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Perennial Ryegrass (*Lolium perenne* L.)

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Summary

A protocol that facilitates rapid establishment of *Agrobacterium*-mediated transformation for perennial ryegrass is described. The synthetic green fluorescent protein (sgfpS65T) reporter gene is introduced in combination with the *npt*II selectable marker gene into axillary bud derived embryogenic calli of perennial ryegrass (*Lolium perenne* L.) by co-cultivation with *Agrobacterium tumefaciens* strain AGL0 harboring binary vector pYF132. Following the co-cultivation calli are cultured for 48 h in liquid callus medium containing timentin at 10°C and 70 rpm, which reduces *Agrobacterium* overgrowth. Using green fluorescent protein (GFP) as a nondestructive visual marker allows identification of responsive genotypes and transgenic cell clusters at an early stage. GFP screening is combined with paromomycin selection to suppress wild type cells. Transgenic plantlets ready to transfer to soil are obtained within 4 mo of explant culture. Between 8 and 16% of the *Agrobacterium*-inoculated calli regenerate independent, Southern positive transgenic plants. Reproducibility and efficiency in this perennial ryegrass transformation protocols is controlled by multiple factors including genotype dependent tissue culture and gene transfer response, a short tissue culture-and-selection period and the efficient suppression of *Agrobacterium* following *Agrobacterium*-mediated gene transfer.

Key Words: Ryegrass; *Lolium perenne* L.; *Agrobacterium*-mediated gene transfer; *npt*II; *gfp*; grass transformation.

1. Introduction

Perennial ryegrass is one of the most widely cultivated grasses in the temperate regions ***(1)***. Genetic transformation is a powerful tool for improvement of turf and forage grasses including perennial ryegrass ***(2)***. A range of gene transfer protocols have been described for the production of transgenic perennial ryegrass plants including biolistic gene transfer using DNA-coated microprojectiles ***(3,4)***, silicon carbide fiber–mediated gene transfer ***(5)*** or direct gene transfer into protoplast ***(6)***. Initial protocols required a long tissue culture period, which is

From: *Methods in Molecular Biology, vol. 344:* Agrobacterium *Protocols, 2/e, volume 2*
Edited by: Kan Wang © Humana Press Inc., Totowa, NJ

more likely to result in undesirable somaclonal variation *(7)*. Large numbers of fertile transgenic perennial ryegrass plants were generated with accelerated tissue culture and selection following biolistic gene transfer *(8)*.

Compared to *Agrobacterium*, biolistic gene transfer is usually successful in a wider range of genotypes *(8)*, and co-transfer of multiple genes is facilitated (*9*). *Agrobacterium*-mediated gene transfer, however, offers potential advantages over biolistic gene transfer. These include preferential integration of T-DNAs into transcriptional active regions *(10,11)* and elimination of selectable marker genes is facilitated by frequent integration of co-transformed T-DNAs into separate chromosomes *(12,13)*. Grasses are not among the natural hosts of *Agrobacterium*, but in the last decade convincing molecular evidence of stable *A. tumefaciens*–mediated gene transfer was presented for rice *(14)*, wheat *(15)*, barley *(16)*, sorghum *(17)*, bentgrass *(18)*, switchgrass *(19)*, zoysiagrass *(20)*, rye, *(21)*, fescue *(22)* and annual ryegrass *(22)*. The transfer of T-DNA and its integration into the plant genome is influenced by the vector-plasmid *(23)*, the bacteria strain *(12,23)*, the addition of *vir*-gene inducing synthetic phenolic compounds *(24)*, culture media composition, culture conditions *(25)* and osmotic stress treatments *(26)* during and before *Agrobacterium* infection, the plant genotype, explant, tissue culture protocol, as well as the suppression and elimination of *Agrobacterium* after co-cultivation *(27)*.

This chapter describes a detailed protocol for stable *Agrobacterium*-mediated genetic transformation of perennial ryegrass. This genetic transformation protocol has supported the generation of transgenic ryegrass plants with 8 to 16 Southern positive, independent events from 100 inoculated freshly induced callus pieces. Using green fluorescent protein (GFP) as a nondestructive visual marker allows identification of transgenic cell clusters at an early stage, tracking their fate and reducing time in tissue culture. GFP was used earlier as reporter gene for rapid establishment and improvement of biolistic transformation in corn *(28)*, rice *(29)*, barley *(30)* and wheat *(31)*. In contrast to results obtained by biolistic transformation we found a close correlation between *gfp*-expression shortly after *Agrobacterium*-mediated gene transfer and stable transformation events. This indicates that the monitoring of successful gene transfer is a time saving step in this protocol and will facilitate its establishment in a different laboratory.

2. Materials

2.1. Agrobacterium *Strain and Plasmid*

Electroporation *(32)* (*see* also **Chapter 3**, Volume 1) was used to introduce the binary vector pYF132 into *Agrobacterium tumefaciens* strain AGL0 *(33)*. pYF132 encodes the selectable marker neomycin phosphotransferase gene (*npt*II) and the sgfpS65T gene both under control of the corn ubiquitin promoter

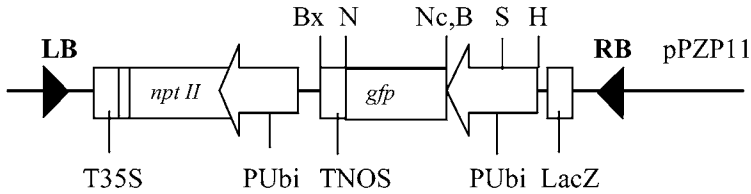

Fig. 1. Diagram of the binary plasmid pYF132 (not drawn to scale). LB, left border; RB, right border; T35S, polyadenylation signal of CaMV35S; P35S, CaMV35S promoter; TNOS, polyadenylation signal of nopaline synthase; PUbi, promoter and first intron of the maize ubiquitin 1 gene; *npt*II, neomycin phosphotransferase; *gfp,* s*gfp*S65T gene; *LacZ,* β-galactosidase complementation factor; Bx, *Bst*XI; *N, Not*I; *Nc, Nco*I; B, *Bam*HI; S, *Sal*I; H, *Hin*dIII; pPZP111, vector backbone pPZP111.

with first intron ***(34)*** and the CaMV 35S and Nos terminator respectively, in the pPZP111 vector-backbone ***(35)*** (*see* **Fig. 1** and **Note 1**).

2.2. Agrobacterium *Culture Media*

1. Luria Broth (LB): 5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.5.
2. Kanamycin monosulfate: 100 mg/mL stock solution in water, filter sterilize (0.2 µm syringe filter) and store 1 mL aliquots at –20°C.
3. Rifampicin: 100 mg/mL stock solution in dimethyl sulfoxide (DMSO), store 1 mL aliquots at –20°C.
4. Liquid culture medium: LB medium with 50 mg/L rifampicin and 50 mg/L kanamycin monosulphate, pH 7.5.
5. Solidified culture medium: LB medium with 10 g/L agar, pH 7.5, with 50 mg/L rifampicin and 50 mg/L kanamycin monosulphate.

2.3. Donor Plant Production to Obtain Tissue Culture Explants

Plants of perennial ryegrass (*Lolium perenne* L.) cultivar Limes (DSV GmbH Lippstadt/Germany) are grown in the greenhouse at 12°C to 16°C during night and 16°C to 20°C during the day with 12/12 h light/dark cycle (*see* **Note 2**). The soil mixture consists a 3:1:1 mixture of topsoil:peat:sand, and plants are fertilized biweekly with Peters-fertilizer (20:20:20 with micronutrients), following the manufacturers recommendations. Light intensity of at least 360 µE/m^2/s at plant height is maintained with sodium vapor lights (Sonagro, Phillips). Tillers are harvested, roots removed and tillers are surface sterilized before excision of axillary buds.

2.4. Perennial Ryegrass Tissue Culture Media

1. Sterilizing solutions: 70% (v/v) ethanol; sodium hypochlorite solution (2.4% active Chlorine) supplemented with a surfactant (0.1% (w/v) of Tween-20).

2. Vitamin stock: Murashige and Skoog (MS) *(36)* Vitamin Mixture 1000X (PhytoTechnology, Shawnee Mission, KS). Dissolve 10.31 g of the premixed vitamins in 100 mL water. Filter sterilize (0.2 µm) and store 1 mL aliquots at –20°C.
3. 2,4-Dichlorophenoxyacetic acid (2,4-D): 2 mg/mL stock solution. Prepare by dissolving in a minimum amount of warm (60°C) 1M KOH and make to volume with water. Store 1 mL aliquots at 4°C.
4. Acetosyringone 100 mM stock solution: Dissolve 196.2 mg of acetosyringone in 10 mL DMSO.
5. Paromomycin sulfate 50 mg/mL stock solution: Dissolve in water, filter sterilize (0.2 µm), and store 1 mL aliquots at –20°C.
6. Timentin 150 mg/mL stock solution: Dissolve in water filter sterilize (0.2 µm) and use immediately.
7. Maltose 10X stock solution: Dissolve 300 g/L maltose in water, pH 5.8, filter sterilize (0.2 µm), and use immediately.
8. Callus induction medium (CIM): 4.3 g basal MS salts *(28)*, 1.5 mL/L 2,4-D stock solution, pH 5.8; 3.0 g/L phytagel, 1 mL/L MS 1000X vitamin stock, 100 mL/L maltose 10X stock solution.
9. Osmotic treatment medium (OTM): CIM plus 72.9 g/L mannitol.
10. Co-cultivation medium, liquid (CCML): 4.3 g basal MS salts, 3.0 mL/L 2,4-D stock solution; 15 g/L glucose; pH 5.2; 2.0 mL/L acetosyringone stock solution (*see* **Notes 3 and 4**), 50 mL/L maltose 10X stock solution.
11. Co-cultivation medium, solid (CCMS): CCML solidified with 3.0 g/L phytagel.
12. Subculture medium, liquid with Timentin (SCML): 4.3 g basal MS salts, 1.5 mL/L 2,4-D stock solution, pH 5.8; 1 mL/L 1000X MS vitamin stock, 1 mL/L timentin stock solution, 100 mL/L maltose 10X stock solution.
13. Callus selection medium (CSM): 4.3 g basal MS salts, 1.5 mL/L 2,4-D stock solution; pH 5.8; 6.0 g/L agarose, 1 mL/L 1000X MS vitamin stock, 1 mL/L timentin stock solution, 1.0 mL/L paromomycin sulphate stock solution (*see* **Note 5**), 100 mL/L maltose 10X stock solution.
14. Shoot and root regeneration and selection medium (SRRSM): 4.3 g basal MS salts, 20 g/L sucrose; pH 5.8; 6.0 g/L agarose; 1 mL/L 1000X MS vitamin stock, 1.0 mL/L timentin stock solution; 1.0 mL/L paromomycin sulphate stock solution (*see* **Note 5**).
15. All media are sterilized by autoclaving at 121°C, 1.5 bar for 15 min. Water was purified with a Milli-Q water purification system. Antibiotics, and vitamins are added to the medium as concentrated, filter-sterilized solutions after autoclaving and at a medium temperature of less than 50°C. Acetosyringone is added after autoclaving. Media containing Timentin are used immediately after preparation, others are stored at room temperature for up to 2 wk.

3. Methods

3.1. Explants and Agrobacterium *Preparation*

1. Surface sterilization: Rinse tillers for 3 min in 70% (v/v) ethanol and for 15 min in 50% sodium hypochlorite solution (1.2% active chlorine) containing approx

0.1% (w/v) of Tween-20 while shaking at 50 rpm, followed by 5 washes with previously autoclaved (121°C, 1.5 bar for 15 min) water.
2. Excise axillary buds approx 4–10 mm in size and place on callus induction medium (CIM). Culture 12 explants per 90-mm Petri-dish at 20 µE/m^2/s and 25°C for 28 to 56 d and subculture to fresh CIM every 14 d.
3. Grow *A. tumefaciens* strain AGL0 harboring vector pYF132 on LB agar culture medium (with antibiotics) at 28°C for 2 d. Transfer one colony of bacteria to 2 mL of LB liquid culture medium (with antibiotics) and grow overnight at 28°C on an orbital shaker at 230 rpm (*see* **Notes 6** and **7**).
4. Measure absorbance at 660 nm of a 1 mL aliquot of the bacterial overnight culture in a spectrophotometer (expected OD_{660} value is 2.0–2.5).
5. Centrifuge 1 mL of the bacterial culture at 13,000*g* for 5 min, discard the supernatant, resuspend the pellet and dilute the suspension to an OD_{660} value of (1.5–2.0) in 1/1 (v/v) LB medium/CCML (without antibiotics) and incubate at 28°C on an orbital shaker at 230 rpm for 2 h before co-cultivation.

3.2. Inoculation, Co-cultivation, Selection, and Regeneration of Transgenic Plants

1. For osmotic treatment place 15–20 embryogenic calli (*see* **Subheading 3.1.2.**) in the center of a 90-mm Petri-dish with OTM medium for 4–6 h prior inoculation with *Agrobacterium*.
2. Inoculate 15–20 embryogenic calli per 90-mm Petri-dish by pipetting approx 200 µL *Agrobacterium* suspension on the calli (*see* **Subheading 3.1.5.**), vacuum treat at 500–800 mbar for 1 min and keep in the laminar flow hood for 10 min.
3. Transfer calli briefly to blotting paper to remove excess *Agrobacterium*, followed by a co-culture on CCMS medium for 44 to 48 h at 22°C and 20 µE/m^2/s.
4. After 44 to 48 h co-cultivation in CCMS medium, rinse explants thoroughly (at least 5 times) in SCML medium and culture approximately 10 calli per 100 mL Erlenmeyer flask with 20 mL SCML medium for 48 h at 10°C and 70 rpm (*see* **Note 7**) in the dark.
5. Blot calli dry on a filter paper and transfer calli to CSM to suppress nontransgenic events. Maintain cultures at 20 µE/m^2/s for 4 wk at 25°C with a subculture to fresh CSM medium after 14 d (*see* **Note 8**).
6. Expression of s*gfp* (S65T) appears as defined fluorescent spots (*see* **Note 8**) under a stereomicroscope equipped with a fluorescent module (*see* **Note 9**). Five to seven days after co-cultivation, the number of GFP expressing signals per callus declines. Approximately 10% of the cells expressing GFP two days after co-cultivation developed into fluorescing calli. Ten to fourteen days after transfer to selection medium green fluorescence of calli under a fluorescent light source and rapid growth of these calli on culture media with paromomycin are co-existing events. In regenerated shoots a strong red autofluorescence from chlorophyll masks the GFP signal. The strong fluorescence in roots is therefore the most reliable indicator for the transgenic character of the regenerated plantlets.
7. Transfer calli to 90-mm Petridish with SRRSM medium and culture at a 16 h photoperiod, 130 µE/m^2/s illumination at 25°C for shoot regeneration for 3 wk (*see* **Note 10**).

8. Transfer 5 regenerating calli to a Magenta box (Sigma-Aldrich) with 40 mL SRRSM medium and culture at a 16 h photoperiod, 130 μE/m^2/s illumination at 25°C for shoot elongation and root formation of transgenic shoots within 3 wk (*see* **Note 10**).
9. It takes approx 7 mo from transplanting until harvest of mature seeds. Rooted transgenic plantlets were transferred to soil after carefully washing off medium from the roots. Topsoil, peat, and sand were mixed in a 3:1:1 ratio for plant growth. To support acclimation plantlets were covered with a Magenta box for the first four days following transfer to soil. Plants are grown in the growth chamber at 12°C to 16°C during night and 16°C to 20°C during the day with 12/12 h light (400 μE/m^2/s) /dark cycle for 3 wk. Then they were vernalized at 4°C 12/12 h light (100 μE/m^2/s) /dark cycle for 10 wk. Following vernalization plants are grown in the growth chamber at 12°C to 16°C during night and 16°C to 20°C during the day with 12/12 h light/dark cycle until end of tillering. After tillering a 16/8 h light/dark cycle is used to produce reproductive tillers. Plants were fertilized biweekly with Peters-fertilizer (20:20:20 with micronutrients; St. Louis, MO), following the manufacturers recommendations. Light intensity of 400 μE/m^2/s at plant height was maintained with metal halide lights until the end of tillering. After tillering sodium vapor lights provided the same light intensity. As soon as immature inflorescenses emerged and before pollination, each transgenic inflorescence is covered together with a wildtype inflorescense with a cellophane bag and pollination within the bags is enhanced with daily agitation of the bags. Under these conditions more than 70% of the transgenic lines were fertile and seed set varied between 10 and 70%.
10. Regenerated plants and their progeny can be assessed for expression of the *npt*II transgene by a commercially available enzyme linked immunosorbent assay (ELISA)-kit (Agdia, Elkhart, IN) (*see* **Note 11**). Southern blot analyses is performed to confirm the stable integration of the transgenes. Northern blot, western blot or ELISA analyses are used to confirm the expression of the GFP or alternative co-transferred transgenes.

4. Notes

1. The binary plasmid used (pYF132) has incorporated bacterial resistance to kanamycin. *A. tumefaciens* strain AGL0 has chromosomal resistance to rifampicin *(33)*.
2. Tissue culture response is highly dependent on donor plant quality. Therefore it is important to avoid stresses including drought, temperatures above 25°C, pests, diseases, and pesticides. Ryegrass is a cross-pollinated species, resulting in a significant genotypic variability within a cultivar. This variability reduces also the reproducibility of tissue culture and transformation response from ryegrass cultivars. In the early transformation experiments cross-pollinating ryegrass populations were used or cell suspension derived from a single explant *(3)*. The identification of inbred lines displaying a good regeneration response from tissue cultures has also been described *(8)*. In contrast to single embryo derived cell suspensions, axillary bud, or immature inflorescense derived callus as target for gene

transfer also allow the introduction of transgenes into an isogenic background but allow avoidance of extended tissue culture periods.

3. Co-cultivation in a medium rich in auxins induces cell division, callus proliferation and maintains tissues in an undifferentiated state, which might enhance transformation competence.
4. The supplementation of CCML and or CCMS medium with Acetosyringone, used prior and during co-cultivation almost doubles the transformation efficiency in perennial ryegrass.
5. Gelling agents may cause precipitation of paromomycin rendering it inactive as a selective agent. Agarose does not cause precipitation of paromomycin.
6. In a series of transformation experiments with the same plasmid, reproducibility can be improved by using 20 µL of an *Agrobacterium* stock stored in glycerol as an inoculum instead of colonies. For the preparation of glycerol stocks a 100 mL *Agrobacterium* suspension can be grown overnight (as a subculture of the culture initiated in 3.1.3 in 100 mL LB medium). After mixing 1:1 with an autoclaved Glycerol solution (30%) the aliquoted suspension can be stored for several months at –80°C.
7. Ryegrass tissue cultures are very sensitive to *Agrobacterium* overgrowth. The described thorough rinsing of the explants for two days on a shaker at 10°C and the use of timentin reduces the potential of *Agrobacterium* overgrowth.
8. The successful *Agrobacterium*-mediated transformation in perennial ryegrass is very genotype dependent. Using GFP as a nondestructive visual marker allows identifying responsive genotypes 2 d after co-cultivation, because there is a good correlation between fluorescent signals shortly after co-cultivation and stable transformation events.
9. The expression of GFP was visualized using a ZEISS stemi SV6 stereomicroscope (Carl Zeiss, Germany) with a 50W mercury lamp, a BP470/20nm excitation filter, and a BP505-530 barrier filter.
10. Paromomycin, compared to other selective agents, is very effective in suppressing elongation of nontransgenic shoots and roots. Effective inhibition of shoot and root growth by paromomycin depends also on the light intensity during regeneration. Under the described selection and culture conditions (130 µE/m^2/s illumination) none of the plantlets growing roots inside the 50 mg/L containing paromomycin medium were nontransgenic escapes. In contrast, under 60 µE/m^2/s instead of 130 µE/m^2/s illumination approximately 30% of the regenerated and root forming plantlets were nontransgenic escapes (Altpeter, unpublished).
11. The removal of the selectable marker or reporter genes from transgenic elite events can be achieved by *Agrobacterium*-mediated co-transformation of unlinked T-DNA's followed by segregation analysis in sexual progenies ***(12,13)***.

Acknowledgments

The author would like to thank Ulf Feuerstein, DSV GmbH, Lippstadt, Germany for providing seeds from perennial ryegrass cultivar Limes; Pal Maliga, Rutgers University, Piscataway, USA for providing vector pPZP111;

Gerad Lazo, Plant Gene Expression Center, Albany, USA for providing *Agrobacterium* strain AGL0; and Jen Sheen (Massachusetts General Hospital, Boston) for the gift of the synthetic *gfp*(S65T) gene.

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