Review

FOOD SAFETY EVALUATION OF CROPS PRODUCED THROUGH GENETIC ENGINEERING – HOW TO REDUCE UNINTENDED EFFECTS?*

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Scientists started applying genetic engineering techniques to improve crops two decades ago; about 70 varieties obtained via genetic engineering have been approved to date. Although genetic engineering offers the most precise and controllable genetic modification of crops in entire history of plant improvement, the site of insertion of a desirable gene cannot be predicted during the application of this technology. As a consequence, unintended effects might occur due to activation or silencing of genes, giving rise to allergic reactions or toxicity. Therefore, extensive chemical, biochemical and nutritional analyses are performed on each new genetically engineered variety. Since the unintended effects may be predictable on the basis of what is known about the insertion place of the transgenic DNA, an important aim of plant biotechnology is to define techniques for the insertion of transgene into the predetermined chromosomal position (gene targeting). Although gene targeting cannot be applied routinely in crop plants, given the recent advances, that goal may be reached in the near future.

KEY WORDS: *genetically modified organisms, plant breeding, risk assessment, gene targeting, homologous recombination*

Since the dawn of agriculture, humans have been manipulating crops to enhance their quality and yield. Via conventional breeding, seed producers have developed many modern crop varieties. One of the most important technology used for genetic modification of crops in the last century was mutation breeding; plants were exposed to gamma rays, protons, neutrons, alpha particles, beta particles, or chemicals, such as sodium azide and ethyl methanesulphonate, in order to induce useful mutations. Of the 2,322 officially released radiation-breeding varieties, almost a half were released over the last 15 years (1). In the meantime, scientists have begun applying genetic engineering techniques to improve crops – around 70 varieties obtained via genetic engineering have been approved to date (2).

In contrast to earlier breeding methods which involved random mutations or mixing of thousands of unknown plant genes, genetic engineering allows the transfer of a single gene or a couple of genes in a much more precise, controllable and predictable way. Nevertheless, this technology has raised questions about its safety to consumers and the environment. Therefore, extensive chemical, biochemical and nutritional analyses are performed on each new genetically engineered variety. In some cases, the concentration of hundreds of cellular metabolites has been determined (Table 1) (3-10). It is important to understand that plants improved through conventional genetic modification, including mutation breeding, undergo no food or environmental safety review prior to being introduced into the marketplace.

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Table 1 *Examples of composition studies of genetically modified crops*

Plant	Trait	Parameter tested	Ref.
Canola	High lauric acid	AA, EA, FA, GL	(3)
Canola GT73	Herbicide resistant	AA, EA, FA, GL, MI, PA, PX, SI	(4)
Maize Bt176	Insect resistant	AA, FA, MI, PX, SU, MT	(5, 6)
Maize MON810	Insect resistant	AA, FA, MI, PA, PX, SU, TF, TI	(7)
Soybean GTS 40-3-2	Herbicide resistant	AA, FA, IF, LE, PA, PX, SR, TI, UR	(8, 9)
Soybean	High oleic acid	AA, FA, IF, MI, PA, PX, SR, TI, VI	(10)

Abbrevations: AA, amino acids; EA, erucic acid; FA, fatty acids; GL, glucosinolates; IF, isoflavones; LE, lectins; MI, minerals; MT, mycotoxins; PA, phytic acid; PX, proximates (e.g. protein, fat, ash, fibre, moisture, carbohydrate); SI, sinapine; SR, stachyose and raffinose; SU, sugars; TF, tocopherol(s); TI, trypsin inhibitor; UR, urease; VI, vitamins.

Although genetic engineering offers the most precise and controllable genetic modification of crops in entire history of plant improvement, the site of insertion of desirable gene cannot be predicted during the application of this technology. Therefore, unintended effects might occur due to activation or silencing of genes, giving rise to allergic reactions or toxicity (11, 12). In that respect, an important aim of plant biotechnology is to establish techniques for the insertion of transgene into the predetermined chromosomal position (gene targeting) that could be obtained only via homologous recombination. Unfortunately, despite a number of promising approaches, no feasible gene targeting technique has been developed for crop plants to date. Given the recent advances, however, this goal may be reached in the near future.

BASIC SAFETY EVALUATION PRINCIPLES FOR FOODS DERIVED FROM GENETICALLY MODIFIED (GM) CROPS

From the very first initiatives to establish globally agreed-upon guidelines for safety assessment of food and food ingredients derived from GM organisms, the comparison with relevant traditionally bred plant varieties has been the leading principle (13). The underlying assumption is that traditional crop varieties currently on the market, although not elaborately lab-tested before being marketed, have gained a history of safe use because they have been consumed for decades. This history of safe use has been used as a baseline for safety assessment of new GM plant varieties derived from established plant lines. The comparative concept for the safety evaluation of foods derived from GM crops has further been elaborated by the Organisation for Economic Co-operation and Development (OECD) and crystallised in the so-called Principle of Substantial Equivalence (14).

Specific international guidance on these issues has been provided in the meantime by the OECD (15, 16), the European Scientific Committee on Foodstuffs (SCF) (17), the United Nations Food and Agriculture Organisation/World Health Organisation (FAO/WHO) (18, 19, 20), and Codex (21). A detailed overview of safety assessment practices related to GM food crops has recently been reviewed by *Kuiper et al*. (22) and *Konig et al*. (11).

Basic safety evaluation of foods derived from GM crops includes molecular characterization of the introduced genetic fragment and of the resulting new proteins or metabolites (11); the analysis of the composition of relevant plant parts with respect to key nutrients and anti-nutrients, including natural toxins and potential allergens (Table 1); potential for gene transfer of specific genes from GM food to microorganisms in particular, in the human and animal gastro-intestinal tract (23, 24); potential allergenicity of the new gene products, or alteration of the intrinsic allergenicity of the GM food organism (25, 26); estimated intake levels of the newly introduced proteins as well as of the final product, including any altered constituent (11); and toxicological and nutritional evaluation of the resulting data and additional toxicity testing (of the whole food) where necessary (11, 22).

UNINTENDED EFFECTS

A desirable gene can be integrated into a plant's genomic DNA using a range of techniques defined as "gene transfer technologies" (reviewed in 27). The two most commonly used methods of DNA delivery are the biolistic or microprojectile bombardment system,

and *Agrobacterium*-mediated transformation, the latter being the most widely used system (28, 29). The biolistic method is based on a physical delivery of DNA-coated gold or tungsten microprojectiles into plant target tissue by acceleration. *Agrobacterium*-mediated transformation exploits the biological ability of this soil-borne bacterium to copy and transfer a specific portion of its DNA (termed T-DNA) present on a tumour-inducing (Ti) plasmid into the plant cell nucleus, where it can be integrated into chromosomes (for the most recent model for T-DNA transfer see 30-33). T-DNA integrates into the plant genome in the absence of any homology with the plant DNA sequences through the process of illegitimate recombination (33-35).

In both gene transfer technologies, insertion of the transgene could theoretically occur randomly along the length of the chromosomes. However, transgenes and, in particular, T-DNA containing transgenes for which more data are available have preferences for gene rich-regions (36-41). The fact that T-DNA integration into genes can cause mutations due to the loss of gene function was soon recognised (37).

A possible consequence of random integration of transgenes into the gene rich-regions of the plant genome is the disruption of endogenous gene function. This disruption may lead to changes in the levels and activities of inherent enzymes, nutrients and metabolites, or in the production of new proteins or toxins. This phenomenon is called "unintended effects". The occurrence of unintended effects is not unique to genetic modification using recombinant DNA technology, it also occurs frequently in conventional breeding (Table 2, ref. 42-49). However, in contrast to genetically engineered plants, varieties improved through conventional genetic modification, including mutation breeding, undergo no food or environmental safety review prior to being introduced into the marketplace.

Assessment of unintended effects

Potential unintended alterations in the composition of GM food crops as a result of genetic modification are one of the key elements of safety assessment (Table 1). For the most recent review, see *Konig et al*. (11). Beside the fact that relevant unintended side effects might remain undetected when only specific compounds or intermediates are analysed in important nutritional and anti-nutritional pathways, compositional analysis of GM plants and their traditional counterparts is very complex (11). Therefore, the development of more general, unbiased methods of analysis is encouraged in order to detect relevant changes in a much larger part of the physiology of the plant (12, 15, 19).

Unbiased fingerprinting approaches at the level of DNA, gene expression, proteins, metabolites and their secondary structures could provide a more thorough insight into any unpredicted changes in the physiology of the plant that might go undetected when focusing on single compounds (50). First, sequence analysis of the insertion point of the genetic fragment might be significant to evaluate whether it is possible to identify any potential side-effects, for example, based on the interruption of regulatory or gene sequences, or the presence of any such sequence in the vicinity. However, there is still limited knowledge about the genetic code of organisms under investigation, especially for regulatory elements (51-53). Second, microarrays enable simultaneous screening of altered gene expressions in a large numbers of genes. However, correct interpretation of results is both difficult and dependent on many different factors. These include experimental setup, available equipment, software,

Table 2 *Examples of unintended effects resulting from conventional breeding and genetic engineering*

Conventional breeding			
Plant	Targeted trait	Unintended effect	Reference
Celery	Pest resistance	Increased furanocoumarins content	(42)
Potato	Pest resistance	Increased glycoalkaloid content, low yield	(43)
Squash	Pest resistance	Increased cucurbitacin content	(44)
Genetic engineering breeding			
Plant	Targeted trait	Unintended effect	Reference
Rice	Carotenoid biosynthetic pathway	Unexpected carotenoid derivatives	(45)
Potato	Expression of soybean glycinin	Increased glycoalkaloid content	(46, 47)
Potato	Expression of yeast invertase	Reduced glycoalkaloid content	(48)
Soybean	Herbicide resistant (glyphosate)	Increased lignin content, low yield	(49)

and knowledge of the organism under investigation (54, 55). Third, given that altered gene expression levels might not correlate directly to shifts in protein levels, the most direct method of investigating unpredicted alterations is proteomic analysis of the tissues of interest (56, 57). However, there are several important setbacks. Setting up an informative system for a single tissue is time-consuming. Furthermore, reliable quantification remains problematic, despite the availability of advanced software. The sensitivity of this approach is affected by slight changes in isolation conditions (53). The fourth direct approach is the analysis of secondary metabolites. Informative systems have been set up for different organisms using gas and liquid chromatography in combination with mass spectrometry or nuclear magnetic resonance (58-60). In theory, identification of large numbers of constituting compounds can be achieved using a combination of these techniques. However, in practice, there are several important drawbacks. These include a lack of reliable data on profile variation for relevant compounds in different matrices of the organism under study, and the standardization of extraction procedures and measurement protocols (53).

It is clear that it is too early to apply profiling methods for the detection of unintended effects in GM food crops on a routine basis. However, despite the technical hurdles, it is also clear that these new developments have the potential to give increased insights into relevant changes in the physiology of plant products resulting from genetic modification or from the application of new and existing food processing techniques (61).

How to reduce unintended effects?

Unintended effects may be predicted on the basis of what is known about the insertion place of the transgenic DNA, the function of the disrupted part of the genome and the function of the inserted gene and its involvement in metabolic pathways. With this in mind, the most straightforward approach to reduce unintended effects might be the integration of transgene into a predetermined chromosomal position via homologous recombination (gene targeting). However, the low frequency of homologous recombination in somatic plant cells remains the major obstacle for this approach. This is because the frequency of integration at random locations by illegitimate recombination in the higher eukaryotes is several orders of magnitude higher than that of targeted integration by homologous recombination (62).

The first reports of successful gene targeting in mouse and plant cells appeared within the space of three years (63, 64). However, by 2001, more than 7000 chromosomal loci had been successfully targeted in mouse embryonic stem cells (65), but only a single targeting event of an endogenous gene was reported for flowering plants (66). In this single study Kempin *et al.* (66) reported knockout of the *AGL5* gene of *Arabidopsis* by homologous recombination. As these researchers obtained only a single targeting event among 750 transformants, they were unable to evaluate the reproducibility of gene targeting or its frequency.

In the meantime, there have been several attempts to determine the parameters that are crucial for efficient gene targeting. Unfortunately, parameters that are known to be important for gene targeting in mammals seem to be of little consequence in plants (67, 68). Researches have therefore focused on the studies of mechanisms and frequencies of various types of homologous recombination in plants. The most widely used approach has been based on the study of intrachromosomal homologous recombination between artificial repeated DNA sequences integrated into plant genome (69, 70). Reciprocal homologous recombination between artificial repeats leads either to deletion or inversion of DNA stretches that subsequently cause altered phenotype of the cells in which recombination has taken place. The most common substrates used to study homologous recombination contained pairs of deletion derivatives of the selectable marker (i.e. *nptII*) or reporter (i.e. *uidA*) gene, integrated into a single chromosomal locus (71, 72). Homologous recombination between these deletion derivatives restores a functional gene and thus provides a marker for such events (i.e. functional *nptII* gene confers kanamycin resistance). This experimental approach makes it possible to see influence of various parameters on the levels of homologous recombination in plant cells (Table 3, ref. 71-77). To date, however, our improved understanding of homologous recombination has not resulted in a significant increase in targeted integration events in plants.

Nevertheless, two important recent advances allowing more accurate estimation of gene targeting frequency at chromosomal loci of *Arabidopsis* (78) and rice (79) have been reported. The first study was based on the modification of an endogenous *Arabidopsis*

gene encoding protoporphyrinogen oxidase (PPO). The acquisition of two specific mutations in PPO renders the gene product highly resistant to the herbicide butafenacil. Two simultaneous mutations are required for high herbicide tolerance and spontaneous resistance has not been observed so far. Gene targeting by homologous recombination at the *PPO* locus is reproducible at a basal frequency of 2.4×10^{-3} (78). However, the integration at an ectopic position in the genome was also found in the study.

Table 3 *Factors (conditions) that increase the frequency of intrachromosomal homologous recombination (IHR) in somatic plant cells*

Stress factors (mutagens)	Increased IHR frequency	Reference
Pathogens	2-fold	(72)
Salicylic acid	2 to 7-fold	(72)
X-rays	2-fold	(71)
Mitomycin C	9-fold	(71)
Heat shock (50 °C)	6.5-fold	(71)
Modified genes		
cim3	4 to 5-fold	(72)
rad50	10-fold	(73)
MIM (overexp.)	2-fold	(74)
rDNA spacer	9-fold	(75)
Heterologous proteins		
RuvC (*E. coli*)	11-fold	(76)
RecA (*E. coli*)	10-fold	(77)

In the second study, targeted disruption of the *Waxy* gene of rice was obtained, and it was reproducible in several independent experiments (79-81). The observed gene targeting frequency at this locus was approximately 0.65×10^{-3} (in the range reported for the *PPO* locus of *Arabidopsis*). The design of this experiment included a positive/negative selection strategy coupled to a PCR-based screen for predicted insertion into the *Waxy* gene. The targeting events detected by PCR were enriched to frequencies in the 10^{-2} range. Two copies of a negative marker that flanked the targeting homologous sequences were used in the targeting vector in order to eliminate random integration events. Indeed, the targeted lines showed neither ectopic targeting events nor random integration of additional copies of the targeting vector. Hopefully, this highly efficient gene targeting strategy will also be used successfully in other important crop plants.

It is clear that the ultimate goal of the routine use of gene targeting technology for basic studies of plant gene functions and for plant biotechnology is still somewhere ahead. Nevertheless, given recent advances, it may be reached in the near future.

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Sažetak

PROCJENA ZDRAVSTVENE ISPRAVNOSTI POLJOPRIVREDNIH KULTURA OPLEMENJENIH GENETIČKIM INŽENJERSTVOM – KAKO UMANJITI NENAMJERNE UČINKE?

Genetičko inženjerstvo primjenjuje se u oplemenjivanju poljoprivrednih kultura u posljednjih dvadeset godina. Temelji se uglavnom na ugradnji jednog ili dvaju novih gena u genom biljaka. Do danas je odobreno za uzgoj oko 70 sorta oplemenjenih tom tehnologijom. U usporedbi sa svim tehnologijama koje se primjenjuju u oplemenjivanju bilja, genetičkim inženjerstvom postižu se najpreciznije promjene u genetičkome materijalu. Međutim, tijekom primjene te tehnologije ne može se predvidjeti mjesto u genomu u koje će se ugraditi željeni gen. Zbog toga može doći do nenamjerne aktivacije ili inaktivacije određenih gena, a to može uzrokovati nepoželjne promjene, primjerice u alergološkim ili toksikološkim značajkama. Zato se prije komercijalizacije provode iscrpne kemijske, biokemijske i nutricionističke analize svake nove sorte oplemenjene tom tehnologijom. Budući da se nenamjerni učinci mogu predvidjeti u određenoj mjeri na temelju spoznaja o mjestu u genomu u koje se željeni gen ugradio, jedan od najvažnijih ciljeva moderne biljne biotehnologije svakako je razvoj tehnika koje će omogućiti ugradnju željenih gena u unaprijed odabrano mjesto u genomu. Ta se metoda naziva "gene targeting". Za razliku od svih ostalih skupina organizama, "gene targeting" još nije metoda koja se može rutinski primijeniti u biljaka. Međutim, uzimajući u obzir nedavna postignuća na tom području, taj će se cilj vjerojatno ostvariti u bliskoj budućnosti.

KLJUČNE RIJEČI: *"gene targeting", genetički modificirani organizmi, homologna rekombinacija, oplemenjivanje bilja, procjena rizika*

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