Review Article

The biosafety of molecular farming in plants

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Abstract

Molecular farming in plants provides an inexpensive and convenient way to produce biopharmaceutical molecules and other valuable proteins on a large scale. Proof of principle has been demonstrated for a large number of recombinant human proteins, subunit vaccines and antibodies (Schillberg *et al*., 2002a, b), and detailed cost evaluations have been described for the production of technical proteins in corn (Kusnadi *et al*., 1997; Evangelista *et al*., 1998). In terms of biosafety, plants have several advantages over traditional production systems, such as the absence of endotoxins, human pathogens and oncogenic DNA sequences. However, other biosafety concerns remain to be addressed. These not only reflect the quality and safety of the final product, but also the wider effects of molecular farming on health and the environment. In this review we discuss the technological basis of molecular farming in plants and identify potential biosafety risks: transgene spread in the environment, recombinant protein accumulation in the ecosystem, contamination of food and feed chains with transgenes and their products, and product quality and safety.

Introduction

Molecular farming is the use of crops for the large-scale production of valuable recombinant proteins. Currently, pharmaceutical proteins are the major focus of this emerging technology (Fischer and Emans, 2000; Daniell *et al*., 2001b; Fischer *et al*., 2003) but plants can also be used to produce food and feed additives, biopolymers, industrial enzymes and research-grade technical proteins such as avidin and β-glucuronidase (Hood, 2002). Plants have many practical and economic advantages over traditional production systems, while edible crops in particular are regarded as among the safest production systems for pharmaceutical proteins (Schillberg *et al*., 2002a,b; Fischer *et al*., 2003). In the vast majority of cases, molecular farming involves the use of transgenic plants, i.e. plants with novel genes stably incorporated into the nuclear genome. Therefore, transgene pollution and recombinant protein toxicity in the environment are safety concerns that must be addressed. Another biosafety issue is the possibility that food and feed crops containing recombinant proteins could contaminate unmodified crops or the environment during harvesting, transport, processing and/or waste disposal, and therefore enter the food chain. Finally, all plant-derived recombinant proteins that are administered to human patients must be compared to their native counterparts and rigorously tested for safety. In this review, we discuss the technological basis of molecular farming in plants, the associated biosafety issues and what is being done to address them.

Molecular farming in plants

Economic advantages over traditional expression systems

Molecular farming in plants has a relatively short history, and the commercial adoption of this technology occurred very recently. Part of the reason for this was initial scepticism in the scientific community that plants would ever become useful expression hosts for human proteins, particularly with the availability of numerous proven alternatives in which proteins are isolated from large-scale cultures of bacterial, yeast or animal cells, or from the milk of transgenic animals. However, plant-based production systems have advantages over all the traditional methods and are now being used not only for the production of valuable pharmaceutical proteins, but also for the production of technical proteins where markets are already established (Kusnadi *et al*., 1997; Hood, 2002). From a commercial point of view, one of the major benefits of plants is the reduced set-up and running costs (Raskin *et al*., 2002). There is no

requirement for expensive equipment, media or skilled personnel. Once a transgenic plant line has been produced, it can be maintained, harvested and processed using existing agricultural infrastructure and unskilled labour. Furthermore, while scaling up production in fermenter-based systems and transgenic animal herds is expensive and limited in scope, production systems based on plants can be scaled up and down simply by increasing or decreasing the amount of cultivated land according to the demands of the market. Most of the costs associated with molecular farming in plants are due to post-harvest processing and compliance with regulatory procedures.

Product safety compared to traditional expression systems

In terms of product safety, animal-based expression systems run the risk of harbouring pathogens and undesirable DNA sequences (e.g. oncogenes) which could contaminate the end-product and pose a threat to human patients. Similarly, recombinant proteins produced in bacteria could be contaminated with endotoxins. Such undesirable entities are generally absent from plants, and this makes plants particularly suitable for the production of protein therapeutics and uniquely suitable for the production of edible vaccines, i.e. subunit vaccines that are expressed within edible plant organs such as fruits and seeds. However, there may be other contaminants that need to be removed before a recombinant protein can be administered to human patients. These include toxic metabolites (such as the alkaloids produced in many tobacco cultivars), allergens and field chemicals such as pesticides and herbicides.

Although separated by millions of years of evolution, the machinery for protein processing in animals and plants is remarkably conserved. Thus, recombinant mammalian proteins produced in plants are folded, assembled and modified in very similar ways to their native counterparts, and are in most cases functionally equivalent. There are, however, certain differences in glycan chain structure that could affect the performance and/or safety of recombinant proteins administered to humans. The biosafety issues concerning plant-specific glycan chains are discussed in more detail later. It should be noted that differences in glycan structures are present when comparing native human proteins and recombinant proteins produced in any eukaryotic expression system. These differences are particularly evident in yeast and insect cells but occur even when murine cells are used as expression hosts.

The diversity of plant-based expression systems

Various plant-based expression technologies can be used for molecular farming, but in most cases the production crop is generated by stable gene transfer to the plant nuclear genome (Giddings, 2001). This is achieved by transformation using either the soil pathogen *Agrobacterium tumefaciens* or by direct transfer procedures such as electroporation or particle bombardment. Prior to transfer, the gene encoding the desired protein is inserted into a suitable expression construct that includes a promoter, regulatory elements that ensure efficient RNA processing and protein synthesis, and a polyadenylation signal. Constitutive promoters are widely used but there are many benefits to the use of restricted promoters. For example, the expression of recombinant proteins specifically in cereal seeds excludes those protein from vegetative organs (a useful strategy if the protein interferes with normal plant growth) and offers enhanced protein stability brought about by desiccation, which facilitates long-term storage. Restricted promoters, as discussed later, also offer biosafety advantages by limiting protein toxicity effects in the environment. While regulatory elements, particularly the promoter, are major considerations in expression construct design, the coding region of the transgene may need to be optimized in order to match the codon preferences of the expression host (especially if the transgene is derived from a prokaryotic source). Finally, due consideration should be given to the destination of the expressed protein. Complex proteins should be directed to the secretory pathway because this provides the most appropriate molecular environment for folding, the formation of disulfide linkages and assembly. It is also the only subcellular compartment within which glycosylation occurs. Targeting is achieved by including in the expression construct an N-terminal signal sequence, which allows the ribosome to dock to receptors on the endoplasmic reticulum (ER). In addition, a C-terminal retrieval signal (e.g. KDEL) can be included so that the recombinant protein accumulates in the ER rather than being secreted to the apoplast (reviewed by Schillberg *et al*., 2002a, b).

A number of crop species have been developed as hosts for molecular farming and each has advantages and disadvantages for different applications (see Stoger *et al*., 2002). Tobacco is a popular choice because of the high total yields that can be achieved, reflecting the large amount of biomass produced in several annual harvests. However, as stated above, a disadvantage associated with many tobacco cultivars is the production of toxic alkaloids, which must be removed during the post-harvest processing steps. This disadvantage is not shared with alfalfa, a feed crop with a similarly high biomass yield. However, the technology for gene transfer and expression in alfalfa lags behind that of tobacco so this crop is not so widely exploited. As stated above, cereals are advantageous because recombinant proteins can be directed to accumulate in dried seeds, which can be stored for several years at ambient temperatures with no loss of protein activity. Conversely, proteins expressed in leafy crops such as tobacco can be very unstable and the leaves must be dried, frozen or processed immediately after harvest. Fruit and vegetable crops are beneficial because recombinant proteins accumulating in edible storage organs can be administered to humans directly with minimal processing. This strategy has been explored in species such as tomato, banana and potato for the delivery of edible vaccines. Oil and fibre crops may be economically advantageous for the synthesis of some proteins because the production costs are offset to a certain degree by the added revenue derived from their secondary products. However, these benefits must be weighed carefully against the disadvantages brought about be the interference of oils and fibres in downstream processing.

Alternatives to transgenic plants are desirable not only for biosafety reasons, but also because long development times are often required to establish the first generation of transgenic producer lines - particularly if problems are encountered with the stability of transgene expression. Several systems have been developed which not only allow the rapid expression of recombinant proteins but also appear to limit the likelihood of transgenes escaping into the environment. These systems, discussed in more detail below, include tobacco transient expression by agroinfiltration, cell suspension cultures, virus-infected plants and transplastomic (chloroplast-transformed) plants (Schillberg *et al*., 2002a, b).

Biosafety issues in molecular farming

The challenges

Interest in biosafety implies the existence of potential risks. Two important questions therefore need to be asked when addressing the biosafety issues in molecular farming:

- Who, or what, is at risk from molecular farming?
- What biological hazards are involved?

Because molecular farming is used to produce pharmaceutical and technical molecules in genetically modified plants, there are two

separate categories of risk. The first applies to all transgenic plants whether or not they are used for molecular farming and concerns the risk that transgenes and/or their encoded proteins could spread in the environment and that non-target organisms could be affected. Ultimately, humans could be affected by the consumption of food containing such genes or recombinant proteins. This is the issue of *gene and protein pollution*. The second category applies to all pharmaceutical products and concerns the risk that such products could be harmful in the human or animal patients to which they are administered. This is the risk of *product safety*. We address these issues below and outline strategies that can be used to reduce the level of risk involved.

Transgene pollution – the problems

Transgene pollution is the spread of transgenes beyond the intended genetically-modified species by natural gene flow mechanisms. The transgenes might spread from transgenic to non-transgenic populations of the same crop species growing nearby, from transgenic crops to related wild species, or even from transgenic plants to microorganisms and animals. Transgene pollution is undesirable because the impact of novel genes on the performance and fitness of other species is unpredictable, and in the worst case scenario could severely disrupt natural and agricultural ecosystems, even leading to extinction in some species. The spread of transgenes to 'natural' crops, i.e. crops that were never intended to be modified, is also undesirable because this provides an early opportunity for foreign DNA sequences and their products to enter animal and human food chains. A further reason for concern is that the spread of proprietary transgenes into wild species places intellectual property in the public domain. For molecular farming applications, two classes of transgene pollution need to be considered:

- The possible spread of primary transgenes, i.e. genes encoding the recombinant proteins intended to be produced in the transgenic plants. The impact of such genes on the survival and fecundity of wild species would be difficult to predict, as would be their effects on the balance of natural ecosystems. However, it is clearly undesirable for proteins, which are designed to have a specific physiological effect when administered to humans or animals, to be expressed in natural populations of plants and microorganisms, or in crops intended for the human and domestic animal consumption.
- The possible spread of superfluous DNA sequences, e.g. selectable marker genes and fragments of the vector backbone. Much of the concern over GM plants has centred on the risk of herbicide resistance markers spreading to weeds and antibiotic resistance markers spreading to human and animal pathogens. There is also concern that most plant transformation procedures are 'messy', i.e. much more genetic information than actually required is introduced into the plant genome, and this may be accompanied by deletions and other modifications to the host plant's DNA. The spread of any recombinant DNA sequence is undesirable because of the unpredictable and potentially negative impact on other organisms and their inter-relationships.

Transgene pollution – the mechanisms

Gene flow from transgenic plants to other organisms has been shown to follow two distinct routes:

Vertical gene transfer

Vertical gene transfer is the movement of DNA between plants that are at least partially sexually compatible, and crops for molecular farming should be chosen with the minimization of gene flow in mind (Box 1). This is the most prevalent form of transgene pollution and occurs predominantly via the dispersal of transgenic pollen, resulting in the formation of hybrid seeds with a transgenic male parent (Eastham and Sweet, 2002). Gene flow from transgenic to non-transgenic populations of the same crop could occur by this method if the two populations were close enough for wind- or insect-mediated pollen transfer. Very high rates of gene flow from crops to related wild species have also been documented along this route. For example, King (1996) noted that 50% of wild strawberries growing near a field of cultivated transgenic strawberries contained marker genes from the transgenic population. Similarly, herbicide resistance genes have introgressed from transgenic oil seed rape to its weedy cousin *Brassica campestris* by hybridization (Mikkelsen *et al.* 1996). As discussed below, a number of potential solutions to the problem of transgene pollution have been based on preventing the spread of transgenic pollen, either by physical or genetic containment. However, hybrid seeds could also be generated with the transgenic plant as the female parent if the transgenic crops were fertilized by wild type pollen. In this case, transgene pollution would occur via seed dispersal, either during growth, harvesting or during transport. Seed dispersal from fully transgenic plants could also result in the colonization of natural ecosystems.

Horizontal gene transfer

Horizontal gene transfer is the movement of genes between species that are not sexually compatible and may belong to very different taxonomic groups. The process is common in bacteria, resulting in the transfer of plasmid-borne antibiotic resistance traits from harmless species or strains to pathogenic ones, but there are few examples of natural gene transfer between bacteria and higher eukaryotes. *Agrobacterium tumefaciens* and related species represent a special case where gene transfer occurs naturally from bacteria to plants. There is a perceived risk that horizontal gene transfer from transgenic plants to bacteria in the soil or in the digestive systems of herbivores could yield new bacterial strains expressing marker genes and/or primary transgenes. These traits could have unpredictable effects on relationships between different organisms, e.g. they could render harmless bacteria pathogenic, or could be passed on to pathogenic species making them more difficult to control. There is a specific concern that antibiotic resistance markers and transgenes encoding pharmaceutical proteins could be acquired by human pathogens.

The risks of horizontal transgene transfer from plants to microbes are considered to be vanishingly small because of the lack of evidence, over millions of years of evolution, that natural plant genes have followed this route. Recently, Kay *et al*. (2002) have demonstrated horizontal transfer of marker genes from the chloroplasts of transplastomic tobacco plants to opportunistic strains of *Acinetobacter* spp., but transfer was achieved only under highly idealized conditions in which the bacteria were modified to contain a sequence homologous to the plant's transgene. No gene flow was demonstrated to wild type strains of the bacterium. Even if gene transfer from plants to bacteria

Box 1. Biosafety issues: what not to look for in a platform crop for molecular farming

- abundant pollen production
- abundant seed production
- small, easily dispersed seeds
- important food/feed crop
- widely planted throughout the world
- often grown as open-pollinated varieties
- spontaneously mates with wild relatives
- high frequency of gene flow by outcrossing

did occur in nature, it would be necessary for the transgene to be maintained in the recipient bacterial population. In the case of antibiotic resistance markers there might be strong selective pressure for transgene maintenance due to the widespread use of antibiotics. However, since all natural plants are already liberally covered with antibiotic-resistant bacteria, these would seem to be a much more likely source of resistance genes that could jump to human pathogens (Smalla *et al*., 2000). It has been demonstrated that DNA can be taken up from saliva by oral bacteria, and that cells lining the gastrointestinal tract can take up and incorporate DNA from the gut. Again, however, there is a conspicuous lack of evidence that such mechanisms have resulted in the stable incorporation of a plant gene into a bacterial population. Studies with glyphosate-resistant transgenic plants showed that the DNA was completely digested in the gastric environment within a few minutes. Antibiotic resistance genes are the focus of attention because of their potentially strong and general selective advantage in human pathogens. Other transgenes, with much more specific therapeutic applications, would not provide the same benefits as antibiotic resistance and would likely be eliminated even if transfer from plants to bacteria were inevitable. These seemingly insurmountable barriers indicate that horizontal gene transfer is unlikely to represent a significant hazard, and biosafety research has therefore focused on ways to prevent transgene pollution by vertical transfer.

Transgene pollution – possible solutions

The prevention of transgene pollution requires gene flow from transgenic plants to be abolished and there are two ways to achieve this. In the case of marker genes and other superfluous sequences, which serve no useful purpose once a line of transgenic plants has been generated, one approach is to eliminate the genes (or never incorporate them in the first place) and therefore remove the source of pollution (Hohn *et al*., 2001; Hare and Chua, 2002). This strategy cannot be used to prevent primary transgene pollution since the primary transgene, encoding the recombinant protein of interest, must be maintained in the transgenic population. Here the only solution is containment, which may be achieved by physical or genetic means.

Minimum required genetic modification

The standard method for producing a transgenic plant line is to introduce the primary transgene along with a selectable marker, which allows the propagation of transformed plant material at the expense of nontransformed material. The use of selectable markers is perhaps one of the major issues in biosafety, since traditional markers which exploit herbicide or antibiotic resistance as selectable traits each represent significant environmental or health threats. It is also standard practice to transform plants with plasmid vectors containing the expression cassette. This results in the integration of vector backbone sequences along with the functional transgenes. Not only are such sequences superfluous to requirements, but they also have numerous undesirable effects in transgenic plants, acting as triggers for *de novo* methylation and promoting extensive rearrangement of the foreign DNA sequences prior to integration (Kohli *et al*., 1999). They may also carry additional functional DNA sequences such as selectable markers, promoters and origins of replication used in bacteria, which could become active in environmental organisms polluted by gene flow.

Ideally, it would be possible to produce transgenic plants carrying just the primary transgene, without recourse to marker genes and other superfluous sequences (Box 2). However, the negative impact of these sequences have been established only in the last few years, and only recently have efforts been made to dispense with them. In the case of *Agrobacterium*-mediated transformation, it has been realized that inefficiency in the T-DNA processing step results in the co-transfer of vector sequences in 30-60% of transformation events depending on plant species, *Agrobacterium* strain and transformation method (Wenck *et al*., 1997). Since plasmids are prerequisite for this mode of gene transfer, the only way to guarantee clean transformation (transformation without vector sequences) is to flank the T-DNA with counterselectable marker genes that kill any plant cells containing them (Ramanathan and Veluthambi, 1995; Kononov *et al*., 1997). With direct DNA transfer methods, vector sequences are generally present in all transformants because whole plasmids are used in the transformation procedure. An efficient and practical alternative is to carry out transformation using minimal cassettes, i.e. linear constructs containing just the promoter, open reading frame and polyadenylation signal (Fu *et al*., 2000). Not only does this avoid vector backbone integration but it appears to address another problem specific to direct transfer methods, which is the formation of large, highly complex, multicopy transgene loci containing many rearrangements (Kohli *et al*., 1999; Jackson *et al*., 2001; Twyman *et al*., 2002). Such loci are undesirable because they tend to be unstable, and in many cases contain inverted repeats or truncated transgenes that have the potential to form DNA secondary structures or to express hairpin RNAs, both of which can trigger transgene silencing (Plasterk and Ketting, 2000; Hammond *et al*., 2001). In contrast, transformation with minimal cassettes leads to the generation of very simple integration patterns with the majority of transgenic loci represented by a single transgene copy (Fu *et al*., 2000).

Dispensing with selectable markers is more difficult because stable transformation is a rare event and markers are required to identify the very few transformed plant cells in a large background of nontransformed ones. It is possible, although quite laborious, to screen plant cells for the incorporation of a primary transgene using the polymerase chain reaction, without relying on any type of marker. However, most 'marker-free' transformation strategies involve removal of selectable markers *after* transformation has been achieved (Box 2). An alternative approach is to use an innocuous scorable marker gene such as *gusA* (encoding the bacterial enzyme β-glucuronidase) or *gfp* (encoding the jellyfish green fluorescent protein). Even better, a bacterial gene or preferably a plant gene can be used as an innocuous selectable marker, i.e. a gene that would have no conceivable negative effects in wild populations. Examples of such markers include growth regulators (e.g. *ipt* or *CKI1*) and metabolic markers (e.g. *manA* or *BADH*) under inducible control. Such markers could be used to restrict the growth of plants under non-permissive conditions but would not affect the growth or reproduction of wild plants (Zuo *et al*., 2000; Daniel *et al*., 2001a).

Box 2. Transformation strategies to eliminate non-essential sequences

Elimination of vector backbone
- Use counterselectable markers (*Agrobacterium*)
- Clean DNA transformation (bombardment)

Elimination of marker genes
- Marker-free transformation (detection by PCR)
- Independent cointegration and segregation
- Transposon-mediated repositioning and segregation
- Marker excision by site-specific recombination (e.g. Cre-*loxP*, FLP/*frt*)
- Marker excision using the λ *attB* system
- Use of innocuous genes if markers cannot be avoided

Simplification of transgenic loci
- Position-specific transgene integration
- Screening for low-copy-number transformants
- Clean DNA transformation to favour simple locus structure
- Post-transformation locus streamlining by site-specific recombination

Elimination of non-essential genetic information

Where the use of conventional markers is inescapable, an acceptable strategy is the elimination of these genes after transformation, leaving transgenic plants containing the primary transgene alone. This can be achieved either by segregation or recombination, the former requiring independent cointegration of the marker and primary transgene and the latter requiring the use of site-specific recombination systems such as Cre-*lox*P or FLP-*FRP*.

It is surprisingly difficult to persuade separate transgenes to integrate at different loci allowing segregation in later generations. Where two separate plasmids are used to coat microprojectiles, cointegration at the same locus is the predominant outcome (usually as a highly complex concatemer). The introduction of separate binary vectors into *Agrobacterium*, and even the use of different *Agrobacterium* strains for co-infection, also generally results in co-integration, although this depends on the strain. For example, Komari *et al*. (1996) were able to achieve marker gene segregation in a small number of R_1 transgenic plants following a transformation strategy involving co-infection with two different *Agrobacterium* strains. More recently, it has been shown that particle bombardment with minimal cassettes can yield a large number of independent cointegration events, resulting in efficient marker gene segregation in later generations (Pawan Agrawal, pers. comm.). An alternative and rather elegant way to achieve the same goal is to clone the primary transgene and marker gene in a single construct, but enclose the marker gene within the active elements of a transposon such as *Activator*. Integration is followed by transposition, resulting in the relocation of the marker gene to a different genomic site. As discussed above, the marker can then be eliminated by crossing (Goldsbrough *et al*., 1993).

The need for crossing can be avoided by building a marker excision strategy into the transformation construct. In most cases, this involves the use of a two-component site-specific recombination system such as Cre-*loxP* (Dale and Ow, 1991). Cre is a recombinase that recognizes short sequences known as *loxP*. If two *loxP* sites are in the same orientation, Cre recombinase activity will excise any DNA between them, so marker genes flanked by *loxP* sites can be efficiently excised from transgenic plants if Cre is present. Cre can be expressed transiently (Gleave *et al*., 1999) or crosses can be carried out between primary transgenic lines and Cre-transgenic lines to generate hybrids containing both *cre* and the *lox*P-flanked marker, allowing the marker gene to be removed. Where this strategy is used, further crossing is required to remove the *cre* transgene. More recently, the *attB* system from bacteriophage λ has been developed for use in transgenic plants because spontaneous recombination occurs at a high frequency, leading to marker removal (Zubco *et al*., 2000).

Site-specific recombination has also been used to reduce the complexity of multicopy transgenic loci generated by particle bombardment. As discussed above, such loci are prone to transgene silencing and structural instability, and are unsatisfactory from a biosafety perspective because the complex organization means that uncharacterized transcripts and proteins could be produced with unpredictable effects. Simplification is possible either by inserting the transgene at a predefined locus or by streamlining the locus structure after transformation. Both these processes can be achieved using a site-specific recombination system such as Cre-*lox*P. Site-specific integration of transgenes can occur if the genome contains a recombinase recognition site such as *lox*P that has been introduced in a previous round of transformation. Transgene integration occurs at a low efficiency if an unmodified recombination system is used because the equilibrium of the reaction favours excision. However, high-efficiency Cre-mediated integration has been achieved in tobacco using mutated *lox*P sites (Albert *et al*., 1995). Post-integration locus simplification in transgenic wheat has been achieved by incorporating a single *lox*P site within the transgene. Cre expression facilitated recombination between the tandemly-arranged *lox*P sites until only one remained, reducing the transgenic locus to a single copy. This resulted in increased transgene expression accompanied by reduced methylation at the transgenic locus (Srivastava *et al*., 1999).

Containment of essential transgenes

For indispensable primary transgenes, the only way to avoid transgene pollution is by containment, which aims to prevent seed and pollen dispersal, prevent the survival of dispersed seeds and pollen, or prevent gene flow from viable pollen. The containment may be physical and artificial (i.e. transgenic plants can be maintained in greenhouses or the flowers/fruits may be concealed in plastic bags in the field) or it may be based on isolation (a sufficient distance between the transgenic crop and any potential recipients of the pollen will minimize the chances of outcrossing). Isolation zones around transgenic crops can be barren, but a more suitable alternative for insect-pollinated crops is to provide a zone of non-insect-pollinated plants which would discourage the insects from leaving the transgenic zone. Barrier crops, i.e. a border of non-GM plants of the same species as the transgenic crop, are also useful as these can absorb much of the pollen released by transgenic plants and can then be destroyed after flowering.

Biological containment provides an additional barrier to gene flow and many different strategies have been employed (Box 3). In some cases, natural genetic barriers have been exploited. For example, molecular farming in self-pollinating species (e.g. rice, wheat, pea) or crops with no sexually compatible wild relatives near the site of production provide a first level of defence against gene flow. Cleistogamy (self-fertilization before flower opening) is an extension of the above, and could be engineered into crops used for molecular farming by modifying the architecture of flower development. In practice, however, there is always a residual risk of outcrossing. Another potential strategy, yet to be fully explored, is the exploitation of apomixis (embryo development in the absence of fertilization). Transformation strategies can also be adapted to take advantage of natural barriers. An example of this approach is genomic incompatibility, which is suitable for polyploid plant species. Many cultivated crops are polyploid but have distinct genomes, only a subset of which are compatible with related wild species for interspecific hybridization. In the case of wheat, only the D genome is compatible with wild Aegilops species. Therefore, wheat plants used for molecular farming should carry the transgene(s) on the A or B genomes, a fact that can be established by fluorescence in situ hybridization (FISH).

Box 3. Biological containment methods to prevent transgene pollution

- Use of self-pollinating crops, exploitation of cleistogamy
- Asynchronous flowering times, atypical growing seasons
- Use of crops lacking wild relatives that are compatible for hybridization
- Strengthening of hybridization barriers between compatible species
- Apomixis
- Interference with flower development
- Male sterility (interference with pollen development)
- Seed sterility
- Maternal inheritance (plastid transformation)
- Transgene integration on incompatible genomes
- Transgenic mitigation
- Conditional transgene excision

These natural mechanisms may be replaced or augmented with artificial strategies, which include male sterility, chloroplast transformation, conditional transgene excision and transgene mitigation. Male sterility is achieved by interfering with pollen development, often through the expression of a ribonuclease that prevents the differentiation of the male reproductive organs (e.g. Mariani et al., 1990). For example, Bayer Crop Sciences have developed and commercialized a male-sterile variety of oilseed rape expressing barnase. The barnase inhibitor (barstar) is also expressed, but it is controlled by an inducible promoter allowing propagation of the transgenic line under laboratory conditions but not in the field. This strategy prevents outcrossing by pollen dispersal but not by pollen immigration, so there remains the possibility of transgene pollution by seed dispersal.

An alternative to male sterility is chloroplast transformation, i.e. the introduction of foreign DNA into the chloroplast genome rather than the nuclear genome. This limits gene flow because there are no chloroplasts in the pollen of most crop plants, and in some species the chloroplast genome is degraded during generative and sperm cell development. There are several advantages to molecular farming by chloroplast expression in addition to the biosafety benefits, including the high yields of recombinant protein that are possible, the absence of position effects and transgene silencing phenomena which can frustrate molecular farming enterprises in nuclear transgenic plants, and the opportunity to carry out multigene engineering using operons (Daniell *et al*., 2002). Thus far, the technology is limited to tobacco, tomato and potato, although transformed chloroplasts in these species have been used successfully for the production of diverse products, including biopolymers, vaccines and human growth hormone (see recent review by Maliga, 2003). One possible disadvantage is that proteins produced in chloroplasts are not glycosylated so this system cannot be used for the production of complex glycoproteins (Daniell *et al*., 2002). It is also notable that chloroplast inheritance is not strictly maternal in some species, so while gene flow by pollen dispersal may be staunched, it may not be eliminated. As with male sterility, chloroplast transformation does not prevent transgene pollution by volunteer seed dispersal.

Another genetic barrier to transgene flow is seed sterility, which is achieved by using suicide genes to destroy the developing plant embryo. This mechanism is employed in Monsanto's notorious 'terminator technology' in which a ribosome inhibitor protein is expressed under the control of an embryonic promoter, but in a manner that is regulated by tetracycline. Among several variations of the technique originally discussed in a patent application assigned to Pine Land Corporation, one involved the tetracycline-depended expression of Cre recombinase, which would lead to the excision of the suicide gene if it was flanked by *lox*P sites. A 'recoverable block of function' system, based on the constitutive expression of barnase and the inducible expression of barstar, has also been developed (Kuvshinov *et al*., 2001).

Transgenic mitigation can also be used to prevent the spread of transgenes to wild plants. This involves the inclusion of a tightly linked transgene that confers a trait that is selectively neutral to the crop but disadvantageous to wild plants. Examples might include dwarfing genes or genes that control seed dormancy or shattering. A more sophisticated strategy is conditional transgene excision. In this strategy, plants are created with the transgene flanked by *lox*P sites. A *cre* transgene is also present, and this is expressed under the control of a cell-specific or inducible promoter, such that the transgene is physically removed before flowering. If the *cre* transgene is also present within the *lox*P sites, then this transgene will be removed from the plant at the same time, but only when its task is complete. One potential drawback of this approach is that incomplete transgene excision will leave a residual population of transformed cells from which gametes could arise.

Alternative production systems

As stated above, the problem of gene flow from transgenic plants reflects the fact that the transgenes are stably incorporated and form part of the plant's genome. As well as transgenic plants and transplastomic plants, however, it is possible to produce recombinant proteins by transient expression (Kapila *et al*., 1996) or through the use of viral vectors (Porta and Lomonossoff, 2002). In each of these cases, there is no permanent modification. Transient expression is carried out using tobacco leaves that have been infiltrated with *Agrobacterium*, while plant viruses do not stably integrate into the plant genome. Transient expression of therapeutic proteins has been shown to be feasible on a laboratory scale (Fischer *et al*., 1999b). A further alternative is the use of plant suspension cultures in sealed, sterile reactor vessels (Fischer *et al*., 1999a; Doran, 2000). The advantages of containment may be outweighed by the increased production costs reflecting the need for expensive equipment and skilled personnel, but for the farming of valuable pharmaceuticals such an investment may be justified.

Protein pollution – the problems

The recombinant proteins produced by transgenic plants constitute another form of pollution and represent a further threat to the environment. One immediate concern is the possible negative effect of recombinant proteins on non-target organisms, particularly insects and microorganisms that interact directly with the plant and herbivores that may eat transgenic plant material laced with biopharmaceutical molecules. Such proteins might have direct toxicity effects, or they might accumulate in the food chain and therefore affect animals that do not interact with the transgenic plants at all. Toxicity may result from direct consumption (e.g. the ingestion of toxins by aphids, and knock-on effects to ladybirds and birds further up the food chain), by simple exposure to the plant (e.g. the effects of pollen on butterflies and moths), from the exudation of recombinant protein into the rhizosphere or leaf guttation fluid (most likely to affect microorganisms) and by the consumption of dead and decaying plant material by saprophytes. Many recombinant proteins expressed in plants are directed to the secretory pathway in order to fold or assemble properly. Such proteins accumulate in the apoplast, the space beneath the cell wall, but there is some leakage into the guttation fluid and root exudate that may change the biochemical environment on the exposed leaf surface or in the rhizosphere. The long-term effects of recombinant pharmaceuticals accumulating in the soil and in drainage water have not been investigated and provide scope for all manner of unseen hazards. Finally, the processing of transgenic plants will produce waste containing residual recombinant proteins. An important biosafety issue, particularly for large-scale enterprises, is what to do with this waste plant material. A pertinent danger is that such material will be allowed to decay in the environment, providing further opportunities for both protein pollution and transgene escape.

Protein pollution – possible solutions

Like transgene pollution, protein pollution can be addressed to a certain extent by physical containment, since this restricts the impact of protein toxicity to a very localized environment and limits knock-on effects to non-target organisms. However, further barriers to protein toxicity can be put in place by controlling transgene expression, and therefore limiting the availability of the protein to interacting organisms.

Controlling gene expression

The exposure of non-target organisms to recombinant proteins can be minimized by restricting expression to particular tissues. For example, a number of promoters have been identified that restrict gene expression to seeds or fruit. This would prevent the consumption of the protein by insects and other animals feeding on green plant tissue and would likewise avoid other forms of contact, such as the exposure of pollinating insects to recombinant proteins expressed in pollen grains. By avoiding transgene expression in roots, leaching of the recombinant protein into the soil (and consequent disruption of the rhizosphere) would also be prevented. If restricted expression strategies were used in combination with effective management (e.g. specific harvesting times) then vegetative transgenic material could safely decay in the environment with no risk of protein toxicity. An alternative strategy is to bring the transgene under inducible control, such that the recombinant protein would be expressed only when the plant was exposed to a certain chemical inducer (Zuo and Chua, 2000). One of the most promising developments in this area is the use of inducible expression systems to prevent recombinant protein expression until after the crop has been harvested, as has been shown for recombinant glucocerebrosidase (Cramer *et al*., 1999). An effective waste-management policy would then be necessary for the waste generated by product processing and extraction.

Controlling protein accumulation and activity

In addition to the control of gene expression, the protein can also be targeted to a specific intracellular compartment. This would not necessarily protect herbivores from exposure to the protein, but it might limit adventitious contact. For example, by adding a KDEL tetrapeptide sequence to the C-terminus of the recombinant protein, there is efficient retrieval from the Golgi apparatus to the endoplasmic reticulum (ER) (Munro and Pelham, 1987). This helps to prevent proteins being secreted to the apoplast, phloem or xylem, where contact with the plant's environment, including microbes and insect pests, becomes more likely. This is also the case for recombinant proteins containing a heterologous transmembrane domain, which are anchored in the plasma membrane or in the vacuolar membrane depending on other targeting information (Schillberg *et al*., 2001). The chloroplast or vacuole are alternative destinations for recombinant proteins produced in plants (Jiang and Sun, 2002). Recombinant proteins can also be produced as inactive precursors that have to be processed by proteolytic cleavage before they attain full biological activity. This strategy, which was used for the expression of hirudin, not only limits the extent of protein toxicity in the environment, but also protects the host plant from any negative effects the recombinant protein might have on growth or development.

Contamination of the food chain during processing

We have discussed ways in which transgenic plant material, or products derived therefrom, could enter the food chain of humans or domestic animals. These include transgene spread to food and feed crops, contact between transgenic plants and non-target organisms, adventitious herbivory of transgenic plants and leaching of recombinant proteins into the environment through poor waste management. Another major source of contamination is the unintentional mixing of transgenic and non-transgenic crops during harvesting, transport, refining and processing, which has resulted in some highly-publicized incidents including the discovery of recombinant DNA in Linda McCartney organic food products and the discovery of unregistered StarLink maize in maize products. A more pertinent example in molecular farming is the recent ProdiGene incident in which stray maize plants expressing pharmaceutical proteins were found growing among a soyabean crop.

The problem of contamination is compounded by the use of existing facilities to process both food/feed crops and crops used for molecular farming. Ideally, there should be a clear distinction between transgenic plant material used for molecular farming and any normal plant material being processed in the same facility, which is intended for human or domestic animal consumption. A rigorous series of regulatory practices should be in place from the farm to the factory, ensuring complete isolation of transgenic material during growth, harvesting, transport, storage, processing, extraction and waste disposal, and this should supported by validated procedures for cleaning shared equipment. The accidental mixing of transgenic and non-transgenic harvest products is more likely when those products appear visually identical. Therefore, an important step towards identity preservation is the use of non-commercial crop varieties, visually striking varieties (e.g. white tomatoes) or non-food/feed crops that could not possibly be introduced into food or feed processing by misidentification (e.g. dandelion).

Product safety

The impact of plant-produced proteins on human and animal health is of paramount importance in molecular farming, particularly when dealing with pharmaceuticals. While recombinant proteins produced in plants are in most regards identical to their native counterparts, there are two potential areas of concern.

- The purified protein may be contaminated with toxic substances from the plant or applied to the plant, e.g. plant-derived metabolites, allergens, field chemicals (e.g. herbicides, pesticides, fungicides), fertilizers, dung and manure.
- The product itself, due to intrinsic properties, may be harmful.

One aspect in which plant-derived recombinant proteins can differ significantly from their native counterparts is glycosylation. While most of the protein post-translational modification steps in plants and animals are conserved, there are minor differences in the structures of glycan chains, such as the absence of terminal sialic acid residues and the presence of the plant-specific residues α–1,3-fucose and β–1-2 xylose in complex glycans (Cabanes-Macheteau *et al*., 1999). There is a concern that such differences could render plant-derived proteins inactive, make them harmful or make them immunogenic. Glycan chains play a number of roles including structural (helping proteins to fold correctly), chemical (making proteins more soluble), biochemical (making proteins more stable by protecting them from proteases) and functional (allowing proteins to be recognized by other molecules, including cell surface receptors). Even minor differences can have profound effects on the protein's stability, biodistribution and biological activity. For example, the normal glycoform of the hormone erythropoietin has a half-life of 6-8 hours but a variant lacking the terminal sialic acid residue has a half-life of less than 5 minutes (Fukada *et al*., 1989). Studies have shown that recombinant antibodies with plant-specific complex glycans do not provoke an immune response in mice (Chargelegue *et al*., 2000). It is also possible to modify the glycan structure and make it more similar to the pattern normally found in humans by co-introducing transgenes encoding human glycosyltransferase (Bakker *et al*., 2001). Targeting recombinant proteins to the ER-lumen using a KDEL retrieval sequence could also be beneficial here. As well as improving the yields of unstable proteins and preventing secretion into the environment, ER-retention also prevents the replacement of high-mannose glycan core structures with plant-specific xylose and fucose residues, because these modifications take place in the Golgi apparatus.

Conclusions

Molecular farming provides an opportunity for the economical and large-scale production of pharmaceuticals, industrial enzymes and technical proteins that are currently produced at great expense and in small quantities. We must ensure that these benefits are not outweighed by risks to human health and the environment by transgene pollution, the accumulation of recombinant proteins and the contamination of food and feed crops. In this review, we have summarized strategies that are employed or could be implemented to reduce the risk of transgene escape from genetically modified plants, and to limit the negative impact of recombinant proteins on non-target organisms and the environment in general. Primary goals include the production of clean transgenic plants with the minimum of additional genetic information required to achieve high level recombinant protein expression, and the introduction of physical and biological containment methods that limit the potential for transgenes to spread in the environment. The production of well-characterized transgenic plants will allow more effective risk assessment and transgene tracking. The use of controlled expression systems in combination with containment will help to prevent the indiscriminate pollution of the environment with recombinant proteins and the possible introduction of such proteins into the human food chain. Risks associated with the products of molecular farming include contamination with undesirable plant products and innate properties of the recombinant protein itself, often reflecting differences in glycan structure. These risks can be addressed through rigorous comparisons with equivalent products isolated from their natural sources or produced in other systems, and comprehensive testing and safety monitoring from the field through to the extraction, clarification and isolation of the pure pharmaceutical product.

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