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Transgenic *Lolium* and *Festuca*

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ABSTRACT

Genetic engineering enables us to exchange genes among species beyond the taxonomic barrier for controlling and expanding the genetic variation of organisms. Although genetic modification of organisms can be accomplished by other asexual techniques, such as artificial mutation and somatic hybridization, so far only genetic engineering can accurately control target phenotypes by controlling foreign gene expression under the control of the optional promoter in the host genome. This indicates that, for plant breeding programs, genetic engineering can produce epoch-making breeding materials with valuable traits that are difficult to generate by conventional breeding. We present here a comprehensive summary of information on the genetic engineering of *Lolium* and *Festuca* species.

Keywords: forage grass, genetic engineering, molecular breeding, transformation

CONTENTS

TRANSFORMATION SYSTEMS 14
TRANSGENIC PLANTS 15
AIMING AT PRACTICAL USE OF TRANSGENIC PLANTS 16
REFERENCES 17

TRANSFORMATION SYSTEMS

Gene transfer can be performed with polyethylene glycol, particle bombardment, whiskers, or *Agrobacterium tumefaciens*. During the last decade, transformation systems in both *Lolium* and *Festuca* species were developed with all of these gene transfer methods. In the 1990s, polyethylene glycol-mediated transformation systems were used to produce transgenic plants of *Lolium* and *Festuca* species (Wang *et al.* 1992, 1997). This method, however, is no longer in general use since it requires delicate and complicated manipulation of protoplasts. In the late 1990s, particle bombardment- and whisker-mediated gene transfer methods became established as alternative transformation systems in *Lolium* and *Festuca* species, because these methods can directly use callus and suspension cells as targets without any troublesome protoplast culture (Spangenberg *et al.* 1995; Ye *et al.* 1997; Dalton *et al.* 1998, 1999; Altpeter *et al.* 2000; Takahashi *et al.* 2002). Although the whisker-mediated gene transfer method is the simplest, cheapest, and fastest method of direct gene transfer, compared with gene transfer mediated by particle bombardment, it still has a low transformation frequency and can treat only limited amounts of target tissue (Dalton *et al.* 1998). Particle bombardment is therefore currently the predominant gene transfer method for *Lolium* and *Festuca* species. Some research groups have concentrated their efforts on establishing *Agrobacterium*-mediated transformation systems in *Lolium* and *Festuca*, even though these two species are not primary hosts of the *Agrobacterium* (Bettany *et al.* 2003; Wang and Ge 2005; Bajaj *et al.* 2006; Cao *et al.* 2006; Sato and Takamizo 2006; Ge *et al.* 2007). Because the *Agrobacterium*-mediated gene transfer method produces low-copy-number transgenes and results in stable gene expression in the transgenic plants, this method, as well as the particle bombardment method, will prevail in the near future.

For genetic transformation to be considered for incorporation into breeding programs, high throughput and practical transformation systems are indispensable. A high-throughput transformation system comprises high transformation efficiency together with an efficient supply of a large amount of homogenous, regenerable target tissues. However, the majorities of *Lolium* and *Festuca* species are outcrossing plants and are self-incompatible, and there are many genotypes within each cultivar, indicating that the regeneration potential strongly depends on the genotype. Further, since regenerable genotypes are rare in *Lolium* and *Festuca* species, screening for a single, highly regenerable genotype is essential to obtain a large amount of homogenous regenerable target tissues. Takahashi *et al.* (2004) screened regenerable genotypes from 11,317 genotypes of Italian ryegrass through tissue culture of mature seeds of 12 cultivars, and the screened genotypes were aseptically preserved and propagated *in vitro*. Highly regenerable calli were routinely induced by isolating meristems from the screened genotypes and were incorporated into a high-throughput particle bombardment-mediated transformation system (Takahashi *et al.* 2002). Similarly, Bajaj *et al.* (2006) screened perennial ryegrass genotypes with high embryogenic potential during tissue culture, and they have maintained these genotypes as plants *in vitro*. Embryogenic calli derived from meristematic regions of the vegetative tillers were subjected to *Agrobacterium*-mediated gene transfer, and a large number of transgenic plants were efficiently produced. In each of the two above-mentioned transformation systems, maintenance of highly regenerative genotypes as plants *in vitro* is a common feature. This allows a large amount of single-genotype–derived regene-

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rable calli to be introduced at any time. This is advantageous, since transgenic plants derived from a single genotype can be precisely evaluated by comparing them with the original, non-transgenic plants preserved *in vitro* as a control. These transformation systems are effective in outcrossing plants – especially annual plant species such as Italian ryegrass (Cao *et al*. 2007).

TRANSGENIC PLANTS

Crop productivity is limited by unfavorable environment (Boyer 1982). Therefore, conferring stress tolerance on crops, including forage and turf grasses, is considered to be the most critical strategy for stable production of the crops, and genetic engineering is expected to be a powerful tool for the production of stress tolerant transgenic plants. Modification of fructan metabolism is a key approach to developing stress-tolerant grass species, since fructan is the main carbohydrate accumulated in C3 temperate grasses and is associated with winter hardiness and the sustaining of regrowth immediately after defoliation; it also contributes to the nutritive value of feed. To functionally analyze fructan synthesis in ryegrass, Ye *et al.* (2001) produced a transgenic Italian ryegrass constitutively expressing a *Bacillus subtilis sacB* gene. The transgenic plants accumulated a small amount of bacterial levan; levels of high-molecular-weight native fructan were decreased, and the pattern of accumulation of oligosaccharides in the range of 5 to 35 degrees of polymerization was distorted. Growth of the levan-accumulating *sacB*-transgenic ryegrass plants slowed down at the onset of the reproductive stage, and flowering plants were stunted and had narrower leaves and poorly developing root systems. Such aberrant phenotypes may be due to the accumulation of bacterial-type levan in plants. Unfortunately, the levan-accumulating *sacB*-transgenic ryegrass plants were not evaluated for performance under any type of stress. Hisano *et al.* (2004) produced transgenic perennial ryegrass plants overexpressing the wheat-derived fructan-metabolism–related genes *wft1* and *wft2*, which encode sucrose: fructan 6-fructosyltransferase (6-SFT) and sucrose:sucrose 1-fructosyltransferase (1-SST), respectively. Significant increases in fructan content were detected in transgenic plants expressing 6-SFT or 1-SST. Freezing tolerance of the transgenic plants was confirmed at a cellular level by freezing tests conducted by evaluating the electrical conductivity of detached leaves. Unlike in transgenic plants expressing the *sacB* gene, no aberrant development was observed in the transgenic plants that accumulated a greater amount of fructan than non-transgenic plants.

Freezing-tolerant transgenic tall fescue was also produced with the *Agrobacterium tumefaciens* isopentenyl transferase (*ipt*) gene (Hu *et al.* 2005). Freezing tests on detached leaves indicated increased tolerance to freezing in transgenic plants at a cellular level. Field trials of the transgenic plants showed higher tillering propensity and a more pronounced stay-green phenotype than were seen in non-transgenic plants under low temperatures. Li *et al.* (2004) also produced transgenic Italian ryegrass plants containing the *ipt* gene. The transgenic plants displayed a stay-green phenotype, although stress tolerance was not evaluated.

Wu *et al.* (2005) produced salt-tolerant transgenic perennial ryegrass plants by introducing the rice vacuolar Na^+/H^+ antiporter gene (*OsNHX1*). The Na^+/H^+ antiporter gene pumps Na^+ from the cytoplasm into vacuoles to maintain a higher K^+/Na^+ ratio in the cytoplasm than in the vacuoles, thus protecting the cell from sodium toxicity. Transgenic plants expressing the antiporter gene survived after stress treatment for 10 weeks with a nutrient solution containing 350 mmol/L NaCl, whereas no nontransgenic plants survived. Similarly, Zhao *et al*. (2007b) produced transgenic tall fescue plants expressing the *Arabidopsis*-derived vacuolar Na^+/H^+ antiporter gene (*AtNHX1*). Higher germination rate and biomass and less deleterious effects were observed in the transgenic plants when compared with non-transgenic plants under saline condition. The transgenic plants grown hydroponically were not affected by NaCl concentration below 200 mM whereas nontransgenic plants showed progressive chlorosis, reduced leaf size and growth inhibition under increased NaCl condition. Also, Qiao *et al*. (2007) isolated a Na^+/H^+ antiporter gene (*AeNHX1*) from root tissue of *Agropyron elongatum* that has a strong tolerance to salt stress, and transgenic tall fescue plants expressing the *AeNHX1* gene showed normal growth after irrigation with 300 mM NaCl solution at 5-day intervals for 45 days and higher relative dry weight than that of nontransgenic plants showing chlorosis and wilting.

The gene encoding transcription factors responsive to abiotic stresses are promising tool for producing multi abiotic stress tolerant transgenic plants since many stress tolerance-related genes are simultaneously regulated by the transcription factor. Wu *et al*. (2006) showed that transgenic turf-type tall fescue plants highly expressing the stress tolerance-related *CBF1* gene from *Arabidopsis* showed higher prominent performance under high salinity condition than nontransgenic plants, and their multiple stress tolerances such as low and high temperatures, dehydration and high salinity stresses were shown by analysis of electronic conductivity using *in-vitro* detached leaves. Similarly, the transcription factor *Arabidopsis DREB1A/CBF3* gene was also introduced into tall fescue by Zhao *et al*. (2007a). The transgenic plants highly contained the compatible osmolyte proline, and showed higher drought survival than nontransgenic plants under withholding water condition.

Recently, Lee *et al.* (2007) produced transgenic tall fescue plants tolerant to the stress associated with reactive oxygen species (ROS). They used *Agrobacterium*-mediated transformation to produce transgenic plants co-expressing 2 foreign genes encoding anti-oxidative enzymes – cassava CuZn-superoxide dismutase (SOD) and pea ascorbate peroxidase (APX) – under the control of the oxidative-stress–inducible promoter sweet potato peroxidase anionic 2 (SWAP2). Expression of the transgenes in the transgenic plants was upregulated during ROS-related stresses. Lower amounts of ROS were detected in transgenic plants exposed to the stresses than in stress-exposed non-transgenic plants, resulting in decreased chlorophyll degradation and cellular damage. This indicates that anti-oxidative defense in transgenic tall fescue was improved by co-overexpression of the genes encoding CuZn-SOD and APX.

In addition to research on abiotic stress tolerance, transgenic research on biotic stress tolerance has been reported. Xu *et al.* (2001) produced transgenic perennial ryegrass plants expressing an untranslatable ryegrass mosaic virus (RgMV) coat protein (CP) under the control of the rice *Act1* promoter. After inoculation with RgMV, the most resistant transgenic line showed no immuno-detectable RgMV-CP in the primary transformants or their sexual progeny. Molecular analysis clearly showed that the RgMV resistance of the transgenic line is triggered by targeted RNA degradation, resulting in post-transcriptional gene silencing (PTGS). Takahashi *et al.* (2005) targeted enhanced resistance to the fungal pathogen crown rust (*Puccinia coronata*) in Italian ryegrass. They introduced the rice chitinase (*RCC2*) gene into Italian ryegrass. Bioassay of detached leaves indicated increased resistance to crown rust in transgenic plants highly expressing the *RCC2* gene. The most resistant transgenic plants had approximately 8.7 times the chitinase activity of the nontransgenic plants. Another approach was employed by Dong *et al*. (2008) for production of transgenic tall fescue plants resistant to fungal disease carrying bacteriophage T4 lysozyme. Lysozymes have 1,4-β-*N*-acetylmuramidase that hydrolyze peptidoglycan in bacterial cell walls and have membrane-disturbing activity for antifungal activity. The conferred resistance to fungal diseases, gray leaf spot and brown patch which are caused by *Magnaporthe grisea* and *Rhizoctonia solani*, respectively, were clearly observed in the transgenic tall fescue. Similarly, they also produced transgenic tall fescue expressing the alfalfa β-1,3-glucanase *AGLU1* gene that degrades the major structural component β-1,3-glucan in fungal cell walls, truncated

frog dermaseptin SI gene that is cytolytic to bacteria, yeast, filamentous fungi and protozoa, or rice *Pi9* gene that is R gene resistant to the rice blast (Dong *et al*. 2007). The excellent performances of the transgenic plants expressing the *AGLU1* gene and dermaseptin SI gene were shown as high resistance to both *M. grisea* and *R. solani*. A transgenic plant containing rice Pi9 gene genomic region was resistant to the turfgrass isolates of *M. grisea*.

Grass pollen – ryegrass pollen in particular – is a major cause of hay fever and seasonal asthma. A number of allergenic proteins have been identified and characterized from ryegrass pollen, and some antisense strategies have been employed to down-regulate the expression of these allergenic proteins. Lol p 5 is the most important and widespread grass pollen allergen, since it reacts with IgE antibodies in more than 90% of patients allergic to grass pollen. Bhalla *et al.* (1999) targeted Lol p 5 and produced transgenic *L. rigidum* plants expressing the Lol p 5 gene in the antisense orientation under the regulatory control of the pollen-specific promoter Ory s1 (from rice). Western blot analysis and slot-blot analysis with the serum of a patient with a grass allergy confirmed a dramatic reduction in allergenicity in the transgenic pollen. Similarly, Petrovska *et al.* (2004) generated and analyzed transgenic ryegrass plants with antisense expression of the major pollen allergens Lol p 1 and Lol p 2 under the control of the pollen-specific promoter Zm13 (from maize). Molecular analysis revealed down-regulation of the target gene expression, resulting in reduced accumulation of the target proteins in transgenic pollen.

Dry matter digestibility is one of the most important traits in forage grasses. Even slight improvement in forage digestibility can have a significant impact on animal performance, such as in beef and milk production. The major factor limiting forage digestibility is the existence of lignin in cell walls. Lignins are phenolic heteropolymers associated with cellulose and hemicellulose in cell walls; they limit the utilization of these cell wall polysaccharides and, concomitantly, the energy uptake from forage grasses by livestock. Therefore, altering the lignin content and improving forage digestibility are crucial issues for efficient production of livestock products. Chen *et al.* (2003) isolated a gene encoding the key lignin biosynthetic enzyme cinnamyl alcohol dehydrogenase (CAD) from tall fescue and introduced the isolated gene back into tall fescue with constructs for either sense or antisense expression of the *CAD* gene. Severely reduced levels of *CAD* gene transcription were detected in a transgenic plant carrying sense *CAD* transgenes, and significantly reduced endogenous CAD enzymatic activities were detected in a transgenic plant carrying antisense *CAD* transgenes. A significant reduction (14–15%) in the Klason lignin content and increased (7.2–9.5%) *in vitro* dry matter digestibility were found in the CAD down-regulated plants when compared with control plants. Chen *et al.* (2004) isolated another lignin-biosynthesis–related gene encoding caffeic acid *O*-methyltransferase (COMT) from tall fescue, and they produced transgenic tall fescue plants with either sense or antisense *COMT* gene constructs. They obtained two transgenic plants with sense *COMT* gene constructs in which the *COMT* gene was co-suppressed. These COMT down-regulated transgenic plants showed drastically reduced COMT enzymatic activity, a significant reduction (28–29%) of Klason lignin content, and increased (9.8–10.8%) *in vitro* dry matter digestibility. Buanafina *et al.* (2006, 2008) focused on ferulic acid esters that play a key role in crosslinking hemicellulose in cell walls. The crosslinks hinder degradation of the cell wall by ruminant microbes. Buanafina *et al.* (2006, 2008) produced transgenic Italian ryegrass and tall fescue plants expressing a fungal ferulic acid esterase that releases both monomeric and dimeric ferulic acids from hemicellulose. Ferulic acid esterase expression in transgenic plants resulted in the release of monomeric and dimeric ferulic acids from the cell walls and increased *in vitro* dry matter digestibility compared with that of non-transgenic plants.

The nutritional value and digestibility of forage grasses decline significantly as maturity progresses. This is largely caused by the high amounts of poorly digestible compounds in floral stems, such as lignins and cell wall compounds coupled to lignin. Delaying flowering by extending the vegetative growth phase is therefore another option for improving digestibility in forage grasses. Later flowering is also expected to result in higher density and persistence of the sward and is thus attractive for the amenity use of grasses. Jensen *et al.* (2004) isolated a floral repressor gene, *LpTFL1*, from perennial ryegrass to manipulate the transition to flowering in red fescue (*F. rubra* L.). Transgenic red fescue plants expressing *LpTFL1* at low to intermediate levels flowered approximately 2 weeks later than control plants, whereas transgenic plants with very high expression of *LpTFL1* remained non-flowering after exposure to strong vernalization conditions in 2 successive years. Van der Valk *et al.* (2004) introduced the *Arabidopsis* TALE-homeobox gene *ATH1* into perennial ryegrass. In the *ATH1*-expressing transgenic plants, heading was delayed, and in many cases the transgenic plants never flowered at all. These transgenic plants showed a leafy phenotype, and when they eventually headed they generally produced a reduced number of inflorescences.

Another approach to improving forage quality is through the accumulation of nutritionally useful proteins in the leaves of forage grasses. For instance, forage containing the sulfur-containing amino acids methionine and cysteine are attractive in ruminant animal nutrition, since such forage leads to efficient growth in beef animals, milk production in dairy cows, and wool growth in sheep. Wang *et al.* (2001) produced transgenic tall fescue expressing the gene encoding sulfur-rich sunflower albumin SFA8 under the control of the cauliflower mosaic virus 35S promoter or the light-regulated wheat *Cab* promoter. SFA8 accumulated at levels of up to 0.2% of the total soluble protein in the leaves of the transgenic plants. However, the level of SFA8 that is required before it becomes nutritionally useful is estimated to be approximately 4% of the total leaf protein. Wang *et al.* (2001) suggested that strategies for further accumulation of SFA8 protein in transgenic plants could include the use of stronger promoters or the development of chloroplast transformation techniques.

A unique type of research was attempted with turf-type perennial ryegrass. Turf-type perennial ryegrass is often used for winter over-seeding of dormant warm-season turfgrass species on golf courses and playing fields. In late spring to early summer, warm-season turfgrass starts growing and turns green. Ideally, perennial ryegrass should decline at this time, since persistence of the ryegrass disturbs the growth of the warm-season turfgrass. Chen *et al.* (2005) proposed using a genetic engineering approach to selectively remove the perennial ryegrass when it is no longer needed. They produced transgenic perennial ryegrass plants expressing an *E. coli argE* gene that encodes an *N*-acetylornithinase. The transgenic plants were selectively eliminated by application of the pro-herbicide *N*-acetyl-PPT since *N*-acetylornithinase expressed in the transgenic plants converts *N*-acetyl-PPT to the herbicide PPT.

AIMING AT PRACTICAL USE OF TRANSGENIC PLANTS

Considering safety and the influence of escaped transgenic plants on natural ecosystems, careful judgment is necessary for practical use of transgenic plants. Care is particularly needed in wind-pollinated and outcrossing *Lolium* and *Festuca* species, because transgenic plants of these species are likely to become aggressive weeds: transgenic plants can easily hybridize with volunteers in the environment, and transgenic seeds will spread widely (Giddings 2000). In fact, Wang *et al.* (2004) reported that, in tall fescue, transgenic pollen reached and pollinated recipient plants up to 150 m from a central transgenic plot. As a first step to solving the issue of transgene flow through transgenic pollen, Takamizo

et al. (2005) proposed utilizing a maternal inheritance trait through cytoplasmic male sterility (CMS), in which viable pollen cannot be produced. They have developed CMS Italian ryegrass and tall fescue lines as transgene recipients. If a complete CMS trait is introduced into a transgenic line, there is no longer a risk of hybridization by transgenic pollen dispersal. Also, transgenes integrated into the plastid genome are inherited maternally, minimizing the possibility of outcrossing transgenes to related weeds (Daniell *et al.* 2005). Plastid transformation will be a key next-generation technology and is expected to be established in *Lolium* and *Festuca* species.

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