantity and/or quality of regulatory factors.

The modular architecture of many promoters (see review by Dynan, 1989) makes it possible to adjust promoter strength and activity pattern, by altering *cis*-acting regulatory DNA elements. The addition of strong enhancer elements, leader sequences or introns to an expression cassette often leads to an increase in promoter activity. For example, the activity from the CaMV 35S promoter in maize protoplasts was strengthened three-fold by a

duplication of the 162-bp enhancer element in the 5′ upstream region of the promoter (Omirulleh et al., 1993). A six-fold improvement in CaMV 35S promoter strength in maize cells was obtained when 131 nucleotides of the 35S untranslated leader sequence was included in the expression cassette (Pierce et al., 1987). An even higher activation of the CaMV 35S promoter was observed when various introns were included in the expression cassette (Callis et al., 1987, Chibbar et al., 1993, Clancy et al., 1994, Maas et al., 1991, McElroy et al., 1991, Tanaka et al., 1990, Vasil et al., 1989). For example, positioning the *Act1* intron 1 between the CaMV 35S promoter and the reporter gene increased expression 40-fold in rice protoplasts and 65-fold in maize cells (McElroy et al., 1991). Improved versions of the CaMV 35S promoter containing a duplicated enhancer and/or an intron are often used for construction of cereal vectors (Hayakawa et al., 1992, Pang et al., 1996).

The pEmu promoter

A strong and constitutively active promoter, pEmu, was assembled by adding a set of enhancer elements to the 5′ end of a truncated *Adh1* promoter linked to its first intron (Last et al., 1991). The enhancer elements of the reconstituted promoter included six copies of the 41-bp anaerobic responsive element (ARE) from the maize *Adh1* promoter (Walker et al., 1987) and four copies of the 40-bp octopine synthase (OCS) enhancer (Ellis et al., 1987). The pEmu promoter was 10- to 50-fold stronger than the CaMV 35S promoter when tested for transient expression in protoplasts of several cereals (Last et al., 1991) and 400 times stronger than the CaMV 35S promoter in sugarcane protoplasts (Rathus et al., 1993). The pEmu promoter has been used to develop transgenic rice (Chamberlain et al., 1994, Li et al., 1997) and sugarcane (Bower and Birch, 1992).

Other constitutive promoters

Other constitutive, but less used, promoters in stable cereal transformation include the cassava vein mosaic virus (CVMV) promoter (Verdaguer et al., 1996), the rice GOS2 (Hensgens et al., 1993), the maize *ZmdJ1* (Baszczynski et al., 1997), and the mannopine synthase (MAS) and the nopaline synthase (NOS) promoters isolated from the Ti plasmid of *Agrobacterium tumefaciens* (Chan et al., 1993, Hensgens et al., 1993, Meijer et al., 1991).

Wilmink et al., (1995) have compiled information on promoter performance in two dicot and six monocot systems. As might be expected, these data suggest that monocot promoters perform better in cereals than in tobacco or carrot. However, an objective comparison of promoter strengths determined in different plant systems is difficult, due to differences in cell lines or tissues used for the analysis, differences in vector constructions, variations in transformation frequencies, etc. Schledzewski and Mendel, (1994) compared promoter strengths by normalizing transient expression

assays by including a second reporter gene as an internal standard for transformation efficiency. It was found from their study that the monocot promoters, pEmu, the rice *Act1* 5′ region and the maize *Ubi1* 5′ region, were consistently stronger (6- to 15-fold) than the CaMV 35S promoter in barley and maize cells. The best performance was obtained from pEmu and the *Ubi1* 5′ region in barley cells, whereas the promoter strengths from the *Ubi1* 5′ region and the *Act1* 5′ regions were highest in maize cells. In contrast, the CaMV 35S promoter was more than 10 times stronger than the monocot promoters in tobacco cells.

4.2 Tissue-specific Promoters for Cereal Improvement

Development of new traits in plants by genetic engineering often requires access to promoters that will direct transgene expression in a tissue-specific, developmental or inducible manner. The use of highly specific promoters, as compared to constitutive promoters, will conserve energy needed to express the transgene in tissues where the transgene product is desired. Tissue-specific gene expression patterns are usually established as a result of several interacting processes executed at various levels of gene regulation. Due to the complexity of gene regulatory mechanisms, it may be preferable to use promoters from homologous or closely related plant species for genetic engineering purposes in cereals. The late development of cereal transformation techniques has delayed analysis of cereal promoters in monocot systems. Most of the characterized cereal promoters have, therefore, been analyzed by transformation of dicot plants, or studied by transient gene expression assays of transformed cereal protoplasts or particle-bombarded cereal tissues. However, not all regulated promoters can be analyzed by transient expression (Matsuoka and Sanada, 1991, Russell and Fromm, 1997). Although the expression pattern of many monocot promoters appears to be faithfully reproduced in dicot species (see for example Colot et al., 1987, Lamppa et al., 1985, Marcotte et al., 1989), the study of promoter performance in cereals will, nevertheless, give more relevant information about regulatory sequences, *trans*-acting factors, and temporal and spatial gene expression patterns during plant development. Promoters shown to confer a tissue-specific expression pattern in transgenic cereals are listed in Table 1.

Seed-specific promoters

The obvious target for improvement of agricultural performance in cereals are various aspects of grain quality. Most of the main storage proteins and enzymes involved in production or degradation of starch are highly tissue-specific, and display a developmentally regulated expression program during grain filling or germination. The expression of promoters of the main

TABLE 1
Tissue-specific promoters functional in transgenic cereals.

Promoter	Source	Reporter gene	Transgenic plant	Expression pattern	Reference
Seed-specific:					
Wx	Rice granule-bound starch synthase gene (*Wx*)	*wx cDNA*	Rice	Endosperm, pollen	Itoh et al., 1997
ZmGBS	Maize granule-bound starch synthase gene (*Wx*)	*uidA*	Maize	Endosperm, pollen	Russell & Fromm 1997
OsAGP	Rice small subunit of ADP-glucose pyrophosphorylase gene	*uidA*	Rice	Endosperm	Russell & Fromm 1997
ZmZ27	Maize zein gene	*uidA*	Maize	Endosperm	Russell & Fromm 1997
Gt1	Rice glutelin gene	*uidA*	Rice	Endosperm	Zheng et al., 1993
1Ax1	Wheat high-molecular-weight glutenin gene	*1Ax1*	Wheat	Endosperm	Altpeter et al., 1996
Dy10	Wheat high-molecular-weight glutenin gene	*Dy10:Dx5*	Wheat	Endosperm	Blechl & Anderson 1996
Ltp2	Barley lipid transfer protein gene	*uidA*	Rice	Aleurone	Kalla et al., 1994.
Stress-induced:					
Adh1-Adh1 intron 1	Maize alcohol dehydrogenase 1 gene	*uidA*	Rice	Root caps, anthers, scutellum, endosperm, shoot and root meristems of embryo; induced by anaerobisis in roots	Kyozuka et al., 1991
RC24	Rice basic chitinase gene	*uidA*	Rice	Root and stem; wound-induced	Xu et al., 1996
Osgrp1	Rice glycine-rich cell-wall protein gene	*uidA*	Rice	Cell differentiation and elongation regions of roots, young leaves and stem; wound-induced	Xu et al., 1995

Continued on next page

TABLE 1
Continued

Promoter	Source	Reporter gene	Transgenic plant	Expression pattern	Reference
Pin2-Act1 intron 1	Potato proteinase inhibitor II gene	*uidA*	Rice	Vascular tissue; systemically induced by wounding, methyl jasmonate and abscisic acid	Xu et al., 1993
COMT	Maize caffeic acid *O*-methyltransferase gene	*uidA*	Maize	Lignin producing tissues; induced by wounding and by elicitors	Capellades et al., 1996
Vst1	Grapewine stilbene synthase gene	*vst1*	Rice	Induced by wounding, elicitor treatment and UV irradiation.	Stark-Lorenzen et al., 1997
Light-regulated:					
PhyA	Oat phytochrome A gene (*phyA*)	*phyA*	Rice	Etiolated tissues	Clough et al., 1995
LHCP	Rice light harvesting chlorophyll *a/b*-binding protein gene of photosystem II	*uidA*	Rice	Green tissues of leaves, stems and flowers; light-induced	Tada et al., 1991
PEPC	Maize phosphoenolpyruvate carboxylate gene	*uidA*	Rice	Mesophyll cells; light-induced	Matsuoka et al., 1994
PPDK	Maize pyruvate orthophosphate dikinase gene	*uidA*	Rice	Mesophyll cells; light-induced	Matsuoka et al., 1993
RbcS	Maize Rubisco small subunit gene	*uidA*	Rice	Mesophyll cells; light-induced	Matsuoka et al., 1994
RbcS	Rice Rubisco small subunit gene (*rbcS3C*)	uidA	Rice	Mesophyll cells; light-induced	Kyozuka et al., 1993
RbcS	Tomato Rubisco small subunit gene	*uidA*	Rice	Mesophyll cells; light-induced	Kyozuka et al., 1993.
Other:					
H3	Wheat histone H3 gene	*uidA*	Rice	Meristems of shoots, roots and young leaves, anther wall, pistil, coleoptile and embryo	Terada et al., 1993

Continued on next page

TABLE 1
Continued

Promoter	Source	Reporter gene	Transgenic plant	Expression pattern	Reference
RolC	Ri plasmid of *Agrobacterium rhizogenes*	*uidA*	Rice	Vascular tissues	Matsuki et al., 1989
RTBV	Rice tungro bacilliform virus	*uidA*	Rice	Phloem	Bhattacharyya-Pakrasi et al., 1993
pca55	Maize gene	*barnase*	Wheat	Tapetum	De Block et al., 1997
pE1	Rice gene	*barnase*	Wheat	Tapetum	De Block et al., 1997
pT72	Rice gene	*barnase*	Wheat	Tapetum	De Block et al., 1997
Osg6B	Rice gene (*osg6B*)	*uidA*	Rice	Tapetum	Yokoi et al., 1997
αAmy8	Rice α-amylase gene	*uidA*	Rice	Mature leaves, stems, sheaths and roots	Chan et al., 1993
Cab-6	Pine chlorophyll *a/b* (Cab) binding protein	*uidA*	Rice	Photosynthetic tissues; light-independent	Yamamoto et al., 1994

storage protein genes, such as the wheat high-molecular-weight (HMW) glutenin, the wheat low-molecular-weight (LMW) glutenin, the barley hordein B, the maize zein, and the rice glutelin gene was first studied in heterologous transgenic plants, such as tobacco and petunia (Colot et al., 1987, Marris et al., 1988, Schernthaner et al., 1988, Takaiwa et al., 1991, Thomas and Flavell, 1990, Ueng et al., 1988). Most of the promoters tested directed an endosperm-specific expression pattern that was identical to that in their natural host. However, the rice glutelin *Gt3* promoter or the maize zein gene introduced into tobacco and petunia, respectively, showed some activity in vegetative tissues in addition to seeds (Leisy et al., 1989, Ueng et al., 1988), indicating that the spatial control of these promoters was not functional in the dicot plants. The seed-specific monocot promoters also appeared to be less active in dicot plants as compared to their natural host (Colot et al., 1987, Schernthaner et al., 1988, Zheng et al., 1993). Promoter deletion analysis performed in dicot plants have revealed several DNA elements with possible role in tissue-specific expression (see review by Vellanoweth and Okita, 1993). Most of the *cis*-acting DNA elements were located within 500 bp upstream of the transcriptional start site.

A more accurate determination of regulatory sequences in the 5′ upstream region of the rice *Gt1* promoter active in transgenic rice was reported by Zheng et al., (1993). Their data showed that high level activity, temporal and spatial regulation of the *Gt1* promoter required 5.1 kb of the 5′ upstream region. The *Gt1* promoter was used to direct expression of a phytoene synthase gene from *Narcissus pseudonarcissus* to the endosperm of transgenic rice (Burkhardt et al., 1997). Phytoene synthase is involved in biosynthesis of provitamin A, and development of transgenic rice expressing this enzyme constitutes one of the steps towards improvement of the nutritional quality of milled rice. Promoters from HMW glutenin genes in wheat have been used to express a hybrid glutenin gene (Blechl and Anderson, 1996) and the HMW glutenin subunit 1Ax1 gene (Altpeter et al., 1996) in wheat.

Stress-induced promoters

Promoters induced by various environmental factors, such as temperature stress, anaerobic growth conditions or pathogen attack, all have potential for use in the development of crops resistant to these various stress conditions. Temperature-induced promoters found functional in transgenic cereals include a cold-induced promoter from barley (Molina et al., 1996) and a heat-shock-induced promoter (*Gmhsp 17.5-E*) from soybean (Lyznik et al., 1995). Promoter induction by anaerobic stress has been identified for the maize alcohol dehydrogenase-1 (*Adh1*) promoter, which has been intensively studied (Howard et al., 1987, Kyozuka et al., 1991, Zhang and Wu, 1988) and used for construction of various cereal expression vectors (Fromm

et al., 1990, Klein et al., 1988). Reporter gene expression driven by the maize *Adh1* promoter linked to its first intron in transgenic rice shows the same tissue-specificity as the endogenous maize *Adh1* gene (Zhang and Wu 1988), which reinforces the notion that promoters from one cereal species are likely to be expressed in the same way in other cereals. The *Adh1* promoter, when linked to a GUS gene, is very active in roots of transgenic rice exposed to anaerobic stress, whereas a low level of marker gene activity is present in maize pollen, root caps and seeds. Very little *Adh1* promoter activity has been observed in leaves under anoxic conditions. Induction of *Adh1* promoter activity in various plant tissues is mediated by an array of sequence elements present within the 5′-flanking region of the promoter (Kyozuka et al., 1991, 1994, Walker et al., 1987). An anaerobic responsive element (ARE) was identified in the −140 to −99 region of the *Adh1* promoter and found to confer induced activity from a truncated CaMV 35S promoter, in response to anoxia in maize protoplasts (Walker et al., 1987). The *Adh1* promoter linked to various marker genes has been introduced into different cereals (Fromm et al., 1990, Nehra et al., 1994; Vasil et al., 1992, Zhang and Wu, 1988).

Promoters induced by elicitors, wounding or pathogen infection are attractive for genetic engineering strategies to enhance plant disease resistance. Wound-induced promoters functional in transgenic cereals have been isolated from a rice basic chitinase gene (*RC24*; Xu et al., 1996), a rice glycine-rich cell-wall protein gene (*Osgrp1*; Xu et al., 1995), a lignin biosynthetic enzyme gene (*COMT*; Capellades et al., 1996) and a potato proteinase inhibitor II gene (*pin2*; Thornburg et al., 1987). The *pin2* promoter linked to the first intron of *Act1* is highly induced in transgenic rice by wounding, methyl jasmonate and abscisic acid (Xu et al., 1993), which are all signals of pathogen attack. Therefore, this promoter could be used to drive expression of insect and/or pathogen resistance genes in cereals (Xu et al., 1993). The *rolC* promoter, which is active in vascular plant tissues, could be part of a strategy to control virus propagation in plants (Matsuki et al., 1989).

Light-regulated promoters

Several light-responsive promoters of photosynthetic genes, such as those encoding the light harvesting chlorophyll *a/b* binding protein (LHCP), phosphoenolpyruvate carboxylate (PEPC), pyruvate orthophosphate dikinase (PPDK), and ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit (RbcS) have been isolated from several plants and studied in transgenic cereals (Kyozuka et al., 1993, Matsuoka et al., 1993, 1994, Tada et al., 1991). Rubisco is the most abundant protein found in plant leaves, which has attracted the use of the *rbcS* 5′ regions for high-level transgene expression in plant photosynthetic tissues, but also for targeting of proteins

to the chloroplast. Both the tomato and the rice *rbcS* promoters are able to confer the same light-induced expression pattern on a GUS reporter gene in transgenic rice plants, where the GUS activity is confined to the photosynthetic mesophyll cells (Kyozuka et al., 1993). The level of light-induction is much higher from the monocot promoter, which suggests there are differences in regulatory mechanisms controlling *rbcS* expression in monocot and dicot plants. Due to the differences in distribution of photosynthetic cells in C_3 and C_4 plants (see review by Nelson and Langdale, 1992), some promoters of photosynthetic genes in C_4 plants confer a different cell-type expression pattern or expression level on reporter genes in C_3 plants, like rice (Matsuoka et al., 1994). One promoter sensitive to light has so far been isolated and studied in transgenic cereals (Bruce et al., 1989, Clough et al., 1995).

5. Modification of Heterologous Genes for Optimization of Gene Expression in Cereals

Gene expression levels in eukaryotes are affected by several factors that act after transcription of the pre-mRNA. These factors are involved in processing and modification of the pre-mRNA, nucleocytoplasmic mRNA transport, translation, and eventually protein modification, transport and folding. The transcriptional and translational machineries in animals and plants are essentially the same when function, regulatory sequences and factors involved are compared. However, different organisms have adapted specific mechanisms for gene regulation. These distinct features may cause problems when genes are transferred from one organism to another, and/or when chimeric constructs are assembled. Genes introduced into plants do not always behave as predicted, because sequences or structures on, or encoded by the transgene are inadvertently misinterpreted by the host. In the following section, we will discuss the influence from various components of a gene expression cassette on post-transcriptional events in cereals, and how these factors should be considered for genetic engineering strategies.

5.1 Intron Influence on Gene Expression in Cereals

The majority of all protein-coding genes in higher eukaryotes contain one or more introns, which play an important role in gene regulation (Buchman and Berg 1988, Simpson et al., 1992). Several studies in cereals have shown that inclusion of an intron between the promoter and the reporter gene can have a positive effect on levels of gene expression (Callis et al., 1987, Clancy et al., 1994, Luehrsen and Walbot, 1991, Mascarenhas et al., 1990, Maas et al., 1991, McElroy et al., 1990, Tanaka et al., 1990, Vasil et al., 1989). The stimulatory effect mediated by certain introns appears to be

more prominent in monocot than in dicot plants (Tanaka et al., 1990). Therefore, most of the cereal transformation vectors contain introns, and the most frequently used introns are listed in Table 2. One of the first reports on intron-enhanced gene expression in cereals came from studies on the *Adh1* gene (Callis et al., 1987). These experiments showed that expression of a CAT reporter gene linked to the *Adh1* 3′ polyadenylation region and driven by the *Adh1* promoter, was enhanced 100-fold in stably transformed maize cells, when the *Adh1* intron 1 was included in the expression cassette. Stimulated reporter gene expression was obtained only with a subset of the *Adh1* introns, and only when the stimulatory intron was part of the transcriptional unit, and preferably placed at the 5′ end of the marker gene. The fact that orientation and position of an intron within the expression cassette is crucial for enhancement of gene expression, suggests that introns differ from transcriptional enhancer elements. This hypothesis has been supported by the finding that extensive deletions of intron sequences do not impair intron-mediated enhancement of gene expression (Clancy et al., 1994, Luehrsen and Walbot, 1991, 1994a). The stimulatory effect of the *Adh1* intron 1 on gene expression varies with promoter and reporter gene used (Callis et al., 1987), and is dependent on maize cell type (Gallie and Young, 1994). Some studies have reported negative effects from the presence of an intron on an expression cassette (Last et al., 1991, Maas et al., 1991, McElroy et al., 1991, Rathus et al., 1993, Wilmink et al., 1995). Therefore, intron function cannot always be predicted, but needs to be tested empirically for each expression cassette.

Current evidence in animal and plant systems indicates that intron function is mediated by the splicing process *per se*, or combined with an interrelated mechanism like RNA capping or polyadenylation, that cause an increase in mRNA stability and transport from the nucleus (Callis et al., 1987, Huang and Gorman, 1990, Luehrsen and Walbot, 1991, Mascarenhas et al., 1990). Thus, intron-stimulated gene expression requires that introns are accurately recognized and excised, and these processes may be regulated by various factors. For example, splicing of the stress-induced transcripts of the soybean *Gmhsp26-A* and the maize *Bronze-2 (Bz2)* genes is inhibited when plants are treated with heavy metals (Czarnecka et al., 1988, Marrs and Walbot, 1997).

A few studies in cereals have suggested that certain exon and intron sequences act together to stimulate gene expression (Clancy et al., 1994, Luehrsen and Walbot, 1991, Maas et al., 1991, Mascarenhas et al., 1990). Mascarenhas et al., (1990) demonstrated that the second intron of the maize *Adh1* gene could stimulate heterologous gene expression, only when cognate exon sequences surrounding the intron were present on the expression cassette. The combination of the first exon and intron of the maize *Shrunken-1* gene stimulated reporter gene expression 1000-fold in rice and maize protoplasts (Maas et al., 1991). Since the intron alone increased pro-

TABLE 2
Introns used in cereal expression vectors

Intron	Promoter	Reporter gene	Cereal tested in	Fold increase[1]	Reference
Maize *Adh1* intron 1	CaMV 35S	*cat*	Maize protoplasts	8- to 21-fold	Callis et al., 1987
exon 1 + intron 2	CaMV 35S	*cat*	Maize protoplasts	12-fold	Mascarenhas et al., 1990
intron 6	CaMV 35S	*cat*	Wheat protoplasts	31-fold	Oard et al., 1989
Rice *Act1* intron 1	CaMV 35S	*uidA*	Rice cells	40-fold	McElroy et al., 1991
	CaMV 35S	uidA	*Maize cells*	65-fold	McElroy et al., 1991
Shrunken-1 intron 1	CaMV 35S	*cat*	Maize protoplasts	23- to 28-fold	Vasil et al., 1989
Bronze-1 intron	CaMV 35S	*cat*	Maize protoplasts	6.2-fold	Callis et al., 1987
Maize *hsp70* intron 1	CaMV E35S	*pgfp*	Maize protoplasts	ND[2]	Pang et al., 1996
Potato ST-LS1 intron 2	CaMV E35S	*pgfp*	Maize protoplasts	1.4-fold	Pang et al., 1996
Castor bean catalase intron 1	CaMV 35S	*uidA*	Rice calli	10- to 40-fold	Tanaka et al., 1990
	CaMV 35S	*uidA*	Rice plants	80- to 90-fold	Tanaka et al., 1990
Soybean phaseolin intron 3	CaMV 35S	*nptII*	Rice plants	ND[2]	Peterhans et al., 1990.

[1]Measured relative activity from control constuct lacking intron. [2]Not determined

moter strength 100-fold, and only a 10-fold increase in promoter strength was mediated by the exon, the combined effect from the exon and the intron appeared to be multiplicative. These data support the cumulative evidence from animal systems implying an essential role for exon sequences in splicing of introns (Berget, 1995, Watakabe et al., 1993). RNA processing studies in tobacco protoplasts have suggested that a high AU content and the presence of 5′ and 3′ splice sites are both necessary and sufficient for accurate recognition by the splicing machinery (Goodall and Filipowicz, 1989, 1991). In contrast to animals and yeast, these experiments did not reveal any role for polypyrimidine or conserved branchpoint sequences in splicing of plant introns. However, this suggestion has been challenged by later studies (Liu and Filipowicz, 1996, Simpson et al., 1996). The observation that plant introns have an AU content that is usually 11–19% higher than those of surrounding exons (Goodall and Filipowicz, 1989, White et al., 1992) has led to the hypothesis that the differential AU content of exons and introns, together with the presence of splice sites, serve as recognition signals for RNA processing in plants (Goodall and Filipowicz, 1989, Lou et al., 1993, Luehrsen and Walbot, 1994b). This model has been supported by studies where splicing efficiency is improved when the AU content of the intron, or the GC content of surrounding exons, are increased (Carle-Urioste et al., 1994, 1997, Luehrsen and Walbot, 1994a). Thus, recognition of introns in plants appears to be determined by different factors, such as the AT-content of the intron, the GC-content of exons and the sequence of the splice sites, all acting in concert with each other (Carle-Urioste et al., 1994). This model may also explain how some GC-rich cereal introns (Goodall and Filipowicz, 1991), or introns with unusual splice sites (Båga et al., 1995) are accurately recognized by the splicing machinery in cereals.

Studies on pre-mRNA processing have revealed some incompatibilities between splicing in monocot and dicot species. Some monocot genes are poorly expressed in dicot systems, due to failure or slow processing of the encoded pre-mRNA (Goodall and Filipowicz, 1989, 1991, Keith and Chua, 1986, Tanaka et al., 1990). On the other hand, many dicot introns are perfectly recognized and spliced in monocot systems, exemplified by efficient splicing of the first intron of the castor bean catalase gene in rice plants (Tanaka et al., 1990), and the third intron of the soybean phaseolin gene in rice cells (Peterhans et al., 1990). The observation that the dicot splicing machinery is more discriminating appears to be associated with a higher requirement for AT-rich introns to compensate for the overall lower GC content of these genes, as compared to monocot genes (Goodall and Filipowicz, 1991).

As discussed above, the intron effect is dependent on several interacting factors, such as cell type, type of promoter, reporter gene, exon, intron and splice site sequences. The variation in intron stimulation of gene expression could be the result of the foreign and chimeric nature of the expression cassette. For example, sequences that resemble splice sites or contain a high

AU content, both of which are recognized as RNA processing signals in the plant, may be carried by the expression vector. Luehrsen and Walbot (1994b) showed that the *Bz2* transcript containing an insertion of an AU-rich sequence was improperly processed. New introns, alternative splicing, and premature polyadenylation of the mRNA were obtained as a result of insertion of AU-rich, but not GC-rich, sequences into various regions of the *Bz2* mRNA. To optimize gene expression in cereals, construction of expression cassettes could be aided by employment of computer programs designed to detect possible splice sites and introns (Kleffe et al., 1996).

5.2 Enhanced Translation by Leader and Polyadenylation Sequences

Studies in animals, yeast and plants have shown that mRNA stability, mRNA transport to cytoplasm and translation efficiency are influenced by the 5′- and 3′-untranslated regions (UTR) of the mRNA (see reviews by Abler and Green, 1996, Kozak, 1991, Rothnie, 1996, Sonenberg, 1994). Therefore, the sequences surrounding a coding region on an expression cassette can play an important role in determining the amount of transgene product produced in transgenic plants. The 5′ untranslated regions of several plant RNA viruses have been found to enhance translation of reporter RNA in both animal and plant systems, presumably by recruiting ribosomes to the mRNA and promoting initiation of translation (Gallie et al., 1987a,b). The presence of the 67-bp tobacco mosaic virus (TMV) leader sequence (Ω), at the 5′-end of the GUS mRNA, increases GUS activity three-fold to 11-fold in electroporated maize or rice protoplasts (Gallie et al., 1989). Similarly, enhanced translation is also mediated by the wheat *Em* 5′-UTR, which stimulates gene expression 10-fold in rice protoplasts, when placed between the CaMV 35S promoter and the GUS reporter gene (Marcotte et al., 1989).

Translation in eukaryotes is usually initiated from the first AUG codon on a transcript, except when the first AUG codon is in a poor sequence context (Kozak, 1991). Hensgens et al., (1992) observed that translation of a GUS coding region in tobacco could start at two different AUG codons, separated by 23 codons. The translational start at the first AUG codon was 10 times more efficient than initiation at the second start site in tobacco leaves, whereas in roots, translation was only initiated from the second AUG codon. When the second AUG codon was preceded by an out-of-frame AUG codon, a six times lower level of GUS activity was produced in both roots and leaves of the transgenic tobacco. The presence of short open reading frames in front of the coding region of a reporter gene has also caused negative affects on gene expression levels in other studies (Damiani and Wessler, 1993, Putterill & Gardner, 1989).

Recognition of initiation codon on the leader sequence is affected by sequence context of the AUG codon in animal systems (Kozak, 1989), whereas sequences surrounding the AUG codon in plants have been

suggested to have a less important role (Luehrsen and Walbot, 1994c). Compilation of sequences around the start codons of 967 maize, rice, wheat and barley genes currently in the TransTerm database (Dalphin et al., 1997) produces a $^{G}/_{A}C^{G}/_{C}$AUGGCG consensus sequence (Table 3), which corresponds closely to the $^{G}/_{A}{}^{C}/_{A}{}^{G}/_{C}$AUGG$^{C}/_{A}$G consensus sequence derived from analysis of 85 maize genes (Luehrsen and Walbot, 1994c). As previously noted, U residues around the AUG start codon of cereal genes are avoided and are very rare at the −1 position (Table 3; Luehrsen and Walbot, 1994c). The consensus sequence for 1837 dicot genes present in the TransTerm database yields the $A^{A}/_{C}$AAUGGC$^{G}/_{U}$ consensus sequence (Table 3), which resembles the consensus AACAAUGGC proposed for plant genes by Lütcke et al., (1987). The consensus sequences of both dicot and monocot genes show that the AUG codon is likely to be followed by an alanine codon (GCN). This was also confirmed by inspection of peptides deduced from the 967 cereal sequences in the TransTerm database, where 52% of the barley and 46% of the rice, maize and wheat encoded protein sequences were initiated by a Met-Ala dipeptide. Inspection of stop codons used in cereal and dicot genes reveals a difference in the preferred termination codon (Table 4). The UGA translational stop codon is most frequent for cereal genes (45.5%), whereas dicot genes prefer the UAA stop codon (45.5%). A cytosine residue following the stop codon is avoided in both monocot and dicot genes.

The signals for mRNA processing and polyadenylation differ significantly between yeast, animals and plants, suggesting that 3′ end formation in these systems occurs by different mechanisms (see review by Rothnie, 1996). This is supported by studies that show that animal poly(A) sequences are non-functional in plants (Hunt et al., 1987). Wu et al., (1994) compared the polyadenylation profile of a CAT mRNA expressed from the CaMV 35S promoter in protoplasts of maize endosperm and tobacco leaves. No difference in polyadenylation in either system was observed when the reporter gene was flanked by the 3′ end of the endosperm-specific zein gene or the 3′ end from the CaMV transcription unit. The authors concluded that the 3′ processing mechanism is the same in different tissues of both monocot and dicot species. So far, there has been no data to suggest any difference between monocot and dicot poly(A) signals in cereals. In fact, most cereal expression vectors carry functional poly(A) sequences, that are derived from genes from the *Agrobacterium* Ti plasmid or dicot genes.

The effect of different polyadenylation sequences on expression levels of a NPTII gene driven by the CaMV 35S promoter in transgenic tobacco was compared by Ingelbrecht et al., (1989). Depending on which polyadenylation sequence was used, a 60-fold difference in NPTII activity was observed. It is interesting to note that different levels of gene stimulation by the polyadenylation sequences were found when the constructs were tested by transient versus stable expression. Very little is known about the effect

TABLE 3

DNA sequence context of dicot and cereal start codons

	Dicot start codon context									Cereal start codon context								
	-3	**-2**	**-1**	**+1**	**+2**	**+3**	**+4**	**+5**	**+6**	**-3**	**-2**	**-1**	**+1**	**+2**	**+3**	**+4**	**+5**	**+6**
G	20	7	16	0	0	100	69	15	27	42	13	31	0	0	100	69	15	51.
A	61	46	48	100	0	0	13	20	22	40	26	24	100	0	0	14	19	12.
U	10	18	13	0	100	0	13	11	41	9	9	4	0	100	0	10	7	18.
C	9	29	23	0	0	0	5	54	10	9	52	41	0	0	0	7	59	19
	A	$^{A}/_{C}$	**A**	**A**	**U**	**G**	**G**	**C**	$^{G}/_{U}$	$^{G}/_{A}$	**C**	$^{G}/_{C}$	**A**	**U**	**G**	**G**	**C**	**G**

Summary of start codon context of dicot and monocot genes from the TransTerm database (Dalphin et al., 1997). The analysis of the dicot start codons included 932 *Arabidopsis thaliana*, 228 *Glycine max*, 257 *Nicotiana tabacum*, 203 *Pisum sativum* and 217 *Solanum lycopersicum* genes. Cereal start codons were analysis from 332 *Zea mays*, 275 *Oryza sativa*, 149 *Triticum aestivum* and 211 *Hordeum vulgare* genes. DNA sequence of consensus start codon context is shown below

TABLE 4

Stop codon usage in cereal and dicot genes

	UAAA	UAAC	UAAG	UAAU	UAGA	UAGC	UAGG	UAGU	UGAA	UGAC	UGAG	UGAU
Cereal	8.0	2.7	10.0	7.0	11.6	3.9	5.5	5.8	11.5	2.3	14.6	17.1
genes		**27.7**				**26.8**				**45.5.**		
Dicot	16.8	3.4	13.0	12.3	10.6	1.2	3.2	4.4	13.4	2.0	9.4	10.4
genes		**45.5**				**19.4**				**35.1**		

Summary of four base stop codon context of dicot and cereal genes from the TransTerm database (Dalphin et al., 1997). The analysis of the dicot stop codons included 1019 *Arabidopsis thaliana*, 278 *Glycine max*, 297 *Nicotiana tabacum*, 235 *Pisum sativum* and 257 *Solanum lycopersicum* genes. Cereal stop codons were analysis from 375 *Zea mays*, 294 *Oryza sativa*, 178 *Triticum aestivum* and 237 *Hordeum vulgare* genes

different polyadenylation sequences have on gene expression levels in cereals. An eight-fold increase in transient CAT expression from an *Adh1* promoter-*Adh1* intron 1-CAT fusion was obtained in electroporated maize protoplasts, when the reporter gene was linked to the NOS 3′ end instead of the *Adh1* 3′ end. For some cereal genes, the 3′ sequences have been implicated in tissue-specific expression. For example, it has been suggested that the 3′ region of maize *rbcS* has a role in repression of *rbcS* expression in mesophyll cells (Viret et al., 1994). The 5′ leader and the 3′ UTR of a barley α-amylase have both been suggested to direct gene expression to aleurone and endosperm tissues of barley (Gallie and Young, 1994).

5.3 Modification of Gene Sequences for Optimal Gene Expression

Plants genes have, like many genes from other organisms, a biased codon usage and base composition (Campbell and Gowri, 1990, Murray et al., 1989), which may be of importance for expression of foreign genes. Monocot nuclear genes use a set of 38 preferred codons, with the exception of the highly expressed genes, which favor only 32 codons (Campbell and Gowri, 1990). The bias in codon usage for highly active genes in yeast and *Escherichia coli* matches the level of isoaccepting tRNA species in the cells (Bennetzen and Hall, 1982, Sharp and Li, 1986). A correlation has also been found between the tRNA population of the maize endosperm and the codon usage of the zein gene, which encodes the main storage protein in this tissue (Viotti et al., 1978). The high level of glutamine, alanine and leucine codons on the zein transcript is well adapted to the most abundant isoaccepting tRNA species in the maize endosperm, but not to the tRNA population of the embryo. However, little is known about the distribution of tRNA species in various plant tissues or species. Experiments in yeast have shown that the occurrence of many rare codons on a transcript causes destabilization of the transcribed mRNA (Hoekema et al., 1987), but there is no direct evidence in plants that the presence of rare codons renders the transcript unstable (Abler and Green, 1996).

The G + C content of plant genes is high in comparison to genes from most other sources. A 44 to 70% G + C value has been reported for monocot genes, of which the majority are in the 60 to 70% range (Matassi et al., 1989). Genes from dicots have a more narrow distribution of G + C residues, with values ranging from 40 to 56% (Matassi et al., 1989). The main difference in base content of monocot and dicot coding regions relates to an overall higher G + C content at the third position of the codon, especially for highly expressed genes (Fennoy and Bailey-Serres, 1993, Murray et al., 1989). This difference in codon usage between monocot and dicot genes is also seen when homologous sequences are compared (Matassi et al., 1989). It has been explained that the high G + C content in monocot genes correlates with the isochore pattern of the genome (Matassi et al., 1989).

The importance of codon usage and G + C content of coding regions, for optimal gene expression in plants, has been demonstrated by the development of transgenic dicot and monocot plants expressing the *Bacillus thuringiensis* δ-endotoxin (*Bt* toxin) gene. The first transformation experiments with dicot plants using a truncated variant of the AT-rich *Bt* toxin gene under the control of strong promoters, resulted in production of an unstable toxin transcript, and consequently, a low production level of the insect control protein (Fischhoff et al., 1987, Perlak et al., 1990). Perlak et al., (1990) showed that toxin production encoded by a hybrid truncated *Bt* toxin gene, *CryIA(c)*, could be increased more than 50-fold by changing the codon usage and raising the G + C content of the coding region, without altering the encoded protein sequence. These modifications also removed potential RNA processing sequences, such as ATTTA sequences (Ohme-Takagi et al., 1993) and polyadenylation signals. Similar modifications to other *Bt* toxin genes were made to allow efficient transcription and translation in monocot systems (Fujimoto et al., 1993, Koziel et al., 1993, Nayak et al., 1997). A modified *CryIA(b)* gene driven by the CaMV 35S promoter fused to the first intron of the castor bean catalase 1 gene was used to develop insect-resistant japonica rice (Fujimoto et al., 1993). Nayak et al., (1997) obtained insect-resistant indica rice by introduction of a reconstructed *CryIA(c)* gene driven by the *Ubi1* 5′ region. Transgenic maize expressing an improved *CryIA(b)* gene under the control of the constitutive CaMV 35S promoter and tissue-specific promoters, respectively, have also been obtained (Koziel et al., 1993).

Development of transgenic barley expressing a heat-stable (1,3-1,4)-β-glucanase gene during germination was possible by modification of the coding sequence of the transgene (Jensen et al., 1996). The improvement of the hybrid bacterial (1,3-1,4)-β-glucanase gene involved adaptation of the codon usage to that of the barley (1,3-1,4)-β-glucanase isoenzyme EII coding region. Similarly, several different modifications of the AT-rich GFP gene have been made to improve expression levels in plants (Haseloff et al., 1997, Pang et al., 1996, Reichel et al., 1996, Rouwendal et al., 1997). For example, Rouwendal and coworkers (1997) increased GFP expression by removing a cryptic splice site, and by changes at the third position of the codons to increase the G + C content of the gene from 32 to 60 %. It is possible that some of the sequence changes made to the GFP gene had a stabilizing effect on the GFP transcript, similar to that which occurred with modifications made to the *Bt* toxin genes.

6. Silencing of Gene Expression in Cereals

The introduction of transgenes into animals or plants has led to some unanticipated findings, where total or variegated silencing of inserted or

endogenous genes has been observed (see recent reviews by Baulcombe, 1996, Bingham, 1997, Stam et al., 1997). The unpredictable behavior of transgenes has become a serious concern for development of transgenic cereals, in which gene silencing appears to be a problem (Finnegan and McElroy, 1994, Itoh et al., 1997, Register et al., 1994). To date, no detailed studies on gene silencing mechanisms in monocots have been reported, but it is presumed to occur by similar mechanisms as in animals and dicot plants. Nevertheless, it will be of vital importance to learn more about the phenomenon in order to develop effective strategies for generation of transgenic crops, which require uniform and predictable agricultural performance. The regulatory mechanisms may also reveal how multicopy genes, that are part of polyploid genomes in cereals, have evolved to escape gene inactivation and how gene expression is regulated in tissues of different ploidy levels. It is interesting to note that in bread wheat, a hexaploid species, stability of transgene integration and expression has been shown for several sexual generations (Altpeter et al., 1996, Blechl and Anderson, 1996, Srivastava et al., 1996, Vasil and Anderson, 1997).

The gene silencing phenomenon was discovered in transgenic tobacco, in which expression of a transgene was lost after going through a second transformation round (Matzke et al., 1989). Inactivation of transgene expression was only seen in plants containing sequences homologous to introduced genes. Later experiments have shown that gene silencing occurs at both the transcriptional and post-transcriptional levels, and in general involves homology restricted to promoter or coding regions (Park et al., 1996). It is still unclear whether the two types of homology-dependent silencing are overlapping or whether they use different mechanisms to down-regulate gene expression (Stam et al., 1997).

Homologous gene silencing involving promoter sequences is frequently correlated with increased methylation of cytosine residues of the coding region or promoter sequences of suppressed genes. Transgenes that lack corresponding sequences on the host genome may become inactivated through methylation caused by insertion into a heavily methylated region on the chromosome and spreading of the methylation pattern into the transgene (Pröls and Meyer, 1992). In addition, altered methylation status of transgenes or host genes may result from tissue culture effects occurring during regeneration of transgenic plants (Brown, 1989, Phillips et al., 1994). It was recently reported that tissue culture media containing the antibiotics hygromycin, kanamycin or cefotaxime induce DNA hypermethylation in tobacco plants (Schmitt et al., 1997). The effect of these antibiotics on the DNA methylation pattern was found to be both time- and dose-dependent. It is likely that the antibiotic agents studied will cause similar effects during regeneration of transformed cereals.

The involvement of methylation in gene silencing in cereals has been verified by restriction analysis of genomic DNA using different methy-

lation-sensitive restriction enzymes (Ronchi et al., 1995). Further evidence that methylation causes gene silencing in cereals was provided by experiments showing reactivation of suppressed genes by treatment of transgenic plants with the demethylating agent, 5-azacytidine (Meijer et al., 1991, Ronchi et al., 1995). Once the methylation pattern of the genomic DNA is established, it is generally maintained through cell divisions, thereby resulting in an epigenic gene regulation. The silenced phenotype can sometimes display an unstable inheritance and become gradually lost after several generations (Matzke et al., 1989, Neuhuber et al., 1994). The degree of methylation and silencing appears to be correlated with the copy number of the inserted gene, and is therefore particularly serious when direct transformation methods have been used to generate the transgenic plants. These gene delivery methods, such as particle bombardment, generally lead to insertion of several copies of the transgene at a single locus (D'Halluin et al., 1992, Register et al., 1994, Spencer et al., 1992) and could thereby, to a greater extent than *Agrobacterium*-based methods, provoke gene silencing. Recent developments of transformation methods for cereals using *Agrobacterium* (Chan et al., 1993, Cheng et al., 1997, Hiei et al., 1994, Ishida et al., 1996, Tingay et al., 1997) may lessen this problem.

Several models involving direct or indirect DNA:DNA, DNA:RNA and RNA:RNA interactions have been put forward in an attempt to explain gene inactivation occurring at the post-transcriptional level (see reviews by Baulcombe, 1996, Stam et al., 1997). A hypothesis is that gene shut-down is triggered by an initial high expression level from the transgene (Elmayan and Vaucheret, 1996). This may explain why the strong CaMV 35S promoter is often subject to post-transcriptional inactivation in dicot plants. It also raises the question of whether a promoter that is very active during the early stages of plant development actually increases the recovery rate of transgenic plants compared to less active promoters. It has so far been impossible to demonstrate any such correlation due to the limited number of transgenic plants generated and the wide variety of constructs and plant systems used.

Several experiments performed with dicot plants and animals suggest that the severity of gene silencing can be decreased by placing matrix attachment regions (MAR) or scaffold attachment regions (SAR) on each side of the expression cassette (Allen et al., 1993, 1996, Poljak et al., 1994, Schöffl et al., 1993). It is believed that the MAR or the SAR sequences will allow the enclosed DNA to form an independent loop domain that will be insulated from negative effects exerted by the surrounding chromatin (see review by Spiker and Thompson, 1996). A positive effect of these elements in plants was demonstrated when a 140-fold increase in GUS gene expression was noted in transgenic tobacco cell lines when tobacco SAR elements were added to a GUS expression vector (Allen et al., 1996). In these experiments, a more prominent effect of the SAR sequences was found in stably

transformed cells as compared to transiently transformed cells, which suggested that SAR-mediated gene enhancement required integration of the transgene into the chromosome. Although most data from dicot plants show an average increase in marker gene expression when SAR or MAR sequences are used, this enhancement is more evident in transgenic plants with a low copy number of the transgene. Thus, the variability in transgene performance from different transformants does not appear to be diminished by the presence of the SAR/MAR elements. The effect of these sequences in monocot plants has not been assessed so far.

7. Effects of Transgene Methylation on Gene Expression and Stability

Methylation of plant genes has an active role in genome defense, differentiation and development (see reviews by Richards, 1997, Stam et al., 1997, Yoder and Bestor, 1996). In cereals, there is convincing evidence that the tissue-specific expression of the seed storage proteins genes, hordein in barley and zein in maize, is regulated by cytosine methylation (Bianchi and Viotti, 1988, Lund et al., 1995, Sørensen et al., 1996).

Transient expression assays in wheat tissues and barley cell lines have suggested that the methylation pattern of introduced genes affects reporter gene expression levels (Graham and Larkin, 1995, Rogers and Rogers, 1995). In these studies, methylation of adenine residues at *dam* sites, in contrast to deoxycytosine methylation, had a stimulatory effect on gene expression. The enhancement of expression levels varied between the promoters studied and was independent of the initial promoter strength. For example, transient GUS gene expression driven by the *Act1* 5′ region in wheat embryos was stimulated 50-fold by adenine methylation at *dam* sites on the introduced DNA. In contrast, no increase in GUS activity by *dam* methylation of a CaMV 35S promoter-*uidA* cassette was obtained, which the authors correlated to fewer *dam* sites in the promoter region.

Transient transformation studies in barley have indicated that the DNA methylation status of introduced genes affects transgene stability in the host plant (Rogers and Rogers, 1992). It was observed that dA-methylation of *dam* sites (GATC) on introduced DNA was associated with increased instability of transgenes, whereas a lack of dA methylation combined with the presence of dC methylation increased transgene stability over two generations. Instability or poor transmission of transgenes has been observed in transgenic cereals (Peng et al., 1995, Spencer et al., 1992); however, these studies did not correlate these problems to the methylation status of the introduced DNA. Selective loss of transgene in successive generations was reported in *Arabidopsis thaliana* to be due to change in ploidy level of the progeny plants (Mittelsten Scheid et al., 1996). Therefore, whether specific methylation patterns of introduced genes trigger transgene instability remains to be investigated.

8. Future Prospects for Cereal Improvement by Genetic Engineering

Research in the early part of this decade was focused on expanding and improving the transformation technology for elite cultivars of important cereals such as wheat, maize, rice and barley. This objective has been achieved for most of the important cereal crops. We have now reached a stage where incorporation of useful traits into cereals has started to produce plants with improved agronomic performance and/or end-use quality. The grain producers and food-processing industries demand a uniform and consistent performance from agricultural crops. This is a challenge for biotechnology to overcome for the benefit of agriculture. Therefore, it is important to focus cereal research towards the molecular aspects of transgene technology in order to gain understanding of what a plant phenotype means in genetic terms.

A number of issues need to be addressed so that improvements can be made in the transgene technology for cereals. Promoters play an important role in the production of transgenic plants, because these DNA sequences direct transgene expression in a spatial, temporal or inducible manner. Due to the differences in gene regulatory mechanisms in different plants, it is desirable that promoters are analyzed in the plant species in which they are intended for use. It is now possible to perform a detailed study of promoter function by using transgenic cereals. These studies will reveal relevant information about promoter function in cereals during development and upon exposure to various environmental stress conditions, which may have a great influence on crop performance. Studies on interactions between host regulatory factors and *cis*-acting DNA regulatory elements on expression cassettes can provide valuable information which can be used to construct synthetic promoters. Promoters directing a desired gene expression pattern in the plant could be obtained by linking of various modules of well-defined regulatory elements.

The experience with development of insect-resistant plants expressing a modified *Bt* toxin has demonstrated that insertion of a foreign sequence into plants may cause several problems at the level of gene regulation, that will affect the amount of transgene product produced. Introduction of heterologous genes into cereals will require optimization of DNA sequences of the genes for expression in cereals. These alterations may include removal of RNA instability determinants, changes in codon usage, insertion or removal of intron sequences and optimization of regulatory sequences. To avoid unforeseen complications with unrelated gene sequences, it may be preferable to use promoters and gene sequences isolated from cereals. However, the problem with homologous sequences causing transgene silencing and/or co-suppression in cereals needs to be solved. Studies on gene expression and regulation in cereals that are polyploid may provide some answers to the mechanisms that have naturally evolved to prevent co-suppression and/or

gene silencing in these plants. Replacement of direct DNA delivery techniques with *Agrobacterium*-mediated transformation methods will lower the number of inserted transgene copies, thus reducing the level of gene silencing. The effect of MAR or SAR sequences in expression cassettes has so far not been studied in cereals, but data from dicot plants suggest that these elements may overcome some of the problems associated with multiple copy insertion as well as transgene silencing. Development and optimization of site-specific recombination systems for plants to target genes to specific locations on the genome could also reduce the number of inactive transgenes. For example, transgenes could be targeted to regions with active genes, which are known to be organized in clusters on the plant chromosomes (Gill et al., 1996). Promising results in transgenic cell lines using the yeast FLP/FRT site-specific recombination system (Lyznik et al., 1996) indicate that this may be a realistic possibility. Despite the various factors related to gene expression and regulation that need to be elucidated, transgenic cereals will play an important role in agriculture in the next century.

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