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DNA Transfer and Gene Expression in Transgenic Cereals

RICHARD I.S. BRETTELL AND FIONA R. MURRAY

CSIRO Division of Plant Industry, Institute of Plant Production and Processing, GPO Box 1600, Canberra ACT 2601, Australia

Introduction

This decade has seen rapid advances in the development of methods for the routine transfer of genes to cereals, and there are now examples of genetic transformation for all major cereal crop species. Until recently, the two methods which have found wide application are direct gene transfer to protoplasts, and bombardment of intact tissues with DNA-coated microprojectiles. There is now substantial evidence that Agrobacterium can provide an alternative means of transferring genes to rice, and has further potential as a vector for cereal transformation.

For practical application of the technology it is essential to have transformed plants which show continued expression and stable inheritance of transgenes that alter the plant phenotype. Studies of the behaviour of transgenes in cereals have been hampered by the relatively low transformation rates obtained using current transformation methods. However, continued development of existing strategies together with the promise of alternative methods suggests this difficulty will be overcome by enhanced efficiency of gene transfer.

The significance of cereals as the principal source of food throughout the world means that they are an obvious target for genetic engineering, although the acceptance of genetically modified cereal products by the consumer remains to be tested. Despite the technological limitations described in this review, there are now examples where genes have been introduced to alter an agronomic or quality trait of a cereal. The characteristics that are receiving most attention are resistance to pests and diseases, and components of grain quality. Both are amenable to manipulation by the introduction of single genes. An example of pest resistance is the introduction of the Bt toxin gene from *Bacillus thuringiensis* into maize, providing protection against damage caused by the European corn borer (Koziel *et al.*, 1993; Armstrong *et al.*, 1995). The transfer to rice of a chitinase gene with activity against fungal cell walls

Abbreviations: GUS, β-glucuronidase; *gus*, gene coding for β-glucuronidase (*uidA* or *gusA*); HPT, hygromycin phosphotransferase; *hpt*, gene coding for hygromycin phosphotransferase (including *hph*)

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has produced lines with enhanced resistance to sheath blight caused by the fungus *Rhizoctonia solani* (Lin *et al.*, 1995). Specific pathogen resistance genes have recently been cloned and characterised (Staskawicz *et al.*, 1995) and provide a further opportunity for engineering disease resistance. Cereal genes in this category that have been isolated include those corresponding to the *Xa-21* locus for resistance to bacterial blight in rice (Wang *et al.*, 1995) and the *Cre-3* locus for resistance to cereal cyst nematode in wheat (E.Lagudah and O. Moullet, personal communication). In terms of grain quality, the target genes depend on the end use of the grain. In wheat, attention is focussed on genes altering protein and starch composition (Lazzeri and Shewry, 1993; Anderson *et al.*, 1994). In barley, the principal targets are genes that have a role in defining malting quality, such as those coding for α-amylase and β-glucanase (McElroy and Jacobsen, 1995).

This review will first consider the merits of the different methods that have been applied for transforming cereals, before discussing the conditions necessary to ensure stable expression of the transferred genetic material.

Methods for transforming cereals

MICROPROJECTILE BOMBARDMENT

Compared to other methods for plant transformation, microprojectile bombardment or biolistics is a relatively recent innovation. Microscopic particles of tungsten or gold are coated with DNA and then fired into target cells (Sanford *et al.*, 1987). In a proportion of the cells the DNA will be transferred to the nucleus (Yamashita, Jida and Morikawa, 1991; Hunold, Bronner and Hahne, 1994) providing an opportunity for integration into the genome. The particles need to be sufficiently small to penetrate individual cells without destroying their integrity and viability. Initial experiments were made using gunpowder charges to accelerate the DNA-coated metal particles, however consistent results were obtained using helium gas at pressure and this is the preferred propellent in most devices used today (Sanford *et al.*, 1991; Birch and Franks, 1991; Finer *et al.*, 1992; Brown *et al.*, 1994 ; Nabulsi *et al.*, 1994; Songstad *et al.*, 1995). Another approach has been to use a high-voltage electrical discharge through a water droplet to create a shock wave and accelerate a thin sheet carrying the DNA-coated particles (McCabe *et al.*, 1988). Overall, microprojectile bombardment has so far proved to be the most versatile method for cereal transformation and has been used to transform all major species (*Table 1*).

Particle bombardment efficiently delivers DNA into the surface layers of the target material. In multicellular material, potentially transformed cells are connected to and surrounded by non-transformed cells. Consequently, the successful recovery of transformed cultures depends on an efficient and discriminating means of selecting for the proliferation of those cells which carry the introduced DNA. As with other methods of transformation, selection has been achieved using herbicides or antibiotics. Among the widely applied selectable marker genes in cereals, is the *bar* gene from *Streptomyces hygroscopicus* which codes for phosphinothricin N-acetyl transferase (PAT) and confers resistance to phosphinothricin based herbicides such as bialaphos and Basta (Spencer *et al.*, 1990; Wilmink and Dons, 1993; Dennehey *et al.*, 1994). Alternative strategies have employed mutant plant genes for resistance to herbicides

Table 1. Chronology of development of methods for the genetic transformation of cereals. The dates refer to the first substantiated example of stable gene transfer using a given method for the different species. References are provided in the text.

	Microprojectile bombardment	Direct gene transfer to protoplasts	Agrobacterium	Other methods
Rice	1991	1988	1994	
Wheat	1992	1994		
Barley	1994	1995		
Maize	1990	1988		1992
Sorghum	1993			
Oats	1992			
Rye	1994			

such as the sulphonylureas (Fromm *et al.*, 1990; Wilmink and Dons, 1993; Chamberlain *et al.*, 1994) or glyphosate (Vasil *et al.*, 1991). Effective selection of transformed tissues and plants has also been achieved with antibiotics such as kanamycin, geneticin, paromomycin and hygromycin B and corresponding bacterial genes for resistance, *nptII* and *hpt* (Vasil *et al.*, 1991; Bower and Birch, 1992; Li *et al.*, 1993; Hagio *et al.*, 1995; Torbert *et al.*, 1995).

The recovery of transformed plants from the bombarded tissues depends on the ability to regenerate plants from the target cells. Initial success was achieved using established embryogenic suspension cultures as target tissue, for example with maize (Gordon-Kamm *et al.*, 1990; Fromm *et al.*, 1990), rice (Cao *et al.*, 1992), wheat (Vasil *et al.*, 1992) and oats (Somers *et al.*, 1992). However, such long term cultures have a number of disadvantages: they are difficult to establish and the longer they are maintained, the greater is the likelihood that deleterious mutations will have accumulated in the cell lineages which will eventually produce the regenerated plants. In subsequent experiments, embryogenic callus tissues from solid culture media have also been found to provide suitable target materials (Bower and Birch, 1992; Somers *et al.*, 1994; Wan, Widholm and Lemaux, 1995). To obviate the difficulties associated with maintaining embryogenic cultures, there is currently a trend towards using material directly from the plant for bombardment. Immature embryos have been found to be a suitable source of actively dividing tissues for different species such as rice (Christou, Ford and Kofron, 1991, maize (Koziel *et al.*, 1993), barley (Ritala *et al.*, 1994; Wan and Lemaux, 1994), sorghum (Casas *et al.*, 1993), wheat (Vasil *et al.*, 1993; Weeks, Anderson and Blechl, 1993; Becker, Brettschneider and Lörz, 1994; Nehra *et al.*, 1994), triticale (Zimny *et al.*, 1995) and rye (Castillo, Vasil and Vasil, 1994). The target is the part of the embryo, the scutellum, which in the intact cereal grain is the tissue in contact with the developing endosperm. The cells in the surface layers of the scutellum will undergo somatic embryogenesis when a cereal embryo at an appropriate stage of development is placed on a culture medium with the embryo axis in direct contact with medium. Histological studies in barley have confirmed that cells in the scutellar epithelium and sub-epithelial layers participate in the production of embryogenic tissue, and thus provide a suitable target for transformation (Ryschka, Ryschka and Schulze, 1991). An alternative approach has been to target the developing shoot meristem of very young embryos in maize, resulting in the production of

chimaeric meristems from which germline transformants can be recovered (Lowe *et al.*, 1995). This was achieved by culturing sections of the shoot apex on medium containing cytokinin to stimulate shoot proliferation. The method appears to be adaptable to a range of genotypes, including elite inbred lines.

Frequencies of transformation reported for microparticle bombardment are variable, but most commonly lie within the range of 0.1%–1.0% for the percentage of shot explants yielding a transformed cell line. The numbers can often be confounding, as multiple plantlets can be regenerated from a single embryogenic culture. It is therefore important to distinguish between clones and independently transformed plants. The highest frequencies, greater than 5%, have been quoted for rice (Christou, Ford and Kofron, 1991), while wheat transformation appears to be at least ten times less efficient (Weeks, Anderson and Blechl, 1993; Becker, Brettschneider and Lörz, 1994).

Another target that has been used with some success is the immature inflorescence which has been shown to be capable of initiating embryogenic cultures in a number of cereal species (Brettell, Wernicke and Thomas, 1980; Ozias-Akins and Vasil, 1982; Rangan and Vasil, 1983). Barcelo *et al.* (1994) were able to recover transgenic tritordeum plants from inflorescences given a short period of preculture, with the highest frequency of 17 transformants for 178 inflorescences achieved when the tissue was placed in culture the day prior to bombardment. An alternative approach has been to target microspores. These are immature pollen grains, which, although they only have a haploid chromosome complement, are able to divide and regenerate plantlets. This approach has been tried in a number of laboratories, particularly with barley, and there is evidence that it can be used for the recovery of transformed plants. In one example, transformed plants were recovered at a frequency of 1 in 10^7 microspores bombarded (Jähne *et al.*, 1994). It has yet to be demonstrated whether this approach of using gametic cells will be generally applicable for transforming a range of cereal species.

DIRECT GENE TRANSFER TO PROTOPLASTS

Protoplasts are single plant cells from which the cell wall has been removed, and they can provide a large and uniform population of target cells for the introduction of DNA. Plants were first regenerated from tobacco protoplasts more than twenty years ago (Nagata and Takebe, 1971). The technique has since been extended to a range of cereals, following initial success with rice (Abdullah, Cocking and Thompson, 1986; Yamada, Yang and Tang, 1986; Kyozuka, Hayashi and Shimamoto, 1987). However, the recovery of plants from protoplasts is still technically demanding and cannot be achieved routinely for all species.

Populations of protoplasts are prepared by digesting plant tissues in a solution containing a mixture of cellulytic and pectolytic enzymes. DNA, as circular or linearised plasmid, can be introduced into isolated protoplasts following treatment with polyethylene glycol to facilitate the transport of macromolecules through the plasma membrane (Krens *et al.*, 1982; Potrykus *et al.*, 1985; Maas and Werr, 1989). Alternatively the protoplasts can be subjected to electroporation whereby an electric field is used to drive the uptake of DNA (Fromm *et al.*, 1985; Nagata, 1989; Larkin *et al.*, 1990). As techniques have been developed to regenerate plants from cultured

cereal protoplasts, direct gene transfer has been used to produce transgenic plants of rice (Toriyama *et al.*, 1988; Shimamoto *et al.*, 1989; Zhang *et al.*, 1988) and maize (Rhodes *et al.*, 1988).

Application of direct gene transfer to protoplasts is still severely limited by the difficulties encountered in regenerating plants from the cultured protoplasts. In cereals, regeneration of plants has only been achieved from protoplasts isolated from embryogenic cultures, and generally from those kept in liquid suspension. There are no confirmed reports of regeneration of plants from protoplasts isolated directly from an intact cereal plant. Embryogenic suspension cultures are difficult to establish and maintain, and the length of time required to establish suitable cultures appears to favour the accumulation of deleterious mutations. Thus for example, a common feature of barley regenerated from protoplasts is a high frequency of albino, chloroplast-deficient and therefore inviable plants. With wheat and barley, successful regeneration of plants from protoplasts has been achieved independently in a number of laboratories (Harris *et al.*, 1988; Ren *et al.*, 1989; Vasil, Redway and Vasil, 1990; Chang *et al.*, 1991; Jähne, Lazzeri and Lörz, 1991; He, Yang and Scott, 1992; Li *et al.*, 1992; Qiao *et al.*, 1992; Ahmed and Sagi, 1993; Pauk *et al.*, 1994), although the plants recovered in many of these cases lacked vigour and were sterile. While transformed plants have been obtained from protoplasts (He, Yang and Scott, 1994; Funatsuki *et al.*, 1995), there are to our knowledge still no examples where significant numbers of fertile transgenic wheat or barley plants have been recovered by this method. In maize, successful regeneration of plants is limited to a few genotypes but has led to the recovery of transformed plants (Rhodes *et al.*, 1988; Golovkin *et al.*, 1993; Sukhapinda *et al.*, 1993). Rice, on the other hand, appears to be more flexible, with protoplast transformation finding wide application (Toriyama *et al.*, 1988; Zhang *et al.*, 1988; Shimamoto *et al.*, 1989; Datta *et al.*, 1990, 1992; Fujimoto *et al.*, 1993; Shimamoto *et al.*, 1993, Uchimiya *et al.*, 1993; Chamberlain *et al.*, 1994).

AGROBACTERIUM

Genetic manipulation of plant tissues is far from being a recent innovation. Crown gall disease, which affects a number of dicotyledonous species, results from the introduction of foreign DNA into plant cells at sites of wounding, from a Ti (tumour inducing) plasmid harboured by the pathogenic bacterium *Agrobacterium tumefaciens* (Binns and Thomashow, 1988). The Ti-plasmid contains genes which alter the plant host's metabolism in favour of the pathogen. This naturally occurring genetic engineer has been exploited to mediate the introduction into target plant cells of a number of 'genes of interest' following their insertion into the segment of DNA, the T-DNA, transferred from the Ti-plasmid to the host plant.

Agrobacterium has found wide application as a vector for plant transformation, including species that are outside the natural host range of this bacterium. However, cereals have shown a notable reluctance to submit to transformation by Agrobacterium, although it has been demonstrated that infectious viral sequences can be introduced into cereal tissues through a process described as agroinfection (Grimsley *et al.*, 1987; Dale *et al.*, 1989). In these examples genomes from Geminiviruses, maize streak virus or wheat dwarf virus, were cloned into the T-DNA of *Agrobacterium tumefaciens*. Inoculation of a host plant with the bacteria resulted in systemic viral infection.

Attempts to modify this process and use viral vectors for the stable transformation of cereal tissues were unsuccessful, and it was concluded by some authors that the chances of transforming cereals with Agrobacterium were minimal, on the grounds that cereals lack the necessary wound response (Potrykus, 1990). It was argued that transformation would only occur at a wound site and any competent cells at or adjacent to the wound would be unlikely to survive the damage necessary to expose the cells to Agrobacterium.

There have been a number of isolated examples of cereal transformation using Agrobacterium; for example in rice (Raineri *et al.*, 1990; Chan, Lee and Chang, 1992; Chan *et al.*, 1993), maize (Gould *et al.*, 1991), wheat and barley (Deng, Lin and Shao, 1990; Mooney *et al.*, 1991). A lack of confirmation of these results in other laboratories cast some doubt on the validity of these experiments. However, in the past year a comprehensive study has been made in which transformation of japonica rice was achieved through co-cultivation of immature embryos, scutellum callus or suspension cells with *Agrobacterium tumefaciens* (Hiei *et al.*, 1994). Supporting the contention that DNA transfer was occurring in an analogous manner to that in dicotyledonous plants, sequence data showed that the boundaries of the T-DNA were similar to those found in transgenic tobacco with respect to short duplicated sequences. Expression and inheritance of the transgenes, coding for GUS and HPT, were demonstrated over three generations, providing further evidence for stable integration of the transgenes.

These results have since been extended to maize (Y. Hiei and co-workers, unpublished data), and provide an exciting prospect for transformation of other small grain cereals. Agrobacterium mediated transformation would provide an attractive alternative to microprojectile bombardment, particularly in species such as wheat and barley where high rates of transformation have been claimed by few laboratories. In the case of japonica rice, Hiei *et al.* (1994) reported an efficiency of transformation of scutellum-derived callus pieces of between 12% and 29%, which is comparable to that obtained for dicotyledonous plants. This efficiency of transformation would certainly rival the best that can be achieved for rice transformation by microprojectile bombardment.

OTHER METHODS FOR CEREAL TRANSFORMATION

Plant cells present many obstacles to the introduction of exogenous DNA, not least of which is the presence of a thick cellulosic cell wall. In addition extracellular nucleases, which degrade any unprotected DNA molecules, can be produced in abundance. Depending on the tissue, plant cells with large vacuoles and other compartments may also have correspondingly small nuclei as a target for gene transfer. In the absence of really efficient methods for cereal transformation, a number of other approaches have been tried in order to overcome these barriers to introducing exogenous gene sequences into the nucleus where integration into genomic DNA can occur.

One of the more promising approaches has been the use of silicon carbide fibres with an average diameter less than 1 µm (Kaeppler *et al.*, 1990; Wang *et al.*, 1995). The method involves vortexing a mixture of DNA, silicon carbide fibres and plant cells. The silicon carbide fibres act to pierce the plant cell wall allowing entry of DNA. This method has been used to produce fertile, transgenic maize plants following treatment of embryogenic suspension cultures (Frame *et al.*, 1994). However, it is not

clear that the method offers any obvious advantages over particle bombardment with the exception of the low cost of setting up the system. Another development has been the use of fine laser beams to puncture holes in the cell wall and plasma membrane, and this technique has recently been applied to rice transformation (Guo, Liang and Berns, 1995)

Among other methods which have been applied to cereals are injection of developing inflorescences with DNA solutions (de la Pena, Lörz and Schell, 1987), application of DNA to florets near the time at which pollination occurs providing an opportunity for transformation by the so called 'pollen tube pathway' (Luo and Wu, 1988), treating floral organs with Agrobacterium (Hess, Dressler and Nimmrichter, 1990), electrophoresis of DNA into seed tissues (Ahokas, 1989), and electroporation of intact tissues and cells (Li *et al.*, 1991; D'Halluin *et al.*, 1992; Klöti *et al.*, 1993; Zaghmout and Trolinder, 1993; Zhou, Stiff and Konzak, 1993; Laursen *et al.*, 1994). Of these methods, electroporation of intact cells and tissues appears to be the most promising. The other approaches have a poor record: the experiments have not been satisfactorily reproduced, and the evidence for integration of the DNA into the plant genome has been lacking. Moreover, most observations have been confined to first generation plants, and inheritance of putative transgenes has not been examined. It has been suggested that some of the results may have been due to artefact, such as the transformation of endophytic microorganisms suggested by Langridge *et al.* (1992). The market is always open for novel techniques for cereal transformation, but those investing effort in applying a new method should be cautious until it has been satisfactorily reproduced in other laboratories. Meanwhile it appears that existing methods can provide routes for achieving reliable and routine transfer of genes into cereal crop species.

Gene expression in transgenic cereals

STRATEGIES FOR INTRODUCING GENES INTO CEREALS

None of the methods described in the preceding section for delivering DNA into plant cells are so efficient that transformed plants can be routinely recovered without some additional process to enrich or select for the transformed cells. The selection of transformed cells is generally achieved in tissue culture through the use of a selectable marker gene in combination with the corresponding selective agent. For cereals, both antibiotics and herbicides have been shown to be effective (Wilmink and Dons, 1993).

The strategy for transfer of a gene of interest is to introduce this gene at the same time as the selectable marker gene. For methods such as microprojectile bombardment and direct gene transfer to protoplasts which do not involve the construction of a specialised plasmid vector, the two sets of DNA sequence can simply be mixed prior to the transformation. For example, in the case of electroporation of protoplasts, the two types of DNA are added and mixed into the electroporation buffer just before the electric field is applied. Under these conditions, a high proportion of the transformants selected with the selectable marker gene are also found to carry one or more copies of the non-selected gene of interest. This frequency of co-transformation is commonly recorded as greater than 50% and in some studies with microprojectile bombardment has been reported to be as high as 85%–90% (Barcelo *et al.*, 1994; Wan and Lemaux,

1994). To ensure co-integration of a gene of interest and the selectable marker gene, other researchers have adopted the strategy of using single plasmids containing the two genes (e.g. Fromm *et al.*, 1990; Becker, Brettschneider and Lörz, 1994; Cooley, Ford and Christou, 1995; Lin *et al.*, 1995).

For all methods of plant transformation, the transfer of exogenous DNA is essentially a random process with respect to the site of insertion into the genome. However, there is evidence from work with Agrobacterium that integration occurs preferentially into transcriptionally active sites (Koncz *et al.*, 1989). For the few examples in cereals in which a thorough study has been made of the inheritance of transgenes, the data indicate that multiple copies are frequently inserted at a single locus (Spencer *et al.*, 1992; Register *et al.*, 1994; Cooley, Ford and Christou, 1995; Peng *et al.*, 1995).

In the development of a new transformation procedure, the inclusion of a reporter gene can help gauge the efficiency of gene transfer. The most widely used reporter is the β-glucuronidase (*gus*) gene whose product hydrolyses a range of β-glucuronide substrates. Thus transformed cells can be visualised following incubation in the presence of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) which is cleaved to produce an insoluble blue precipitate (Jefferson, Kavanagh and Bevan, 1987). However, there are differences in opinion as to its reliability as a marker in cereals. In rice, the *gus* gene has been used to visualise the tissue specificity of expression determined by a range of promoter sequences (Kyozuka *et al.* 1993, 1994; Itoh *et al.*, 1995; Terada *et al.*, 1995). However, in sorghum and wheat, there are instances in which gene activity has not been detected even though presence of the *gus* gene has been confirmed by gel blot hybridisation (Casas *et al.*, 1993; I.K. Vasil, personal communication). Alternative reporters include firefly luciferase (de Wet *et al.*, 1987; Chia, Chan and Chua, 1994), aequorin from the jellyfish *Aequorea victoria* (Baulcombe, Chapman and Cruz, 1995; Niedz, Sussman and Satterlee, 1995), and *trans*-acting factors that regulate anthocyanin biosynthesis (Goff *et al.*, 1990; Bodeau and Walbot, 1995). However, the ideal reporter gene is still elusive. This would exhibit low background activity, have only moderate stability *in vivo*, have no detrimental effects on metabolism, and be easy to assay quantitatively (McElroy and Brettell, 1994)

REGULATION OF GENE EXPRESSION IN TRANSGENIC CEREALS

The application of gene transfer technology for cereal crop improvement will depend on a clear understanding of the molecular elements that regulate gene expression in plants. Promoters that have found general use in broad-leaved plants for driving constitutive levels of gene expression do not necessarily perform as well in cereals. For example, when a *gus* gene controlled by the 35S promoter from cauliflower mosaic virus was introduced by electroporation into protoplasts isolated from five different cereal cell lines, GUS activities ranged from 0.3% to 10.8% of the activity in *Nicotiana plumbaginifolia* protoplasts (Last *et al.*, 1991). The low efficacy of the 35S promoter in cereals is also seen with DNA introduced by microprojectile bombardment, both in transient expression (Schledzewski and Mendel, 1994) and in the selection of transformed cultures (Z.Y. Li, N.M. Upadhyanya, A.J. Gibbs and P.M. Waterhouse, unpublished results). This suggests that there may be differences between cereals and dicotyledons in the recognition of promoter sequences, due for example to

different affinities for transcription factors. This is further supported by observations that some cereal promoters show poor expression in cells of dicotyledons (Ellis *et al.*, 1987; Yamaguchi-Shinozaki *et al.*, 1990).

Promoter sequences are now available which have been found to give suitable levels of constitutive expression in transformed cereal tissues (McElroy and Brettell, 1994). Not surprisingly many of these have been derived from cereal genes. Examples that have been widely tested are promoters from the rice actin 1, *Act1*, gene (Zhang, McElroy and Wu, 1991) and a maize ubiquitin gene, *Ubi1* (Christensen, Sharrock and Quail, 1992). The strategy of including an intron in the transcribed portion of the gene has commonly been used to further enhance expression (Callis, Fromm and Walbot, 1987; Luehrsen and Walbot, 1991; McElroy *et al.*, 1991; Li *et al.*, 1995). Levels of gene transcription can also be increased by the addition of *cis*-acting elements such as in the pEmu promoter where ocs-elements from Agrobacterium have been combined with the *Adh1* promoter from maize (Last *et al.*, 1991).

The application of genetic engineering to modify characters such as grain quality and disease resistance in cereals will depend on promoters that control gene expression in a tissue specific manner. Tissue specific gene expression is desirable because the expression of transgenes in tissues where they are not required may drain the resources of the plant and result in deleterious effects such as stunting, increased susceptibility to pathogen attack and reduction in yield. For disease resistance, expression of resistance genes in tissues colonised by the pathogen may be sufficient for protection. Thus for barley yellow dwarf virus which is limited to phloem tissues of the host plant, a promoter such as the *rolC* promoter from *Agrobacterium rhizogenes* might be a suitable candidate when constructing genes that interfere with viral replication. This promoter shows a specificity of expression limited to vascular and embryogenic tissues (Matsuki *et al.*, 1989).

Similarly for genes influencing grain composition, such as those coding for seed protein or for enzymes involved in starch biosynthesis, it will be necessary to limit expression of transgenes to the developing endosperm. In a recent series of experiments with wheat, Blechl, Weeks and Anderson (1995) demonstrated expression of an intro-duced chimaeric Dy10-Dx5 high molecular weight glutenin gene linked to a wheat glutenin promoter. Following transformation by microprojectile bombardment, the majority of lines resistant to the selective agent bialaphos exhibited co-expression of the new storage protein gene in endosperm. The transgene was inherited over two genera-tions and levels of novel protein produced were comparable to those produced by the native glutenin genes. This result has been substantiated by studies on tritordeum, a hexaploid hybrid between tetraploid durum wheat and a diploid wild barley. A tritor-deum line was transformed with a glutenin Dx5 gene, again under the control of a wheat glutenin promoter (P.A. Lazzeri, P. Barcelo, F. Barro, A.S. Tatham, R. Fido and P.R. Shewry, unpublished results). The expression of the glutenin subunit is shown in *Figure 1*.

In contrast to broad-leaved dicot plants, there are relatively few studies of the regulation of gene expression in transgenic cereals (McElroy and Brettell, 1994). In those cases where tissue specific promoters have been introduced from a dicotyledon, the pattern of gene expression in the cereal is consistent with that observed in the source plant. Thus a tomato *rbcS* promoter was active in mesophyll cells of transgenic rice (Kyozuka *et al.*, 1993), although activities were less than those recorded for a rice

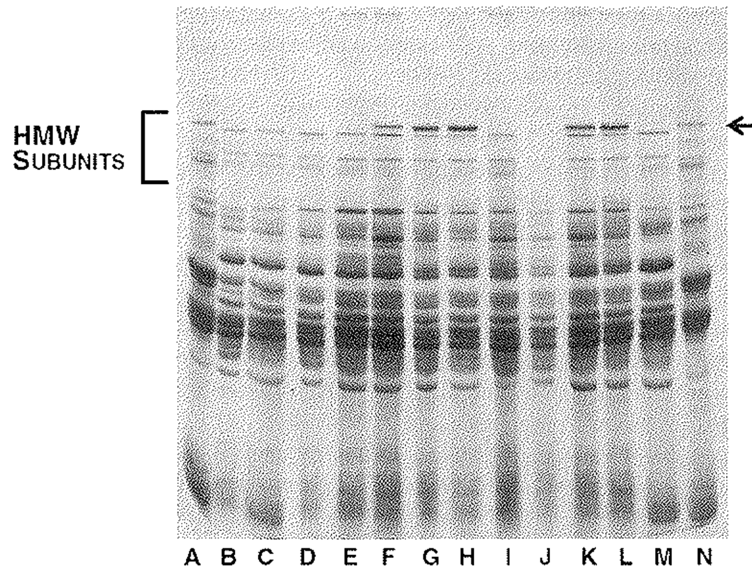

Figure 1. Expression of a wheat high molecular weight glutenin subunit in seeds of transgenic tritordeum. Lanes A and N are bread wheat controls, the presence of the 1Dx5 subunit is seen in lanes F, G, H, K and L (courtesy Paul A. Lazzeri).

rbcS promoter attached to the same *gus* reporter gene. The promoter of a barley aleurone-specific gene has been shown to confer aleurone cell-specific expression in transgenic rice (Kalla *et al.*, 1994). The regulation of expression by the promoter from a rice α-amylase gene has similarly been studied in transgenic rice (Itoh *et al.*, 1995). To ensure consistent levels of transgene expression, the available data would support a strategy of seeking homologous promoters for cereals, before utilising promoters from other plant genera.

GENE INACTIVATION IN TRANSGENIC CEREALS

Many of the descriptions of cereal transformation are confined to the expression of selectable marker and reporter genes, and to material growing in the glasshouse for one or two generations following gene transfer. As transgenic plants are grown more widely and examined under field conditions, it is found that examples are accumulating for transgenes being subject to progressive inactivation. This phenomenon has been discussed in detail in a recent review (Finnegan and McElroy, 1994), and it is clear that cereals provide no exception. For one study where a set of transgenic rice plants carrying multiple copies of the *nptII* gene was examined in the field, no plants could be found which expressed the antibiotic resistance coded by the transgene (Schuh *et al.*, 1993). In oats, six out of fifteen lines with a transgene for GUS showed aberrant segregation ratios with a higher than expected number of plants scoring negative for activity of the enzyme (Somers *et al.*, 1994). In a study of the inheritance of *gus* and *nptII* genes in rice, irregular expression of both genes was found in two of three families examined (Peng *et al.*, 1995). With transformed rice plants shown to contain at least one copy of both *gus* and *bar*, over 90% of the plants expressed bar but only 50% expressed gus (Cooley, Ford and Christou, 1995). In wheat, a study of six

independent lines transformed with *gus* and *bar* revealed differences in the stability of expression of the two transgenes (V. Srivastava, V. Vasil and I.K. Vasil, unpublished results). One line showed unstable expression of *bar*, but for five of the six lines GUS activity was not detectable in the T_2 generation even though presence of the *gus* gene was confirmed.

There is clear evidence that copy number and the position of a transgene in the plant genome will influence the level at which it is expressed (Hobbs, Kpodar and Delong, 1990; Linn *et al.*, 1990; Assaad, Tucker and Signer, 1993). In a recent study of transgenic rice, all plants with one or two copies of *gus* driven by the 35S promoter showed expression, whereas none of the plants with more than ten integrated copies of the gene showed GUS activity (Cooley, Ford and Christou, 1995). The insertion of multiple copies of a given sequence is associated with reduced gene expression and is commonly observed when DNA is introduced by any of the techniques that employ direct gene transfer, e.g. PEG treatment of protoplasts, particle bombardment (Peng *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Somers *et al.*, 1992; Barcelo *et al.*, 1994; Register *et al.*, 1994; Cooley, Ford and Christou, 1995; Dalton *et al.*, 1995). The use of these techniques may therefore be a contributory factor to the high incidence of uneven expression of transgenes in cereals. Another feature which may determine the tendency of a transgene to be silenced is the extent to which it can be recognised as foreign in its new position in the plant genome. Disruption of compositionally homogeneous chromatin by the integration of foreign DNA may mark a region of the chromosome for inactivation (Meyer and Heidmann, 1994). The size and GC content of the transgene, the structure of the promoter, and the 3' sequences may all be important parameters in this regard. Such factors may explain the high incidence of inactivation seen in cereals for the *gus* reporter gene, compared to some of the selectable marker genes commonly used.

Inactivation of transgenes is frequently associated with specific methylation of DNA (Finnegan, Brettell and Dennis, 1993; Meyer and Heidmann, 1994). The occurrence of methylation as a cause or consequence of transgene inactivation, however, is still a matter of debate. However, the following experiments support a direct role for methylation. The long term expression and methylation status of *hpt* and *gus* genes were examined in transgenic pearl millet cells obtained by microprojectile bombardment. During long term culture expression of the *hpt* gene was maintained while there was a gradual decrease in measurable GUS activity, which could be recovered by exposing cells for two weeks to 10μM of the demethylating agent 5-azacytidine (Lambé, Dinant and Matagne, 1995). The inactivation of the *gus* gene was correlated with progressive methylation, revealed by isoschizomeric restriction enzymes, which differ in their ability to cut at sites with methylated cytosine residues. A further example suggesting the involvement of methylation is provided by experiments with rice. In one family of japonica rice transformed with the *gus* gene driven by a modified 35S promoter, consistent uneven expression of GUS was observed in leaves and roots when visualised with a histochemical X-Gluc stain. Rows and blocks of cells with intense blue staining were interspersed with non-staining cells, resulting in a 'spotty' phenotype (B.Witrzens and R. Brettell, unpublished). When seeds of this rice line, homozygous for the *gus* transgene, were germinated on culture medium containing the demethylating agent 5-azacytidine at a concentration of 60μM, uniform blue staining was observed in root tissues in contact with the medium indicating

that GUS activity had been restored. The effect was, however, transitory and was not observed in the leaf tissues.

The precise mechanisms by which foreign DNA sequences are recognised and inactivated remain obscure. The situation is complicated in that gene silencing can be due to transcriptional inactivation or to post-transcriptional events (Flavell, 1994; Matzke and Matzke, 1995). In other plant species, transcriptional inactivation is likewise frequently associated with specific methylation of the transgene, as seen in experiments with *Petunia hybrida* where a chimaeric maize *A1* gene was introduced into a white flowered strain to produce plants with brick-red flower colour (Meyer *et al.*, 1987). Variegation and loss of flower colour was observed among plants in a field trial, and inactivation of the transgene was correlated with increased cytosine methylation in the 35S promoter region derived from cauliflower mosaic virus (Linn *et al.*, 1990; Meyer and Heidmann, 1994). There may be multiple mechanisms by which plants are able to recognise and specifically inactivate exogenous DNA sequences, and these may involve specific DNA-DNA or DNA-RNA interactions (Matzke and Matzke, 1995).

A better understanding of transgene inactivation will help formulate strategies for improving transgene stability under field conditions (Finnegan and McElroy, 1994, 1996 [in the press]). These include selection for single copy transgene insertion events and development of site-specific recombination systems. The inclusion of matrix attachment regions with the introduced gene(s) may mitigate the effects of sequences adjacent to the site of integration (Allen *et al.*, 1993; Mlynárová *et al.*, 1994). Another strategy that is being developed is transposon-mediated delivery of a transgene. For example a gene of interest can be introduced into barley on the *Ds* transposable element from maize. In the presence of an active maize *Ac* element, transposition of single copies of the gene can be induced (D. McElroy, personal communication). Such a system has the dual advantages of providing a means of ensuring single copy integrations, while at the same time enabling the gene of interest to be separated from the selectable marker gene whose presence in the field may not be desirable.

Concluding remarks

The past five years have seen rapid advances in the application of gene transfer technology to cereals. Methods have been developed for genetic transformation of all major crop species, and information is becoming available about the behaviour of transgenes in transgenic cereal plants. Relative efficiencies of transformation appear to be greater with rice and maize than with wheat, barley and oats; however, this may to some extent reflect the amount of research effort expended on the different species.

While there are only a limited number of examples of genes other than selectable markers being expressed in transgenic cereals, the information to hand suggests that the constraints on stable expression are not fundamentally different to those observed in broad-leaved plant species such as tobacco for which transgenic plants have been available for more than a decade. A proportion of transgenes seem to be subject to inactivation, which may depend on position of integration in the genome, copy number and sequence organisation of the introduced DNA. Further refinement of the transformation technology will make it feasible to generate greater numbers of independent

transformants and select those that have the required levels of stable expression.

The agronomic performance of transgenic cereals is insufficiently researched, but will be an important consideration in future studies. By analogy with other species, in most cases it will be necessary to undertake a programme of back-crossing for the following reasons. There is wide genotypic variation in tissue culture response among the cereals, meaning that a particular method of transformation can be limited to certain cultivars or strains. Further, the accumulation of undesirable somaclonal variation (Larkin and Scowcroft, 1981) is associated with an extensive phase of tissue culture which is a feature of all current methods of cereal transformation. Thus, even if transformation can be achieved for the cultivar of interest, it is recommended that the primary transformant is crossed to parental seed grown material to remove any deleterious mutations.

The next decade will be decisive in determining the contribution gene transfer technology can make to crop improvement. The technology must be viewed as an adjunct to, rather than a replacement of, existing breeding methods. The precise tailoring of individual genes provides a powerful additional tool, but does not obviate the necessity of exhaustive testing of any new breeding material generated. The adoption of genetic engineering for cereals will also strongly depend on public acceptance of the technology, and demonstration that genetic modification can result in safe cereal products which provide, directly or indirectly, most of the nutrition for the world's population.

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