GENE TRANSFER TO RYEGRASSES: DOWN-REGULATION OF MAJOR POLLEN ALLERGENS IN TRANSGENIC PLANTS

X.L. Wu, X.D. Ye, Z.Y. Wang[1], I. Potrykus and G. Spangenberg[1*]
Institute for Plant Sciences, Swiss Federal Institute of Technology, CH-8092 Zürich, Switzerland
[1]Present address: Plant Sciences and Biotechnology, Agriculture Victoria, La Trobe University, Bundoora, Victoria, 3083, Australia
[*]Author for correspondence

ABSTRACT
The objective of this study is to generate transgenic ryegrass plants with a down-regulation of two major pollen allergenic proteins *Lol p* I and *Lol p* II. Antisense vectors for *Lol p* I and *Lol p* II cDNAs driven by the pollen-specific promoter *Zm13* have been constructed. Corresponding transgenic plants have been recovered by particle inflow gun-mediated microprojectile bombardment of embryogenic suspension cells from perennial and Italian ryegrass using chimeric *Act1-hph* gene as a selectable marker. Their transgenic nature has been demonstrated by Southern hybridization analysis. The analysis of reproductive development and accumulation of pollen allergens in the transgenic ryegrasses is in progress.

KEYWORDS
Lolium perenne, perennial ryegrass, *L. multiflorum*, Italian ryegrass, microprojectile bombardment, transgenic plants, pollen allergens.

INTRODUCTION

The genus *Lolium* L., ryegrasses, consists of eight diploid (2n = 2x = 14 chromosomes) species indigenous to temperate regions of Europe and Asia (Jauhar 1993). Their geographic distribution has been further extended by man through introductions in the Americas, North Africa, Australia and New Zealand. Particularly two closely related and interfertile species, *L. multiflorum* (Italian or annual ryegrass) and *L. perenne* (English or perennial ryegrass), are key forage grasses in temperate climates throughout the world. Grass pollen allergy is a major environmental disease that afflicts 20% of the population in cool temperate climates. Ryegrasses - due to their abundant pollen production and wide distribution - are responsible for a major portion of grass pollen allergies worldwide. The functional role *in planta* of the major ryegrass pollen allergens *Lol p* I and *Lol p* II is unknow. The enabling technologies for the genetic manipulation of ryegrasses have been established (Wang et al., 1993, 1994; Spangenberg et al., 1995; Ye et al., 1997). Transgenic ryegrass plants expressing selectable marker hygromycin phosphotransferase (*hph*) gene have been obtained by microprojectile bombardment to embryogenic suspension cells in *L. perenne* (Spangenberg et al., 1995) and *L. multiflorum* (Ye et al., 1997). They allowed to generate materials suitable for studying the functional role of the major ryegrass pollen allergens, and to explore the potential for the production of hypoallergenic ryegrass cultivars.

MATERIALS AND METHODS

Establishment and Maintenance of Suspension Cultures: Seeds from perennial ryegrass (*Lolium perenne* L. cv "Citadel") and Italian ryegrass (*L. multiflorum* Lam. ssp. *westerwoldicum* cv. "Andy"), kindly provided by B. Boller (FAL-Zürich-Reckenholz, Switzerland), were used for the establishment of embryogenic suspension cultures as described by Wang et al. (1993).

Vectors for Transformation: Plasmids pZIGSAR and pZIIGSAR bearing cDNA sequences from *Lol p* I and *Lol p* II in antisense orientation under control of the maize pollen-specific promoter *Zm13* were constructed. Plasmid pZm13GUS bearing a chimeric *gusA* gene driven by the *Zm13* promoter (Hamilton et al., 1992) was kindly provided by J. Mascarenhas. *Lol p* I and *Lol p* II cDNAs were made available by A. Sidoli. A chimeric modified *hph* gene under control of rice *act1* 5' regulatory sequences in pAcH1 (Bilang et al. 1991) was used as selectable marker gene. Plasmid pAcH1 was supplied by R. Bilang. *Act1* 5' regulatory sequencs from pCOR117 (McElroy et al., 1991) were kindly made available by R. Wu and D. McElroy.

Transforming DNA and Selection of Stable Transformed Colonies and Recovery of Transgenic Plants: Stable transformation experiments were carried out by mixing plasmids pZIGSAR, or pZIIGSAR or pZm13GUS with plasmid pAcH1. Approximately 200 mg suspension cells plated as a 2 cm diameter monolayer on a filter paper disk were used as target for microprojectile bombardment. The cost-effective particle inflow gun (Finer et al., 1992) was used as microprojectile acceleration device. The bombardment parameters were as following: 500 µm baffle mesh size; 12 cm baffle and 15 cm target-bombardment distances; 6 bar bombardment pressure; one shot with a 10 µl DNA-spermidine bound particle suspension per target using 0.5 mg gold particles coated with 20 µg plasmid DNA (10 µg for each plasmid) (Spangenberg et al., 1995; Ye et al., 1997). After a 3 - 4 day post-bombardment osmotic treatment on solid MSP medium supplemented with 0.25M sorbitol and 0.25M mannitol without hygromycin (Hm), filter paper disks supporting bombarded embryogenic suspension cells were transferred into liquid AAF medium with 25 mg/l or 50 mg/l Hm for *L. multiflorum* or *L. perenne*, respectively. Cells selected in Hm-containing liquid AAF medium were kept at 25°C in the dark on a gyratory shaker at 60 rpm. After 2 weeks, the culture medium was completely replaced by fresh one containing 50 mg/l (for *L. multiflorum*) or 100 mg/l (for *L. perenne*) Hm and maintained for further 2 weeks before plating onto Hm-containing solid MSP medium for proliferation in presence of 100 mg/l (for *L. multiflorum*) or 200 mg/l (for *L. perenne*) Hm for 3 - 4 weeks. The resistant calli obtained were transferred onto regeneration medium MSK (MS medium supplemented with 0.2 mg/l kinetin) without Hm. The regenerated shoots were transferred for rooting onto half strength MS hormone-free medium solidified with 0.3% Gelrite (Duchefa, No. G1101, Haarlem, The Netherlands). All cultures were kept at 25°C under a photoperiod of 16 h fluorescent light (40 µmol $m^{-2}s^{-1}$). After 4 - 5 weeks, rooted plantlets were hardened off, transferred to soil and grown to maturity under greenhouse conditions (16/8 h photoperiod; 23°C/18°C; fluorescent light 145 µmol $m^{-2}s^{-1}$).

DNA Isolation, Gel Electrophoresis and Hybridization Experiments: Total cellular DNA was isolated from freeze-dried leaf material from greenhouse-grown plants. Isolation and digestion of genomic DNA was performed according to Lichtenstein and Draper (1985). Restriction enzyme analysis, gel electrophoresis, DNA blotting, Southern hybridizations were carried out following standard protocols (Sambrook et al., 1989). Hybridization probes (*hph* gene, 1,344 bp *Bam*HI-fragment of pAcH1, *gusA* gene 1,800 bp SmaI + BamHI-fragment of pZm13GUS, *Lolp*I and *Lolp*II cDNA) were DIG-labeled (DIG-11-dUTP, Boehringer Mannheim, Germany) by random priming.

RESULTS AND DISCUSSION

Microprojectile bombardment experiments using embryogenic suspension cells of both ryegrasses were carried out. Co-transformations of either a chimeric *gusA* gene (pZm13GUS) or antisense *Lol p* I (pZIGSAR) and antisense *Lol p* II (pZIIGSAR) full length cDNA sequences driven by the maize pollen-specific *Zm13* promoter with the *Act1-hph* selectable marker gene were performed. Fig. 1 illustrates all steps for the production of transgenic perennial ryegrass plants from the plated suspension cells prior to microprojectile bombardment with pZm13GUS, pZIGSAR or pZIIGSAR (Fig. 1A), through the recovery of hygromycin resistant callus clones (Fig. 1C) after selection, to the regeneration of corresponding plants currently being vernalized (Fig. 1G). Over 1,500 dishes each containing approximately 200 mg fresh weight embryogenic suspension cells (Fig. 1A) of perennial ryegrass were subjected to microprojectile bombardment. Bombarded suspension

cells were subjected to a liquid selection scheme using a stepwise increase in the hygromycin concentration, and lead to the recovery of approximately 300 hygromycin resistant calli. *In vitro* growing plantlets have been obtained from 92 regenerating hygromycin resistant calli (Fig. 1D-F). Currently 21 transgenic perennial ryegrass plants (Fig. 1G) from transformation experiments with pZm13GUS, pZIGSAR, or pZIIGSAR are being vernalized. Analogous experiments were carried out for Italian ryegrass. From over 360 bombarded dishes of Italian ryegrass suspension cells with the three chimeric gene constructs, 90 transgenic Italian ryegrass plants were generated. After an initial PCR screening using primers designed to amplify *hph*, *gusA* or transgene specific *Lol p* I and *Lol p* II sequences, the transgenic nature of the perennial and Italian ryegrass plants was confirmed by Southern hybridization analysis. Representative results are shown for 8 independent transgenic Italian ryegrass plants (Fig. 2). Over 80% of plants transformed with the chimeric *hph* gene (Fig. 2A) were co-tranformants carrying the chimeric *gusA* gene or antisense *Lol p* I or antisense *Lol p* II genes (Fig. 2B).

ACKNOWLEDGEMENTS

Thanks are due to H. Waldner for constructing a particle inflow gun, and to J. Finer for providing the required information for its construction. P. Frick and K. Konja are gratefully acknowledged for carefully growing plants in the greenhouse. This work was funded by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung (SNF grant 31-40315.94).

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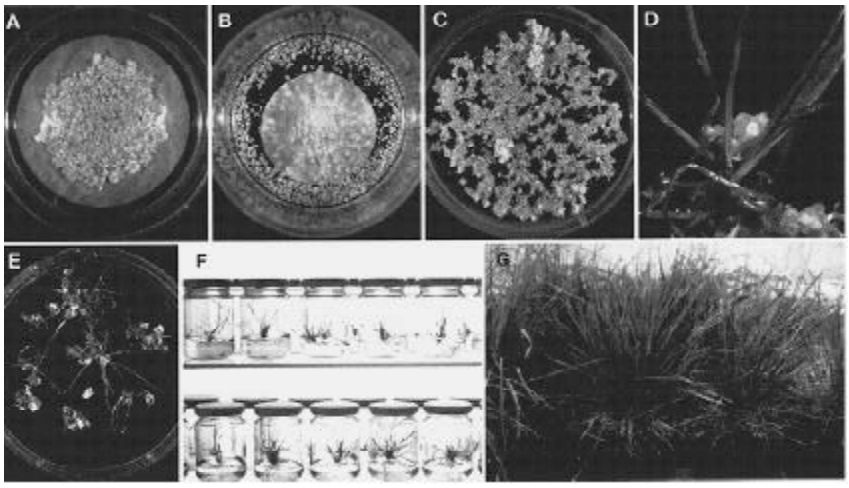
Figure 1

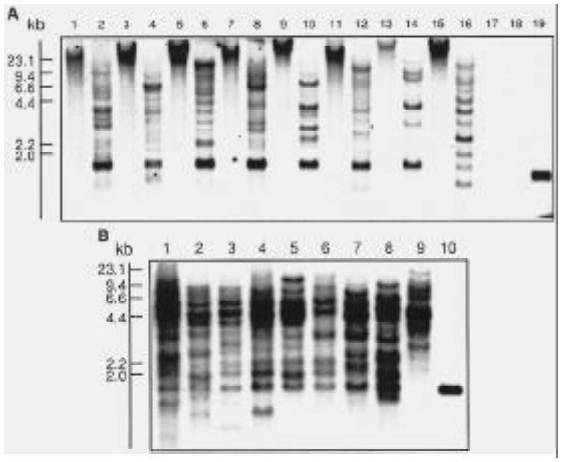
Transgenic *Lolium perenne* plants from particle inflow gun-mediated microprojectile bombardment of embryogenic suspension cells.



A) Suspension cells of *L. perenne* plated on filter paper disk prior to microprojectile bombardment; B) Embryogenic suspension cells of *L. perenne* in liquid AAF medium containing 50 mg/l Hm 2 weeks after bombardment with pAcH1-coated microprojectiles; C) Hygromycin (Hm) resistant calli of *L. perenne* obtained from microprojectile bombarded embryogenic suspension cells using a chimeric *hph* gene (pAcH1) and followed by selection on MSP in presence of 200 mg/l Hm; D) Shoot differentiation from Hm resistant callus of *L. perenne* 2 weeks after transfer onto medium MSK for regeneration; E) Regenerating Hm resistant calli of *L. multiflorum* 10 weeks after bombardment of embryogenic suspension cells with pAcH1-coated microprojectiles; F) Transgenic *L. perenne* plantlets growing *in vitro* 12 weeks after microprojectile bombardment of embryogenic cells; G) Transgenic soil-grown *L. perenne* plants 4 months after bombardment of embryogenic suspension cells.

Figure 2

Southern hybridization analysis of transgenic plants of *L. multiflorum* from microprojectile bombardment of embryogenic suspension cells.



A) Hybridized with *hph* probe. Lanes 1-16): 8 independent Hm resistant pAcH1-transformants, undigested (odd-numbered lanes) and BamHI-digested (even-numbered lanes) total cellular DNA samples isolated from 8 Hm resistant *L. multiflorum* plants; 17-18) non-transformed *L. multiflorum* negative control; 19) 10 pg BamHI-digested pAcH1 as positive control. B) Hybridized with *Lol p* II cDNA. Lanes 1-8): 8 independent pZIIGSAR-transformed plants of *L. multiflorum* (same plants as in A); 9) non-transformed *L. multiflorum* negative control; 10) 10 pg *Xho*I and *Hind*III-digested pZIIGSAR as positive control.