

Transgenic ryegrasses (*Lolium* spp.) with down-regulation of main pollen allergens

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Abstract

Ryegrass pollen (*Lolium* species) is a widespread source of air-borne allergens and is a major cause of hayfever and seasonal allergic asthma, which affect approximately 25% of the population in cool temperate climates. The main allergens of ryegrass pollen are the proteins Lol p 1 and Lol p 2. These proteins belong to two major classes of grass pollen allergens to which over 90% of pollen-allergic patients are sensitive. The functional role *in planta* of these pollen allergen proteins remains largely unknown. Here we describe the generation and analysis of transgenic plants with reduced levels of the main ryegrass pollen allergens, Lol p 1 and Lol p 2 in the most important worldwide cultivated ryegrass species, *L. perenne* and *L. multiflorum*. These transgenic plants will allow the study of the functional role *in planta* of these pollen proteins and the determination of potential for development of hypo-allergenic ryegrass cultivars.

Abbreviations: AFLP – amplified fragment length polymorphism; BAC – bacterial artificial chromosome; DIG – digoxigenin; GUS – β -glucuronidase; *hph* – hygromycin phosphotransferase; LG – linkage group; MES – 2-N-morpholinoethanesulfonic acid; PAGE – polyacrylamide gel electrophoresis; PBS – phosphate-buffered saline; PCR – polymerase chain reaction; PMSF – phenylmethylsulfonyl fluoride; RFLP – restriction fragment length polymorphism; SDS – sodium dodecyl sulphate

Introduction

Hayfever and seasonal allergic asthma are major type I allergic diseases affecting up to 25% of the population in cool temperate climates around the world (Ong et al. 1993; Tamborini et al. 1995). These allergies are largely attributable to pollen allergens from wind-pollinated grass species. Perennial ryegrass (*Lolium*

perenne L.) and Italian ryegrass (*L. multiflorum* Lam.) are the world's most important temperate pasture grasses, and are widely sown for forage and turf while being amenable to genetic modification (Jauhar 1993; Spangenberg et al. 1998). These ryegrasses (*Lolium* spp.) are the dominant source of allergenic pollen because of their wide distribution and abundant production of air-borne pollen during flowering (Smart et al.

1979). A range of allergens have been identified in ryegrass pollen (Singh et al. 1991; Ong et al. 1993; Smith et al. 1994). In perennial ryegrass pollen, at least 17 allergenic proteins ranging in size from 12 to 89 kDa have been detected (Ford and Baldo 1986; Singh et al. 1991; Sidoli et al. 1993; Knox and Suphioglu 1996).

Two different proteins designated, Lol p 1 and Lol p 2 (named according to World Health Organisation rules) have been identified as the main allergenic determinants in ryegrass pollen (King et al. 1996). These allergens exist in multiple isoallergenic forms representing variants that appear to be immunologically indistinguishable (Johnson and Marsh 1965). Lol p 1 is the major ryegrass pollen allergen to which 95% of patients showed increased levels of IgE antibodies in their sera (Kahn and Marsh 1986). The natural allergen is an acidic, glycosylated 35 kDa monomeric protein containing intramolecular disulfide bridges (Singh et al. 1991; Johnson and Marsh 1965). Lol p 1 exists in multiple isoforms of the same size, with polymorphism appearing in at least eight positions in the amino acid sequence (Perez et al. 1990; Griffith et al. 1991). Lol p 1 contains a 29 amino acid IgE-binding region at the C terminus (Esch and Klapper 1989 ab) as well as the 7 cysteine residues strictly conserved in group 1 grass pollen allergens (Singh and Bhalla 2003), and has been localised to the cytosol of the pollen grain (Singh et al. 1991).

Lol p 2 is the second most important ryegrass pollen allergen to which 45% of grass pollen allergic patients are reactive (Freidhoff et al. 1986). Lol p 2 is an acidic protein and is present in at least two isoforms, Lol p 2A and Lol p 2B (Sidoli et al. 1993). Lol p 2A has been purified, sequenced and found to consist of 97 amino acid residues (Ansari et al. 1989).

While the mode of interaction of pollen proteins with the human immune system has been studied in great detail (Freidhoff et al. 1986; Beiber 1996; Stingl and Maurer 1997), the functional role *in planta* of these pollen allergens remains largely unknown (Cosgrove et al. 1997; Li and Cosgrove 2001).

Significant efforts have been made in developing diagnostic and therapeutic reagents for designing new and more effective immuno-therapeutics for treatment of hayfever and seasonal allergic asthma caused by grass pollen allergens. However, an alternative strategy to mitigate these environmental diseases of high socio-economic impact would be to reduce the amount of pollen allergens produced by the source

plant through targeted down-regulation of the expression of pollen allergen encoding genes (Spangenberg et al. 1997, 1998). Previously, transgenic plants of the weedy grass species, annual ryegrass (*L. rigidum*), putatively carrying an antisense *Lol p 5* chimeric gene for down-regulation of this pollen allergen have been reported (Bhalla et al. 1999).

Here we describe, for the first time, the generation and analysis of transgenic plants with reduced levels of their main pollen allergens, Lol p 1 and Lol p 2 in the most important worldwide cultivated ryegrass species, *L. perenne* and *L. multiflorum*. These plants will allow us to determine the functional *in planta* role of these pollen allergens and to explore the potential for the generation of hypo-allergenic ryegrass cultivars. Furthermore, we report the isolation and characterisation of the perennial ryegrass *Lol p 2* promoter for directing pollen specific expression in plants, including the specific down-regulation of expression of pollen allergens.

Materials and methods

Plant material

Seeds of perennial ryegrass (*Lolium perenne* L.) cv. Kelvin and Italian ryegrass (*Lolium multiflorum* Lam.) cv. Andy were used for the induction of embryogenic callus cultures and the establishment of corresponding cell suspensions as previously reported (Spangenberg et al. 1995, 1998; Wang et al. 1993; Ye et al. 1997).

Transformation vectors

Transformation vectors pZIGSAR and pZIIGSAR containing chimeric *Lol p 1* and *Lol p 2* genes, respectively, in antisense orientation under control of the maize pollen specific *Zm13* promoter (Hamilton et al. 1992) were generated (Spangenberg et al. 1998, 2001). The 260 bp 5'-upstream region of *Zm13* was fused with 860 bp *Lol p 1* or 320 bp *Lol p 2* coding fragments. A chimeric β -glucuronidase (*gusA*) gene driven by an enhanced CaMV 35S promoter was also included as a reporter gene in the transformation vectors.

A 952 bp 5' upstream region of *Lol p 2* was cloned into the pZm13GUS vector after excising the 260 bp *Zm13* promoter in order to generate the pLP2GUS vector.

Plasmid pAcH1 carrying a chimeric *hph* gene under the control of the rice actin 1 promoter was used as a selectable marker (Bilang et al. 1991; Spangenberg et al. 1995, 1998).

Production of transgenic ryegrass plants

Transgenic perennial (*L. perenne*) and Italian (*L. multiflorum*) ryegrass plants were generated by biolistic transformation of single-genotype derived embryogenic cell suspensions as described earlier (Spangenberg et al. 1995, 1998; Wang et al. 1993; Ye et al. 1997).

Molecular analysis of transgenic ryegrass plants

Genomic DNA isolation and PCR analysis: DNA was extracted from putative transgenic perennial and Italian ryegrass plants and non-transformed control plants using a protocol for DNA extraction from small amount of leaf tissue as previously reported (Spangenberg et al. 1995; Ye et al. 1997). Thermocycling conditions were as follows: 94 °C for 5 min followed by 30 cycles of 94 °C denaturation for 1 min, 60 °C annealing for 30 sec, 72 °C extension for 1 min (with 5 sec/cycle autoextension), and 72 °C final extension for 10 min. The thermostable DNA polymerase used was Dynazyme (Finnzymes Oy, #F501L). The presence of the *gusA* gene was demonstrated by PCR-amplification of a 270 bp fragment using the forward primer 5'-CTTTAACTATGCCGGGATC-CATCG-3' and the reverse primer 5'-TAACCT-TACCCGGTTGCCAGAGG-3'. The presence of the *hph* gene was demonstrated by PCR-amplification of a 375 bp fragment using the forward primer 5'-GCTGGGGCGTCGGTTTCCACTATCGG-3' and the reverse primer 5'-CGCATAACAGCGGTCAT-TGACTGGAGC-3'. PCR products were separated on 1.2% (w/v) agarose gels and visualised by ethidium bromide staining (0.5 µg/ml).

Southern hybridisation analysis: For Southern hybridisation analysis, genomic DNA was extracted from lyophilised plant material using a CTAB-based protocol as previously described (Spangenberg et al. 1995; Ye et al. 1997). DNA samples (10 – 15 µg) were digested with *Bam*HI, *Hind*III, *Eco*RI, or *Xho*I restriction enzymes. The resulting DNA fragments were separated on a 1% agarose gel and transferred to Hybond N (Amersham Pharmacia Biotech) membranes. Hybridisation was performed according to the manufacturer's instructions and incorporation of

DIG-dUTP into DNA probes and detection of bound probes was performed using the DIG Luminescent Detection Kit (Roche Diagnostics) following the supplier's protocol. Hybridisation conditions were: 4 x SSC, 50% (v/v) formamide, 0.1% (w/v) N-lauroyl-sarcosine, 0.02% (w/v) SDS, and 2% (v/v) blocking solution at 42 °C. Membranes were washed twice in 2 x SSC/0.1% (w/v) SDS for 5 min at 25 °C then 0.2 x SSC/0.1% (w/v) SDS followed by 0.1 x SSC/0.1% (w/v) SDS, both for 15 min at 68 °C.

SDS-PAGE and western blot analysis: Total proteins were extracted by grinding mature anthers from ryegrass plants in PBS buffer (10 mM phosphate buffer, pH 7.2; 150 mM NaCl) containing 1 mM PMSF followed by centrifugation at 14,000 rpm for 20 min at 4 °C. Soluble proteins in the supernatant were quantified by Bradford protein assay (Bio-Rad). Proteins were separated on SDS/15% PAGE gels on a Mini-Protean II system (Bio-Rad). The gels were either stained with Coomassie brilliant blue R250 or used in western blot analysis.

For western blotting, the proteins separated by SDS/PAGE were transferred onto nitrocellulose membranes. The blots were probed with primary antibodies or human sera overnight at 4 °C. The primary antibodies, including polyclonal rabbit anti-Lol p 1 or polyclonal rabbit anti-Lol p 2, were diluted 1:1000 by using PBS. The antibodies used were raised against the recombinant allergens Lol p 1 and Lol p 2 and are thus specific anti-Lol p 1 and anti-Lol p 2 antibodies (Tamborini et al. 1995). The sera of patients allergic to grass pollen were used at a dilution of 1:10. Binding of polyclonal antibodies was detected with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad) at a dilution of 1:1000, while binding of human sera was detected with mouse anti-human IgE antibodies conjugated to alkaline phosphatase (Southern Biotech) at a dilution of 1:1000. Secondary antibody detection was carried out for 2 hours at 25 °C. The colour reaction was developed by using an alkaline phosphatase conjugate substrate kit (Bio-Rad). Equal loading of total proteins from samples of transgenic and non-transformed control plants for the western analysis was ensured by quantifying total protein content and Coomassie staining of replicate gels for each extraction.

Construction and screening of ryegrass genomic DNA library

A Lambda-DASH®II (Stratagene) genomic library constructed from 4-week-old perennial ryegrass cv. Barlano was screened with a (³²P) dCTP-labelled 320 bp *Lol p 2* probe. Positive plaques from the tertiary screen were amplified and purified phage DNA was analysed by restriction endonuclease digestion followed by Southern hybridisation analysis using standard protocols (Sambrook et al. 1989).

Genetic mapping

RFLP loci detected by the *Lol p 1* and *Lol p 2* cDNAs were genetically mapped using the perennial ryegrass p150/112 reference mapping family (Bert et al. 1999). The marker segregation data was analysed in combination with data from heterologous anchor RFLP, AFLP and genomic DNA-derived simple sequence repeat (LPSSR) markers as described by Jones et al. (2002ab). The *Lol p 1* and *Lol p 2* cDNA sequences (GenBank accession numbers X57678 and X73363) were used to detect nucleotide similarity through BLASTN searches of the Rice_genome_japonica_TIGR database with a P value cut-off of 0.001 in the Gramene comparative mapping resource (<http://www.gramene.org/>). Details of the three highest matching BAC sequences were recorded, including name, E value of match and chromosomal location.

Results

Characterisation of the main pollen allergens

The organisation and genetic map positions of genes for the main pollen allergens *Lol p 1* and *Lol p 2* in perennial and Italian ryegrasses were determined by RFLP analysis using genomic DNA samples digested with different restriction enzymes and perennial ryegrass *Lol p 1* and *Lol p 2* gene specific hybridisation probes (Figure 1A–C). These analyses revealed that *Lol p 1* (Figure 1A) and *Lol p 2* (Figure 1B) are encoded by conserved small gene families in both *Lolium* species, with the *Lol p 1* and *Lol p 2* RFLP loci (xlolp1 and xlolp2) mapping to the lower end of linkage group 4 (LG4) and close to the upper end of LG5, respectively (Figure 1C). Comparative genomics analysis with the *Lol p 1* cDNA sequence detected

three BACs assigned to rice chromosome 3 (with highest match to BAC OSJNBa0009C08 with an E value of 3.6×10^{-8}), while the *Lol p 2* cDNA sequence detected two BACs (with highest match to BAC OSJNBa0082M15), assigned to rice chromosomes 10 and 3, respectively.

Accumulation of *Lol p 1* and *Lol p 2* allergens in mature pollen grains from dehiscing anthers of perennial and Italian ryegrass plants was determined. Cross species conservation of *Lol p 1* and *Lol p 2* was demonstrated by immunodetection, using antibodies raised against the recombinant perennial ryegrass *Lol p 1* and *Lol p 2* allergens respectively, of their natural counterparts in perennial and Italian ryegrasses (Figure 1D).

These results support the use of *Lol p 1* and *Lol p 2* – encoding cDNA sequences and corresponding antibodies raised against the recombinant perennial ryegrass allergens as suitable molecular tools for the production and analysis of transgenic plants for down-regulation of the two main pollen allergens in both ryegrass species.

Production of transgenic perennial and Italian ryegrasses

The transformation vector pZm13GUS which carries a chimeric *gusA* reporter gene under the control of the maize *Zm13* promoter (Hamilton et al. 1992) was constructed (Figure 2). This vector was used in the transformation of perennial and Italian ryegrass embryogenic suspension cells and tobacco protoplasts for the recovery and analysis of corresponding transgenic plants to confirm its suitability in conferring pollen-specific transgene expression in heterologous plant systems including both target ryegrass species (Figures 3 and 4).

Transformation vectors pZIGSAR and pZIIGSAR were generated with *Lol p 1* and *Lol p 2* cDNA sequences in antisense orientation under the control of the *Zm13* promoter for down-regulation of the pollen allergens in perennial and Italian ryegrasses. A chimeric *gusA* gene under the control of the CaMV 35S promoter was also included in these vectors (Figure 2).

Single genotype-derived embryogenic cell suspensions of perennial and Italian ryegrasses were established from embryogenic calli obtained from mature seed embryos (Figure 3A–C). Ryegrass embryogenic suspension cells were subjected to biolistic transformation using microprojectiles coated with plasmid

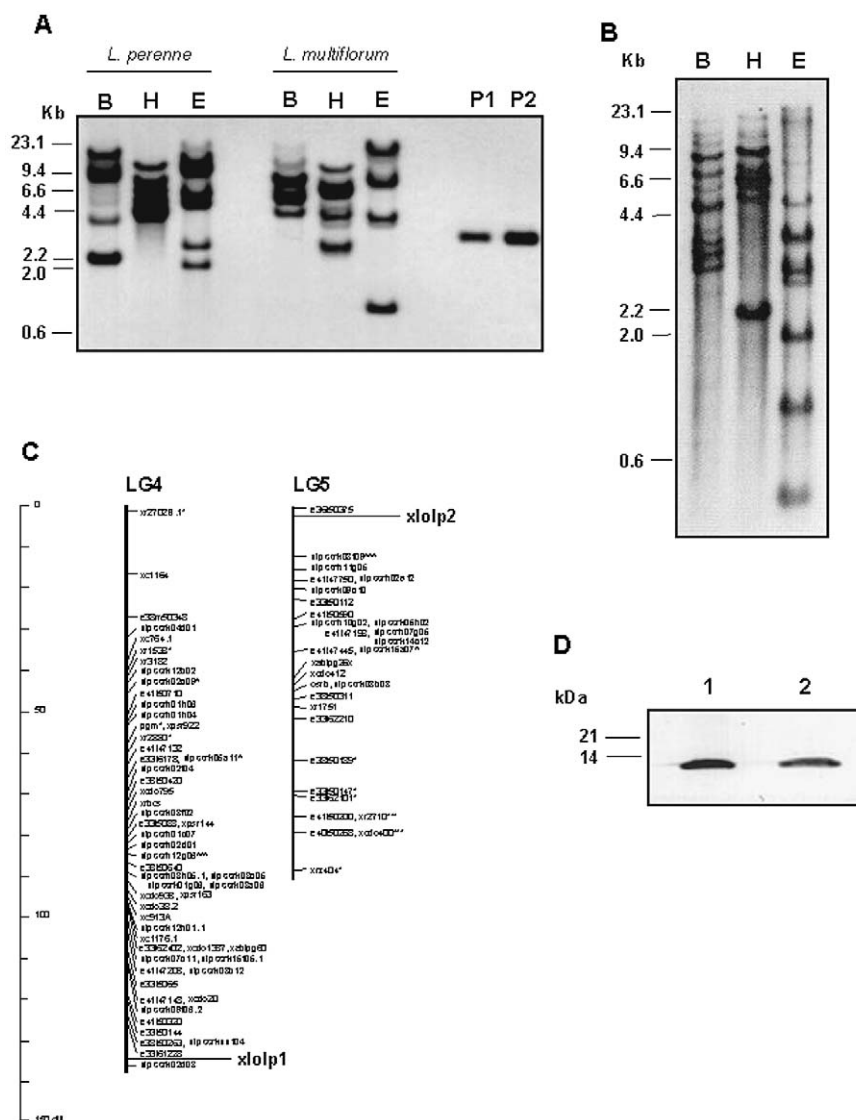


Figure 1. Characterisation of main pollen allergens in perennial and Italian ryegrasses. A) Southern hybridisation analysis of *L. perenne* and *L. multiflorum* plants. Genomic DNA isolated from perennial and Italian ryegrass plants, digested with *Bam*HI, *Hind*III and *Eco*RI and hybridized with *Lol p 1* probe; lanes P1 and P2 represent 10 pg and 20 pg of *Lol p 1* plasmid DNA, respectively (A); (B) Southern hybridisation analysis of *L. perenne* plants. Genomic DNA isolated from perennial ryegrass plants, digested with *Bam*HI, *Hind*III and *Eco*RI and hybridized with *Lol p 2* probe (B); (C) Map positions of *Lol p 1* and *Lol p 2* RFLP loci assigned to perennial ryegrass LG4 and LG5, respectively; (D) Western blot analysis of pollen protein extracts from non-transformed perennial and Italian ryegrass plants detected with polyclonal anti-*Lol p 2* antibody raised against recombinant allergen.

DNA from pZm13GUS or pZIGSAR or pZIIGSAR bearing the chimeric *gusA* reporter gene or *Lol p 1* or *Lol p 2* chimeric genes in antisense orientation under the control of the maize pollen-specific *Zm13* promoter (Figure 2, Figure 3C). A chimeric *hph* gene under the control of the rice actin 1 promoter on pAcH1 was used as the co-transformed selectable marker gene and allowed for the recovery of hygro-

mycin (Hm) resistant calli in perennial and Italian ryegrasses (Figure 3D). Upon transfer to regeneration medium the Hm resistant calli produced multiple green shoots (Figure 3E-F), and the *in vitro* regenerated plantlets (Figure 3G) lead to the establishment of putative transgenic perennial and Italian ryegrass plants grown in soil under containment glasshouse conditions (Figure 3H). A total of 7, 6 and 8 putative

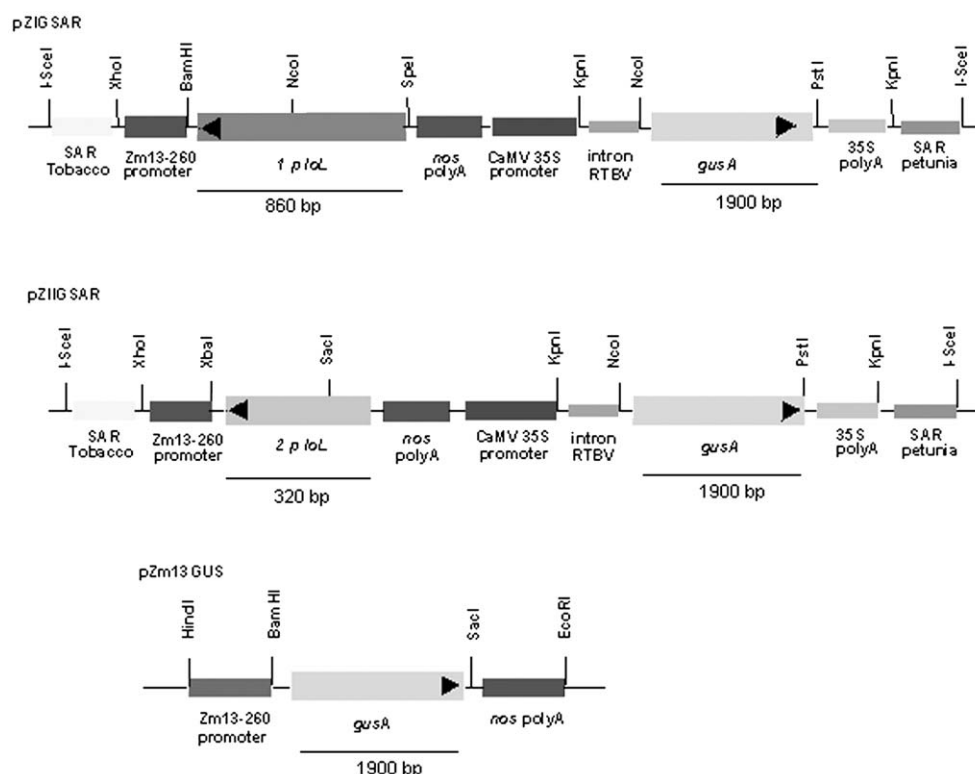


Figure 2. Plasmid maps of transformation vectors pZIGSAR with chimeric antisense *Zm13:Lol p 1* gene; pZIIGSAR with chimeric antisense *Zm13:Lol p 2*; and pZm13GUS with chimeric *Zm13:gusA* reporter gene under the control of the maize pollen-specific *Zm13* promoter used in biolistic transformation of perennial and Italian ryegrasses.

transgenic perennial ryegrass plants and 18, 24 and 30 putative transgenic Italian ryegrass plants were obtained from transformation experiments using pZm13GUS, pZIGSAR and pZIIGSAR, respectively. On average, one Hm resistant callus was recovered from every third bombarded dish supporting 200 to 250 mg fresh weight of suspension cells.

Molecular analysis of transgenic ryegrasses

Perennial and Italian ryegrass plants derived from co-transformation experiments with pZm13GUS, carrying a chimeric *Zm13:gusA* reporter gene, and pACh1, containing a chimeric *Act1:hph* selectable marker gene, were subjected to an initial PCR screening using *hph* and *gusA* specific primers. This analysis revealed the expected 375 bp *hph* amplification product in all DNA samples tested from putative transgenic perennial and Italian ryegrass plants confirming a tight Hm-*in vitro* selection of transformants (Figure 4A), as well as the presence of a 270 bp *gusA* PCR product in *gusA* co-transformed samples representing

co-transformation efficiencies of the non-selectable chimeric *gusA* gene in the 40–60% range (Figure 4B). The transgenic nature of the PCR-positive perennial and Italian ryegrass plants was unequivocally demonstrated by Southern hybridisation analysis using undigested and *Bam*HI-digested genomic DNA samples and the *hph* hybridisation probe (Figure 4C) or undigested and *Eco*RI + *Hind*III-digested genomic DNA samples and the *gusA* hybridisation probe (Figure 4D). For most DNA samples, complex hybridisation patterns were revealed, indicating the integration of multiple and rearranged copies of the transgene (Figure 4C,D).

Similar PCR screening and Southern hybridisation analyses were carried out on DNA samples from putative transgenic tobacco plants derived from direct gene transfer to protoplast with pZm13GUS (data not shown).

Pollen grains and samples from vegetative (i.e., leaves, stems, roots) and other reproductive organs (i.e., anthers) from *Zm13:gusA* transformed perennial and Italian ryegrass plants as well as tobacco plants

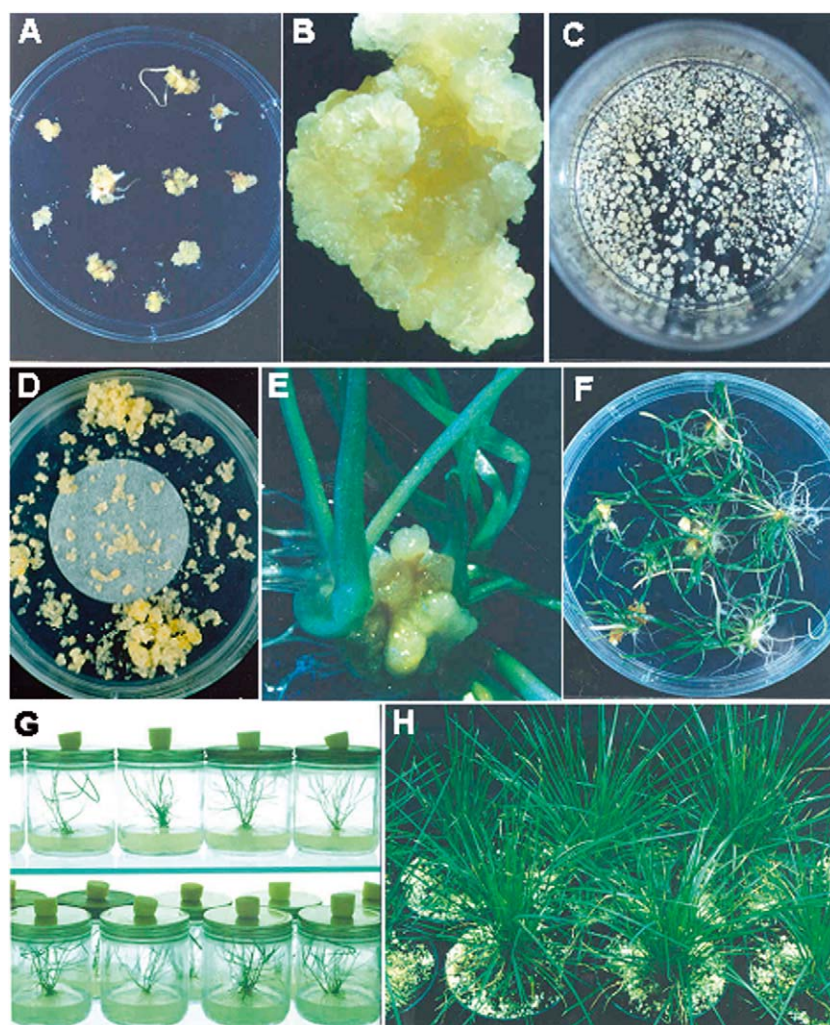


Figure 3. Production of transgenic perennial ryegrass plants for down-regulation of Lol p 1 and Lol p 2 pollen allergens through biolistic transformation of embryogenic suspension cells. (A) Embryogenic, friable, yellowish calli derived from plated embryos, 8 weeks after embryo isolation; (B) Embryogenic callus used to initiate single genotype-derived cell suspension; (C) Single genotype- derived embryogenic cell suspension 3 month after initiation; (D) Hygromycin (Hm) resistant calli obtained after bombardment of embryogenic suspension cells of perennial ryegrass with pZIGSAR- and pAch1-coated microprojectiles; (E–F) Shoot differentiation on Hm resistant callus of perennial ryegrass 4 weeks after transfer onto MSK medium for regeneration; (G) Transgenic perennial ryegrass plantlets growing *in vitro* 14 weeks after microprojectile bombardment of embryogenic cells; (H) Transgenic soil-grown perennial ryegrass plants for down-regulation of Lol p 1 and Lol p 2 pollen allergens 5 months after biolistic transformation of embryogenic suspension cells.

were histochemically stained for GUS activity. GUS activity was detected exclusively in pollen grains, thus confirming the suitability of the *Zm13* promoter in conferring pollen-specific transgene expression in heterologous plant systems including both target ryegrass species (Figure 4E).

Down-regulation of main pollen allergens

Following PCR screening using primers specific to *hph*, *gusA* and *Lol p 1* or *Lol p 2* chimeric genes, transgenic perennial and Italian ryegrass plants transformed respectively with the *Lol p 1* or *Lol p 2* antisense vectors, were identified and their transgenic nature was confirmed by Southern hybridisation analysis. Representative results from Southern hybridisation analyses demonstrating the stable integra-

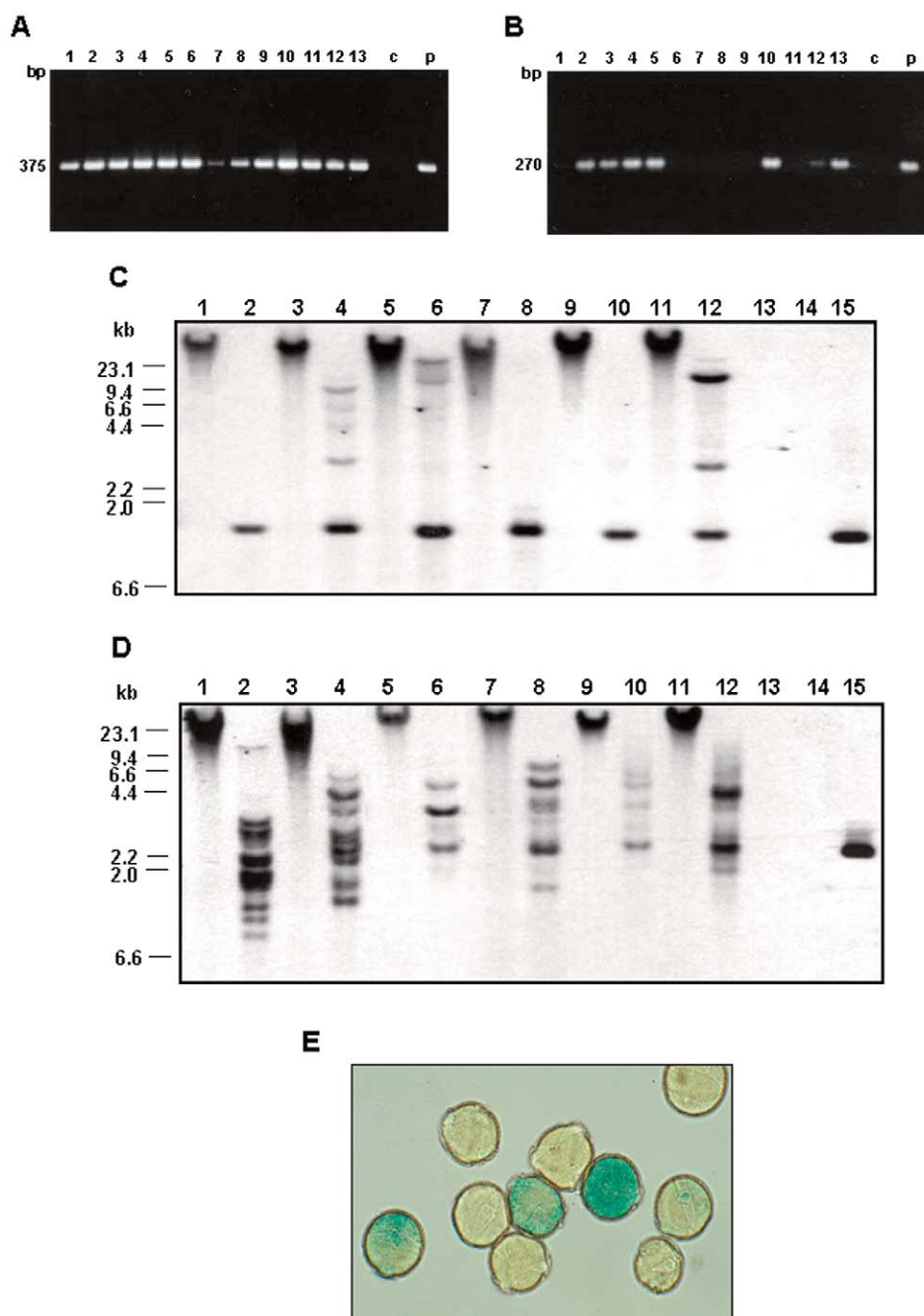


Figure 4. Analysis of transgenic ryegrass plants for assessment of *Zm13* pollen-specific promoter. A-B) PCR screening of transgenic perennial ryegrass plants using primers specific to *hph* (A) and *gusA* (B) genes; C) Southern hybridisation analysis with undigested (odd-numbered lanes) and *Bam*HI-digested (even-numbered lanes) genomic DNA samples isolated from transgenic perennial ryegrass plants and hybridized with the *hph* probe. Lanes: (1–12) six independent [PCR positive samples from (B)] pZm13GUS-transformed perennial ryegrass plants; (13–14) non-transformed perennial ryegrass plant; (15) 10 pg *Bam*HI-digested pACh1; D) Southern hybridisation analysis with undigested (odd-numbered lanes) and *Eco*RI + *Hind*III-digested (even-numbered lanes) genomic DNA samples isolated from transgenic perennial ryegrass plants and hybridized with the *gusA* probe. Lanes: (1–12) six independent [as in (C)] pZm13GUS-transformed perennial ryegrass plants; (13–14) non-transformed perennial ryegrass plant; (15) 10 pg *Eco*RI + *Hind*III-digested pZm13GUS; E) Histochemical GUS staining of pollen grains from transgenic pZm13GUS plant.

tion of the *hph* (Figure 5A), antisense *Lol p 1* (Figure 5B) and antisense *Lol p 2* (Figure 5C) chimeric genes into the genome of independent transgenic ryegrass plants are shown (Figure 5A–C).

Accumulation of *Lol p 1* and *Lol p 2* pollen allergens in transgenic perennial and Italian ryegrass plants carrying chimeric antisense *Lol p 1* and *Lol p 2* genes was determined by SDS/PAGE and western blot analysis using polyclonal anti-*Lol p 1* or anti-*Lol p 2* antibodies and comparatively assessed with non-transformed control ryegrass plants. Total protein extracts from mature anthers of antisense *Lol p 1* and antisense *Lol p 2* transgenic ryegrass plants and non-transformed control plants, ensuring loading equal amounts of total protein on SDS/PAGE followed by western blot analysis with anti-*Lol p 1* or anti-*Lol p 2* immunodetection, allowed for the identification of transgenic ryegrass plants with a significant reduction in the *Lol p 1* and *Lol p 2* allergen levels (Figure 5D,E). The hypo-allergenic character of pollen from transgenic antisense *Lol p 1* ryegrass plants was further confirmed by immunoblots using IgE antibodies from sera of ryegrass pollen-sensitised allergic patients, and compared with *Lol p 1* allergen levels detected in pollen protein extracts from control non-transformed perennial and Italian ryegrasses (Figure 5F). The down-regulation of *Lol p 1* or *Lol p 2* pollen allergens in transgenic perennial and Italian ryegrass plants carrying chimeric antisense *Lol p 1* or *Lol p 2* genes, respectively was consequently demonstrated.

Isolation and characterisation of a pollen-specific promoter

A perennial ryegrass genomic library was constructed and screened with a 320 bp *Lol p 2* cDNA probe leading to the identification of a genomic clone encoding *Lol p 2* and to the isolation and DNA sequencing of 952 bp *Lol p 2* 5' regulatory sequences (Genbank #AY533648). The *Lol p 2* promoter sequence was subjected to sequence analysis against the PLACE database (Higo et al. 1999) revealing different putative pollen-specific regulatory elements including the maize *Zm13* Q element, the tomato pollen 1 LAT52 element and the GTGANTG10 element (Figure 6A). In order to assess whether the *Lol p 2* promoter directs pollen-specific gene expression, the transformation vector pLP2GUS carrying a chimeric *gusA* gene under the control of 952 bp *Lol p 2* 5' regulatory sequences was generated and transgeni-

cally expressed *in planta* (Figure 6B,C). Transgenic tobacco plants expressing *Lol p 2:gusA* grown until flowering produced pollen revealing exclusively strong GUS activity in pollen grains (Figure 6C), thus confirming the pollen-specificity of the perennial ryegrass *Lol p 2* promoter as a further tool for the production of transgenic ryegrasses with hypo-allergenic pollen.

Discussion

We describe the generation and analysis of transgenic perennial and Italian ryegrasses with reduced levels of the major pollen allergens, *Lol p 1* and *Lol p 2*. The *Lol p 1* and *Lol p 2* proteins are the two most important ryegrass pollen allergens, responsible for elevated levels of IgE antibodies in up to 95% and 45%, respectively, of grass pollen-sensitised allergic patients (Kahn and Marsh 1986; Freidhoff et al. 1986). These allergens bind to IgE molecules in grass pollen-sensitised patients and trigger the release of mediators leading to symptoms of hayfever and seasonal allergic asthma (Freidhoff et al. 1986; Singh and Bhalla 2003).

The main pollen allergens in perennial and Italian ryegrasses, *Lol p 1* and *Lol p 2*, are encoded by small, sequence-conserved gene families in both *Lolium* species. Other pollen allergenic proteins have also been reported to correspond to gene families (Griffith et al. 1991; Roberts et al. 1993; Xu et al. 1995). Although the *Lol p 1* and *Lol p 2* cDNAs detect small multigene families in *Lolium* taxa, single polymorphic bands were detected as segregating features in the one-way pseudo-testcross F_1 mapping family (Jones et al. 2002a), with the *xlolp1* and *xlolp2* loci assigned to perennial ryegrass LG4 and LG5. The comparative genomics analysis using the *Lol p 1* nucleotide sequence detected a BAC assigned to rice chromosome 3, consistent with conserved synteny between the lower region of ryegrass LG4 and rice chromosome 3 previously demonstrated by comparative genetic mapping analysis (Jones et al. 2002a).

In addition, the natural counterparts of *Lol p 1* and *Lol p 2* in both perennial and Italian ryegrasses were detected by antibodies raised against the corresponding recombinant perennial ryegrass allergens, demonstrating cross-species epitope conservation in these pollen allergens. A significant level of conservation in immunoreactivity has been found for group 1 allergens between species belonging to different grass

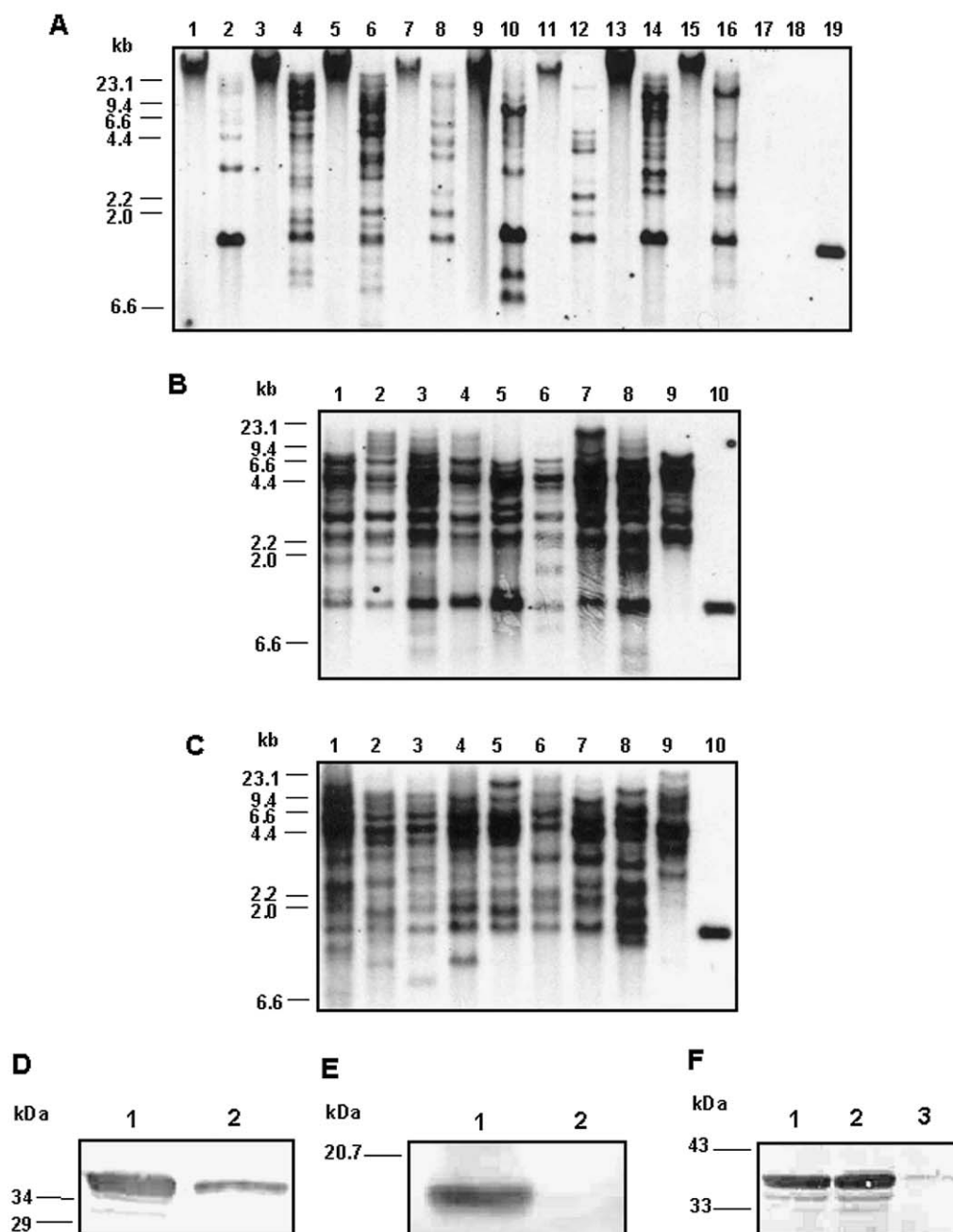


Figure 5. Down-regulation of main pollen allergens Lol p 1 and Lol p 2 in perennial and Italian ryegrasses. (A) Southern hybridisation analysis with undigested (odd-numbered lanes) and *Bam*HI-digested (even-numbered lanes) genomic DNA samples isolated from transgenic perennial ryegrass plants for down-regulation of Lol p 1 and Lol p 2 hybridized with the *hph* probe. Lanes: (1–16) eight independent pZIGSAR-transformed ryegrass plants; (17–18) non-transformed ryegrass control plant; (19) 10 pg *Bam*HI-digested pAcH1 (19); (B) Southern hybridisation analysis with *Xho*I + *Hind*III-digested genomic DNA samples isolated from transgenic Italian ryegrass plants for down-regulation of Lol p 1 hybridized with *Lol* p 1 probe. Lanes: (1–8) six independent pZIGSAR-transformed ryegrass plants; (9) non-transformed ryegrass control plant; (10) 10 pg *Xho*I + *Hind*III-digested pZIGSAR; (C) Southern hybridisation analysis with *Xho*I + *Hind*III-digested genomic DNA samples isolated from transgenic Italian ryegrass plants for down-regulation of Lol p 2 and hybridized with *Lol* p 2 probe. Lanes: (1–8) six independent pZIGSAR-transformed ryegrass plants; (9) non-transformed ryegrass control plant; (10) 10 pg *Xho*I + *Hind*III-digested pZIGSAR; (D) Western blot analysis of pollen protein extracts from transgenic Italian ryegrass plant carrying an antisense *Lol* p 1 transgene (2) compared to non-transformed control plant (1) and probed with anti-*Lol* p 1 antibodies; (E) Western blot analysis of pollen protein extracts from transgenic Italian ryegrass plant carrying an antisense *Lol* p 2 transgene (2) compared to non-transformed control plant (1) and probed with anti-*Lol* p 2 antibodies; (F) Western blot analysis of pollen protein extracts from non-transformed perennial ryegrass control plant (1), Italian ryegrass control plant (2) and transgenic antisense *Lol* p 1 ryegrass plant (3) probed with IgE antibodies from serum of grass pollen allergic patient.

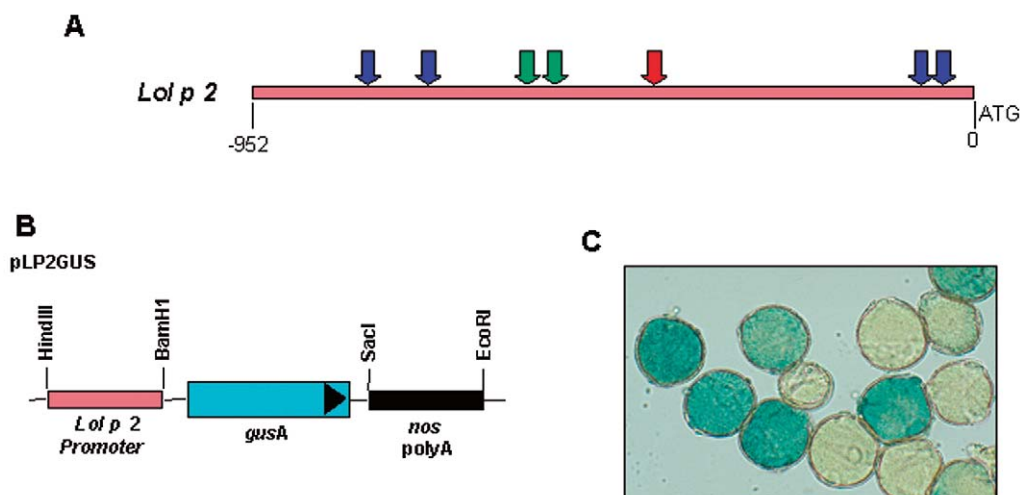


Figure 6. Analysis of *Lol p 2* pollen-specific promoter from perennial ryegrass. (A) Pollen-specific promoter elements of the 952 bp *Lol p 2* 5' regulatory sequence; *Zm13* Q element (blue arrowheads), LAT52 element (green arrowheads) and GTGANTG10 element (red arrowhead); (B) Transformation vector pLP2GUS carrying chimeric *gusA* reporter gene under control of 952 bp *Lol p 2* promoter; (C) Histochemical GUS staining of pollen grains from transgenic pLP2GUS plant.

genera (Laffer et al. 1994; Muller et al. 1996; Singh and Bhalla 2003).

Analysis of transgenic ryegrass plants bearing a chimeric *gusA* reporter gene under the control of the maize *Zm13* promoter demonstrated integration of multiple transgene copies. The multiple bands observed could be due to multiple insertions of rearranged and/or partial copies of the *gusA* and *hph* transgenes in the genome of the transgenic ryegrass plants. Similar results have been reported for transgenic plants of perennial ryegrass (Spangenberg et al. 1995, 1998) and Italian ryegrass (Ye et al. 1997) obtained from biolistic transformation of embryogenic cell suspensions. However, simpler transgene integration patterns have been described for transgenic perennial ryegrass plants obtained by *Agrobacterium*-mediated transformation (Altpeter et al. 2000a, b). Expression of *Zm13:gusA* transgenes in pollen grains from transgenic perennial and Italian ryegrasses and tobacco plants confirmed the pollen specificity of the maize *Zm13* promoter in both monocot and dicot species. Transient expression of a chimeric *Zm13:gusA* reporter gene has been previously shown in *Tradescantia* (Spiderwort) and maize pollen (Hamilton et al. 1992). This demonstrated the potential utility of the *Zm13* pollen-specific promoter to direct expression of chimeric antisense *Lol p 1* and *Lol p 2* genes for antisense-mediated silencing of these pollen allergen genes in transgenic ryegrasses. In ad-

dition, the isolation and characterisation of the perennial ryegrass *Lol p 2* promoter demonstrating its pollen-specificity provides a further tool to enable even more targeted manipulation of pollen allergens in transgenic ryegrasses.

The introduction of the *Lol p 1* and *Lol p 2* transgenes in antisense orientation led to a reduction in accumulation levels of *Lol p 1* and *Lol p 2* allergens in pollen of transgenic ryegrass plants, as demonstrated by immunoblots using polyclonal antibodies raised against the recombinant *Lol p 1* and *Lol p 2* allergens as well as with sera from grass pollen-sensitised patients. Antisense silencing of the *Lol p 5* allergen in annual ryegrass has been previously achieved (Bhalla et al. 1999). Recent advances in RNAi mediated silencing in plants (Wesley et al. 2001; Helliwell and Waterhouse 2003) promoted opportunities for the design and deployment of hairpin vectors targeting the simultaneous silencing of multiple main pollen allergens in *L. perenne* and *L. multiflorum*.

Initial studies suggest that the *Lol p 1* pollen allergens correspond to a novel class of β -expansins putatively involved in invasion of the pollen tube into maternal tissues by loosening cell walls of the grass stigma and style (Cosgrove et al. 1997; Li and Cosgrove 2001). The functional role of *Lol p 2* *in planta* is unknown. The production of transgenic ryegrasses with down-regulation of the main pollen allergens re-

ported here promotes the opportunity for detailed studies to enhance our understanding of the functional role of these conserved pollen proteins in *Lolium* species and to assess the potential for the development and release of hypo-allergenic ryegrass cultivars for hay fever prevention (Spangenberg et al. 1998, 2001).

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