TRANSGENIC PLANTS

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Keywords: transgenic plants, transformation, *Agrobacterium tumefaciens*, edible vaccines, biopharmaceuticals

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Summary

Transgenic plants, or plants which express foreign gene products, can be generated by a variety of procedures, such as *Agrobacterium*-mediated transformation or biolistic delivery. Recent advances in these technologies have resulted in the development of commercially successful disease and herbicide resistant plants which both increase crop yield and reduce costs for the farmer. Safe and inexpensive production of recombinant proteins in large quantities have also been produced in transgenic plants, as well as

plants which possess enhanced nutritional traits. In this section, current techniques employed in plant transformation are investigated. Transgenic plants resistant to plant viruses, insects and herbicides are discussed. The manufacturing of proteins in plants, including edible vaccines, immunotherapeutic agents such as antibodies, and biopharmaceuticals are examined. The development of nutriceuticals in transgenic plants is also discussed. Finally, future directions of transgenic plant research and obstacles which remain to be overcome are considered.

1. Introduction

There is virtually no place on earth where the term 'transgenic plant', referring to plants that contain foreign genetic material, is unfamiliar. Transgenic plants were first developed and introduced as crops in the early 1980's. Since transformed plants were found to be fertile and the foreign gene of interest could be continued throughout the progeny, the enormous commercial potential of transgenic plants and their role in crop improvement was fully realized. The first genetically modified crops were soybean and corn, and appeared on the US market in 1996. Since then, transgenic plants have been commercialized in many other countries. Transgenic plants which exhibit increased pest and disease resistance can prevent global production losses which are currently greater than 35 percent. Transgenic plants also present enormous possibilities to become one of the most cost-effective and safe systems for the large-scale production of proteins for industrial, pharmaceutical, veterinary and agricultural uses. In these cases, the plant derived protein must be biologically identical to its native counterpart and be produced at levels high enough to be purified by relatively simple procedures.

This article focuses on three areas for development of transgenic plants. First, the use of transgenic plants for crop improvement, including resistance to plant viruses and other pests as well as tolerance to various herbicides will be described. Next, the employment of transgenic plants as 'protein factories' for the production of edible vaccines and biopharmaceuticals will be discussed. Finally, the development of transgenic plants for the nutritional enhancement of foods will be examined.

2. Transformation of Plants

Plant transformation, meaning the stable integration of the gene of interest into the plant genome, was originally conducted using a modified strain of *Agrobacterium tumefaciens,* the bacterial strain responsible for crown-gall disease **[***see also - Genetic engineering of plant cells]*. *Agrobacterium tumefaciens* harbours a large tumour-inducing (Ti) plasmid and during infection causes a mass of mainly undifferentiated cells to form on a plant's stem at the soil line (crown). The transfer DNA (T-DNA) portion of the Ti plasmid and its delimiting right and left border sequences become integrated into the nuclear genome of a susceptible plant cell that is in contact with the bacterium. The T-DNA encodes enzymes for synthesizing plant hormones that stimulate cell division and the proliferation of undifferentiated cells into the tumour. Vectors used for transformation today lack the genes for hormone-synthesizing enzymes and therefore can introduce foreign DNA into a nuclear chromosome of a plant cell with minimal damage.

Insertion of DNA into a plant by *A. tumefaciens* involves insertion of a foreign gene between the borders of the T-DNA, which in turn is cloned within a small plasmid (Figure 1). The construct is then transformed into a modified version of *A. tumefaciens* which lacks the virulence genes. Upon infection, the T-DNA is transferred into the plant cell, and the gene of interest is incorporated into the host chromosome. The plant cell can then be regenerated from tissue culture into a mature transgenic plant by transferal through a series of culture media with different hormone contents.

A number of problems exist with this mode of transformation. Primarily, the restricted host range of *Agrobacterium* renders infection of monocots difficult. For this reason, other transformation procedures have been developed. Maize, for example, is commonly transformed by particle bombardment, a procedure in which high velocity microprojectiles carrying DNA can be 'shot' with compressed gas using a 'gene gun' into plant tissue.

In addition to this, foreign gene expression in nuclear transformed plants can vary markedly from one transgenic plant to another. Chromosomal position effects are partially responsible for this problem, since the insertion of the transgene into the plant genome is uncontrolled. Other difficulties include the ability of nuclear transformed plants to express more than one transgene. Since many agronomic traits are in fact multigenic and stem from the action of several genes, the production of transformants expressing multiple genes is a painstakingly long process.

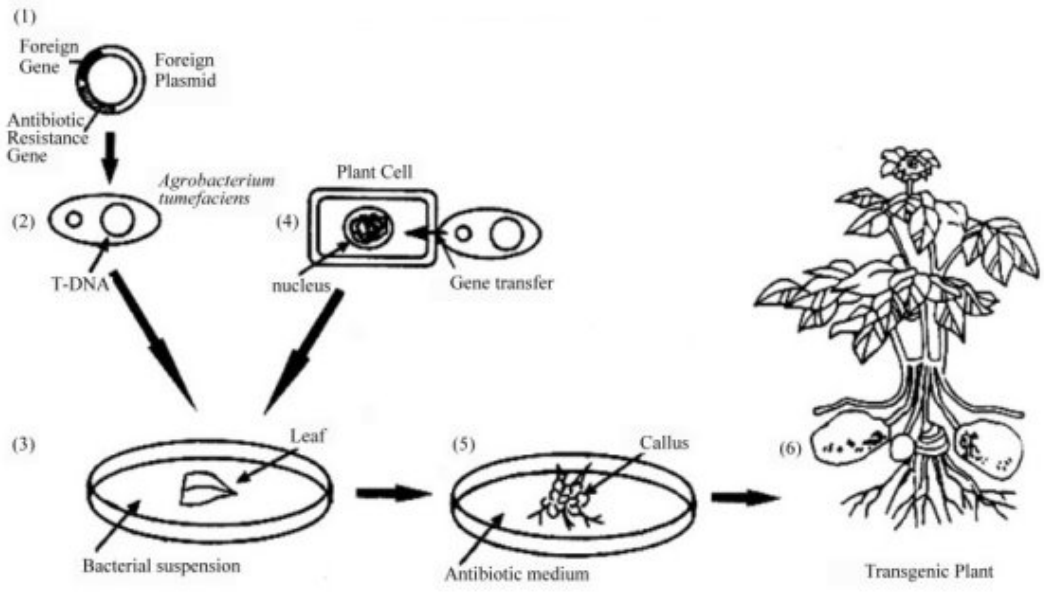

Figure 1: Stages involved in generation of transgenic plants by *Agrobacterium* - mediated transformation. 1.Gene of interest is cloned into foreign plasmid which contains an antibiotic resistance gene. 2. Plasmid is transformed into *Agrobacterium tumefaciens.* 3. Cut leaf is exposed to a suspension of *Agrobacteria* containing the gene of interest. 4. The gene of interest is integrated into the genomic DNA of individual leaf cells. 5. The leaf is exposed to an antibiotic to kill non-transformed cells. The urviving cells form a callus which then sprouts roots and shoots. 6. The plantlets

produced from the callus are transferred to soil. Mature transgenic plants generated now contain the foreign gene of interest.

More recently, genes have been introduced directly into the plastid genome. This was first accomplished for *Chlamydomonas reinhardtii* by biolistic transformation. Plastid transformation is unique from nuclear transformation as the transgene is incorporated directly into the plastid genome by homologous recombination and can be predictably directed to a specific site within the plastid chromosome. Recently, two new procedures involving polyethylene glycol and direct *in situ* injection have also been developed for plastid transformation.

Since chloroplast genes are arranged on operons [*see also - Molecular Biology]*, chloroplast transformation can be used to produce multicistronic mRNAs, and in the future, traits determined by multiple genes can be expressed in chloroplasts. Transgene expression levels can be several fold higher in chloroplast-transformed plants than in their nuclear-transformed counterparts, and lack the same variation in expression levels. The sequestration of foreign proteins in chloroplasts prevents their adverse interactions with the cytoplasmic environment and protects the cell from the accumulation of potentially toxic proteins. Since chloroplasts are not present in pollen, transgenes cannot be transferred to nearby sexually compatible crops to produce 'superweeds'. The ability of chloroplast transformation to overcome several major problems associated with conventional nuclear technologies has created unprecedented opportunities for plant biotechnology in the future.

3. Herbicide and Disease-Resistant Crops

3.1. Plant Virus-Resistant Crops

3.1.1. Coat protein-mediated resistance

Transgenic plants which carry nucleotide sequences derived from plant viruses have been constructed and are capable of protecting against viral diseases (Table 1). The presence of a viral sequence or gene product in a plant can interfere with infection, resulting in cross-protection against the challenger virus. This process is thought to act in a similar manner to that of classical cross-protection, in which infection of plants with a virulent strain of the virus is suppressed by the prior inoculation with a mild strain of the same virus. However, in the case of classical cross-protection, the use of mild strains of the virus may be of disadvantage in agriculture, since viral strains which are seemingly harmless to one crop type may cause severe damage to another, or may act synergistically in conjunction with another virus to create a more severe disease condition.

Virus Gene Product	Virus	Transgenic Plant
Coat Protein	TMV	Tobacco
	PVX	Potato
	A1MV	Alfalfa
	TSWV	Tomato
	TGMV	Tomato

	PAMV	Potato
	PVY	Potato
RNA	PVX	Potato
	TGMV	Tomato
	PLRV	Potato
	PAMV	Potato
	TEV	Tobacco
Replicase	BMV	Tobacco
	PEBV	Pea
	PVX	Potato
	TMV (54K protein)	Tobacco
Movement Protein	SMV	Soybean
	PLRV	Potato
	PVX	Tobacco
Protease	SMV	Soybean

Table 1: Transgenic Plants Resistant to Plant Viruses

Genetically engineered cross-protection was first demonstrated in 1986, when transgenic tobacco plants expressing the coat protein of tobacco mosaic virus (TMV) were found to have suppressed or delayed symptoms upon viral infection. Since resistance could be overcome by inoculation with TMV RNA alone, it appeared that protection was mediated by the presence of the coat protein in these transgenic plants. This phenomenon became known as coat protein-mediated resistance (CPMR), and is characterized by the suppression or delayed development of symptoms during virus infection. Protection was also conferred against a number of related viruses containing a high degree of sequence homology with the coat protein of TMV.

The fact that resistance could be broken by inoculation of the transgenic plant with naked TMV RNA suggested that cross-protection takes place at the level of coat protein expression by preventing uncoating of the virus. However, similar studies conducted on transgenic plants expressing the coat protein of PVX demonstrated that protection was maintained even when the plants were challenged with PVX RNA alone. This suggests that these transgenic plants exhibit a mechanism of protection different from the TMV transgenic plants. In both of these transgenic plant systems, the level of expression of the coat protein was directly correlated with the extent of protection.

Transgenic plants expressing the coat proteins of plant viruses which differ extensively in structure, morphology, genome organization and replication strategies have been generated as well. Transgenic plants expressing the coat protein of alfalfa mosaic virus (AlMV), an isometric virus with a tripartite genome, have been demonstrated to be resistant to virus infection. Transgenic tobacco lines expressing the coat protein of tomato spotted wilt virus (TSWV), an enveloped, multicomponent, ambisense RNA virus, were shown to be resistant to infection. Similarly, transgenic plants expressing the coat protein of tomato golden mosaic virus (TGMV), a geminivirus, were generated and have demonstrated resistance against viral infection.

In addition, a number of plants which express either sense or antisense RNA sequences possess the same amount of resistance as those constructs that were translationally

efficient. For example, plant lines expressing the antisense transcript of the coat protein of tomato golden mosaic virus (TGMV) were protected against virus infection. Furthermore, plants expressing the coat protein, or corresponding sense and antisense RNAs of potato leafroll virus (PLRV), a negative-stranded RNA luteovirus, are resistant as well.

Transgenic potato plants which express antisense RNA, full-length and truncated forms of the potato aucuba mosaic virus (PAMV) coat protein were tested for their ability to protect against PAMV infection. Only plants expressing a construct which lacked the N-terminal domain of the coat protein lost the ability to protect against PAMV infection, suggesting that the N-terminal domain of PAMV coat protein is the active element in cross-protection.

3.1.2. Resistance conferred by other gene products.

Since the initial studies with TMV, numerous examples of cross-protection with viral coding sequences other than those encoding the coat protein have been established in other virus-host systems (Table 1). Expression of the replicase of Brome Mosaic Virus (BMV), pea early browning virus (PEBV) as well as Potato Virus X (PVX) has all demonstrated to confer resistance against infection. However, it is uncertain which portion of the replicase protein is responsible for protection. More recently, tobacco plants transformed with the sequence containing an additional open reading frame encoding a 54 K protein located within the replicase gene of TMV were confirmed to be resistant to infection.

Transformed plants expressing a defective movement protein of TMV or protease of soybean mosaic virus (SMV) were also demonstrated to be resistant to viral infection. Transgenic potato plants expressing mutant PLRV movement protein exhibit a broad range protection against virus infection. These plants were found to be resistant to PLRV and unrelated PVY and PVX.

Transgenic plants have also been generated which express ribozymes directed against specific viral sequences in an attempt to inhibit virus infection and spread. Transgenic plants which express mammalian antibodies directed against plant viral proteins have been shown to reduce the incidence of infection with some degree of success. Finally, plants expressing sequences coding for the defective-interfering particles (DI particles) of cymbidium ringspot virus (CyRSV) demonstrated a high level of resistance against infection by the corresponding viruses. It appears, then, that the expression of a variety of viral gene products can disrupt one or several stages of the viral life cycle during infection.

A number of mechanisms have been proposed to explain cross-protection. These include: competition between the initial infecting virus and the challenging virus for host factors, prevention of uncoating or re-encapsidation of the challenging strain by free viral coat protein originating from the protecting virus already present in the host plant, the blocking of putative receptor sites by free viral coat protein expressed within the host cells, and the inhibition of replication or translation of the challenging virus by RNA:RNA annealing of the transgenic transcripts to the challenger viral RNA.

Transgenic plants expressing the CP of one potyvirus and infected with various potyviruses resulted in progeny whose virions contained as much as 25 percent transgenic CP, suggesting that in this case, cross-protection acts at the level of re-encapsidation. In another study, a number of TMV CP mutants were expressed in a PVX vector prior to challenge inoculation with TMV. Mutant CP which were deficient in virion formation but competent to assemble into helical aggregates still protected against the challenging TMV. In contrast, CP mutants which were incapable of helical aggregation or unable to bind to viral RNA could not cross-protect. Furthermore, CP mutants with enhanced intersubunit interactions were superior at cross-protection. These results suggest again that the protecting CP recoats the challenge virus RNA as it disassembles.

Transgenic plants expressing foreign RNA transcripts have been shown to exhibit homology dependent gene silencing, a phenomenon in which a cellular RNA-specific degradation system eliminates both infecting as well as transgenic RNAs. When an untranslatable form of the TEV CP gene was expressed in transgenic lines, some plant lines exhibited initial susceptibility, yet were able to recover later from TEV infection. Upon analysis, these plants were found to have much lower transgene RNA levels than prior to virus challenge.

Nuclear run-off assays indicated that a post-transcriptional reduction in specifc RNA levels had taken place. Similarly, transgenic tobacco plants expressing either full-length or an N-truncated form of TEV CP were initially susceptible to TEV infection. However, 3-5 weeks after a TEV infection was established, transgenic plants recovered, and new virus-free plant tissue emerged.

A TEV-specific resistant state was induced in the recovered tissue. Steady-state transgene RNA levels were 12-22-fold less than transgene mRNA levels in un-inoculated transgenic tissue. It was proposed that this reduction in transgene transcript accumulation is mediated by a cytoplasmic activity that targets specific RNA sequences for inactivation. Tobacco plants transformed with the polymerase of PVX were also found to be highly resistant to PVX infection. The resistant lines expressed RNA polymerase at much lower levels than those lines which were fully susceptible. It was concluded that homology-dependent gene silencing and transgenic resistance to PVX stems from the same RNA-based mechanism in which RNA with sequence homology to the silenced transgene is degraded by a double-stranded RNase produced by the plant.

None of these models for cross-protection can singly satisfy all of the experimental data compiled to date; perhaps cross-protection is the cumulative result of the simultaneous action of a number of these mechanisms which are effective at different stages of infection. Complementation studies using transgenic plants expressing the coat protein or other nonstructural gene products have been used to further elucidate the mechanisms behind cross-protection in more detail. For example, the coat protein of AlMV plays a role in virus uncoating, the balance of plus/minus strand RNA synthesis, virus movement and symptom formation. Thus expression of the viral coat protein in transgenic plants may disrupt the virus life cycle at multiple levels. The precise mechanism of resistance, therefore, would be dependent on the virus-host system.

3.2. Resistance against Other Pathogens

The expression of foreign genes in plants has been used to employ a number of active defence mechanisms which can protect plants against infection by viruses as well as a variety of other pathogens **[***see also - Crop protection through pest resistance genes***]**. For example, expression of ribosome-inactivating proteins (RIP's) such as the pokeweed antiviral protein (PAP), a 30 kD protein isolated from *Phytolacca americana* in transgenic plants, results in the inhibition of protein synthesis in cells which are infected. Pokeweed antiviral protein inhibits translation by catalytically removing a specific adenine residue from the large rRNA of the 60S subunit of eukaryotic ribosomes. Transgenic plants expressing this protein possess a high level of resistance against a wide spectrum of viruses and other pathogens including TMV, PVX and the fungal pathogen *Rhizoctonia solani*. Increases in pathogenesis-related (PR) proteins, but no increase in salicylic acid levels was observed, suggesting that PAP may elicit a signal transduction pathway that is independent of salicylic acid (SA).

Salicylic acid was overproduced in transgenic plants by transforming tobacco with two bacterial genes coding for enzymes that convert chorismate into SA. This resulted in constitutive expression of the PR proteins, and the plants were resistant to viral and fungal infection in a manner that resembled systemic acquired resistance (SAR).

Similarly, the hrmA gene from *Pseudomonas syringae* expressed in tobacco plants activated the pathogenesis-related genes, and the plants exhibited high levels of resistance to multiple pathogens including tobacco vein mottling virus (TVMV), TEV and black shank fungus *Phytophthora parasitica* in addition to *Pseudomonas syringae*. Finally, transgenic potato plants expressing the phage T4 lysozyme gene were shown to be resistant to the plant-pathogenic enterobacterium *Erwinia carotovora*. Roots from potato lines expressing T4 lysozyme gene exhibited higher levels of killing. T4 lysozyme was shown to be released from the root epidermis cells and was active in the fluid on the root surface.

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Biographical Sketch

Kathleen Laura Hefferon took her B.Sc. at the University of Toronto in 1985, majoring in microbiology. Her M.Sc.—thesis title "Characterization of the structure and expression of the muscle phosphorylase gene in the rat"—at the Department of Biology, York University, was followed in 1995 by a Ph.D. specializing in Molecular Virology at the University of Toronto. After postdoctoral research at the University of Georgia, USA (on late gene expression and host specificity of baculoviruses) in 1996 and at Cornell University (on the role of GP64 in baculovirus infection) in 1997, she was Project Manager and Research Associate at Cornell University (1998-2001).