agronomie (1995) 15, 309-319

Plant breeding (review)

Transformation of Poaceae and gene expression in transgenic plants

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(Received 22 May 1995; accepted 4 July 1995)

Summary — All major Poaceae have been transformed in recent years. This review gives a brief overview of the methods used for transformation and for achieving controlled gene expression in transgenic plants and discusses the problems that are still encountered when transformation technology is applied for practical purposes.

transformation / monocot / gene expression / transgenic plant

Summary — Transformation des *Poaceae* et expression de gènes dans les plantes transgéniques. *Les espèces les plus importantes de la famille des* Poaceae *ont été transformées au cours de ces dernières années. Cet article résume les différentes méthodes utilisées pour la transformation et le contrôle de l'expression des gènes dans les plantes transgéniques. Les problèmes liés à l'utilisation des techniques de transformation en recherche appliquée sont également discutés dans cet article.*

transformation / monocotylédones / expression génique / plante transgénique

INTRODUCTION

The establishment of transformation protocols for monocots in general and particularly for cereals and grasses has lagged behind transformation of many dicots. However, after a very long period of development, many Poaceae, including all the economically important ones, have finally been transformed during the last 6 years (table I). However, the transformation of most of the species is still inefficient and is reported only for a few isolated cases. The notable exceptions are rice (*japonica* varieties) and maize where relatively high transformation efficiencies can be reached routinely, and the commercialization of the first transgenic maize is to be expected soon. In general, transformation efficiencies are very genotype dependent and, for the establishment of the transformation methods, lines or cultivars that show particularly good tissue culture responses have been used. These lines, however, are usually not the economically important ones and transfer of transformation technology to lines of agronomical interest, has, in most cases, yet to be accomplished.

This brief review will provide a subjective overview of the latest achievements in the field of the transformation of Poaceae and the conclusions that can be drawn from this work. The references given are meant to provide illustrative examples rather than a complete list of the published work. The discussion will be restricted to

points relevant for stable transformation and to examples where proof for stable integration of a transgene has been provided by molecular genome analysis.

PLANT MATERIAL

The choice of a suitable starting material was crucial to the development of the transformation technology. Tissues or cells with the competence to take up, integrate and express DNA, and to develop into a mature, fertile plant, had to be found.

Following the early work in dicot transformation, also for cereals, the transformation of protoplasts has been successful (table I). Protoplasts have been obtained from embryogenic cell suspensions, which, with different methods of DNA delivery (see below), were later used directly for transformation. The use of embryogenic cell suspensions offers a variety of advantages over material that must be isolated for each experiment directly from plants, but the initial establishment of such a culture is time-consuming and requires experience. Once such a culture has been established, material for transformation experiments can be readily amplified and large-scale experiments can be performed relatively easily. The uniformity of the starting material allows the introduction of DNA into a large number of cells simultaneously and simple selection schemes can be employed because transgenic cells do not have to be selected from the non-transgenic tissue in which they are embedded. The major disadvantage of this approach is the long time that the plant material should be kept under very artificial tissue culture conditions. During this time, parts of the plant genome are not under a functional selection and mutations or epigenetic changes can accumulate, resulting in phenotypically aberrant plants (albinos, sterile plants, *etc*). These problems can be partially overcome by either continuous establishment of new cultures, which are then only used during a short period of time (about 3 months), or by cryopreservation of cultures in a 'young' state early after establishment (Lynch *et al,* 1994; Wang *et al,* 1994). However, the period required for establishment of a culture may be long enough to cause mutational changes. A further disadvantage of such cell cultures is the apparently greater genotype dependence of the method.

To avoid these long tissue culture phases, regenerable tissue has been isolated directly from plants. The most widely used explants are immature, zygotic embryos (table I). DNA delivered either directly to the scutellum or to calli derived from the scutellum is expressed and integrated at least in some cells. Selectable, antibiotic- or herbicide-resistant calli and (under appropriate conditions) plants can develop from such cells. Plant regeneration from scutellum-derived calli still shows a strong dependence on the genotype of the donor plant, although these effects may be less pronounced than with cell suspensions. It has been reported for rice that regeneration (and transformation) is equally effective with a wide range of different genotypes including *indica* rice varieties which are otherwise very difficult to transform (Cooley *et al,* 1995). In our hands, however, *japonica* rice lines still respond about 100 times more efficiently than the recalcitrant *indica* lines; similar genotype dependencies were observed in work with wheat with some cultivars showing almost no suitable tissue culture response at all. It remains to be seen whether these adverse genotype effects can be overcome by variation of the tissue culture conditions or whether they really provide an intrinsic block.

A high percentage of the plants derived from scutellum are phenotypically normal although problems with fertility may still be encountered. The disadvantages of the method for routine use are the requirement for a continuous supply with appropriate seed material and the necessity to isolate and handle a large number of immature embryos, particularly in those cases where the transformation efficiencies are low (*eg*, 1 plant per 500 treated embryos; Weeks *et al,* 1993). Selection for transgenic tissue may also be more difficult.

Other plant materials like microspores (Jähne *et al,* 1994), inflorescence (Barcelo *et al,* 1994) and apical meristems (Lowe *et al,* 1995) have occasionally been used with success. The future will show how generally applicable these alternatives are.

DNA DELIVERY

Several methods have been used to deliver DNA to isolated plant cells or plant tissues (table I). For protoplasts, treatment with polyethylene glycol (PEG) or electroporation leads to uptake of DNA into a large number of cells without damaging these cells too much to interfere with their viability. Cells that are still surrounded by a cell-wall,

ie intact suspension cells or cells in tissue, require a treatment that can overcome this barrier.

The present method of choice is the delivery of DNA bound to microcarriers that are accelerated by different methods, so that they can penetrate through the cell wall and enter cells by mechanical force. Several different methods to link DNA to the microcarriers and to accelerate these particles have been successfully used and have also been commercialized. In general, gold particles as microcarriers have less adverse effects on cell viability than tungsten particles, and particle acceleration by pulses with inert gases is preferable over the previously used gun powder explosions (reviewed by Christou, 1993). The most widely used particle gun is the PDS-1000/He device (Kikkert, 1993) which is available from Du Pont. We have used a simpler particle inflow gun, which was home-made according to Finer *et al* (1992), with success.

Surprisingly, DNA can also be introduced into intact cells by electroporation (table I; Klöti *et al,* 1993; Songstad *et al,* 1993). This method could have the advantage that the cells that take up DNA are less stressed than by particle bombardment (Hunold *et al,* 1994). However, so far, electroporation conditions have not been optimized for much of the plant material in question and too few examples have been reported for a comparison of the 2 methods.

Other methods to introduce DNA into cells or tissue involve microinjection (Simmonds *et al,* 1992; Lusardi *et al,* 1994), DNA application to growing pollen tubes (Luo and Wu, 1988), and shaking cells with small silicon carbide crystals (whiskers) in a DNA solution (Kaeppler *et al,* 1992; Frame *et al,* 1994). The last method is experimentally very simple and does not require any expensive equipment; the general applicability of the other methods (assessed by Potrykus, 1990) remains to be proved.

One reason for the delay between transformation of monocots and dicots was the apparent unresponsiveness of monocots to treatment with *Agrobacterium*. *Agrobacterium*-mediated infection of cereals with DNA viruses provided the first indications that agrobacteria can in fact transfer DNA to cereals (Grimsley *et al,* 1987) and recently transgenic rice plants with stably integrated, *Agrobacterium*-transferred DNA have been described (Chan *et al,* 1993; Hiei *et al,* 1994). Attempts to repeat this success with other cereals are underway in many laboratories but have to our knowledge only resulted in transient expression events.

In monocots, the applicability of *Agrobacterium*-mediated gene transfer would not overcome the requirement for regenerable starting material, but may provide a more efficient and less destructive method of DNA delivery to this material. Furthermore, integration patterns after *Agrobacterium*-mediated transformation are usually simpler than with all methods of direct DNA delivery where rearrangements and truncations of the transfected DNA are very frequent and where integration of multiple copies at one or more loci is the rule. It is generally believed that the expression patterns of single copy transgenes are more stable and predictable than those of multiple copies (Spencer *et al,* 1992; Cooley *et al,* 1995).

For applications, genes besides the selectable marker must be introduced. These genes can be located on the same DNA fragment or can be supplied on separate fragments. Cotransformation rates between 20% (Peng *et al,* 1990; Rathore *et al,* 1993) and 100% (Goto *et al,* 1993; Barcelo *et al,* 1994; Wan and Lemaux, 1994) have been reported for genes provided as unlinked DNA fragments. With linked genes, rates of 100% are normal (Cooley *et al,* 1995).

SELECTION OF TRANSGENIC PLANT TISSUE

Transgenic cells or tissues are regenerated in the presence of selective agents. Four genes that encode proteins detoxifying antibiotics or herbicides have mainly been used for cereals and grasses (table I):

1) The *npt*II gene of the *Escherichia coli* transposon Tn5 confers resistance to kanamycin and related compounds like geneticin (Bevan *et al,* 1983). Kanamycin was used initially for some cereals. In cases where endogenous resistance against kanamycin and/or interference with regeneration were encountered, geneticin proved to be a valuable alternative.

2) The *hpt*I gene isolated from *E coli* confers resistance to hygromycin (Gritz and Davies, 1983) and was mainly used for rice transformation and for some grasses. Selection conditions can be used that are very tight and almost exclusively allow growth of transgenic material.

3) Genes coding for methotrexate-resistant dihydrofolate reductases (DHFR) have been isolated from *E coli* (Pattishall *et al,* 1977) and a mouse DHFR gene was modified to encode a resistant protein (Eichholtz *et al,* 1987). The gene allowed effective selection of transgenic wheat and rice.

Table I. Transgenic Poaceae.

Species	*Starting material*	*Selection*	*Method*	*Comments*	*Reference*
Barley *Hordeum vulgare*	IE callus; microspore-derived embryos IE Cultured microspores	Bialaphos No PPT	Biolistics Biolistics Biolistics	Albinism, high cotransformation and coexpression 1 out of 227 plants transgenic Only 4 plants of 12 express linked genes	Wan and Lemaux, 1994 Ritala *et al,* 1994 Jähne *et al,* 1994
Fescue, red *Festuca rubra*	Protoplasts (from cell suspensions) Suspension cells	PPT, Hyg Hyg	PEG Biolistics	Fertility unclear	Spangenberg *et al,* 1994 Spangenberg *et al,* 1995a
Fescue, tall *F arundinacea*	Protoplasts (from cell suspensions) Suspension cells	PPT Hyg	PEG Biolistics	Fertility unclear	Wang *et al,* 1992 Spangenberg *et al,* 1995a
Maize *Zea mays*	Protoplasts (from cell suspensions) Suspension cells Suspension cells, callus Callus culture Suspension cells IE callus IE Suspension cells Suspension cells	Km, PPT Bialaphos PPT Hyg Bialaphos, glufosinate PPT Km Bialaphos Basta ®	PEG Biolistics Biolistics Biolistics Biolistics (PIG) Biolistics Electroporation Electroporation Silicon carbide fibers	Selection and regeneration of GUS positive clones 80% cotransformation, 20% coexpression Unexpected segregation Osmotic pretreatment Wounded or enzyme-treated E Whole cell electroporation < 10% as efficient as biolistics	Omirulleh *et al,* 1993 Gordon Kamm *et al,* 1990 Fromm *et al,* 1990 Walters *et al,* 1992 Vain *et al,* 1993 Koziel *et al,* 1993 D'Halluin *et al,* 1992 Montain Laursen *et al,* 1994 Frame *et al,* 1994
Oat *Avena sativa*	Callus and suspension cells	PPT	Biolistics	Most lines sterile	Somers *et al,* 1992
Perennial ryegrass *Lolium perenne*	Suspension cells	Hyg	Biolistics	Fertility unclear	Spangenberg *et al,* 1995b

Table I. Cont.

Species	*Starting material*	*Selection*	*Method*	*Comments*	*Reference*
Rice	Protoplasts (from cell suspensions)	Hyg	PEG	Japonica rice	Shimamoto *et al,* 1989
Oryza sativa	Protoplasts (from cell suspensions)	Hyg	PEG	Indica rice	Datta *et al,* 1990
	IE	Hyg, bialaphos	Biolistics	Several genotypes	Christou *et al,* 1991
	IE	Hyg, bialaphos	Biolistics	Genotype independent	Cooley *et al,* 1995
	IE (callus)	Hyg	Biolistics		Li *et al,* 1993
	IE	Geneticin	*Agrobacterium*		Chan *et al,* 1993
	Best with scutellum callus	Hyg	*Agrobacterium*	Integration borders analysed	Hiei *et al,* 1994
	Half embryos	Hyg	Electroporation		Xu and Li, 1994
					Review: Ayres and Park, 1994
Rye	IE callus (1 to 5 months old)	PPT	Biolistics		Castillo *et al,* 1994
Secale cereale					
Sorghum	IE	Bialaphos	Biolistics	No transgene expression	Casas *et al,* 1993
Sorghum bicolor					
Sugar cane	Callus culture	Geneticin	Biolistics		Bower and Birch, 1992
Saccharum officinarum	Cell clusters from callus	GUS staining	Electroporation	Electroporation of intact cells	Arencibia *et al,* 1995
Tritordeum	Inflorescence	Geneticin	Biolistics	High coexpression	Barcelo *et al,* 1994
Hordeum x Triticum					
Turf grasses:					
Creeping bentgrass	Protoplasts (from cell suspension)	Geneticin	Electroporation		Asano and Ugaki, 1994
Agrostis alba					
Redtop	Callus	GUS staining	Biolistics		Zhong *et al,* 1993
Agrostis palustris	Callus	Bialaphos	Biolistics	No seeds	Hartman *et al,* 1994
Wheat	Callus culture	Basta®	Biolistics	Slow (12–15 months)	Vasil *et al,* 1992
Triticum aestivum	IE, callus	Bialaphos	Biolistics	Fast (5–6 months)	Weeks *et al,* 1993
	IE, callus	Basta®	Biolistics	Fast (7–9 months)	Vasil *et al,* 1993
	IE, callus, scutellum	PPT, geneticin	Biolistics	50% escapes with PPT, 20% with geneticin	Nehra *et al,* 1994
	IE	PPT	Biolistics		Becker *et al,* 1994
	Protoplasts (from cell suspension)	PPT	Electroporation		He *et al,* 1994
					Review: Maheshwari *et al,* 1995

The starting materials, selective agents and methods of DNA delivery used for the generation of transgenic plants are listed. Abbreviations used: IE: immature, zygotic embryo; PPT: phosphinothricin; Hyg: hygromycin; Km: kanamycin; PEG: polyethyleneglycol; PIG: particle inflow gun; GUS: β-glucuronidase.

Selection for methotrexate resistance has a potential advantage over the other selection schemes mentioned in which a selective agent has to be detoxified by a transgene-encoded enzyme. Low enzyme activity or low accessibility of the selective agent to the enzyme may result in incomplete detoxification. The balance between transgene expression and concentration of the selective agent could be more critical than in the case of methotrexate where plant cells containing low levels of the resistant DHFR may also survive in high concentrations of methotrexate.

4) The *bar* gene of *Streptomyces hygroscopicus* (Murakami *et al,* 1986) and the *pat* gene from *S viridiochromogenes* (Wohlleben *et al,* 1988) both confer resistance to the herbicide compound phosphinothricin (PPT) and its derivatives Basta® and bialaphos. The gene was useful for selection in a wide variety of Poaceae. Selection can be accomplished either by addition of the compound to the growth medium or by spraying the plantlets. The precise selection scheme must be optimized for each species and each starting material. Concentrations of PPT that would allow a 100% effective selection can interfere with the regeneration capacity of the tissue (*eg,* in wheat transformation; Nehra *et al,* 1994). Suboptimal amounts therefore are being used which also allow growth of some untransformed material. In such cases, it is important to continue selection throughout plant regeneration. Nevertheless, the *bar* gene presently seems to be the selective gene of choice for all Poaceae, except rice where selection with hygromycin is very well established.

In a few cases, transgenic material has been selected by a sublethal GUS staining (Omirulleh *et al,* 1993; Arencibia *et al,* 1995). After a brief exposure to the reagents required for staining, some cells are still viable and can further proliferate and regenerate. This method is necessarily inefficient and only recommendable if no other selection system is available or if the presence of a selectable marker in a transgenic plant is undesirable. The detection of the activity of genes regulating anthocyanin synthesis would not require a staining process but the expression of these anthocyanin genes may interfere with viability of the cells (unpublished observations; also mentioned in McElroy and Bretell, 1994). The recently described green fluorescent protein from *Aequorea victoria* may provide an alternative visible selectable marker in the future (Chalfie *et al,* 1994).

GENE EXPRESSION

The expression of transgenes is affected by control signals within the transferred DNA but also by sequences surrounding it. These latter position effects make any assessment of the function of transferred expression signals in transgenic Poaceae difficult. In most cases, only very few plants have been analysed with a given construct. Since the variations between independent transgenic plants and even between different plants derived from the same initial transformation event can be considerable, the low numbers of such plants that have been analysed prevent a meaningful statistical evaluation. Most of the data referring to the comparison of expression signals are therefore derived from transient expression systems (Last *et al,* 1991; McElroy *et al,* 1991; Chibbar *et al,* 1993).

For the expression of the selectable marker gene, promoters are required that express at a high level in most of the plant cells. The most commonly used constitutive promoters are the cauliflower mosaic virus (CaMV) ^{35}S promoter, the maize ubiquitin 1 promoter and the rice actin 1 promoter (reviewed in McElroy and Brettell, 1994).

The CaMV ^{35}S promoter is the most widely used promoter in dicot transformation and was naturally also tested in the early attempts of monocot transformation. It was found to be active in cereals and grasses and was used in its wild-type form for the expression of selectable and screenable marker genes. However, widely different activity levels have been reported for different expression systems with a general tendency towards low expression levels. The promoter was therefore modified by duplication of its own enhancer (Omirulleh *et al,* 1993), by insertion of other transcriptional enhancers (Olive *et al,* 1990; Last *et al,* 1991) or by combination with intron sequences (Tanaka *et al,* 1990; see also below). In mature plants, the promoter may exhibit some tissue specificity for the vascular tissue but it is also reported to be active in more or less all cells (Battraw and Hall, 1990; Terada *et al,* 1990). In our hands, an intron-enhanced CaMV ^{35}S promoter is mainly active in the mesophyll cells of old rice leaves and weak in the vascular tissue and the epidermis, but shows considerable variation during plant development.

The maize ubiquitin 1 promoter together with the first intron of the gene (Christensen *et al,* 1992) seems to be the strongest monocot expression signal at least in protoplasts. It is now

widely used for cereal transformation (Toki *et al,* 1992; Weeks *et al,* 1993). It is regarded as a constitutive promoter; however, in transgenic plants, transgenes were shown to be active mainly in young tissue and transgene activity was inducible by stress (Cornejo *et al,* 1993).

Gene expression cassettes have also been constructed with the rice actin 1 promoter together with the first intron of the gene (McElroy *et al,* 1991). Very high activities could again be obtained in single cell systems and constitutive expression was observed in transgenic plants (Zhang *et al,* 1991).

A very strong constitutive promoter has been constructed by a combination of several multiplied enhancer elements (pEmu: Last *et al,* 1991).

For purposes of tissue-specific or inducible expression, a number of specific promoters mainly from maize and rice are available, which are functional in transgenic plants (Kyozuka *et al,* 1991, 1993; Schäffner and Sheen, 1991; Gotor *et al,* 1993; Koziel *et al,* 1993; Xu *et al,* 1993; Zheng *et al,* 1993; see also McElroy and Brettell, 1994; Terada *et al,* 1995). Tissue-specific dicot promoters have also been used in monocots but expression levels are often quite low and expression specificities may be different (Luan and Bogorad, 1992; Kyozuka *et al,* 1993). It remains to be seen whether monocot promoters in heterologous monocot plants will always show a better predictable activity than dicot promoters.

It has become practice to include introns in gene expression cassettes for transformation of Poaceae. This is based on experiments in protoplasts that showed that the presence of introns can enhance gene expression up to 100-fold (Callis *et al,* 1987; McElroy *et al,* 1990; Last *et al,* 1991; Maas *et al,* 1991; Chibbar *et al,* 1993). It should be noted that these data are frequently derived from comparisons of constructs that varied by more than just the presence or absence of an intron and that very different stimulation factors have been observed in different expression systems (Oard *et al,* 1989; Vasil *et al,* 1989; Last *et al,* 1991; Luehrsen and Walbot, 1991; Rathus *et al,* 1993). The effect of an intron can drastically depend on the promoter with which it is combined (Last *et al,* 1991) and in some gene expression systems, introns that lead to an enhancement of expression in cereals even reduced gene expression (*eg,* sugarcane; Rathus *et al,* 1993). The molecular basis of expression enhancement by introns in unclear. Post-transcriptional effects on the processing of primary transcripts (including splicing, polyadenylation and transport to the cytoplasm) are probable but the presence of position-dependent enhancer elements cannot be excluded for some cases. The most effective region for intron insertion into expression constructs seems to be the 5' of the coding region either upstream or in the open reading frame. It is therefore crucial for efficient translation that the intron is properly removed by splicing. Generally, monocot and dicot plant introns seem to be spliced efficiently in monocots while monocot introns are not well recognized in dicots (Goodall and Filipowicz, 1989; Peterhans *et al,* 1990; McElroy *et al,* 1991). The relatively poor knowledge about splicing in plants and particularly in monocots make it advisable to test intron-containing constructs before routine use. Another element usually present in expression cassettes is the polyadenylation signal. In dicots, very large effects on the expression efficiencies of different signals have been reported (Ingelbrecht *et al,* 1989). In monocots only a few have been compared and so far no significant differences have been observed (McElroy *et al,* 1995).

Sequences in the coding region of the transgene may have a negative effect on expression efficiency. In genes derived from bacteria, the codon usage can often be very different from that of the plant (Murray *et al,* 1989; Campbell and Gowri, 1990) and clusters of rare codons may slow down translation. Furthermore, prokaryotic genes are not adapted to the RNA-processing machinery of the plant nucleus and may accidentally contain features that cause aberrant processing or degradation of the RNA. Modification of the coding sequence by excessive mutagenesis to remove such potentially deleterious features has been shown to cause strong increases of expression of the respective genes (Perlak *et al,* 1991; Fujimoto *et al,* 1993; Koziel *et al,* 1993).

TRANSGENE INHERITANCE AND STABILITY OF GENE EXPRESSION

Data on the fate of a stably integrated transgene in cereals over several generations have only been published for very few cases. While inheritance of a gene usually follows the Mendelian rules (Christou *et al,* 1991; Castillo *et al,* 1992; Toki *et al,* 1992; Vasil *et al,* 1992), transgene expression in the offspring of a transgenic plant can be quite unpreditable (Meijer *et al,* 1991; Spencer *et al,* 1992; Walters *et al,* 1992; Murry *et*

al, 1993; Register *et al,* 1994). The analysis is frequently complicated by the presence of more than 1 transgene copy. Genes may be physically present but gene activity may nevertheless be lost (Gordon-Kamm *et al,* 1990; Jähne *et al,* 1994). Loss of gene activity can occur in the primary transformant or in the offspring, and different linked genes can be affected differently. It has been found in several cases, that an unselected gene is not or only poorly expressed, although the linked selectable gene is strongly expressed (Spencer *et al,* 1992; Nehra *et al,* 1994; Register *et al,* 1994). Expression characteristics of 2 different, non-selectable genes can also be different (Cooley *et al,* 1995). Transgenic plants can also become chimaeric for the expression of a transgene (Gordon-Kamm *et al,* 1990).

At present, no general conclusions about the frequency of loss of gene activity and about the mechanisms causing such losses can be made. In dicots, silencing effects are often (but not always) related to the presence of multiple copies of a gene or a promoter but in these better examined cases the actual mechanism and the frequency of occurrence are unknown. If duplication of sequences also causes silencing in monocots, the use of homologous promoters or introns could cause problems for long-term gene stability, which could be avoided by the use of virus-derived signals. A variety of monocot DNA viruses (Geminiviruses and Pararetroviruses) could provide such signals but only very few have so far been tested in transgenic plants (Medberry and Olszewski, 1993; Yin and Beachy, 1995).

ACKNOWLEDGMENTS

We wish to thank all our colleagues at the ETH for sharing their experiences of the transformation of rice, wheat and grasses and C Fischer for translation of parts of the manuscript into French. JF Gratefully acknowledges support by the Swiss National Foundation.

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