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Review

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Current Status of Wheat Transformation

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Abstract

Traditionally, genetic variability is generated by an extensive crossing program, which is complemented by strict selection to identify useful new recombinants. Plant biotechnology offers many opportunities for breeders to solve certain breeding problems at the molecular level. The tissue culture methodology and the genetic modification of economically important monocotyledons have undergone a revolution in the last decade. As the production of transgenic plants is a complex procedure, including the uptake of DNA molecules into the cells, the integration of foreign nucleotide sequences into the host genomic DNA and the expression of new genes in a controlled way, and as there are still many unsolved questions, further development is necessary. The methodology opens up the possibility of introducing novel genes that may induce resistance to diseases and abiotic stresses, allow the modification of dough quality and the dietetic quality of proteins, and increase the levels of micronutrients such as iron, zinc, and vitamins. In the present review, the authors would like to summarise the most important advances in wheat transformation.

Introduction

The unique properties of wheat flours and the wide geographical distribution of wheat (FAO, 2001), makes it one of the three most important crops in the world. With the help of the genetic variation present in wheat germplasm and in sexually compatible, closely related species, traditional plant breeding has brought enormous increases in wheat production through selection and crossing procedures (Bedő et al., 1998; MaIntosh, 1998). However, advances in plant biotechnology now make it possible to use genes originating from organisms, which are not sexually compatible with wheat. The introduction of genes to produce genetically modified (GM) crops assists in improving the quality of wheat and reducing the enormous yield losses attributed to weeds, pests and pathogens (Lazzeri et al., 1997; Pellegrineschi et al., 2000; Rasco-Gaunt et al., 1999; Vasil, 1998). Successful transformation is a two-step process, involving the integration of foreign DNA and the regeneration of fertile plants. Difficulties at all levels of the wheat transformation process suggest that comprehensive criteria should still be used to ensure success. One such set of criteria was proposed by Potrykus (Potrykus, 1991). The efficiency of stable transformation is strongly dependent on genotype, explant source, and medium composition. The first transgenic wheat plants were obtained by Vasil et al. and the results were published in 1992 (Vasil, 1992). Several transgenic wheat varieties have been produced with different introduced genes during the last decade. Some of these new genes, for instance HMW-GS, improved the bread- and pasta-making quality (Barro et al., 1997; Blechl et al., 1998; He et al., 1999; Rooke et al., 1999). Other genes enhanced resistance against fungi (Dahleen et al., 2000; Pellegrineschi et al., 2000), insects (Alpeter et al., 1999; Stoger et al., 1999), viruses (Karunaratne et al., 1996) and herbicides (Vasil et al., 1992). Male sterility has been achieved in transgenic plants (De Block et al., 1997). Plants with new starch characteristics were produced with the

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introduction of the wheat starch branching enzyme gene (Baga et al., 1999). The efficiency of wheat transformation is commonly reported at around 1% (Alpeter et al., 1996). After successful transformation the instability of transgene expression has been a major problem in some transformation experiments (Barro et al., 1998; Cannell et al., 1998). The partial suppression of transgenes such as endogenous HMW-glutenin genes has also been observed in wheat (Alvarez et al., 2000; Blechl et al., 1998). Transformation by microprojectile bombardment of wheat tissue is the most widely used DNA delivery method. This was first reported by Vasil et al. (1992), and shortly afterwards reproducible wheat transformation from immature embryo bombardment was demonstrated by numerous laboratories for wheat (Alpeter et al., 1996; Becker et al., 1994; Nehra et al., 1994; Srivastava et al., 1996; Weeks et al., 1993) and for tetraploid durum (Bommineni et al., 1997; He et al., 1999). One improvement compared with the original reports was achieved by applying an osmotic shock just before bombardment (Alpeter et al., 1996, Blechl and Anderson, 1996; Rasco-Gaunt et al., 2000). Other methods were also used for DNA delivery with more or less success. One report has been published on successful wheat transformation using *Agrobacterium* (Cheng et al., 1997), but it is not currently thought that *Agrobacterium* is ready to replace the biolistic method in most laboratories. He and Lazerri (He and Lazzeri, 1998) optimised the conditions for cell electroporation, while Serik et al. (1996) reported successful gene delivery into mature wheat embryos by silicon carbide fibre-mediated DNA transformation. The pollen tube pathway was used by Chong et al. (1998) for wheat transformation, but with little success.

In this paper, the current status of wheat transformation in the world is discussed, with the emphasize on Hungary.

Tissue culture methodology

For efficient genetic modification, high regeneration capacity from elite wheat varieties is a primary requirement. The efficiency of stable transformation is still strongly dependent on genotype, explant source, and medium composition.

Genotype is a primary factor for successful cereal transformation. Efforts are being made to extend the technology to elite genotypes, which are either agronomically important breeding lines or currently commercial varieties (Iser et al., 1999; Pauk et al., 1994; Tang et al., 1999; Zhang et al., 1999). Several commercial wheat genotypes (mainly Japanese) were tested for callus induction and regeneration capability from anther and immature embryo cultures (Machii et al., 1998). The regeneration of some elite CIMMYT bread wheats from immature embryos was also solved (Fennell et al., 1996).

A variety of explants have been used in attempts to establish regenerable tissue cultures of wheat, including whole seed (caryopsis), mature and immature embryos, isolated scutellum, immature inflorescence, immature leaf, mesocotyl, apical meristem, coleoptilar node, and root. The best results have been obtained by culturing immature embryos. Regenerable cell suspension cultures have been established from embryogenic callus tissues of wheat. Protoplasts isolated from such suspensions have been successfully cultured to regenerate plants (Ahmed and Sagi, 1993; Yang et al., 1993). Transgenic wheat can also be recovered using immature inflorescences (Dudits et al., 1975; Rasco-Gaunt and Barcelo, 1999). Shoot meristem cultures can be obtained in a wide range of genotypes (Lemaux et al., 1999; Zhang et al., 1999). A number of laboratories use long-term embryogenic callus culture as the target tissue. This is used mainly for rice, as it is more problematic in most other cereals because of loss of regenerability, increase in albinism, loss of fertility and frequent occurrence of abnormal phenotypes among regenerants (Gordon-Kamm et al., 1999; Lemaux et al., 1999). There has been considerable discussion about the genetic control of regeneration ability in wheat and suggestions have been made as to the location of these traits on different chromosomes (Ben et al., 1997; Ben et al., 1995; Galiba et al., 1986).

Several projects have focused on the examination of medium parameters (Perl et al., 1992; Rasco-Gaunt et al., 1999; Zhang et al., 1999) and culture conditions (Takumi and Shimada, 1996). The influence of DNA delivery methods on regeneration (Gless et al., 1998; Rasco-Gaunt et al., 2000) has also been studied. Generally MS (Murashige and Skoog, 1962) is the main salt formulation used for cereal species from the Triticeae. 2,4 dichloroacetic acid is the auxin commonly used, with or without a low concentration of cytokinins to induce callus formation. For shoot regeneration, higher concentrations of cytokinins are used, with or without auxin supplementation (Barro et al., 1998; Rasco-Gaunt et al., 2000). The addition of zeatin at 5 or 10 mg/L to regeneration media also had a positive effect on regeneration (Barro et al., 1999). The influence of auxin type molecules (indole acetic acid -IAA, naphthyl acetic acid -NAA), the concentration of macroelements in the regeneration media, the incubation temperature and the light intensity during tissue culture phases were studied by Tams et al. (2000). A number of other medium com-

ponents have been found to enhance callus proliferation and/or regeneration, and the recovery of transformants. Rasco-Gaunt et al. (1999) have observed that high concentrations of sugar and a supplement of silver nitrate increase the plant regeneration frequency of bread wheat, suggesting that they protect the explants from damage caused by the transformation procedure. Other studies have involved the effects of osmotic treatment using high sugar or sugar alcohols (Chen et al., 1998; Rasco-Gaunt et al., 2000), various carbon sources (Zhang et al., 1999), metal ions such as silver and high levels of copper (Cho et al., 1999; Zhang et al., 1999). During DNA delivery the influence of different parameters, such as the quantity of plasmid DNA, spermidine concentration, Ca ion concentration, amount of gold particles, acceleration pressure, bombardment distance, the osmotic condition of tissues and the type of auxin, on transient GUS expression was studied by Rasco-Gaunt et al (1999).

Selection of transformants

Transformed cells are connected to and surrounded by non-transformed cells, so the recovery of transformed cultures depends on an efficient means of selecting for cells which carry introduced DNA. Current cereal transformation systems make use of marker genes for the selection of transgenic plants. These are typically resistance genes that confer tolerance to toxic substances such as antibiotics or herbicides, so that wild-type cells can be inhibited or killed while allowing transgenic cells to proliferate.

The first generation of selectable marker genes conferred resistance to antibiotics such as the aminoglycosides, kanamycin, geneticin and paromomycin (Chen et al., 1998; Cheng et al., 1997). The Sat3 gene, which confers resistance to streptomycins, was recently introduced by Jelenska et al. (2000). Genes conferring resistance to herbicidal compounds provided an alternative to antibiotics for selection. The most commonly used herbicide resistance gene *bar* or *pat* (phosphinotricin acetyl transferase) (De Block et al., 1995) confers tolerance to phosphinotricin (PPT) based formulations, such as gluphosinate, bialaphos, Basta and Herbiace by detoxifying these compounds. There are differences in their effectiveness in maize and in wheat (Dennehey et al., 1994). PPT-based selection has been used in all transformable cereals (Barro et al., 1998; Brettschneider et al., 1997; Iser et al., 1999; Kim et al., 1999; Kohli et al., 1999; Pauk et al., 1998; Rasco-Gaunt et al., 1999). Although widely used and efficient, this selection system may have side effects, as indicated by Bregitzer et al. (1998). To optimise wheat transformation frequencies Murray et al. (1998) used four selection systems. Each of the *bar*, *hpt* and *nptII* genes driven by either the Emu (Chamberlain et al., 1994) or Ubi1 (Christensen et al., 1992) promoters have been used in combination with bialaphos, hygromycin B and geneticin or paromomycin selective agents. The antibiotic paromomycin in combination with *nptII* gene successfully recovered transgenic plants and it became the favoured selection system. Less commonly used herbicide selection systems in cereals are those which exploit tolerance to glyphosate or sulphonylurea compounds. Resistance to these herbicides has been obtained by using genes encoding the mutant target enzymes EPSPS/GOX and ALS (Zhang et al., 2000; Wan and Lemaux 1994). A further herbicide marker, the cyanamide-based selection system, was used by Weeks (Weeks, 1998) in wheat.

Scorable marker genes are used as visual markers in transient expression studies for evaluating DNA delivery, but are generally not used as selectable markers for transformation because their use is more complicated. In the early years of plant transformation, the β-glucuronidase marker gene (uidA/GUS) (Jefferson, 1987) and CAT (chloramphenicol acetyltransferase) (Bruce, 1989; Chibbar et al., 1991) were used as indicators of DNA transfer into plant cells. The reporter gene usually codes a protein having enzyme activity, so quantitative detection can be easily carried out 48 hours after transformation. The enzyme activity should be so small that it does not effect the development of the plant; it should be resistant to proteases and stable over a wide pH and temperature range. The detection limit should be small and the half-life period should be short. Only a few reporter genes meet all these requirements. There are several alternative visual markers, such as the anthocyanin and luciferase systems, which have also been used for cereal transformation. Anthocyanin may have a detrimental effect on the transformed cell, but pigment accumulation in the plant tissues is easily visible (Chawla et al., 1999; Marrs et al., 1995). Luciferase (LUC) does not seem to have a toxic effect on cereal cells, but the cost of the equipment required to measure LUC activity is high (Baruah-Wolff et al., 1999; Fromm et al., 1990). The luciferase enzyme catalyses the ATP-dependent oxidation of the luciferin substrate. Green fluorescent protein (GFP), which originates from jelly-fish, is a more recent scorable marker that is increasingly applied in plant transformation. This marker allows the non-destructive, visual identification of transgenic cells by standard fluorescent microscopy. The system is tissue- and genotype-independent, shows low toxicity and is cellau-

tonomous. Recent reports on GFP indicate that its use in cereal genetic transformation has considerable promise (Ahlandsberg et al., 2000; Pang et al., 1996; Tamás et al., 1999; Vain et al., 1998).

Positive selection (Haldrup et al., 1998) is a strategy that gives transgenic cells a metabolic advantage over non-transgenic cells. These new marker systems are designed to be neutral in terms of consumer (animal or human) and environmental impact. The most advanced positive selection system employed to date in cereals is the mannose selection system, which has recently been used for wheat transformation by Reed et al. (1999). This system uses the phosphomannose isomerase (*manA* or *pmi*) gene as the selectable gene and mannose as the selective agent (Negretto et al., 2000). An alternative approach to the positive selection of transgenic cereal lines, based on the modification of in vitro culture performance, has recently been introduced by Lowe et al. (2000). The system has been shown to allow the visual selection of maize and wheat transformants. The *ipt* (isopentenyl transferase) gene is also an alternative to the most widely used selectable marker genes (Ebinuma et al., 1997). Several other positive selection strategies, which have been shown to function in dicots, have yet to be tested in cereal systems. These strategies usually work by catalysing the production of different enzymes (Ebinuma et al., 1997; Kunkel et al., 1999; Sugita et al., 1999).

As the presence of selectable marker genes is not always desirable in the transformants, attention has been devoted to the development of a technology for the selective elimination of marker sequences from the transgenic plant. This may be achieved by normal segregation (Komari et al., 1996), but more predictable systems use site-specific recombination, or gene replacement mechanisms.

Promoters

For driving either scorable or selectable genes, promoters are essential. There are three major classes of promoters currently used in biotechnology. Constitutive promoters are mainly used to drive the expression of selectable and scorable marker genes for the identification of transgenic tissues in vitro. They are also used for driving the gene of interest, if this needs to be expressed at a high level in all tissues and at all developmental stages of the plant. The promoters used in cereal transformation are the 35S promoter isolated from the tobacco cauliflower mosaic virus (CaMV 35S) (Odell et al., 1985), rice Act1 (Barro et al., 1998; Mc Elroy et al., 1991; Zhong et al., 1996), maize polyubiquitin Ubi1 sequences (Barro et al., 1998; Christensen et al., 1992; Stoger et al., 1999; Weeks et al., 1993) and the pEmu promoter (Last et al., 1991; Li et al., 1997). For enhancing gene expression several introns were tested, such as rice Act1 intron1 (Mc Elroy et al., 1991), or adh1 intron1 (Barro et al., 1998; Chibbar et al., 1993). A promoter recently isolated from sugarcane (ScBV) is a promising addition to the wheat constitutive promoter repertory (Tzafrir et al., 1998).

Tissue-specific and developmentally-regulated promoters allow the expression of transgenes only in specific tissues or under certain developmental conditions. Wheat HMW glutenin subunit promoters (HMWG 1A × 1 and 1D × 5 genes) have been used to drive endosperm specific expression in transgenic bread wheat (Alpeter et al., 1996; Barro et al., 1997) and durum wheat (He et al., 1999; Lamacchia et al., 2000). In the case of GBSS (granule bound starch synthase) promoters, expression was observed in both the endosperm and pollen grains (Russell and Fromm, 1997). Anther tapetum and pollen grains are tissues of interest for creating nuclear male sterile lines for hybrid production (De Block et al., 1997). Rice sucrose synthase (Rss) and the rice tungro bacilliform virus (RTBV) sequences are active in phloem (Bhattacharyya-Pakrasi et al., 1993; Stoger et al., 1999). Light-regulated promoters specific for meristematic cells or chlorophyll-containing tissues are listed by Baga et al. (1999). These include: chlorophyll a/b binding protein (LHCP), phosphoenolpyruvate carboxylate (PEPC), pyruvate orhophosphate dikinase (PPDK), and ribulose-1,5-bisphosphate carboxylase (Kyozuka et al., 1993; Matsuoka et al., 1994).

Inducible promoters are a third class of promoters that are only expressed under specific induction conditions. Among the pathogen/wound induced promoters, Vst1 (stilbene promoter from grapevine) has been characterised in wheat (Leckband and Loerz, 1998) and is able to drive transgene expression after fungal infection and wounding. Rice basic chitinase (RC24) and rice glycine-rich cell-wall protein (Osgrp1) are two other wound-induced promoters (Xu et al., 1995; Xu et al., 1996). A second group of inducible promoters are chemically induced, such as herbicide safeners and alcohol-activated systems (Caddick et al., 1998; Greenland et al., 1997). Stress-induced promoters are also used in developing resistance in plants against cold and heat shock, anaerobic stress and pathogens (Kyozuka et al., 1991; Lyznik et al., 1995; Molina et al., 1996; Vasil et al., 1992).

Transformation methods and worldwide results

Direct gene transfer

The first transformation technique successfully applied in wheat was polyethylene-glycol (PEG) treatment (Fromm et al., 1986; Lrz et al., 1985). The disadvantage of this method is that the regeneration ability of the protoplast is very low, so only a few varieties and lines could be used for transformation. This method is reproducible only in rice and maize (Barcelo and Lazzeri 1998; Birch, 1997; Golovkin et al., 1993). Several other conditions limit the utility of this technique. Cereal suspension cultures lose their embryogenic potential (DiMaio and Shillito, 1989) and accumulate genetic abnormalities (Karp, 1991) over time. As a result of these limitations, protoplast transformation is not the method of choice for producing populations of transgenic plants.

During cell/tissue electroporation high-voltage electrical pulses are used to allow DNA uptake through cell membranes from a surrounding buffer solution. The technique works in all major cereal species; however, the transformation efficiency is not sufficiently high, except for maize and rice (Laursen et al., 1994; Xu and Li, 1994). The optimum conditions for DNA transfer into mature emb ryos of barley via electroporation were recently determined by Grel et al. (2000). The method has advantages over protoplast transformation. Callus tissues or primary explants such as immature embryos or inflorescence tissues are usually targeted (He et al., 2000). One disadvantage could be that the target tissue preparation is critical in this technique and the amount of DNA delivered into the target cells is less than with particle bombardment.

Silicon carbon fibre vortexing is an other direct gene transfer technique to be developed. In plants the technique was first applied in suspension cultures (Frame et al., 1994), but it has also been applied in organised tissues such as embryos and embryo-derived callus (Serik et al., 1996; Matsushita et al., 1999; Petolino et al., 2000). Despite the simplicity of the method it is not widely used because the production of embryogenic suspensions is difficult and time-consuming and the microscopic SiC fibres are hazardous to human health.

There are reports on the production of transgenic wheat by the pollen tube method (Chong et al., 1998), which is a very attractive method, but little success has been achieved so far.

Biolistic particle delivery is a mechanical method of transformation that uses gas pressure (originally gunpowder explosion) to introduce DNA-coated microcarriers into intact plant cells, tissues and organs. The method may be highly advantageous when major biological barriers exist to either *Agrobacterium* or protoplast-mediated transformation. Other advantages are that it does not need a long tissue culturing methodology. DNA macromolecules can be introduced into all living tissues and cells and the number of transgenic cells and plants can be increased compared to the methods used before. Particle bombardment (biolistic transformation) is currently the most widely used technique for direct gene transfer. It was developed by Sanford in 1988 (Sanford, 1988).

After the helium-driven gun (Kikkert, 1993), which is a widely-used, standard particle bombardment device, a hand-held particle gun device has been developed recently, which allows transgene delivery to tissues of intact plants (Chaure et al., 2000). The direct delivery of DNA by microprojectile bombardment is thus far the most reliable and satisfactory method for the production of fertile transgenic wheat plants (Alpeter et al., 1996; Barro et al., 1997; Blechl and Anderson, 1996; De Block et al., 1997; Ortiz et al., 1996). The first commercial GM cereal varieties were produced by this method. In wheat, an efficiency of 9.7% (Zhang et al., 2000) was reported when immature embryos were transformed. There are two major requirements for efficient transformation. The first is the efficient delivery of particles into large numbers of target cells. The second is a high level of division and regeneration in the targeted cells. A number of authors have studied the influence of different particle types, sizes, and different procedures for the precipitation of DNA onto particles (Harwood et al., 2000; Rasco-Gaunt et al., 1999). Most of these studies have been performed using the PDS-1000/He gun (BioRad). In cereals, two types of target systems are used for bombardment. In the first method, primary explants are bombarded immediately or soon after isolation. The cells are induced to become embryogenic and regenerate (Barro et al., 1997). The second technique uses with pre-established proliferating embryogenic cultures. Bombardment is followed by further proliferation and regeneration (Gordon-Kamm et al., 1990; Vasil et al., 1992). Modifications to culture procedures, such as the plasmolysis of the tissues prior to bombardment or culture on high-osmotic media (Finer et al., 1999), help the targeted tissues to tolerate the damage/stress of the bombardment process.

Agrobacterium-mediated gene transfer

Agrobacterium tumefaciens-mediated transformation is another possible method for cereal transformation. *Agrobacterium tumefaciens*, a soil bacterium, can genetically transform plant cells with a segment of DNA from tumour-inducing plasmid (Ti plasmid) with the resultant production of a crown gall, which is a plant tumour. Crown gall is a disease that causes considerable damage to perennial crops. Initially this technique was only used successfully for dicots, but there was a breakthrough in the middle of the 90s, when reports were published of stable transformed monocots such as rice, barley, wheat, and maize (Cheng et al., 1997; Hiei et al., 1994; Tingay et al., 1997). The successful transformation of wheat, one of the most important crops, has still not been confirmed in other publications. However, as *Agrobacterium tumefaciens* is a soil-borne microorganism occurring everywhere in nature, it seems to be very promising as a gene mediator. In rice and maize, transformation efficiencies in the range of 5-20% are regularly obtained (Cheng et al., 1998; Ishida et al., 1996; Toki, 1997). In barley and wheat, efficiencies of 4% (Tingay et al., 1997) and 1.6 % (Cheng et al., 1997) have been reported. It is important to note that model genotypes developed for tissue culture were used in each case. There are genotype limitations to tissue culture and the regeneration system. A wide range of factors, influence the bacterial and plant cells involved in the process (Gheysen et al., 1998; Hansen and Chilton, 1999; Hiei et al., 1997). These primary components need to be analysed and optimised for each species and to some extent at the cultivar level within species. The preferred tissue source in cereals is the scutella of immature embryos (Cheng et al., 1997; Ishida et al., 1996; Tingay et al., 1997), or embryogenic cultures derived from them (Hiei et al., 1994). In wheat and barley the methodology is still under development. Currently, the efficient application of *Agrobacterium* transformation (>1%) is confined to a very limited range of model genotypes, which have exceptional responses in tissue culture. In wheat, this genotype is Bob White, which probably has higher amenability to *Agrobacterium* infection than typical agronomically elite germplasm. The advantages of the method are its simplicity and efficiency, the production of plants with simple integration patterns, the integration of a small number of copies of large segments of DNA with defined ends into the chromosome with little rearrangement, and the high quality and fertility of both monocotyledonous and dicotyledonous transgenic plants.

Cereal transformation experiments in Hungary

In this chapter the cereal transformation experiments carried out in Hungary are summarised on the basis of the different techniques. A brief summary of the results of regeneration studies is given first, followed by the results of transformation work in different laboratories.

The efficiency of stable transformation is still strongly dependent on genotype (Felföldi and Purnhauser, 1992), explant source and medium composition. The effects of the cytokinin, auxin, aminoacid and carbohydrate concentrations in the induction and regeneration media have been widely studied in Hungary as well. Karsai et al. studied the effect of induction medium and maltoze concentration on in vitro androgenesis of hexaploid winter wheat and achieved 9.1% green plantlet regeneration (Karsai et al., 1994). She also established that total plant regeneration was affected by environmental factors and genotype × environment interaction (Karsai et al., 1993). The effect of incubation time and medium composition on plant regeneration from microspore-derived embryo-like structures in wheat anther culture was recently studied by Puolimatka and Pauk (Puolimatka and Pauk, 2000), who conclude that there is an optimum time for maximum plant regeneration and that embryo-like structures should be transferred from induction to regeneration medium no later than 7 weeks after anther isolation. Studies on regeneration from callus and protoplast culture were also carried out (Pauk and Purnhauser, 1993; Pauk and Szarka, 1991). Certain microelements, especially biologically active heavy metals, may increase plant regeneration significantly. The use of the ethylene inhibitor Ag (Purnhauser et al., 1987) significantly improved shoot regeneration in different crop species. Purnhauser was the first to report that Cu applied at a higher concentration than in the original MS medium effectively promoted shoot regeneration in poorly-regenerating wheat callus cultures and in other crop species (Purnhauser, 1991; Purnhauser and Gyulai, 1993). The effect of Al on the androgenic response of different wheat × triticale crosses was investigated by Karsai et al. using anther culture and it was found that *in vivo* Al treatment at the seedling stage resulted significantly higher embryo induction and slightly higher green plant regeneration rate compared with the control populations studied (Karsai et al., 1994). Barnabás et al. recently found that the application of *in vitro* Al selection significantly increased the probability of obtaining doubled haploid lines with significantly higher tolerance, compared to the original genotype (Barnabás et al., 2000).

Wheat ovary, glume, floret and anther co-cultures were found to produce embryo-like structures. Investigations were made on the effect of co-culture explant type, duration and initiation time on the culture of microspores and it was concluded that a positive co-culture effect on wheat microspore embryogenesis was obtained with non-embryogenic glume explants (Puolimatka and Pauk, 1999). The effect of 2,4-D and kinetin on plant regeneration in wheat was also studied (Fekete and Pauk, 1989). The results showed that callus culture regeneration was significantly increased by using 2,4-D and kinetin in various combinations, so that 86% of the calluses regenerated plantlets. The influence of auxin-type molecules (IAA, NAA), the concentration of macroelements in the regeneration media, the incubation temperature and the light intensity during tissue culture phases were studied by Tamás et al. (2000), who established a tissue culture method for plant regeneration from immature scutella of elite winter wheat varieties.

Hungarian scientists were among the first to develop a reproducible protoplast-plant system in rice (Jenes and Pauk, 1989). In the course of this work they found a genotype suitable for protoplast transformation experiments and reproducible plant regeneration system for protoplasts. The experiment was carried out with PEG treatment using GUS and *nptII* genes. Gene transfer during PEG treatment depends on various parameters, such as the incubation time and diluting time of the PEG solution (Bittencourt et al., 1995). The medium used for protoplast proliferation and regeneration also affects gene transfer (Jenes and Pauk, 1989). It was established that the regeneration ratio for protoplasts isolated from an embryogenic suspension culture was low and genotypedependent (Pauk et al., 1994). Though many factors influence and limit the utility of the method, several results were achieved. PEG-mediated GUS transfer efficiency was studied for rice (Bittencourt et al., 1995) and barley (Jenes et al., 1994) protoplasts, while herbicide- (Dudits et al., 1993) and MTX (methotrexate) -resistant (Golovkin et al., 1993) transgenic maize plants were produced from protoplasts by direct uptake. The regeneration of fertile maize plants from protoplast-derived transgenic callus tissues was also carried out by Omirulleh et al. (1993). In this work the plasmid DNA contained the GUS gene, under the control of the CaMV 35S promoter, linked to the wheat alpha-amylase gene. Vectors carried either the *nptII* or PAT gene as selective marker. The GUS activity was expressed in the leaf epidermis, the mesophyll, the vascular bundles, the cortex and the vascular cylinder of the root. The activity of different promoters, such as the *Arabidopsis thaliana* cyclin promoter fused with β-glucuronidase (Peres et al., 1999) and the H4 promoter (Bilgin et al., 1999), was also studied in maize plants produced by direct DNA uptake into the embryogenic cell suspension protoplasts. For checking and establishing the stability of the integrated DNA a Southern DNA hybridization method was suggested by Jenes et al. (1992). In wheat Ahmed et al. (1997) studied the factors which affect the transient expression of vector constructs in protoplasts. It was found that the protoplast culture medium plays an important role in the degree of transient gene expression. Treated protoplast cultured in KM medium giving a higher GUS activity than those in the GM medium. Yakovleva and Dudits (Yakovleva and Dudits, 1993) studied the effect of an aminoxy analogue of putrescine on wheat cell cultures (*Triticum monococcum* L.), which revealed altered polyamine metabolism. Under conditions of severe growth inhibition the level of free putrescine in the cell suspension culture decreased.

An expeditious and highly efficient technique of microinjection has been developed with the aim of introducing exogenous DNA into egg cells and zygotes of wheat in Martonvásár (Pónya et al., 1999). A mechanical dissection method and a novel immobilization approach were used to microinject around 15 egg cells of wheat per hour. By exposing the protoplasts to a high-frequency alternating-current field for immobilization, a significantly higher transient expression rate could be achieved for the injected genes (46 and 52% for egg cells and zygotes, respectively) than reported thus far for plant protoplasts.

Transformation techniques have become routine for many dicotyledonous species. Unfortunately the transformation of cereals has been more difficult. In 1996 Jenes et al. (1996) developed a new particle bombardment instrument (Genebooster), as the biolistic method seemed to be the most efficient method for the transformation of cereals. The results achieved in banana (Sági et al., 1995), maize (Golovkin et al., 1993; Omirulleh et al., 1993) and rice (Jenes et al., 1996; Pauk et al., 1996) transformation, let wheat to become the next target for particle bombardment. Recently, several interesting characteristics of bread and durum wheat have been modified by genetic engineering, including dough mixing properties (He et al., 1999; Shewry, 2000). In view of the functional and economic importance of the HMW subunits it is not surprising that the HMW subunit genes have been identified as targets for expression in transgenic wheats. According to Popineau et al. (2001), overexpression of the 1A×1 and 1D×5 subunits modified glutenin aggregation, but the glutenin properties were much more affected by the expression of the 1D×5

transgene. In order to improve the functional properties of the flour, the Australian bread wheat line L88-6 was transformed at IACR-Rothamsted, Harpenden, UK (Barro et al., 1997). The transgenic B73-6-1 spring wheat line contains extra copies of a native HMW glutenin gene (1D ×5) so the flour contains a higher quantity of this protein. The technological and rheological properties of these two lines were studied in a Hungarian field experiment in Martonvásár (Rakszegi et al., 2000). The results of the field experiment support those of Rooke et al. (1999), who showed that the overexpression of subunit 1D×5 results in an over-strong type of dough suitable for blending with poor grade flour or for the development of novel end uses. A successful wheat transformation experiment was also carried out in Hungary. The first Hungarian herbicide-resistant wheat genotype generated using an *in vitro* method was reported by Pauk et al. (1998). The *bar* marker gene was introduced using a particle gun device into immature embryo-derived calli of different ages, followed by marker gene selection. The six independent transformants, in which the expression of the *bar* gene was confirmed by a PAT assay, were transplanted into the greenhouse. These plants showed resistance against 0.1% bialaphos spraying. The transformants matured fertile seeds under safe greenhouse conditions. Tamás et al. (C. Tamás, personal communication) are also using a biolistic instrument for the production of transgenic wheat plants. The target plant materials are immature embryos of winter bread wheat genotypes originating from the Martonvásár breeding programs. The plasmid used for bombardment contains the genes of interest, which consist of various HMW glutenin genes, and genes for improving stress resistance. The selectable gene is *nptII*, while the *Emu*, *Ubi1* or *CaMV35S* promoters have been used in combination with paromomycin and kanamycin selective agents. Besides glutenin, gliadin alsáo has an effect on wheat quality. Vibók et al. (1999) isolated γ-gliadin gene from a wheat genomic library and its tissuespecific effect was studied in transgenic rice (*Oryza sativa*). A hybrid promoter construct was created from γ-gliadin promoter and rice actin1 promoter. A β-glucuronidase reporter gene (*gusA*) was fused downstream of the hybrid promoter. This construct was introduced into rice embryos by particle bombardment. Transgenic rice plants carrying the hybrid promoter linked to *gusA* were regenerated from bombarded embryos and grown for further studies.

In Hungary five geneguns are already operating in different plant research institutes (Agricultural Biotechnology Center, Gödöllő; Agricultural Research Institute of the HAS, Martonvásár; Lornd Eötvös University, Budapest; Szent István University, Gödöllő; Cereal Research Non-Profit Company, Szeged) and will hopefully advance domestic genetic research.

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