TRANSGENIC VEGETABLE CROPS FOR MANAGING INSECT PESTS AND FUNGAL AND VIRAL DISEASES

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Summary

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Vegetable crop species are grown worldwide to provide a source of fiber, nutrients and vitamins in the human diet. Genetic transformation for the introduction of foreign genes to enhance resistance to insect pests and fungal diseases has been accomplished for at least 19 vegetable crop species belonging to 8 botanical families. Although some reports of genetically engineered vegetable crop species are limited to expression of selectable marker genes, there are many reports, described here, that have demonstrated the expression of genes which encode potentially useful agronomic and horticultural traits. These include enhanced resistance to insect pests through the expression of *Bacillus thuringensis* crystalline endotoxins and trypsin inhibitors. Enhanced resistance to fungal pathogens has been achieved through the expression of antifungal proteins and various other antimicrobial compounds, while virus resistance has been achieved through coat-protein mediated expression. Transgenic vegetable crops with enhanced resistance to pests and diseases should become a part of an integrated pest management program in the future.

1. Introduction

Vegetable crop species are grown worldwide and provide an important source of fiber, nutrients and vitamins in the human diet. They are consumed fresh or may be eaten after cooking, processing of pickling, and constitute an important part of the meals of billions of people. The crops may be grown under field conditions or under controlled environment conditions, such as in greenhouses. A large number of vegetable crop species have been genetically transformed [see also – *Crop protection through Pest-resistant Strains*], and they belong to at least 8 different taxonomic families.

Most crops are annual or infrequently biennial plants (such as carrot); a few species are perennial (such as asparagus and watercress). The edible portions of these plants represent the complete spectrum of botanical features, including root (beet, carrot), stem (asparagus), tuber (potato), leaf (cabbage, chicory, lettuce, spinach, watercress), flower (broccoli, cauliflower) and fruit (cucumber, eggplant, pepper, tomato).

Significant progress has already been made using conventional breeding strategies to produce horticulturally improved, high-yielding and nutritionally-enhanced cultivars of virtually all of the vegetable crops presently grown under cultivation.

In addition, resistance to insect pests and diseases, and enhanced tolerance to environmental stresses, have been incorporated using conventional breeding methods [see also – *Conventional Plant Breeding for Higher Yields and Pest Resistance*]. This has resulted in vegetable crop species being cultivated in a wide range of environments and niches throughout the world.

With the advent of recent techniques in genetic engineering that now permit the introduction into plants of foreign genes through transformation; these methods have been utilized to introduce additional genes to potentially enhance the horticultural quality of vegetable crops. In this chapter, the general approaches used to transform vegetable crop species and examples of crops with specific traits to enhance insect pest and fungal and viral disease resistance are described.

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2. Genetic Engineering Technologies

2.1. Tissue culture selection

The first step in developing transgenic plants [see also – *Transgenic* Plants] is to have a procedure to regenerate an entire plant from individual transformed cells. Plant tissue culture relies on the ability of individual plant cells to regenerate into whole functional plants (totipotency). Plant cells or organs are cultured under sterile conditions on defined nutrient media supplemented with specific concentrations of plant growth regulators under controlled environmental conditions. The most widely used medium is Murashige and Skoog's medium. Optimum conditions must be defined for each crop species and sometimes each tissue type. Plantlet regeneration may subsequently occur through somatic embryogenesis or organogenesis. Once transformants are identified after appropriate selection, they are then multiplied and regenerated into clones of identical plantlets. Examples of the vegetable crop species that have been genetically engineered to enhance pest and disease resistance are given in Table 1.

Family	**Crop**	**Transformation method**	**Novel protein introduced**	**Reference**
Asteraceae	Lettuce (*Lactuca sativa* L.)	*A. tumefaciens*	Virus coat protein, neomycin phosphotransferase	Pang et al. (1996); Dinant et al. (1997)
Chenopodiacea	Spinach (*Spinacia oleracea* L.)	*A. tumefaciens*	Virus coat protein, neomycin phosphotransferase	Yang et al. (1997a)
Convolvulaceae	Sweet potato (*Ipomoea batatas* (L) Lam.)	*A. tumefaciens*	trypsin inhibitor, snowdrop lectin, neomycin phosphotransferase,β-glucuronidase	Newell et al. (1995)
		Electroporation	Virus coat protein, hygromycin phosphotransferase	Nishiguchi et al. (1998)
Cruciferaceae	Broccoli (*Brassica oleracea* L.var. *italica)*	*A. tumefaciens*	*B. thuringiensis* Cry IA, neomycin phosphotransferase	Metz et al. (1995a)
		A. rhizogenes	*B. thuringiensis* Cry IA, neomycin phosphotransferase, β-glucuronidase	Christey et al. (1997)
		A. tumefaciens	*B. thuringiensis* Cry IC, hygromycin phorphotransferase	Cao et al. (1999)
	Cabbage (*Brassica oleraceae* L. var. *capitata)*	*A. tumefaciens*	*B. thuringiensis* Cry IA, neomycin phosphotransferase	Metz et al. (1995a)
		A. rhizogenes	*B. thuringiensis* Cry IA, neomycin phosphotransferase, β-glucuronidase	Christey et al. (1997)
		A. tumefaciens	*B. thuringiensis Cry* IAb3, neomycin	Jin et al. (2000).

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			phosphotransferase	
		A. tumefaciens	*Aspergilus niger* glucose oxidase, hygromycin phosphotransferase	Lee et al. (2000)
	Cauliflower (*Brassica oleracea* var. *botrytis)*	*A. tumefaciens*	Virus coat protein, hygromycin phosphotransferase, neomycin phosphotransferase, β-glucuronidase	Passelegue and Kerlan (1996)
		A. rhizogenes	*B. thuringiensis* Cry IA, neomycin phosphotransferase, β-glucuronidase	Christey et al. (1997)
		A. tumefaciens	trypsin inhibitor, neomycin phosphotransferase	Ding et al. (1998)
		A. tumefaciens	antibacterial peptides, neomycin phosphotransferase	Braun et al. (2000)
		A. tumefaciens	*B. thuringiensis* Cry 9Aa, hygromycin phosphotransferase	Kuvshinov et al. (2001)
	Chinese cabbage (*Brassica campestris* L.)	*A. tumefaciens*	virus coat protein, neomycin phosphotransferase	Jun et al. (1995)
		A. tumefaciens	*B. thuringiensis* Cry IAb, Cry IAc, neomycin phosphotransferase	Xiang et al. (2000)
		A. tumefaciens	*B. thuringiensis* Cry IC, hygromycin phosphotransferase	Cho et al. (2001)
	Rutabaga (*Brassica napobrassica* L.)	*A. tumefaciens*	*B. thuringiensis* Cry IA, neomycin phosphotransferase	Li et al. (1995)
	Watercress (*Rorippa nasturtium-aquaticum* L.)	*A. tumefaciens*	*B. thuringiensis* Cry II a3, neomycin phosphotransferase	Jin et al. (1999)
Cucurbitaceae	Cucumber (*Cucumis sativus* L.)	*A. tumefaciens*	chitinase, neomycin phosphotransferase	Raharjo et al. (1996)
		A. tumefaciens	chitinase, neomycin phosphotransferase	Tabei et al. (1998)
	Squash (*Cucurbita pepo* L.)	*A. tumefaciens*	virus coat protein, neomycin phosphotransferase	Clough and Hamm (1995)
Leguminosae	Bean (*Phaseolus vulgaris* L.)	Biolistic	antisense viral RNA, neomycin phosphotransferase, β-glucuronidase	Aragao et al. (1996; 1998)
	Pea (*Pisum*	*A. tumefaciens*	α-amylase,	Schroeder et al.

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	sativum L.)		phosphinothricin acetyltransferase	(1995)
		A. tumefaciens	β-glucuronidase, neomycin phorphotransferase	Polowick et al. (2000)
Solanaceae	Eggplant (*Solanum melongena* L.)	*A. tumefaciens*	*B. thuringiensis* Cry III B, β-glucuronidase	Chen et al. (1995)
		A. tumefaciens	*B. thuringiensis* Cry III B, neomycin phosphotransferase	Lannacone et al. (1995); Arapaia et al. (1997)
		A. tumefaciens	*B. thuringiensis* Cry III B, neomycin phosphotransferase, β-glucuronidase	Billings et at. (1997)
		A. tumefaciens	*B. thuringiensis* Cry III A, neomycin phosphotransferase, β-glucuronidase	Jelenkovic et al. (1998)
		A. tumefaciens	*B. thuringiensis* Cry IAb, neomycin phosphotransferase	Kumar et al. (1998)
	Pepper (sweet) (*Capsicum annuum* L.)	*A. tumefaciens*	virus coat protein, neomycin phosphotransferase	Zhu et al. (1996)
	Pepper (hot) (*C. annuum* L.)	*A. tumefaciens*	viral satellite RNA, neomycin phosphotransferase	Kim et al. (1997)
		A. tumefaciens	virus coat protein, neomycin phosphotransferase	Cai et al. (2003)
	Tomato (*Lycopersicon esculentum* Mill.)	*A. tumefaciens*	chitinase, glucanase, neomycin phosphotransferase	Jongedijk et al. (1995)
		A. tumefaciens	virus coat protein, neomycin phosphotransferase	Gielen et al. (1996)
		A. tumefaciens	viral replicase, neomycin phosphotransferase	Gal-On et al. (1998)
		A. tumefaciens	virus coat protein, neomycin phosphotransferase	Kaniewski et al. (1999)
		A. tumefaciens	chitinase, neomycin phosphotransferase	Tabaeizadeh et al. (1999)
		A. tumefaciens	*B. thuringiensis* Cry IAc, neomycin phosphotransferase	Mandaoker et al. (2000)
		A. tumefaciens	defensin, neomycin phosphotransferase	Parashina et al. (2000)
		A. tumefaciens	polygalacturonase-inhibiting protein, neomycin phosphotransferase	Powell et al. (2000)
		A. tumefaciens	chitinase, neomycin phosphotransferase	Gongora et al. (2001)

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Umbelliferae	Carrot (*Daucus carota* L.)	*A. tumefaciens*	chitinase, neomycin phosphotransferase	Gilbert et al. (1996)
		A. tumefaciens	lysozyme, neomycin phosphotransferase	Takaichi and Oeda (2000)
		A. tumefaciens	thaumatin-like protein, hygromycin phosphotransferase, phosphinothricin acetyltransferase	Chen and Punja (2002)

Table 1: A summary of vegetable crop species which have been genetically transformed to enhance resistance to pests and diseases.

Prior to beginning the research to achieve the genetically engineered plant containing an introduced (foreign) gene, the gene of interest must be isolated and cloned and incorporated into a bacterial vector (usually a plasmid construct) for delivery into the host plant. The gene of interest is linked to a promoter at the 5' end, whose function is to regulate the foreign gene expression by either allowing constitutive expression or inducible expression in response to a stimulus, such as a developmental stage or in response to wounding or infection. The most widely used promoter for transformation of vegetable crop species has been the Cauliflower Mosaic Virus 35S promoter (CaMV 35S), which provides constitutive expression of the gene product throughout the plant. The ubiquitin promoter from maize or Arabidopsis has also been used, for example, in carrot. Pathogen-inducible and wound-inducible promoters have also been described which are expressed at sites of infection or wounds. A terminator sequence is then added to the 3' end of the gene of interest to prevent read-through and in some cases, additional regulatory sequences may be included in the construct.

Selectable marker genes are also included in the construct. These are genes that allow transformed cells expressing them to be selected against a large population of non-transformed cells in tissue culture. The selectable marker gene usually codes for resistance to a herbicide or antibiotic. The most well-known marker used in transformations is the *npt II* gene encoding the enzyme neomycin phosphotransferase, which confers resistance to the aminoglycoside antibiotics kanamycin, neomycin and G-418. This allows for rapid and efficient selection of transformed cells. Other selectable markers that have been used include hygromycin phosphotransferase (*htp*), and phosphinothricin acetyltransferase (*pat, bar*)] or reporter genes [β-glucuronidase (*uid* A, *gus*)] (Table 1). Recently, positive selectable markers which rely on the ability of transformed cells to grow on normally nonutilizable sources of carbon, such as mannose, have been described.

2.2. Gene transfer technologies

There are two approaches for achieving plant transformation, one involving an indirect means of gene transfer using *Agrobacterium* vectors and the second by direct methods using physical or electrical means of gene transfer.

2.2.1. Agrobacterium-mediated transformation

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Agrobacterium tumefaciens, the causal agent of crown gall disease, infects plant cells to make them tumorigenic at the site of infection. Tumor cells contain genetic material unique to the bacterium. Research beginning in the 1970's revealed that *A. tumefaciens* is attracted to wounded plant tissues, attaches itself to the host and transfers a sequence of DNA harboring specific genes (T-DNA) from a large tumor-induding (Ti) plasmid. This process is mediated by a set of bacterial virulence genes on the Ti-plasmid whose expression is induced by host plant phenolic compounds eg: acetosyringone, which are produced at wound sites. The T-DNA enters the plant nucleus, integrates into the host genome and is subsequently expressed. Genes within the foreign DNA are then expressed to produce plant growth regulators (auxins and cytokinins), which cause uncontrollable cell growth and result in the production of tumors. Furthermore, the integration of the T-DNA fragment is also responsible for the synthesis of opines, which are amino acid and sugar derivatives that are metabolized by the *Agrobacterium* while living within the tumor.

By the early 1980's, biotechnologists were exploiting the natural ability of the biological vector to transfer foreign genes into plant cells. *Agrobacterium*-mediated transformation is simple and reliable and is the most commonly used method for vegetable crop transformation; however, it is limited by the bacterium's host range, since gene delivery has been predominantly successful with susceptible dicotyledonous species whereas monocots are generally not infected. Recently, *Agrobacterium*-mediated transformation of some monocots was demonstrated using highly embryogenic tissues and efficient selection protocols. While A*llium* species were previously regarded as recalcitrant to transformation and regeneration, reports of onion transformed by *Agrobacterium* are available and stable transgenic garlic has been devolped.

In contrast, *A. rhizogenes* is a soil bacterium that causes hairy root disease in wounded dicotylendonous plants and is a natural vector with the ability to transfer a specific segment of DNA in a similar manner to *A. tumefaciens*. Recently, there has been interest in using this vector for stable transformation of foreign genes into vegetable crop plants. *A. rhizogenes* carries a large root-inducing (Ri) plasmid harboring a T-DNA region. Integration and expression of Ri T-DNA into plant cells exploits similar mechanisms as *A. tumefaciens*-mediated gene delivery. Transformants can be selected by the development of hairy roots on hormone-free media or by the expression of an inserted foreign gene within the T-DNA region. However, transgenic plants regenerated from hairy roots may show altered phenotypes characterized by changes in life cycle, late flowering, higher growth rates, reduced fertility and morphological changes involving increased rooting, dwarfing and wrinkled leaves. *A. rhizogenes*-mediated transformation has been achieved with several vegetable crops and foreign genes to enhance resistance to insects have been expressed in cruciferous crops, including broccoli, cabbage and cauliflower (Table 1).

2.2.2. Direct methods for transformation

2.2.2.1. Particle bombardment (biolistics)

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This method was invented to overcome the obstacles of transforming plants that were not amenable to *A. tumefaciens* gene delivery. At present, the use of this transformation method is second to that of *A. tumefaciens*. Particle (microprojectile) bombardment involves accelerating DNA-coated microscopic gold or tungsten particles into target cells, where the genetic material integrates into the genome and results in the stable expression of the foreign gene. Microprojectile bombardment is the only transformation technique that can be applied to almost any cell or tissue type. The methodologies are simple and identical regardless of the target cells or DNA used. This method has been used to obtain genetically engineered bean and asparagus plants.

2.2.2.2. Protoplast-mediated transformation

DNA uptake into protoplasts relies on the temporary removal of the plant cell wall which functions as the principal barrier impeding foreign DNA entry into the cell. Removal of the plant cell wall is carried out enzymatically. The resulting protoplast becomes more amenable to DNA uptake by physical and chemical means that create pores in the cell membrane eg. electroporation, thereby allowing molecules to pass inside the cell. Once the foreign gene enters the plant cell, the membrane pores reseal, the cell wall is regenerated and the intact cell is induced to multiply to form callus, from which clones of transgenic plantlets can be regenerated.

An alternative to electroporation is a chemical means of DNA uptake involving polyethylene glycol (PEG). PEG in combination with divalent cations, such as calcium or magnesium, induce DNA uptake into protoplasts. The introduction of DNA into protoplasts by electroporation or PEG-mediated uptake potentially allows for the production of very large numbers of transformed cells; however, success is limited by difficulties in culturing protoplasts and achieving plant regeneration. This method has been used to produce transgenic plants of sweet potato. Additional methods to achieve crop transformation are described by Hansen and Wright in 1999, Newell in 2000, and Songstad *and coworkers in* 1995, although none of them has at the present time been applied to vegetable crop species.

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