

# Reduction in Allergenicity of Grass Pollen by Genetic Engineering

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## Key Words

Pollen allergen · Genetic engineering · Antisense · Downregulation · Grass, transgenic · Grass, hypoallergenic · Allergy, type 1

## Abstract

**Background:** Hay fever and allergic asthma triggered by grass pollen allergens affect ~20% of the population in cool temperate climates. Ryegrass is the dominant source of allergens due to its prodigious airborne pollen production. Lol p 5 or group 5 is among the most important and widespread grass pollen allergen because it reacts with IgE antibodies of more than 90% of grass pollen-allergic patients, contains most of the grass pollen-specific IgE epitopes and elicits strong biological responses. Significant efforts have been made in developing diagnostic and therapeutic reagents for designing new and more effective immunotherapeutic strategies for treatment of allergic diseases. An alternative approach to this problem could be to reduce the amount of allergen content in the source plant. **Methods:** High velocity microprojectile bombardment was used to genetically engineer ryegrass. Antisense construct targeted to one of major allergen, Lol p 5, was introduced. The expression of antisense RNA was regulated by a pollen-specific promoter. Pollen was analysed for IgE reactivity. **Results:** Analysis of proteins with allergen-specific

monoclonal and polyclonal antibodies did not detect Lol p 5 in the transgenic pollen. The transgenic pollen showed remarkably reduced allergenicity as reflected by low IgE binding capacity of pollen extract as compared to control pollen. The transgenic ryegrass plants in which *Lol p 5* gene expression is perturbed showed normal fertile pollen development. **Conclusions:** Our studies showed that it is possible to selectively 'switch off' allergen production in pollen of ryegrass demonstrating feasibility of genetic engineering of plants for reduced allergenicity.

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## Introduction

The discovery of novel techniques to introduce genes into plant cells has given us the opportunity to create, characterize and select plant cultivars which could not be obtained by traditional breeding methods. Antisense RNA has been used experimentally to regulate gene expression and to eliminate gene products in prokaryotic and eukaryotic organisms including yeast, animal and plant cells [1]. Antisense technology is based on blocking the information flow from DNA via RNA to protein. This technology is practically applicable to crop plants to suppress the gene expression [2, 3]. Our laboratory has shown use of antisense in down-regulation of anther and pollen-

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specific genes to create male sterility in *Arabidopsis* [4] and cauliflower (*Brassica oleracea* var. *botrytis*) [5].

Grasses produce prolific amounts of pollen containing allergenic proteins known to cause allergic reactions such as hay fever and allergic asthma in humans during spring and summer. Grass pollen allergy afflicts up to 20% of the population in cool temperate climates [6]. Two different proteins have been described as the major allergens of grass pollen, group 1 and group 5. The most abundant of these is group 1, a 35-kD protein which constitutes approximately 5% of the total extracted pollen proteins. Group 5 is a 31-kD protein and is less abundant but more allergenic than group 1 proteins. Examination of blood sera of grass pollen-allergic patients showed that 90% of patients allergic to grass pollen have IgE recognizing this allergen [7]. Because of clinical significance, most of the studies on grass pollen allergens have been focused on its diagnostic and therapeutic aspects. On the other hand, it would be desirable to produce plants without this allergy-causing protein. In this study, we report reduction in allergenicity of ryegrass pollen by antisense downregulation of pollen allergen production using genetic engineering approach.

## Material and Methods

A commercial genotype of ryegrass (*Lolium rigidum* L.) was used. A pollen-specific promoter, *Ory s I* [8], was used to drive the antisense expression of *Lol p 5*. The 1,507-bp 5' upstream region of *Ory s I* was fused with 0.94 kb of *Lol p 5* cDNA clone in reverse orientation. This vector also carried a plant-selectable marker gene, the neomycin phosphotransferase-II (*NPT-II*) gene. Particle bombardment was performed with a helium-driven DuPont Biolistic Delivery System PDS-1000 (Bio-Rad) at 1100 psi using 1.0  $\mu$  gold particles following manufacturer's instructions. Regeneration and confirmation of transgenic status of plants was carried out as described by Bhalla et al. [9]. Total soluble proteins were used for SDS-PAGE and protein blotting analysis. For Western blotting, the SDS-PAGE-separated proteins were transferred onto a nitrocellulose membrane. The blots were probed with antibody essentially as described by Singh et al. [7] and the colour reaction was developed using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate as substrates. Binding of human IgE with sera of patients allergic to grass pollen was detected using <sup>125</sup>I-labelled anti-human IgE (Bioclone, Australia).

## Results

Three- to 4-week-old calli were used for microprojectile bombardment. For selection of stably transformed tissue and regenerated plants, the concentration of the anti-

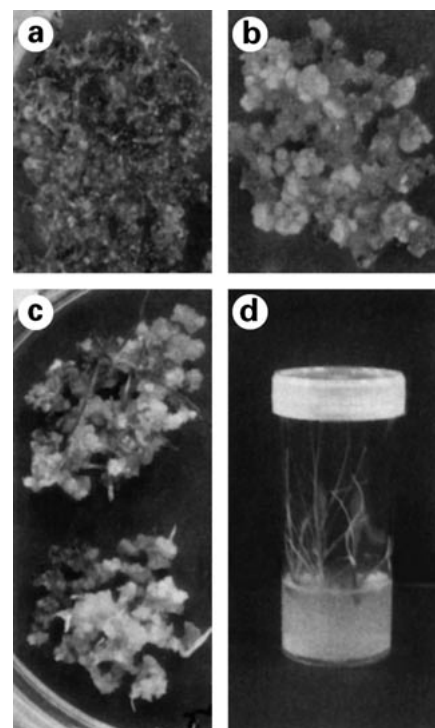


**Fig. 1.** Selection of stably transformed ryegrass tissue and regenerated shoots. **a** Unbombarded callus on selection medium appears dark, hence dead. **b** Bombarded callus on selection medium showing growing white, healthy transformed callus cells. **c** Shoot regeneration from transformed callus. **d** Regenerated shoots on selection medium.

biotic required to inhibit growth and to kill control untransformed callus was determined (fig. 1a). Plants were regenerated from callus lines which grew in the presence of selection medium (fig. 1b, 1c). In order to avoid escapes (untransformed shoots) a transformant selection step was introduced where green regenerated shoots were exposed to a selection medium containing kanamycin and geneticin but without carbohydrate (sucrose) source (fig. 1d). Within 2–4 weeks, most of the untransformed shoots turned white, while transformed shoots remained green on this medium and continued to grow. Transfer of regenerated green shoots to transformant selection medium was very effective at screening out false positives (fig. 1d). *NPT-II* expression was assayed using small leaf pieces as described by Bhalla et al. [9]. PCR amplification of genomic DNA from transgenic plants exhibited a DNA fragment of the expected 690-bp size. No amplification was observed in untransformed ryegrass. Confirmation of stable integration of DNA in regenerated plants was also

done by Southern analysis. The plants were successfully grown under glasshouse conditions.

The transgenic plants grew normally and produced inflorescences comparable to wild-type control plants. These plants were analysed for allergenic protein content by probing the pollen protein blot with Lol p 5-specific monoclonal and polyclonal antibodies. No difference was observed in the overall soluble pollen protein profile of transgenic and wild-type control plants. The use of Lol p 5-specific monoclonal and polyclonal antibodies showed presence of Lol p 5 protein in pollen extract from wild-type plant. In contrast, no such immunoreactive protein could be detected in pollen extract of the transgenic plant. Incubation with anti Lol p 1-specific monoclonal antibody revealed that expression of Lol p 1 was not affected in pollen of transgenic plants, indicating specific inhibition of Lol p 5 production. Slot blot assay was used to analyse sera from a number of grass pollen allergic patients for IgE reactivity to the pollen of the transgenic plant. A dramatic reduction in overall IgE reactivity of pollen extract from the transgenic plant was observed in all the sera tested (fig. 2). Western blot analysis confirmed that this overall reduction in IgE binding observed in slot blot analysis was due to specific inhibition (or absence) of Lol p 5 in the transgenic grass.

Pollen of transgenic plants were also analysed for pollen development and viability. FCR test and SEM of the pollen from transgenic and wild-type control plants showed no difference in pollen viability and morphology. Pollination studies showed that pollen from transgenic plants were able to germinate and penetrate stigmatic tissues.

## Discussion

Significant efforts have been made in developing diagnostic and therapeutic reagents for designing new and more effective immunotherapeutic strategies for treatment of allergic diseases [10, 11]. An alternative approach to alleviate allergic symptoms could be to reduce the environmental load of inhalant allergens by genetic modification of the source plants for reduced production of allergenic proteins. Traditional plant breeding and selection methods have not been successful in decreasing or eliminating these proteins. With the availability of cloned allergens it is now feasible to ablate the function of a specific allergen gene by genetic transformation with targeted antisense construct. The mechanism of antisense action is based on hybridization of antisense sequence to a comple-

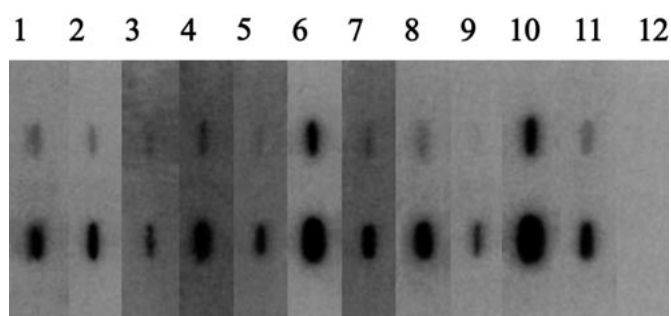


**Fig. 2.** Allergic activity of the transgenic pollen as measured by IgE reactivity of pollen extracts in slot blot analysis. Top row represents transgenic pollen, bottom row untransformed control pollen. Lanes 1–11 were probed with sera from 11 known-allergic patients and lane 12 with sera from a non-allergic patient.

mentary sequence in mRNA molecule coding for a specific protein. When produced in cell, antisense RNA behaves in a dominant manner, i.e. if the sequences have high levels of nucleotide identity, antisense RNA produced by one introduced sequence can ablate the function of all the related genes. This is particularly relevant in case of grass pollen allergens. Both group 1 and group 5 allergens belong to gene families encoding distinct isoforms. However, all these isoforms show a high level of sequence identities implying that antisense DNA construct based on any one of these isoforms should result in antisense-induced depletion of all the isoforms.

Since Lol p 1 and 5 allergens show pollen-specific expression, prerequisite for preparation of antisense construct to deplete these allergens in pollen is the use of a strong pollen-specific gene promoter so that endogenous and antisense RNA are well coordinated during pollen development. Accordingly, we used antisense construct comprising *Lol p 5A* and *Ory s 1* promoter sequences. *Ory s 1* promoter, originally isolated from rice, has been shown to be pollen-specific and its temporal expression pattern reflects that of *Lol p 5* [8].

Recent advances in plant genetic engineering have made the introduction of new genes possible in monocot plants including grasses. The most successful and therefore favoured gene delivery system for monocots involves direct introduction of DNA constructs into intact cells using high velocity microprojectile bombardment. We have used this gene delivery system for introducing an antisense construct targeted to *Lol p 5* gene in ryegrass. In our study, the use of *Ory s 1* promoter with antisense *Lol p 5A* cDNA led to the production of hypoallergenic grass which showed no Lol p 5 production (as indicated by lack

of Lol p 5-specific antibody binding to pollen extract). For most of the allergic patient sera, there was dramatic reduction of IgE binding capacity of transgenic pollen extracts as compared to control extracts.

In conclusion, our results show the potential of antisense approach in reducing the allergenic potential of plants. Such a strategy can have a general applicability for production of transgenic plants depleted of both inhaled

and ingested allergens. In addition, such an approach could also help in elucidating in vivo function of allergen(s) in plants.

## Acknowledgment

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