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Removing allergens and reducing toxins from food crops

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Genetic engineering is being applied to eliminate or substantially reduce plant-derived substances that can be harmful to human health in otherwise nutritious foods. Post-transcriptional gene silencing, most recently via RNA interference, has been used in efforts to remove allergens from rice, soybean, apple, tomato, and peanut. RNA silencing has the potential to simultaneously alter expression of all members of a multi-gene family in a tissue-specific manner with little collateral change in the plant. Similarly, RNA silencing, as well as the overexpression of a beneficial gene, have been used in transgenic cassava to reduce toxic cyanogens. These studies are illustrative of the future application of biotechnology toward production of foods that are of direct value to the consumer.

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Introduction

The current commercially available transgenic crops have been developed with producers in mind. With traits such as herbicide tolerance, and insect and virus resistance, these crops help increase production, reduce input costs, and improve environmental management. A significant portion of the next generation of transgenics will be non-staple and staple food crops that have health, nutritional, and safety benefits for the consumer in both developed and developing countries [1•]. This review will focus on the latest research efforts to use biotechnology to remove plant-derived allergens and reduce toxins from foods through downregulating deleterious genes, or overexpressing preferable genes (Figure 1). It is important to note here the fundamental requirements that distinguish these efforts. For some people allergens can be dangerous at extremely low doses and therefore the ultimate goal is to

completely eliminate the presence of the wild-type allergen from the food before it may be considered safe for all consumers. However, foods containing toxins may be rendered relatively harmless when toxins are decreased to levels that are recognized as safe for consumption.

Food allergy

Food allergy is defined as a hypersensitive response to foods that is mediated mainly as a result of an immunoglobulin E (IgE) mechanism. A food allergy is acquired usually within the first two years of life [2] and sensitization occurs directly by means of the gastrointestinal tract, but sometimes an inhalant allergen like pollen will result in the production of IgE that can cross-react with proteins found in fruits and vegetables [3]. Approximately 5–8% of children less than three years of age and 1–2% of adults have a food allergy [4], and like other allergies, food allergies appear to be increasing [5,6]. The reason for the increase is unclear but may be due to a number of causes ranging from children living in cleaner environments (the hygiene hypothesis) to better diagnoses.

There are eight foods that account for 90% of all food-allergic reactions, namely peanut, tree nuts (walnut, Brazil nut, cashew, etc.), soybean, wheat, milk, egg, fish and shellfish [7]. Currently, the only options available for patients with food allergies are to avoid ingesting the food allergen, seek medical treatment, or eat foods with no or low allergen content.

Plant food allergens

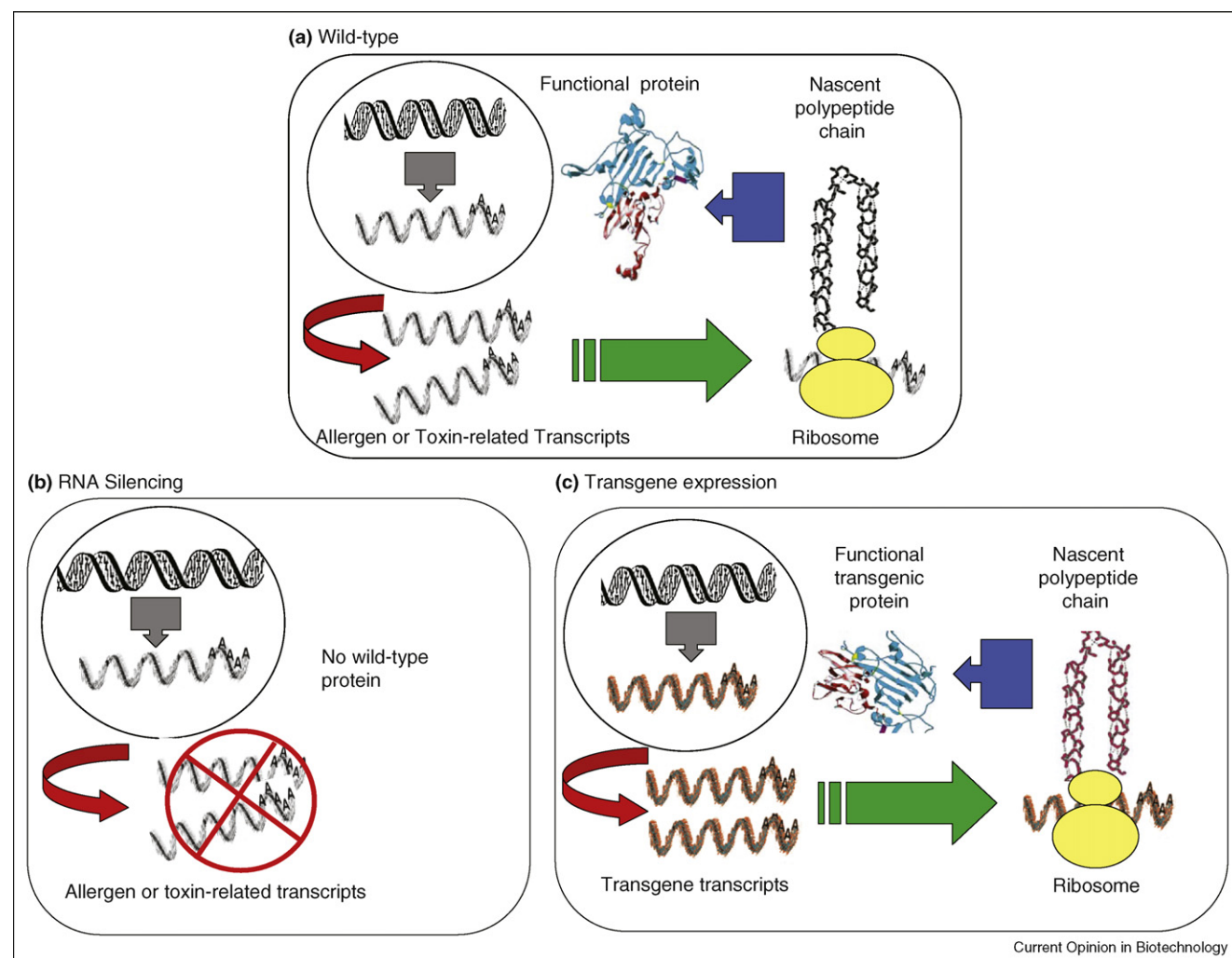
Plant-derived allergens can be classified depending on their structure, function, and biochemical and immunological properties. In cereals and legumes, most of the allergens are either pathogenesis-related (PR) proteins, or seed storage proteins [8]. Additionally, many allergenic proteins are members of three large superfamilies, namely the prolamin, cupin, and cysteine protease superfamilies. For a review of the structural, biological, and evolutionary relationships of plant allergenic proteins, see [9].

Post-translational gene silencing to eliminate allergens from food crops

The strategy used to reduce plant food allergens has relied on post-transcriptional gene silencing (PTGS) that results in sequence-specific mRNA degradation thereby preventing translation (Figure 1). The first attempts to eliminate plant allergens were in rice [10] and soybean [11] where seed-specific promoters drove antisense or sense constructs, respectively. Although the rice transgenics and their progeny had substantially reduced levels of the targeted 14–16 kDa allergens, none of them were

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Figure 1



Strategies to eliminate allergens or reduce toxins through genetic engineering. **(a)** Normal flow of genetic information from DNA to RNA to protein. **(b)** Post-transcriptional gene silencing of unwanted allergen or toxin-related protein products. **(c)** Expression of a transgene to produce desired transgenic protein. Gray arrow indicates transcription, red arrow indicates RNA transport out of the nucleus and into the cytoplasm, green arrow indicates translation, and the blue arrow indicates protein folding.

allergen-free, probably due to insufficient sequence homology between the antisense constructs and members of the multi-gene family that encoded these allergens [12]. By contrast, complete knockdown of the soybean allergen, Gly m Bd 30K also known as P34, was obtained in one co-suppression line. Significantly, P34 elimination was maintained over three generations and there were no observable plant morphological or reproductive differences between transgenic plants and wild type.

Since P34 is not encoded by a large multi-gene family with divergent sequences, success was probably achieved owing to near complete sequence identity between the transgene and the coding regions of the endogenous gene. However, as described for rice above, and the examples to

follow, many allergens are encoded by members of multi-gene families, making silencing of all members technically challenging. Therefore, the most recent and preferred application of PTGS for allergen reduction has been RNA interference (RNAi) because it has improved control when compared to antisense technology [13] and has been used to successfully shut-off expression of all genes in a family [14,15]. Additionally, all the studies to be described below have utilized a version of the CaMV 35S promoter for constitutive expression of the RNAi constructs.

An RNAi approach was first used to downregulate the major apple allergen Mal d 1, a member of a family of at least 18 genes (loci) that displays IgE cross-reactivity to

the birch pollen allergen Bet v 1, a PR-protein. Five independent lines at the plantlet stage showed up to a 10-fold reduction in Mal d 1 leaf expression with no phenotypic abnormalities observable when compared with wild type [16]. However, it is crucial that future studies evaluate the transgenics' reproductive capacities, levels of Mal d 1 in the fruit, and affect of Mal d 1 down-regulation on disease resistance before it is known whether this approach is viable.

More recently, two tomato allergens, Lyc e 1 (profilin) and Lyc e 3 (non-specific LTP), each having two isoforms, were successfully inhibited through expression of RNAi vectors [17,18]. Three independent transgenic lines had Lyc e 1-reduced tomato fruits at levels that were 10-fold lower than wild type. However, their phenotypes were dramatically different from wild type, showing extreme dwarfing under greenhouse conditions, as well as delayed flowering, and severe yield reductions. These deleterious phenotypes are illustrative of the physiological disruption that resulted from the downregulation of profilin that functions in regulating the organization of the cytoskeleton. Therefore, along with this approach, expression of a non-allergenic profilin must be added to tomato so that proper cellular function is maintained. By contrast, no detectable levels of Lyc e 3 were found in transgenics that were phenotypically normal, producing seed that allowed for testing in subsequent generations. One homozygous line showed no detectable Lyc e 3 on immunoblots, and skin prick tests revealed highly reduced allergenicity. Importantly, these plants did not show upregulation of other tomato allergens [19]. These results are promising for the production of hypoallergenic tomato.

The most recent use of RNAi to reduce allergen expression has been in peanut, undoubtedly the most dangerous of food allergens because it is responsible for the majority of food-induced anaphylactic shock [20]. The focus for allergy reduction has been on an allergen that is recognized by the serum IgE from more than 90% of peanut-sensitive patients [21], and one of the best characterized, Ara h 2 [22–26]. It is a seed storage protein in the prolamin family (conglutin-7) that shows weak trypsin inhibitor activity [27], and is encoded by two homeologous genes, *ara h 2.01* and *ara h 2.02* [28].

The first report examined transgenics containing an *ara h 2.02* RNAi construct generated from a genomic clone [29]. Transgenics had variable yields, producing anywhere from 2 to 32 pods, compared with the wild type average of 25 pods. Seeds from two independent primary transgenic lines had up to a 25% reduction of Ara h 2 and a decrease in IgE binding capacity as compared with wild type.

The second report, generated transgenics containing an RNAi vector consisting of portions of *ara h 2.01* [30]. This RNAi approach was able to decrease expression of Ara h

2, as well as another severe allergen, Ara h 6. Also a conglutin, Ara h 6 has 63% sequence homology with Ara h 2 and is encoded by three genes [28]. The three independent transgenic lines examined all showed reduced IgE binding, and the two lines that were tested had seed yields and germination rates that were not different from wild type. One transgenic line showed complete silencing of both Ara h 2 and Ara h 6. Importantly, decreasing the expression of Ara h 2, which as mentioned earlier is a weak trypsin inhibitor, did not make peanut more susceptible to *Aspergillus flavus*, the fungus that produces aflatoxin and can be a major problem in peanut production. However, a recent study using a quantitative proteomics approach (spectral counting analysis) showed that there were protein changes detectable in these lines [31^{••}]. One transgenic line showed increases in Ara h 10 (oleosin 1), agglutinin, and Ahy-3 (arachin), as well as a decrease in Ara h 1 (conarachin) that was also displayed by a second line. The third transgenic line showed an increase in 13-lipoxygenase.

These results point to the importance of being able to accurately detect changes in plant protein profiles. It is imperative that any transgenics produced are further characterized to determine the stability and cause of the variable and concomitant protein changes detected among the lines and their ultimate effect not only on allergenicity but also on agronomic traits as well.

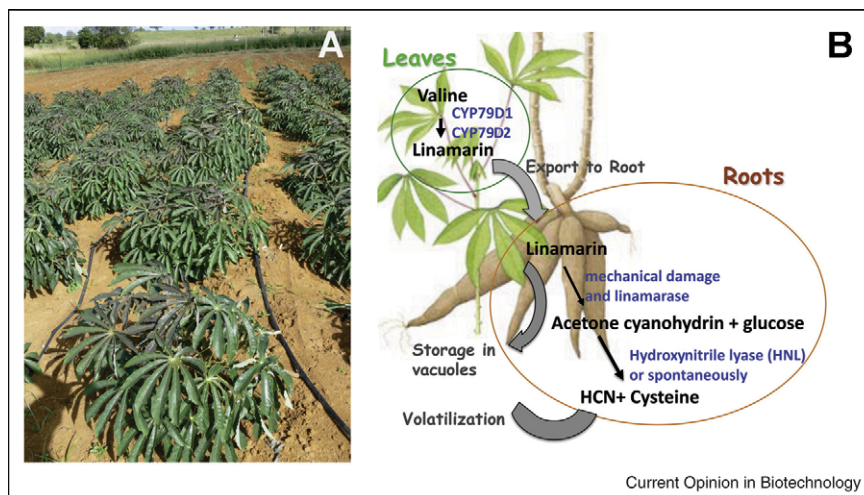
Toxins in food crops: cassava as a test case

Cassava is the main staple food for more than 500 million people in the tropical and subtropical regions of the world (Figure 2A) [32]. However, cassava contains sufficient plant toxins to cause acute human toxicity or permanent disability if the food is not properly processed before consumption [33]. The cyanogenic glycosides linamarin and lotaustralin account for this toxicity and are present in all plant parts except the seed [34].

Cassava roots are the primary plant organ consumed and are a major source of calories (starch), particularly in subSaharan Africa. The cassava root cortex can contain between 15 and 1500 mg cyanogen equivalents/kgfw [35]. The outer peel (2–5 mm) of the root has a high cyanogen content (average of 577 mg/kgfw) that protects the root from herbivory [36].

Chronic exposure to cyanogens can cause hyperthyroidism and neurological disorders including tropical ataxic neuropathy [37,38]. Acute exposure to high cyanogen levels may result in permanent paralysis of the legs (konzo). Cyanide poisoning following consumption of poorly processed cassava is exacerbated by insufficient amounts of sulfur-containing amino acids (cysteine) in the diet [39] because reduced sulfur or sulfur-containing amino acids are substrates for the enzymes rhodanese and β -cyanoalanine synthase that detoxify or assimilate cyanide into amino

Figure 2



Cassava and cyanogenesis. (A) Cassava in the field. (B) Linamarin synthesis and metabolism. Downregulation of CYP79D1 and CYP79D2 or expression of hydroxynitrile lyase in the roots leads to reduced cyanogens levels.

acids, respectively. In the absence of sufficient cysteine, cyanide is converted to cyanate. The brain is particularly sensitive to cyanate poisoning.

Cyanogenic glycosides and cyanogenesis in cassava

The physiology and biochemistry of cyanogenesis in cassava has been elucidated (Figure 2B) [40]. Briefly, two cytochrome P450s (CYP79D1 and CYP79D2) catalyze the first-dedicated step in cyanogen synthesis from either valine (linamarin), the preferred substrate, or isoleucine (lotaustralin). In linamarin synthesis, the end product of the cytochrome P450 catalyzed reactions is the hydroxynitrile intermediate, acetone cyanohydrin [40]. Acetone cyanohydrin is subsequently glycosylated to produce linamarin that is stored in vacuoles.

Cyanogenesis in cassava is initiated by tissue disruption (Figure 2B). Rupture of the plant cell vacuole releases linamarin that is subsequently hydrolyzed by linamarase [41,42]. The products of linamarin hydrolysis are acetone cyanohydrin and glucose. Importantly, acetone cyanohydrin will spontaneously decompose to produce cyanide at pH >5.0 or temperatures >35 °C [43]. Acetone cyanohydrin can also be enzymatically broken down by hydroxynitrile lyase (HNL). Cassava roots, however, have much lower linamarase activities than leaves and very little or no root HNL activity, presumably accounting for the accumulation of potentially toxic levels of acetone cyanohydrin in poorly processed cassava roots [43,44].

Engineering reduced cyanide levels in cassava foods

Transgenic strategies have been used to engineer cyanogen levels in cassava plants and processed foods using two

different approaches: (1) acceleration of cyanogen turnover and cyanide volatilization during processing by elevation of root HNL activity [45,46], and (2) reduction (99%) in root cyanogen levels by suppression of CYP79D1 and CYP79D2 expression in leaves [47,48].

For the first approach, a cassava cDNA encoding HNL, driven by a constitutive double 35S promoter, was expressed in cassava [46]. The HNL activity of transgenic roots was 13-fold greater than that of wild-type plants. Analyses of acetone cyanohydrin levels in processed transgenic roots indicated that cyanogenesis was completed in as short as three hours versus the normal 1–3 days. Additionally, plants over-expressing HNL had normal root linamarin levels and linamarase activity indicating that beneficial herbivore deterrence should not be compromised in these transgenic plants.

In the second approach, expression of CYP79D1 and CYP79D2 genes was selectively inhibited either in leaves or roots of transgenics through the use of cab1 or patatin promoters, respectively, driving antisense constructs [47,48]. Reduced CYP79D1 and CYP79D2 expression in leaves resulted in decreased leaf linamarin content ranging from 6% to 40% of wild-type levels. Surprisingly, roots of these transgenic plants had a 99% reduction in linamarin content even though root CYP79D1 and CYP79D2 transcript levels were unaffected. By contrast, transgenic plants whose expression of the CYP79D1 and CYP79D2 genes was inhibited in the roots had wild-type root linamarin levels [48]. These results indicate that the linamarin present in roots was synthesized in the leaves and transported to the roots.

Surprisingly, when cab1-CYP79D1/D2 antisense plants were transferred from growth medium containing nitrate and ammonia to potting soil, many of the plants died and those that survived produced severely stunted roots with reduced free amino acids levels (T. Zidenga, personal communication). When the same transgenics were grown on medium in which the ammonia was replaced with nitrate, the plants failed to produce strong roots [48]. By contrast, patatin-CYP79D1/D2 antisense plants grew normally when grown in modified growth medium lacking ammonia or in potting soil.

The above results lead to a model where linamarin is transported to the roots and is either stored in the vacuole, or the linamarin nitrile group is assimilated into amino acids. At present, the mechanism by which linamarin is partitioned between root vacuoles and amino acid metabolism remains unknown. Future transgenic studies are aimed at reducing cyanogen levels in the cassava root cortex by redirecting linamarin from storage to amino acid and protein synthesis [49^{••}].

Conclusions

It has been clearly demonstrated that genetic engineering can produce food crops that are hypoallergenic or are rendered harmless owing to detoxification. The advantage of this approach is that it can make foods that were once unsafe available as good sources of nutrition and calories while also paving the way for better consumer understanding and acceptance of genetically modified foods. Additionally, with the current swift advancement of plant genomics, proteomics and metabolomics there will be both a store of novel genes to evaluate and improved genetic engineering strategies for continued food crop improvement.

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