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Safe genetically engineered plants

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Abstract

The application of genetic engineering to plants has provided genetically modified plants (GMPs, or transgenic plants) that are cultivated worldwide on increasing areas. The most widespread GMPs are herbicide-resistant soybean and canola and insect-resistant corn and cotton. New GMPs that produce vaccines, pharmaceutical or industrial proteins, and fortified food are approaching the market. The techniques employed to introduce foreign genes into plants allow a quite good degree of predictability of the results, and their genome is minimally modified. However, some aspects of GMPs have raised concern: (a) control of the insertion site of the introduced DNA sequences into the plant genome and of its mutagenic effect; (b) presence of selectable marker genes conferring resistance to an antibiotic or an herbicide, linked to the useful gene; (c) insertion of undesired bacterial plasmid sequences; and (d) gene flow from transgenic plants to non-transgenic crops or wild plants. In response to public concerns, genetic engineering techniques are continuously being improved. Techniques to direct foreign gene integration into chosen genomic sites, to avoid the use of selectable genes or to remove them from the cultivated plants, to reduce the transfer of undesired bacterial sequences, and make use of alternative, safer selectable genes, are all fields of active research. In our laboratory, some of these new techniques are applied to alfalfa, an important forage plant. These emerging methods for plant genetic engineering are briefly reviewed in this work.

1. Introduction

Agriculture is based on profound modifications of plants to make them suitable for utilization by humans. In fact, most cultivated plants are 'domesticated', that is, they are very different from their wild ancestors, and in many cases they are no longer able to survive on their own outside the agricultural environments.

Genetic improvement of plants (or plant breeding) has long been carried out by crossing selected plants and recovering useful combinations of traits in the progeny. Genetic engineering (GE) is a modern addition to the plant breeders' toolkit, that has been applied to plants since the mid-1980s. GE allows one to endow plants with genes and traits that they cannot acquire otherwise. In other words, genes from any living organism, and also synthetic genes, can now be introduced into plants to improve many economic traits and obtain new products from them. A plant in which a gene has been introduced by GE is called a genetically modified plant (GMP) or transgenic plant. The fact that the genetic code is universal allows one to transfer genes between different organisms because the encoded protein (its aminoacid sequence) will not change.

In 2005, GMPs were cultivated worldwide on more than 90 million hectares (James 2005), an area that has grown constantly since 1996. The most widespread GMPs are herbicide-tolerant soybean and canola, and insect-tolerant corn and cotton (for an updated review of plant genetic engineering methods and accomplishments see Christou and Klee (2004)).

The so-called second generation of GMPs is that of plants in which the quality of the product has been changed and improved; examples can be rice with increased provitamin A level, canola varieties with modified fatty acid composition, or lysine-rich soybean. New GMPs that produce pharmaceutical or industrial proteins or vaccines are approaching the market (Ma *et al* 2005), and they have been defined as 'third-generation' GMPs.

The two most used techniques that allow one to introduce foreign genes into plants are *Agrobacterium*-mediated transformation and the particle delivery system, or gene gun. The first method employs the natural ability of the soil bacterium *Agrobacterium tumefaciens* to transfer specific DNA sequences to the plant nuclear chromosomes. A sophisticated interaction between the bacterium and the plant cell involving tens of genes on each side results in the stable integration of bacterial sequences (transferred DNA, T-DNA) into the plant genome (Lacroix *et al* 2006). For genetic engineering purposes, the wild-type T-DNA is replaced with useful genes, and suitable strains of *Agrobacterium* introduce them into the plant genome.

With the gene gun, the DNA sequences to be delivered are attached to the surface of microscopic gold particles (0.6–1 μm diameter), that are shot into the plant cells with a helium pressure-driven machine. Once in the plant cell, the DNA is integrated into the nuclear or the chloroplast genome.

GE techniques allow a quite good degree of predictability of the results, since only one or a few genes are introduced into a given individual, so that its genome, containing several thousands of genes, is minimally modified. However, the cultivation and use of GMPs and their products have created concern and have been criticized.

In this paper, we examine the main features of GMPs that have raised concerns, and present strategies that can be adopted to make GMPs more acceptable.

2. Critical remarks to GMPs

Several aspects of GMPs have been criticized, from their biological novelty to the possible economical, social, and political implications of their cultivation. We will concentrate on some scientific aspects:

- (1) control of the insertion site of the introduced DNA sequences into the plant genome and of its mutagenic effect;
- (2) presence of a selectable marker gene (SMG) conferring resistance to an antibiotic or an herbicide, linked to the useful gene;

- (3) insertion of undesired bacterial plasmid sequences; and
- (4) gene flow from transgenic plants to non-transgenic crops or wild plants.

These features are briefly discussed and some of the available solutions presented.

2.1. Control of the insertion site of the introduced DNA sequences into the plant genome and of its mutagenic effect

When the DNA sequences are introduced into the plastid genome (plastome) the insertion site can be chosen so that mutations do not occur. Plastome genetic engineering is now applicable to several plant species (Daniell *et al* 2005) but the nuclear genome remains the commonest destination of the foreign DNA in plants. In this case, the insertion is usually random; in fact, T-DNA insertion can be used as a mutation technique to demonstrate the function of genes (see for example Alonso *et al* 2003).

Does this pose a risk? Mutations continuously occur in the genomes of all organisms. If a mutation is deleterious it tends to be selected out from populations. When transgenic plants are produced, the phenotypically normal ones are chosen, thus excluding mutations in essential genes. In any case, regulatory agencies require that the insertion site is determined before the release of GMPs, so that if the insertion occurred within or close to a known or putative gene, the effect of the insertion on the expression of that gene can be assessed.

Research on techniques that allow one to choose the insertion site in the plant nucleus is progressing (Tzfira and White 2005, Wright *et al* 2005), and the frequency with which targeted insertions are obtained was recently significantly increased in model systems. It was demonstrated that, by creating double strand breaks at a target site, the frequency with which foreign sequences are integrated at that site can be increased to levels of practical utility. Artificial endonucleases capable of cutting the DNA at any predetermined site will soon be available (Li *et al* 2007).

2.2. Presence of a selectable marker gene (SMG) conferring resistance to an antibiotic or an herbicide, linked to the useful gene

An SMG is generally introduced into the plant cells with useful gene(s) to allow only the cells that have integrated and express the foreign sequences to generate a plant. Most SMGs confer on the plant cells a resistance to an antibiotic or a herbicide that is present into the tissue culture media. The SMGs are often linked to the useful gene(s) and remain in the genetically engineered plant even though their usefulness is limited to the *in vitro* plant regeneration phase.

These genes have been a matter of concern. The presence of antibiotic resistance genes in transgenic plants might increase the probability that these genes are transferred to pathogenic bacteria (horizontal gene transfer), thus limiting the clinical use of specific antibiotics. Herbicide resistance genes have also been used as SMGs, and they are themselves useful genes in herbicide-tolerant transgenic crops. Concern has been expressed that these genes may be transferred to wild, sexually compatible plants thus giving rise to herbicide-resistant weeds. Transgenic, herbicide-resistant plants may also survive in the field, becoming weeds of the succeeding crop.

Do these SMGs pose risks? According to the vast majority of experts, they do not (Dale *et al* 2002, Gay and Gillespie 2005). However, the European Community has recommended that genes for resistance to clinically important antibiotics are no longer present in GMPs. Fortunately, many alternatives are available.

Obviously, the best solution is to avoid the presence of SMGs, that is, producing marker-free transgenic plants. This can be accomplished in two ways: (i) introducing the useful gene

without an SMG and identifying the (rare) transgenic plant individuals among the regenerated plants using the polymerase chain reaction (PCR; see for instance (de Vetten *et al* 2003, Popelka *et al* 2003)); (ii) removing SMGs after the selection of transgenic cells/plants. The latter task can be accomplished in two ways: co-transformation (reviewed in Ebinuma *et al* 2001) or post-transformation excision of SMG sequences.

Co-transformation aims at introducing the useful gene and the SMG at different chromosomal sites. To accomplish this objective each gene is inserted in a separate transformation vector, or in the same vector but in separate transferred DNA (T-DNA) regions. Plants are then regenerated under selection pressure, and consequently they will contain the SMG; among the plants obtained, those also containing the useful gene are identified by molecular analyses. If the two genes are at different chromosomal sites (independent) their separation is accomplished by crossing the transgenic with non-transgenic plants and selecting the plants containing only the useful gene among the progenies. This method is simple but requires high co-transformation efficiency and can be expensive; a limitation is that it is not easily applicable to clonally propagated plants.

Post-transformation excision of SMGs can be based on transposition (Yoder and Goldsbrough 1994), intrachromosomal recombination (ZubKo *et al* 2000) or site-specific recombination (Hare and Chua 2002). Several elegant implementations of the latter method have been recently developed, that cannot be reviewed here. In brief, the DNA sequences to be excised (containing the SMG) are flanked by short sequences that are recognized by enzymes (recombinases) that cut out the intervening DNA, that is then lost. These methods suit both sexually and asexually propagated plants.

In our laboratory, we are working on a marker-free transformation system for alfalfa (*Medicago sativa* L.), an important forage crop. The feasibility of transformation without SMGs (markerless transformation) is being estimated by using selectable genes that play the part of an useful gene, or by the use of a reporter gene. With the first approach, selection is not applied during the first round of regeneration after the *Agrobacterium* transformation treatment, but rather during a second round of regeneration, to quickly obtain an estimate of the percentage of transgenic events among all the regeneration events (somatic embryos). With the second approach, the regeneration events are screened for reporter gene expression by a histochemical reaction, and a second regeneration cycle is not required. Our first experiment, still underway, indicates that 1.4% of the embryos regenerated without selection are transgenic.

We are also attempting the co-transformation approach to marker-free alfalfa: in two experiments, 5.6 and 6% co-transformation efficiency was obtained, and independent segregation of the two genes was demonstrated in the progeny of one co-transformed plant. Co-transformation rates need to be improved for routine use of this method in alfalfa GE, and we are designing experiments with this objective.

Selection systems based on the replacement of sucrose as a carbon source in the growth media with a sugar that the plant cells are unable to utilize (xylose, mannose, galactose, or arabinose) have been successfully employed in some species (see for instance LaFayette *et al* 2005). With this approach (improperly referred to as 'positive selection') SMGs are bacterial genes that confer on the plant cell the ability to metabolize the 'unusual' carbohydrates.

Some alternative selection systems employing genes that confer resistance to phytotoxic substances other than antibiotics or herbicides are available (for a recent review see Rosellini *et al* 2006). In our laboratory, the efficiency of the *hemL* gene from *Synechococcus*, conferring resistance to gabaculine, was compared with that of the conventional antibiotic resistance gene *NptII*, and found to be significantly higher (Rosellini *et al* 2007). The gene homologous to *hemL* was isolated from alfalfa with the objective of developing a plant-derived SMG that can be considered completely safe.

2.3. Insertion of undesired bacterial (plasmid) sequences

The presence of sequences derived from non-plant organisms in the transferred DNA is considered by many a shortcoming of GMPs. Bacterial genes are expressed in all the transgenic plants presently on the market, and viral or bacterial (*Agrobacterium*) promoters and terminators drive their expression in most cases. Several research groups have developed useful genes, selectable markers, promoters, terminators, and T-DNA border sequences derived from plant genomes (see for example Rommens *et al* 2005). The search for plant sequences to replace non-plant sequences in transformation vectors appears to be worthwhile for a better public attitude toward GMPs.

For delivery into the plant cells, the DNA sequences are cloned into bacterial plasmids. A plasmid is a large circular DNA molecule that can be replicated and maintained in bacterial cells. With both *Agrobacterium* and the gene gun methods, DNA sequences flanking those of interest are often found integrated into the transgenic plant genome (Martineau *et al* 1994, DeBuck *et al* 2000, Christou and Klee 2004); these sequences are not requested for expression of the introduced useful gene.

Plastome transformation represent a solution to this problem; in fact, only the desired sequences precisely integrate into the plastid genome by homologous recombination (Maliga 2002). Any undesired sequences integrated by chance into the nuclear genome are easily removed by backcrossing the transgenic plants with an untransformed plants used as pollen parent.

For nuclear transformation, the gene gun method allows delivery of the desired, 'clean' sequences, though at an increased cost, because the cleaning of the DNA sequences requires restriction enzyme digestion of the plasmid that contains them, electrophoresis and purification of the DNA to be transferred (Fu *et al* 2000, Altpeter *et al* 2005). In the case of *Agrobacterium* transformation, understanding of the mechanisms of the generation of the DNA fragment transferred to the plant cell (T-DNA) is not yet complete, but recently new insight has been gained on the functioning of T-DNA border sequences that can improve the process by reducing the frequency of undesired transfer of plasmid sequences flanking the T-DNA region (Podevin *et al* 2006). Site-specific recombination can also be exploited for the removal of unwanted bacterial plasmid sequences. In any case, selection of 'clean' transgenic plants through molecular techniques and sequencing of the DNA regions flanking the insert is required before the release of transgenic plants.

2.4. Gene flow from transgenic plants to non-transgenic crops or wild plants

In one way or another, transgenes can 'escape' the cultivated field. Hybridization with wild, sexually compatible plants has been observed in the case of canola (*Brassica napus*) and red fescue (*Festuca rubra*). This is considered not dangerous for transgenes that are on the market today, but it may become risky when the transgenes encode for pharmaceutical proteins. This emerging technology holds the promise of providing less expensive drugs and vaccines, but unintended exposure to such proteins should be absolutely avoided.

Several ways to prevent or reduce gene flow are available. The genetic strategies are discussed by Daniell (2002) and Lee and Natesan (2006). An effective gene containment approach is the introduction of the gene(s) into the plastome. Because in most crop plants plastid inheritance is strictly maternal, the transgene is not transmitted to the progeny through pollen (that flies away from the cultivated field), but only through the egg cells (that remain in the cultivated field). Plastome transformation provides a good gene containment level.

A second system is the so-called 'genetic mitigation' approach, that relies upon the introduction, linked to the useful gene, of a second gene that decreases the fitness (the ability

to survive and reproduce in the wild) of the plants that originate from the hybridization of crop plants with wild relatives. This system was modelled by Haygood *et al* (2004) and appears to be very effective, at least when the useful gene does not significantly increase the fitness of the hybrids.

A third group of methods, collectively defined ‘Genetic Use Restriction Technology’ (GURT) or ‘terminator’, prevent gene escape by inhibiting seed germination of the transgenic plants. This technology was strongly criticized for its possible negative impact on agriculture of less developed countries, and has not been implemented; however, it can be very useful in special cases, for example for GMPs producing bioactive molecules, such as vaccines, that necessitate very strict containment.

Pollen sterilization and flowering control are other containment strategies that have had definitive experimental demonstration and also commercial applications.

3. Conclusions

Based on the available technology and fast research developments, it can be stated that, in the near future, GMPs will be designed in such a way that the introduction of unwanted sequences, the randomness of DNA integration and the risks of gene escape will be minimized or avoided.

The research efforts toward ‘cleaner’ plant GE is providing insight into basic mechanisms of DNA recombination, mutation, bacteria–plant interaction and nuclear–plastid gene interaction. In the near future, our ability to control the genetic transformation process will result in GMPs in which the modifications of the genome are minimal and completely known, so that unexpected effects are largely avoided. This will hopefully imply higher *perceived* safety of GMPs. In our opinion, genetically engineered plants have received undeserved bad press, especially in Europe, and public perception of their risks and benefits should change to the advantage of the latter. In Europe, the development of plant biotechnology research and industry has been slow, mostly due to an unfavourable public attitude. A ‘European way’ to GMPs that takes into account their public perception may help to catch up.

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