ABSTRACT

Chen, Xi. Genetic Transformation of Perennial Ryegrass for Chemical Induced Elimination (Under the direction of Dr. Rongda Qu.)

Perennial ryegrass (*Lolium perenne* L.) is an important forage and turfgrass species, widely distributed throughout the world, including North and South America, Europe, New Zealand, and Australia. As a turfgrass, it is widely used in winter overseeding of warm-season turfgrasses. Summer persistence is a major problem for overseeding use of the species.

To solve this problem through biotechnology approach, we introduced an *E.coli argE* gene into perennial ryegrass. *ArgE* gene encodes N-acetylornithinase (NAO) which is involved in the arginine biosynthesis pathway. It has been shown that NAO can deacetylate N-acetyl-phosphinothricin (N-acetyl-PPT), a chemical which is non-toxic to plants, and produce phosphinothricin (PPT), the active ingredient of herbicide Basta and Finale.

The objectives for this project are (1) to develop an efficient transformation system for perennial ryegrass, (2) to introduce *argE* gene into perennial ryegrass and (3) to determine if transformed plants can be killed using N-acetyl-PPT.

Three approaches were used to develop target materials for bombardment transformation: suspension lines from liquid culture, selected embryogenic calli and embryogenic calluse lines from solid culture medium.

A total of 200 plates of selected calli induced from mature seeds were bombarded and 51 hygromycin B resistant calli were recovered, among which a total of 47 green plants were regenerated from 22 independently transformed resistant calli. Although a

few resistant calli were obtained, no transgenic plants were produced from callus lines and suspension cell lines.

Presence of the transgenes into plant genomes was demonstrated by PCR as well as Southern hybridization analysis. When N-acetyl-PPT was painted on transgenic leaves, leaf damage ranged from yellow to completely dead and the whole transgenic plants were killed in N-acetyl-PPT spray experiment. It suggested that the *argE* gene function as expected in transgenic perennial ryegrass plants.

Genetic Transformation of Perennial Ryegrass for Chemical Induced Elimination

by

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Chair of Advisory Committee

BIOGRAPHY

Xi Chen was born in Tianjin, China in 1976. He received his B.S degree in Biochemistry in 1999 from Lanzhou University, Lanzhou, China. In 2000, he came to the U.S. to pursue a Master of Science degree in Department of Crop Science at North Carolina State University.

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Table of Contents

List of Tables

List of Figures

Introduction

It is now nearly two decades since the first transgenic plants were successfully generated. Plant genetic transformation involves the introduction and integration of desired genes into the genome of a plant cell, and in most cases, regeneration of a whole plant from the transformed cell. Through genetic transformation, a series of useful traits has been introduced into crops. These include crops resistant to diseases and insects, tolerant to herbicides and environmental stresses, and crops able to produce pharmaceuticals, food additives and other beneficial chemicals. Some of the transgenic crops, especially those resistant to herbicides and insects, performed so well that they are taking the seed markets in cotton, soybean and corn in the US as successful cases in which genetic transformation is integrated into crop breeding and applied to agricultural production.

The advances in generating useful transgenic crops have come from three research areas: basic research in plant molecular biology and physiology; the development of transformation technologies; and improvement in plant tissue culture to regenerate transformed plant cells or tissues.

According to Birth (1997), the essential requirements for a transformation system include:

1. Availability of a target tissue including cells competent for plant regeneration
2. A method to introduce DNA into those regenerable cells; and
3. A procedure to select and regenerate transformed plants at a satisfactory frequency

Although dicotyledonous plant species are commonly transformed using *Agrobacterium*-based transformation systems, early work suggested that monocotyledonous species were recalcitrant to infection by *Agrobacterium*. Thus, protoplast transformation became the first approach for monocot transformation. Protoplasts can be induced to take up DNA either by polyethylene glycol treatment or by electroporation. Both methods have proven to be suitable for stable transformation of cereal protoplasts and transformed cell lines were obtained (Fromm et al., 1986; Rhodes et al., 1988; Lazzeri et al., 1992). Although protoplast transformation methods had some success, the procedure is tedious and regeneration of transgenic plants remains difficult for many species. In addition, sterility of transgenic plants was frequently a problem with the method. As a result, this method has been largely replaced by the biolistic, and in certain cases, *Agrobacterium*-mediated approaches for monocot transformation. However, for several types of experiments such as the analysis of promoter function and gene expression, protoplast transformation remains a useful alternative (Fisher & Hain, 1995).

The invention of the particle bombardment technique (Sanford et al., 1987) was a major development in plant genetic manipulation as it enabled transformation of many plant species not amenable to *Agrobacterium*- or protoplast- based gene transfer. Particle bombardment is one of the most widely used plant transformation method and has been applied to a broad range of species, especially monocots. The method is based on the delivery of DNA into plant cells such as suspension cultures, tissue in culture, whole plant parts, etc using dense, microscopic particles as carriers. These gold or tungsten particles, typically1μm in diameter, are coated with plasmid DNA containing plant gene

expression cassettes and accelerated into target cells by either gunpowder, gas or air pressure, or by electrical discharge to penetrate the rigid cell wall barrier.

For the transformation of cereals, the choice of appropriate target tissue is of major importance as there are only a few types of cells competent for both transformation and plant regeneration. The most common tissues used for this purpose are immature embryos, embryogenic suspension cells and embryogenic callus cultures. For stable transformation and regeneration, suspension cells were used as target tissue in maize (Gordon-Kamm et al., 1990), rice (Cao et al., 1992) and oat (Somers et al., 1992). However, the regeneration ability of the cells is often significantly reduced during maintenance of the suspension. Alternatively, embryogenic callus has been considered as a target tissue because the time needed for establishment of cultures and plant regeneration is shorter for callus cultures than for suspension cultures. Using callus cultures, it was possible to regenerate transgenic sugarcane (Bower & Birth, 1992), wheat (Vasil et al., 1992), maize (Waters et al., 1992) and barley (Wan & Lemaux, 1993).

Immature embryos are often used as a target tissue to initiate regenerable callus and to perform genetic transformation in cereals. The time necessary for the preparation of the target tissue is comparatively low and the risk of somaclonal variation is reduced as the period in culture is shortened to a few weeks. In cereals, scutellar tissue of immature embryos of rice (Christou et al., 1991), maize (Koziel et al., 1993) and wheat (Weeks et al., 1993) have been successfully used as the target tissue for stable transformation and subsequent regeneration of transgenic plants. Considerable variation in the frequency of transformation of immature embryos, ranging between 0.00% and 1.71%, were found among wheat cultivars (Takumi & Shimada, 1997).

The soil-borne microorganism *Agrobacterium tumefaciens* has been utilized routinely for transformation of dicotyledonous plants. It appeared until recently that monocotyledons were beyond the range of this transformation method because most are not among the natural hosts of *Agrobacterium*. A valuable extension to *Agrobacterium* utility occurred when it was conclusively proven that monocots, such as rice, could indeed be successfully transformed by the bacterium at relatively high frequencies (Hiei et al., 1994)

Protocols for efficient *Agrobacterium*-mediated transformation were reported for rice subspecies Japonica (Hiei et al., 1994), Javanica (Dong et al., 1996) and Indica (Rashid et al., 1996). The key factors involved in the various protocols include the use of tissue that consists of actively dividing, embryonic cells, such as calli induced from scutella; co-cultivation of cells with *Agrobacterium* in the presence of acetosyringone, a potent inducer of the *Agrobacterium* virulence genes that are involved in plant cell infection and T-DNA transfer, composition of media, bacterial strains and vectors, and genotype, etc.

Efficient *Agrobacterium*-mediated transformation was also reported in maize (Ishida et al., 1996). Immature embryos were inoculated with *Agrobacterium* and the frequency of transformation was quite high, varying between 5% and 30%. Transgenic barley plants were obtained from immature embryos infected with *Agrobacterium* (Tingay et al., 1997). *Agrobacterium* transformation of wheat immature embryo and embryogenic calli has also been reported (Cheng et al., 1997).

Silicon carbide whiskers have been used to introduce DNA into plant cells. The essence of the technology is the microscopic needle-like silicon-carbide whiskers which

are approximately 0.6 microns in diameter and vary from 5-80 microns in length to deliver plasmid DNA into intact suspension cultured cells. Collisions between the silicon carbide whiskers and the suspension cells lead to cell penetration, DNA uptake, and subsequent stable transformation of the plant cells. This approach has produced fertile transgenic maize (Frame et al., 1994), Italian ryegrass, perennial ryegrass, tall fescue and creeping bentgrass (Dalton et al., 1998). However, the method is limited to fine suspension cultures of cells that can be readily penetrated by the whiskers, and thus is often associated with inability to regenerate plants.

Promoters play an essential role in transgene expression in transgenic plants. Large numbers of potentially useful promoters have been isolated from cereals, and their effectiveness has been examined in transient expression assays as well as in stably transformed plants. Commonly used promoters for plant transformation include the cauliflower mosaic virus (CaMV) 35S promoter, which is a constitutive promoter, suitable for driving expression of foreign genes in dicotyledons; and the maize *ubi1* and rice *Act1* promoters, near-constitutive promoters, which drive strong expression of transgenes in monocotyledons. Promoters are also available to drive expression of transgenes in particular tissues of the plant, or at certain developmental stages (Grosset et al., 1997).

Scorable markers or reporter genes have been utilized since the early days of plant transformation as indicators that transformation has indeed taken place, either for transient transgene expression assays or stable transformation analysis. The two most frequently used scorable markers are *GUS* gene from *E. coli* and luciferase gene (LUC) from firefly. The *GUS* gene encodes beta-glucuronidase, the expression of which can be

readily evaluated by histochemical assays (Jefferson et al., 1987), while luciferase activity can be easily examined by fluorometry (DeWet et al., 1987). More recently, the gene of green fluorescent protein (GFP) from jellyfish has become an important *in vivo* reporter in plants. GFP allows visualization of transgenic cells by excitation with ultra-violet or blue light without the need to supply a substrate to the tested tissue (Sheen et al., 1995).

The establishment of an efficient plant transformation system requires careful choice of an appropriate selectable marker gene. The neomycin phosphotransferase II gene (*nptII*) in concert with antibiotic kanamycin has become the most widely used selectable marker gene system in dicotyledonous plants. For cereals and grasses, antibiotic hygromycin B has been used more successfully as a selection agent. Hygromycin B resistance is conferred by the *hph* gene isolated from *E. coli* coding for the enzyme hygromycin phosphotransferase (Waldron et al., 1985). Alternatively, herbicide resistance genes, such as the Basta-resistant *bar* gene and Roundup-resistant CP4 gene, have also been successfully used for transgenic plant selection. In wheat and barley, phosphinothricin (PPT, the active ingredient of herbicide Basta) resistance conferred by the *bar* gene has proved to be useful for the selection of the transformed plants. The *bar* gene encodes the enzyme phosphinothricin acetyltransferase to detoxify the herbicide by chemical modification (Wilmink & Dons, 1993). The CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme has a reduced affinity for glyphosate when compared to the native plant EPSPS enzyme. As a result, transgenic plants expressing the CP4 EPSPS enzyme become tolerant to glyphosate (Delannay et al., 1995).

Mannose-6-phosphate isomerase (MPI) is a recently developed selectable marker. This enzyme, encoded by *manA* gene from *E coli,* can convert mannose-6-phosphate to fructose-6-phosphate. Non-transgenic plant cells cannot metabolize mannose as a carbon source. Thus, only transformed cells expressing *manA* can grow on medium with mannose as the only sugar source and the growth of untransformed plant cells is inhibited (Hansen, 1999).

Advances in genetic transformation of turfgrass, like other major crop species, have been made due to the development of plant regeneration from embryogenic tissue cultures. Transgenic turf grasses were first obtained by direct gene transfer to protoplasts (Horn et al., 1988). With the development of biolistic techniques, bombardment of embryogenic cell cultures has become the main method for producing transgenic grasses. Successful transformation has been reported for tall fescue (*Festuca arundinacea*) (Wang et al., 1992), orchard grass (*Dactylis glomerata*) (Horn et al., 1988), creeping bentgrass (*Agrostis palustris*) (Zhong et al., 1993), red fescue (*Festuca rubra*) (Spangenberg et al., 1995), Italian ryegrass (*Lolium multiflorum*) (Ye et al., 1997), and perennial ryegrass (*Lolium perenne*) (Spangenberg et al., 1995).

Perennial ryegrass is a cool-season perennial bunchgrass native to Europe, temperate Asia, and North Africa. It is widely distributed throughout the world, including North and South America, Europe, New Zealand, and Australia as a forage grass as well as a turfgrass species. In the United States, perennial ryegrass is used for forage predominately in the coastal Northwest, irrigated intermountain valleys of the West, the Midwest, and Northeast (Jauhar 1993). Perennial ryegrass is commonly used as turfgrass for winter overseeding of warm-season turfgrass species, mostly in golf courses in the

South and the transition zones, because it grows well in winter and can withstand traffic at low mowing height (Mohr et al., 1998).

Genetic improvement of perennial ryegrass by conventional plant breeding methods has been fruitful in the past decades. Biotechnological approaches can introduce agronomically useful genes and help enlarge the germplasm pool for the conventional breeding efforts. Gene transfer to perennial ryegrass has been reported in the past several years through a variety of transformation methods: direct gene transfer to protoplasts, biolistic-and whiskers-mediated transformation of embryogenic suspension cells and calli.

One prerequisite for generation of transgenic plants from protoplasts is the availability of a reproducible and efficient plant regeneration system from these cells. Significant progress has been made in the establishment of plant regeneration system from embryogenic suspension cells and corresponding protoplasts of perennial ryegrass (Wang et al., 1993).

Plant regeneration from cell suspension-derived protoplasts in perennial ryegrass was first reported by Dalton (1988). The first fertile plants from protoplasts in perennial ryegrass were obtained by Wang et al. (1993). The potential of embryogenic suspension cultures and corresponding protoplasts for producing fertile, well-performing plants which can be integrated into breeding programs was demonstrated (Stadelmann et al., 1998).

Wang et al. (1997) reported transgenic perennial ryegrass plants produced by direct gene transfer to protoplasts. Twenty kanamycin-resistant callus clones regenerating green plants were obtained in Italian ryegrass and perennial ryegrass after PEG-mediated

transformation of cell suspension-derived protoplasts with a binary vector carrying a chimeric *nptII* gene driven by the *nos* promoter as selectable marker and a chimeric GUS reporter gene under control of the CaMV 35S promoter. An average transformation frequency of $5x10^{-6}$ was estimated.

In whiskers-mediated transformation, Dalton et al. (1998) obtained one perennial ryegrass transgenic plant by silicon carbide whiskers transformation. However, the number of transgenic plants generated was rather small. The usefulness of this method in grass transformation needs further evaluation.

For the biolistic approach, stable transformation and long-term expression of the chimeric *gusA* gene in callus line of *L. perenne* obtained by microprojectile bombardment of nonnorphogenic suspension cells was reported (van der Mass et al., 1994). A CaMV 35S-driven *hph* gene was used as a selectable marker for these experiments. Bombarded perennial ryegrass suspension cells were selected on solid medium containing 150 mg/L hygromycin. On average, 5.5 hygromycin-resistant calli were obtained from 250 mg of fresh-weight bombarded suspension cells. The stable integration of one to five *gusA* and *hph* gene copies in the genome of the hygromycin-resistant perennial ryegrass callus lines was demonstrated by Southern hybridization analyses. GUS activity was still detected in 40% of the callus lines after 1 year.

Transgenic perennial ryegrass plants were obtained by microprojectile bombardment of embryogenic suspension cells using a chimeric *hph* gene construct driven by the rice *Act1* 5' regulatory sequences (Spangenberg et al., 1995). Different treatments of the target cells and different DNA-particle delivery parameters were evaluated. Pre-bombardment osmotic treatment of the suspension cells for 30 min in

liquid medium containing 30 g/L sucrose and supplemented with 0.25 M mannitol and 0.25 M sorbitol led to a significant increase in the frequency of transient *gusA* expression. On average one hygomycin-resistant callus was recovered in 26% of the bombarded dishes. Plants were regenerated from 23% of the hygromycin resistant calli.

Transgenic perennial ryegrass and Italian ryegrass plants were also regenerated from suspension cells bombarded with two plasmids containing selectable *hph* gene and nonselectable *gusA* gene, respectively (Dalton et al., 1999). By PCR analysis, co-transformation frequencies of the GUS gene varied from 33% to 78% of transformants, while histochemical staining of leaf tissue from soil-grown plants showed that the co-expression frequency varied from 37% to 50%.

Large numbers of independently transformed fertile perennial ryegrass plants from forage- and turf-type cultivars were produced by Altpeter et al. (2000). To reduce tissue culture period, young embryogenic calli from mature embryos, immature embryos and young inflorescences were used as targets for the biolistic gene transfer. Transgenic plants expressing *npt* II gene driven by the maize *ubi1* promoter were selected. In total, 83 independent transgenic perennial ryegrass plants were produced. Transgene expression was confirmed by ELISA or western blot and transgene integration, by Southern hybridization. Transformation frequency was highly affected by genotype, explant, selection regime and the duration of the callus induction period. Using same transformation protocol, untranslatable Ryegrass mosaic virus coat protein (RgMV-CP) gene was introduced into perennial ryegrass to explore the potential of RNA-mediated virus resistance. A significant influence of the transgenic line, virus strain and the period after inoculation on the RgMV level was observed (Xu et al., 2001).

We intend to use a biotechnology approach to improve perennial ryegrass. Perennial ryegrass is widely used for the overseeding of quality bermudagrass lawns, golf courses and sports fields in the South. Most golf course greens today are overseeded with perennial ryegrass for primary winter cover. However, when summer comes, many perennial ryegrass plants are not completed killed by the heat as expected and become weeds in the course sites. Our goal was to transform perennial ryegrass with *argE* gene to help solve this problem.

The *argE* gene encodes *N*-acetylornithinase (NAO; EC 3.5.1.16) in the *E. coli* biosynthetic pathway, which converts N_2-acetylornithine into acetate and ornithine (Meinnel et al., 1992). This gene has been successfully expressed in tapetum tissue in transgenic tobacco plants to develop a system of inducible male sterility, which is based on the deacetylation of the non-toxic compound N-acetyl-L-phosphinothricin (N-acetyl-PPT) and consequent production of phosphinothricin (PPT), an herbicide, in the anther tissue (Kriete et al., 1996). We consider this gene be a good candidate for perennial ryegrass transformation for chemical induced elimination to target its summer persistence problem.

The objectives for this project are: (1) to develop an efficient transformation system for perennial ryegrass; (2) to introduce *argE* gene into perennial ryegrass and (3) to determine if transformed plants can be killed using N-acetyl-PPT.

Materials and Methods

1. Plant materials

1.1 Induction of callus using mature seed and immature infloresence as explants

Three turf-type perennial ryegrass cultivars, 'Majesty', 'Roadrunner' and 'Lisabelle', were used. Mature seeds were dehusked by constant stirring in 50% sulfuric acid for 30 min, rinsed five times with distilled water followed by a rinse with 95% ethanol, and surface-sterilized with stirring in 100% Clorox® (5.25% sodium hypochlorite) plus 0.1% of Tween 20 (Fisher, Pittsburgh, PA) for 30 min, then rinsed with sterile distilled water six times. Sterilized seeds were soaked in sterile distilled water overnight at room temperature to facilitate embryo excision. To improve callus induction of mature seed culture, sterilized seeds were chopped into small pieces to suppress germination.

For experiments using immature inflorescences, perennial ryegrass 'Brightstar' was grown in the field of Pure Seed Testing Inc (Rolesville, NC). Young inflorescences wrapped in leaf sheath, were collected and surface sterilized in 50% Clorox® for 20 min, and rinsed five times with sterile distilled water. Immature inflorescences were between 1.0 and 1.5 cm long at the time of excision, and were cut into 2 to 3 mm segments to stimulate callus formation.

1.2 Culture conditions

The chopped seeds or inflorescences were plated on the callus induction medium containing MS basal medium components (Murashige and Shoog, 1962) supplemented

with 30 g/L sucrose, 2 mg/L 2,4, dichlorophenoxyacetic acid (2, 4-D), 0.1 mg/L 6-benzylaminopurine (BAP) and 3 g/L phytagel (Bradley et al., 2001). All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. The pH was adjusted to 5.8 with 0.2 N KOH prior to autoclaving. All the components were added to the medium before autoclaving except BAP, which was added to the autoclaved medium as a sterile stock after the medium was cooled to 50°C.

Callus induction was conducted in a culture chamber (I-36NL, Percival, Boone, IA) in the dark at 25°C for 6 weeks. Induced calli were excised from the explant and subcultured under the same conditions for an additional 4 weeks before transformation.

1.3 Suspension cultures

After 10 weeks of callus induction, embryogenic calli, derived from a single seed, were transferred to a 125-ml flask containing 30 ml AA liquid medium (Müller and Grafe, 1978) supplemented with 2 mg/L 2, 4-D, 20 g/L sucrose and 30 g/L D-sorbitol. The suspension cultures were maintained in the dark at 25°C on an orbital shaker at 120 rpm. Once a week, the culture medium was replaced with fresh medium.

It took 4 to 8 weeks for the suspension cultures to become established. Suspension cells established in individual flask were considered a line. The regeneration ability of a suspension line was tested by transferring cells to a regeneration medium containing MS basal medium, 30 g/L sucrose, 3 g/L phytagel and 2.5 mg/L BAP. Environmental conditions in the incubator (CU-32L, Percival) used for regeneration included a 16-hr light /8-hr dark cycle with a constant 25°C temperature. Cultured cells of suspension lines with good regeneration ability were devided and transferred into other flasks to

multiply. These lines were subsequently used for transformation experiments. A suspension line was terminated if its cell clusters failed to regenerate on regeneration medium.

2. Transformation

2.1 Plasmids

The plasmid pRQ213 contains the coding region of *argE* gene, obtained by PCR, driven by the maize *ubi1* gene promoter (Figure 1A). The plasmid pAC1 contains the selectable *hph* gene, encoding the enzyme hygromycin phosphotransferase driven by the 35S promoter and the *GUS* reporter gene driven by rice *Act1* gene promoter (Figure 1B). Plasmid pAcH1 (Spangenberg et al., 1995) contains a *hph* gene driven by the rice *Act1* promoter (Figure 1C).

2.2 Transformation by the biolistic method

Transformation experiments were performed using a PDS-1000/He biolistic® particle delivery system (Bio-Rad, Hercules, CA). Three mg of 1μm gold particles suspended in 50 μL of sterile 50% glycerol solution, were coated with 5 μL of plasmid (1 μg/μL), mixed with 50 μL of 2.5 M $CaCl_2$ and 20 μL of 0.1 M spermidine according to the manufacturer's instruction.

Based on preliminary experiments using *GUS* gene expression levels as an indicator, 1550 psi (pound per square inch), two shots per target plate were chosen for all bombardment experiments. Five hours before biolistic gene transfer, calli were transferred to subculture medium supplemented with 0.5M mannitol for osmotic

treatment and retransferred to mannitol-free callus medium 12-16 hours after the biolistic gene transfer.

Plasmids pAC1 and pRQ213 or pAcH1 and pRQ213 were mixed in 1:1 ratio, respectively and used for bombardment experiments.

2.3 Selection of Hyg B-resistant calli and recovery of putative transgenic plants

After one week of biolistic transformation, the calli were transferred to a selection medium (MS basal medium, 30 g/L sucrose, 2 mg/L 2,4-D, 0.1 mg/L BAP, and 3 g /L phytagel) containing 150 mg/L hyg B. After six weeks selection in the dark at 25°C, the surviving calli were transferred to fresh selection medium containing 250 mg/L hyg B for further selection of eight weeks. After the second round of selection, actively growing, hyg B resistant calli were transferred either to pre-regeneration medium (MS basal medium supplemented with 1 mg/L NAA, 1 mg/L BAP, and 5 mg/L ABA) for two weeks or were plated directly on the regeneration medium (MS basal medium, 30 g/L sucrose, 2.5 mg/L BAP, and 3 g/L phytagel). Regenerated shoots were transferred for rooting in a half-strength MS, hormone-free rooting medium with or withour 25 mg/L hyg B in a Magenta box (Magenta Corp., Chicago, IL). Six weeks later, rooted plants were transplanted into potting soil (Metro-Mix 200, Scotts, Marysville, OH), one per pot. Plants were covered with transparent lids for a week after transplanting for better survival.

All plantlets that were derived from a single resistant callus and survived the rooting medium selection were considered to represent the same transformation event.

2.4 Growing condition of transgenic ryegrass plants in greenhouse

Transgenic perennial ryegrass plants were directly transferred from the laboratory to the greenhouse at the NCSU Phytotron's facility. The environmental conditions inside the greenhouse included a daytime temperature at a constant 30°C and a constant nighttime temperature of 26°C. Natural sunlight served as light source.

3. Transgene assays

3.1 Assay of *GUS* reporter gene expression

GUS assay buffer was prepared and filter sterilized according to Jefferson (1987). Two days after bombardment with plasmids pAC1 and pRQ213, callus samples were immersed in GUS assay buffer overnight and examined under a stere microscope for the observation of blue spots.

3.2 PCR analysis of putative transgenic plants

Genomic DNA was extracted from 10 mg leaf tissues of each non-transformed and putative transgenic perennial ryegrass plant based on the protocol of Dellaporta et al. (1983) and dissolved in 25 µl TE buffer. PCR was carried out in 50µL reaction with 1.5mM $MgCl_2$, 1.25U of Plantum® Taq polymerase (Invitrogen, Carlsbad, CA), using 1µl genomic DNA and 1 µM each oligonucleotide primers (5'-GTGTCAACAATGAAAAACAA-3' and 5'- CCTACGTTTTAATGCCAGCA-3') designed to amplify an *argE* gene fragment. DNA was amplified in a MiniCycle™ thermocycler (MJ Research, Watertown, MA) (denaturation first at 94°C for 3 min, then 35 cycles of the following: denaturation at 94°C, 1 min; annealing at 50°C, 1 min;

extension at 72°C, 1 min). PCR products were analyzed by electrophoresis in 1.0% agarose gels.

3.3 Southern hybridization analysis of putative transgenic plants

Genomic DNA was extracted from leaf tissues of non-transformed and putative transgenic perennial ryegrass plant based on the protocol of Dellaporta et al. (1983) with the addition of a DNase-free RNase A treatment (Sigma, 0.5 mg/mL, 37°C, 30 min). Twenty μg genomic DNA from each sample, digested with restriction endonucleases *EcoR*I or *Hind*III (Promega, Madison, WI) for overnight, was separated by electrophoresis in a 1.0% agarose gel. Plasmid DNAs equivalent to one copy in a 2C perennial ryegrass genome (Arumugannthan at al., 1999) were used as positive controls (24.7 pg for pRQ213 in 0.1 μg/μl herring DNA). The fractionated DNA was transferred to GeneScreen™ hybridization transfer membrane (NEN Research Products, Boston, MA) according to instructions of the manufacturer. Hybridization was carried out following standard protocols (Sambrook at al., 1989). The *argE* gene probe was a 1.4 kb *EcoR*I fragment of pRQ213, which covers the full length coding region of the *argE* gene and the *NOS* terminator. The fragment was labeled with [^{32}P] dCTP (PerkinElmer Life Sciences, Boston, MA) by random hexamer priming with the Prime-It® II Random Primer labeling Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

3.4 Northern hybridization analysis of putative transgenic plants

Total RNA was extracted from leaf tissue. Fresh tissue was homogenized with a motar and pestle in liquid nitrogen, then suspended in Tris,HCl, pH9.0 and mixed with

an equal volume of phenol. The aqueous phase was treated with equal volume of phenol again, precipitated with 20% KoAc and isopropanol, and resuspended in sterile water. To prevent RNA degradation, the tubes used for RNA preparation were dipped into liquid nitrogen and kept in –80°C immediately. Approximately 20 μg of total RNA was separated by formaldehyde agarose gel electrophoresis and transferred to GeneScreen™ hybridization transfer membrane (PerkinElmer) according to instructions of the manufacturer. The hybridization and washing procedures were the same as in Southern hybridization.

Poly (A) + RNA was isolated from about 400 μg total RNA using PolyATract® mRNA isolation system IV (Promega). The hybridization procedures were the same as in Northern hybridization for total RNA.

3.5 Leaf painting and plant spray with N-acetyl-PPT

The non-toxic compound N-acetyl-PPT was kindly synthesized by Dr. Binghe Wang's lab at Department of Chemistry, NCSU. Two leaves were chosen randomly in every control (non-transgenic) plant from tissue culture and putative transgenic perennial ryegrass plants, and a segment of every leaf was marked using a laboratory marker pen (VWR Scientific, San Francisco, CA) and generously painted on both sides using a cotton swab with 3 mg/mL N-acetyl-PPT solution containing 0.1% Tween 20 as surfactant. After five to seven days, the leaves were evaluated for damage.

In spraying experiments, approximately 10 ml 3 mg/ml N-acetyl-PPT solution containing 0.1% Tween 20 as surfactant was used to spray each control and putative transgenic perennial ryegrass plant. After seven to ten days, the damage was evaluated.

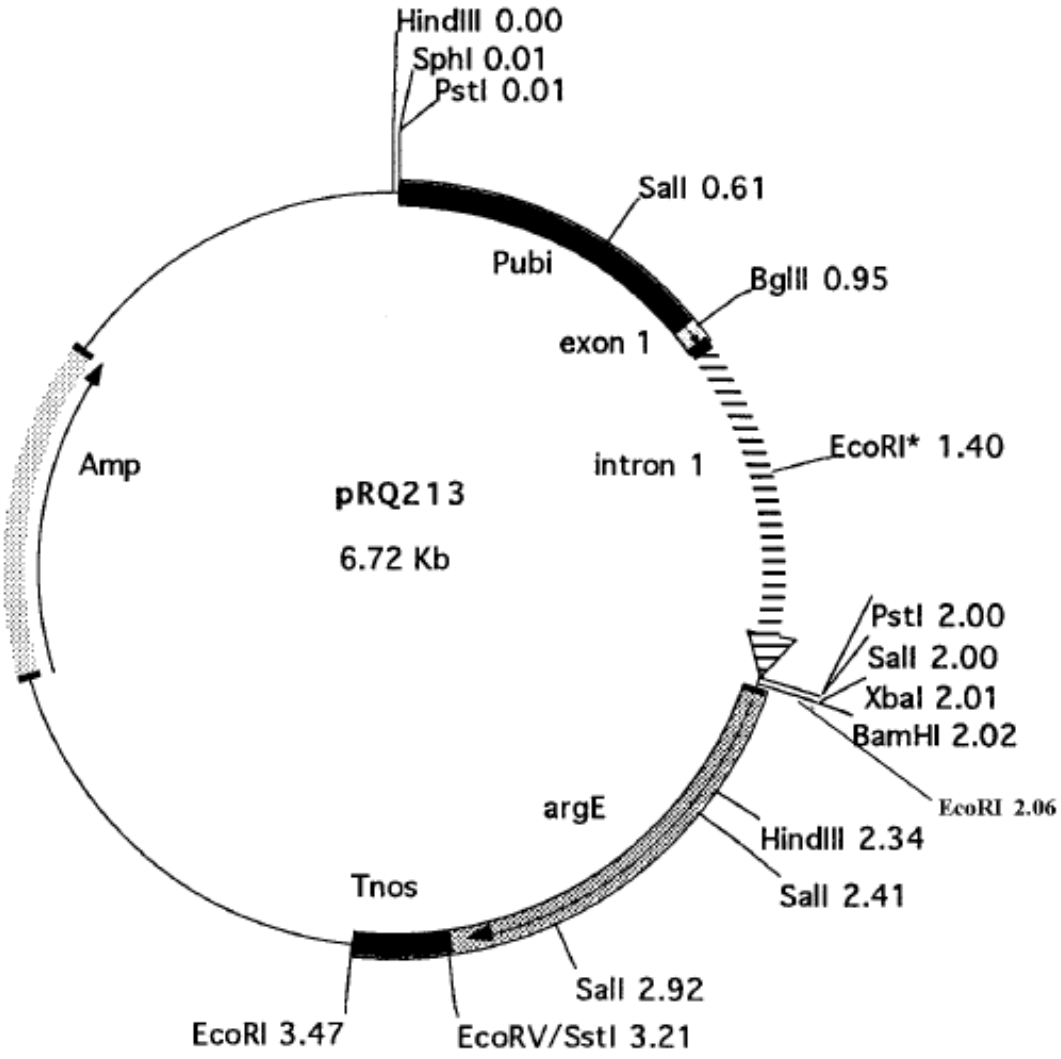


Fig. 1. Plasmids used in biolistics transformation experiments.

A. Plasmid pRQ213

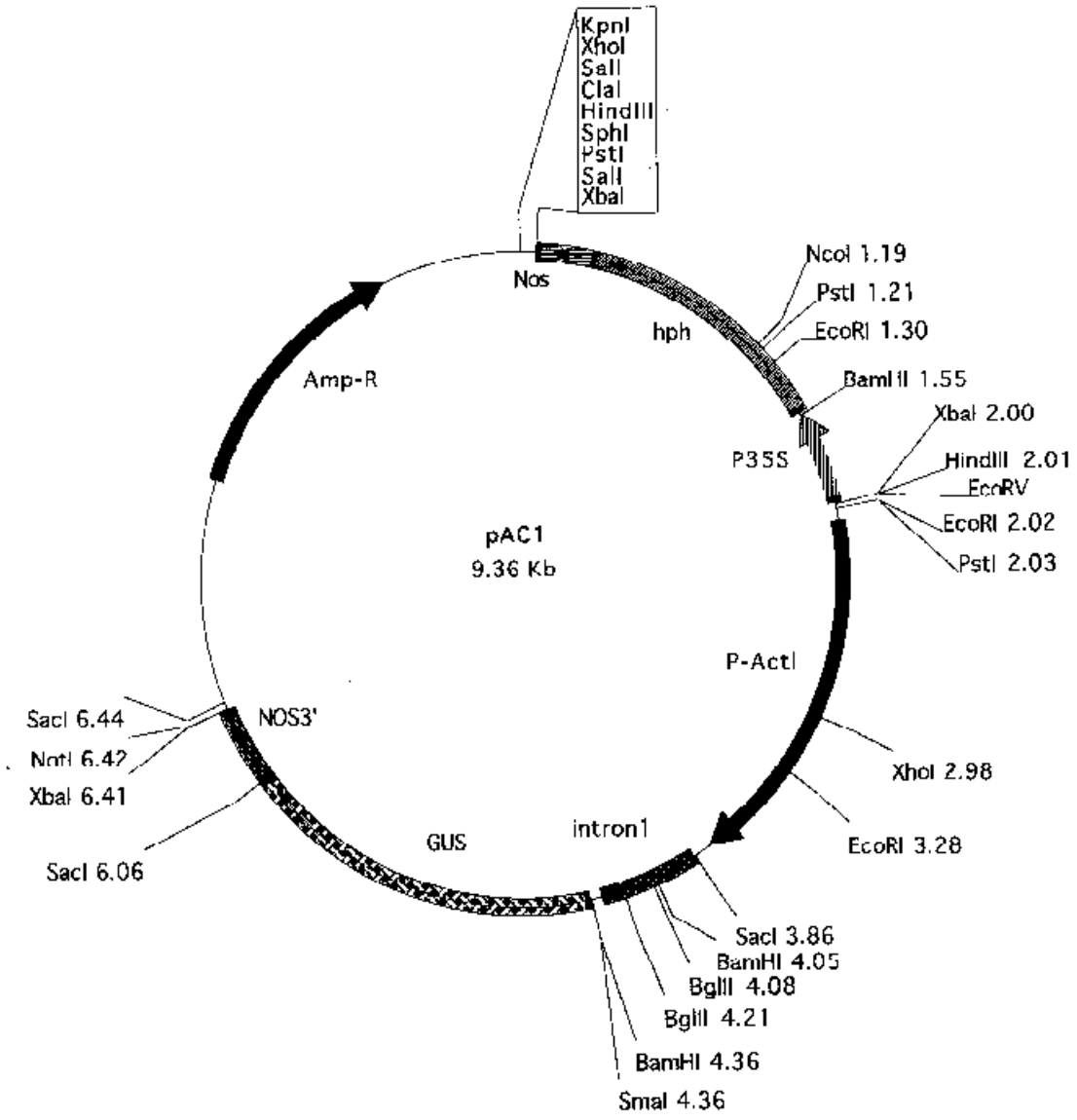


Fig. 1. B. Plasmid pAC1

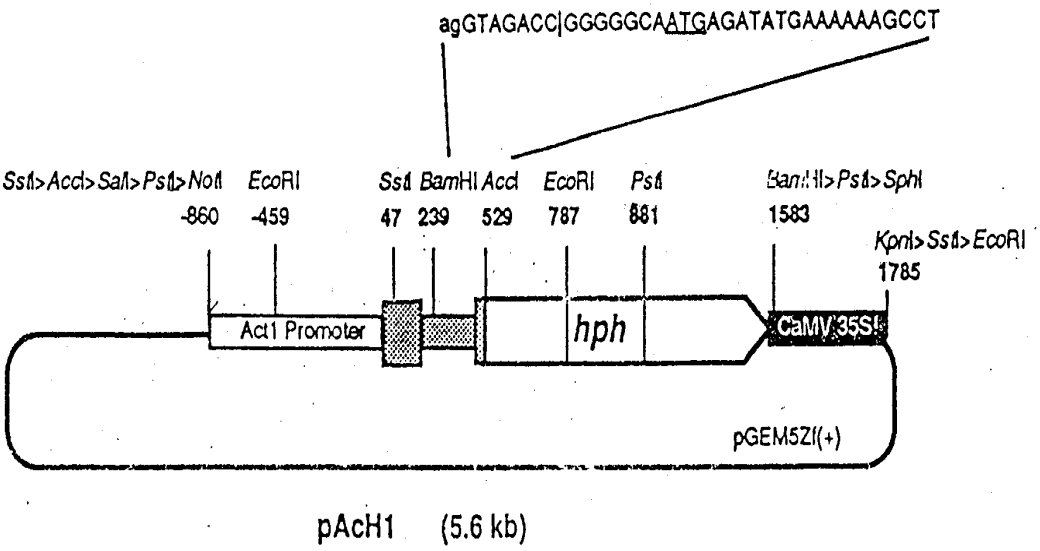


Fig. 1. C Plasmid pAcH1

Legend:

Figure 1. A. Gene construct and restriction map of plasmid pRQ213.
B. Gene construct and restriction map of plasmid pAC1.
C. Gene construct and restriction map of plasmid pAcH1.

RESULTS:

Sequence analysis of the cloned *E.coli argE* gene

Primer argE1 (5' GTGTCAACAATGAAAAACAA 3') and primer argE2 (5' CCTACGTTTTAATGCCAGCA 3') were designed to amplify the *argE* gene from genomic DNA extracted from *E. coli*. Primer argE1 is located at the 5' untranslated region of the gene while primer argE2 is downstream of the translation termination codon. The PCR product was cloned into the pCR®2.1 vector. The plasmid DNA was extracted and the PCR amplified fragment was subjected to sequence analysis at the DNA sequencing & synthesis facility of Iowa State University. The result shows that the PCR-amplified fragment is identical to the *argE* gene reported (GenBank number: X55417.1 GI:40956) (Meinnel et al., 1992). The amplified fragment is 1170 bp in length and encodes a protein of 383 amino acids. The following is the amplified sequence of *argE* gene and the derived amino acid sequence. The underlines indicate the two primers. And the translation iniation and termination codons are red-colored.

DNA sequence of amplified fragment

```
                                                                  50
GTGTCAACAATGAAAAACAAATTACCGCCATTTATCGAGATTTACCGCGC
                                                                 100
TCTGATTGCCACACCTTCAATAAGCGCCACGGAAGAGGCACTCGATCAAA
                                                                 150
GCAATGCAGATTTAATCACTCTGCTGGCGGACTGGTTTAAAGATTTGGGC
                                                                 200
TTCAATGTGGAAGTGCAGCCTGTTCCAGGAACTCGCAACAAATTCAATAT
                                                                 250
```

GCTGGCAAGTATCGGACAGGGGGCTGGCGGCTTGTTGCTGGCGGGGCATA

300

CCGATACGGTGCCATTTGATGACGGTCGCTGGACGCGCGATCCGTTTACA

350

CTGACGGAGCATGACGGCAAGCTTTACGGCTTAGGCACCGCCGACATGAA

400

AGGCTTTTTTGCGTTTATCCTTGATGCGCTACGCGATGTCGACGTCACGA

450

AACTGAAAAAACCGCTCTACATTCTGGCGACTGCTGATGAAGAAACCAGT

500

ATGGCCGGAGCGCGTTATTTTGCCGAAACTACCGCCCTGCGCCCCGGATT

550

GCGCCATCATTGGCGAACCGACGTCACTACAACCGGTACGCGCACATAAA

600

GGTCATATCTCTAACGCCATNCGTATTCANGGCCAGTCGGGGCACTTCCA

650

GCGATCCAGCACGCNGAGTTAACGCTTTCGAACTAATGCACGACGCCTTC

700

GGGCATATTTGCAATTGCGCGATACCNTGAAAGAACGTTATCACTACGAA

750

GCGTTTACCGTGCCATACCCTACGCTCAACCTCGGGCATATTCACGGTGG

800

CGACGCTTCTAACCGTATTTGCGCTTGCTGTGAGTTGCATATGGATATTC

850

GTCCGCTGCCTGGCATGACACTCAATGAACTTAATGGTTTGCTCAACGAT

900

GCATTGGCTCCGGTGAGCGAACGCTGGCCGGGTCGTCTGACGGTCGACGA

950

GCTGCATCCGCCGATCCCTGGCTATGAATGCCCACCGAATCATCAACTGG

1000

TTGAAGTGGTTGAGAAATTGCTCGGAGCAAAAACCGAAGTGGTGAACTAC

1050

TGTACCGAAGCGCCGTTTATTCAAACGTTATGCCCGACGCTGGTGTTGGG

1100

GCCTGGCTCAATTAATCAGGCTCATCAACCTGATGAATATCTGGAAACAC

1150

GGTTTATCAAGCCCACCCGCGAACTGATAACCCAGGTAATTCACCATTTT

TGCTGGCATTAAAACGTAGG

Sequence of amino acids

```
1    mknklppfie iyraliatps isateealdq snadlitlla dwfkdlgfnv
51   evqpvpgtrn kfnmlasigq gaggllagh tdtvpfddgr wtrdpftlte
101  hdgklyglgt admkgffafi ldalrdvdvt klkkplyila tadeetsmag
151  aryfaettal rpdcaiigep tslqpvrahk ghisnairiq gqsghssdpa
201  rgvnaielmh daighilqlr dnlkeryhye aftvpyptln lghihggdas
251  nricaccelh mdirplpgmt lnelngllnd alapvserwp grltvdelhp
```

```
301  pipgyecppn hqlvevvekl lgaktevvny cteapfiqtl cptlvlgpgs
351  inqahqpdey letrfikptr elitqvihhf cwh
```

Establishment and transformation of suspension lines

Three approaches were used to develop target materials for bombardment transformation: suspension lines from liquid culture, selected embryogenic calli and embryogenic callus lines from solid culture medium.

In suspension line approach, calli were induced from three cultivars of perennial ryegrass and used to develop suspension lines as described in the Materials and Methods section. The regeneration ability of established suspension line was tested by transferring cells to regeneration medium approximately two months after initiation of the suspension lines. Only 15% of the suspension lines were regenerable (Table1).

Variety	Majesty	Roadrunner	Brightstar
No. of suspension lines	60	36	16
No. of regenerable lines	6	8	2

Table1. Regeneration test of suspension lines of perennial ryegrass

The regenerable suspension lines were used for transformation. Two combinations of plasmids were used in these experiments: (1) pRQ213 and pAC1 and (2) pRQ213 and pAcH1. After bombardment, the calli were transferred to selection medium containing 150 mg/L hygB. After six weeks selection in the dark, growth of most of the cultured cells was totally inhibited and the cultures turned dark brown. Surviving calli were

transferred to a second selection medium containing 250 mg/L hyg B for an additional eight weeks of selection. Most of the calli that survived the first selection were eliminated during the second cycle of selection. Among a total of 91 plates of suspension cells bombarded, only three hyg B-resistant calli were obtained and none was regenerable (Table 2).

Plasmid used	Cultivar	No. of plates bombardment	No. of resistant calli	No. of regenerated plants
RQ213+AC1	Majesty	12	1	0
	Roadrunner	14	0	0
	Brightstar	7	0	0
RQ213+ACH1	Majesty	24	1	0
	Roadrunner	28	1	0
	Brightstar	6	0	0
Total		**91**	**3**	**0**

Table 2. Transformation results of perennial ryegrass suspension cells

Transformation of select young embryogenic callus

Callus was initiated from two different explant sources - mature embryos and young infloresences of four cultivars (see below). The calli could be divided into three major morphological categories: A) soft and watery (Figure 2A), B) yellowish or pale, compact (Figure 2B) and C) yellowish, or off-white, compact, translucent and friable (Figure 2C and 2D). In transient GUS gene expression assays, only type C cells showed relatively high number of blue spots implying transformation competency of this type of callus (Figure 3); few blue spots were observed from other types of callus. Thus, the type C callus was selected for further bombardment experiments. A total of 200 plates of type C calli induced from mature seeds were bombarded. The same two-step hyg B selection scheme was used for selection of putative transgenic calli. After eight weeks selection on

the 250 mg/L hyg B, the transformed cells grew into a white or light yellow colored callus, and were easily distinguished from dead tissues in the plate (Figure 4). Fifty-one hyg B-resistant calli were obtained , among which 22 regenerated into green plants (independent transformation events) and a total of 47 green plants were recovered (Table 3). All the putative transgenic plants recovered were transformed with plasmid pRQ213 containing the *argE* gene and pAcH1 containing the *hph* gene. Eighteen plates of selected calli induced from young inflorescences were bombarded and no resistant callus was recovered. By cultivars, 44 putative transgenic plants (from 19 independent transformation events) were obtained from 'Majesty', 3 from 'Lisabelle' (3 independent transformation events) and none were recovered from 'Roadrunner' and 'Brightstar'.

After eight weeks of selection on medium containing 250 mg/L hyg B, resistant calli were transferred to regeneration medium. Four to five weeks later, regenerated shoots (Figure 5A) were transferred to a rooting medium. Both leaves and roots of resistant plants grew well in the rooting medium (Figure 5B). All of the green putative transgenic plants survived transplantation to soil. All appeared normal in morphology and growth, and could not be distinguished from wild type plants.

Tissue culture and transformation of callus lines

In the process of selection of young embryogenic callus, some type C calli were subcultured every four weeks to develop callus lines. Very often, the calli began to lose the embryogenic structure during subculture and turned soft. In the transformation experiments, four resistant calli were obtained from 27 plates bombarded, but no transgenic plant was recovered from the resistant calli (Table 4).

Plasmid used	Cultivar	No.of callus line developed	No. of plates bombardment	No. of resistant calli	No. of regenerated plants
pRQ213+pAcH1	Majesty	52	27	4	0

Table 4. Transformation results of perennial ryegrass callus lines

Leaf painting and plant spray experiments

Leaves of putative transgenic plants and non-transformed plants were painted with 3 mg/mL N-acetyl-PPT solution. As shown in Figure 6, the damage of transformed leaves ranged from yellow to completely dead, no sign of damage was observed on the non-transgenic plant leaves. The result suggested that the introduced *argE* gene be functional, as expected, and caused the damage in the painted leaves of the transgenic plants. Twenty-five of the total 44 transgenic plants were subjected to leaf painting experiments and the results are shown in Table 5. Other plants are still too young to be tested.

Two transgenic plants, TM2 and TM9, which showed obvious damage in leaf painting experiment, were chosen for a further spray experiment. Tillers were taken from these two plants and vegetatively propagated. Figure 7 shows that the results of transgenic and non-transgenic ryegrass plants ten days after spray of N-aectyl-PPT. The transgenic plants turned yellow and were dying and no sign of damage was observed on both control plants: a non-transgenic perennial ryegrass (Figure 7A) and a bermudagrass (Figure 7B).

Molecular analyses of the transgenic plants

Figure 8 shows the PCR results of 14 transgenic plants after electrophoresis in 1.0% agarose gels. Positive control from pRQ213 and all the transgenic plants tested showed a 1.17 kb band of the amplified *argE* gene. No band was observed in negative control from non-transgenic plant.

Southern hybridization analysis was performed to confirm the *argE* gene integrated into plant genome. The results of six individual plants transformed with *argE* gene are shown in Figure 9. When hybridizations using *EcoRI* digested genomic DNA were analyzed, each of the six plants displayed an expected 1.4 kb band.

No conclusive Northern hybridization result was obtained probably due to the low expression of the bacterial gene.

Explant	Plasmid used	Cultivar	No. of plates bombardment	No. of resistant calli	No. of regenerable calli	No. of regenerated plants
Mature seed	pRQ213+ pAC1	Majesty	18	2	0	0
		Roadrunner	12	1	0	0
		Lisabelle	2	0	0	0
	pRQ213+ pAcH1	Majesty	71	41	19	44
		Roadrunner	32	4	0	0
		Lisabelle	65	3	3	3
Young inflorescence	pRQ213+ pAC1	Brightstar	6	0	0	0
	pRQ213+ pACH	Brightstar	12	0	0	0
Total			218	51	22	47

Table 3. Transformation results of perennial ryegrass young embryogenic calli

No. of transgenic plant	TM1	TM2	TM3	TM4	TM5	TM6	TM7	TM8	TM9
Damage level*	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+ +
No. of transgenic plant	TM11	TM12	TM13	TM14	TM15	TM16	TM17	TM18	TM19
Damage level	+ +	+ +	+ +	+ +	+ +	+	+	+ +	+ +
No. of transgenic plant	TM20	TM21-1	TM22-1	TM22-2	TM23-1	TM23-2	TM23-3	TM23-4	TM23-5
Damage level	+ + +	+ +	+	+	+ + +	+ +	+ +	+ + +	+ + +

* Damage level: '+' indicates that the leaves turned yellow to yellow green.
'+ +' indicates that the leaves turned brown in the painted region.
'+ + +' indicates that the half or even the whole leaf turned brown.

Table 5. Damage level of transgenic ryegrass plants after leaf painting

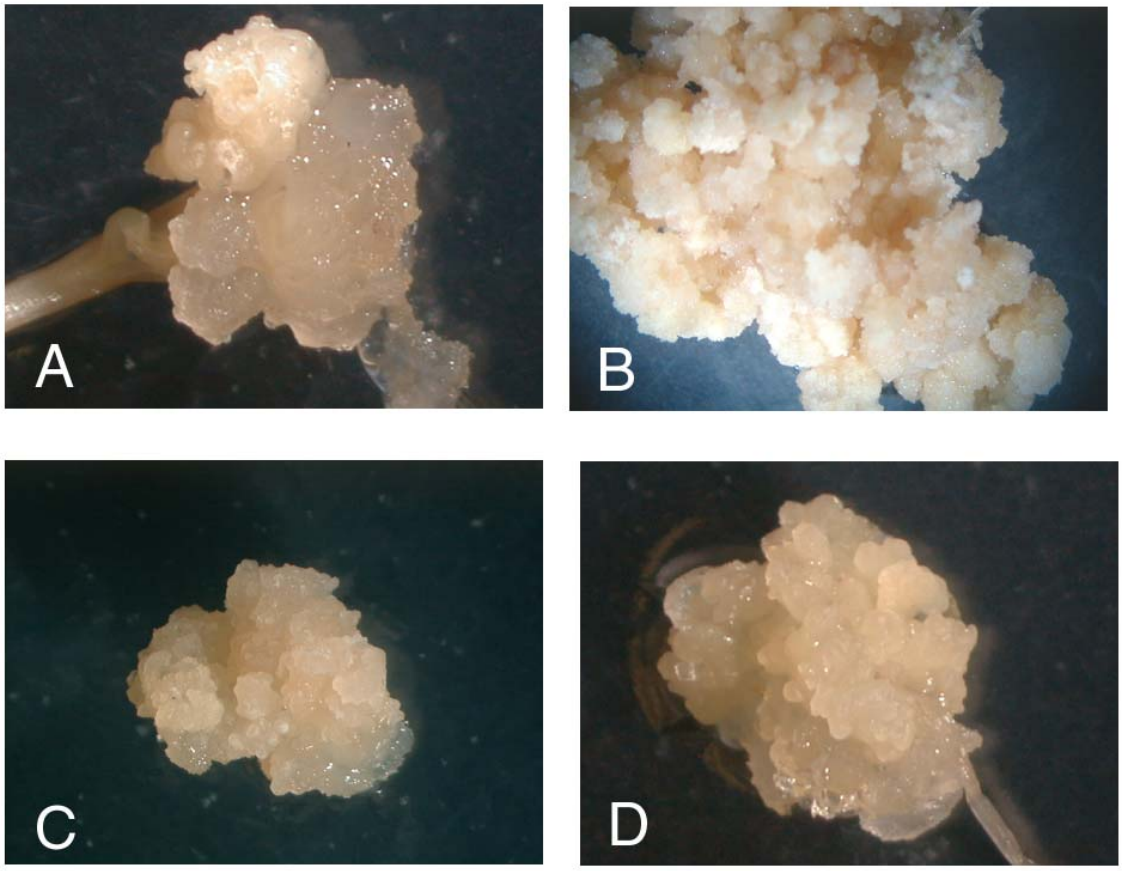


Fig. 2 Callus types of perennial ryegrass

Legend:

Figure 2.Three types of calli structure.

A. Type A callus showing soft and watery structure.
B. Type B callus showing pale and compact structure.
C. and D. Type C callus showing yellowish or off-white, compact, translucent and friable structure.

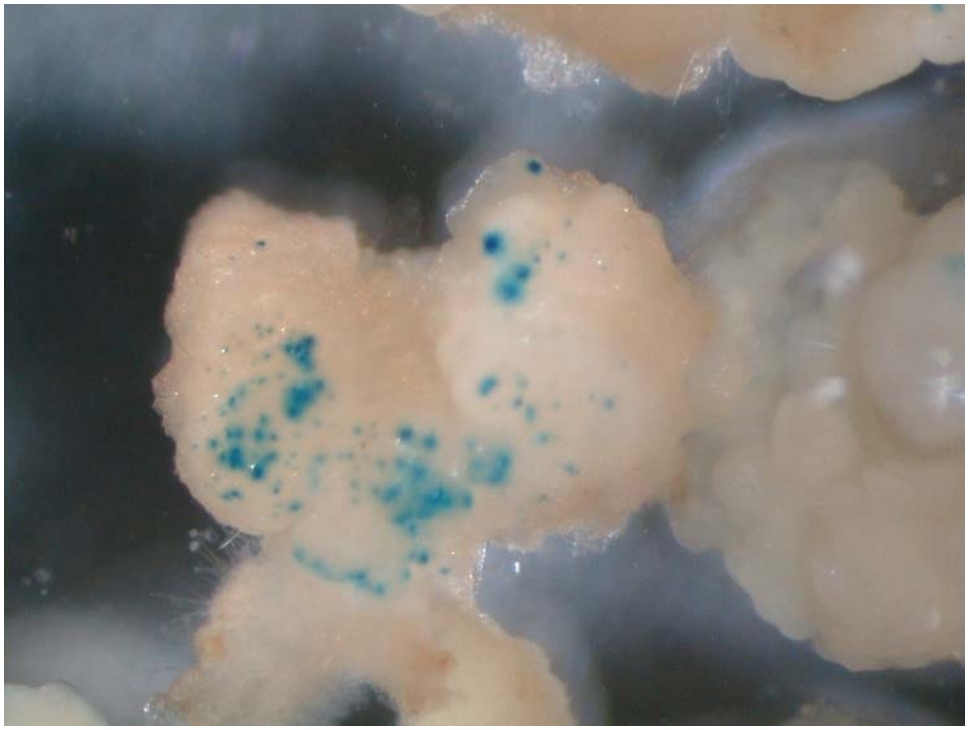


Fig. 3. Transient expression of the GUS reporter gene on type C callus.

Legend:

Figure 3. Transient expression of the GUS reporter gene. Type C callus of cultivar 'Majesty' was bombarded with plasmids pRQ213 and pAC1. GUS assay experiment showed the blue spots on the callus.

Fig. 4. Resistant calli on 250 mg /L hyg B selection medium

Legend:

Figure 4. Growing resistant calli of ‘Majesty’ six weeks after being transferred to the selection medium containing 250 mg/L hyg B.

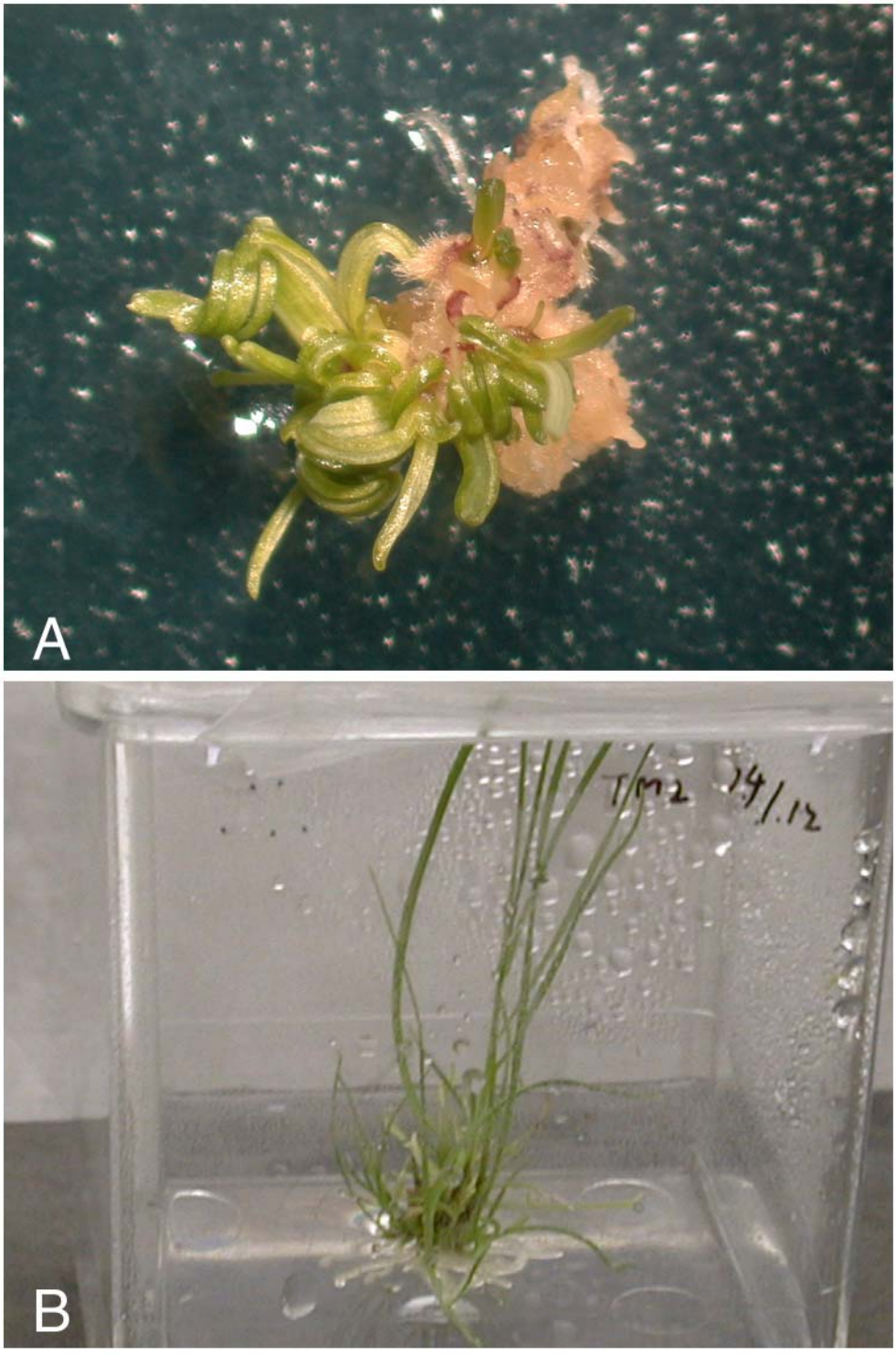


Fig. 5. Regenerated perennial ryegrass plants.

A. Regenerated shoots from resistant callus.
B. Regenerated ryegrass plant on the rooting medium.

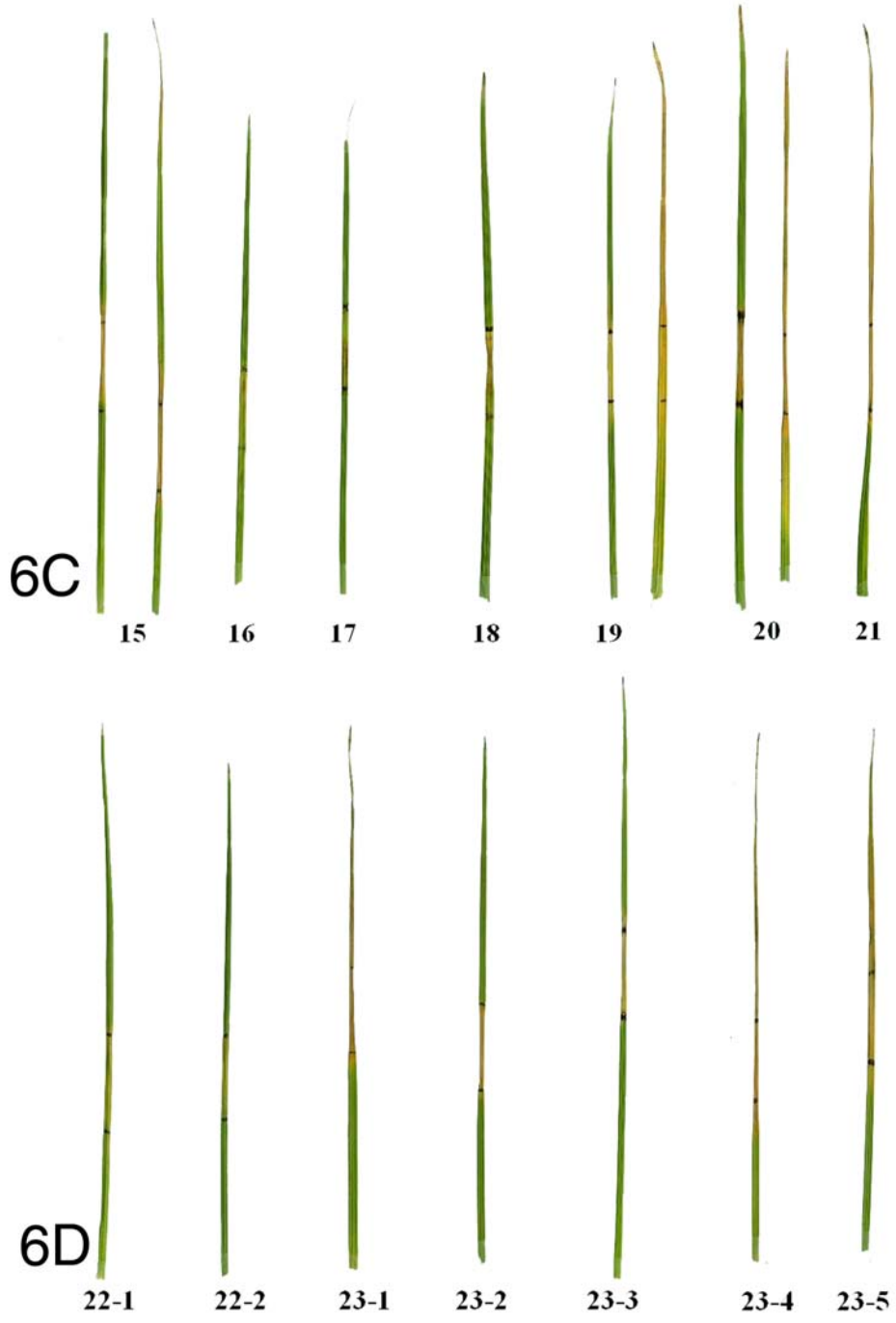
Legend:

Figure 5. Regenerated perennial ryegrass plants.

A. Regenerated shoots from resistant callus on regeneration medium.
B. Regenerated ryegrass plant on the rooting medium.


6A
C
1
2
3
4
5
6


6B
7
8
9
11
12
13
14


6C
15
16
17
18
19
20
21
6D
22-1
22-2
23-1
23-2
23-3
23-4
23-5

Legend:

Figure 6. A-D. Leaf painting experiment. A segment of every leaf was marked using a laboratory marker pen and generously painted on both sides using a cotton swab with 3 mg/ml N-acetyl-PPT solution containing 0.1% Tween 20 as surfactant. After a week, the leaves were collected to evaluate damage level. Numbers of the transgenic plants are indicated. C: non-transgenic control plant.

Fig. 7 Result of spray experiment with N-acetyl-PPT.

Legend:

Figure 7. Spray experiment.

A. Two *argE* gene-transformed plants, TM2 and Tm9, on the left, and one non-transgenic control plant, on the right 10 days after N-acetyl-PPT spraying.

B. Bermudagrass control.

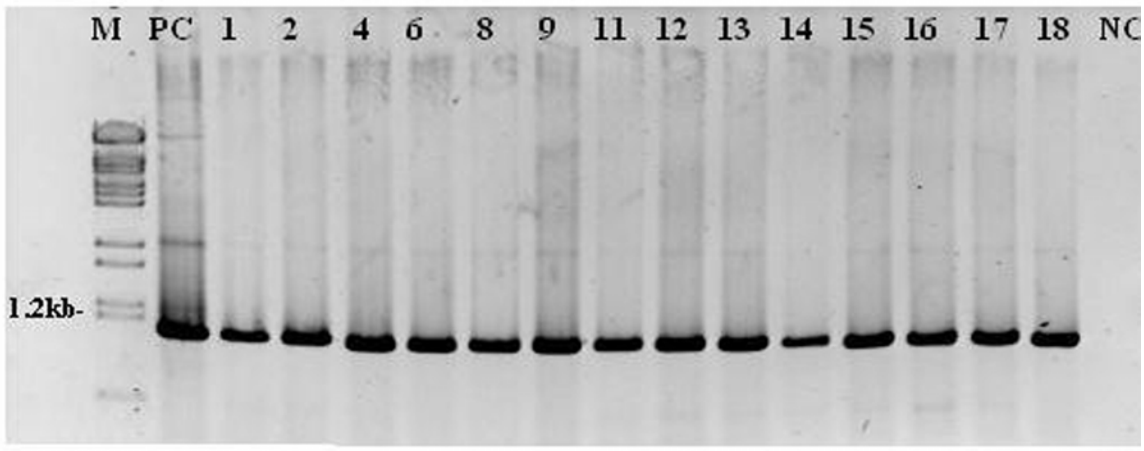


Fig. 8. *ArgE* gene amplified by PCR in transgenic perennial ryegrass plants.

Legend:

Figure 8. Numbers of transgenic plants are indicated. PC: positive control. NC: Negative control.

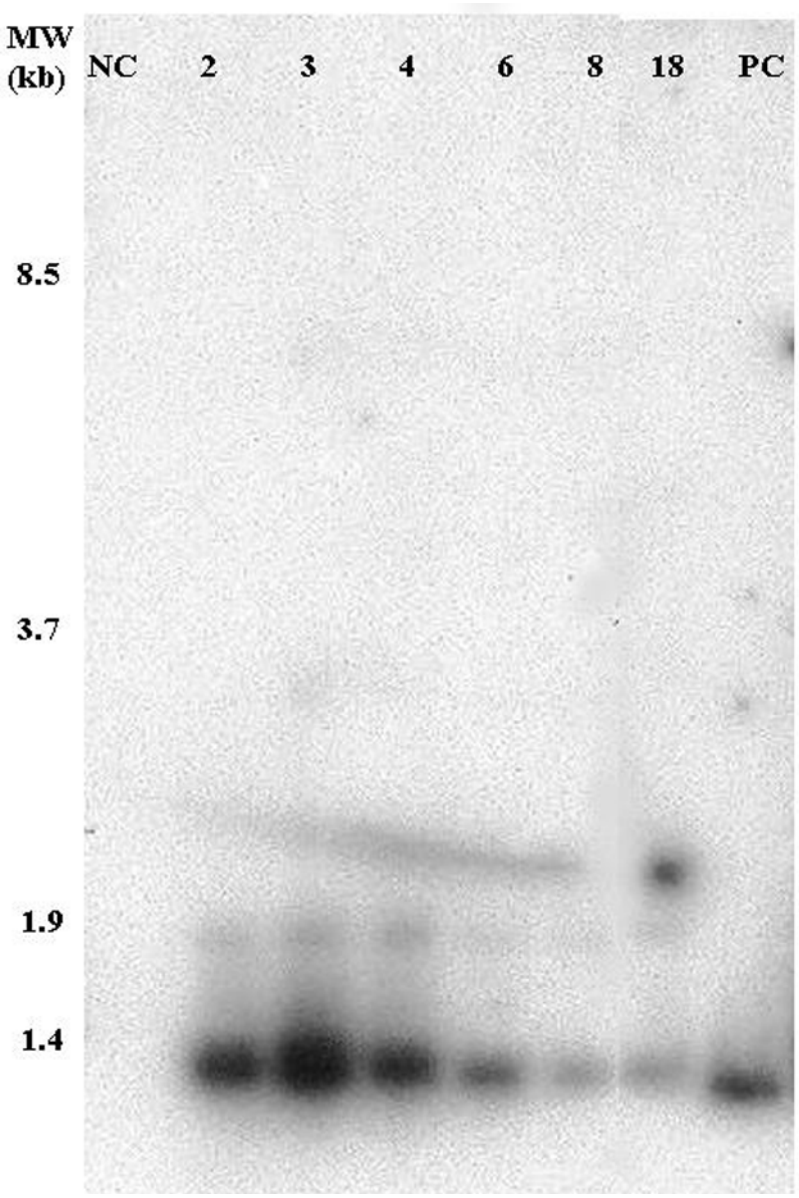


Fig. 9 Autoradiograph of Southern hybridization.

Legend:

Figure 9. Autoradiograph of Southern hybridization analysis of DNA from six transgenic plants. The probes used were [^{32}P] dCTP labeled *EcoRI* digested plasmid pRQ213 fragment containing *argE* gene and *NOS* terminator region. NC: negative control (non-transgenic plant), PC: positive control (*EcoRI* digested pRQ213 fragment of plasmid pRQ213).

Discussion

Genetic transformation can introduce genes from other species, families or even kingdoms into crops, including turfgrasses, for their improvement. In this study, I have successfully developed a protocol to transform perennial ryegrass and demonstrated the utility of using an *E. coli argE* gene as a chemical-induced suicide gene to solve summer persistence problem of the winter overseeding perennial ryegrass.

Successful plant transformation depends on target plant cells which are affected by genotype, explant, culture medium and the age of callus in culture.

Mature seed is often chosen as explant to induce callus for turfgrass transformation because of the good quality of the callus induced, as well as the year-round availability of the explant. Comparitive experiments revealed cultivar differences in tissue culture responses, and 'Roadrunner', 'Brightstar' and 'Majesty' were among the best in callus induction and regeneration (Bradley et al., 2001). Longitudinally slicing of caryopses of perennial ryegrass prior to plating profoundly improved the callus induction rate as well as its regeneration ability (Bradley et al., 2001).

Cultivar also plays an important role in transformation competency of the callus induced. In this study, only 'Majesty' can be transformed at a relatively high frequency. 'Lisabelle' had low transformation efficiency, while no transformants were obtained from 'Roadrunner' and 'Brightstar'.

Transgenic plants from suspension cell lines of perennial ryegrass by the biolistic method have been reported (Spangenberg er al., 1995; Dalton et al., 1999), but a rapid loss of regeneration potential of highly embryogenic perennial ryegrass suspensions was observed (Wang et al., 1997). This is in agreement with the results presented in this

study, in which the suspension cells were apt to lose regeneration ability during prolonged subculture. In addition, for some reasons, the suspension cultures I generated were not competent for transformation.

Young embryogenic callus has been used as target cells for the production of a large number of independently transformed perennial ryegrass plants (Altpeter et al., 2000) although their variety ('Lisabelle') did not work well in this experiment. In this study we identified a unique type of sector from certain calli, which is compact, granular, off-white, and translucent, and it is the best source for successful perennial ryegrass transformation in my experiments. The key to obtain stable transformation is to deliver the foreign gene(s) into actively dividing cells of the callus. When bombardment approach is used, these cells have to be on the surface of the target tissues. It seems the sectors we identified meet these requirements. Moreover, cells from this type of sectors also have good regeneration potential.

The age of the callus seems also important for successful transformation. In general, calli of 2 to 3 months are the best for the biolistic transformation.

Perennial ryegrass is a major turfgrass species and the yearly sale is around 200 million pounds. A major use of perennial ryegrass is for winter overseeding on bermudagrass in golf courses in the South so to keep the courses green and more playable year-round. A main problem for winter overseeded perennial ryegrass is its summer persistence, i.e., the plants are not killed by the summer heat when they are supposed to. Consequently, the overseeded plants become weeds on the bermudagrass lawn. To help solve this problem, we introduced the *argE* gene from *E. coli* into perennial ryegrass. The product of *argE* gene converts N-acetyl-PPT, a non-toxic chemical, into PPT

(phosphinothricin), the active ingredient of herbicides 'Finale', 'Liberty', and 'Basta'. This gene has been used for chemical-induced male sterility in plants (Kriete et al., 1996).

In this study, *argE* gene was introduced into perennial ryegrass for chemical induced elimination of summer persistent plants. The gene was integrated into the genome and expressed properly as shown by the Southern blot analyses. From the Southern blot picture, the six lanes showed the same pattern, which suggested that these transgenic ryegrass plants maybe came from one independent transformation event. Also, leaf painting and spray experiments with N-acetyl-PPT suggested that the gene function as expected in transgenic perennial ryegrass plants.

Although total RNA and Poly A(+) RNA were extract to do Northern hybridization, no conclusive result was obtained probably due to the low expression of the bacterial gene in plant. From the results of leaf painting and spray experiments, it suggests that maybe the low amount of the product of *argE* gene lead the damage of plants after applying N-acetyl-PPT.

Although the *argE* gene participates in the biosynthetic pathway from glutamate to arginine in *E. coli,* no phenotypic changes in the transgenic perennial ryegrass plants were observed. It suggests that the expression of *argE* gene in perennial ryegrass may not substantially interfere with the normal amino acid metabolism.

This study successfully demonstrates the concept of introducing an induced suicide gene into perennial ryegrass to solve the problem of summer persistence. Because of the outcrossing nature of perennial ryegrass, to breed a cultivar with this trait, several

independently transformed lines will need to be crossed to each other followed by further selection.

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