

Knocking out expression of plant allergen genes

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Abstract

Pollen of many grasses, trees, and weeds are the source of inhalant allergic proteins while various other plant products are allergenic only upon their ingestion as a food source. Allergenic proteins of pollen are exposed to human immune system after their rapid release from pollen upon coming in contact with moist surface of nasal mucosa. The advent of molecular cloning and ability to genetically transform plants now offer unprecedented opportunities to produce hypoallergenic plants by targeted switching off allergen production. Gene silencing strategies that operate at post-transcriptional level are highly suitable for blocking allergen production. We have demonstrated the concept of allergen gene silencing through antisense approach by producing ryegrass plants that do not produce major allergen in its pollen. 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 the *in vivo* function of allergen(s) in plants and contribution of an allergen to overall allergenic potential of an allergen source.

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1. Introduction

Allergens are defined by their ability to cause the induction of hypersensitivity response when encountered by an immune system of sensitive individuals. Inhalation or ingestion of potential allergens leads to production of allergen-specific IgE antibodies. The incidence and severity of allergic disorders is steadily increasing worldwide. Exposure to common environmental antigens is the cause of allergic conditions such as hay fever, allergic asthma, and eczema affecting up to 25% of the population in developed countries.

Pollen grains of various weeds, trees, and grasses are significant source of inhalant allergens. In various regions of the world, the pollen of grass genera is the causative factor of respiratory allergy in more individuals than any other flowering plant family. At least 40% of type 1 allergic patients are sensitized against grass

pollen allergens thus making grass pollen the most frequent elicitor of allergic symptoms [1]. Ryegrass and closely related grasses of subfamily *Pooideae* are distributed in cool temperate regions in North America, Europe, and Southern parts of Australia. In warm temperate and sub-tropical environment, pollen of Bermuda grass (subfamily, *Chloridoideae*) is an important source of allergens. Atopic individuals can also suffer from ingested food-based allergens. Food allergy affects 8% of children under 3 years of age and approximately 2% of the adult population. The perceived risks of allergies associated with foods containing biotechnology-based modifications have also highlighted the allergy problem. Most of the inhalant or food allergens of plant origin are proteins ranging from 10 to 50 kDa.

The advent of molecular biology era has led to isolation and cloning of genes encoding these allergens and potential applications of recombinant allergens in allergy diagnosis and therapy [2–6]. Besides, there is the exciting possibility of blocking allergen production by knocking out genes encoding these proteins. Any

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strategy that focuses on knocking out single genes, for example, the genetic selection of mutagenized plant populations is unlikely to lead to significant reduction in allergen production because most of the allergen proteins are present as isoforms encoded by genes belonging to multigene families. Therefore, post-transcriptional gene silencing such as use of antisense or RNAi based constructs are only feasible approaches to remove allergen production. Sequence micro-heterogeneity between different isoforms is a commonly observed feature of allergens. However, most of the genes encoding isoforms have conserved stretches of identical nucleotide sequences. It is possible to silence all the isoforms of a target allergen by using an interfering construct that will have significant homology to common nucleotide sequences shared by all the isoforms.

In grasses, two different proteins designated as belonging to groups 1 and 5 have been described as the major allergens [2]. More than 95% of grass pollen allergic patients show sera IgE antibodies that react with these allergens [3,4]. Group 1 allergens, 30–35 kDa proteins, are present in the cytoplasm of the pollen grain but are released rapidly upon hydration [3]. The cDNAs encoding group 1 allergens from various grass genera have been cloned [2]. Group 5 allergens are highly IgE reactive proteins of molecular mass 28–32 kDa and were initially identified as IgE reactive fusion proteins in grass pollen cDNA expression libraries [4]. Group 1 and group 5 together account for most of the IgE binding capacity of crude pollen extracts. Both group 1 and 5 allergens exist as multiple isoforms encoded by multigene families [2,7].

In our laboratory, we have demonstrated the concept of allergen gene silencing through antisense approach by producing ryegrass plants that do not produce major allergen, Lol p 5, in its pollen [8]. The mechanism of antisense action is based on hybridization of antisense sequence to complementary 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 for group 5 grass pollen allergens. For example, two *Lol p 5* isoforms, *Lol p 5a* and *Lol p 5b*, show only 70% identity at the nucleotide level but both their sequences share long stretches of identical sequences, implying that the antisense DNA construct based on any one of these two isoforms should result in depletion of both isoforms. In our experiments an antisense construct based on *Lol p 5A* regulated by a pollen-specific promoter was used to genetically engineer grass plants. The transgenic pollen showed remarkably reduced allergenicity as reflected by the low IgE binding capacity of pollen extract as compared to control pollen [8]. Our results thus provided proof of the

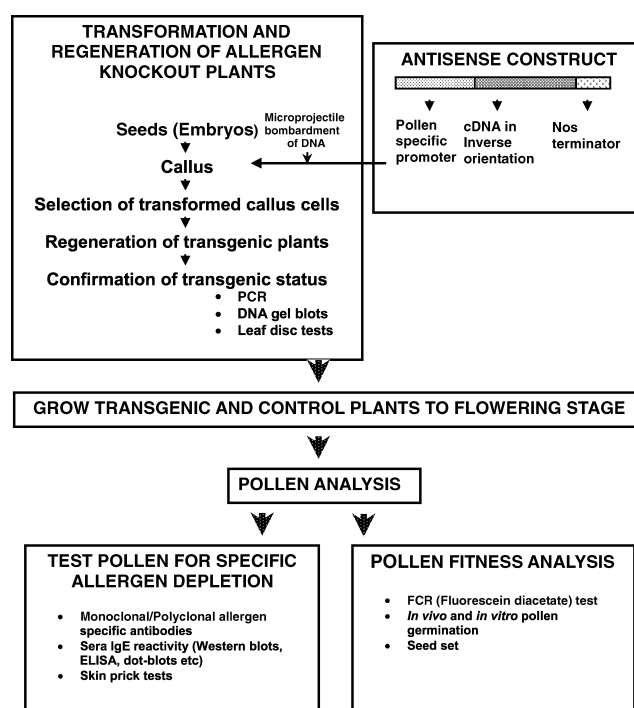


Fig. 1. Schematic representation of knocking out pollen allergen gene in ryegrass plants using antisense approach.

principle for the concept of allergen removal through gene silencing. This principle is potentially applicable for removing inhalant or food allergens in plants amenable to genetic transformation.

In the following sections, we will briefly describe the design of antisense construct for the silencing of *Lol p 5* and the methodologies for the generation of hypoallergenic ryegrass plants lacking *Lol p 5* production in pollen (Fig. 1).

2. Description of method

2.1. Generation of antisense construct

Since grass allergens (such as *Lol p 1* and *5*) show pollen-specific expression, pre-requisite for the preparation of antisense construct is the use of a strong pollen-specific gene promoter so that production of endogenous and antisense RNA is well coordinated during pollen development to deplete the allergen in pollen. Accordingly, we used antisense construct comprising *Lol p 5A* and *Ory s1* promoter sequences. *Ory s1* promoter, originally isolated from rice, has been shown to be pollen specific and its temporal expression pattern reflects that of *Lol p 5* [9]. In our study, the 1507 bp 5' upstream region of *Ory s1* was fused with 0.94 kb (nt 1–943, [4]) of *Lol p 5* cDNA clone in reverse orientation and cloned into a derivative of pUC 19 vector. However,

the smaller size of cDNA can be used for allergen knockout studies as smaller cDNA of 100 bp has been shown to be effective for antisense mediated gene silencing [10]. Antisense orientation of the allergen cDNA should be confirmed by sequencing. The vector should carry a plant selectable marker gene. Neomycin phosphotransferase-II (*NPT-II*) gene and hygromycin phosphotransferase (HPT) are commonly used antibiotic selectable markers in plant transformation studies. For ryegrass, our plasmid vector contained the *NPT-II* gene.

2.2. Gene delivery

For grasses, microprojectile bombardment with a helium-driven DuPont Biolistic Delivery System PDS-1000 (Bio-Rad) at 1100 psi using 1.0 µm gold particles achieved successful gene delivery [8]. However, bombardment parameters such as the size of gold or tungsten particles and pressure need to be standardized for each plant type to obtain optimum gene delivery in the cells. *Agrobacterium* mediated gene delivery is more suitable for rice and dicot plants such as ragweed, rapeseed, birch, etc., as these plants are considered to be more amenable to *Agrobacterium* infection. *Agrobacterium* mediated gene delivery protocol for rice and *Brassica* crops has been published [11,12]. For *Agrobacterium* mediated gene delivery, the cloning of antisense gene construct in a binary vector is required.

2.3. Transformation and regeneration of transgenic plants

It is well established that plant regeneration and transformation is very much genotype dependent. Therefore, it is not possible to give a general method applicable to all plant types. The following described method is based on our experience with ryegrass. However, the concepts described here should be generally applicable.

Embryogenic calli induced from seeds of ryegrass are used for plant regeneration. For this, ryegrass seeds are first surface sterilized in 13% (v/v) sodium hypochlorite for 20 min followed by five time rinsing in sterile distilled water. The seeds are then placed onto callus induction medium (MS medium [13] containing 20 g/l sucrose, 5 mg/l 2,4-D, and 6 g/l agar, Sigma Chemical). The pH of the tissue culture media is adjusted to 5.8 before autoclaving at 121 °C for 20 min. The effect of different concentrations of 2,4-D for callus induction should be evaluated in order to produce a high quality regenerable callus. An efficient regeneration system for production of viable green shoots is pre-requisite for transformation. The *GUS* reporter gene can be used initially to assay transient expression in callus to optimize microprojectile bombardment conditions. Three- to four-week-old calli are usually found to be ideal targets for bombardment. For bombardment, calli are spread out

in the middle of a petri dish containing agar-solidified MS medium. Following bombardment, the calli are allowed to recover for 2–3 days at room temperature before transferring them on to a selection medium (callus induction medium with 50 mg/l kanamycin, since *NPT-II* is used as the selectable marker gene) for 4 weeks, followed by transfer to regeneration medium (MS medium with 2 mg/l BAP). Shoots usually start to appear within 6–8 weeks on this medium. For selection of stably transformed tissue and regenerated plants, the concentration of the antibiotic required to inhibit growth and to kill control untransformed callus is determined [14]. Plants are regenerated from callus lines that grow in the presence of selection medium [14]. To avoid escapes—untransformed shoots—a transformant selection step is advised where green regenerated shoots are exposed to a medium containing kanamycin but without carbohydrate (sucrose) source [14]. In our hands, this seems to be very effective for grasses, rice, and *Brassica* crop plants. Within 2–4 weeks, most of the untransformed shoots turn white, while transformed shoots remain green on this medium and will continue to grow. Regenerated ryegrass shoots are rooted on MS medium without hormone, followed by transfer to soil and are grown under greenhouse conditions (18 h light at 25 °C).

2.4. Confirmation of transformation

It is essential to confirm the transgenic status of the regenerated plants by the following tests mentioned below:

2.4.1. Leaf disc test

For ryegrass plants, a leaf disc assay is used as a diagnostic marker for *NPT-II* expression (if *NPT-II* gene is used as selectable marker). For this, leaves from green regenerated shoots are carefully removed from root induction medium. Using aseptic techniques, the leaves are cut into small sections of approximately 4–5 mm in length with underside down are carefully pressed into the surface of agar (0.7%) containing 50 mg/l kanamycin. Leaf sections from wild-type tissue culture regenerated plants are used as controls. Untransformed leaf pieces do not swell on this medium and turn white within 7 days while leaf pieces from transformed plants expand in size and remain green.

2.4.2. DNA analysis

Genomic DNA of the putative transgenic plants is analysed by PCR and DNA blot analyses. Genomic DNA is usually extracted from leaf tissues using the CTAB method [15]. PCR amplification of genomic DNA using *NPT-II*-specific primers (For example; primer 1: 5'GAGGCTATTCGGCTATGACTG3' and primer 2: 5'GGAGCGGCGATACCGTAAAGC3'; internal primers of *NPT-II* gene designed to amplify the

690 bp DNA fragment or similar primer set can be designed) should exhibit a DNA fragment of the expected size. No amplification should be observed in untransformed plants. Confirmation of stable integration of transgene DNA in regenerated plants should be done by DNA blot analysis [16]. DNA blot will indicate the number of gene insertions in the transgenic plants.

2.5. Analysis of transgenic plants carrying antisense Lol p 5 construct for the allergen knockout

The following studies are performed using either mature fully developed anthers or pollen.

2.5.1. Protein analysis using SDS–PAGE and protein blotting

Analyse the transgenic plants for allergenic protein content by probing the pollen protein blot with 5-specific monoclonal and/or polyclonal antibodies. For this, total proteins are extracted from mature anthers of transgenic and wild-type plants and analysed by SDS–PAGE. Total proteins are extracted by grinding mature anthers in PBS (10 mM phosphate buffer, pH 7.2, 150 mM NaCl) containing 1 mM PMSF, followed by centrifugation at 14,000 rpm for 10 min. Soluble proteins in the supernatant are quantified by Bio-Rad assay (Bio-Rad). Proteins are separated on 15% SDS–PAGE gels using the Mini-Protean II system (Bio-Rad). The gels are stained with Coomassie brilliant blue R250. For Western blotting, the SDS–PAGE separated proteins are transferred onto a nitrocellulose membrane (Schleicher & Schuell) and the blots are probed with antibody essentially as described by Singh et al. [4]. In our study, we used polyclonal antibodies diluted 1:50 using 0.5% BSA in PBS; monoclonal antibodies and sera of patients allergic to grass pollen at dilutions of 1:5. Binding of polyclonal antibodies is detected using anti-rabbit Ig-conjugated alkaline phosphatase (Promega) while binding of monoclonal antibodies is detected with anti-mouse Ig-conjugated alkaline phosphatase (Promega). The colour reaction is 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 can be detected either using ¹²⁵I-labelled anti-human IgE (Bioclone, Australia) or alkaline phosphatase labelled antibodies coupled with chemiluminescence detection. The overall soluble pollen protein profile between transgenic and wild-type control plants should be compared first. It is expected that the overall protein profile remains unchanged except the targeted protein. In case of ryegrass, no difference was observed in the overall soluble pollen protein profile of transgenic and wild-type control plants. Further, use of Lol p 5-specific monoclonal and polyclonal antibodies showed the presence of Lol p 5 protein in the pollen extract from wild-type plant while

no such immunoreactive protein could be detected in pollen extract of the transgenic plant [8]. Moreover, incubation of the Western blot with anti-Lol p 1-specific monoclonal antibody revealed that expression of Lol p 1 was not affected in the pollen of transgenic plants, indicating specific inhibition of Lol p 5 production.

2.5.2. Protein immunolocalization test

Immunocytochemistry can also be used to confirm results obtained by Western blot study. For this, anthers can be fixed in 4% paraformaldehyde in 50 mM Pipes buffer, pH 8.0, overnight. After dehydration of the samples in graded ethanol and xylene series, samples are embedded in paraffin. Sections of 6–8 µm thickness are attached to poly-L-lysine coated slides, deparaffinized with xylene, and rehydrated through graded ethanol series. Sections are incubated in 5% skim milk in PBS for 1 h, washed twice with PBS, and incubated overnight with monoclonal antibodies diluted 1:5 with 1% BSA in PBS. Binding of monoclonal antibodies can be detected with anti-mouse Ig-conjugated alkaline phosphatase (Promega). The colour reaction is developed using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate as substrates.

2.5.3. Analysis of IgE reactivity

Western blot and/or slot blot analyses have been used to measure the allergen induced production of IgE antibodies in the sera of susceptible individuals [17]. While Western blot analysis will clearly show the reduction of IgE binding to the protein, slot blot will give an indication on the overall IgE binding reduction and binding to a large number of patient sera can be examined. We have used both methods to analyse the transgenic ryegrass pollen extract and found a dramatic reduction in the overall IgE reactivity of pollen extract from transgenic plant in a number of sera from grass pollen allergic patients, indicating that Lol p 5 is a major contributor to the IgE reactivity in grass allergic patients.

2.5.4. Pollen fitness analysis

Effect of allergen gene knockout on pollen fitness can be checked following anther dehiscence using pollen viability and pollination tests. The fluorochromatic reaction is routinely used as a test for pollen viability. For this, fresh pollen grains from wild-type and transformed plants are incubated in a drop of fluorescein diacetate (Sigma) solution (0.2 mg/ml final concentration in 10% sucrose) and viewed with an epifluorescence microscope under UV excitation. Viable pollen grains appear fluorescent green while non-viable or dead pollen are pale yellow. Pollen viability of the transgenic plants should be comparable to that of the control plants. Further, effect of allergen knockout on pollen should also be checked by pollination studies to examine pollen germination, pollen tube growth, and seed set.

3. Concluding remarks

Recent advances in recombinant DNA technology and plant biotechnology have provided an opportunity to address some of the fundamental questions underpinning pollen allergy. Plant genetic engineering has made the introduction of foreign or synthetic genes possible in 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 while *Agrobacterium*-mediated transformation is now more routine for dicot plants and some monocot plants such as rice. These gene delivery systems have been used to explore the possibility of removing allergen from ryegrass [8,14] and rice plants [18,19] via antisense approach to obtain hypoallergenic plants. Rice allergenic protein of 14–16 kDa important for food allergy was targeted to reduce the allergenic protein content of rice grain. No major differences in the amount of major seed proteins were observed between the transgenic and control rice plants while a significant reduction in 16 kDa seed protein was found in the transgenic plants. By using modified promoter, a further reduction in the allergenic protein was observed, indicating that the rice allergen synthesis in mature rice seeds can be repressed by the antisense approach, even though the allergenic proteins are products of a multi-gene family [19]. However, to completely remove this protein from the rice grain needs further improvement, as even a small amount of residual allergens might elicit an allergic reaction in the patients. Therefore, the choice of a strong seed-specific promoter to drive antisense expression is required in such a case. In our laboratory, we targeted one of the major allergens of ryegrass pollen and showed that it was possible to silence all the isoforms of the target allergen by using antisense construct. In our study, the use of *Ory sativa* promoter (pollen-specific promoter) with antisense Lol p 5A cDNA led to the production of hypoallergenic grass that showed no Lol p 5 production, as indicated by lack of Lol p 5-specific antibody binding to pollen extract. For most of the grass allergic patient sera tested, there was a dramatic reduction in IgE binding capacity of transgenic pollen extracts as compared to control extracts, confirming that Lol p 5 is the major immunoreactive allergen of grass pollen [8].

Antisense technology is based on blocking the information flow from DNA via RNA to protein. This technology now has been applied to crop plant to modify a variety of traits such as flower colour, fruit ripening, producing longer lasting flowers [20], and generating male sterility for hybrid seed production [12,21,22]. Despite the extensive application of gene suppression using antisense transgenes, its precise mechanism is not clear. It has been generally assumed that the transcripts of the antisense gene and the target gene form an RNA duplex, which is then

degraded by double strand-specific nucleases, resulting in suppression of the target gene. The other method of post-transcriptional degradation of target mRNA is by co-suppression where suppression of the gene is caused by sense transgene [23]. Recently, gene silencing by sense transgene was found to be correlated with the production of small antisense RNAs (asRNAs) of 21–28 nucleotides, which were preferentially generated from the 3' region of the transgene [24]. Further, efficiency of gene silencing can be improved by hairpin RNA (hpRNA) approach [25]. The use of sense transgene and hairpin RNA still needs to be explored in allergen knockout studies. Knocking out expression of allergen gene will provide not only hypoallergenic plants but also contribute towards delineating the contribution of individual allergens towards overall allergenic potential of allergen source materials.

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