Research Focus

Genetic engineering for removing food allergens from plants

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Genetic engineering has great potential for improving the safety of plant-based foods by eliminating allergenic components. A recent publication by Dodo *et al.* demonstrates that RNA interference can mediate the silencing of a major allergen protein from peanuts. Three additional papers (two by Le *et al.* and one by Lorenz *et al.*) report the removal of allergenic proteins from tomato fruits. This research highlights the potential for using genetically engineered hypoallergenic plants in a new approach to alleviating food allergy symptoms.

Introduction

An allergy is generally defined as an adverse immune-mediated hypersensitivity to normally harmless environmental substances, called allergens. Allergic diseases, such as hay fever, allergic asthma, dermatitis, urticaria (hives), angioedema and anaphylaxis, are serious health problems that affect nearly a quarter of the population worldwide [1–3]. An allergen is defined by its ability to induce a specific immune response in genetically pre-disposed individuals through the production of elevated levels of a specific immunoglobulin E (IgE), a class of antibodies that play a crucial role in allergy because of their ability to bind to specific receptors on mast cells (Figure 1).

The allergens that elicit serious allergic reactions include several proteins of plant origin, which are delivered to the human mucosal immune system through either inhaled pollen or ingested plant-based foods. This encounter initiates a cascade of reactions that leads to clinical symptoms of an allergy (Figure 1). The symptoms of respiratory allergy can be effectively controlled by anti-allergy drugs or by specific immunotherapy [4–6]. There is no well-established therapy, however, for symptoms of food allergy, and avoidance of food allergens is usually the only available approach for dealing with the symptoms of food allergy. Food allergy is more common in children than in adults, and the allergenic foods implicated in more than 90% of such children are egg whites, cow's milk, soybeans, peanuts, tree nuts, certain fruits and fish [7–9]. Although the majority of children outgrow their allergies to milk and eggs, this is not the case with nuts, fish and shellfish, which account for most symptoms of food allergy in adults. An allergy to particular foods can also be triggered by the presence of allergenic structures that are shared by pollen and food. For example, individuals who are allergic to birch pollen often show food allergy to fruits from plants of the Rosaceae family, such as apples, cherries, apricots and pears. Likewise, individuals who are allergic to mugwort (*Artemesia*) pollen show allergic reactions to carrots and celery [10]. Such an association is called a pollen–food syndrome (Box 1).

Towards hypoallergenic peanuts

The peanut (*Arachis hypogea*) allergy is one of the most severe food allergies and is the most common cause of life-threatening anaphylaxis [8]. An allergic reaction to peanut can be triggered by exposure to trace amounts (0.1 – 10 mg), although a more typical threshold amount for triggering a reaction is approximately one peanut kernel. Because of the frequent use of peanuts and peanut products in food preparations, exposure to peanuts is very difficult to avoid and accidental ingestion is quite common. The peanut allergy is typically long term, with only ~20% of young children outgrowing it by school age. Several peanut proteins have been implicated as allergens [11]. The three proteins considered as important peanut allergens belong to legume seed-storage protein families. Ara h 1, a 63.5-kDa glycoprotein, shows homology to vicilin proteins in other legume seeds. Ara h 2 belongs to a conglutin storage protein family, and Ara h 3 is a legumin-type storage protein that has high sequence similarity to glycinin, a storage protein of soybean seeds. In peanut plants, these allergenic proteins are detectable only in the embryonic axes and cotyledons of seeds. Of these three allergenic proteins, the Ara h 2 (17.5-kDa) glycoprotein is the most potent allergen, with nearly 50-fold greater potency than Ara h 1. Overall, allergen proteins comprise ~5% of the total cellular protein of peanut seeds [11,12].

In a recent issue of the *Plant Biotechnology Journal*, Dodo *et al.* [13] reported the application of RNA interference (RNAi) technology for the silencing of Ara h 2 in peanut. This first-reported attempt to use genetic engineering to produce hypoallergenic peanuts resulted in a significant reduction of Ara h 2 and a subsequent decrease in peanut allergenicity. The gene-silenced transgenic peanut plants were produced by *Agrobacterium*-mediated transformation with an intron-spliced Ara h 2 RNAi construct under the control of the CaMV 35S promoter. Hypocotyl explants were used for *Agrobacterium* infection and for subsequent regeneration by tissue culture and selection of transgenic plants. Peanut plants that escaped antibiotic selection served as non-transgenic controls that would identify any changes that might arise as a result of the tissue-culture regeneration step. The transgenic peanut plants were similar to both tissue-culture-regenerated

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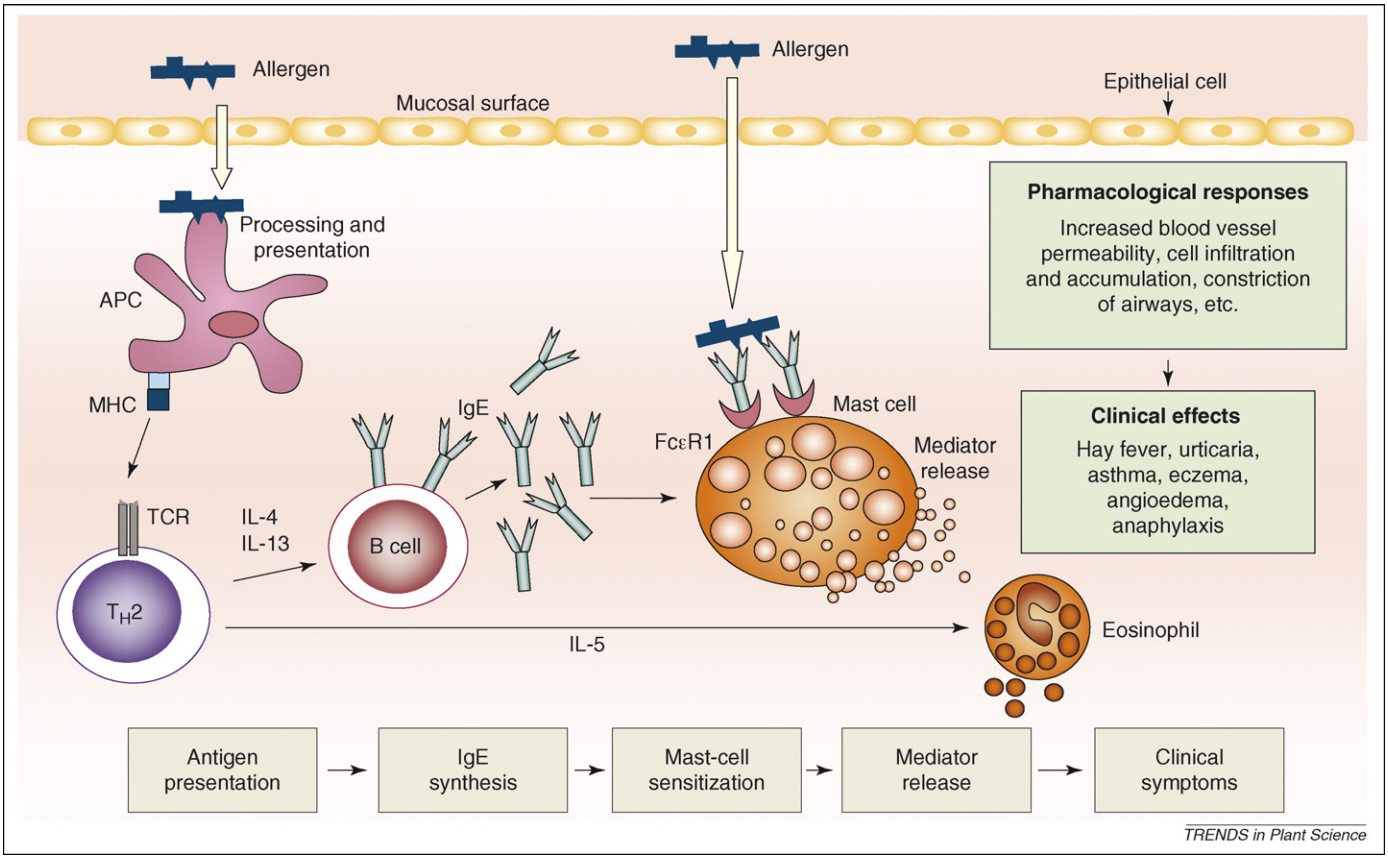

Figure 1. Schematic overview of allergy mechanisms. Allergen molecules enter via mucosal surfaces and are taken up by local antigen-presenting cells (APCs; e.g. dendritic cells). The allergens taken up by APCs are processed and presented to T helper 2 (T_H2) cells. T_H2 cells secrete cytokines (e.g. interleukins such as IL-4 and IL-13), which predominantly stimulate B cells to produce allergen-specific immunoglobulin E (IgE) and also mediate the stimulation of other proinflammatory cells, such as eosinophils. IgE sensitizes mast cells by binding to Fcε receptors (FcεRs). Following subsequent exposure, the allergen cross-links IgE molecules, leading to mast-cell degranulation and the secretion of mediators that are responsible for allergic inflammation. These mediators lead to clinical symptoms of allergy. This process is described in more detail by Bhalla and Singh [4].

non-transgenic and seed-raised wild-type plants in terms of their appearance, growth and reproduction. Crude peanut extract from the transgenic plants showed up to 25% reduction in Ara h 2 content. Immunoassays using individual seed extracts confirmed, however, that about one-quarter of the seeds produced by the transgenic plants were either free of Ara h 2 or contained a significantly reduced amount of the allergen. Frequently, however, only one of the two seeds in a peanut pod showed silencing of Ara h 2 production. This is not unexpected because heterozygosity of the transgene in the first transgenic-plant (T_0) generation results in only 50% of seeds inheriting the silencing construct. Even with the gene-silencing construct segregating in seeds, the pooled seed extract from T_0 plants showed a significant decrease in allergenicity. Dodo *et al.* point out that the ratio of seeds that are free from Ara h 2 will be higher in the T_1 and T_2 progeny of the transgenic T_0, because of an expected increase in the homozygosity of the RNAi transgene. These data show that T_0 plants, which produce only half of their seeds with reduced allergen content, are not suitable as a source of hypoallergenic peanuts. Homozygosity of the transgene occurring in T_1 and subsequent generations will ensure that almost all of the seeds produced will inherit the RNAi construct and thus will be allergen free.

Box 1. Pollen–food syndrome

Individuals who suffer from pollen allergy often show adverse reactions following the ingestion of certain plant-based foods. This pollen–food syndrome is caused by immunoglobulin E (IgE) cross-reactive structures that are shared by pollen and food allergens [10,25,26]. In a majority of allergy sufferers, the symptoms of food allergy triggered by the ingestion of allergens similar to those present in pollen are usually restricted to the oral cavity; they typically include itching and occasional swelling of the lips, mouth, tongue and throat. For this reason, the reaction is also known as oral-allergy syndrome (OAS). This syndrome occurs in a high percentage (50–70%) of individuals with a pollen allergy. For the majority of patients, the symptoms are minor and of short duration; a small percentage of patients, however, show severe symptoms of food allergy, including anaphylaxis. Some studies have demonstrated that immunotherapy for pollen allergy can reduce or eliminate OAS symptoms [27,28]. Cooking and processing of foods often inactivates allergens, reducing the chances of triggering such allergic reactions.

Eliminating allergens from tomatoes

Tomatoes (*Lycopersicon esculentum*) are consumed worldwide. Tomato is a particularly relevant allergen source in European Mediterranean regions, where tomatoes form an important part of the diet. Tomato allergy is usually observed in individuals who are also allergic to birch pollen. This is due to an oral-allergy syndrome that is attributable to the presence of similar allergenic components in both sources (Box 1). The allergenic proteins in tomato fruit include Lyc e 1, Lyc e 2 and Lyc c 3. Lyc e 1

corresponds to profilin, a ubiquitous protein found in all eukaryotic cells. Plant profilins have been described as pan-allergens that are present in several food sources, such as celery, carrots, soybeans, apples, tomatoes and hazelnuts, and in the pollen of diverse genera, such as birch, olive, ragweed and grass genera [14]. Nearly a quarter of individuals who are allergic to tomato show enhanced levels of anti-profilin IgE antibodies, which comprise up to 42% of the total IgE antibodies directed against components of tomato fruit extracts. Le *et al.* [15] used an RNAi strategy to silence the allergens Lyc e 1.01 and Lyc e 1.02, two highly similar isoforms of tomato profilin. The RNAi construct that targeted Lyc e 1.01 and Lyc e 1.02 was under the control of the constitutive 35S promoter. Immunoassays showed a 10-fold reduction in Lyc e 1 accumulation in transgenic fruits. The transgenic plants exhibited severe growth retardation along with yield reduction, however, and some transgenic lines did not bear any fruit. There was a clear correlation between the degree of gene silencing and the severity of the dwarf phenotype. These results highlight the obstacles in removing certain plant allergenic proteins that perform essential cellular housekeeping functions.

A non-specific lipid transfer protein (ns-LTP), which belongs to a multi-gene family of highly conserved cysteine-rich proteins, has been identified as an allergen in many fruits, including tomatoes, plums and apples [16]. In tomato, Lyc c 3, a 9-kDa IgE-reactive polypeptide, has been identified as a ns-LTP.

Le *et al.* [17] also targeted Lyc e 3 for downregulation by the RNAi approach. The level of Lyc c 3 in transgenic fruits was decreased to less than 0.5% of that in wild-type fruits. The fruit extracts from these transgenic tomatoes were further tested for allergenicity using a skin prick test (SPT). SPT involves placing a small amount of allergen extract on a marked area of skin and then making a small prick with a sterile lancet. If the substance is allergenic to the tested individual, a swelling known as a wheal becomes apparent within 15–20 min. Four out of five allergic individuals showed dramatically reduced wheal reaction to Lyc c 3-silenced tomato fruits when compared with wild-type fruit [18]. The suppression of ns-LTP remained stable in T_2 plants and, in contrast to the Lyc e 1-silenced plants, the plants lacking Lyc c 3 were indistinguishable from wild-type plants. The fruits of Lyc c 3-silenced plants were unaltered in size or number per plant, showing that removal of Lyc c 3 protein has no effect on either plant reproduction or fruit yield. These results demonstrate that plants that are genetically modified for low allergen character can successfully match the yield of their unmodified counterparts.

The reports on peanuts and tomatoes discussed here build on previous successes of genetic-engineering-based silencing of Gly m Bd 30K, the major food allergen from soybean [19], Mal d1 from apples [20] and a 16k-Da allergenic protein from rice grains [21].

Perspectives

The research discussed here has provided initial glimpses into how genetic engineering can enhance the safety of plant-based food products through the silencing of allergen-encoding genes. Before these exciting possibilities can be realized, some significant challenges remain. We do not know whether most food-plant allergens are equally amenable to reduction by genetic engineering. In theory, with the availability of robust gene-silencing technologies such as RNAi, we essentially have all the tools required to target virtually any plant allergen whose sequence has been determined and hence to create designer hypoallergenic foods [22–24]. Post-transcriptional gene silencing approaches such as RNAi make it possible to achieve the silencing of multi-gene families by introducing a single RNAi-inducing construct using a genetic-engineering approach [23]. RNAi is particularly suitable for blocking allergen production because most allergen-encoding genes exist as multi-gene families; it is almost impossible to block allergen production by mutation breeding, which knocks out a single gene at a time. Control of tissue-specific suppression is another advantage of RNAi over gene silencing approaches that are based on mutation breeding.

The real bottleneck for creating hypoallergenic foods rests in the essential requirement for some of the major allergenic proteins for normal plant function. This has been amply demonstrated in the case of profilin, which is both an important pan-allergen for humans and a protein that is essential for plant cell function. On the other hand, the successful production of developmentally normal Ara h 2-deficient transgenic peanuts and ns-LTP-deficient transgenic tomatoes shows that many food allergens can be reduced or eliminated without any associated yield or growth penalty. It is very unlikely that plant foods can be engineered to be completely free of allergens, but the removal of a few immunodominant allergens might reduce the severity of allergic reactions, substantially improving the safety of foods. It is hoped that, over time, hypoallergenic food products from genetically engineered plants will reach market shelves. The availability of foods with enhanced safety profiles might help to increase the consumer acceptability of genetic engineering. Genetically engineered hypoallergenic plants might one day be established as a useful adjunct to allergen avoidance as a strategy for the management of food allergy symptoms.

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References

1 Wüthrich, B. (1989) Epidemiology of the allergic diseases: are they really on the increase? *Int. Arch. Allergy Appl. Immunol.* 90 (Suppl 1), 3–10
2 Gerth van Wijk, R. (2002) Allergy: a global problem. *Allergy* 57, 1097–1110
3 Devereux, G. (2006) The increase in the prevalence of allergy and asthma: food for thought. *Nat. Rev. Immunol.* 6, 869–874
4 Bhalla, P.L. and Singh, M.B. (2008) Biotechnology based allergy diagnosis and vaccination. *Trends Biotechnol.* 26, 153–161
5 Nelson, H.S. (2007) Allergen Immunotherapy: where is it now? *J. Allergy Clin. Immunol.* 119, 769–779
6 Valenta, R. and Niederberger, V. (2007) Recombinant allergens for immunotherapy. *J. Allergy Clin. Immunol.* 119, 826–830
7 Breiteneder, H. and Clare Mills, E.N. (2005) Plant food allergens-structural and functional aspects of allergenicity. *Biotechnol. Adv.* 23, 395–399

8 Lee, L.A. and Burks, A.W. (2006) Food allergies: prevalence, molecular characterization, and treatment/prevention strategies. *Annu. Rev. Nutr.* 26, 539–565
9 Sicherer, S.H. and Sampson, H.A. (2007) Peanut allergy: emerging concepts and approaches for an apparent epidemic. *J. Allergy Clin. Immunol.* 120, 491–503
10 Egger, M. *et al.* (2006) Pollen-food syndromes associated with weed pollinosis: an update from the molecular point of view. *Allergy* 61, 461–476
11 Kang, I.H. *et al.* (2007) Temporal and spatial expression of the major allergens in developing and germinating peanut seed. *Plant Physiol.* 144, 836–845
12 de Leon, M.P. *et al.* (2007) The peanut allergy epidemic: allergen molecular characterisation and prospects for specific therapy. *Expert Rev. Mol. Med.* 9, 1–18
13 Dodo, H.W. *et al.* (2007) Alleviating peanut allergy using genetic engineering: the silencing of the immunodominant allergen Ara h 2 leads to its significant reduction and a decrease in peanut allergenicity. *Plant Biotechnol. J.* 6, 135–145
14 Ferreira, F. *et al.* (2004) Allergic cross-reactivity from gene to clinic. *Allergy* 59, 243–267
15 Le, L.Q. *et al.* (2006) Reduced allergenicity of tomato fruits harvested from Lyc e 1-silenced transgenic tomato plants. *J. Allergy Clin. Immunol.* 118, 1176–1183
16 Breiteneder, H. and Radauer, C. (2004) A classification of plant food allergens. *J. Allergy Clin. Immunol.* 113, 821–830
17 Le, L.Q. *et al.* (2006) Design of tomato fruits with reduced allergenicity by dsRNAi-mediated inhibition of ns-LTP (Lyc c e 3) expression. *Plant Biotechnol. J.* 4, 231–242
18 Lorenz, Y. *et al.* (2006) Skin prick tests reveal stable and heritable reduction of allergenic potency of gene-silenced tomato fruits. *J. Allergy Clin. Immunol.* 118, 711–718
19 Herman, E.M. *et al.* (2003) Genetic modification removes an immunodominant allergen from soybean. *Plant Physiol.* 132, 36–43
20 Gilissen, L.J. *et al.* (2005) Silencing the major apple allergen Mal d 1 by using the RNA interference approach. *J. Allergy Clin. Immunol.* 115, 364–369
21 Tada, Y. *et al.* (1996) Reduction of 14-16 kDa allergenic proteins in transgenic rice plants by antisense gene. *FEBS Lett.* 391, 341–345
22 Bhalla, P.L. and Singh, M.B. (2004) Knocking out expression of plant allergen genes. *Methods* 32, 340–345
23 Kusaba, M. (2004) RNA interference in crop plants. *Curr. Opin. Biotechnol.* 15, 139–143
24 Small, I. (2007) RNAi for revealing and engineering plant gene functions. *Curr. Opin. Biotechnol.* 18, 148–153
25 Sicherer, S.H. (2001) Clinical implications of cross-reactive food allergens. *J. Allergy Clin. Immunol.* 108, 881–890
26 Bohle, B. (2007) The impact of pollen-related food allergens on pollen allergy. *Allergy* 62, 3–10
27 Bolhaar, S.T. *et al.* (2004) Efficacy of birch-pollen immunotherapy on cross-reactive food allergy confirmed by skin tests and double-blind food challenges. *Clin. Exp. Allergy* 34, 761–769
28 Alonso, R. *et al.* (2007) An observational study on outgrowing food allergy during non-birch pollen-specific, subcutaneous immunotherapy. *Int. Arch. Allergy Immunol.* 143, 185–189

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Letters

Cisgenesis and intragenesis, sisters in innovative plant breeding

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In a recent issue of *Trends in Plant Science*, Caius Rommens *et al.* [1] provided a valuable overview of intragenic modification in the context of other plant breeding approaches. These authors defined an intragenic plant as a genetically modified plant that only contains genetic elements from within the sexual compatibility group. Intragenesis, an innovative gene technology breeding method, creates new genes with desired traits by isolating functional genetic elements such as promoters, coding parts or terminators of existing genes, rearranging them *in vitro*, and inserting this new 'intragenic' DNA combination back into the plant. According to Rommens *et al.* [1], this approach mimics traditional plant breeding, with the added advantage that the sequence of the inserted DNA is far better known than is DNA used in traditional plant-breeding technologies such as introgression breeding. Therefore, they state that intragenesis is at least as safe as traditional breeding. Consequently, they argue that intragenic crops should be cleared through the regulatory process at the same oversight level as traditionally bred crops. We agree with this main conclusion of the paper, but we question the definition of one of the described methods for crop improvement, cisgenic modification.

In 2006, we introduced the term cisgenesis with the following definition, 'A cisgenic plant is a crop plant that has been genetically modified with one or more genes (containing introns and flanking regions such as native promoter and terminator regions in a sense orientation) isolated from a crossable donor plant' [2]. We repeated this definition in two other publications [3,4]. Although Rommens *et al*. [1] refer to one of our papers, in their Figure 1 they changed the definition of cisgenesis to the 'transfer of traits from related *but sexually incompatible* species' (italics by us). According to the legend of this figure, however, cisgenic plants can also contain traits that 'resemble native traits', implying that these would be from within sexually compatible species. This is confusing. We have been very clear about cisgenesis: cisgenic plants contain only genes from within the same species or from sexually compatible species as used in traditional breeding, and any reference to traits from sexually incompatible species should not be made in conjunction with cisgenic modifications.

There are two differences between intragenesis and cisgenesis. The first concerns regulatory elements: in

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