The Triticeae crops comprise wheat, barley, rye and triticale, which together provide a major portion of the world’s food and feed. The growing demand for human nutrition and renewable energy requires an intensification in application-oriented research and the establishment and utilization of current biotechnology in these crops. Genetic transformation provides an important means both to elucidate gene function, and to engineer crop plants in a directed and precise way. This review covers a range of issues surrounding the production of stable transgenic lines within the Triticeae. Some quality aspects of transgenesis of particular relevance to the Triticeae are also discussed.

Key Words: Gene transfer, Agrobacterium-mediated transformation, embryogenic pollen, transgene homozygosity, recipient genotype, transgene integration.

Introduction

The small-grain temperate Triticeae cereals include the hexaploids bread wheat (*Triticum aestivum*) and triticale (*×* Triticosecale, derived from the cross between the tetraploid macaroni wheat *T. turgidum* conv. *durum* and cereal rye, *Secale cereale*), the tetraploid macaroni wheat, and the diploids barley (*Hordeum vulgare*) and cereal rye. Bread wheat and barley represent two of the most important crops worldwide, and taken together, this group of species provides a substantial proportion of the world’s food and feed. More recently, it has also been identified as a source of bioenergy. A substantial proportion of barley production supplies the malting industry. Barley has attracted a deal of research attention, as its genome represents a suitable model for the small-grain cereal group, and its diploid status is advantageous for both classical and molecular genetic investigations.

Conventional breeding has provided much of the impressive gains in crop productivity made over the last century, but increasingly, further improvement is becoming dependent on a range of enabling technologies, which includes wide hybridization, induced mutagenesis, the exploitation of haploidy and marker-assisted breeding. Recombinant DNA technology has had little impact to date in the temperate small-grain cereals, but in principle offers the opportunity to tailor crop plants to future demand. This review covers the state-of-the-art with respect to the generation of stably transgenic plants in the Triticeae.

An exploration of the many applications of transformation in the areas of both gene function identification and crop improvement lies largely beyond the scope of this paper; instead we seek to inform the reader concerning current transformation vectors, gene transfer methodologies and some further aspects of transgene technology which are of particular relevance to this vital group of crop species.

Transformation vectors

Basic principles

The effect of a transgene on plant phenotype is highly dependent on the nature of the expression cassette. Over-expression is most easily achieved by fusing an appropriate promoter to the transgene open reading frame. A non-native promoter can be employed to induce ectopic expression within a tissue or during a period of development, where the endogenous gene is not expressed. Putative gene functions can best be verified by the transgenic complementation of a loss-of-function mutant, as demonstrated, for example in barley (Stein et al. 2005). The level of endogenous gene expression can be manipulated by transgenesis either via co-suppression (which arises when transcript abundance exceeds a certain threshold), achieved by introducing multiple copies of an over-expression construct; or by antisense route technology, in which the transgene consists of a full (or partial) DNA sequence complementary to that of the target gene. This latter strategy relies on *in planta* hybridization between the native and the transgenic transcript, since double-stranded RNA molecules are recognized and degraded by the host plant’s post-transcriptional gene silencing machinery. When the transgene consists of an inverted...
repeat of the target sequence which results in the autonomous formation of hairpin RNA molecules, the technique is commonly referred to as RNA interference (RNAi). These knock-down approaches tend to reduce the level of target gene expression rather than completely abolish it. However, reduction (rather than the complete loss) of gene expression can be useful, especially where a loss-of-function mutant is lethal. Moreover, by judicious choice of promoter, gene expression can be down-regulated within a particular tissue or developmental stage, so allowing for a greater level of precision in the identification of the target gene’s effect on phenotype. A further advantage of RNAi over knock-out mutants is that it allows for several members of a gene family to be targeted for down-regulation, provided a conserved DNA sequence is used. As an example of an RNAi approach in the Triticeae, Travella et al. (2006) were able to manipulate the expression of both a phytoene desaturase (Pds) and an ethylene insensitive 2 (Ein2) gene in wheat, leading to a clear association of phenotype with transcript level.

While the early successes in cereal transformation were achieved using biolistic transformation (the bombardment of gene transfer recipient cells with metallic particles coated in plasmid DNA), more recently it has been established that monocotyledonous plants are also susceptible to transformation by Agrobacterium tumefaciens. However, most of the available binary vectors have been developed for use in dicotyledonous species, and are of rather limited value in the monocotyledons, because the promoters and other required elements are not sufficiently functional in the latter. Thus, generic vectors adapted to the monocotyledons have been developed (Miki and Shimamoto 2004, CAMBIA, BRACT). A set of vectors designed to have a modular configuration has been described by Himmelbach et al. (2007). This design both allows them to be used to transform monocotyledonous plants, and simplifies the insertion of transgene sequences by the presence of GATEWAY™ recombination sites (Life Technologies, Carlsbad, USA). A number of such vectors are now available for the scientific community, designed to carry a diverse range of both ubiquitous and specific promoters to drive the expression of either effector genes or RNAi constructs. In addition, a variety of selectable marker expression cassettes can be readily exchanged between compatible custom vectors (DNA Cloning Service, Hamburg, Germany).

**Promoters**

The phenotypic effect of a transgene is heavily dependent upon the choice of promoter used to drive its expression. Promoters able to generate ubiquitous expression were widely used during the development of transformation technology. The effectiveness of maize Ubi-1, cauliflower mosaic virus 35S and rice Act1 has been well characterized in transgenic barley and wheat (Stoeger et al. 1999, Furtado and Henry 2005, Vickers et al. 2006, Primavesi et al. 2008). Although such promoters are classed as neither tissue nor developmental stage specific, the level of transgene expression they induce can nevertheless vary greatly. In the Triticeae cereals, most of the tissue-specific promoters that have been functionally verified in the context of recombinant DNA are associated with expression in the grain. Promoters derived from the endosperm storage protein genes rice Gla-B1 (Patel et al. 2000, Xue et al. 2003, Huang et al. 2006), barley Hor3-1 and Hor2-4 (Cho et al. 1999, Patel et al. 2000), and wheat α-gliadin (Vickers et al. 2006), γ-gliadin (Van Herpen et al. 2009, Piston et al. 2009) and Glu-A1-1, B1-1 and D1-1 (Lamacchia et al. 2001, Schuenemann et al. 2002, Brinch-Pederson et al. 2003) have been associated with high levels of endosperm-specific transgene expression in barley and wheat. Particularly strong endosperm-specific reporter gene expression was achieved in barley using the oat Glo1 promoter (Vickers et al. 2006), while aleurone-specific expression could be induced by the incorporation of the promoters of either a high-pl α-amylase (Jensen et al. 1996, Nuuttila et al. 1999) or the trypsin inhibitor T1 (Joensuu et al. 2006) gene. In the developing grain, Furtado et al. (2009) were able to identify that the promoter of a barley bifunctional α-amylase/subtilisin inhibitor gene (Isa) drove the pericarp-specific expression of the green fluorescent protein reporter gene in barley. In contrast, the promoter of a wheat early maturing embryo (Em) gene was functional in both barley and wheat. Endosperm transfer cell-specific expression was induced in barley and wheat by the wheat Pr60 promoter (Kovalchuk et al. 2009), the rice PR602 and PR9 promoters (Li et al. 2008) and the maize Mrp-1 promoter, the latter also driving transgene expression in the vascular tissue of the spike (Barrero et al. 2009).

A few tissue-specific promoters have been identified, however, which are effective in tissues other than the grain. Kempe et al. (2009) were able to show, in experiments aimed at inducing male sterility, that the rice Tap promoter can drive tapetum-specific expression of two compatible barnase gene fragments in transgenic wheat. The leaf epidermis also represents a tissue of interest, since this is where the primary reaction between the plant and its foliar pathogens occurs. Altpeter et al. (2005) demonstrated the epidermis specificity of the TaGstA1 promoter in wheat, and this same promoter is also effective in barley (Himmelbach et al. 2007). The Leml promoter drives expression in the bracts of both barley (Skadsen et al. 2002) and wheat (Somleva and Blechl 2006), whereas the HvLem2 promoter was shown to drive preferential gene expression in the lemma, palea and coleoptile in the presence of salicylic acid (Tilahun et al. 2006).

**Gene transfer methodologies**

The Triticeae cereals have long been considered as difficult to transform, both because their somatic tissue cannot be easily induced to form de novo shoots, and because of their limited amenability to Agrobacterium-mediated transformation. Both these problems have been largely overcome by improvements in cell culture techniques and methods used...
for gene transfer. A schematic overview of transformation methods available for the Triticeae species is presented here as Fig. 1. Explants (isolated ovule, immature embryo, embryogenic pollen culture) used for Agrobacterium-based transformation of barley in the authors’ laboratory are illustrated in Fig. 2.

**Immature embryos and derived callus**

Wheat was the first Triticeae species to be stably transformed (Vasil et al. 1992). This was achieved by the bombardment of embryogenic callus derived from immature zygotic embryos with metal particles coated in plasmid DNA. These earliest transgenic wheat lines were sterile, so this important technical landmark was followed by both an intensive effort to optimize the method (Becker et al. 1994, Nehra et al. 1994, Rasco-Gaunt et al. 2001), and to adapt it for use in barley (Wan and Lemaux 1994), cereal rye (Castillo et al. 1994, Popelka et al. 2003), triticale (Zimny et al. 1995) and macaroni wheat (Bommineni et al. 1997). Increasing the susceptibility of monocotyledons to infection by Agrobacterium has been hampered by the specialization of the pathogen to the dicotyledons. Nonetheless, breakthroughs in Agrobacterium-mediated transformation of rice by Hiei et al. (1994) and in maize by Ishida et al. (1996) were achieved on the basis of highly totipotent scutellum tissue of immature embryos, improved co-culturing conditions and hypervirulent Agrobacterium strains (Komari et al. 1996). Stable transformed wheat and barley lines produced by means of Agrobacterium were first reported by Cheng et al. (1997) and Tingay et al. (1997), respectively. Current barley transformation protocols claim an efficiency of over 10% (i.e. ten independent transgenic regenerants per 100 inoculated embryos) (Matthews et al. 2001, Coronado et al. 2005, Hensel et al. 2008), a significant advance over the rate achieved by biolistic transformation. However, the efficiency of current Agrobacterium-based methods achievable in wheat (Wu et al. 2003, Hu et al. 2003, Hensel et al. 2009), cereal rye (Popelka and Altpeter 2003) and triticale (Nadolska-Orczyk et al. 2005) remains comparable to the results obtained by biolistic transformation.
Alternative sources of explants for transformation

Although immature zygotic embryos are the commonest explant used in cereal transformation (because of their readiness to generate somatic embryos in vitro), other somatic tissues have also been tested for their suitability as explants. These include the inflorescence primordia of Tritordeum (the allohexaploid hybrid between macaroni wheat and Hordeum chilense) (Barcelo et al. 1994) and barley shoot meristems (Zhang et al. 1999). However, biolistic transformation from either of these explant types has so far failed to result in any successful application even though the transformation experiments date back over a decade. The zygote in principle represents an ideal target for transformation, but physical access to it is hampered by its being embedded deep within the pistil. While microinjection of plasmid DNA into isolated barley zygotes did very rarely result in reporter gene expression in transgenic lines (Holm et al. 2000), Holm et al. (2006) took advantage of an efficient in vitro regeneration system based on barley ovules isolated at the zygote stage and, in combination with Agrobacterium-mediated gene transfer, were able to achieve a transformation efficiency of up to 3%. This method also allowed the generation of transgenic lines whithout use of a selectable marker gene, however with the transformation efficiency being markedly reduced. Further optimization of this approach has raised the transformation efficiency to 8% (Kumlehn et al. 2009).

Isolated immature pollen and embryogenic pollen

The isolation of transgene homozygotes derived from somatic tissue requires a determination of transgene copy number (usually performed by DNA gel blot hybridization), in combination with a segregation analysis in the subsequent (T₁ and T₂) generations. This process adds substantial time to the transformation procedure itself. Transgene homozygosity is required to ensure that genetically uniform plant materials can be obtained via sexual reproduction, so that phenotypic evaluation of the transgene effect can be made over time and space. At the same time, of course, it also allows for any transgenic line which shows breeding potential to be readily incorporated into a conventional breeding programme.

A possible strategy to avoid the need to select for homozygotes among the T₁ (the progeny of a primary (T₀) transgenic) is to target immature pollen for transformation, since these are produced in large numbers by the plant, and contain the host genome in its haploid state. Provided that transformed pollen cells can be made to proliferate in vitro and subsequently be converted into haploid plants, then chromosome doubling (whether spontaneous or chemically induced) will result in instant homozygosity for the transgene. However, the cell wall of the immature pollen grain (consisting of the intine and the exine) represents a significant physical barrier to transgene introduction (Stoeger et al. 1995). In addition, the gene transfer process imposes a substantial level of stress (either through wounding where biolistic transformation is attempted, or response to pathogen infection in methods employing Agrobacterium), while the need to interrupt the normal pollen maturation process and to confer regenerability by the imposition of temperature and/or nutritional stress can also be injurious to the host cell. All of these factors have resulted in little progress being made towards the development of robust transformation protocols based on immature pollen as explant. Nevertheless, stably transgenic barley plants have been obtained through biolistic transformation of immature pollen followed by embryonic development (Jaehne et al. 1994, Carlson et al. 2001). Shim et al. (2009) were able to show that about one third of the transgenic regenerants derived from biolistic gene transfer to immature barley pollen were instantly homozygous for the transgene, one third were hemizygous, and the remainder (haploids) were successfully converted to homozygous transgenics via colchicine-induced chromosome doubling. In a related approach, Salmenkallio-Marttila et al. (1995) electroporated protoplasts isolated from 3–4 week old embryogenic barley pollen cultures, but all the resulting transgenic regenerants were hemizygous for the transgene and were shown to be clones derived from a single transformation event. More recently, Kumlehn et al. (2006) developed a method of Agrobacterium-mediated gene transfer to embryogenic barley pollen cultures and obtained numerous transgenic lines, more than half of which were haploid. Among the spontaneously diploid regenerants, only a few were homozygous for the transgene. The transformation efficiency was as high as 3.7 transgenic lines per donor spike, i.e. well above that reported in other studies based on the transformation of embryogenic pollen cultures. It also represented the first demonstration that winter, as opposed to spring, barley cultivars were able to be transformed. The ultimate value of this particular method was demonstrated by its application in approaches to functional gene analysis (Stein et al. 2005, Radchuk et al. 2006).

Selectable markers

Preferential or even exclusive development of transgenic cells, tissues or plants is necessary to establish and utilize viable genetic transformation systems. To fulfil this requirement, a selectable marker gene is typically linked and co-introduced with the gene of interest to render the transgenic cells resistant to a respective selective pressure. In many early attempts to transform Triticeae cereals, kanamycin resistance conferred by the E. coli neomycin phosphotransferase (NPTII) gene was used, e.g. in barley by Lazzeri et al. (1991) and Funatsuuki et al. (1995), and in wheat by Cheng et al. (1997). In later studies however, it was found that the selection systems based on the Streptomyces spec. phosphinothricin acetyltransferase genes pat and bar conferring resistance to the herbicide Phosphinothricin (barley: Wan and Lemaux 1994, Tingay et al. 1997, Horvath et al. 2000, wheat: Becker et al. 1994, Nehra et al. 1994, Wu et al. 2003) as well as on the E. coli hygromycin phosphotransferase (HPT) gene which confers resistance to the antibiotic Hygromycin B (barley: Matthews et al. 2001, Coronado et al. 2005,
Hensel et al. 2008, wheat: Hensel et al. 2009) are more effective. The 5-enol-pyruvyl shikimate-3-phosphate synthase (EPSPS) encoded by the aroA gene from Agrobacterium strain CP4 confers resistance to the non-selective herbicide Glyphosate. This selectable marker system was implemented for the production of transgenic wheat by Zhou et al. (1995) using biolistic gene transfer and by Hu et al. (2003) employing Agrobacterium. Another selection system that has been utilized in biolistic transformation of wheat rests on the rice acetolactate synthase (ALS or AHAS) gene (Ogawa et al. 2008, Kempe et al. 2009). ALS is a target enzyme for a number of non-selective herbicides to which the mutated rice als allele that is utilized as a selectable marker gene confers resistance, since its product is not inhibited by these herbicides while its particular genetic modification does not compromise ALS enzyme function. Reed et al. (2001) and Wright et al. (2001) reported the employment of the E.coli phosphomannose isomerase (PMI) gene manA as a valuable alternative selectable marker in barley and wheat, respectively. PMI converts mannose-6-phosphate, which cannot be utilized for carbon assimilation by most plant species, into fructose-6-phosphate. Therefore, cells expressing the manA gene can preferentially develop on medium containing mannose as a carbon source.

**Transformation efficiency is dependent on the recipient’s genotype**

The recipient genotype is a major factor in determining transformation efficiency, because both transformability and the regeneration ability are under genetic control. The barley cultivar “Golden Promise” (Wan and Lemaux 1994) and the wheat breeding line “Bobwhite SH 98 26” (Pellegrineschi et al. 2002) have been found to be among the most amenable genotypes for transformation based on immature embryo explants. The production of transgenic lines from pollen explants is as yet confined to a small number of barley cultivars (Kumlehn et al. 2006, Shim et al. 2009). Frequently however, it would be desirable and sometimes even essential to transform genotypes other than the model ones. For example, candidate genes anticipated to confer tolerance to abiotic stress or resistance to a pathogen can best be analysed in the context of a susceptible plant genetic background. In surveys of both wheat and barley germplasm, several elite lines have proven amenable to transformation, albeit often at the cost of transformation efficiency (Takumi and Shimada 1997, Wu et al. 2003, Hensel et al. 2008). In particular, the German winter wheat cultivar “Certo” appears relatively easy to transform both via biolistic (Varshney and Altpeter 2001) or via gene transfer by means of Agrobacterium (Hensel et al. 2009). Surprisingly, the isolated ovules of four barley cultivars which responded only poorly to tissue culture nonetheless proved quite amenable to Agrobacterium-mediated gene transfer, implying that this particular transformation method is less genotype dependent than the others used to create transgenic Triticeae plants (Holme et al. 2008).

**Quality aspects of transgenic materials**

**Transgene integration**

The generation of true transgenic lines in the Triticeae remains a non-trivial task, and care needs to be taken to avoid the selection of false positives. These can escape identification when PCR is relied upon as the sole means of establishing whether or not an individual is transgenic. The presence of the transgene amplicon does not unambiguously prove its integration in the plant genome, since the sequence may also be present either as free plasmid DNA, in persisting Agrobacterium or in unnoticed contaminating microbes. Expression of the transgene in planta may also represent insufficient evidence for integration, since many bacteria can express transgenes driven by viral or plant promoters. A number of techniques are appropriate to validate transgene integration. Prominent among these are the expression of an intron-containing selectable marker and/or reporter gene (since bacteria are unable to splice out introns), the use of DNA gel blot hybridization to show that the transgene has been incorporated within a stretch of host DNA, and evidence for sexual transmission through subsequent generations (see also Langridge et al. 1992).

Both the location and pattern of transgene integration is critical for transgene expression. In the allohexaploid genome of bread wheat, biolistic transformation leads to a random distribution of transgene integration sites, and transgene expression is more strongly influenced by the identity of the promoter than by any position effect (Jackson et al. 2001). Similarly in barley, both biolistic transformation and Agrobacterium-mediated gene transfer are non-selective in terms of the genomic location of the integration site (Salvo-Garrido et al. 2004). However, while multiple integration events induced by biolistic transformation can result in a variety of transgene configurations, Agrobacterium tends to produce tandem arrays, if two or more copies are integrated at a single site (Stahl et al. 2002). The advantages and disadvantages of Agrobacterium-based methods compared to biolistic transformation remain controversial (Altpeter et al. 2005). However, a broad consensus is that biolistic transformation is useful for transient expression experiments, whereas a respective agroinfection-based system has yet to be established for the Triticeae cereals. Agrobacterium-mediated transformation is much preferred for fixed (non-transient) expression, since it is associated with a smaller mean transgene copy number (Cheng et al. 1997, Hu et al. 2003, Travella et al. 2005, Kumlehn et al. 2006). The cre/loxP system of bacteriophage P1, which allows for site specific recombination events in genomic DNA, has been heavily used to produce knockout animals, but its use in higher plants is still limited. Srivastava et al. (1999) were able to demonstrate its utility in inducing single copy integration events in wheat. By flanking the bar gene by lox recombination sites, a four copy locus was converted to eventually contain only a single copy transgene. In another approach, Yao et al. (2006) succeeded in increasing the proportion of low-copy
integration events in wheat lines induced by biolistic transformation through the use of linearized minimal transformation cassettes.

**Chimerism**

In principle, the transfer and integration of heterologous DNA occurs within a single cell, which then proliferates, differentiates and finally regenerates into a homogeneously transgenic plant. Deviations from this expectation can occur when non-transformed cells contribute to the regenerating plant because the selection regime was insufficiently stringent. Moreover, non-transgenic cells may profit from attenuation of the selective agent that is detoxified by neighbouring transgenic cells. However, chimerism will not be sexually transmitted to the progeny. The only consequence may be that the segregation of transgenics within T1-families can deviate from what is expected according to the Mendelian rules. A so far hardly considered scenario includes that a regenerant could be mosaic for two or more independent transformation events, if more than one cell has been independently transformed. Those plants are typically not recognized in routine procedures, since their progeny segregates in a fashion similar to that of non-chimeric primary transgenics carrying uncoupled copies of the transgene.

**Conclusions**

Our current capacity to transform the Triticeae model barley lags behind that of other model plants (in particular *Arabidopsis thaliana*), but has reached a practical level. That of the other Triticeae crop species, however, remains unsatisfactory, although all of these species are in principle transformable both by means of biolistics and *Agrobacterium*. The full potential of transformation to advance genetic understanding and drive crop improvement in the Triticeae has yet to be realised, due to a poor level of transformation efficiency, a high dependence on genotype and a limited array of cell- and tissue-specific promoters. Some desirable future goals include the establishment of chemically inducible expression systems, the targeting of sequence-directed transgene integration and the development of reliable methods for the integration of long (>>10 kb) DNA sequences to allow the engineering of biochemical pathways. It can be foreseen that genetic transformation technologies will make an increasing contribution to progress in both applied research and crop improvement in the Triticeae.

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