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Review

Genetic modification and plant food allergens: risks and benefits

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Abstract

Plant genetic engineering has the potential to both introduce new allergenic proteins into foods and remove established allergens. A number of allergenic plant proteins have been characterized, showing that many are related to proteins which have potentially valuable properties for use in nutritional enhancement, food processing and crop protection. It is therefore important to monitor the allergenic potential of proteins used for plant genetic engineering and major biotechnology companies have established systems for this. Current technology allows gene expression to be down-regulated using antisense or co-suppression and future developments may allow targeted gene mutation or gene replacement. However, the application of this technology may be limited at least in the short term by the presence of multiple allergens and their contribution to food processing or other properties. Furthermore, the long-term stability of these systems needs to be established as reversion could have serious consequences.

Keywords: Reviews; Food allergy

Contents

1. Introduction

The major crops that we grow and consume today are the product of centuries, or even millennia, of plant breeding. This started with the subconscious selection of genotypes with high yields and good agronomic properties by early agricultural societies, followed by deliberate selection and the development of scientific plant breeding over the past century. For example, hexaploid bread wheat probably originated

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about 9–10 000 years ago and has since been selected to give a vast number of cultivars adapted to give high yields in environments ranging from Scandinavia to Argentina, including highland areas in the tropics and sub-tropics [1].

Plant breeding is based on the selection of the best plants within a variable population, which is usually generated by crossing two parental lines with different characters. In some cases the aim is to achieve a complete rearrangement of genes from the two parents to give a wide range of novel combinations. In others, the aim may be to introduce (introgress) only one or a small number of genes from one line to the other, for example, to introduce genes for pathogen resistance from exotic or wild relatives. However, in all cases the success of plant breeding is ultimately determined by the existence of variation in the crop species itself or in related species from which it can be transferred by crossing.

It is not surprising, therefore, that the search for useful variation by screening germplasm collections and wild relatives has become an important element of crop improvement. Furthermore, two approaches have also been used to directly create variation.

The first is mutagenesis, which was introduced in the 1960s. A range of physical and chemical mutagens were used to generate novel variation which was then selected and incorporated into breeding programmes. Despite initial optimism, the results were generally disappointing. Most mutations proved to be deleterious or associated with deleterious side effects and were also recessive which would limit their use in polyploid species such as bread wheat. Furthermore, it is almost impossible to determine how many mutant genes are present in the mutagenised populations or in cultivars derived from mutation breeding. Mutation breeding was readily accepted by regulatory bodies and consumers, and some commercially successful cultivars were released. It is unlikely that it would be accepted so readily if it were a new technique now.

The second approach to introduce novel variation is, of course, by genetic modification. Unlike mutation breeding, this has been the subject of intense public debate, particularly in relation to impacts on the environment and on human diet and health. In the present article we will outline the principles of plant genetic modification and discuss the advantages and disadvantages. We will then discuss the potential impact of genetic modification on plant food allergens, either to unintentionally introduce new allergenic proteins or to remove existing allergens.

2. Plant genetic modification

Plant genetic modification is based on the introduction of a DNA sequence into plant cells in a form that allows it to be incorporated into the chromosomes (integration), stably inherited and expressed in a specific fashion. The sequence usually comprises two parts, with a central coding region which is transcribed to make a protein and flanked by regulatory promoter sequences which control the mechanics and specificity (i.e. level, cell and tissue type, timing) of expression.

The advantages over classical plant breeding are clear. Firstly, the coding region can be derived from any source ranging from the recipient plant through related genotypes and species to microbes and animals, by-passing fertility barriers that limit conventional plant breeding. It is also possible to mutate the coding sequence to alter the properties of the encoded pattern (biological activity, nutritional quality, functional properties) or even to synthesise genes encoding patterns designed ab initio. Secondly, only single defined genes are transferred, or small numbers of genes, which can be readily identified and monitored in the progeny. This contrasts with conventional and mutation breeding in which it is not possible to identify or precisely quantify the existence of gene transfer or mutation. Thirdly, it is possible to precisely control the level and pattern of transgene expression, and to decrease the expression of endogenous genes. This is of great potential value for manipulating allergen levels in transgenic plants and will therefore be discussed in some detail.

3. Transgenic plants and food allergens

3.1. Introduction of novel allergens

Many transgenic products will ultimately be consumed by humans, whether they have been introduced to improve specific aspects of food quality (e.g. aspects of nutritional quality, processing quality, flavour enhancements, nutraceuticals, post-har-

vest storage) or to confer improved agronomic performance or resistance to the plant (e.g. resistance to pathogenic fungi in vegetable crops).

The possibility that some of these proteins will prove to be allergenic is of great concern to consumers and regulatory authorities. This concern is not entirely unfounded. Firstly, many known protein allergens have biological activities, which could have applications in transgenic plants. Many proteins with potential antimicrobial or antifungal properties, and hence biotechnological applications, are known allergens. The biotechnology industry will have to be careful in choosing safe proteins to use. Some examples of potentially allergenic proteins are:

(i) 2S albumins are important storage proteins in seeds of many dicotyledonous plants, including legumes, composits, crucifers and many nuts [2,3]. They may also have biological activity, particularly in crucifers, being inhibitors of trypsin in Kohlrabi (*Brassica hapus* var. *rapifera*) and black mustard (*B. nigra*) and of α-chymotrypsin in charlock (*Sinapsis arvensis)* [4–6] and as membrane active antifungal proteins in radish (*Raphanus sativus*) [7,8]. However, they are also major allergens in the related oriental mustard (Bra j **1**) and yellow mustard (*Sinapis alba*) [9,10] and in a range of other species including Brazil nut, cotton seed, castor bean, walnut and chickpea [11–15].

2S albumins which are rich in cysteine or methionine residues also occur in seeds of some species, notably methionine-rich albumins in Brazil nut, sunflower, cotton and amaranthus [16–19] and cysteine-rich components in pea and quinoa [20,21]). The methionine-rich albumins are attractive sources of methionine for expression in transgenic plants to improve sulphur-deficient species such as legumes. However, at least one of these, the Brazil nut albumin, is known to be a major allergen [13]. This is discussed in more detail below.

(ii) Lipid transfer proteins (LTPs) are a family of small (90–93 amino acid residues) proteins present in seeds and other plant organs [22].

Antifungal LTPs have been purified from various tissues of a range of species, including seeds of radish and onion [23–26]. Some LTPs are also allergens in fruit of the family Rosaceae notably peach and apple [27–29].

(iii) The PR (pathogenesis-related) proteins are synthesised by plants in response to microbial pathogens or chemical elicitors (notably salicylic acid). They were initially identified in tobacco leaves responding by pre-sensitising to inoculation with tobacco mosaic virus but have since been identified in many other species (see [30,31]). They are a complex of proteins with various biological activities and may combine to provide a broad spectrum of resistance to pathogens. A number of plant allergens are known to be related to members of the PR protein complex, including endochitinases in chestnut and avocado [32,33] and Bet v **1** (birch pollen)-related allergens in apples, cherries, celery and carrots [34,35].

(iv) Bakers' asthma is associated with the inhalation of wheat flour and is a major respiratory allergy in workers in the flour milling and baking industries. The most important allergenic proteins appear to be low M_r inhibitors of α-amylase and/or trypsin which are present in wheat, rye and barley grain [36–40]. Inhibitors of this group are active against α-amylases and proteinases from various organisms, including digestive α-amylases of some insect pests [41]. Genetically modified (GM) tobacco plants expressing a barley trypsin inhibitor (BTI-CMC) or a wheat α-amylase inhibitor (WMAI-1, Syn 0.28) have been shown to be lethal in leaf disc assays to larvae of two species of the Lepidoptera [41]. However, it should be noted that WMAI-1 also appears to be allergenic, reacting with IgE from pooled sera from patients with Bakers' asthma [36]. It has also been demonstrated that a related α-amylase inhibitor of wheat reacted with IgE from children with dietary rather than respiratory allergy to wheat. The association of this group of inhibitors with respiratory and dietary allergy to wheat and with dietary allergy to rice (see below) suggests that their use in transgenic crops and food would not be acceptable to consumers, regulatory authorities or the industry.

Although the above discussion is far from exhaustive it does highlight the importance of considering allergenic potential when identifying novel proteins to improve the resistance or quality of crop plants. This has also been highlighted by the well-publicised problems experienced with the Brazil nut 2S albumin.

A broad survey of 2S albumin fractions from twelve species from eleven plant families showed that the Brazil nut fraction contained over 17 mol % methionine [2]. Subsequent studies showed that the

fraction contained at least six related proteins with large subunits of M_r about 9000 and small subunits of M_r about 3000 [16,42,43]. Expression of the protein in GM plants resulted in increases in total seed methionine at about 20% in *Arabidopsis* [44], 30% in tobacco [45], 33% in oilseed rape [46] and 300% in narbon bean (*Vicia narbonensis*) [47]. Substantial increases in methionine were also obtained in transgenic soybean ([48], R. Jung, personal communication). However, the commercial use of this gene was halted when it was demonstrated that the Brazil nut albumin is strongly allergenic to humans [13]. Furthermore, IgE fractions from patients allergic to Brazil nut were shown to react with proteins of the expected molecular mass in extracts of GM soybean [49].

Although the experience with the Brazil nut protein highlights the potential problems of introducing allergens in GM plants, it also demonstrates that the plant biotechnology industry is well aware of such potential hazards, the problem being detected by testing procedures already in place. Not surprisingly, the industry has now installed systems to ensure that potential allergenic proteins are identified and the work discontinued before material reaches the food chain, and preferably before transgenic plants are even produced.

Fig. 1 summarises a typical procedure developed by one major plant biotechnology company [50], in which sequence comparisons, stability to proteolytic digestion, immunoassays and skin prick tests are combined to identify allergenic potential. Criteria for assessing the allergenic potential of GM foods are also discussed in detail in a recent paper [51]. The routine use of such criteria and systems should ensure that GM foods have low added allergenicity,

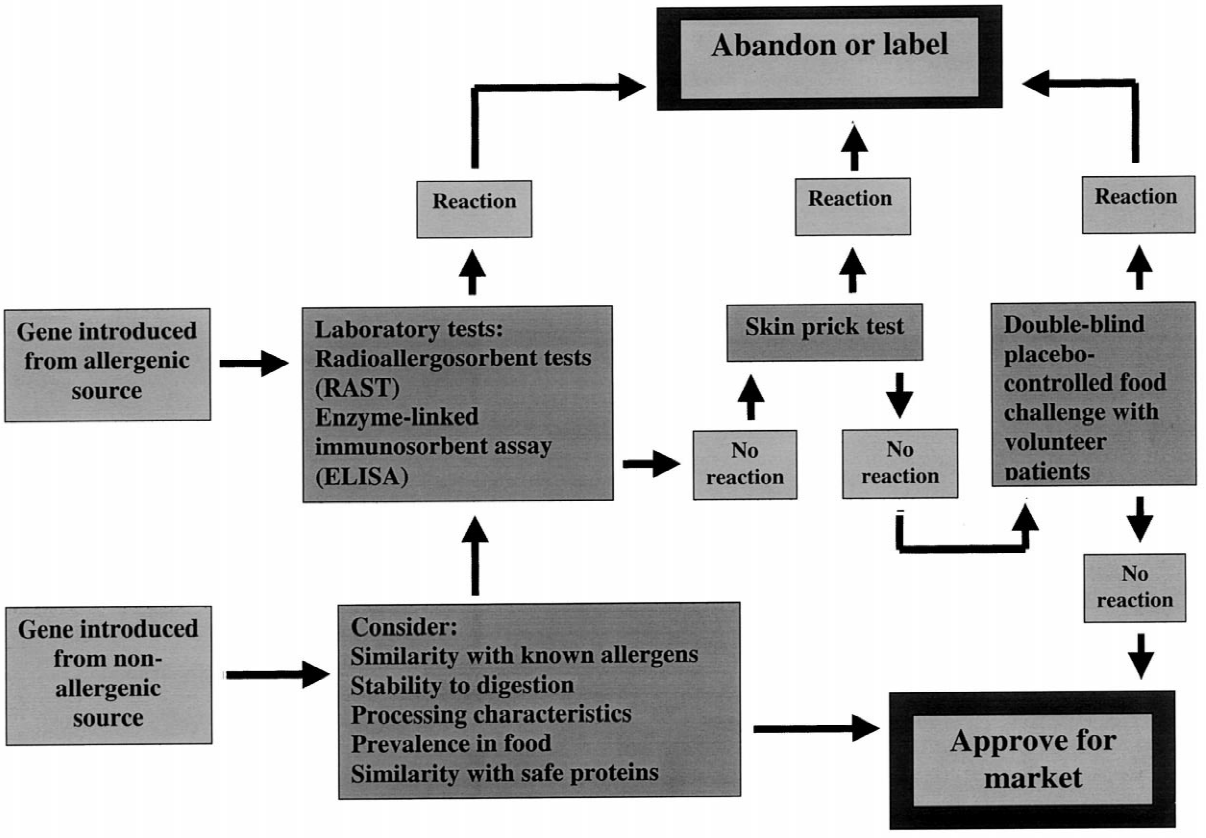

Fig. 1. Flow diagram showing the assessment and testing of possible allergenicity in GM foods. Redrawn from Astwood et al. [50], with the publisher's permission.

certainly when compared with new varieties and types of crops produced by conventional procedures for which no testing for allergenicity is required.

3.2. Removal of allergenicity by silencing of endogeneous genes

Amid the debate about the increased risk of increased allergenicity in GM food, it is often forgotten that GM technology could be used to reduce or remove allergenic proteins in the plant.

There are two methods of silencing endogeneous genes in plants using genetic modification. Both require the identification and cloning of the gene and its reintroduction of all or part of it into the plant. In the first method, a chimaeric gene is produced using part of the gene of interest in reverse orientation downstream of the promoter sequence. The promoter may derive from the same gene, but usually is a more powerful one. When this chimaeric gene is reinserted into the plant it produces RNA of the reverse and complementary sequence of that produced by the endogeneous gene. This so-called antisense RNA [52] interferes with the accumulation of sense (ie. normal) RNA from the target gene, preventing the sense RNA from acting as a template for protein synthesis. Use of a powerful promoter to drive expression of the antisense gene ensures that the antisense RNA is present in much greater quantities than the sense RNA and hence causes efficient suppression of protein synthesis.

In the second method, called co-suppression [53], one or more additional copies of all or part of the sense gene is re-introduced into the plant. In most cases, this leads to increased production of the protein. In others, however, gene silencing occurs with neither the endogenous nor introduced gene being expressed. It is not yet clear why this silencing happens, and its effect may be exerted either at the level of transcription, preventing the production of an RNA molecule from the DNA template, or post-translationally, causing degradation of the RNA molecule before it can act as a template for protein biosynthesis. However, both co-suppression and antisense gene silencing have been used to produce genetically modified (GM) plants in which the trait is stably inherited.

Both co-suppression and antisense have been applied to the extension of fruit shelf-life. Most famously, tomato was one of the first crop biotechnology products to be marketed with lines produced by Grierson and co-workers in collaboration with Zeneca [54] and the ''FlavrSavr'' tomato produced by Calgene in 1994. The ''FlavrSavr'' variety contained an antisense gene to the fruit ripening gene polygalacturonase. This prevented softening and deterioration of the fruit at the later stages of ripening, allowing it to be left on the vine for longer, deepening its colour and developing its flavour. Unfortunately, Calgene introduced the trait into a poor-flavour variety. Consequently, ''FlavrSavr'' never proved popular and has been withdrawn.

At the same time as Calgene was developing ''FlavrSavr'', Zeneca was using co-suppression to silence the same polygalacturonase gene. However, Zeneca chose to introduce the trait into a tomato used for processing and this proved to be much more successful. These tomatoes have a higher solid content than conventional varieties, reducing waste and processing costs in paste production and giving the paste a thicker consistency. This product is on the market in many countries and proved popular in the UK from its introduction in 1996 until 1999 when most retailers withdrew it in response to anti-GM hostility.

The ability to down regulate gene expression using antisense and co-suppression techniques led to the proposal that genetic engineering could be used to remove allergens and various toxins from plants. Both techniques can give almost complete gene inactivation. However, to the best of our knowledge this has only been attempted with one allergen, which is present in rice grains.

The major dietary allergens in rice grain have M_r ranging from about 14–16 000 and are inhibitors of α-amylase from human saliva [55]. Sequence comparisons show a relationship to the α-amylase/trypsin inhibitors of cereals discussed above [56]. More than 10 different but homologous cDNA clones were identified, indicating the presence of a multigene family [56–59]. Nevertheless, Tada et al. [60] showed that antisense expression of a single sequence resulted in substantial decreases in the total allergen content of transgenic rice seed, from about 300 mg/seed (20 mg) to 60–70 mg/seed. This was

associated with a decrease in sense transcripts to about 20% of the wild type levels. The levels of reduction were also stable for up to three generations. Similarly, antisense technology has been used to down-regulate expression of the Lol p**5** allergen in the pollen of ryegrass (*Lolium perenne*) [61]. The protein was undetectable by immunoblotting with allergen-specific antibodies and the pollen showed reduced allergenicity.

These studies, and the use of antisense and co-suppression of gene expression in other systems, demonstrate, the feasibility of reducing or eliminating allergens by genetic engineering. However, the ultimate success of this approach could be limited by several factors.

(i) Incomplete suppression of gene expression could lead to reduction, as in rice, rather than complete elimination. This could result from the presence of multigene families comprising members with varying degrees of identity with the sequences used for down-regulation or from inherent ''leakiness'' of the suppression system used.

(ii) The long term stability of the gene suppression systems is still not established. Any reversion could have serious clinical consequences. The risk of incomplete gene silencing or that the silencing could break down might make it unwise for someone with a severe food allergy to risk eating a GM food from which the allergenic protein had been removed in this way. However, the adoption of GM varieties in which the allergenic protein gene expression has been suppressed would be expected to reduce significantly the numbers of cases of severe allergic reaction occurring due to accidental exposure.

(iii) Many plant foods contain multiple allergens with no sequence homology. For example, immunoblotting with IgE antibodies from patients with atopic allergy to soybean identified at least sixteen reactive proteins ranging in M_r from 14 to 70 000 [62] and subsequent studies have shown that they include an M_r 30 000 thiol proteinase [63], a Kunitz soybean trypsin inhibitor [64] and the α-subunit of the β-congycinin 7S globulin storage protein [65]. These proteins have no sequence homology and multiple transgenes would be required to effect their removal.

(iv) A further limitation will arise when the allergens, or closely related proteins, contribute to the functional (processing) properties of the crop. For example, wheat gluten proteins have been implicated in respiratory and dietary allergy to wheat, including exercise-induced anaphylaxis [66–68]. Furthermore, the putative IgE-binding epitope identified by Tanabe et al. [69] as involved in dietary allergy (Gln.Gln.Gln.Pro.Pro) forms part of a repetitive sequence with related proline and glutamine-rich repeats present in many other gluten proteins.

Wheat gluten proteins are responsible for the visco-elastic properties that allow dough to be processed into bread, pasta, noodles and a range of other foods. Removal of a substantial number of gluten proteins to eliminate allergenicity would therefore severely disrupt the processing properties. A similar situation could occur in soybean where the 7S globulins (β-conglycinin) account for up to half of the total storage proteins (the remainder being 11S glycinins) [70] and undoubtedly contribute to the functional properties [71] that allow the wide use of soybean proteins in food processing.

3.3. Removal of allergenic epitopes

An attractive alternative to removing whole allergenic proteins is to remove only the epitopes responsible for the allergenicity. The allergenic sequences in the major Ara h **1** and Ara h **2** allergens of peanuts have been identified by binding to synthetic overlapping peptides [72,73], a method which is suitable for the identification of sequence epitopes but not conformational epitopes. The latter would require a protein engineering approach with the expression of mutant proteins in heterologous systems. Based on this information it should be possible to design homologous, non-allergenic proteins with similar structures, stability and biological and functional properties. This approach has been successfully used to remove conformational epitopes from the major allergens of apple [74] and cherry [75].

The next stage will be to use genes encoding such proteins to replace the endogenous allergen genes. It is still not possible to carry out targetted gene replacement in plants although this has been achieved in some animal and microbial systems. However, a promising new technology offers the possibility of making targetted gene mutations using

hybrid molecules (chimeras) of DNA and RNA [76–78]. This system called chimeroplasty, could be used to switch off gene expression by mutation of regulatory sequences or to replace individual amino acids involved in the active sites of enzymes or in allergenic epitopes.

Although this technology is still in its infancy it could have a major impact on plant genetic engineering in the future.

4. Conclusions

Plant genetic engineering is a powerful tool, which is already having a major impact on crop production with approximately 70 million hectares of GM crops being grown commercially in 1999. The use of these crops has already led to less use of chemical insecticides and weedkillers, adoption of more environmentally sustainable agricultural practices (such as conservation tillage), higher yields, and foods which are more nutritious, safer and have longer shelf lives [79–81]. There is no doubt that product quality will become an increasingly important target in the future, with an emphasis on enrichment with compounds that contribute to a healthy diet (e.g. polyunsaturated long chain fatty acids, vitamins, soluble fibre). The industry is well aware that this work could result in the introduction of allergens and has established systems and tests to ensure that this does not occur. Furthermore, it may also be possible to use genetic engineering to reduce the levels of allergens or allergenic epitopes present in foods, and perhaps in some cases to eliminate them completely.

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