Advances in cereal gene transfer

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Over the past five years, transgenic strains of various major cereals have been produced, with transformation of rice and maize being most common. A majority of the cereal transformants obtained to date has been generated by the particle bombardment technique, but *Agrobacterium*-mediated transformation is rapidly becoming the method of choice. Rice, the plant in which transformation-related technology is most advanced, appears to be the model monocotyledon for basic and applied studies.

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Current Opinion in Plant Biology 1998, 1:161–165

http://biomednet.com/elecref/1369526600100161

 ISSN 1369-5266

Introduction

The development of methods for the genetic transformation of cereals was delayed for some time as compared to the initial success in dicotyledonous species. The major cause of the delay was the fact that transformation mediated by the soil bacterium *Agrobacterium tumefaciens* was not readily applicable to cereal plants. Cereals have been transformed primarily by direct transformation methods, such as particle bombardment, which has been the most popular technique to date. It has been demonstrated, however, that *A. tumefaciens* can efficiently transform cereals such as maize and rice. With advances in gene transfer technology, more and more genes have been transferred to cereals for various purposes. In this review, we shall discuss the latest developments in the transformation of cereal plants. We shall focus on advances in gene transfer techniques for cereals and we shall also mention specific genes that have recently been introduced into cereal plants, as well as various issues related to the integration and expression of foreign genes.

Methods of gene delivery

Several years ago, most reports of gene transfer to cereals dealt with the transient expression of foreign genes. Now, by contrast, the number of papers that describe production of transgenic cereals is increasing rapidly. Two species, rice and maize, have been heavily favored in such studies because of their economic importance and the availability of tissue culture technology. In the transformation of cereals, immature embryos have been the most popular starting material and the *bar* gene, which encodes phosphinothricin acetyltransferase, has been the most widely used selective marker.

Direct transformation methods

In the majority of recent studies, particle bombardment was used to transform plant materials directly. Target tissues are bombarded with highly accelerated particles coated with DNA for the transfer of genes to cereals. Since the prior culture of protoplasts is not necessary, this technique has been preferred to methods that involve electroporation or polyethyleneglycol. Convenient systems for particle bombardment have been commercially available for several years, and methods have been optimized for various plant species.

Maize has been used more than rice in the development of new transformation techniques [1–3] and for assays of promoters [4–6]. In one new technique, magnetic particles are used in combination with magnetic selection after bombardment to increase the frequency of transformation (number of independent transformants / number of treated tissue pieces × 100%) [2]. Studies in rice have focused predominantly on efforts to produce transformants with agronomically important genes [7•,8•,9,10].

There are some reports of particle bombardment for transformation of other cereals. Considerable variations in the frequency of transformation of immature embryos, ranging between 0.00% and 1.71%, were found among wheat cultivars [11]. Fluorescence *in situ* hybridization was used to localize transgenes delivered to wheat, barley and triticale [12]. An analysis of transgenic wheat with the gene for a seed storage protein has also been described [13].

Particle bombardment is especially powerful for the analysis of the transient expression of foreign genes in plant cells because intact, fully developed tissues can be targeted. The expression of chimeric genes, consisting of promoters, other controlling elements and reporter genes, can be conveniently assessed after bombardment of various tissues. Results of such analyses should be interpreted with care because tissues are no longer intact after they have been bombarded. This technique is probably more suitable for studies of positively regulated gene expression than of negatively regulated expression. Transient expression can also be exploited in studies of the tissue-specific accumulation of particular proteins, such as a γ-zein in the endosperm in maize [14], or the activities of genetic elements, such as transposition of transposons Ac/Ds in intact cells of barley [15].

Use of other direct transformation methods has been limited. There are two reports of tests of ‘silicon carbide

whisker' methods, in which silicon carbide fibers act as needles for the microinjection of DNA into plant cells, in wheat [16], rice [17] and maize[18], respectively. Methods involving rice protoplasts have also been described [19••,20•].

Agrobacterium-mediated transformation

The soil phytopathogen *A. tumefaciens* has been utilized routinely for transformation of dicotyledonous plants. The advantageous features of *Agrobacterium*-mediated transformation include the transfer of pieces of DNA (T-DNA) with defined ends and with minimal rearrangement, the transfer of relatively large segments of DNA, the integration of small numbers of copies of genes into plant chromosomes, and the high quality and fertility of resultant transgenic plants. The background and underlying mechanisms of such transformation have been discussed in recent reviews [21,22]. It appeared until recently that monocotyledons were beyond the range of this technology. Various attempts to infect monocotyledons with *Agrobacterium* were made in the 1970s and 1980s, but no conclusive evidence of integrative transformation was obtained until quite recently.

Efficient protocols for *Agrobacterium*-mediated transformation were reported for Japonica rice in 1994 [23], and subsequently for Javanica [24] and Indica [25] rice. A key point in the various protocols is the use of tissue that consists of actively dividing, embryonic cells, such as calli induced from scutella. Such cells are co-cultivated with *Agrobacterium* in the presence of acetosyringone, a potent inducer of the *Agrobacterium* genes that are involved in the transfer of DNA. Transgenic rice has also been efficiently produced from immature embryos of Japonica and Indica rice [26], and at a lower frequency from isolated shoot apices [27]. The conditions that support the active growth of plant cells in tissue culture facilitate selection and shorten the time required for the transformation procedure [28]. *Agrobacterium*-mediated transformation has been used to monitor the tapetum-specific expression of the promoter of the rice *Osg6B* gene in rice [29•].

Efficient *Agrobacterium*-mediated transformation is now also possible in another important cereal, namely, maize [30••]. Immature embryos are inoculated with *Agrobacterium* and the frequency of transformation can be quite high, varying between 5% and 30%. Transgenic barley plants were recently obtained from immature embryos that had been infected with *Agrobacterium* [31••]. Furthermore, transgenic wheat plants were obtained from immature embryos and embryogenic calli that had been infected with *Agrobacterium* (see note added in proof). The frequencies of transformation were somewhat lower than frequencies in studies with rice and maize but are likely to increase as a result of improvements in methodology.

'Agroinfection', the process by which the sequence of a viral genome can be introduced into a higher plant via *Agrobacterium*, with the resultant systemic infection of the host plant by the virus, was exploited in a study of the transfer of T-DNA in meristematic cells of maize that harbored intracellular *Agrobacterium* [32] and in a study of the functions of an *Agrobacterium* virulence gene during agroinfection [33]. Early transcription of genes of *Agrobacterium* T-DNA in tobacco and maize has been analyzed and it seems that the difficulties encountered in attempts to transform maize might involve integration of the T-DNA and not the entry of T-DNA into cells or targeting of the T-DNA to the nucleus [34•].

'Super-binary vectors' have been used in the development of methods for the *Agrobacterium*-mediated transformation of rice and maize. They carry a DNA fragment from a strain of *Agrobacterium* that functions very efficiently in transformation [23,30••]. Moreover, novel super-binary vectors, which include two separate T-DNAs and are suitable for co-transformation, have recently been constructed [35••]. Co-transformation, with marker genes and other genes on different DNA molecules, might have a number of advantages. For example, construction of polynucleotides might be simplified. Marker genes and other genes might segregate independently and transgenic plants, free from selective markers, might be obtained in later generations. Both the efficiency of transformation and the frequency of the unlinked integration of transgenes were high when rice was treated with the new co-transformation vectors. Thus, marker-free transformants of cereal plants can now be produced efficiently.

Genes that have been introduced into cereal plants

Various genes have been transferred to cereal plants. Production of transgenic plants that carry genes related to agronomically important traits has been reported mainly in rice, primarily because methods of transformation have been well established for this cereal.

Some of the genes that have recently been studied are related to resistance to disease and insects. A grapevine stilbene synthase gene, which is involved in biosynthesis of a phytoalexin (inducible antimicrobial compound), from grapevine was introduced to rice and an enhanced resistance of the transformants to the fungus *Pyricularia oryzae* was observed [36•]. Transgenic rice expressing a coat protein of rice dwarf virus has been recovered [10]. It has also been reported that elite Indica rice expressing CryIAc endotoxin from *Bacillus thuringiensis* were proven to be resistant against yellow stem borer [9].

Some other traits have been introduced in attempts to improve the quality of crops. A gene for phytoene synthase from daffodil was transferred to rice and the accumulation of phytoene, which is a key precursor in biosynthesis of provitamin A, was detected in the endosperm [7•]. This technology should be useful for creation of nutritionally

improved rice. The possible modification of seed storage proteins and of the quality of grains has been examined in recent studies. Expression in maize of a mutant gene for α-zein, with a defect in appropriate processing, resulted in the floury2 phenotype, characterized by a soft, starchy endosperm [37]. A recombinant gene for high molecular weight glutenin was transferred to wheat with the consequent modification of gluten polymers in the endosperm [14].

Efficient methods for the control of self-fertilization of wheat have long been needed. A novel nuclear male-sterility system, which had been demonstrated in tobacco and maize, was successfully tested in wheat [38••]. In this study, a gene for a ribonuclease under the control of tapetum-specific promoters from rice or maize, was introduced into wheat by particle bombardment. Each transgene was expressed specifically in the tapetum and male sterile wheat plants were obtained.

Transformation is a powerful tool for the dissection of basic aspects of metabolic pathways. For example, rice plants with only 65% of the ribulose-1,5-bisphosphate carboxylase found in wild-type plants were produced by introduction of an antisense gene. The rice used nitrogen with enhanced efficiency during photosynthesis at saturating levels of CO_2 and high irradiance [8•].

Promoters used in transformation

Large numbers of potentially useful promoters have been isolated from cereals, and their effectiveness has been examined in transient expression assays in various cereal tissues and stably transformed cell lines after particle bombardment. For example, the CM3 promoter of barley was specifically expressed in the outermost layer of the endosperm in maize [39]. The promoter of the gene for a hydroxyproline-rich glycoprotein in maize was active in the meristems of young shoots, pericarp, styles, auricles and cortical cells in the root tips of maize [40]. Transcription from this promoter was induced by ethylene in maize. The promoter of a gene for maize ubiquitin was combined with an intron from the maize alcohol dehydrogenase (*Adh*) gene and the gene was strongly expressed in immature embryos of wheat [41]. Rice cells were transformed with the gene encoding green fluorescent protein (previously isolated from jellyfish) under the control of the promoter of a gene for maize ubiquitin and expression of the gene was induced at elevated temperatures [42].

The studies cited above and others support the hypothesis that cloned promoters generally retain the expression profiles of their native genes both in the original species and in other species. Such a hypothesis can be extended to transgenic plants. Examples include expression of tapetum-specific promoters from maize and rice in the wheat tapetum [38••]; the *Osg6B* promoter in the rice tapetum [29•]; the promoters of a maize *waxy* gene, a maize gene for a 27 kDa zein, a rice gene for ADP-glucose pyrophosphorylase and a rice gene for the seed storage protein glutelin 1 in maize endosperm [5]; and the promoter of a maize *DnaJ*-related gene in maize [4].

Promoters connected to selectable marker genes were compared after transformation of Indica rice. The promoter of a maize gene for ubiquitin and the promoter of a maize *Emu* gene were more effective than the 35S promoter of cauliflower mosaic virus and the promoter of a rice gene for actin [43]. Activity of promoters may be different from plant to plant and choice of promoters which can be expressed properly in cereals is important.

Integration and expression of transgenes

In general, *Agrobacterium*-mediated transformation results in integration of small numbers of copies of transgenes in plant genomes in both dicotyledons and monocotyledons. By contrast, direct transformation tends to create more complicated patterns of integration [22,38••]. Fewer than three copies of transgenes were introduced into rice and maize by *Agrobacterium* in a majority of transformants examined [30••,44]. Stable inheritance of transgenes up to the fourth generation of rice plants after transformation by *A. tumefaciens* has been demonstrated [44]. Thus, in this respect, *Agrobacterium*-mediated transformation is the method of choice.

An analysis of transgenic rice plants produced from protoplasts led to the suggestion that one factor that contributes to the complex patterns of integration of foreign genes in transformants is the strong activity of topoisomerase I or II [20•]. Massive rearrangements, including deletion and translocations, were found at sites of integration and the recognition sites for topoisomerases were identified in the rearranged sequences.

A method for reducing the number of copies of transgenes incorporated after particle bombardment has been reported [38••]. Wheat tissues were treated, before bombardment, with niacinamide, which is an inhibitor of poly(ADP-ribose)polymerase (PARP). PARP is an enzyme which modifies nuclear-associated proteins including histones, topoisomerases and PARP itself. The majority of the transformants produced contained fewer than three copies of transgenes whereas all transformants obtained by the conventional method contained more than five copies.

The expression of transgenes and even of native genes is sometimes unexpectedly suppressed. This phenomenon is known as gene silencing. Most often, sequences homologous to the transgene are involved in this process [45]. The inactivation of gene expression is known as co-suppression when homologous coding sequences are involved. Gene silencing and co-suppression have been studied mainly in dicotyledonous plants but a few recent papers have dealt with this problem in rice. Silencing of the *waxy* gene was observed in rice that

had been transformed with a cloned *waxy* gene [19••]. Cytosine methylation was implicated in the silencing of genes encoding β-glucuronidase in transgenic rice [46]. Similar transcriptional silencing of transgenes and 5-azacytidine-mediated reactivation of the genes have been reported in rice [47•]. Such studies extend our understanding of gene silencing to monocotyledonous species. Gene silencing is probably an intrinsic mechanism for the control of gene expression in higher plants, and further studies will surely be of fundamental importance in plant biotechnology.

Conclusions

Methods for gene transfer to cereals have become increasingly successful and sophisticated in recent years. Transformation by particle bombardment is now a routine technique in major cereals. *Agrobacterium*-mediated transformation, which is the preferred method for dicotyledons but was once believed not to be applicable to monocotyledons, is now used very efficiently in both rice and maize. It is likely that this method will soon be extended to include other cereals. Thus, we can anticipate that a growing number of genes will be transferred to cereals by *Agrobacterium*. Particle bombardment methods will probably also be improved and will remain very important. Many agriculturally useful genes have already been transferred to various cereals and the variety of promoters that can properly control the expression of transgenes in cereals is also increasing. Gene silencing in cereals, however, cannot be ignored and particular attention should be given to the phenomenon in the future.

Rice is now unique among crop plants, having a small genome and being well characterized. Methods for transformation of most genotypes are available and the plant is of obvious economic importance. We predict that the functions of many plant genes will soon be tested in rice before they are tested in any other plant.

Note added in proof

Cheng *et al.* [48] reported the first successful transformation of wheat by *Agrobacterium*, with molecular and genetic analyses of transformants. Wheat appears to be another cereal species in which *Agrobacterium*-methods will be the method of choice for the production of transgenic plants.

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