New Biotechnology • Volume 27, Number 5 • November 2010

Review

Inactivation of allergens and toxins

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Plants are replete with thousands of proteins and small molecules, many of which are species-specific, poisonous or dangerous. Over time humans have learned to avoid dangerous plants or inactivate many toxic components in food plants, but there is still room for ameliorating food crops (and plants in general) in terms of their allergens and toxins content, especially in their edible parts. Inactivation at the genetic rather than physical or chemical level has many advantages and classical genetic approaches have resulted in significant reduction of toxin content. The capacity, offered by genetic engineering, of turning off (inactivating) specific genes has opened up the possibility of altering the plant content in a far more precise manner than previously available. Different levels of intervention (genes coding for toxins/allergens or for enzymes, transporters or regulators involved in their metabolism) are possible and there are several tools for inactivating genes, both direct (using chemical and physical mutagens, insertion of transposons and other genetic elements) and indirect (antisense RNA, RNA interference, microRNA, eventually leading to gene silencing). Each level/strategy has specific advantages and disadvantages (speed, costs, selectivity, stability, reversibility, frequency of desired genotype and regulatory regime). Paradigmatic examples from classical and transgenic approaches are discussed to emphasize the need to revise the present regulatory process. Reducing the content of natural toxins is a trade-off process: the lesser the content of natural toxins, the higher the susceptibility of a plant to pests and therefore the stronger the need to protect plants. As a consequence, more specific pesticides like Bt are needed to substitute for general pesticides.

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The dangers of nature and food

Toxic substances abound in living beings, plants included. Humans use plants (or products made from them) as a source of food, fiber, fuel, tools or drugs and therefore are constantly exposed to toxins and allergens of plant origin. The plant world can thus be viewed as a 'minefield'. A short walk both in cultivated fields and wild areas in many places in Italy, for which I have some experience, and more generally everywhere in the world, allows one to meet plants which have caused poisoning or even fatalities in humans or animals (see some examples in Table 1). For instance, castor bean (*Ricinus communis*) is common in southern Italy and produces ricin, a poison among the most potent known to man. The lethal oral dose in humans is approximately eight beans; even half a bean was enough to cause death [1]. Other highly toxic encounters in Mediterranean countries are oleander (*Nerium oleander*) and most plants in the Ranunculaceae, Scrofulariaceae and Solanaceae (nightshade) families. For references on common toxic plants in Italy [2]; for North America [3]; for a general treatise [4]; for a recent compilation [5]; for a website [6]. The common names for several members of the Solanaceae are quite explicit in their message: angel's trumpet or devil's weed (*Datura stramonium*), the apple of Sodom (*Solanum sodomeum*), bittersweet nightshade or poisonberry (*Solanum dulcamara*), black nightshade or devil's little tomatoes (*Solanum nigrum*) and deadly nightshade (*Atropa belladonna*). Some of the fruits or flowers are quite attractive in appearance and therefore become more dangerous for people raised in urban settings and who are unaware of the risks, children in particular, for example [7–9]. One author suggests that 'about 2% of plant species can severely poison people who happen to ingest them', with alkaloids being the major cause [10]. Some toxins are quite widespread among plants, like cyanogenic glucosides, which are reported in at least 2500 different species [11]. Many toxic plants are weedy, wild plants which need not human's intervention to survive.

Likewise, many crops have dangerous substances (Table 1), some in edible part and some in organs not used as food. For instance potato tubers or ripe tomato fruits usually have low levels of glycoalkaloids, but leaves, diseased tubers and fruits (a small berry) of potato or leaves and immature fruits of tomato are more

TABLE 1

Examples of wild and crop plants with toxic substances and their effects

Common name	Latin name	Toxic substance[a]	Effect[b]	Dose[c]|content[d]
Giant fennel	*Ferula communis*	Prenylated coumarins	Lethal	
Jimson weed	*Datura stramonium*	Atropine (and other alkaloids)	Lethal	100 seeds|0.1 mg/seed
Tobacco	*Nicotiana tabacum*	Nicotine	Lethal	1 mg/kg|3–6%
Apple of sodom	*Solanum sodomeum*	Solasonine, solanidine	Toxic	30 mg/kg|0.3 mg/g
Castor bean	*Ricinus communis*	Ricin/ricinoleic acid	Lethal	Half a seed
Pepper	*Capsicum* spp.	Capsaicin	Lethal	|0–2 mg/g
Tomato	*Solanum lycopersicum*	Tomatine	Toxic	
Potato	*Solanum tuberosum*	Solanine	Lethal	|3–6 mg/kg
Cassava (Yucca)	*Manihot esculenta*	Cyanogenic glucosides	Paralysis–stunting	|15–400 mg HCN/kg
Soybean	*Glycine max*	Protease/amylase inhibitors	Toxic	
Almond	*Prunus dulcis*	Cyanogenic glucosides	Lethal	20 seeds|29 mg/kg
Brussel sprouts	*Brassica oleracea*	Glucosinolates	Lethal-goiter	|1–2 mg/g
Cotton	*Gossypium hirsutum*	Gossypol	Cardio/hepatotoxic	0.3–3 mg/kg|10 mg/g
Vetch	*Lathyrus sativus*	Oxalyl-diaminopropionic acid	Neurotoxin/paresis	|0.3–3.2%
Lima bean	*Phaseolus lunatus*	Cyanogenic glucosides	Lethal	|2–3 mg HCN/kg
Poppy	*Papaver somniferum*	Morphine	Lethal	100 mg|10 mg/g
Bamboo	Several species	Cyanogenic glucosides	Toxic	|1–8 g HCN/kg

[a] Only the main toxic components are listed. Most of the plants in the table are mentioned in [14], but see also [1–13]. For the giant fennel toxicity, see [147], for Jimson weed [148], for pepper [149]. Many other toxic substances can often contaminate plants or food, but are not considered in this list.
[b] The effect is obviously dependent on the dose. When a substance or a plant is defined as lethal in the table, there are reports in the literature of fatal cases for humans or grazing animals (e.g. [147]). For other examples of toxicity in animals, see http://www.ansci.cornell.edu/plants/ (plant poisonous to livestock and other animals).
[c] The lethal dose reported is usually the minimum observed and may not be always lethal. The dose is expressed as the amount of plant (e.g. number of seeds) or as the amount per kg of body weight able to cause the effect.
[d] The content refers to the main active principle causing the toxic effect and it is expressed per plant part or weight.

toxic. Cases of severe poisoning, sometimes fatal, are reported in the literature for several of the edible plants listed in Table 1 (e.g. [12,13]; for a compilation [14]).

Most of the toxic substances in plants are known to men since time immemorial and were identified by modern science according to their chemical characteristics (alkaloids, glucosides, aminoacids, proteins, lipids, etc.). Their toxicology and mode of action have been described (for a comprehensive compilation see [4,5]). Although some (e.g. digitoxin) have a long history of use as pharmaceuticals and are still used today, most have been abandoned because of the short interval between therapeutic and toxic dose.

The ability of humans to survive and thrive depends on their capacity to recognize and avoid or inactivate most of these toxic compounds. Especially for plants used as food, this is achieved by a combination of proper storage and processing (e.g. maceration and fermentation) among which cooking is the most prominent for its major effect of heat inactivation. Knowledge in this context can be likened to a precise map handed down from generation to generation through culture and education, warning of the dangers of the minefield, while technology becomes similar to a metal detector to reveal, avoid or inactivate toxic substances. Knowledge and technology buffer us from the toxic effect of nature and allow a far wider spectrum of plants or plant parts to be used to our benefit than those 'naturally' available. The widespread belief in the superior goodness of nature and the evil of manipulations by human is causing harm and death (e.g. [15–17]).

Cultivated plants seem to have fewer toxins than their wild relatives, as the result of selection for better-tasting plants [18]. For example, the wild potato *Solanum acaule* has three times more glycoalkaloids than cultivated potato and is more toxic [19–21]. Cultivated Brassicaceae (cabbage, broccoli and cauliflower), when compared to wild species, have less glucosinolates, a major class of secondary metabolites [22,23] and this affects the survival of herbivore insects and their parasitoids [24,23]. The wild bean *Phaseolus lunatus* contains about three times cyanogenic glucosides when compared to the cultivated bean [25]. Wild and cultivated beans have different levels of antinutritional factors [26]. Cyanogenic glucosides in white clover (a forage crop) act as a deterrent against herbivores [27], but cultivars devoid of cyanogenic glucosides have been bred to obtain better palatability for grazing animals. Similar reductions have been reported for other crops [28]. The issue is complicated by the effect of the environment and pest pressure [29]. Whether this is a general rule remains to be demonstrated, but it seems an acceptable hypothesis and might contribute to the general susceptibility of crop plants to pests. Nevertheless, the point remains that humans can clearly tolerate at least low levels of toxins in their diet without ill effects. In fact, the ability to safely consume a low level of toxins has been a key element in the survival of all omnivores. The most appealing explanation for the observed crop–wild differences is that humans selected loss of function mutations leading to a reduced toxin content during the domestication process on the basis of feeding 'tests'. Most presumably it was a long process of trial and error (or trial and death). At least in one case it seems that not only the overall quantity of toxic glucosinolates is reduced, but also that inducibility by wounding is lost in the cultivated species [23].

Thus many crops still produce the same kind of toxins as their wild relatives, albeit in lower quantity, at least in edible parts. This means that the biosynthetic capacity is there. Indeed sometimes crops are fatal for humans [12,13,30]. Moreover, toxin content might increase spontaneously or during the breeding process, the so-called 'unintended effects'. Cases are known where commercial varieties caused health problems for this reason: rashes from celery [31,32], vomiting, stomach cramps, diarrhea or collapse from zucchini [33,34], potato [35] and bottle gourd [36]. Therefore testing for known toxins is routinely performed in crops known to contain toxic compounds, irrespective of the breeding method used. A problem relevant both to the developing and developed world is mycotoxin contamination of foodstuffs. Mycotoxins are not actually produced by plants, but are a byproduct of fungal growth on plants or foods. While there are several strategies (both conventional and transgenic) to control mycotoxins, this is outside the scope of my review. Other authors discuss mycotoxins in this issue (W. Parrot, B. Chassy).

Improving food safety and food security

The presence of toxic substances is still problematic for a few crop plants, which might be ameliorated by a further reduction, as well as for wild plants, in those cases for which a rapid domestication process might be desirable, such as for some biofuel crops [37]. To give a perception of the relevance of crop amelioration in economical as well as human terms, I provide three examples: rapeseed, cassava and cotton.

Rapeseed is widely grown and the annual production in 2007 was 50 Mt. The seeds are used mainly for oil production. After extraction, the resultant meal (35 Mt/year) is a good source of protein for animal feed, but its use is often limited by the amount of glucosinolates that can be ingested because of their toxicity. Glucosinolates themselves are not toxic, but upon cell disruption, they are hydrolyzed by plant myrosinases (specific esterases) and their hydrolysis products have been shown to be deleterious to rat, pig, poultry, rabbit, cow, sheep and fish, with effects on health, growth, productivity and reproduction (reviewed in [38]). In several cases, high-level intake results in increased mortality. Part of the negative effects on animals can be reduced by iodine supplementation, because some of the glucosinolates hydrolysis products interfere with thyroid hormone production. Classical breeding was used to create varieties low in glucosinolates: the so-called 'double zero' varieties are low in (but not devoid of) both erucic acid and glucosinolates. Also several treatments are available to reduce glucosinolate content [38]. Processing like heat inactivation further reduces the toxicity of glucosinolates, but also reduces lysine availability and thus the quality of the feed [39]. Thus genetic engineering gives a possibility of improving the meal through selective removal/reduction of glucosinolates in seeds beyond the reductions already obtained by breeding. The problem of toxicity might be less relevant in developed countries where most varieties have already a reduced glucosinolate content, but further improvements at the genetic level can translate into increased feed utilization efficiency, even in developed countries, making intensive agriculture more sustainable.

Cassava is a staple food for around 700 million people in the world, mainly Africa and Latin America. The starchy tuberous roots are poor in protein and contain varying amounts of two cyanogenic glucosides (linamarin and lotaustralin) which can be converted to HCN upon hydrolysis of the glucoside. Chronic

exposure to sublethal levels of HCN is responsible for konzo (irreversible paralysis of legs [40–42]), goiter and cretinism, stunting of children [42] and possibly Tropical Ataxic Neuropathy [43]. Some of these effects are exacerbated by diets poor in iodine and/or protein. On the history and sufferings connected to goiter due to iodine deficiency, I recommend the book by Hetzel [44]. Both bitter and sweet cassava (with a reduced content of cyanogenic glucosides) are available [45], but the preference of consumers and farmers depends also on traits such as cooking quality, starch texture and resistance to disease. Therefore the availability of plants combining certain characteristics with reduced cyanogen content might be better achieved by transgenesis rather than breeding. Given the rising consumption of cassava, especially in Africa [46] there is the case for improving varieties as well as education on the methods to process cassava tubers to remove cyanogens [46].

The third example is cotton, a crop primarily grown for fiber with an annual production in the range of 25–28 Mt of fiber in recent years. Interestingly, for each kg of fiber the plant produces 1.65 kg of seed (41–46 Mt/year) which contains 21% oil and 23% protein. The meal left after oil extraction contains high-quality protein (8–10 Mt/year), but it is unsuitable for consumption by monogastric animals, humans included, because of the presence of gossypol, a cardio- and hepato-toxic terpenoid [47]. It is therefore used as feed for ruminants, which are less sensitive to gossypol, either as meal after oil extraction or more rarely as whole seeds. Costly chemical, biological and physical procedures (see [48] for some references) are used to remove gossypol from cottonseed products to allow their use as food for non-ruminant animals, including solvent extraction with different solvents, ferrous sulfate or calcium hydroxide treatment, microbial fermentation and mechanical processing. It is clear that the development of varieties without gossypol would completely eliminate the need for gossypol removal and could potentially satisfy the daily protein requirement for half a billion people. A glandless cotton mutation was discovered in 1954 and immediately attracted the attention because gossypol accumulates in epidermal glands, located in seeds and aerial plant parts. Several commercial glandless varieties were developed by conventional breeding but they turned out to be extraordinarily susceptible to several insect pests, presumably because they lack protective terpenoids [49,50].

Targeting the genes rather than the proteins

The overwhelming majority of toxins are either protein themselves or are synthesized by proteins. The dogma of molecular biology states that 'DNA makes mRNA and mRNA makes protein'. This is normally represented as: Gene → mRNA → Protein. If we target the gene or the mRNA coding for a certain protein, then we end up not making the protein at all or making a nonfunctional protein. Therefore, the most sensible approach to reduce/inactivate a toxin in a living being is targeting the gene coding for (i) the toxin (if this is a protein synthesized through mRNA/ribosomes), (ii) a component of the specific machinery/pathway responsible for its production/accumulation (as is the case for toxic metabolites) or (iii) a regulator of the expression of the toxin, either directly (for a toxic protein) or indirectly (if it is a metabolite). Other strategies are the pharmacological or physical inactivation of the protein (e.g. by heat through cooking and food processing) or the stimulation of its degradation, but these strategies will not be dealt with here. I shall focus on inactivation at the gene/mRNA level as a safe and cheap alternative. The power of this approach is that mutations are inherited and usually quite stable. All the progeny of a plant with a disrupted gene will carry the same inactive allele. This implies that protein inactivation through gene inactivation is a once-for-all approach and needs not to be repeated at each generation or harvest. In a few cases, mutations could revert to the original status, but this is a spontaneous process whose frequency depends on the type of mutation. Selecting the appropriate mutation can make the reversion frequency extremely low. The next question is: how it is possible to inactivate a gene or its corresponding mRNA?

Direct and indirect gene inactivation strategies

Mutations arise spontaneously in any organism and by several means. Some of the causes are inevitable, such as background radiation, the endogenous production of reactive oxygen species or the mutagenic effect of DNA replication and cell division, while others can be induced or strengthened by environmental conditions. Mutation frequency can be enhanced for experimental purposes by various treatments: UV, X- and γ-rays, chemical mutagens and mitogens (indirectly), just to name a few. Mutants arise for instance because transposons can move around and 'jump' into genes. Similar results can be obtained by natural transposons or T-DNA/engineered transposons [51–54]. Genes have been inactivated through mutation (broadly defined as base changes, insertion or deletion) all the time. A mutation can involve just a single base or entire chromosomes. The importance of this process is particularly evident during domestication whereby the expression of certain genes was altered. For instance, loss of shattering, a trait of great importance in agriculture, is attributed to a disruption in the development of the abscission zone between grains and pedicles [55,56]; for more examples, see [57,58]. Many mutations involved in domestication are recessive, consistent with a loss of function and are deleterious in the wild (see contribution by P. Raven in this issue). Whether a similar phenomenon applies to the reduction in toxin content that happened during domestication, it is too early to tell for the lack of molecular data, but it seems quite a plausible mechanism.

Mutations resulting in inactivation of a protein can be classified into two broad categories (Fig. 1): mutations in the targeted gene and mutations involving another gene, but which affect the targeted gene via an RNA intermediate. The first class of mutations strike at the gene itself (box in Fig. 1a) thereby compromising the ability to produce a functional/stable mRNA or affecting the functionality or stability of the corresponding protein. The other class (RNA-mediated, Fig. 1b) interferes with the expression of the target gene by means of a double-stranded RNA (dsRNA), but leaves the gene sequence unchanged. This second class is collectively referred to as post-transcriptional gene silencing (PTGS), different variants of which are possible (antisense, RNAi, miRNA, hpRNA, etc.) and often involve epigenetic changes [59–61]. To be precise, the direct inactivation of a gene coding for a regulator (e.g. transcription factor) of a metabolic pathway is a protein-mediated strategy and therefore should be classified as an 'indirect gene inactivation', but for the sake of simplicity it will be treated as a direct gene inactivation strategy, because the targeted gene is directly inactivated.

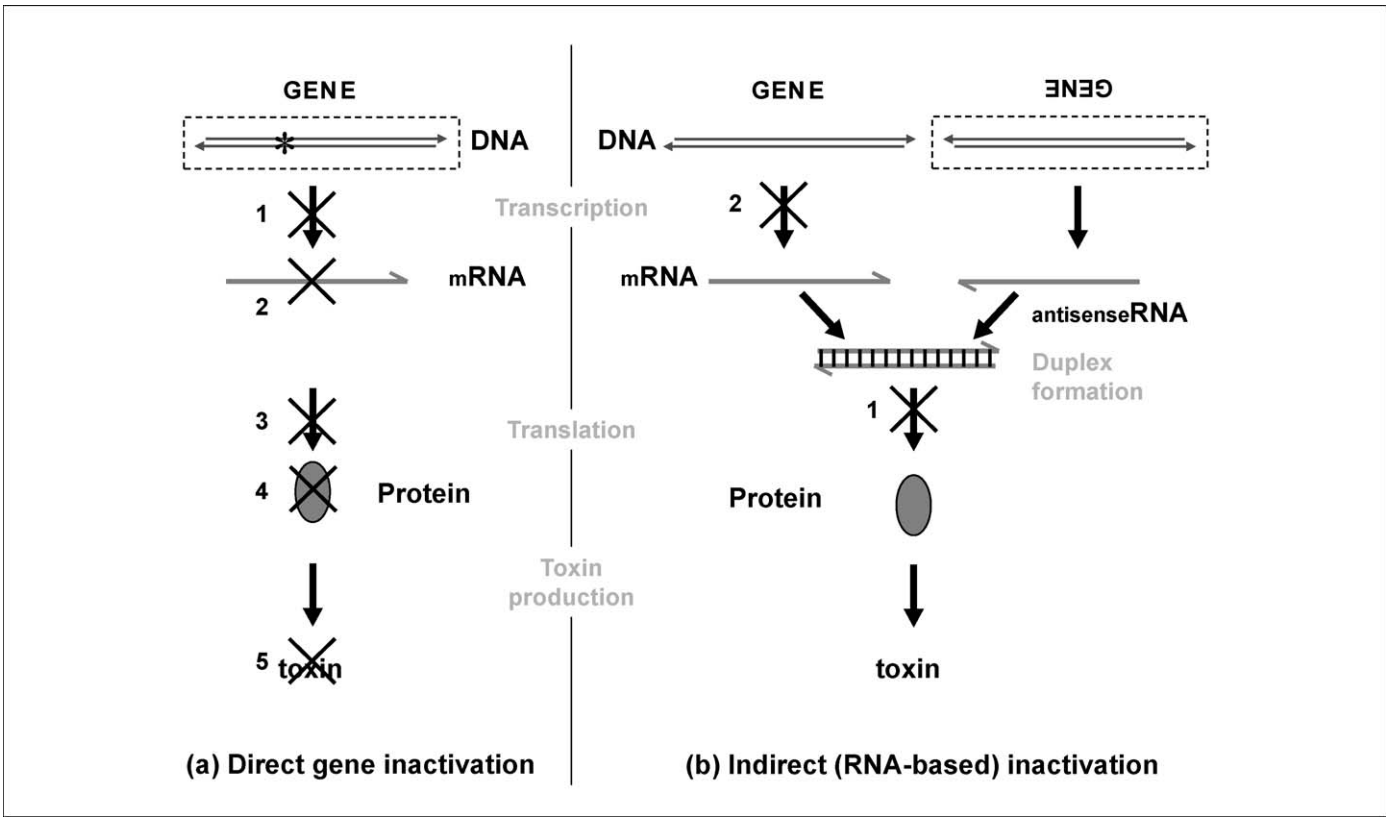

FIGURE 1

Classification of gene inactivation strategies. Strategies can be broadly assigned to either to **(a)** direct or **(b)** indirect category. The former indicates all those situations where the gene itself (within dashed box) is inactivated by the mutation, which is depicted as an asterisk at the DNA level and representing any change in the DNA sequence; large × represent all the potential levels where the inactivation may reveal itself: transcription (1), mRNA processing or stability (2), translation (3), protein folding or stability (4) or function (5). Indirect strategies (b) leave the original gene intact, but introduce another gene (dashed box) which produces an RNA molecule complementary to the mRNA of the gene that is going to be silenced. For this reason the introduced gene is depicted in the antisense orientation and the RNA produced is called antisenseRNA, often abbreviated in asRNA. The mRNA (sense) and the antisenseRNA pair together forming a duplex (dsRNA) which inhibits translation directly (1) or prevents transcription (2, indirectly, at the chromatin level, via the production of small RNAs).

The different methods to obtain a mutant are listed in Table 2, together with advantages/disadvantages of each method. It is noteworthy to stress that different methods might end up exactly in the same result – lack of a (functional) protein – and could be mediated by the same or a similar change at the DNA level, irrespective of the agent performing the modification (be it a human being or a bacterium or the plant itself) or the method by which the mutation is produced. It is therefore hard or impossible to distinguish natural/non-natural mutations (see contributions by W. Arber and by W. Parrott in this issue). Moreover what is relevant is the phenotype, the effect of the modification, and not the method used for achieving it. It is plausible that different direct

TABLE 2

Kinds of mutation and their advantages/disadvantages

Origin	Advantages[a]	Disadvantages[b]
Spontaneous mutation	No/little regulation	Low frequency/restricted choice
Induced mutation	No/little regulation	Low frequency/restricted choice
Mutagenic oligonucl.	Specific, quick, little/no regulation	Restricted choice
Transposon	May be specific	May be reversible, single target, low frequency
T-DNA insertion	Specific/irreversible	Single target, low frequency
Antisense RNA	Specific, dominant, sequence-based, many targets	Silenced gene intact (reversible), may be leaky
RNAi (hpRNA)	Specific, dominant, sequence-based, many targets	Silenced gene intact (reversible), may be leaky
miRNA	Specific, dominant, sequence-based, many targets	Silenced gene intact (reversible), may be leaky

[a] *Advantages*: specificity of inactivation might have different degrees of intensity and might concern different tissues. Both depend on the construct and the transformation event. Irreversibility depends also on the technique and on the event. 'Sequence-based' means that only the sequence is required to obtain the desired mutant (and the frequency of the mutant is usually high.

[b] *Disadvantages*: by frequency it is meant the number of mutants with the desired phenotype compared to the number of mutants generated. Leaky means that small amount of toxin might still be produced (e.g. [63]) in the tissue.

mutations (e.g. a deletion spanning the whole gene, the insertion of a T-DNA or of a transposon or a point mutation) produce the same effect by affecting a similar target, like, for instance (1) the promoter region (eliminating transcription) or (2) the coding region (introducing an early stop codon or a missense mutation, affecting protein stability, folding or activity) or (3) a splice site (abolishing the splicing) or (4) determinants of mRNA stability (causing rapid mRNA degradation). The extent (length of the DNA involved), nature (insertion, deletion or change) and site of action (transcription, splicing, mRNA stability, translation, protein folding or stability or catalysis) of the mutation can be very different. Similarly for indirect mutations, the origin of the asRNA, its length, position and extent of pairing with the mRNA can vary greatly between different indirect strategies. Also the ultimate level of action for the asRNA can be different: in some cases the duplex formation targets the mRNA for destruction and inhibits translation, in other cases the small RNA fragments can lead to an alteration in the methylation pattern of the gene and ultimately in the silencing of transcription.

No one method suites all situations (of pros and cons)

Gene inactivation is an excellent means to study gene function and it has been applied to basically all processes in living organisms since the discovery of mutations and their hereditability. More recently, systematic insertional mutagenesis was applied to *Arabidopsis* (e.g. [53,54,62] and other plants to study gene function in all aspects of their biology. This paper deals only with strategies aiming at inactivating toxin and allergens.

In the case of direct gene inactivation, some methods like X-rays or T-DNA insertion very often cause irreversible mutations which are stably inherited. Both characteristics are obviously advantageous for breeding. Other mutations, caused by chemical mutagens, spontaneous to base change or transposon insertion, might be more prone to reversion and less desirable compared to stable ones. Certain methods (insertional mutagenesis with T-DNA or transposons) are ineffective or slow when multiple gene codings for similar proteins need to be inactivated at the same time. In these cases, approaches like RNAi or antisense are more effective. Another big advantage of this approach is that knowledge of the sequence is the only requirement. Once the target gene is known, the construction of a transgenic organism affected in the expression of the gene is relatively easy. However, in the case of indirect gene inactivation, the target gene remains intact and therefore the phenotype might revert completely when the 'interfering' gene is inactivated or removed. Very interestingly, RNA-based inactivation methods allow for gene inactivation in specific tissues or developmental stages, as well as multiple targets, goals much more difficult (but not impossible in principle) to achieve with other methods.

In short, the best method depends on the specific combination of trait/crop one wants to achieve. The strong regulation required for mutants produced by some method is of course a self-imposed disadvantage that has no scientific basis (see contribution by H. Miller in this issue).

Examples of inactivation of toxins in transgenic plants

The seed-specific inactivation of the biosynthetic pathway for gossypol is the most striking example of the potential of biotechnology for toxin inactivation. Sunilkumar *et al.* [63] cloned a fragment of δ-cadinene synthase, the first step in gossypol biosynthesis, into a hpRNA vector and obtained tissue-specific silencing of the corresponding gene by restricting the expression with the seed-specific α-globulin B gene promoter. All transgenic seeds show a strong reduction in the level of gossypol, within the limits approved by the World Health Organization (WHO). The trait strictly co-segregates with the transgene and is stably maintained in the RNAi lines. The levels of gossypol and other protective terpenoids (hemigossypolone and heliocides) in leaves are not altered. Earlier attempts to reduce gossypol via antisense RNA did not yield a strong reduction or were unconvincing (see [63,64] for other references).

The authors demonstrated that it is possible to disrupt gossypol biosynthesis in seeds (and in seeds only) by interfering with the expression of a biosynthetic gene during seed development. Targeted gene silencing can thus be used to modulate biosynthetic pathways in a specific tissue to obtain a desired phenotype. Traditional breeding was unable to achieve this goal. Most remarkably, the authors hope to get reduced-gossypol cotton through regulatory approval process in the U.S., but, due to the very high costs (estimated in the range of 50 M$, see contribution by I. Potrykus in this issue) they 'do not know where the money is going to come from' (K. Rathore, pers. commun.). The foregone benefits of a delay in delivering this variety to farmers are evident with around a billion hungry people on the planet.

Another example is the reduction of glucosinolates in *Arabidopsis*. Several groups have recently identified regulators of the biosynthetic pathway [65–71]. Overexpression and gene inactivation/silencing studies have revealed that Myb28, 29 and 76 control the aliphatic pathway. Myb28 is responsible for the basal transcription of the biosynthetic genes together with Myb29. Inactivation of the former effectively eliminates long-chain aliphatic glucosinolates, while inactivation of the latter reduces the amount of short-chain glucosinolates. Elimination of both gene functions results in the complete loss of aliphatic glucosinolates. Myb76 seems to be relevant in the induction of the pathway following wounding, but does not play a major role in the basal transcriptional regulation. By contrast, Myb34, 51 and 122 control the aromatic (indolic) branch. There appears to be a complex cross regulation between the two branches because a reduction in flux in one branch stimulates the flux in the other one. Even though *Arabidopsis* is not a crop, research findings with this species are easily transferred to other brassicas (e.g. [72]). A precise manipulation of glucosinolate content in seeds needs a better understanding of the full regulatory circuitry and transport. As for cotton, seed-specific silencing might be a desirable approach to avoid an overall increase in pest sensitivity.

As a third example there is again cassava. Different transgenic strategies have been attempted to reduce cyanogenic glucosides [73–77]. Antisense inhibition or RNA interference in leaves of the first step of cyanogen biosynthesis reduces linamarin levels by 60–94% in leaves and by 99% in roots. These plants however are impaired in growth or tuber formation in the absence of a reduced nitrogen source, presumably due to the role of cyanogen hydrolysis in aminoacid biosynthesis [73,77]. A more promising strategy is expressing the leaf-specific enzyme hydroxynitrile lyase (HNL) in roots to accelerate cyanogenesis and cyanide volatilization

during processing [74]. Several other examples of reductions of toxin have been published, but they have little relevance to food (nicotine in tobacco [78], morphine in Poppy [79,80]). Of interest is the reduction of antinutritional factors like phytic acid in maize [81] for environmental benefits, even if it decreases germination.

Room for improvement of orphan crops

Lathyrus sativus is a hardy tropical/subtropical legume also known as grass or Indian pea. Beans from this so-called 'famine crop' are an important source of nutrition for poor people in Asia and Africa, but contain a neurotoxin: oxalyldiamino-propionic acid (ODAP). This compound causes lathyrism, a lower limbs paralytic disease prevalent among adults in Central India who consume large quantities of seeds for several months [82]. Safe content for ODAP is <0.2%, while content in germplasm ranges between 0.3 and 3.3 [83]. Soaking and boiling of seeds reduce ODAP levels but effective detoxification often results in a decrease of nutritional quality. Classical breeding and tissue culture approaches have already produced varieties with greatly reduced ODAP levels (see references in [84], but the substantial outcrossing rate for this crop means that low ODAP lines must be multiplied in isolation and provided to farmers every year [85]. A biosynthetic pathway has been proposed for ODAP [86] and it is thus feasible to attempt its silencing only in the seed using a transgenic approach, as done for gossypol biosynthesis in cotton. Antisense or RNAi construct, due to their dominance, would reduce the need for segregation in seed production.

Other examples are two millet species, fonio and pearl millet, which are cultivated for food in sub-Saharan Africa and India with an annual production of 22 Mt (80% of the world total). High consumption of these two species is known to cause goiter (see references in [87]) with its burden of suffering [44] due to the flavonoids apigenin and vitexin, respectively in fonio and pearl millet, which are strong inhibitors of thyroid peroxidase. Available knowledge allows one to attempt the targeted inactivation of the biosynthetic pathway in seeds and suggest that genetic engineering approaches are more reasonable than conventional ones [87].

Trade-offs for toxin reduction

Reduction in toxin content usually comes with a price: plants become more susceptible to pests [70,71,27,88] sometimes to the point of making them unsuitable for cultivation [49,50]. Several natural pesticides are quite general in their mode of action [89] and natural pesticides account for 99.99% of our dietary pesticide intake [90]. For example, benzoxazinones, secondary metabolites from cereals, are important in the defense against insects, fungi and bacteria [91,92] and the same is true for the glucosinolates/myrosinase system in brassicas [93]. Similarly cyanogenic glucosides seem generally toxic against insects and animals [11,94,95] and protect plants from herbivores [27,28], even though several insects might have evolved specific resistance. On the contrary, accumulating new pesticides into a plant increases pest resistance (e.g. cyanogenic glucosides [96]). This strategy is indeed the key to the success of insect resistance based on Bt toxins engineered into cotton and maize [97], as well as many other species (e.g. [98,99]). The environmental and safety price bargained through the more precise tools of genetic engineering is expected to be substantially lower than those obtained with classical genetic approaches, because of the use of pesticides (e.g. Bt or avidin, see [100]) targeting only specific classes of pests, and a much wiser alternative to the application of synthetic chemicals.

Plant-derived allergens

Allergens are of widespread occurrence and one might not be aware of their presence until experiencing their effects. It is not only a nuisance and/or a cost, but it could be a deadly threat. Minute amounts of allergens might cause a life-threatening event called an anaphylactic reaction. This might occur after ingestion, skin contact, injection or inhalation of an allergen. In the UK alone, allergens in food are reported to have caused 48 deaths over a 7-year period between 1999 and 2006 [101]. Half of the eight foods accounting for 90% of all food-allergic reactions (milk, egg, fish, shellfish, peanut, tree nut, soy, and wheat) are of plant origin [102]. Products containing them are quite widespread and difficult to avoid in a standard diet. Beyond them, pollen is the major cause of respiratory allergy, with at least 40% of type 1 allergic patients who are sensitized against grass pollen allergens.

Contrary to common perception, transgenic plants never caused allergic reactions to consumers. In one case a gene for a 2S albumin from the Brazil nut (a known allergenic food) was expressed in soybean [103]. The resulting transgenic soybean was tested for allergenicity and it was ascertained that the 2S albumin is indeed a major Brazil-nut allergen. The development of this product was abandoned, no product was ever commercialized or released and no consumer suffered any allergic reactions. This was not a serendipitous finding, because if a gene used for transgenesis comes from a plant containing allergens, the transgene has to be checked for allergenicity. A similar situation was found for transgenic peas expressing the bean α-amylase inhibitor [104]. The transgenic peas elicited an immune response in mice upon feeding, but the reaction could be ascribed to changes induced in the plant by the transformation and regeneration procedure or by the changes detected in the α-amylase inhibitor between bean and pea [105] regarding the glycosylation pattern and the removal of amino acid residues of the protein. The guidance rules adopted in the EU require a risk analysis for potential allergenicity for any gene that is being used for transformation [106,107].

Examples of inactivation of allergens in transgenic plants

There are several examples of manipulations for the reduction of plant allergens content (apple, peanut, wheat, soybean, ryegrass and birch). In this paper I discuss one example each from soybean and apple. Several papers describing or reviewing other cases are available [108–112] (M. Schenk, Birch pollen allergy: molecular characterization and hypoallergenic products, Ph.D. thesis, Wageningen University, 2008 (http://www.library.wur.nl/wda/dissertations/dis4391.pdf)).

In the US/Europe: 5–8% of babies and 2% of adults are reported to be allergic to soybeans. The dominant soybean allergen is a protein named P34 or Gly m Bd 30 K, with more than 65% of soy-sensitive patients reacting only to it. Mutagenesis and breeding allowed the removal of some soybean allergens [113,114], but not the dominant allergen P34. Transgenic soybeans without P34 were readily obtained by gene silencing [115,116]. Apart P34, the authors found no difference in composition, development, struc-

ture, polypeptide pattern or ultrastructure when comparing the silenced line with control plants. However, using the very same words of the authors, 'regulatory difficulties and the lack of acceptance of GM soybeans by the baby food and formula industry makes using such an allergen-suppressed soybean difficult at the present time', a euphemism to mean nearly impossible. Therefore an alternative approach was used to achieve the same goal: identify soybeans lacking the allergen. The entire USDA national soybean germplasm collection was screened and out of more than 16,000 accessions screened, they found 12 lines (2 of which are cultivated soybean) with no P34 allergen [117]. Based on the sequence analysis, it is possible to guess the reason why these soybean plants lack the allergen. It is however possible that the expression of many other genes is altered with concomitant unintended effects (e.g. expression of new allergens). By contrast, the suppressed soybean line was thoroughly investigated by 2D gel electrophoresis and the only change detected concerns the targeted polypeptide out of the 1400 examined. Beyond any logic, the approval for the transgenic event will be far more complicated and costly than for the conventional mutant lines (E. Herman, pers. commun.).

Apple allergy is dominated by protein Mal d 1, which is also found in birch pollen. Allergenicity depends on the amount of specific Mal d 1 isoforms, whose quantity varies among apple cultivars. Because of this, classical breeding might be used to create new hypo-allergenic cultivars, but this is complicated by the fact that Mal d 1 is encoded by a gene family comprising at least 18 members (loci) arranged in several gene clusters. The expression of Mal d 1 in apple was inhibited by RNAi [118] and this translated into a reduced *in vivo* allergenicity. In another study [119], the allelic diversity of the seven Mal d 1 genes was investigated in several apple cultivars. It is clear that few alleles associate strongly with differences in allergenicity, suggesting that the production of new varieties by breeding is a feasible target. However, it takes over 15 years to produce a marketable cultivar out of a cross and therefore the direct production of clones with reduced amount of an allergen by transformation of existing cultivars seems a reasonable shortcut, except for the exorbitantly high hurdles associated with present regulatory regime.

It is often feared in non-scholarly sources that plant biotechnology would inadvertently introduce new allergens in foods. The examples presented here, as well as the available literature, make it clear that biotechnology is part of the solution to allergies rather than a cause of increased concern.

An example of insanity in regulation: percent similarity is not everything

Biosafety regulations require that if a protein shares at least 35% identity over 80 amino acids to an allergen, then any transgenic plant or product expressing it must be labeled as 'potential allergen', even if there is no evidence for any allergenicity [107], unless it can be proved that the protein is not an allergen. Phaseolin is a protein from bean which is not recognized as an allergen or listed in the official allergenonline.com website, even if it shares a substantial similarity (53% identity) to β-conglycinin, a minor soybean allergen. Moreover phaseolin is safely eaten by around one billion people everyday. The 27 kDa γ-zein is a storage protein from maize which is also not recognized as allergenic and consumed by hundreds of millions of people everyday. Zeolin, a chimera between phaseolin and 89 amino acids of γ-zein has been produced [120] and expressed in transgenic cassava (C. Fauquet, pers. commun.). However, zeolin-expressing cassava should be labeled as a 'potential allergen' because the similarity of phaseolin to β-conglycinin is well above the limit and it would be impossible to demonstrate that zeolin cannot be an allergen. Actually it would only be possible, as well as difficult and expensive, to demonstrate that the risk is below a certain level. This cassava shows a 350% improvement in protein content and a 55% reduction in cyanogenic glucoside, an unintended but welcome effect. It would be made freely available in developing countries if regulations would allow it. The labeling requirement, an obviously impossible (as well as ridiculous) task in places like Africa, makes this transgenic cassava another victim of present day regulation and a rather enlightening example of its insanity.

To stress the point, let us take an example of poetry (the first verses of Dante's Paradise, Canto I, v. 1–3): 'The glory of Him who moveth everything/Doth penetrate the universe, and shine/In one part more and in another less.' If we now substitute 40% of the letters in the words (changes underlined), we could get the following as one of the many examples: The story of him who believeth everything/Does infiltrate diverse lies and causes/one part of farmers or another to die. Obviously the result is not poetry any longer and the meaning is substantially different. A similar thing happens with protein sequences. Two proteins could have 80% identity and yet perform different functions or have different structures. Conversely, proteins with little or no sequence identity could have similar structures or perform similar functions. The % of sequence identity is often a poor indicator of the protein properties and it is unreasonable to rely on it for predictions, if other evidence is at hand.

Conclusions

Plant-derived allergens and toxins are ubiquitous, abundant and essentially unavoidable components of our diet and environment. Tools are available to reduce them at the genetic level, either by conventional or transgenic approaches. However, strategies must be reasonable, that is accept some level of risk, and effective, that is the benefits have to be balanced against cost. For instance, it is unreasonable to require demonstration that zeolin is not an allergen when both phaseolin and zein are not. Similarly, it is unfair to demand multigenerational feeding tests on insect resistance Bt maize but not on maize varieties more resistant to several insects because accumulate more benzoxazinones [121]. Overcautious regulation goes in the opposite directions on both issues: a zero risk tolerance requires endless testing (and infinite costs) to obtain approval for innovative products as substitutes of older technologies. Moreover, reducing the content of natural toxins is often a threshold issue (the dose makes the poison). Accepting low levels of toxins seems a sensible option [89,90] and even a beneficial choice [122].

The insanity of present regulation is more evident with so-called 'loss of function' mutations, that is mutations inactivating gene function, such as many of those mentioned in this review, but similar arguments can be put forward for other kinds of genetic changes. The fact that genetic engineering easily achieved something that conventional breeding was unable to do – for example

maintain gossypol in leaves, where it is useful, and eliminate it in seeds [63], see also [123,124] – is the demonstration of the higher precision of this technology, not a proof of its unnaturalness, because it is conceivable that screening a larger number of conventional mutants might eventually deliver the same phenotype. An overcautious attitude might kill the technology altogether and its associated benefits. Comparing the techniques adopted for reducing toxins and allergens, usually transgenesis shows superior characteristics: it is not only more efficient in obtaining the desired phenotype (both in time and trial numbers) but also more precise. Natural null mutants for the P34 soybean allergen [117] have a frequency of 2/14,000 in cultivated soybean, that is 0.014%, and the exact reason for the lack of P34 is uncertain. Conversely, the frequency of soybeans coming out of a transformation showing P34 cosuppression is in the 10–20% range (E. Herman, pers. commun.). The possibility of 'unintended effects' is obviously smaller for the transgenic mutant, because a detailed analysis revealed only one change in composition (one protein missing out of around 1400 examined), the reason of which is the transgene. In other words safety testing of transgenic varieties must be compared against testing of varieties developed by conventional means.

Breeding approaches allowed in the past the creation of new varieties with lower toxin levels: erucic acid and glucosinolates in brassicas [39], cyanogenic glucosides in clover, cassava, almonds and cotton just to name a few [27,46,49,125,126]. Transgenesis is another tool which can be employed for the same purpose (e.g. [63,73]) and seems particularly suited for reducing the allergenic content of foods and plants in general, especially in fruit trees, where the use of conventional means, like mutagens or crosses among natural variants, is discouraged for practical reasons (e.g. the method takes too long a time or would alter the peculiar characteristics of the cultivar).

Other specific problems still await a solution or optimization. Several legumes must be heat treated before consumption especially for monogastric animals because they contain one or more toxic compounds: trypsin inhibitors, amylase inhibitors and lectins (in legumes [127]), saponins, vicine and convicine (pyrimidine glucosides from broad beans) responsible for favism in humans [128], just to name a few. The possibility of reducing single or multiple toxins in food and feed could improve food safety, food security and conversion efficiency. Other compounds like phytate are not toxic, but reduce availability of phosphate and iron in legumes and, to a lesser extent, in cereals [81]. The evident consequence of this further domestication is the need to substitute general pesticides for new, more specific pesticides like Bt to counter plant pests. Several new plant toxic proteins with insecticidal properties have potential in this respect [129–131] some of which are commonly found in foods we already eat (e.g. [132]) and we know how to inactivate them. A particular appealing strategy is the use of RNAi in plants to silence pest genes [133,134].

Sometimes it could be desirable to modulate the content of specific compounds. Glucosinolate hydrolysis products seem also to be responsible for the anticarcinogenic activity of brassica vegetables in humans [135], but the beneficial dose window of glucosinolate hydrolysis products can be rather narrow. It is amazing how fully acceptable is a new 'superbroccoli' variety obtained by conventional breeding through a cross with a wild variety [136] with a 10-fold increase in a specific glucosinolate content and a 100-fold inducing potency of a marker of phase II detoxification enzymes in mammalian systems. This is obviously considered to be a good thing by the popular press [137]. Another variety, named 'Booster Broccoli™', with a smaller but substantial increase in sulforafane, has just been launched on the market and its purported non-GM status is highlighted together with the benefits of a high sulforafane diet [138]. One wonders what would the reaction be if a transgenic canola (engineered for instance for herbicide tolerance) with minor alteration in glucosinolate profile was to be introduced in the market.

It is conceivable that new almond or peach varieties might accumulate much more cyanogenic glucosides and new potato varieties might accumulate more or new glycoalkaloids. From a few cases in the past [31–35] we know classical breeding can cause problems and yet, in the EU, new varieties with a real toxic potential (e.g. potato) require no regulatory scrutiny (no compulsory measurement of toxic compounds and no safety tests) before release, cultivation or commercialization if they are produced by conventional means. And we also know that conventionally bred crops might present far more changes at the genomic level than transgenic ones [139–144] or might contain new allergens [145]. Therefore there is a strong case for demanding a more science-based regulation (see also contribution by H. Miller in this issue).

Gene technology could further improve food safety, food security and wellbeing as well as reduce environmental impact of agriculture and other human activities. Regulation is a major obstacle because (rewording an Italian common way of saying) 'where logic ends, biotech regulation begins'. Technology is of course a constant source of new problems and challenges as it has been since the beginning of human society. As examples, think of the dangers of moving at high speed or, more recently, the hypothesis that the rise in allergies is linked to a reduced microbial exposure [146]. But rather than reverting to older and less safe technologies, we need to think of more technology as the solution. To state it more humorously in the words of F. Salamini: 'Everybody wants to return to nature, but not by foot'.

Note added in proof

An interesting approach to insect 'resistance' is reported in Ref. [150].

Acknowledgements

I thank several colleagues and friends (especially W. Parrott, J. Gressel, D. Kershen, K. Ammann, F. Salamini, G. Fico, A. Vitale, D. Ederle, T. Maggiore, K. Petroni, F. Rossi, G. Succi, L. Gilissen, N. Bisht, K. Rathore, D. Shah, D. Carputo, B. Parisi, C. Faquet, E. Herman, R. Goodman) for support, information, personal communications, help with literature or reviewing the manuscript. Massimo e Monica Delledonne helped by graciously providing a few days of peace. I acknowledge the support of the Scientific Council for Ag-Biotechnology of the Lombardy Region and the University of Milan for access to several books. A particular thanks to I. Potrykus for everything.

I dedicate this paper to Giovanna Ferro-Luzzi and Bruce N. Ames for their inspiring work and examples.

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