EXPLORING NEW TRANSGENES FOR PYRAMIDING RESISTANCE TO POTATO TUBER MOTH IN POTATO

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Abstract of thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

Exploring new transgenes for pyramiding resistance to potato tuber moth in potato

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Potato tuber moth, *Phthorimaea operculella* (Zeller) (PTM), is a major insect pest of potato crops. The larvae mine into foliage and exposed tubers, making them difficult to control with insecticide applications or cultural methods. This thesis describes strategies to genetically engineer potato plants resistant to PTM. It includes the construction of chimeric *cry* genes, the insertion of these genes into standard binary vctors, and the subsequent transformation of potato using *Agrobacterium*-mediated gene transfer. Multiplex-PCR was performed to confirm the presence of the selectable marker gene and specific *cry* gene(s) in all regenerated lines. Multiplex RT-PCR was performed to confirm the transgene expression in transgenic lines. Southern blot and ELISA analyses were performed for transgenic lines to reveal the gene copy number and Cry protein level respectively.

In transgenic potato it is often desirable to couple high-level expression in foliage with no expression in the edible tubers, especially for resistance to pests that primarily infest foliage. To accomplish this, a light inducible *Lhca3* promoter for transcriptional control of *cry*1Ac9 and *cry*9Aa2 genes for resistance to PTM was successfully used. Significantly inhibited larval growth of PTM on excised greenhouse-grown leaves was observed in 51% of the *cry*1Ac9-trangenic lines and 84% of the *cry*9Aa2-transgenic lines. RT-PCR analysis identified several

transgenic lines with high levels of *cry* gene mRNA in leaves and no to low levels in tubers.

This thesis has also demonstrated the effectiveness of two further *cry* genes, *cry*1Ba1 and *cry*1Ca5, for PTM resistance in transgenic potato. This is important to assemble a suite of genes capable of effecting PTM control to allow future evaluation of different approaches toward deployment of PTM resistance management strategies. Over 90% of the *cry*1Ca5-transgenic lines gave 100 % larval mortality of PTM on excised greenhouse-grown leaves and tubers bioassays. 40-50% of the *cry*1Ba1-transgenic lines gave 50 to 100% of larval mortality of PTM on excised greenhouse-grown leaves and tubers bioassays.

An experimental approach to simulate *cry* gene pyramiding in potatoes was designed and used to assess the interaction of three different *cry* genes, *cry*1Ac9, *cry*9Aa2 and *cry*1Ba1 genes, in pair wise combinations. It is important to investigate how the expression of two dissimilar *Bt* toxins interact to confer insect resistance in transgenic plants before developing pyramided plants. The results showed that all combinations of the three *cry* genes were largely consistent with additive impacts on PTM larvae, although results from the combination of the *cry*1Ac9 and *cry*9Aa2 genes were suggestive of slight synergistic effects. Potato lines transgenic for both *cry*1Ac9 gene and *cry*9Aa2 gene were developed to investigate the effects of gene pyramiding. The feasibility of two strategies for *Agrobacterium*-mediated transgene pyramiding in potato were also evaluated.

The results of this thesis demonstrated that potatoes transgenic for *cry* genes offer a valuable approach to develop elite plant material for potential use as an additional component in integrated pest management of PTM.

Keywords: Potato tuber moth (PTM), *Phthorimaea operculella* (Zeller), pyramiding, *Bacillus thuringiensis* (*Bt*), *cry*1Ac9 gene, *cry*9Aa2 gene, *cry*1Ba1 gene, *cry*1Ca5 gene, *Lhca3* promoter, CaMV *35S* promoter, transgenic potatoes.

Contents

List of tables

List of figures

Abbreviations

bp	base pair
Bt	*Bacillus thuringiensis*
CaMV	cauliflower mosaic virus
°C	degrees centigrade
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbant assay
g	gram
GI	growth index
hr	hour
ICP	insecticidal crystal protein
kb	kilobase
kDa	kiloDaltons
L	litre
mg	milligram
min	minute
ml	millilitre
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
nm	nanometer
nM	nanomolar
ng	nanogram
PTM	potato tuber moth
PCR	polymerase chain reaction
RT-PCR	reverse transcription PCR
RNA	ribonucleic acid
Tris	tris(hydroxylmethyl)-methylamine
T-DNA	transfer DNA
µl	microlitre
µg	microgram

Source : Foot, M. 1975: Potato tuber-moth, *Phthorimaea operculella* (Zeller), life-cycle.

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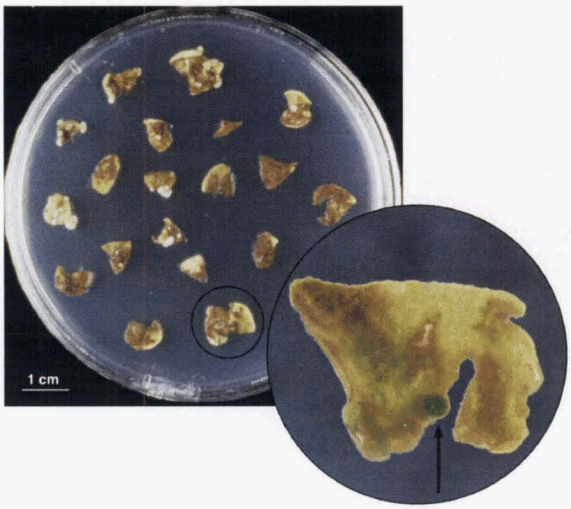
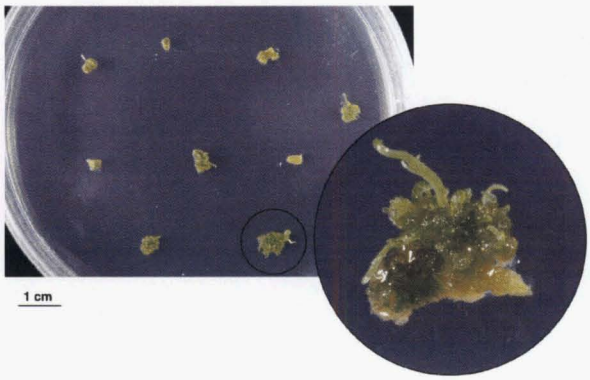


A. Selection of kanamycin-resistant cell colonies on leaf segments of 'Iwa' 3-6 weeks following co-cultivation with *Agrobacterium tumefaciens*. Arrow indicates a transformed green cell colony.



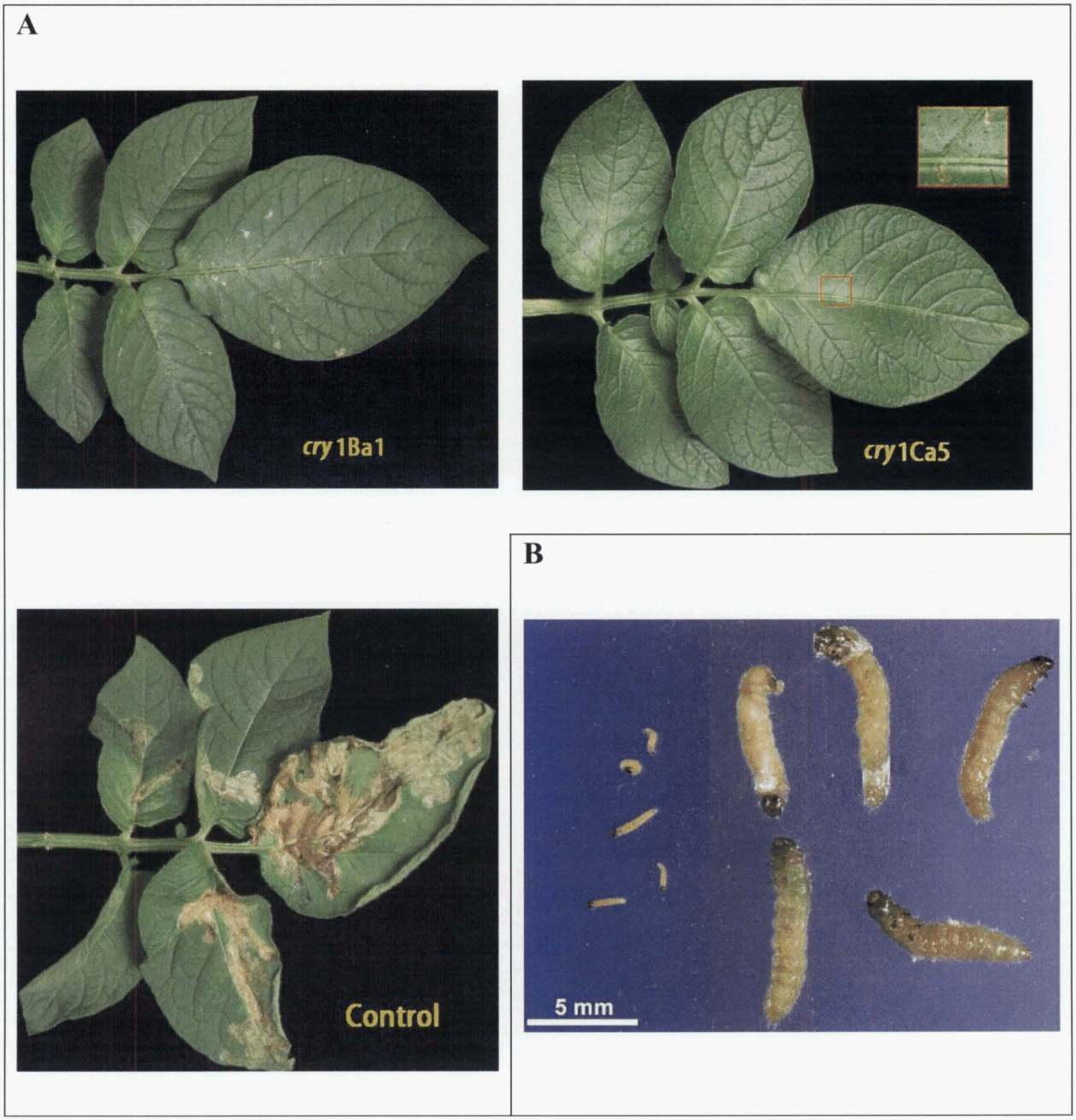
B. Kanamycin-resistant cell colonies growing on regeneration medium.

C. Shoot proliferation from the shoot-regenerated cell colony on multiplication medium.

D. Root development from a non-transgenic 'Iwa' shoot (left) and a transformed 'Iwa' shoot (right) 2 weeks following transfer to kanamycin-supplemented multiplication medium. Note the extensive root growth below the surface of the medium from the transformed shoot.

Transgenic potato lines in the containment greenhouse.

Examples of 'off-type' transgenic potato lines in the containment greenhouse.



Bioassays of potato tuber moth larvae (PTM) on transgenic potato plants.
A. . Detached leaves from representative plants transformed with the *cry*1Ba1 gene, *cry*1Ca5 gene and non-transformed 'Iwa' control plant, showing the extent of damage after infestation with 5 PTM larvae for 9 days. **B.** Differences in size and development of PTM larvae recovered from transgenic plants containing the *cry*1Ba1 gene (left) and non-trangenic 'Iwa' control leaves (right), after 9 days infestation.

Chapter 1. Literature Review

1.1 Introduction

The potato, *Solanum tuberosum* L., is the most important non-cereal world food crop. It is very widely grown on a world scale and ranks fourth in food production following maize, wheat and rice (Table 1.1). The potato continues to be adopted in most developing countries where it is becoming an increasingly important source of rural employment, income and food (Hawkes, 1990; Raman, 1994). World potato production from developing countries has increased by over 10 million metric tons in last five years (FAOSTAT, 2004).

The potato is one of the major vegetable crops grown in New Zealand for fresh use and for processing (Petrie & Bezar, 1998). In New Zealand, nearly 11,300 hectares were harvested in 2004, yielding 500,000 Mt (FAOSTAT, 2004). The adaptability of the potato enables it to be grown in most parts of New Zealand in a wide variety of soils and climates (Logan, 1983). Most of the potatoes grown in New Zealand are consumed in the domestic market. The New Zealand market for fresh potatoes is relatively stable. New Zealand's fresh potato exports go mostly to South Pacific Islands, Pacific Rim markets and Middle East (Petrie & Bezar 1998).

The potato tuber moth (PTM), *Phthorimaea operculella* (Zeller), Lepidoptera: Gelechiidae) is one of the most economically damaging pests of potatoes in areas such as the Middle East and North Africa (Raman & Palacios, 1982; Ebora & Sticklen, 1994; Lagunes *et al.*, 1996) and New Zealand (Atkinson *et al.*, 1956; Foot, 1979). Other countries where PTM is an important pest include India (Trivedi & Rajagopal, 1992) and Australia (Horne & Rae, 1995), while in North America, PTM is of importance in Southern and Central California (Oatman & Platner, 1974). In Europe it causes the greatest damage in the Mediterranean region (Spain, Italy, Greece, Southern France and Cyprus) (Izhevskiy, 1986). In New Zealand, PTM is most prevalent in the southern part of the North Island (Claridge, 1972).

Table 1.1 World production of major crops in year 2004 (Source: FAOSTAT, 2004).

Crop	Production (Mt)
Maize	705,293,226
Wheat	624,093,306
Rice	608,496,284
Potatoes	328,865,936

1.2 Biology of potato tuber moth

The potato tuber moth (PTM) is an oligophagous insect. Its host plants are mainly in the *Solanaceae* family (Fenemore, 1988; Das & Raman, 1994). PTM usually attacks potato foliage and tubers, but will also feed on tobacco (*Nicotiana tabacum* L.), tomato (*Lycospersicon esculentum* Mill.) and eggplant (*Solanum melongena* L.) (Atkinson *et al.*, 1956 ; Fenemore, 1980). However, the potato is an ideal host plant for PTM since the larvae can fully utilize all parts of the plant (Broodryk, 1971).

The adult moths are small with narrow wings and are greyish brown. The moth's body length is about 10 mm and the wingspan is about 13 mm (Atkinson *et al.*, 1956; Foot, 1975a). They are most active at dusk and dawn but during the day can be seen flying within potato crops (Foot, 1975a). Females lay from 100 to 200 eggs/individual (Fenmore, 1980) on rough surfaces such as potato tuber eyes or the abaxial surfaces of leaves (Traynier, 1975). The eggs are pearly white when first deposited and gradually turn yellow on maturity and become black just before hatching. Hatching occurs 3 –6 days after oviposition, depending on temperature (Traynier, 1975; Foot, 1979).

The importance of PTM as a pest relates to the biology of the larvae. These are slender caterpillars with dark heads and a pinkish or greenish colour (Atkinson *et al.*, 1956; Foot, 1975a). They mine leaves, petioles and tubers and feed by tunnelling between the abaxial and adaxial surfaces of leaves causing transparent leaf blisters (Foot, 1975a). In tubers, they mine and form a gallery either just beneath the skin or deep in the flesh, which their coat with silk threads and eject their frass to the outside (Atkinson *et al.*, 1956; Foot, 1975a). Such potatoes are low in quality and market value and create sites for infection by microorganisms (Das & Raman, 1994; Plaisted *et al.*, 1994).

PTM larvae mature in 16 – 24 days, depending on temperature (Foot, 1975a). When fully grown, they leave their host and pupate in a silken, greyish cocoon, covered by soil particles and usually found in the soil near the base of plants, in dead leaves, other sheltered sites or on stored potatoes (Atkinson *et al.*, 1956; Foot, 1975a). A new generation of moths emerges from the cocoons in 6-9 days (Foot, 1975a). In New Zealand, usually during summer, a generation may be produced in five to six weeks and in the North Island, five to six generations may be passed through in the field during a season (Atkinson *et al.*, 1956). Infestations and damage are more common in a hot, dry climate (Atkinson *et al.*, 1956; Foot, 1979).

1.3 Management of potato tuber moth

There are a number of methods used to reduce crop loss from PTM; these include cultural management practices (Foot, 1974b & 1975b), biological control (Foot, 1979; Salama *et al.*, 1995a & 1995b; Kroschel *et al.*, 1996a & 1996b) and the use of broad-spectrum insecticides (Foot, 1974a; Shrivastava & Deole, 1989; Raj & Trivedi, 1993; O'Connor, 2002). Also, a partially-sterile male technique (Makee & Saour, 1997 & 1999) and breeding resistance to PTM into commercial cultivars from wild species (Ortiz *et al.*, 1990; Raman *et al.*, 1994; Malakar & Tingey, 1999) have been evaluated.

Cultural control is an important management practice for PTM from infested fields to stored tubers (Das & Raman, 1994). These practices include planting uninfested

tubers, planting time, keeping potatoes well hilled at least 5 cm over the tubers, cultivating or irrigating to prevent deep cracks in soils, removing foliage with herbicide application before harvest, harvesting tubers as soon as possible after they have matured, immediately removing of tubers from the field, storing the tubers below 10°C, screening the storage area for egg-laying moths, and fumigating or steam cleaning sacks before they are reused (Foot, 1974b & 1975b; Hanafi, 1999). However many of these practices are difficult to implement, labour intensive operations and are highly dependent on weather.

Biological control involves releasing a number of predators and parasitic enemies of PTM that have been recorded from different parts of the world (Trivedi & Rajakopal, 1992). In New Zealand, the braconid parasite *Apanteles subandinus* was released in the late 1960s into the lower North Island and has since become widely established throughout both North and South Islands but has not given any effective control of PTM (Foot, 1979).

Granulosis virus (Baculoviridae) can be used for biological control of the PTM (Kroschel *et al.*, 1996a & 1996b). However, the persistence of this virus in the field is limited by direct sunlight since ultraviolet (UV) radiation reduces virus activity, and repeated application at frequent intervals is required to overcome this problem (Kroschel *et al.*, 1996b).

The bacterium *Bacillus thuringiensis* (*Bt*) is used as a biopesticide to control PTM (Salama *et al.*, 1995a). Different strains of *Bt* are commercially available to control PTM in the field and during tuber storage, but *Bt* has very low persistence to UV irradiation in the field which limits its effectiveness (Salama *et al.*, 1995b). In experiments, aqueous suspensions of *Bt* were less effective than dusting with a sand mixture onto the potato (Lal, 1987 & Raman *et al.*, 1987). However dusting with the *Bt* / sand mixture can affect the water content of tubers in storage and larvae inside the tubers are not inhibited by this treatment (Kroschel & Koch, 1996).

Insecticides are used as essential practice by most of the potato cultivating countries to control the PTM. Most of the insecticides recommended belong to the organophosphate, synthetic pyrethoid or carbamate chemical groups (Foot, 1974a; Trivedi & Rajagopal, 1992; Raj & Trivedi, 1993; O'Connor, 2002). In New Zealand, the details of the main recommended insecticides to control PTM are given in Table 1.2.

Table 1.2 Information on insecticides recommended for use on potato crops in New Zealand for the control of potato tuber moth (O'Connor, 2002).

Insecticide	**Chemical group**	**Acute oral LD_{50}**	**Acute dermal LD_{50}**
Azinphos-methyl	Organo-phosphate	10-13 mg/kg (rats)	280 mg/kg (rats)
Baythroid	Synthetic pyrethroid	1213-1410 mg/kg (rats)	5000 mg/kg
Carbaryl	Carbamate	400 mg/kg (rats)	500 mg/kg (rats)
Deltamethrin	Synthetic pyrethroid	>5000 mg/kg (rats)	2000 mg/kg (rabbits)
Karate Zeon	Synthetic pyrethroid	467-955 mg/kg (rats)	>1800 mg/kg (rabbits)
Methamidophos	Organo-phosphate	30 mg/kg (rats)	50-110 mg/kg (rats)
Orthene	Organo-phosphate	866 mg/kg (rats)	>2000 mg/kg (rabbits)
Endosulfan	Sulphurous acid and ester of cylic diol	80-110 mg/kg (rats)	359 mg/kg (rabbits)

Efficacy of the chemicals is restricted when they are applied while PTM larvae inhabit mines. The larvae do not ingest the active substance until they emerge from mines. As pointed out by Foot (1974a), there is no point in using chemicals, having only an indirect effect on tuber infestation, if a large moth population is generated in a nearby field. Heavy infestation of PTM still requires the use of repeated application of chemicals (O'Connor, 2002) while frequent use of pyrethroids could soon result in the development of resistance in PTM (Cisneros, 1984). Symington (2003) reported that recommended pesticides for use against PTM such as permethrin, endosulfan and methamidophos interfere with the effectiveness of the parasitoid *Orgilus lepidus* to control PTM in Australia.

However, the realisation of the risks of using insecticides in potato production have resulted in an increased awareness of serious problems such as: development of PTM resistance to insecticides, persistence of residues in tubers for consumption, destruction of beneficial organisms, human intoxication, and contamination of the environment (Raman *et al.*, 1994).

Integrated Pest Management (IPM) is becoming more important in controlling pests without relying totally on chemical insecticides. The IPM strategy involves the complementary use of cultural, biological and chemical control methods. Under IPM, insecticides are applied only if pheromone trapped PTM populations exceed the appropriate threshold level (Raman, 1982; Hanafi, 1999). However, pheromone trapping of PTM is affected by climatic factors such as wind speed (Krambias, 1976). In New Zealand, Herman (2000) reported that pest population-based spray threshold under IPM would not be feasible to control PTM because sometimes few or no PTM larvae in the foliage can be associated with more infested tubers at harvest, while relatively large populations of PTM larvae in foliage can be associated with tuber damage at harvest.

Cultivating pest-resistant cultivars is one of the best approaches in IPM. This can reduce the need for chemical treatments and increase the effectiveness of alternative methods of control. Development of pest-resistant potato cultivars has

been reported to have had limited success by Raman and Palacios (1982), Chavez *et al.* (1988), Ortiz *et al.* (1990), Arnone *et al.* (1998). In these studies, genes with resistance to PTM expressed in hybrids were obtained through traditional breeding with wild species. However, major constraints to breeding relate to the tetrasomic nature of inheritance with the potential of four different alleles per locus, ensuring high heterozygosity and severe inbreeding depression in elite parental potato clones (Struik *et al.*, 1997). Other difficulties in traditional breeding are lack of availability of resistant germplasm (Hawkes, 1990) and resulting plant populations showing a low resistance frequency at the tetraploid level (Raman *et al.*, 1994).

The management methods discussed above are not providing satisfactory control of PTM. However, the development of potatoes by genetic engineering to insert genes resistance to PTM could provide an additional important component for IPM systems against this pest.

1.4 Genetic engineering

Genetic engineering (GE) deals with the transformation of foreign genes isolated from plants, viruses, bacteria or animals into a new genetic background. GE involves the linking of DNA molecules by *in vitro* manipulations for the purpose of generating a novel organism with desired characteristics (Hartl & Jones, 1998). The ultimate aim is to regenerate plants identical to the parental material, except for the newly inserted genes (Draper & Scott, 1991; Webb & Morris, 1992).

Clonal crops such as potatoes are highly heterozygous and usually suffer from severe inbreeding depression and instant loss of their genetic integrity upon selfing or outcrossing (Conner & Christey, 1994). Therefore, it is impossible to maintain the genetic integrity of a cultivar by combining traits through sexual hybridization. Furthermore, sexual breeding is complicated in potatoes by tetrasomic segregation patterns and incomplete fertility in many tetraploid commercial cultivars (Ebora & Sticklen, 1994). Therefore the transformation of genes by genetic engineering represents the only effective way to produce isogenic lines of commercial clonal cultivars of potatoes (Conner & Christey, 1994).

A major goal of plant GE is the introduction of agronomically desirable traits into crop plants in situations where conventional breeding methods have been unsuccessful (Ebora & Sticklen, 1994). In the meantime, considerable progress has also been made in the identification of genes to be used in the engineering of plants for crop improvements, such as herbicide resistance, virus resistance and insect resistance (Peferoen, 1992).

The major differences between traditional plant breeding and genetic engineering (GE) were described by Peferoen *et al.* (1990) as :

1) in traditional breeding, genetic information can only transfer among individuals from the same or closely related species but in GE approaches, DNA can be transferred into a plant from any organism (microbes, plants and animals),

2) in GE, only well-characterized genes are transferred; in contrast, traditional breeding may transfer traits from wild germplasam into a crop plant, without knowing the genes responsible for this trait, and

3) the breeding process can be considerably faster in GE because a gene is transferred in a single step, while other characteristics of the recipient variety remain unaffected in theory and the amount of backcrossing needed can be considerably reduced.

There are number of methods used for gene transformation of which only four have been successful in plants (Potrykus, 1993). These are *Agrobacterium tumefaciens*-mediated gene transfer, protoplast-based direct gene transfer, microinjection and biolistic approaches. The most widely used method for gene transformation into plants is *Agrobacterium*-mediated gene transfer (Grant *et al.*, 1991; Potrykus, 1993; Gheysen *et al.*, 1998), because it is a very efficient and versatile vector for the stable introduction of genes into plants driven by biological processes such as illegitimate recombination (Hooykaas & Schilperoort, 1992).

1.5 *Agrobacterium*-mediated gene transfer

Agrobacterium species are phytopathogenic, gram-negative, soil-borne bacteria. They cause crown galls or tumours on plants by infecting and transferring a piece of their DNA (transferred DNA, T-DNA) into the nuclear genome of the host plant (Webb & Morris, 1992; Hansen *et al.*, 1994; Zupan *et al.*, 2000). Most of the machinery necessary for this T-DNA transfer resides on a tumour-inducing (Ti) plasmid. This Ti plasmid includes the T-DNA itself, delimited by 25 bp imperfect repeats known as right and left borders (RB and LB respectively) that define the boundaries of the T-DNA and about 35 virulence (*vir*) genes. The combined action of the *vir* genes achieves the delivery of the T-DNA to the nucleus of the host plant cell (Gheysen *et al.*, 1998; Zupan *et al.*, 2000). The T-DNA contains the genes for inducing tumour formation. *Agrobacterium*-mediated transformation systems take advantage of this natural plant transformation mechanism. Removal of all the genes within the T-DNA does not impede the ability of *Agrobacterium* to transfer this DNA, but does prevent the formation of tumours. Any DNA between left and right borders will be transferred to a plant cell (Zupan & Zambryski, 1995).

Vir genes are located outside of T-DNA on the Ti plasmid. These genes are involved in processing of the T-DNA from the Ti plasmid and in T-DNA transfer from the bacterium to the plant cell. Certain Vir proteins promote T-DNA targeting to the nucleus and are probably involved in precise T-DNA integration into the plant DNA (Gheysen *et al.*, 1998). The *vir* genes are capable functioning in a cis or trans-acting manner (Hansen *et al.*, 1994). Some chromosomal genes are important for attachment of the bacterium to the plant cell (Gheysen *et al.*, 1998). Ti plasmids and their host *Agrobacterium* strains that are no longer oncogenic are termed 'disarmed'. Disarmed *A. tumefaciens* strains containing a helper plasmid with the whole of the T-DNA deleted still retain the *vir* functions and the capacity for T-DNA transfer (Hoekema *et al.*, 1983).

There are two key advances that have made *Agrobacterium* transformation the method of choice for plant genetic engineering. These are the development of binary Ti vectors and a range of disarmed *Agrobacterium* strains. The two main

components for successful *Agrobacterium*-mediated gene transfer, the T-DNA and the *vir* region, can reside on separate plasmids. These form the basis of modern plasmid vectors, termed binary Ti vectors (Hoekema *et al.*, 1983). The *vir* gene functions are provided by the disarmed Ti plasmids resident in the *Agrobacterium* strain. The T-DNA, with the gene(s) to be transferred, is provided on the binary vector.

The region between left and right borders of T-DNA on binary vectors is modified by insertion of the genes to be transferred and a selectable marker gene (Mansour *et al.*, 2002). Such vectors are transformed into *E. coli* for replication and manipulation and subsequently transferred by conjugation or electroporation to the disarmed *A. tumefaciens* strains containing a helper plasmid (whole of the T-DNA deleted) that provides the vir functions (Hoekema *et al.*, 1983). On infection of a plant the activated *vir* genes function in trans resulting in transfer of all DNA between left and right borders to a plant genome (Grant *et al.*, 1991; Gheysen *et al.*, 1998). *Agrobacterium*-mediated transformation using binary vectors is the preferred method to develop the transgenic potato plants because the integration of single intact transgenes is usually associated with this method (Conner & Jacobs, 1999).

Most *in vitro* gene manipulation techniques use *E. coli* and consequently binary Ti vectors replicate in both *E. coli* and *Agrobacterium.* Some of the important binary Ti vector types reviewed by Hellens *et al.* (2000).

1.6 Selectable marker genes

The selectable marker gene is important to enable transformed cells to survive in the media while non-transformed cells will not. This is because the media contains selection agent which is toxic to non-transformed plant cells (Draper & Scott, 1991). For this purpose, the neomycin phosphotransferase II (*npt* II) gene from the bacterial transposon Tn5 is widely used as selectable marker gene, which confers resistance to the kanamycin as selection agent in that media through detoxification by phosphorylation (Conner *et al.*, 1991; Webb & Morris, 1992).

Kanamycin tends to bleach tissues first and inhibit further growth of non-transformed plant tissues without causing rapid necrosis. It is therefore the most commonly used selective agent for plant transformation (Draper & Scott, 1991). The use of the selection agent at a critical threshold concentration is important since a too low concentration causes failure to eliminate the non transformed cells and a too higher concentration may lead to the recovery of transgenic lines with higher expression due to the multiple copies of the transgenes which may result in subsequent problems of homology-induced gene silencing (Barrell *et al.*, 1998).

Barrell *et al.* (2002) investigated a range of alternative selectable markers and ranked them in terms of their relative efficiency of recovering transgenic lines in potato transformation as kanamycin (*npt*II) > hygromycin (hpt) > methotrexate (dhfr) > phosphinothricin (bar) > phleomycin (ble). The use of different selectable marker genes will be valuable to pyramid transgenes into same cultivar of clonal crops such as potato (Barrell *et al.*, 1998).

1.7 Regulation of transgene expression

Foreign genes need to have appropriate *cis* regulatory DNA sequences called promoter (5' to the coding region) and polyadenylation signal (3' to the coding region) for effective transgene expression in transgenic plants to ensure efficient transcription, stability and translation of mRNA (Webb & Morris, 1992; Schuler *et al.*, 1998). In eukaryotes, in addition to the promoter sequences other DNA sequences called enhancers that interact with the promoter to determine the level of transcription can also be important (Hartl & Jones, 1998). Certain sequences from *A. tumefaciens* and *A. rhizogenes* have been used as promoters such as those from *nos* (nopaline synthase), *ocs* (octopine synthase), *mas* (mannopine synthase) genes (Webb & Morris, 1992). However, cauliflower mosaic virus (CaMV) *35S* promoter is now most widely used and considered as constitutive promoter since showed no tissue-specificity of expression is observed (Odell *et al.*, 1985) and it is active during most stages of plant development (Peferoen, 1992).

Promoters can be classified according to the gene expression such as constitutive gene expression (Odell *et al.*, 1985); tissue-specific gene expression, for example phloem-specific promoters (Shi *et al.*, 1994), seed-specific promoters (Ishimoto *et al.*, 1996) and pollen-specific promoters (Koziel *et al.*, 1993); and gene expression induced by environmental stimuli such as light-sensitive promoters (Nap *et al.*, 1993) and heat-shock promoters (Moore *et al.*, 1998). In addition to these, there are other classes also have been reported, such as chemically inducible promoters (Cao *et al.*, 2001) and wound-induced promoters (Peferoen *et al.*, 1990; Garbarino & Belknap, 1994). As an alternative approach Moore *et al.* (1998) established a promoter and transcription factor system pOp/LhG4 that allows trans gene expression only after crossing of reporter and activator plants.

Constitutive promoters like the CaMV *35S* promoter have been used to drive gene expression in the majority of potato transgenic plants developed to date (Conner *et al.*, 1991; Douches *et al.*, 1998 & 2002; Lagnaoui *et al.*, 2000; Mohammed *et al.*, 2000; Davidson *et al.*, 2002a & 2004a). However, continuous gene expression in all plant tissues is claimed to increase the risk of the pest over coming the resistance mechanism and may also result in yield penalties as the plant directs more resources than necessary to its defences (Schuler *et al.*, 1998). Also, it is gene expression which raises the most concern for food safety (Conner *et al.*, 1997). Hence, an alternative promoter is important for potato transformation with only a foliar specific expression/function for when transgene expression is only necessary in the foliage.

The most effective approach to achieve this involves the isolation of appropriate promoters from potatoes. A good candidate is the potato *Lhca*3.St.1 gene encoding the apoprotein 2 (type III chlorophyll a/b binding protein) of the light harvesting complex of Photosystem I, isolated from the potato cultivar Bintje (Nap *et al.*, 1993). This promoter is active in leaves, stems and other green parts of potato and tobacco plants while no expression has been observed in roots, tubers and under ground stolons (Nap *et al.*, 1993). *Lhca*3.St.1 promoter gives higher expression than the doubled cauliflower mosaic virus *35S* (dCaMV) (Nap *et al.*, 1993; Conner

et al., 1999; Annadana *et al.*, 2001), which in turn is stronger than the single CaMV *35S* promoter.

Transgenic plants show different gene-expression level between independently derived lines, even when the same transgene and promoter are used (Grant *et al.*, 1991; Conner & Jacobs, 1999). Gene silencing and copy number can influence in gene expression. In some cases, transgenes have become inoperative, an effect known as gene silencing (Senior & Dale, 1996). There are several causes of gene silencing and these seem to rely on homology either between different transgenes or between transgenes and endogenous genes (Matzke & Matzke, 1995). However, different types of gene silencing have been proposed such as position effect, environmental conditions, homology dependent gene silencing, transcriptional gene silencing, co-expression and antisense (Senior & Dale, 1996).

Generally, position effects result from the random integration of the transgene into different sites of the plant genome in which the surrounding endogenous plant DNA can influence the expression of the inserted genes. Plants exhibiting undesired transgene expression resulting from such effects have to be discarded (Grant *et al.*, 1991; Conner & Christey, 1994). Mlyanárová *et al.* (1994, 1995 & 1996) demonstrated that position effects can be reduced in transgenic plants by the placement of a matrix-associated region (MAR) on either side of transgenes that insulate transcriptional regulation from the effects of surrounding chromatin.

Sometimes T-DNA transfer to plant cells results in transformation events in which multiple copies of the transgenes are integrated into one or more sites in the plant genome (Conner *et al.*, 1997; Gheysen *et al.*, 1998). Transgene expression remains relatively high and stable when the insertion of the T-DNA occurs as one intact DNA fragment into low/single copy DNA at a single locus (Conner & Jacobs, 1999).

1.8 The use of *Bacillus thuringiensis* for engineering insect-resistant plants

Bacillus thuringiensis (*Bt*) is an aerobic, motile, gram-positive, endospore-forming bacterium that produces crystals during its sporulation (Schnepf *et al.*, 1998; Shelton *et al.*, 2002). It was initially isolated in Japan by Ishiwata and formally described by Berliner in 1915 (Shelton *et al.*, 2002). These crystals are also called protoxins, insecticidal crystal proteins (ICPs) or δ (delta) endotoxins (Ebora & Sticklen, 1994). Different ICPs from *Bt* strains are toxic to different groups of insects (Schnepf *et al.*, 1998). For this reason, *Bt* is the most widely used biologically produced pest control agent.

Initially, crystal proteins were characterised based on their insecticidal activities for the primary ranking of their corresponding genes using a systematic based nomenclature (Höfte & Whiteley, 1989). However, now they are characterised based on hierachical clustering using amino acid sequence of crystal proteins referred to as sequence based nomenclature (Crickmore *et al.*, 1998). To date more than 100 *cry* genes have been isolated and sequenced (*Bacillus thuringiensis* Toxin Nomenclature, 2005).

Bt directly causes mortality in insects through a toxic pathway of Cry proteins. Upon ingestion by susceptible insects, crystal proteins are dissolved under the alkaline conditions of the insect midgut and protoxins of 27-140 kDa are released (Gleave *et al.*, 1992a & 1992b). This protoxin is then cleaved by midgut proteases into a toxic core fragment and passes through the peritrophic membrane. This activated toxin binds to a receptors in the brush border membrane of midgut epithelial cells, creating pores in the cell membrane, leading to cell lysis, and eventually insect death (van Rie *et al.*, 1990a & 1990b; Schnepf *et al.*, 1998; Ferré and van Rie, 2002).

Various Cry proteins have shown more toxicity against certain insects when they are ingested in combination rather than alone; this is attributed to the synergistic interaction of toxin potency among various *Bt* toxins (Lee *et al.*, 1996). *Bt* ICPs are active against a variety of species from different orders such as Lepidoptera (moths

and butterflies), Coleoptera (beetles), and Diptera (flies and mosquitoes) (Peferoen, 1992). The larvae of PTM are affected by various strains of *Bt* (Salama *et al.*, 1995a). The efficiency of *Bt* as a conventional insecticide is limited by instability of the protein when exposed to UV light (Salama *et al.*, 1995b) and poor retention on plant surfaces in wet weather. Alternatively, insertion of genes encoding Cry proteins into plants via GE approaches is being realised to control insect pests.

Several *cry* genes have been introduced into plants since the first transgenic tobacco plants were developed with *cry* genes (Peferoen, 1992). Unmodified crystal protein genes (native *cry* genes) do not express efficiently in transgenic plants (Schnepf *et al.*, 1998). Therefore, considerable modification to the *Bt* genes, such as truncation of improper splice sites, poly(A) signals, ATTTA sequences, mRNA degradation signals and transcription termination sites, as well as codon-modification, has proved necessary in order to get adequate expression to confer insect resistance on transgenic plants (Gleave *et al.*, 1998; Fujimoto *et al.*, 1993; Beuning *et al.*, 2001; Perlak *et al.*, 1991; Vaeck *et al.*, 1987; van der Salm *et al.*, 1994).

Currently, engineered crops such as potato, cotton and maize with modified *cry* genes for insect resistance have been commercialised (Shelton *et al.*, 2002), and other crops including cereals, leafy vegetables, root crops, forage crops and trees are also being engineered to express *cry* genes (Schuler *et al.*, 1998).

1.9 Potato plants with engineered resistance to potato tuber moth

The development of transgenic potatoes with resistance to PTM has involved three approaches, exploiting different mechanisms of actions (Barrell *et al.*, 1998). These include the transfer and expression of genes encoding: 1) protease inhibitors, 2) biotin-binding proteins and 3) *cry* genes from *Bacillus thuringiensis*.

1.9.1 Potato plants expressing protease inhibitors

Protease inhibitors irreversibly bind to protease enzymes in the insect gut and interfere with protein digestion, thereby preventing normal growth and development (Ryan, 1990). Using mid-gut extracts from PTM larvae, a wide range of protease inhibitors were tested by *in vitro* screening of inhibition of proteolysis. Of 28 inhibitors investigated, two were identified with significant activity against PTM mid-gut protease activity: bovine pancreatic trypsin inhibitor (BPTI) and α_1-antitrypsin inhibitor (α_1-AT) (Christeller *et al.*, 1992). The coding regions of genes for spleen inhibitor (which is highly homologous to BPTI) and α_1-AT were placed under the control of the *35S* promoter and inserted into the binary vectors pART27 (Gleave, 1992) and pBIN19 (Bevan, 1984), and transformed into the Iwa potato using *Agrobacterium*-mediated transformation. Thirty independently derived lines for each vector, confirmed as being transgenic, were analysed by western analysis for levels of expression. The highest expressing lines accumulated between 0.3 and 0.8% of total protein as the inhibitor and were multiplied by *in vitro* micro-propagation and transferred to the greenhouse for PTM bioassays. No resistance to PTM larvae was evident in the foliage of greenhouse-grown plants upon inoculation with neonate larvae (Christeller *et al.*, 1995). Further bioassays using excised leaves confirmed that these lines expressing proteinase inhibitors failed to confer any improved resistance to PTM larvae. This was attributed to the larvae responding to the ingestion of the protease inhibitors by producing, second-tier proteases (Christeller *et al.*, 1995; Jongsma *et al.*, 1995).

1.9.2 Potato plants expressing biotin-binding proteins

Biotin-binding proteins, such as avidin and streptavidin, cause a lethal vitamin deficiency in insects when ingested at low doses (Morgan *et al.*, 1993; Markwick *et al.*, 2001). The binary vectors pLA2 containing an avidin gene and pSAVα2 containing a streptavidin gene were used for *Agrobacterium*-mediated transformation of tobacco and apple (Murray *et al.*, 2002; Markwick *et al.*, 2003). Both the avidin and streptavidin genes were under the transcriptional control of the *35S* promoter. The coding regions of the avidin and streptavidin genes were cloned as translational fusions to the leader sequence from the potato proteinase inhibitor gene (PPI-I and PPI-II respectively) to target the accumulation of the biotin-binding proteins to the plant cell vacuole so that they would not be self-toxic. The transgenic tobacco plants exhibited over 90% of mortality to PTM larvae and the transgenic apple plants exhibited high level of resistance to lightbrown apple moth larvae (*Epiphyas postvittana*) (Markwick *et al.*, 2003).

Takla (2001) and Meiyalaghan *et al.* (2005b) used the binary vectors pLA2 and pSAVα2 respectively for potato transformation. A total of 40 and 65 independently derived lines for each vector, respectively, were confirmed as being transgenic for both the *npt*II marker gene and the gene encoding biotin-binding proteins. Avidin and streptavidin contents in the leaves of these lines were determined using an ELISA assay. Avidin was detected in nearly all avidin-transgenic lines, although the concentrations were highly variable between independently derived transgenic lines and ranged from 0.4 to 2.5 nmoles/g fresh weight (Takla, 2001). All the lines producing avidin exhibited high mortality of PTM larvae, with most lines inducing complete mortality (Takla, 2001). In contrast to the avidin-transgenic lines, streptavidin was detected in only a relatively small proportion of streptavidin-transgenic lines (13 of 65). For the streptavidin-positive lines the concentration ranged from 0.8 to 42.6 nmoles/g fresh weight, all of which exhibited high mortality of PTM larvae (Meiyalaghan *et al.*, 2005b).

Overall, transgenic potato lines accumulating avidin or streptavidin showed a range of off-type characteristics such as marginal leaf curl, leaf wrinkling, reduced vigour, abnormally small and/or deformed tubers, or a combination of these traits (Takla, 2001; Meiyalaghan *et al.*, 2005b). It was observed that virtually all transgenic lines with higher levels of avidin or streptavidin and higher PTM larvae mortality, exhibited the most severe morphological differences relative to their non-transformed controls. They were attributed this to the accumulation of biotin-binding proteins that were not adequately targeted to the plant vacuoles and resulted in some phytotoxic effects on the transgenic potato lines. This is surprising as exactly the same binary vectors, with the leader sequences for vacuolar targeting derived from potato, did not result in such effects when transferred to tobacco (Murray *et al.*, 2002).

1.9.3 Potato plants expressing *cry* genes

The availability of a variety of ICPs from *Bt* active against PTM has enabled the development of resistance in a range of potato varieties. van Rie *et al.* (1994) described PTM as susceptible to *cry*1A(b), *cry*1B and *cry*1C genes since PTM larvae have receptors for these toxins in the midgut epithelium. Transgenic potatoes have been developed with a range of *cry* genes active against PTM (Table 1.3). These *cry* genes have been modified before inserted into plant genome to increase the gene expression in transgenic potatoes.

Table 1.3 *Bt* transgenic potatoes with expressing crystal proteins for control of PTM.

***Bt* gene**	**Promoter**	**Cultivars transformed**	**Resistance**	**Reference**
*cry*1Ab	TR2'	Berolina, Kennebec	Almost 100% mortality	Peferoen *et al.* 1990.
	TR2'	Kennebec, Bintje, Yesmina	Almost 100% mortality	Jansens *et al.* 1995.
	35S, d *35S*	Cruza 148, Sangema, LT-8	Almost 100% mortality	Canedo *et al.* 1997.
	35S	Kufri Badshan, Kufri Ashoka, Kufri Sindhuri, Kufri Lauvkar, Kufri Jawahar	Almost 100% mortality	Chakrabarti *et al.* 2000.
*cry*1Ac	*35S*	FL1607	10% mortality after 48hrs bioassay	Ebora *et al.* 1994.
*cry*1Ac9	*35S*	Ilam Hardy, Iwa, Russet Burbank, Red rascal, Rua, White Delight	Inhibited larval growth and failure to reach pupation	Davidson *et al.* 2002a, 2002b, 2004a, 2004b.
cry5	*35S*, GSP, Patatin	Lemhi Russet, Atlantic, Spunta	Mortality in tubers up to 100%, in leaves up to 96%	Douches *et al.* 1998, 2002; Westedt *et al.* 1998; Li *et al.* 1999; Lagnaoui *et al.* 2000; Mohammed *et al.* 2000.
*cry*9Aa2	*35S*	Iwa	Inhibited larval growth and failure to reach pupation	Takla 2001.

1.10 Resistance management for insect pests on transgenic plants

Pest resistance to insecticides is a well-known phenomenon and over 500 species of insects have developed resistance to one or more pesticides (Shelton *et al.*, 2000). These cases of resistance are not limited to synthetic insecticide but also to *Bt* toxins. Proteolytic processing of protoxins, improved repair of damaged midgut cells and modification of a *cry* protein-binding site are identified as three different mechanisms of resistance to *Bt* in insect pests (Ferré & van Rie, 2002). There are several reports on the development of insect resistance to *Bt* formulations in laboratory and field. Colorado potato beetle, cottonwood leaf beetle, tobacco budworm, cotton leaf worm and European corn borer have shown the development of *Bt* resistance in laboratory screening, where as Diamondback moth (*Plutella xylostella*) has developed resistance in the field to *Bt* sprays (Tabashnik, 1994; Ferré & van Rie, 2002). To date, there are no cases of insect resistance to *Bt*-transgenic plants in the field. Although, to prevent insects from developing resistance to transgenic plants, it is important to implement the resistance management strategies before pest-resistant crops are widely and intensively deployed.

Various resistance management strategies (Table 1.4) in the context of *Bt* have been reviewed in detail (McGaughey & Whalon, 1992; Tabashnik, 1994; Gould *et al.*, 1994; Roush, 1997b; McGaughey *et al.*, 1998; Gould, 1998 & 2003; Shelton *et al.*, 2000). These strategies include 1) mixtures of toxins with different mechanisms, either within the same plant or in different plants, or expressed serially over time; 2) rotations to alternative toxins temporally to reduce the frequency of resistant individuals; 3) refuges, temporal and spatial, to facilitate survival of susceptible individuals; 4) low doses of toxin that produce sublethal effects, such as reduced fecundity and slowed development, favouring other mortality factors; 5) ultrahigh doses of toxin that kill resistant heterozygotes and homozygotes; and 6) gene regulation of toxin titre, location, and induction. However, the only commercially deployed strategy is use of a high dose of a single gene in combination with a refuge (Shelton *et al.*, 2000).

Table 1.4 Summary of deployment strategies

Strategy	Objective
Refugia with non-transgenic plants	To generate sufficient numbers of susceptible insects to dilute resistant alleles
High levels of expression of a single toxin in all plants	To kill maximum percentage of the most vulnerable stage of totally susceptible insects
Low levels of expression of a single toxin in all plants	To decrease the reproductive rate and growth rate of insect pests by sublethal dose
Multiple gene deployment or gene pyramiding or gene stacking	To reduce the likelihood of resistance development since requires several mutations to occur simultaneously in the same insect
Targeted toxin-gene expression in certain parts of the plant or under certain environmental conditions	To reduce the time periods of insect pests to exposure to a toxin

1.10.1 Refugia

A refuge is an area on non-transgenic crop that is placed either within a crop as a seed mixture (a multi-line approach) or as a separate block with close proximity (Conner *et al.*, 2003). Shelton *et al.*, (2000) have shown that a separate refuge is more effective than a mixed refuge for diamondback moth in *Bt*-transgenic broccoli plants, since more susceptible alleles are conserved in a separate refuge. If such individuals mate with rare resistant homozygous individuals only susceptible heterozygote progeny are produced.

Experiments in movement of PTM within and between crops conducted by Cameron *et al.* (2002) suggest that a refuge should be placed close to transgenic potato crops to dilute potential resistance of PTM to transgenic crops.

1.10.2 Toxin rotation

The large number of naturally occurring *Bt* toxins offers hope of countering resistance through frequent changes in the toxin being expressed in the transgenic plants. The rotations to alternative toxins temporally to reduce the frequency of resistant individuals (Tabashnik, 1989). The key hypotheses of this strategy are: 1) different genes confer resistance to different *Bt* toxins so that cross-resistance to the alternative *Bt* toxin does not occur; 2) Resistance genes to any given toxins are rare, so that the mating of two individuals carrying distinct resistance genes to different toxins would be extremely rare; 3) The large number of *Bt* toxins gives a potentially wide choice of toxins to be used.

For some insects, laboratory data indicate a fair probability of achieving successful resistance management through toxin rotation. As an example, selection of the cabbage looper, *Trichoplusia ni*, with *cry*1Ab toxin did not lead to cross-resistance to the closely related *cry*1Aa and *cry*1Ac toxins (Estada & Ferrè, 1994). Even though a large number of *Bt* toxins is recorded, only a subset of the total number is generally active against a given pest. The members within one of these subsets may share the same receptor(s) on insect midgut tissues. This sharing of receptors in turn may lead to cross-resistance. Given these facts, the choice of possible *Bt* toxins to use in a toxin rotation strategy against a specific pest may become very limited. Therefore, the investigation of cross resistance of PTM to *Bt* toxins and investigating new *cry* genes conferring resistance to PTM are necessary to establish different transgenic potato plants to implement this strategy.

1.10.3 Gene deployment

The deployment of different genes and their level of expression should be based on insect sensitivity and level of resistance development. Higher dosage of toxins kills heterozygous insects (those with both resistance and susceptible alleles). A high dose of toxins sufficient to kill insects with an increasing degree of resistance and even resistant homozygous insects is important. However, experiments deploying a high dose strategy, using commercial formulation of *Bt*, in the field on a DBM population that had already developed a significant resistance, has shown that a refuge area of 25% combined with the high dose strategy was insufficient to prevent further development of resistance (Perez *et al.*, 1997). Whereas, high levels expression of *cry*1C protected transgenic broccoli not only from susceptible or *cry*1A resistance DBM larvae, but also from those selected for moderate levels of resistance of *cry*1C (Cao *et al.*, 2002).

1.10.4 Regulation of gene expression

Activation of transgenes by the use of appropriate promoters is most important for durability and specificity of resistance. In most cases, CaMV *35S* promoter is widely used, which is considered as a constitutive promoter that shows no tissue-specificity of transgene expression. Schuler *et al.* (1998) suggested that continuous gene expression in all plant tissues may increase the risk of the pest resistance. Expression of transgene could be limited spatially and temporally to specific plants parts and at specific times by inducible promoters such as wound-induced promoter (Peferoen *et al.*, 1990) and light-sensitive promoter (Nap *et al.*, 1993) might contribute to the management of resistance development and unfavourable interactions with the beneficial insects. For efficient pest control, it is important that effective levels of insect control proteins are expressed at the site where the insects feed. The use of foliar specific light inducible promoter in transgenic potato plants to express genes conferring resistance to insect pests could be a relatively efficient control of pests such as PTM. Because, PTM easily infest foliage of potato crops prior to the tubers, preventing foliar infestation may be sufficient to indirectly avoid tuber infestation. In addition, that would be preferable from a food safety

point of view since higher expression of Cry proteins in tubers may be a deterrent to public perception of transgenic potatoes (Conner & Jacobs, 1999).

1.10.5 Gene pyramiding

This involves the expression of multiple transgenes simultaneously in a same plant. Theoretical models suggest that cultivars with two dissimilar insect toxin genes pyramided in the same plant have the potential to delay the development of resistance much more effectively than single-toxin plants used sequentially or in mosaics or seed mixtures, even with relatively small and more economically acceptable refuge sizes (Roush, 1997a). When pyramiding *cry* genes it is important to use genes that target different binding site specificity in insects (Frutos *et al.*, 1999; Ferré & van Rie, 2002). When two distinct toxins are expressed in transgenic plants, there is little potential for cross-resistance and insects require at least resistance alleles at two independent gene loci (R and R′) to permit high survival on such plants (Gould, 1998). Such genotypes of insects are exceedingly rare and therefore plants with pyramided transgenes will contribute little to the evolution of adaptation in early phases of selection.

Before deployment of gene pyramiding, an examination of synergistic effect from combination of multiple toxin genes is more important. Indeed, Lee *et al.* (1996) found that a combination of *cry*1Aa and *cry*1A showed a synergistic effect on gypsy moth larvae while a combination of *cry*1Aa and *cry*1Ab exhibited an antagonistic effect. van Rie *et al.* (1994) pointed out that PTM is sensitive to *cry*1Ab, *cry*1B and *cry*1C genes and that larvae have different receptors for these toxins. Therefore, investigation of potential synergistic effects with combinations of *cry* genes would be useful for deploying transgene pyramiding in potato against PTM.

Considerable strategies have been proposed for introducing and expressing multiple transgenes in crops (Berger, 2000). It has been reported that transgenic rice plants expressing three insecticidal genes such as *cry*1Ac, *cry*2A and a snowdrop lectin gene, introduced by particle bombardment, showed higher resistance to rice leaf

folder, yellow stemborer and brown planthopper than plants expressing single transgenes (Maqbool *et al.* 2001). Insecticidal action of the transgenic broccoli plants expressing both *cry*1Ac and *cry*1C, were produced by sexual crosses of broccoli lines with the different genes. These plants exhibited significant mortality of DBM larvae which is resistance to *cry*1Ac or *cry*1C individually (Cao *et al.*, 2002). In contrast, it is difficult to pyramid transgenes in potato by sexual crossing of different transgenic parental lines because potato is vegetatively propagated crop. Pyramiding of genes in clonal crops, that can not be usually crossed sexually, presents new challenge for deployment of gene strategies in agriculture. Therefore, experimental design of strategies for pyramiding in potato using *Agrobacterium*-mediated transformation is important.

Clearly, the current generation(s) of insect-resistant transgenic crops need to employ gene pyramiding strategies. Only if such studies are undertaken in a sound and systematic manner can we protect what McGaughey and Whalon (1992) describe as "the most scientifically, environmentally and sociologically acceptable pest suppression tools of this century..."

1.10.6 Objectives of this work

The overall goal of this work was to evaluate approaches for PTM resistance in potato cultivars by investigating novel transgenic strategies involving alternative transcriptional gene control, deploying new genes and gene pyramiding. This goal was set by aiming to complete the following objectives:

1. Develop a population of independently derived potato lines transgenic for a *cry*1Ac9 gene and a *cry*9Aa2 gene individually under the *Lhca3* promoter as an alternative means of transcriptional gene control (Chapter 2).

2. Develop a population of independently derived potato lines transgenic for a *cry*1Ba1 gene and a *cry*1Ca5 gene individually, as alternative *cry* genes under the transcriptional control of the CaMV *35S* promoter (Chapter 3).

3. Develop an experimental approach to simulate the gene pyramiding for deployment of *cry* genes in clonal crops (Chapter 4).

4. Develop a population of independently derived potato lines transgenic for a *cry*1Ac9 gene and a *cry*9Aa2 gene to investigate the effects of gene pyramiding (Chapter 5).

Chapter 2. Expression of *cry*1Ac9 and *cry*9Aa2 genes under a potato light-inducible *Lhca*3 promoter in transgenic potatoes for tuber moth resistance

2.1 Introduction

Potato tuber moth (PTM) *Phthorimaea operculella* (Zeller) is one of the most damaging insect pests of potato (*Solanum tuberosum* L.) crops in many temperate and tropical countries (Fenemore, 1988). In the field the moths usually lay their eggs on the undersides of leaves or on exposed tubers (Foot, 1979). The larvae, hatching from eggs laid on leaves, cause transparent blotch mines in the foliage. Larvae mine the tubers in the field as well as in storage, reducing tuber quality. The damaged areas can also provide an infection point for pathogens (Plaisted *et al.*, 1994). Various management strategies such as cultural practices, biological and chemical control are used to manage PTM (Foot, 1974a, 1974b & 1975b; Hanafi, 1999). To contribute an additional component to integrated pest management, the development of transgenic potatoes that express the insecticidal toxins produced by different strains of the soil bacterium, *Bacillus thuringiensis* (*Bt*), has been investigated (Jansens *et al.*, 1995; Douches *et al.*, 1998; Rico *et al.*, 1998; Cañedo *et al.*, 1999; Li *et al.*, 1999; Chakrabarti *et al.*, 2000; Davidson *et al.*, 2002a & 2004a).

The Cry1Ac9 toxin (designated under the revised nomenclature of Crickmore *et al.*, 1998), isolated from *Bt* strain, DSIR732, serotyped as var. *kurstaki*, is known to have insecticidal activity against PTM larvae (Gleave *et al.*, 1992a). The gene encoding this protein was codon-modified to increase its expression in plants and confer resistance to PTM larvae in tobacco (Beuning *et al.*, 2001). The subsequent transfer of this modified *cry*1Ac9 gene under the regulatory control of the CaMV35S promoter to potato confirmed efficacy against PTM larvae in the main crop in which this pest causes economic damage (Davidson *et al.*, 2002a & 2004a).

Gleave *et al.* (1992b) cloned and characterized a *cry* gene, encoding a 129 kDa protein from the *Bt* strain, DSIR517, serotyped as var. *galleriae*. It has been designated *cry*9Aa2 (Crickmore *et al.*, 1998) and the encoded protein has been shown to have insecticidal activity against PTM larvae (Gleave *et al.*, 1992b). Gleave *et al.* (1998) modified this *cry*9Aa2 gene into three truncated versions, G7, G10 and G14, with increasing modifications to codon usage. Tobacco plants transgenic for these modified *cry* genes showed resistance to PTM larvae, with higher efficacy associated with more changes to the codon usage (Gleave *et al.*, 1998). The transfer of the G14 version of the *cry*9Aa2 gene under the regulatory control of the 35S promoter to potato provided effective resistance to PTM larvae (Meiyalaghan *et al.*, 2005a).

The application of genetic engineering to many food crops, including potato, has raised public concerns, especially when the transferred gene is expressed in the plant components utilised as food (Conner & Jacobs, 1999). The 35S promoter used for transcriptional control of the *cry* genes in previous investigations in potato (Douches *et al.*, 1998; Rico *et al.*, 1998; Cañedo *et al.*, 1999; Li *et al.*, 1999; Chakrabarti *et al.*, 2000; Davidson *et al.*, 2002a & 2004a) is well known to be constitutively expressed throughout plants (Odell *et al.*, 1985), including potato (Gatehouse *et al.*, 1997). However, it is often desirable to couple high-level expression of the transgene in potato foliage with no expression in tubers. The most effective approach to achieve this involves the isolation of appropriate promoters from potatoes. A good candidate is the potato *Lhca*3.St.1 gene encoding the apoprotein 2 (type III chlorophyll *a/b* binding protein) of the light harvesting complex of Photosystem I, isolated from the potato cultivar Bintje (Nap *et al.*, 1993). A 538 bp fragment homologous to this promoter has been isolated from the potato cultivar Iwa. Apart from a 41 bp insertion starting at 281 bp upstream from the putative transcription start site, the DNA sequence shows 96% identity to the DNA sequence of *Lhca*3.St.1 gene from potato cultivar Bintje (Shang *et al.*, 2000).

This chapter describes the independent transfer of modified *cry*1Ac9 and *cry*9Aa2 genes under a light inducible *Lhca*3 promoter into the potato cultivar Iwa. The resulting putatively transformed lines were characterized using multiplex PCR and Southern analysis. The level of transgene expression in leaves and tubers, was determined using multiplex RT-PCR. All putatively transformed lines were also evaluated in a containment greenhouse for plant phenotypic appearance and resistance to PTM larvae in foliage and tubers.

2.2 Materials and methods

2.2.1 Vector construction

In order to construct an expression cassette with the Iwa *Lhca*3 promoter (Shang al., 2000), the *35S* promoter of pART7 (Gleave, 1992) was replaced with the Iwa *Lhca*3 promoter. Both the Bluescript plasmid harbouring the Iwa *Lhca*3 promoter and pART7 were digested using *Sac*I and *Xho*I restriction endonucleases. The required fragments were extracted from an agarose gel and ligated together using standard methods (Sambrook *et al.*, 1989). The resulting plasmid, pART7cab, was then cleaved with *Eco*RI and *Bam*HI, and ligated with the 2028 bp coding region of the G14 version of *cry*9Aa2 excised from pART27G14 (Gleave *et al.*, 1998) using *Eco*RI and *Bam*HI. The resulting *Lhca*3-*cry*9Aa2-*ocs*3' chimeric gene was cloned as a 3.3 kb *Not*I fragment into pART27 (Gleave, 1992) to produce the binary vector pART27cab9Aa2. The 1.9 kb coding region of the *cry*1Ac9 gene was excised from pART27*cry*1Ac9^{B} (Beuning *et al.*, 2001) and cloned as a *Kpn*I and *Hind*III fragment into pART7cab, thereby placing the *cry*1Ac9 gene under transcriptional control of *Lhca*3 promoter. The resulting *Lhca*3-*cry*1Ac9-*ocs*3' chimeric gene was cloned as a 3.2 kb *Not*I fragment into the binary vector pART27 (Gleave, 1992) to produce pART27cab1Ac9.

The p*nos*-*npt*II-*nos*3' chimeric gene on the binary vectors was used as a selectable marker conferring resistance to kanamycin for the transformation of potato cultivar Iwa. The binary vectors pART27cab1Ac9 and pART27cab9Aa2 were individually transferred to the disarmed *Agrobacterium tumefaciens* strain EHA105 (Hood *et*

al., 1993) using the freeze-thaw method (Höfgen & Wilmitzer, 1988). Prior to co-cultivation with potato tissue, the *Agrobacterium* cultures harboring the binary vectors were cultured overnight at 28°C, on a shaking table, in LB broth supplemented with 300 mg l^{-1} spectinomycin.

2.2.2 Plant material

Virus-free plants were multiplied *in vitro* on a multiplication medium consisting of MS salts and vitamins (Murashige & Skoog, 1962) plus 30 g L^{-1} sucrose, 40 mg L^{-1} ascorbic acid, 500 mg L^{-1}casein hydrolysate, and 7 g L^{-1} agar. The agar was added after pH was adjusted to 5.8 with 0.1 M KOH, then the medium was autoclaved at 121°C for 15 min. Aliquots of 50 ml were dispensed into (80 mm diameter x 50 mm high) pre-sterilised plastic containers (Vertex Plastics, Hamilton, New Zealand). Plants were routinely subcultured as two to three node segments every 3–4 weeks and incubated at 26°C under cool white fluorescent lamps (80–100 µmol $m^{-2}s^{-1}$; 16-h photoperiod).

2.2.3 Potato transformation

Virus-free potato cultivar Iwa was transformed . Fully expanded leaves from the *in vitro* plants were excised, cut in half across midribs, while submerged in the liquid *Agrobacterium* culture. After about 30 sec, these leaf segments were blotted dry on sterile filter paper (Whatman® No. 1, 100 mm diameter). They were then cultured on callus induction medium (multiplication medium supplemented with 0.2 mg L^{-1} napthaleneactic acid and 2 mg L^{-1} benzylaminopurine) in standard plastic Petri dishes (9 cm diameter x 1 cm high) under reduced light intensity (5-10 µmol m^{-2} s^{-1}) by covering the Petri dishes with white paper. After two days, the leaf segments were transferred to the callus induction medium supplemented with 200 mg L^{-1} Timentin™ to prevent *Agrobacterium* overgrowth. Five days later, they were transferred on to the same medium further supplemented with 100 mg L^{-1} kanamycin in order to select the transformed cell colonies. Individual kanamycin-resistant cell colonies (0.5-1 mm diameter), developing on the leaf segments in 3-6 weeks, were excised and transferred on to regeneration medium (potato

multiplication medium with sucrose reduced to 5 g L^{-1}, plus 1.0 mg L^{-1} zeatin and 5 mg L^{-1} gibberelic acid, both filter sterilised and added after autoclaving) supplemented with 200 mg L^{-1} Timentin and 50 mg L^{-1} kanamycin in plastic Petri dishes (9 cm diameter x 2 cm high). The cell colonies were cultured under low light intensity (30-40 μmol m^{-2} s^{-1}) until shoots regenerated. Individual cell colonies, each with regenerated shoots, were transferred to potato multiplication medium containing 100 mg L^{-1} Timentin in plastic containers as described above. Single healthy shoots derived from individual shoot clumps were excised and transferred to multiplication medium containing 100 mg L^{-1} Timentin and 50 mg L^{-1} kanamycin. Individual shoots (one per original shoot clump) that rooted readily in the kanamycin- supplemented medium were labeled and further subcultured on to potato multiplication medium with 100 mg L^{-1} Timentin for micropropagation in plastic containers as described above. All antibiotics were filter sterilised and added, as required, just prior to dispensing the media into culture vessels or dishes.

2.2.4 Screening of putative transformed lines using PCR

Genomic DNA was isolated from in vitro shoots of putative transgenic and control plants based on the method described by Bernatzky & Tanksley (1986). DNA was amplified in a polymerase chain reaction (PCR) containing primers specific for the transgene of interest multiplexed with primers for the endogenous potato actin gene as an internal control (Table 2.1). PCRs were carried out in a Mastercycler (Eppendorf, Hamburg, Germany). The reactions included 2.5 μl 10x buffer (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween® 20), 1.5 μl 25 mM $MgCl_2$, 2.5 μl dNTP (at 2 mM each of dATP, dCTP, dGTP, dTTP), 0.25 μl Red Hot® DNA polymerase at 5 U μl^{-1} (Advanced Biotechnologies, Surrey, U.K.), 0.5 μl of each primer (at 10 μM), 1.0 μl of DNA (10-50 ng) and water to a total volume of 25 μl. The conditions for PCR were: 1 min at 93°C, followed by 35 cycles of 30 s 92°C, 30 s 60°C, 90 s 72°C, followed by a 6 min extension at 72°C. Amplified products were separated by electrophoresis in a 2% agarose gel and visualized under UV light after staining with ethidium bromide.

Table 2.1 Primers for PCR of each gene and expected product size.

Target gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size (bp)
*cry*1Ac9	GCCACAGAATAACAACGTGC	GCATACCGTACACGAACTCG	359
*cry*9Aa2	GCACGGAATTATTGGCGCTTC	CACGATGTCCAACACCATCAA	826
*npt*II	ATGACTGGGCACAACAGACAATCGGCTGCT	CGGGTAGCCAACGCTATGTCCTGATAGCGG	612
Actin	GATGGCAGAAGGCGAAGATA	GAGCTGGTCTTTGAAGTCTCG	1069

2.2.5 Phenotypic evaluation of transgenic lines in greenhouse

All of the putatively transformed lines were transferred to the containment greenhouse using the method described in Conner *et al.* (1994). Two plants were established in each of three PB5 bags (15 cm x 15 cm x 15 cm black polythene bags) per line, with each PB5 bag treated as a replicate, and the bags placed in the greenhouse in a randomised block design. The greenhouse conditions provided heating below 15°C and ventilation above 22°C. Day length was supplemented to 16 h when needed with 500 W metal halide vapour bulbs, and relative humidity was maintained above 60%.

After 6-8 weeks in the greenhouse, the appearance of the foliage from each line was recorded using the categories: phenotypically normal, marginal leaf curl, leaf wrinkling, reduced vigour, and/or stunted plants (Conner *et al.* 1994). Tubers were also evaluated based on their size and appearance at the time of harvest, 14 weeks after planting in the greenhouse. Lines that produced tubers < 10 mm in length (from apical to distal end) and/or were deformed in shape were considered abnormal.

Harvested tubers were immediately transferred into brown paper bags and stored in cool dark storage (temperature 8°C). In this way exposure to light was minimized to avoid inadvertent induction of transgene expression in the tubers.

2.2.6 Insect bioassay with excised leaves

Young, fully expanded leaves from transgenic and non transgenic control lines grown in the greenhouse for 7-8 weeks were used for the PTM larvae bioassays. The insects used in the bioassays were obtained from a laboratory colony maintained as previously described (Davidson *et al.* 2002a). The petioles of excised leaves were inserted through a polystyrene stopper (2 cm diameter x 1.5 cm long with a full length radial slit) into each 25 ml glass vial full of water. The leaf with glass vial was then placed vertically in a transparent plastic jar (500 ml) (Figure 2.1). One leaf with five leaflets was used per replication and the bioassay was

replicated three times per potato line. Five PTM neonate larvae were collectively weighed and placed on the leaflets. Leaf material from each of three replicate plants (which were arranged in a randomized block design on a greenhouse bench) was placed in separate containers. The jar was covered by fine Terylene mesh (pore size approximately 0.25 mm^2) and placed in a controlled temperature room at 22 ± 3°C and under a photoperiod of 16 h light and 8 h dark. Surviving larvae were removed after 9 days and weighed individually. A growth index (GI) for each larvae was calculated as GI = $\log_e$(final weight/mean initial weight).

Figure 2.1 An example of the experimental setup for PTM bioassay with excised leaves

2.2.7 Insect bioassay with tubers

Greenhouse-grown tubers, harvested from transgenic and non-transgenic control lines described above for excised leaf bioassay, were exposed to PTM neonate larvae. For each of three replicates, a 40-60 g whole tuber was punctured approximately 20 times with a 1 mm diameter needle to a depth of 10 mm to facilitate access by the larvae and placed in a 350 ml semi transparent plastic pottle. Ten neonate larvae were placed on to each replicate tuber. The pottle was covered with Terylene mesh (pore size approximately 0.25 mm^2) and placed in a controlled

temperature room at 22 ± 3°C in darkness. The number of surviving larvae and pupae were counted after three weeks.

2.2.8 Reverse transcription (RT)-PCR analyses

Total RNA was isolated from leaves and tubers of greenhouse-grown plants using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. For RNA isolation tubers were taken from dark storage and processed in the shortest possible time (less than 10 min) to minimise light exposure. The RNA samples were treated with DNaseI, Amplification Grade (Invitrogen), according to the manufacturer's directions. Following DNaseI treatment, the RNA samples were tested for the presence of contaminating DNA by PCR amplification with actin gene specific primers as described above. A PCR product of 1069 bp rather than 709 bp is expected if DNA is present, since the primers flank two introns. The multiplex RT-PCR was performed on each DNA-free RNA sample using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq (Invitrogen). The RT-PCR primers used for *cry*1Ac9 and actin were the same as those used for PCR analysis (Table 2.1). For *cry*9Aa2 new primers were designed to conveniently distinguish the RT-PCR product of *cry*9Aa2 from the RT-PCR product of the actin gene. For the *cry*9Aa2 gene the nucleotide sequences of the primers were $^{5'}$GCATCTAATCGCCGTTCA$^{3'}$ and $^{5'}$CGAATTTGGTCCGGACTT$^{3'}$, which generate an expected PCR product of 424 bp. RT-PCRs were carried out in a Mastercycler (Eppendorf, Hamburg, Germany). The reactions included 1 µl SuperScript™ III RT / Platinum® Taq mix, 12.5 µl 2x Reaction Mix (a buffer containing 0.4 mM of each dNTP, 3.2 mM $MgSO_4$), 0.5 µl of each primer (at 10 µM), 1.0 µl of RNA (10-50 ng) and water to a total volume of 25 µl. The conditions for RT-PCR were: 30 min at 55°C (for cDNA synthesis), 2 min at 94°C (to denature the SuperScript™ III RT enzyme), 35 cycles of 15 s 94°C, 30 s 60°C, 90 s 68°C (PCR amplification), followed by a 5 min extension at 68°C. Amplified products were separated by electrophoresis in a 2% agarose gel and visualized under UV light after staining with ethidium bromide.

2.2.9 Southern analysis

Total genomic DNA from young leaves of selected greenhouse-grown plants was extracted based on the method described by Bernatzky & Tanksley (1986). Southern analysis was carried out essentially according to Sambrook & Russell (2001). The genomic DNA was digested using *Hind*III (NEB, Beverly, MA, USA) at 10 units per μg DNA. *Hind*III restricts once and twice within the T-DNA of pART27*cry*1Ac9^{B} and pART27*cry*9Aa2 respectively. Approximately 10 μg of digested DNA was loaded per lane on a 0.8% TAE-buffered agarose gel. Following separation of fragments, the DNA was transferred to Hybond N+ membrane (Amersham, Little Chalfont, UK). Hybridisation was performed with radioactively-labeled DNA probes of *cry*1Ac9 and *cry*9Aa2 consisting of 359 bp and 826 bp fragments (as PCR products described above) encoding the *cry* genes respectively. Both probes were labelled with [α-^{32}P] dATP using the Megaprime DNA labelling system (Amersham, Little Chalfont, UK). Membranes were placed on Kodak X-Omat XK-1 (blue) X-ray film (Eastman Kodak Company, Rochester, NY) with intensifying screens for 19 days.

2.2.10 Enzyme-linked immunosorbent assay (ELISA)

Due to the unavailability of an ELISA kit to detect *cry*9Aa2 protein, ELISA was performed only for *cry*1Ac9-transgenic lines. A QuantiPlate^{TM} Kit for Cry1Ab/Cry1Ac (EnviroLogix Inc., Portland, ME, USA) was used to detect recombinant protein expression according to the manufacturer's instructions. This kit represents a double-antibody sandwich quantitative ELISA with a detection limit of 1.2 μg L^{-1} for Cry1Ac in sample extracts, using the equivalent Cry1Ac calibrators of 1.5, 10 and 25 μg L^{-1}. Weighed leaf samples, taken from the youngest, fully expanded leaves from six-week-old greenhouse plants were ground in Extraction/Dilution Buffer. The sample extracts were allowed to settle and supernatant was used at a dilution of 1:11. Diluted samples were loaded into wells coated with antibody raised against *cry*1Ab toxin. After 15 min of incubation, an enzyme conjugate was loaded into the wells as secondary antibody. After 1 hr incubation, the wells were washed clean of sample extracts with Wash Buffer.

Subsequent addition of substrate and 30 min incubation resulted in a greenish-blue coloration. Finally, addition of Stop Solution turned the well contents yellow and the intensity of colour was measured spectrophotometrically with a ThermoMax™ microplate reader (Molecular Devices, Sunnyvale, Calif.) at 450 nm. The levels of Cry1Ac protein in the extracts were determined by a calibration based on the extrapolation from a standard curve based on known samples and standard absorbance values. The optical density value (OD) of the untransformed Iwa control was subtracted before determining the concentration of Cry1Ac protein.

2.2.11 Statistical analysis

Mean GI for each replicate for each line were analysed with analysis of variance. Percent mortality, pupation and live larvae were analysed with a binomial generalised linear model, with a logit link (McCullagh & Nelder, 1989). Comparisons with the control line were made as part of these analyses, and a probability level of 5% was used throughout to determine significance. Analyses were carried out using GenStat (GenStat Committee, 2003).

2.3 Results

2.3.1 Potato transformation

The two T-DNA vectors outlined in Figure 2.2 were successfully constructed and transformed into potato cultivar Iwa. After 3-4 weeks of placing Agrobacterium co-cultivated explants on callus induction medium, small cell colonies developed along the cut leaf edges and/or leaf surfaces. Most of the cell colonies were green and grew as hard, compact callus while some were pale-green and friable. Following transfer to regeneration medium, most hard green cell colonies produced shoots, whereas the friable calli generally failed. Two to three weeks after the cell colonies were transferred with regenerated shoots to potato multiplication medium, 2-4 fully grown shoots developed from each original cell colony. In kanamycin selection medium, single healthy shoots excised from each shoot clump readily formed roots within a week. A total of 66 independently derived, putative transgenic potato lines were selected (35 lines for *cry*1Ac9 and 31 lines for *cry*9Aa2), on the basis of their ability to grow and root on kanamycin selection medium.

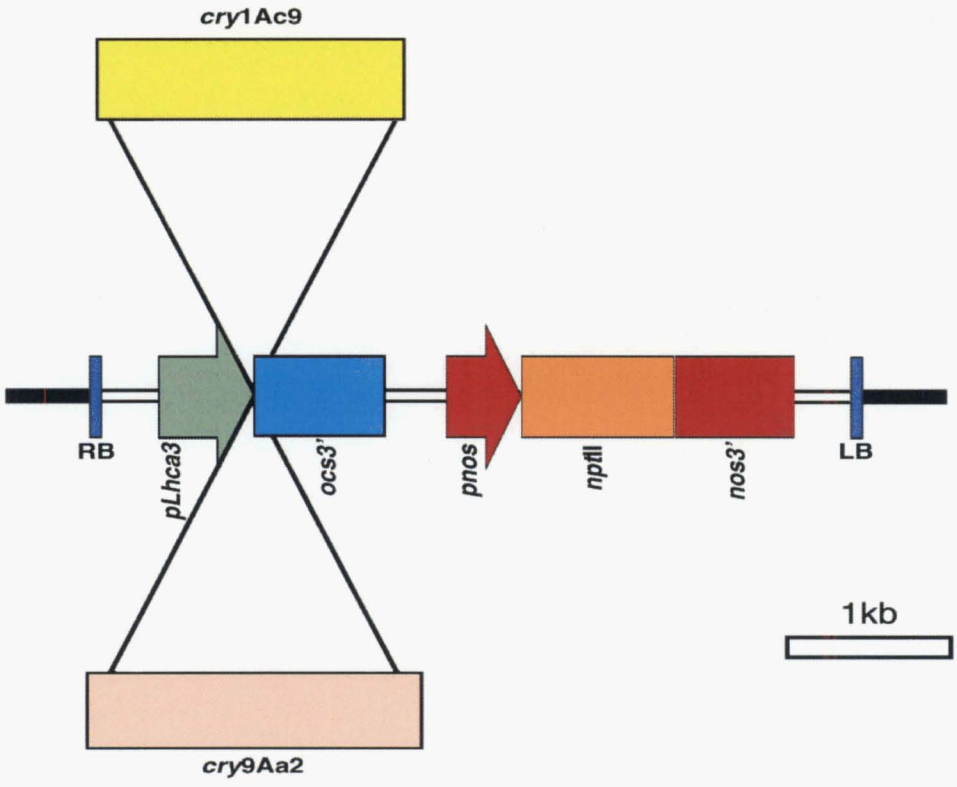


Figure 2.2 Schematic representation of the T-DNA regions of the two binary vectors (pART27cab1Ac9 and pART27cab9Aa2) used in this study.

RB, LB, right and left T-DNA borders respectively.

2.3.2 PCR analysis of regenerated lines

The presence of the *npt*II and *cry* genes in these lines was confirmed using multiplex PCR with an endogenous actin gene as an internal positive control. Since the actin product was expected in both transgenic and non-transgenic potato plants, this allows failed PCR reactions to be conveniently distinguished from a non-transgenic line. PCR products from representative lines are illustrated in Figure 2.3. All 66 putative transgenic lines were PCR positive for both the *npt*II and *cry* genes.

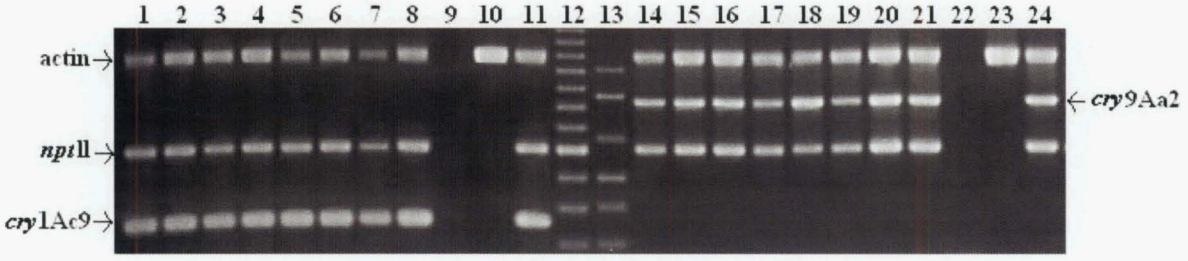


Figure 2.3 PCR analysis of putative transgenic potato (*Solanum tuberosum*) cv. 'Iwa' lines.

Lanes 1-11 represent a multiplex reaction with the *cry*1Ac9 primers producing an expected 359 bp product, the *npt*II primers producing an expected 612 bp product and the actin primers as an internal control producing product 1069 bp. Lanes 14-24 represent a multiplex reaction with *cry*9Aa2 primers producing an expected 826 bp product, the 612 bp *npt*II product and the 1069 bp actin product. Lanes 1-8, lines transformed with pART27cab1Ac9 (#102, #107, #110, #116, #121, #122, #123 and #130 respectively); lanes 9 and 22, no DNA template control; lanes 10 and 23, non-transgenic 'Iwa' control; lane 11, 'Iwa' line I52 known to be transgenic for *npt*II and *cry*1Ac9 (positive control, ex Davidson *et al.*, 2002a); lanes 12 and 13, 100 bp molecular ruler 10380-012 and 1 kb plus molecular ruler 10787-018 (Invitrogen, Carlsbad, California) size markers respectively; lanes 14-21, lines transformed with pART27cab9Aa2 (#7, #12, #17, #18, #22, #27, #28 and #29 respectively); lane 24, 'Iwa' line DG4c known to be transgenic for *npt*II and *cry*9Aa2 (positive control, ex Meiyalaghan *et al.*, 2005a).

2.3.3 Greenhouse evaluation and insect bioassays

The majority of the lines were observed to have a phenotypically normal appearance when grown in the greenhouse. Two lines with the *cry*1Ac9 gene (#117 and #128) and three lines with the *cry*9Aa2 gene (#1, #2 and #26) exhibited a range of off-type characteristics such as marginal leaf curl, leaf wrinkling, reduced vigour, abnormally small and/or deformed tubers, or a combination of these traits.

For the population of *cry*1Ac9-transgenic lines, there was substantial variation in larval GI (Figure 2.4). The larval GI was not significantly less than the non-transgenic control for 17 of the 35 lines. The remaining 18 lines all exhibited a significantly lower larval GI than the control (Figure 2.4), with a few lines (#107, #116 and #123) severely inhibiting the growth of PTM larvae. The mortality of larvae developing on tubers also varied markedly between the transgenic lines. PTM larval mortality on the control tubers was 13% (95% confidence limits of 6-26%), whereas mortality on the transgenic lines ranged from 3% (95% confidence limits of 0.4-24%) to 53% (95% confidence limits of 33-72%). There was little correlation between mortality on tubers and the larval GI on foliage ($r = -0.21$). Of the 18 transgenic lines that exhibited a significantly lower PTM larval GI than the control on foliage, 11 showed no significant difference from the non-transgenic control in larval mortality in the tuber bioassays. PTM bioassay results for tubers of the 8 lines with the lowest larval GI on foliage are shown in Table 2.2. There was little consistency in larval mortality and pupation rates on tubers among these transgenic lines.

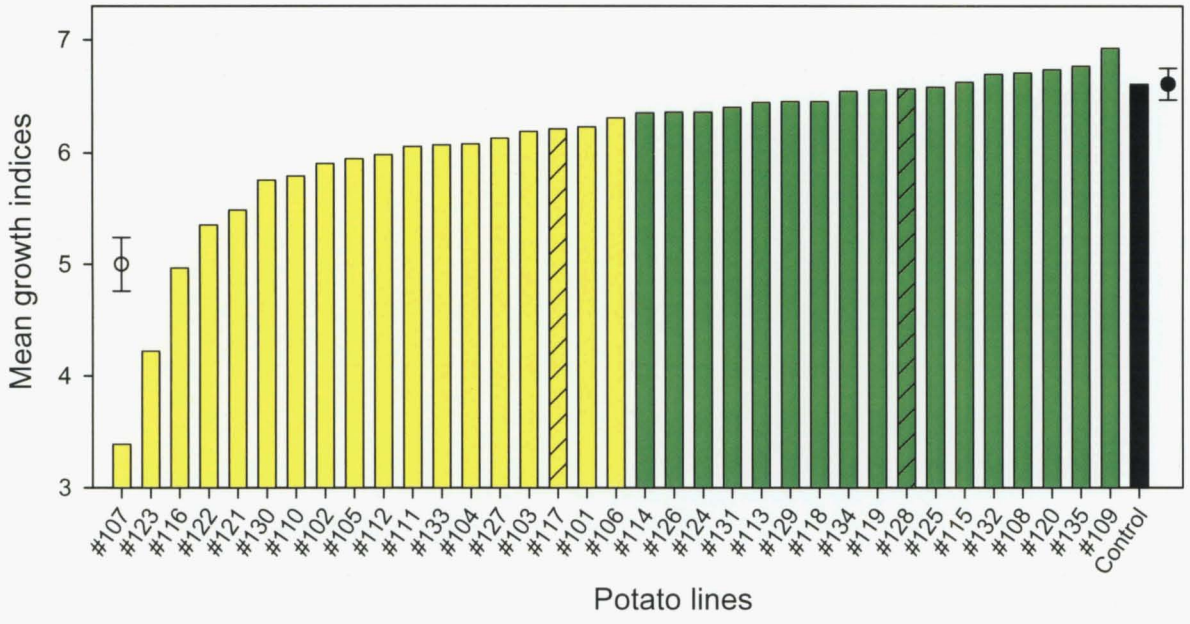


Figure 2.4 The mean growth indices of potato tuber moth (PTM) larvae reared on leaves of *cry*1Ac9-transgenic potato plants.

The error bars represent 95% confidence limits for the mean growth indices for (i) non-transgenic control line (solid circle) (ii) any transgenic line (empty circle). Green bars indicate lines that were not significantly different from the non-transgenic control line at 5% level. Hatched bars indicate lines that were off-type in appearance.

Table 2.2 Performance of 8 selected *cry*1Ac9-transgenic lines in bioassays against PTM larvae.

(95% confidence limits in brackets, df = 75).

Line	Mean GI in leaf bioassay	Larval mortality in tuber bioassay (%)	Individuals remaining as larvae in tuber bioassay (%)	Individuals reaching pupation in tuber bioassay (%)
#107	3.39	23 (10, 44)	70 (49, 85)	7 (2, 25)
#123	4.22	10 (3, 30)	60 (40, 77)	30 (16, 49)
#116	4.97	27 (13, 48)	63 (43, 80)	10 (3, 28)
#122	5.35	33 (17, 54)	63 (43, 80)	3 (0, 22)
#121	5.49	40 (22, 61)	60 (40, 77)	0 (0, 12)
#130	5.75	53 (34, 72)	47 (28, 66)	0 (0,12)
#110	5.79	17 (6,37)	60 (40, 77)	23 (11, 43)
#102	5.90	43 (25, 64)	43 (25, 63)	13 (5, 32)
Control	6.61 (±0.20)	13 (6, 26)	37 (24, 51)	50 (37, 63)

For the population of cry9Aa2-transgenic lines, foliage from 26 of the 31 lines supported larval growth with significantly lower GI than larvae on foliage from non-transgenic control plants (Figure 2.5). The larval growth of PTM on 12 of the transgenic lines was severely inhibited (GI under 5.0). Similar to the results with the cry1Ac9-transgenic lines, the mortality of larvae developing on tubers varied markedly between the transgenic lines. PTM larval mortality on the control tubers was 12% (95% confidence limits of 5-25%), whereas mortality on the transgenic line ranged from 0 to 43% (95% confidence limits of 0-13% and 25-64% respectively). The percent mortality on the tubers was uncorrelated ($r = 0.04$) with larval GI on leaves. Of the 26 transgenic lines that exhibited a significantly lower PTM larval GI on foliage, 21 showed no significant difference from the non-transgenic control in larval mortality in the tuber bioassays. The PTM bioassay results for the 8 lines with the lowest larval GI on foliage are shown in Table 2.3. Amongst these lines, there was little consistency in mortality and pupation rates on tubers. Mortality on tubers of these selected lines was reasonably similar to the control tubers, but pupation was significantly lower than the control except for the transgenic lines #7, #12 and #17.

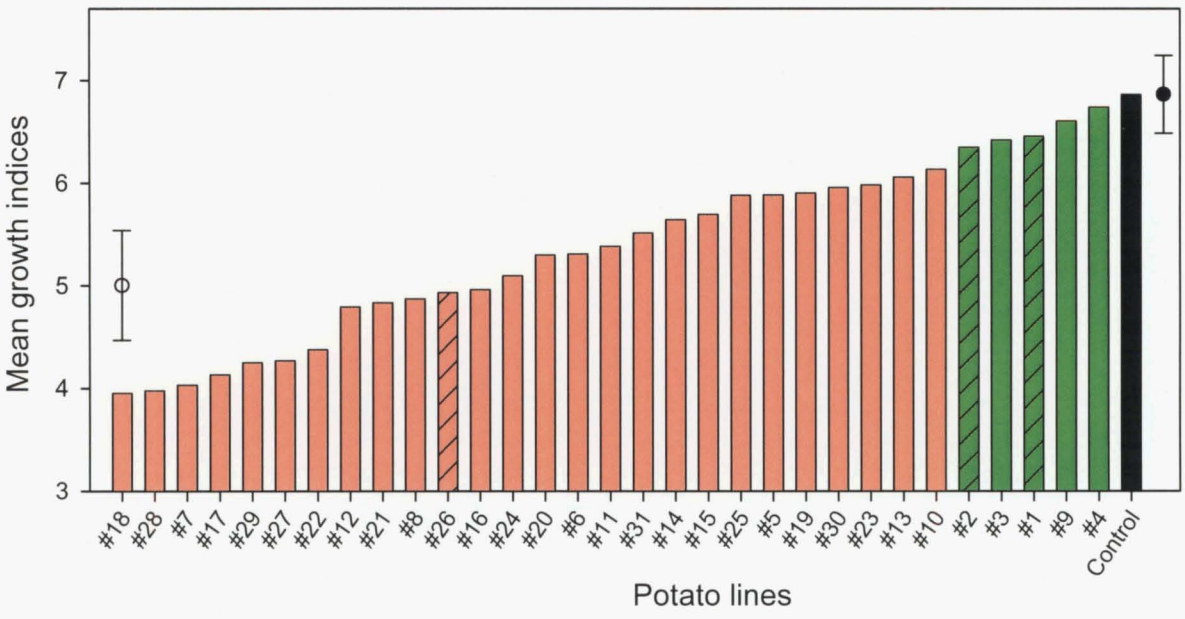


Figure 2.5 The mean growth indices of potato tuber moth (PTM) larvae reared on leaves of *cry*9Aa2-transgenic potato plants.

The error bars represent 95% confidence limits for the mean growth indices for (i) non-transgenic control line (solid circle) (ii) any transgenic line (empty circle). Green bars indicate lines that were not significantly different from the non-transgenic control line at 5% level. Hatched bars indicate lines that were off-type in appearance.

Table 2.3 Performance of 8 selected *cry*9Aa2-transgenic lines in bioassays against PTM larvae.

(95% confidence limits in brackets, df = 67).

Line	Mean GI in leaf bioassay	Larval mortality in tuber bioassay (%)	Individuals remaining as larvae in tuber bioassay (%)	Individuals reaching pupation in tuber bioassay (%)
#18	3.95	20 (8, 41)	47 (30, 65)	33 (18, 52)
#28	3.98	10 (3, 30)	67 (48, 81)	23 (11, 42)
#7	4.03	13 (4, 34)	23 (11, 42)	63 (44, 79)
#17	4.13	10 (3, 30)	10 (3, 27)	80 (61, 91)
#29	4.25	10 (3, 30)	63 (45, 79)	27 (14, 46)
#27	4.27	13 (4, 34)	67 (48, 81)	20 (9, 39)
#22	4.38	13 (4, 34)	60 (42, 76)	27 (14, 46)
#12	4.79	10 (3, 30)	7 (2, 24)	83 (65, 93)
Control	6.87 (±0.46)	12 (5, 25)	13 (7, 25)	75 (62, 85)

2.3.4 RT-PCR analysis of selected transgenic lines

The expression of the *cry* genes in leaves and tubers of transgenic lines was determined by multiplex RT-PCR analysis with an endogenous actin gene (Figure 2.6). The use of the endogenous actin gene as an internal control allows failed reactions to be conveniently distinguished from non-expressing transgenic line and also provides a baseline for standardising gene expression between transgenic lines. Since the primers flank two introns in the actin gene, it also provides a convenient check for DNA contamination of the RNA samples. RT-PCR analysis using actin gene primers produced the expected 709 bp product in all samples. However, RT-PCR actin products also showed a faint 835 bp fragment in some samples.

RT-PCR showed that amplification of the expected 359 and 424 bp fragments had occurred in all the transgenic lines for the *cry*1Ac9 and *cry*9Aa2 genes respectively. These were substantially brighter bands than for the expression of the actin gene in leaf samples and considerably fainter than the actin bands for tuber samples from most transgenic lines in both *cry*1Ac9- and *cry*9Aa2-transgenic lines (Figure 2.6).

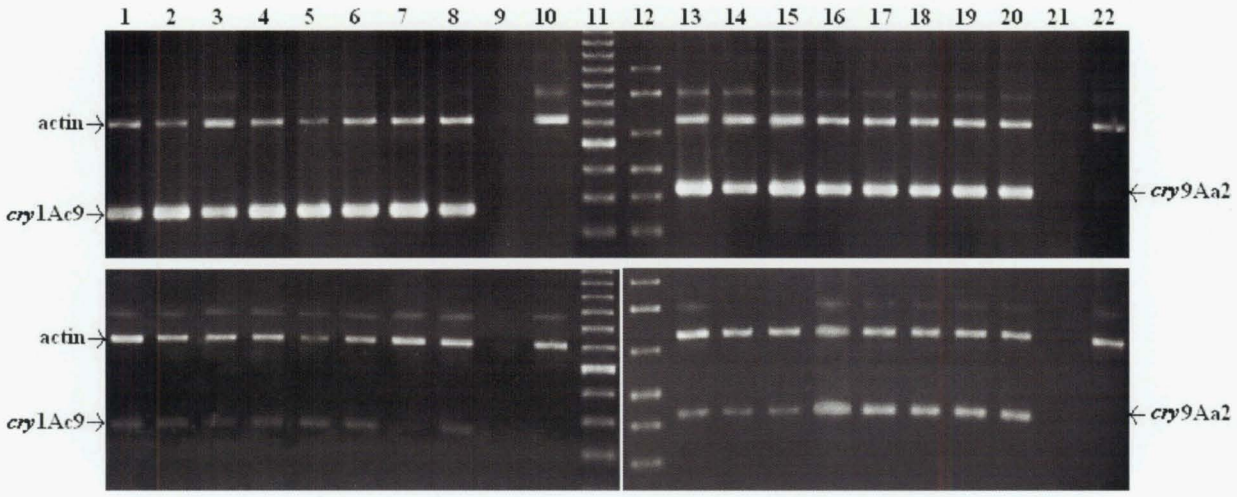


Figure 2.6 Reverse transcription (RT)-PCR analysis of transgenic potato lines.

Upper panel represents RT-PCR analysis of RNA isolated from leaves and lower panel represents RT-PCR analysis of RNA isolated from tubers. Lanes 1-10 represent a multiplex reaction with the *cry*1Ac9 primers producing an expected 359 bp product and the actin primers as an internal control producing an expected 709 bp product. Lanes 13-22 represent a multiplex reaction with *cry*9Aa2 primers producing an expected 424 bp product and the 709 bp actin product. Lanes 1-8, cry1Ac9-transgenic lines #102, #107, #110, #116, #121, #122, #123 and #130 respectively; lanes 9 and 21, no RNA template control; lanes 10 and 22, non-transgenic 'Iwa' control; lanes 11 and 12, 100 bp molecular ruler 10380-012 and 1 kb plus molecular ruler 10787-018 (Invitrogen, Carlsbad, California) size markers respectively; lanes 13-20, cry9Aa2-transgenic lines #7, #12, #17, #18, #22, #27, #28 and #29 respectively.

2.3.5 Southern analysis and ELISA

The transgenic status of eight high performing *cry*1Ac9-transgenic lines and four high performing *cry*9Aa2-transgenic lines was further confirmed by Southern analysis. When restricting the DNA with *Hin*dIII and probing with the *cry* gene, a single band greater than 2.9 and 2.1 kb is expected for each intact insertion of the *cry*1Ac9 and *cry*9Aa2 respectively. Two to nine copies of the *cry*1Ac9 gene and two to six copies of the *cry* 9Aa2 gene were evident in the independently derived transgenic lines (Figure 2.7).

The amount of Cry protein expressed by the *cry*1Ac9 transgenic potato lines was less than 60 ng per gram of fresh leaf tissue, which represents the detection limit of the ELISA method used.

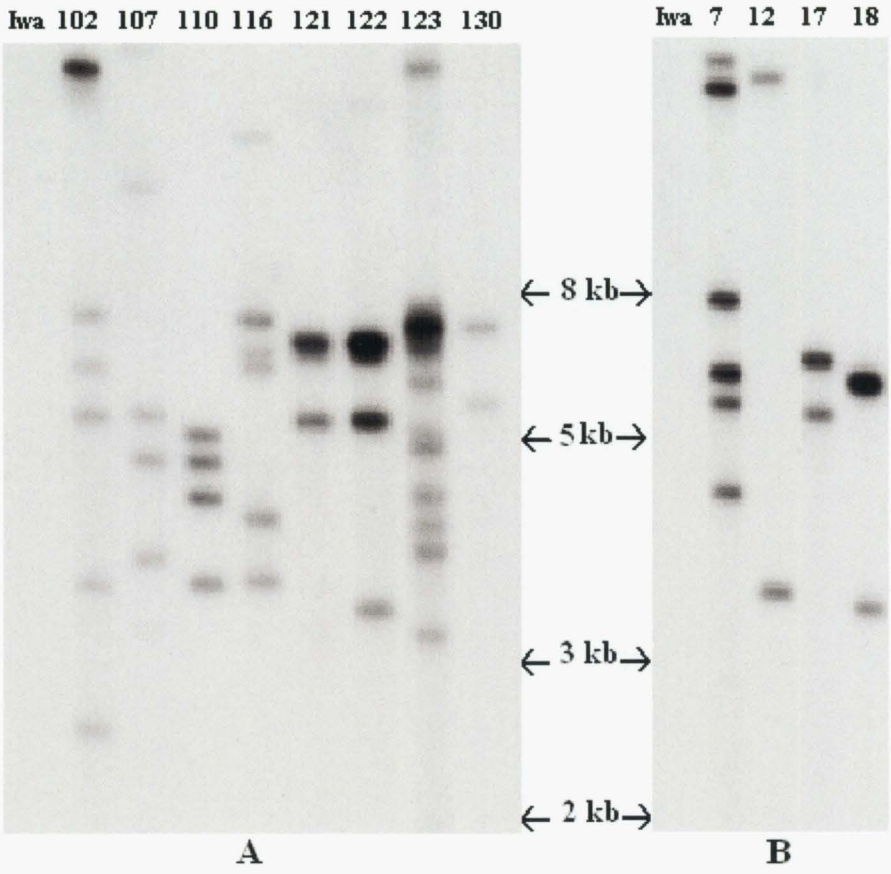


Figure 2.7 Southern analysis of selected transgenic potato lines (labels above the lanes refer to the plant lines analysed).

(A) *cry*1Ac9-transgenic lines. Probe used for hybridization was a 359 bp DNA fragment corresponding to the 3' end of the *cry*1Ac9 sequence. DNA was restricted with *Hind*III. A single band greater that 2.9 kb is expected for each intact insertion of the *cry*1Ac9 gene. (B) *cry*9Aa2-transgenic lines. Probe used for hybridization was a 826 bp DNA fragment corresponding to the 5' end of the *cry*9Aa2 sequence. DNA was restricted with *Hind*III. A single band greater that 2.2 kb is expected for each intact insertion of the *cry*9Aa2 gene.

2.4 Discussion

The development of transgenic plants to confer insect pest resistance is becoming a valuable component of integrated pest management (IPM) programmes. However, the application of genetic engineering to many food crops, including potato, has raised public concerns (Conner & Jacobs, 1999). In potato, it is often desirable to couple high-level expression of the transgene in foliage with no expression in tubers. Constitutive promoters have been used to drive *cry* gene expression in all previous studies targeting PTM resistance in transgenic potatoes (Douches *et al.*, 1998 & 2002; Jansens *et al.*, 1995; Cañedo *et al.*, 1999; Li *et al.*, 1999; Chakrabarti *et al.*, 2000; Mohammed *et al.*, 2000; Davidson *et al.*, 2002a & 2004a). Since PTM generally infests foliage of potato crops prior to the tubers (Foot, 1979), preventing foliar infestation may be sufficient to avoid tuber infestation. Therefore, the use of a foliage-specific promoter for transcriptional control of transgenes targeting PTM in potatoes may provide a valuable component for IPM, while simultaneously avoiding transgene expression in tubers to help allay some public concerns associated with the transgenic technology in potato crops.

This study has investigated the value of the potato *Lhca*3 promoter, known to be light-inducible (Nap *et al.*, 1993; Shang *et al.*, 2000), as a potential foliage-specific promoter for the transcriptional control of *cry* gene expression in potatoes. Analysis of all putative transgenic lines using PCR established the presence of the *npt*II and either *cry*1Ac9 or *cry*9Aa2 genes in all regenerated lines (e.g. Figure 2.3), thereby confirming their transgenic status and a high rate of success for the *Agrobacterium*-mediated gene transfer system of potato using kanamycin resistance as a selectable marker (Barrell *et al.*, 2002).

Neonate larvae are most susceptible to Cry proteins (Roush, 1996) and PTM are usually in this life stage when first encountering potato tissue (Fenemore, 1988). For these reasons, neonate PTM larvae were used in the bioassays. The bioassay method used was based on the assumption that the level of Cry protein in excised leaves was similar to that in the foliage of intact plants. The same assumption has been made in similar studies involving PTM bioassays (Ebora *et al.*, 1994; Jansens

et al., 1995; Westedt *et al.*, 1998; Beuning *et al.*, 2001; Douches *et al.*, 1998 & 2002; Li *et al.*, 1999; Davidson *et al.*, 2002a & 2004a), and has been validated by the observed high correlation between excised leaves and intact plants for growth of PTM larvae in response to *cry* gene expression (Davidson *et al.*, 2002a). In the case of the leaf bioassays, the final larval weights were made toward the end of the exponential growth phase at day 9 and were therefore appropriate for the calculation of a growth rate. After 9 days the rate of larval weight gain starts to diminish as they begin to lay down fatty tissue in preparation for pupation (Beuning *et al.*, 2001). In the case of the tuber bioassays, the numbers of individuals in the larval and pupal stages were recorded after three weeks. The earlier recovery of larvae for weighing was not possible due to difficulties in finding and removing undamaged larvae from the tubers.

From the two populations of independently derived transgenic lines, 16% of the *cry*9Aa2-transgenic lines and 49% of the *cry*1Ac9-transgenic lines failed to exhibit improved resistance to PTM larvae in the bioassay with excised leaves (Figures 2.3 & 2.4). This may be due to insufficient expression or accumulation of Cry protein in the foliage of these transgenic lines to inhibit growth of PTM larvae. The remaining transgenic lines showed significantly lower GI than the non-transgenic control in the PTM bioassays with excised leaves. Larvae recovered from these lines after the 9-day bioassay were small and of substantially lower weight. In our previous studies, PTM larvae with such poor growth in response to *cry* gene expression in transgenic potatoes have failed to reach pupation, resulting in complete disruption to the life cycle (Davidson *et al.*, 2002a).

The majority of the lines that gave good resistance to PTM larvae in foliage did not show significantly higher mortality on tubers than the non-transgenic control tubers (Tables 2.2 & 2.3). However, at the end of the 3-week tuber bioassay, some transgenic lines had higher percentages of larvae and lower levels of pupation than non-transgenic control tubers. This indicates that the *cry*1Ac9 or *cry*9Aa2 gene expression present in tubers of some transgenic lines may be sufficient to slightly slow the development of the PTM larvae. However, lines #110 and #123 with the

*cry*1Ac9 gene and lines #7, #12 and #17 with the *cry*9Aa2 gene showed the low larval mortality and high frequency of pupation similar to that of the non transgenic controls. These lines also exhibited high resistance to PTM larvae in foliage. The difference in insecticidal activity between foliage and tubers of these lines can be attributed to a higher level of *cry* gene expression in foliage and minimal and/or no expression in tubers due to the light-inducible *Lhca*3 promoter (Figure 2.6).

Considerable variation in the level of PTM resistance among the independently derived transgenic potato lines was observed in this study (Figures 2.3 & 2.4). Similar variability in transgene expression among insect-resistant transgenic plants has been commonly reported in other studies (e.g. Peferoen *et al.*, 1990; Van Rie *et al.*, 1994; Beuning *et al.*, 2001), including Iwa potato lines transgenic for the *cry*1Ac9 gene under the control of CaMV *35S* promoter (Davidson *et al.*, 2002a & 2004a). Such variation is usually attributed to unpredictable levels of transgene expression as a consequence of position effects resulting from differences in the integration site of the transgenes within the plant genome (Peach & Velten, 1991; Conner & Christey, 1994) and/or differences in T-DNA copy number (Hobbs *et al.*, 1993). Such variation in transgene expression is therefore not promoter dependent.

The sequence StPoAc58 (GenBank accession X55749) was used to design primers for the potato actin gene. Based on this sequence the primers are predicted to produce a 1041 bp PCR product, including two introns. However, the actin primers produced a 1069 bp PCR product for the potato cultivar Iwa (Figure 2.3). The DNA sequence of this product shows 95% identity to the actin *StPoAc58* sequence. Based on the *StPoAc58* sequence, the 1069 bp fragment of the Iwa actin gene is predicted to contain two introns of 234 bp and 126 bp respectively. In RT-PCR analysis the actin primers produced the expected 709 bp product as a bright band (Figure 2.6), which is equivalent to the genomic fragment minus the two introns. However, sometimes a 835 bp product was also observed as a faint band (Figure 2.6). The latter RT-PCR product is assumed to have retained the 126 bp intron. A reverse primer designed in this intron produced the predicted product size in RT-

PCR analysis (data not shown), therefore confirming the presence of an incompletely spliced mRNA in the RNA samples.

RT-PCR analysis of leaf RNA of selected lines confirmed the high level of transcriptional expression of the *cry*1Ac9 or *cry*9Aa2 genes in the foliage of transgenic plants, as indicated by the higher brightness of the expected products of the *cry* genes relative to the actin gene (Figure 2.6). This high *cry* gene expression in foliage can be attributed to the *Lhca*3 promoter. Other studies have also reported that the *Lhca*3.St.1 promoter gave higher expression in the foliage of transgenic chrysanthemum (Annadana *et al.*, 2001), potato and tobacco (Nap *et al.*, 1993) plants.

RT-PCR analysis of RNA from tubers revealed that a faint band of *cry* mRNA was detectable in the transgenic tubers containing either the *cry*1Ac9 or *cry*9Aa2 gene. Therefore, transcriptional expression of the *cry* gene by the *Lhca*3 promoter was relatively low in tubers. Furthermore, the level of *cry* mRNA was substantially lower than the mRNA of endogenous potato actin gene in tubers as judged by the relative brightness of the expected products from tuber RNA. This suggests that the high expression of the *cry* genes in foliage coupled with their minimal expression in tubers can be attributed to the *Lhca*3 promoter. These results are supported by the PTM bioassay data which established a marked difference between the phenotypic expression of insect resistance in the foliage and tubers of the transgenic potato lines.

Previously, northern analyses confirmed that the *Lhca3*.St.1 gene was expressed predominantly in foliage and not expressed in tubers of potato (Nap *et al.*, 1993). In contrast, the occasional expression of *cry* genes in tubers, observed in this study, could be due to position effects as a consequence of random integration of the transgene into the potato genome (Conner & Christey, 1994). The very low level of *cry* gene expression observed in tubers of transgenic lines using RT-PCR is probably a consequence of brief exposure to light at the time of harvest or during RNA isolation.

The majority of the independently derived transgenic lines with either the *cry*1Ac9 gene or the *cry*9Aa2 gene were observed to have a phenotypically normal appearance when grown in the greenhouse. Two *cry*1Ac9-transgenic lines and three *cry*9Aa2-transgenic lines exhibited a range of off-type characteristics such as marginal leaf curl, leaf wrinkling, reduced vigour, abnormally small and/or deformed tubers, or a combination of these traits. Such abnormal phenotypes are frequently observed among transgenic plants and are attributed to somaclonal variation that arises during the cell culture phase of plant transformation (Conner & Christey, 1994).

In contrast to the observed high *cry*1Ac9 transcriptional expression in leaves (Figure 2.6), the level of Cry protein remained under the detection limit of the ELISA method used (60 ng g^{-1} fresh weight of leaf tissue). A similar result was obtained in previous studies with CaMV *35S* promoter (Davidson *et al.*, 2002a & 2004a). Although the *cry*1Ac9 gene was estimated to range from 2 to 9 copies per genome based on Southern analysis (Figure 2.7), the Cry protein level was not detectable by ELISA. However, our results do indicate that the low level of Cry1Ac9 protein expressed in the foliage is sufficient to dramatically inhibit PTM larval growth rate to a level known to break the PTM life cycle and prevent pupation.

This chapter has established that the expression of either the *cry*1Ac9 or *cry*9Aa2 gene in transgenic potato plants offers protection against PTM larval damage in foliage when expressed under the transcriptional control of the light-inducible *Lhca*3 promoter. Several transgenic lines were identified with high resistance to PTM larvae in the foliage and with no or minimal *cry* gene expression in tubers.

Chapter 3. Transgenic potato lines expressing either a *cry*1Ba1 gene or a *cry*1Ca5 gene are resistant to potato tuber moth

3.1 Introduction

Transgenic potatoes that express the insecticidal toxins produced by different strains of the soil bacterium, *Bacillus thuringiensis* (Bt), offer the potential to contribute an additional component to integrated pest management for the control of potato tuber moth (PTM), *Phthorimaea operculella*. To ensure the effectiveness of the transgenic potato plants with insect resistance, it is important to implement resistance management strategies as soon as a transgenic cultivar is released. Various strategies have been proposed for resistance management, including: a combination of insect resistance genes effecting different modes of action; time- or tissue-specific expression of the insect resistance gene; low transgenic expression in combination with biological control; mixtures, rotation or mosaics of high-expressing transgenic plants with the use of refugia (Gould, 1998). However, the strategy most widely deployed in agriculture involves the use of a high dose of a single gene in combination with one or more refuges (Gould, 1998; Shelton *et al.*, 2000).

For deployment of transgenic approaches to control PTM in potato it is important to assemble a suite of genes capable of effecting PTM control to allow future evaluation of different approaches to the deployment of insect resistance management strategies. To date, several *cry* genes have been demonstrated to be highly effective at controlling PTM when expressed in potato plants. These include: *cry*1Ab (Jansens *et al.* 1995; Cañedo *et al.*, 1999; Chakrabarti *et al.*, 2000), *cry*1Ac (Ebora *et al.*, 1994) *cry*1Ac9 (Davidson *et al.*, 2002a & 2004a), *cry*5 (Douches *et al.,* 1998 & 2002; Westedt *et al.*, 1998; Li *et al.*, 1999; Lagnaoui *et al.*, 2000; Mohammed *et al.*, 2000), and *cry*9Aa2 (Takla, 2001). To further increase the diversity of *cry* genes for control of PTM in potato, this study evaluated the effectiveness of modified *cry*1Ba1 and *cry*1Ca5 genes to confer resistance to PTM

when expressed in transgenic potato. Putatively transgenic lines regenerated from the Iwa cultivar were characterized using multiplex PCR and Southern analysis. All lines confirmed as being transgenic were evaluated in a containment greenhouse for plant phenotypic appearance and their resistance to PTM was evaluated via detached leaf and tuber bioassays.

3.2 Materials and methods

3.2.1 Vector construction

A synthetic sequence identical to modified coding region of *cry*1Ca5 (Strizhov *et al.*, 1996) was ligated into the *Bam*HI site of primary cloning vector pART7 (Gleave, 1992). Restriction analysis using *Xba*I confirmed successful ligation and allowed clones with the desired orientation of the *cry*1Ca5 gene to be selected. The resulting *35S-cry*1Ca5-*ocs* chimeric gene was cloned as 4 kb *Not*I fragment into binary vector pART27 (Gleave, 1992) to produce pART27*cry*1Ca5 (Figure 3.1). A modified coding region of *cry*1Ba1 (C. R. Voisey *et al*, unpublished) was cloned as a *Hin*dIII - *Eco*RI fragment into the pBluescript SK (Stratagene). The resulting plasmid was restricted with *Xho*I and *Eco*RI and the *cry*1Ba1 fragment extracted from an agarose gel, then ligated into the *Xho*I and *Eco*RI sites of pART7 vector (Gleave, 1992). The resulting *35S-cry*1Ba1-*ocs* chimeric gene was cloned as 4.2 kb *Not*I fragment into binary vector pART27 (Gleave, 1992) to produce pART27*cry*1Ba1 (Figure 3.1).

The p*nos-npt*II-*nos*3' chimeric gene on the binary vectors was used as a selectable marker conferring resistance to kanamycin for the transformation of the potato cultivar Iwa. The binary vectors pART27*cry*1Ba1 and pART27*cry*1Ca5 were individually transferred to the disarmed *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.*, 1993) using the freeze-thaw method (Höfgen & Wilmitzer, 1988). *Agrobacterium* cultures harboring the binary vectors were cultured overnight at 28°C, on a shaking table, in LB broth supplemented with 300 mg L^{-1} spectinomycin. The resulting cultures were used for co-cultivation with potato leaf segments.

3.2.2 Potato transformation

Virus-free plants of the potato cultivar Iwa were maintained and transformed as described in Chapter 2.

3.2.3 Screening of putative transformed lines using PCR

This was performed as described in Chapter 2 with primers specific for the transgene of interest multiplexed with primers for the endogenous potato actin gene as an internal control (Table 3.1).

3.2.4 Phenotypic evaluation of transgenic lines in the greenhouse

Full details are given in Chapter 2.

3.2.5 Insect bioassay with excised leaves

Full details are given in Chapter 2.

3.2.6 Insect bioassay with tubers

Full details are given in Chapter 2.

3.2.7 Southern analysis

Total genomic DNA from young leaves of selected greenhouse-grown plants was extracted based on the method described by Bernatzky & Tanksley (1986). Southern analysis was carried out essentially according to Sambrook and Russell (2001). The genomic DNA was digested using *Hin*dIII (NEB, Beverly, MA, USA) at 10 units per µg DNA. *Hin*dIII restricts once and twice within the T-DNA of pART27*cry*1Ca5 and pART27*cry*1Ba1 respectively. Approximately 10 µg of digested DNA was loaded per lane on a 0.8% TAE-buffered agarose gel. Following separation of fragments, the DNA was transferred to Hybond N+ membrane (Amersham, Little Chalfont, UK). Hybridisation was performed with a radioactively-labeled DNA probe of 1.35 kb fragment corresponding to the CaMV *35S* promoter sequence. The probe was labelled with [α-^{32}P] dATP using the

Megaprime DNA labelling system (Amersham, Little Chalfont, UK). Membranes were placed on Kodak X-Omat XK-1 (blue) X-ray film (Eastman Kodak Company, Rochester, NY), with intensifying screens, for 19 days.

3.2.8 Enzyme-linked immunosorbent assay (ELISA)

Due to the unavailability of an ELISA kit to detect Cry1Ba1 protein, ELISA was performed only for *cry*1Ca5-transgenic lines. The EnviroLogix Cry1C Plate Kit (EnviroLogix Inc., Portland, ME, USA) was used to detect recombinant protein expression according to the manufacturer's instructions. This kit represents a double-antibody sandwich quantitative ELISA. Sample extracts were made from weighed leaf samples taken from the youngest, fully expanded leaves from six-week-old greenhouse plants and from weighed tuber samples taken after harvest. The intensity of colour development was measured spectrophotometrically with a ThermoMax™ microplate reader (Molecular Devices, Sunnyvale, Calif.) at 450 nm. The levels of Cry1C protein in the leaf and tuber extracts were determined by a calibration based on the extrapolation from a standard curve based on known samples and standard absorbance values. The OD value of the untransformed Iwa control was subtracted before determining the concentration of Cry1C protein. In order to bring assay results within the range of calibration, the ELISA assay was repeated for leaf samples at dilution of 1:51.

3.2.9 Statistical analysis

GIs for each surviving larva in each replicate for each line were analysed with a mixed model, fitted with residual maximum likelihood (REML) (Patterson & Thompson, 1971) which included random effects to account for between- and within-replicate variability for the lines. Percent mortality, pupation and live larvae were analysed with a binomial generalised linear model, with a logit link (McCullagh & Nelder, 1989). Comparisons with the control line were made as part of these analyses, and a probability level of 5% was used throughout to determine significance. Analyses were carried out using GenStat (GenStat Committee, 2003).

Table 3.1 Primers and expected product size for PCR of each gene.

Target gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size (bp)
*cry*1Ca5	CGATGAGTACGCTGATCACTG	TTTCTGATTGCGCTGCTC	293
*cry*1Ba1	TCACCCAAATCCCTATGGTC	TAAAGAGAGCGTTGACAGCC	474
*npt*II	ATGACTGGGCACAACAGACAATCGGCTGCT	CGGGTAGCCAACGCTATGTCCTGATAGCGG	612
Actin	GATGGCAGAAGGCGAAGATA	GAGCTGGTCTTTGAAGTCTCG	1069

3.3 Results

3.3.1 Potato transformation

The two binary vectors outlined in Figure 3.1 were successfully constructed and T-DNA was transformed into cv. Iwa. After 3-4 weeks of placing *Agrobacterium* co-cultivated explants on callus induction medium, small cell colonies developed along the cut leaf edges and/or leaf surfaces. Most of the cell colonies were green and grew as a hard, compact callus while some were pale-green and friable. Following transfer to regeneration medium most hard green cell colonies produced shoots, whereas the fiable calli generally failed. Two to three weeks after the cell colonies were transferred with regenerated shoots to potato multiplication medium, 2-4 fully grown shoots developed from each original cell colony. Single healthy shoots excised from each shoot clump readily formed roots within a week on kanamycin selection medium. A total of 74 independently derived, putative transgenic potato lines were selected (38 lines for *cry*1Ba1 gene and 36 lines for *cry*1Ca5 gene), on the basis of their ability to grow and root on kanamycin selection medium.

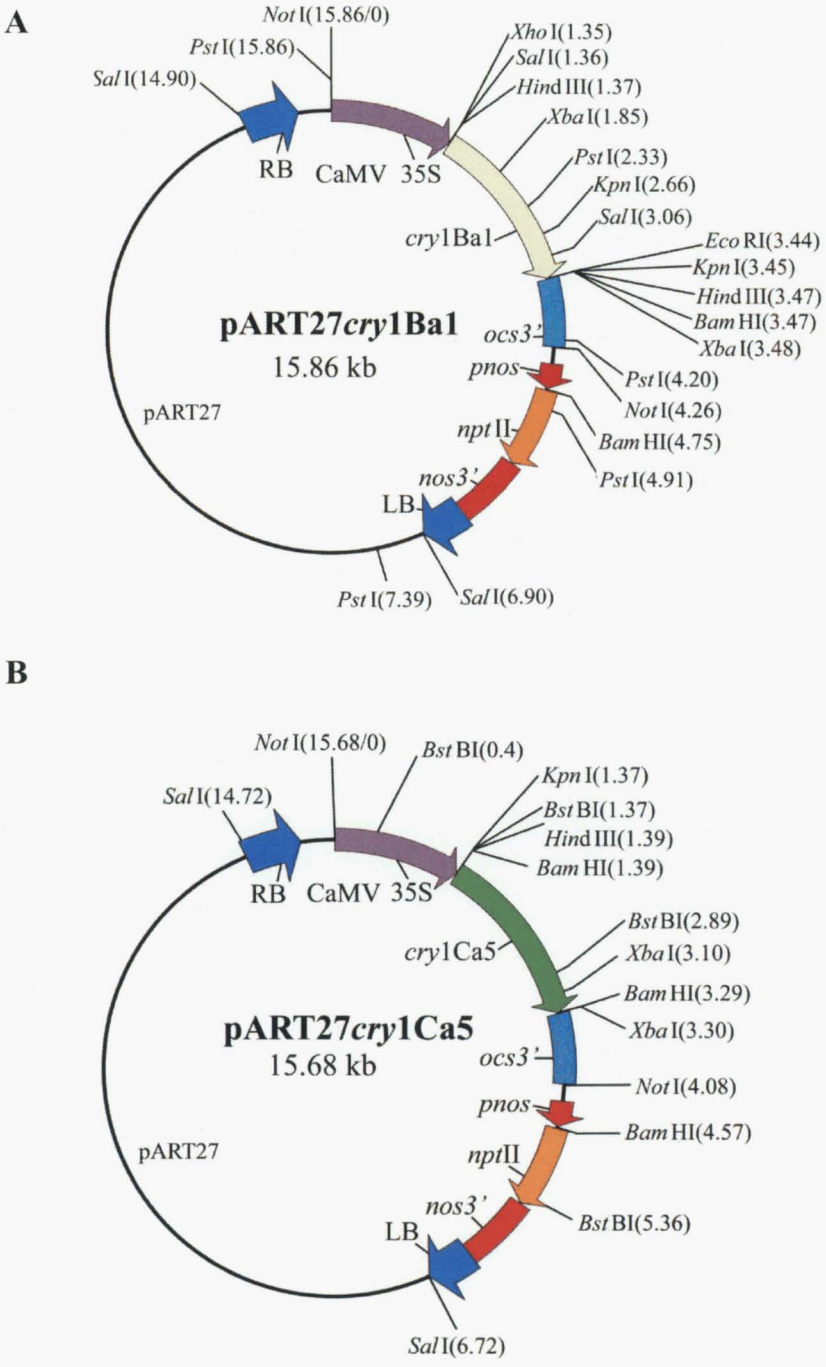


Figure 3.1 Diagrammatic representation of the two binary vectors.

(A) pART27*cry*1Ba1 vector; (B) pART27*cry*1Ca5 vector. The CaMV*35S-cry* gene-*ocs*3' chimeric gene and the p*nos-npt*II-*nos*3' for kanamycin selection are located between the left (LB) and right (RB) borders delimiting the T-DNA of pART27 (Gleave, 1992). The positions of important restriction sites are indicated.

3.3.2 PCR analysis of regenerated lines

The presence of the *npt*II and *cry* genes in these lines was confirmed using multiplex PCR with an endogenous actin gene as an internal positive control. Since the actin product was expected in both transgenic and non-transgenic potato plants, this allows a failed PCR reactions to be conveniently distinguished from a non-transgenic line. PCR products from selected lines are illustrated in Figure 3.2. All 74 putative transgenic lines were PCR positive for both the *npt*II and *cry* genes.

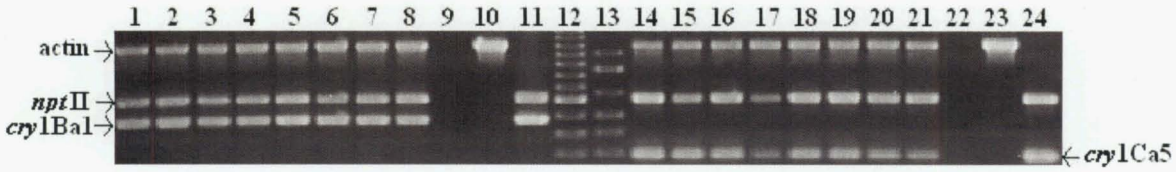


Figure 3.2 PCR analysis of putative transgenic potato (*Solanum tuberosum*) cv. 'Iwa' lines.

Lanes 1-11, represent a multiplexed reaction with the *cry*1Ba1 primers producing an expected 474 bp product, the *npt*II primers producing an expected 612 bp product and the actin primers as an internal control producing product 1069 bp. Lanes 14-24, represent a multiplex reaction with *cry*1Ca5 primers producing an expected 293 bp product, the 612 bp *npt*II product and the 1069 bp actin product. Lanes 1-8, lines transformed with pART27*cry*1Ba1 (M201, M203, M205, M207, M214, M221, M223 and M224 respectively); lanes 9 and 22, no DNA template control; lanes 10 and 23, non-transgenic 'Iwa' control; lane 11, plasmid pART27*cry*1Ba1 (positive control for *npt*II and *cry*1Ba1 genes); lanes 12 and 13, 100 bp molecular ruler 10380-012 and 1 kb plus molecular ruler 10787-018 (Invitrogen, Carlsbad, California) size markers respectively; lanes 14-21, lines transformed with pART27cry1Ca5 (M302, M307, M311, M326, M328, M329, M332 and M334 respectively); lane 24, plasmid pART27*cry*1Ca5 (positive control for *npt*II and *cry*1Ca5 genes).

3.3.3 Greenhouse evaluation and insect bioassays

The majority of the lines were observed to have a phenotypically normal appearance when grown in the greenhouse. Seven lines with the *cry*1Ba1 gene (M202, M205, M210, M213, M222, M231 and M232) and four lines with the *cry*1Ca5 gene (M310, M322, M323 and M336) populations exhibited a range of off-type characteristics such as marginal leaf curl, leaf wrinkling, reduced vigour, abnormally small and/or deformed tubers, or a combination of these traits. The line M213 only produced very small tubers, whereas line M336 failed to produce any tubers. Therefore, tuber bioassay not carried out to these two lines.

The larval GI on leaves of the non-transgenic control line was 6.61, whereas the GI ranged from 0.11 to 6.48 on the *cry*1Ba1-transgenic lines, with no obvious groupings among the lines (Figure 3.3). Larval GI on the *cry*1Ba1-transgenic lines was significantly lower than the control ($P < 0.05$) for all but one line (M220). There was a strong correlation ($|r| \geq 0.75$) between mean GI on leaves, percent mortality on the leaves and percent mortality on the tubers (Figure 3.4). Seven of the higher performing *cry*1Ba1-transgenic lines were selected for further analysis based on their phenotypically normal appearance and high level of resistance to PTM larvae. The PTM bioassay results for these seven lines are shown in Table 3.2. Percentage of mortality for all of these lines was high on both leaves and tubers, in all cases being significantly higher than with the non-transgenic control line. In contrast, none of the larvae reached pupation on the selected lines, whereas nearly 60% of larvae pupated on the control line.

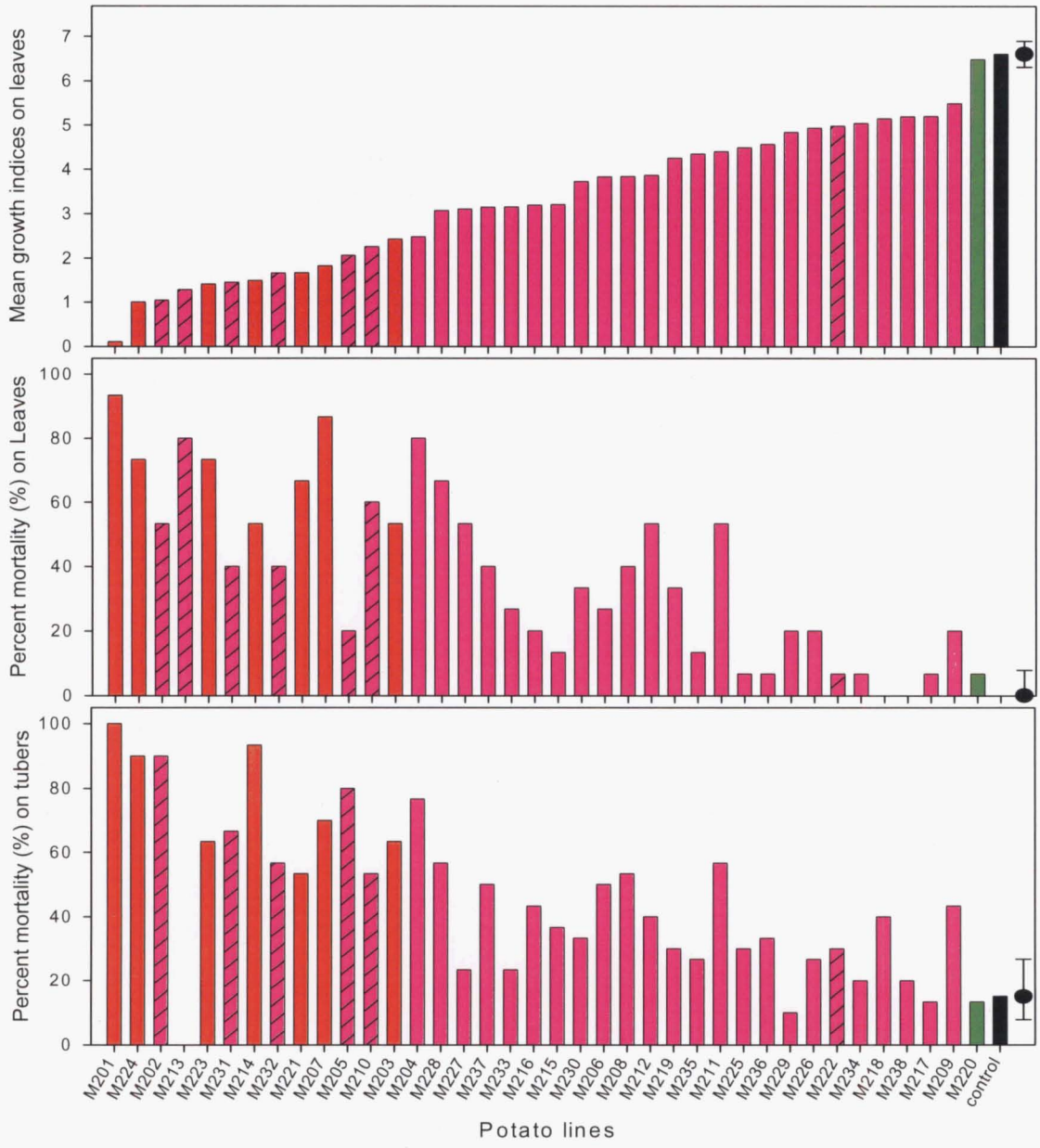


Figure 3.3 The mean growth indices and percent mortality of larvae reared on leaves and percent mortality of PTM larvae reared on tubers of *cry*1Ba1-transgenic potato plants.

The error bars represent 95% confidence limits for the non-transgenic control line (df = 79). Green bars illustrate a line for which the mean GI was not significantly different from the non-transgenic control line at 5% probability level. Hatched bars represented lines identified with a phenotypically abnormal appearance. Red bars indicate lines that were selected for further molecular analysis.

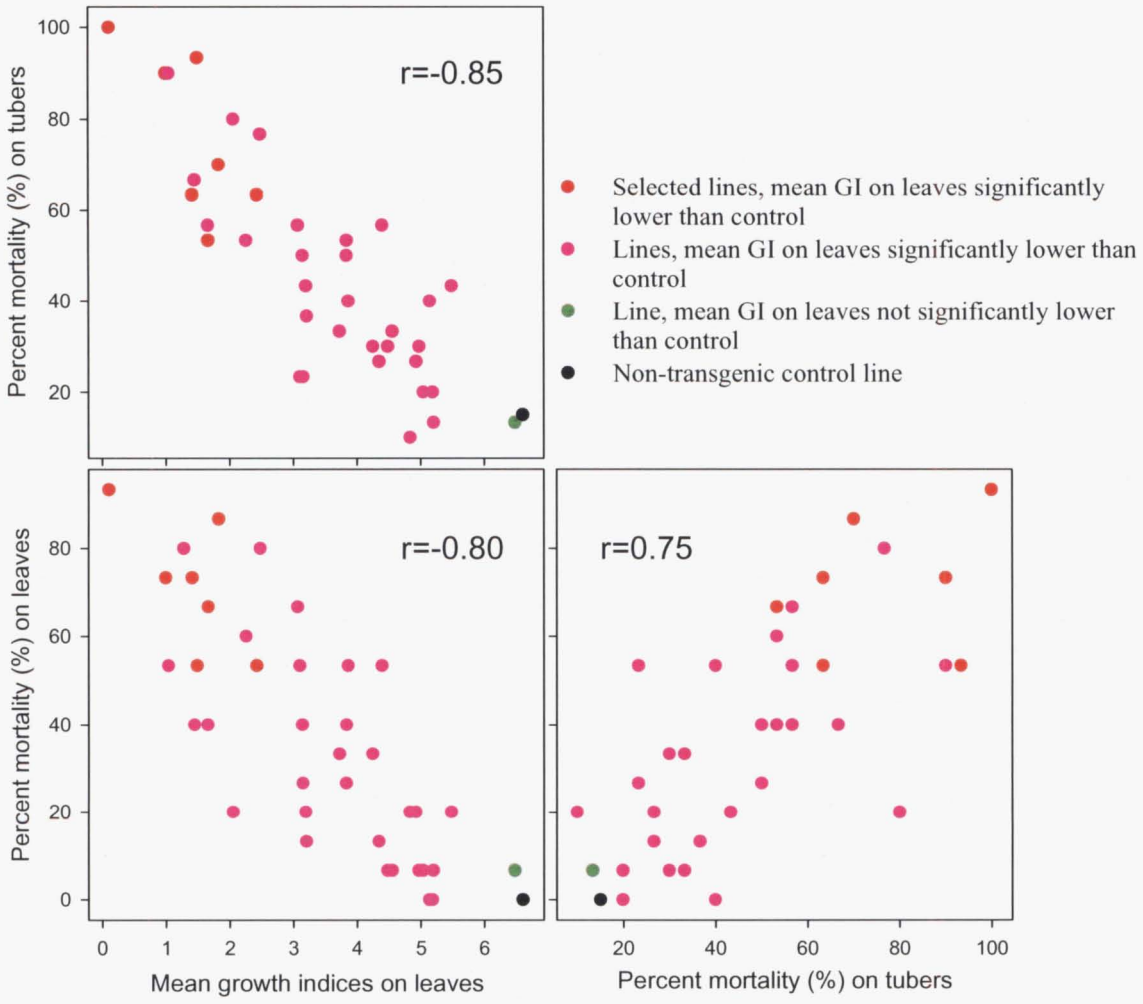


Figure 3.4 Correlation between PTM larval growth index (GI) on leaves, mortality of PTM larvae on leaves and PTM larval mortality on tubers for *cry*1Ba1-transgenic potato lines.

Table 3.2 PTM bioassay results for the seven selected *cry*1Ba1-transgenic lines.

(95 % confidence limits in brackets, df = 79).

Line	Mean GI in leaf bioassay	Larval mortality in the leaf bioassay (%)	Larval mortality in the tuber bioassay (%)	Individuals remaining as larvae in tuber bioassay (%)	Individuals reaching pupation in tuber bioassay (%)
#201	0.11(-1.18,1.39)	93 (65, 99)	100 (90, 100)	0 (0, 11)	0 (0, 7)
#203	2.43 (1.83,3.03)	53 (29, 76)	63 (45, 79)	37 (22, 54)	0 (0, 7)
#207	1.83 (0.78,2.87)	87 (60, 97)	70 (51, 84)	30 (17, 48)	0 (0, 7)
#214	1.49 (0.89,2.09)	53 (29, 76)	93 (76, 98)	7 (2, 22)	0 (0, 7)
#221	1.66 (1.02,2.30)	67 (41, 85)	53 (35, 71)	47 (30, 64)	0 (0, 7)
#223	1.41 (0.73,2.10)	73 (47, 90)	63 (45, 79)	37 (22, 54)	0 (0, 7)
#224	1.00 (0.31,1.68)	73 (47, 90)	90 (73, 97)	10 (3, 26)	0 (0, 7)
Control	6.61 (6.32,6.90)	0 (0, 8)	15 (8, 27)	27 (17, 39)	58 (51, 65)

In the leaf bioassay, larval GI on the *cry*1Ca5-transgenic lines were all zero, except line M315, due to 100% mortality of the larvae. In the tuber bioassay, all *cry*1Ca5-transgenic lines showed 100% mortality except lines M315, M324 and M335. The pupation and survived larval rates of line M315 on tubers were similar to non-transgenic control tuber, whereas lines M324 and M335 showed high larval mortality on tubers at 77% and 84% respectively.

In excised leaf bioassay, extensive leaf damage from the mining activity of PTM larvae was observed after nine days on the leaves of the non-transgenic Iwa controls. In comparison, very little damage was observed on leaves of most *cry*1Ba1-transgenic lines except for line M220 which exhibited similar leaf damage to the control. Very minute or no larval mines were observed on the leaves of all *cry*1Ca5-transgenic lines except line M315 which showed leaf damage similar to control.

3.3.4 Southern analysis and ELISA

The transgenic status of seven *cry*1Ba1 lines and eight *cry*1Ca5 lines, selected based on the higher resistance to PTM larvae and a normal "phenotypic" appearance, was further confirmed by Southern analysis. When restricting the DNA with *Hin*dIII and probing with the 1.35 kb DNA fragment corresponding to the CaMV*35S* promoter sequence, a single band greater than 1.8 kb is expected for each intact insertion of the *cry*1Ba1 and *cry*1Ca5. Two to eight copies of the *cry*1Ba1 gene and one to six copies of the *cry*1Ca5 gene were evident in the independently derived transgenic lines (Figure 3.5, Table 3.3).

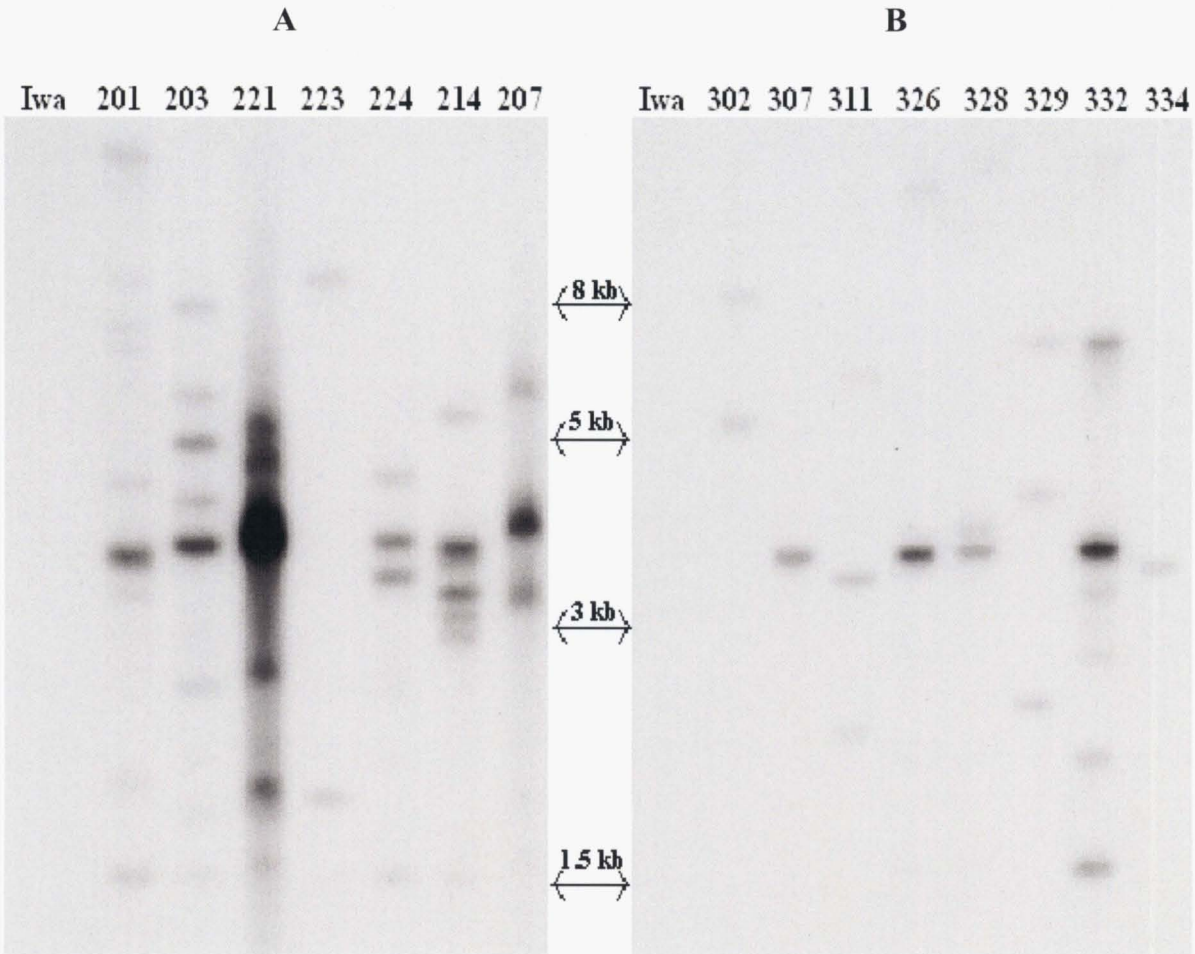


Figure 3.5 Southern analysis of selected transgenic potato lines (labels above the lanes refer to the plant lines analysed).

(A) *cry*1Ba1-transgenic lines. (B) *cry*1Ca5-transgenic lines. DNA was restricted with *Hin*dIII. Probe used for hybridisation was a 1.35 kb DNA fragment corresponding to the CaMV *35S* promoter sequence. A single band greater than 1.8 kb is expected for each intact insertion of the *cry* gene.

The amount of Cry1C protein in leaves and tubers was determined by ELISA tests for the eight selected *cry*1Ca5-transgenic lines. A range of 0.26 to 8.42 µg of protein per g of leaf tissues and 0.23 to 0.78 µg protein per g of tuber tissue was present in these transgenic lines (Table 3.3).

Table 3.3 Molecular characterization of transgenic lines by quantification of Cry1Ca5 protein (µg/g of tissue) and by copy number of *cry*1Ca5 gene.

Line	**Cry1Ca5 protein (µg/g of tissue)**		***cry*1Ca5 gene copy number**
	Leaf	Tuber	
M302	6.31	0.78	2
M307	4.18	0.5	1
M311	6.36	0.63	3
M326	0.26	0.23	2
M328	3.77	*	3
M329	8.42	*	3
M332	2.5	0.37	6
M334	4.3	0.58	1

* ELISA not carried out

3.4 Discussion

Insect pests have the potential to overcome any control mechanisms imposed to limit population growth. Consequently, the possibility that pests will adapt to Cry proteins expressed in transgenic crops presents an ongoing concern that threatens the sustainability of their use. So far, no insect resistance to a *cry*-transgenic crop has been reported from the field. However, more than a dozen pests have been selected in the laboratory for resistance to Cry toxins (Tabashnik, 1994; Frutos *et al.*, 1999; Liu *et al.*, 2001; Ferré & van Rie, 2002). Development of resistance may be due to changes in ICP receptors in the insect mid-gut and insects can adapt to Cry toxins by a general mechanism of alterations in ICP receptors (van Rie *et al.*, 1990b; Ferré & van Rie, 2002). van Rie *et al.* (1994) reported that Cry1A(b), Cry1B and Cry1C ICPs bind to distinct receptors in PTM larval mid-gut and resistance to one ICP does not automatically imply cross resistance to other ICP. Accordingly, growing of mixtures of transgenic potato plants with different *cry* genes and/or alternating transgenic potato plants with different *cry* genes year-by-year may be a valuable approach towards PTM resistance management. Therefore, development and evaluation of transgenic potato plants expressing different ICPs is important.

Many *cry* genes have been transferred to, and expressed in, several different plant species (Schuler *et al.*, 1998). However, considerable modification to the coding regions of these *cry* genes, such as removal of improper splice sites, poly(A) signals, ATTTA sequences, mRNA degradation signals and transcription termination sites as well as codon-modifications, has proved necessary in order to get adequate expression to confer insect resistance on transgenic plants (Gleave *et al.*, 1998; Fujimoto *et al.*, 1993; Beuning *et al.*, 2001; Perlak *et al.*, 1991; Vaeck *et al.*, 1987; van der Salm *et al.*, 1994). The *cry*1Ca5 gene and the *cry*1Ba1 gene used in this study were substantially modified for optimized expression in transgenic plants. The *cry*1Ba1 gene was modified to give an overall GC content of 46 % (C.R. Voisey *et al.*, unpublished data). About 15% of the nucleotides in the *cry*1Ca5 gene were modified to remove 21 potential poly(A) signals, 12 ATTTA motifs, 68 sequence blocks with 6 or more consecutive A/Ts, as well as to change

249 out of 630 codons according to preferential codon usage in dicotyledonous plants (Strizhov *et al.*, 1996).

The successful expression of *cry*1B and *cry*1C genes in transgenic plants has been previously reported to confer resistance for larvae of Lepidopteran insects. Expression of a *cry*1C gene provided effective control of diamondback moth, cabbage worm and cabbage looper in transgenic vegetable brassicas (Cao *et al.*, 1999; Cho *et al.*, 2001) and tobacco cutworm in transgenic tobacco (Christov *et al.*, 1999). Likewise, expression of a *cry*1B gene and *cry*1B-*cry*1Ab gene fusion conferred resistance to southwestern corn borer, sugarcane borer, and the fall armyworm transgenic tropical maize (Bohorova *et al.*, 2001), as well as to striped stem borer in transgenic rice (Breitler *et al.*, 2000 & 2004). Furthermore, *cry* genes identical to those used in this study have been previously shown to confer resistance to Lepidopteran larvae. The *cry*1Ca5 gene provided effective control of Egyptian cotton leafworm and the beet armyworm in transgenic alfalfa and tobacco (Strizhov *et al.*, 1996), whereas the *cry*1Ba1 gene conferred resistance to cabbage white butterfly and diamondback moth in vegetable brassica (Christey *et al.*, 2004).

Neonate PTM larvae were used in the bioassays because PTM are usually in this life stage when first encountering potato tissue (Fenemore, 1988). For the tuber bioassays, the number of individuals in the larval and pupal stages were recorded after three weeks. The earlier recovery of larvae for weighing was not possible due to difficulties of finding and removing undamaged larvae from the tubers. For the leaf bioassay the final larval weights were made toward the end of the exponential growth phase at day 9 and therefore appropriate for the calculation of a growth rate. After 9 days the rate of larval weight gain starts to diminish as they begin to lay down fatty tissue in preparation for pupation (Beuning *et al.*, 2001). The leaf bioassay method was based on the assumption that the level of Cry protein in excised leaves was similar to that in the foliage of intact plants. The observed high correlation between excised leaves and intact plants for growth of PTM larvae in response to *cry* gene expression suggests this assumption is valid (Davidson *et al.*, 2002a).

All transgenic lines with the *cry*1Ca5 gene, except line M315, showed 100% PTM larval mortality in the bioassay with excised leaves. In contrast, 40% of the transgenic lines with the *cry*1Ba1 gene showed more than 50% PTM larval mortality in the bioassay with excised leaves (Figure 3.3). These data suggest that the *cry*1Ca5 gene is more effective for the control of PTM larvae than the *cry*1Ba1 gene used in this study. All *cry*1Ba1-transgenic lines, except line M220, showed significantly lower larval GI than non-transgenic control line (Figure 3.3). Furthermore, the majority of *cry*1Ba1 lines which gave good resistance to PTM larvae in foliage also showed higher larval mortality on tubers (Figure 3.4). However, considerable variation in the level of resistance was observed, presumably as a result of variable levels of *cry* gene expression among the independently derived transgenic potato lines. Such variation is usually attributed to unpredictable levels of transgene expression as a consequence of position effects resulting from differences in the integration site of the transgenes within plant genome (Peach & Velten, 1991; Conner & Christey, 1994) and/or differences in T-DNA copy number (Hobbs *et al.*, 1993).

The majority of the independently derived transgenic lines with either the *cry*1Ca5 or *cry*1Ba1 genes were observed to have a phenotypically normal appearance when grown in the greenhouse. Seven lines from *cry*1Ba1 and four lines from *cry*1Ca5 populations exhibited a range of off-type characteristics such as marginal leaf curl, leaf wrinkling, reduced vigour, abnormally small and/or deformed tubers, or a combination of these traits. Such abnormal phenotypes are frequently observed among transgenic plants and are attributed to somaclonal variation that arises during cell culture phase of plant transformation (Conner & Christey, 1994).

The ELISA results of selected lines revealed that, *cry*1Ca5 gene driven by CaMV *35S* promoter was expressed at levels of about 0.26 – 8.42 µg protein per g of fresh leaf tissues. In contrast, the proteins level was lower in tuber tissues (about 0.23 – 0.78 µg protein per g of tuber tissue) (Table 3.3). Despite this difference in Cry protein accumulation between leaves and tubers, most *cry*1Ca5-transgenic lines exhibited 100% mortality to PTM larvae in both leaves and tubers. In a few lines

(M315, M324 and M335) there was some larval survival on tubers. This is probably a consequence of differences in transgene expression between leaves and tubers. Levels of transgene expression are well known to vary between different plant species and different plant organs (Llewellyn *et al.*, 1994; Pauk *et al.*, 1995; Nilsson *et al.*, 1996). There was no correlation between *cry*1Ca5 gene copy number and amount of Cry1Ca5 protein expressed in leaves and tubers (Table 3.3).

In conclusion, the *cry*1Ca5 and *cry*1Ba1 genes offer valuable additions to the suite of *cry* genes already available for the control of PTM in potato (Jansens *et al.*, 1995; Douches *et al.*, 1998; Rico *et al.*, 1998; Cañedo *et al.*, 1999; Li *et al.*, 1999; Chakrabarti *et al.*, 2000; Davidson *et al.*, 2002a & 2004a). These genes can be either pyramided with other effective *cry* genes, deployed individually within mixtures of transgenic potato plants with other *cry* genes, and/or alternating the use of specific *cry* genes in transgenic potato plants year-by-year. Together these approaches using a range of effective *cry* genes present a range of opportunities to facilitate the development of durable resistance to PTM in potato.

Chapter 4. An experimental approach to simulate transgene pyramiding for the deployment of *cry* genes to control potato tuber moth (*Phthorimaea operculella*)

4.1 Introduction

Insecticidal crystal proteins (ICP) synthesized by the bacterium *Bt* are the most widely used biological insecticides for the control of various insect pests (Schnepf *et al.*, 1998). More recently, genetically engineered crops that express *cry* genes encoding *Bt* proteins have also been produced (Schuler *et al.*, 1998; de Maagd *et al.*, 1999; Shelton *et al.*, 2002; Nap *et al.*, 2003) for the control of crop pests.

The likelihood that pests will develop resistance to *Bt* proteins of biological insecticides and transgenic crops is a continuing concern that threatens the sustainability of their use. Laboratory screening shows that many pests can evolve resistance, but so far the diamondback moth is the only insect species that has evolved resistance to *Bt* biopesticide in the field (Tabashnik *et al.*, 1990; Liu *et al.*, 1996; Ferré and van Rie, 2002). It has been suggested that using a mixture of different toxins could have the potential to delay insect resistance more effectively than the use of a single toxin (Gould, 1998; Ferré and van Rie, 2002; Zhao *et al.*, 2003 & 2005).

Evaluating the interaction among *Bt* toxins is important prior to transgene pyramiding for deployment of *cry* genes to control insects pests. Such interaction studies have shown the synergistic effects of the *Bt* toxins on the certain pests (e.g. Tabashnik, 1992; Poncet *et al.*, 1995; Lee *et al.* 1996; Liu *et al.*, 1998). However, the majority of such studies have been based on the use of either partially or wholly purified crystal proteins and spores of *Bt*. In only a few instances have interactions been investigated *in planta* through the expression of transgenes. These studies have involved broccoli with pyramided transgenes established by sexual crosses between plants that express two different *cry* genes (Cao *et al.*, 2002; Zhao *et al.*, 2003 & 2005).

The PTM is a pest of potatoes, has been the focus of numerous studies to develop transgenic plants through the transfer of *cry* genes into potato (Jansens *et al.*, 1995; Douches *et al.*, 1998; Rico *et al.*, 1998; Cañedo *et al.*, 1999; Li *et al.*, 1999; Chakrabarti *et al.*, 2000; Davidson *et al.*, 2002a & 2004a; Meiyalaghan *et al.*, 2005a & 2005b). No studies have investigated the efficacy of pyramiding *cry* genes in potatoes for resistance to PTM. One of the methods used to develop transgenic plants with more than one *cry* gene is sexual crossing (Cao *et al.*, 2002). Potatoes are a clonal crop with tetrasomic inheritance, high heterozygosity and severe inbreeding depression. Consequently, the genetic integrity of cultivars is instantly lost upon self- or cross-pollination (Conner & Christey, 1994), thus sexual crossing to pyramid transgenes is inappropriate. Re-transformation strategies are required, in which case it is important first to investigate how the expression of two dissimilar *Bt* toxins interact to confer insect resistance in transgenic plants.

This Chapter describes the development of an experimental approach to simulate the pyramiding of insect-resistant transgenes in clonal crops such as potatoes with the aim to identify which combination of *cry* genes would be appropriate for a pyramid strategy. This involved alternating the daily feeding of PTM larvae between isogenic pairs of potato plants established from two transgenic lines with different *cry* genes and a non-transgenic control. In this manner, the pyramiding of *cry*1Ac9, *cry*9Aa2 and *cry*1Ba1 genes in potato has simulated.

4.2 Materials and methods

4.2.1 Plant material

All potato lines used in this study were derived from the cultivar Iwa (Table 4.1). Tubers were stored for 6-12 months at 4°C, then planted in PB5 bags (15 cm x 15 cm x 15 cm black polythene bags) containing a soil mix (Conner *et al.*, 1994) and placed on the top of the benches in a containment greenhouse. The greenhouse conditions provided heating below 15°C, ventilation above 22°C, day length was supplemented to 16 h when needed with 500 W metal halide vapour bulbs, and

relative humidity was maintained above 60%. After 7-8 weeks growth in the greenhouse, leaves from these plants were used for insect bioassays.

Table 4.1 The origin of independently derived isogenic potato lines derived from cultivar Iwa used in the study.

Plant line	Original designation	Chimeric *cry* gene	Source
C	Non-transgenic Iwa	Null	-
D	DG4c	35S-*cry*9Aa2-ocs	Meiyalaghan *et al.*, 2005a
I	Iwa75	35S-*cry*1Ac9-ocs	Davidson *et al.*, 2002a
M	M211	35S-*cry*1Ba1-ocs	Chapter 3

4.2.2 Experimental design

Separate experiments were established for each pair of transgenic lines (for example, lines A and B) with different *cry* genes and the non-transgenic control (for example, line C). This set of three plant lines has six possible pair-wise combinations (A-A, B-B, A-B, A-C, B-C and C-C) and nine ordered-pair wise combinations (A-A, B-B, A-B, B-A, A-C, C-A, B-C, C-B and C-C). For each ordered-pair combination, two plants (one plant per line) were established in each of four PB5 bags, with each PB5 bag treated as a replicate and placed in the greenhouse in a randomised block design (Figure 4.1). The bioassay involved alternating the daily feeding of larvae between two lines of each pair. In this manner, the pyramiding of pairwise combinations of *cry*1Ac9, *cry*9Aa2 and *cry*1Ba1 genes in potato was simulated.

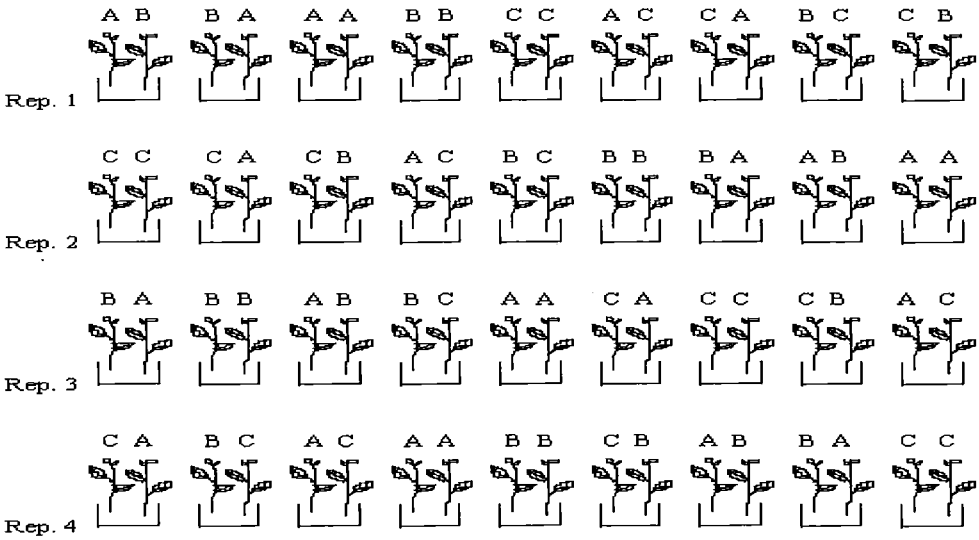


Figure 4.1 Schematic representation of the experimental set up in the greenhouse.

(Rep. = Replicate; A & B = Two different transgenic lines; C = Non-transgenic control).

4.2.3 Insect bioassay with excised leaves

The PTM larvae used in the bioassays were obtained from a laboratory colony maintained as previously described (Davidson *et al.*, 2002a). Newly hatched larvae were fed on non-transgenic potato leaves (cv. Iwa) for the first five days and these 5 d old larvae were used in the bioassay.

One leaf with 3-5 leaflets, excised from the first plant of the paired lines, was placed in the bottom of a 350 ml plastic container. Five PTM larvae (5 d old) were weighed and transferred to the leaflets, after which the containers were sealed and placed in random order in a controlled temperature room at 22 ± 3°C range with a photoperiod of 16 h light: 8 h dark. On the following day, the surviving larvae were recovered and transferred to a plastic container into which were placed 3-5 leaflets, excised from the second plant of paired lines, and the container returned to the above conditions. In this manner, surviving larvae were fed on alternate days with leaves from the two lines for 4 days (for example, for paired lines A-B, the leaves from line A were fed on day one, leaves from line B on day two, leaves from line A

on day three and leaves from line B on day four). The final weight for each surviving larva was recorded after four days. A growth index (GI) for each larva was calculated as GI = $\log_e$(final weight/mean initial weight).

4.2.4 Statistical analysis

The larval mean GIs for each replicate container for each pair of lines were analysed with analysis of variance. This included different contrasts to compare (i) the differences between the lines for the first plant, and between lines for the second plant in factorial combination, (ii) between larvae that were fed the same line twice and two different lines, comparing also between the lines used and the order of the lines fed, and (iii) the mean for two pairs with the same line with the mean of the pair of two different lines (for example, the mean for AB with the mean of (AA and BB)). Analyses were carried out using GenStat (GenStat Committee, 2003). A probability level of 5% was used to determine significance.

4.3 Results

4.3.1 Interaction of *cry*1Ac9 and *cry*1Ba1 in transgenic potato leaves

For the set of I (with the *cry*1Ac9 gene), M (with the *cry*1Ba1 gene) and C (non-transgenic control) lines, mean larval GI varied significantly between the first plant types ($P < 0.001$) and between the second plant types ($P < 0.001$, Figure 4.2A). The interaction was not strong ($P = 0.446$), suggesting that the differences between the second plant types were fairly similar, regardless of the type of the first plant. The mean GI varied between the three pairs with plants of the same type (I-I, M-M, C-C) ($P < 0.001$) and also between pairs where the plants were different (I-M, M-C, I-C) ($P < 0.001$). The order for the pairs where the plants were different was unimportant ($P > 0.628$). For this reason, the data from the same pairs of lines with the alterative order (e.g. I-M and M-I) were pooled to simplify the presentation of data (Figure 4.2A).

The mean GI of the I-I and M-M treatments was not significantly different from that of the I-M pairs (P = 0.785), suggesting additive effects of the *cry*1Ac9 and *cry*1Ba1 genes. Similarly the mean GI of M-M and C-C was similar to the mean GI for M-C pair (P = 0.637) pairs. However, there was some indication (P = 0.098) that the larval mean GI for I-C pair was lower than the mean GI of the I-I and C-C treatments.

4.3.2 Interaction of *cry*9Aa2 and *cry*1Ba1 in transgenic potato leaves

In the experiment using lines D (with the *cry*9Aa2 gene), M (with the *cry*1Ba1 gene) and C (non-transgenic control), the results were similar to the previous combinations (Figure 4.2B) with mean larval GI varying significantly between both first and second plant types (P < 0.001), but the interaction was negligible (P = 0.999). The order of the pairs when the plants were different was unimportant (P > 0.293). The mean larval GI varied between the three pairs where plants were of the same type (D-D, M-M, C-C) (P < 0.001) and also between pairs where the plants were different (D-M, M-C, D-C) (P < 0.001) (Figure 4.2B).

The mean GI of the D-D and M-M treatments was not significantly different from the mean GI of the D-M pairs (P = 0.978), suggesting additive effects of the *cry*9Aa2 and *cry*1Ba1 genes. Similarly, the mean GIs of the D-C pairs and the M-C pairs were not significantly different from the means for the D-D and C-C treatments (P = 0.812), and the M-M and C-C treatments (P = 0.981), respectively.

4.3.3 Interaction of *cry*1Ac9 and *cry*9Aa2 genes in transgenic potato leaves

For the set of I (with the *cry*1Ac9 gene), D (with the *cry*9Aa2 gene) and C (non-transgenic control) lines, mean larval GI varied significantly between both the first plant types and the second plant types (P < 0.001, Figure 4.2C). Similar to the previous experiments, the interaction was not strong (P = 0.289). The mean larval GI varied significantly between the three pairs with plants of the same type (I-I, D-D, C-C) (P < 0.001) and between pairs where the plants were different (I-D, I-C, C-D) (P = 0.01). The order of the pairs where the plants were different was relatively

unimportant for the I-C and C-I pairs ($P = 0.167$), as well as for the D-C and C-D pairs ($P = 0.313$). However, there was some suggestion of a difference for the I-D and D-I pairs ($P = 0.063$), with the larval GI slightly lower for the I-D pair than for D-I pair.

For I-C and D-C pairs, the mean larval GI of the mixed pair was similar to that of the two unmixed pairs ($P > 0.1$). However, for the I-D pair, there was some evidence that the mean GI was lower than that of the I-I and D-D pairs ($P = 0.089$) (Figure 4.2C).

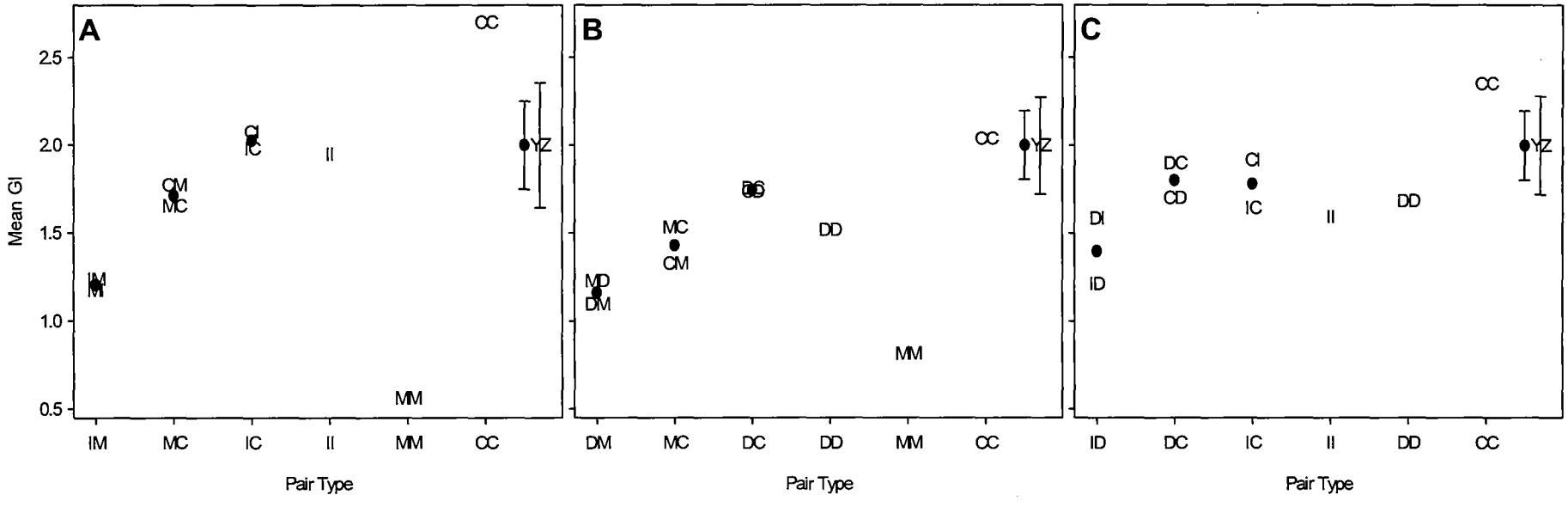


Figure 4.2 Three independent experiments simulating the effects of *cry* gene pyramiding on PTM larval growth when reared on excised leaves from greenhouse-grown potato lines.

(A) Combinations of Iwa75 (I, *cry*1Ac9), M211 (M, *cry*1Ba1) and control Iwa (C). (B) Combinations of Iwa75 (I), DG4c (D, *cry*1Ac9) and control Iwa (C). (C) Combinations of Iwa75 (I), DG4c (D) and control Iwa (C). For each pair indicated on the X-axis, there are two means represented by letters showing means for the two feeding orders for that pair (with the letters showing the order), and a solid symbol (•) representing the mean value for the two possible orders. The error bars are 95% confidence limits (df = 24) for particular means. The limit on the right applies to means indicated by letters on the graph, with the centre positioned arbitrarily and represented by YZ. The limits on the left apply to each of the means indicated by • on the graph, with the centre positioned arbitrarily and represented by •.

4.4 Discussion

Plants that express two *cry* genes encoding dissimilar *Bt* toxins (gene pyramiding or gene stacking) have the potential to delay the appearance of insect resistance more effectively than single-toxin transgenic plants (Gould *et al.*, 1994; Zhao *et al.*, 2003; Gould, 2003). The transgene pyramiding strategies for pest management employed to date include plant transformation using a vector carrying two or more insect resistance genes (Li *et al.*, 1999), co-transformation with two or more insecticidal genes on separate vectors (Hua *et al.*, 1993; Maqbool *et al.*, 2001), sequential transformation of plants with two different insect resistance genes (Greenplate *et al.*, 2003) or the sexual hybridisation of transgenic lines carrying two different insect resistance genes (Cao *et al.*, 2002).

In clonal crops such as potatoes, sexual crossing to pyramid transgenes is inappropriate. Since potatoes possess tetrasomic inheritance, high heterozygosity and severe inbreeding depression, the genetic integrity of cultivars is instantly lost upon self or cross pollination (Conner & Christey, 1994). To pyramid transgenes in such clonal crops therefore requires either simultaneous transformation or re-transformation approaches. It is important first to investigate how the expression of two dissimilar *cry* genes might interact in transgenic plants, since *Bt* toxins can interact, with antagonistic (Del Rincón-Castro *et al.*, 1999; Lee *et al.*, 1996), additive (Poncet *et al.*, 1995) and synergistic effects (Tabashnik 1992; Poncet *et al.*, 1995; Lee *et al.*, 1996; Liu *et al.*, 1998).

This Chapter developed an experimental approach to simulate transgene pyramiding using existing isogenic transgenic lines expressing different *cry* genes. This involved measuring the growth response of insect larvae with alternate daily feeding between pair-wise combinations of plants. In order to investigate interactions between insect resistance genes expressed in transgenic plants, it is important to use transgenic lines that exhibit only partial inhibition of larval growth rather than complete mortality. In this manner, any further enhancement of inhibitory activity on insect growth from the interaction of two transgenes can be

experimentally detected. If one or both transgenic lines induced complete inhibition of larval growth, then any further synergistic effects by a second insect resistance gene would not be observed. For this reason the transgenic lines I (Iwa 75), G (DG4c) and M (M211), expressing the *cry*1Ac9, *cry*9Aa2 and *cry*1Ba1 genes respectively (Table 4.1), were selected on the basis of having moderate resistance to PTM larvae and possessing the normal "phenotypic appearance" of potato cultivar Iwa from which they were all derived. In all cases these *cry* genes are controlled by identical regulatory elements and have been cloned into the same vector system (Davidson *et al.*, 2002a; Meiyalaghan *et al.*, 2005a; Chapter 3). Since they have been transferred to the same potato cultivar and phenotypically selected for the same appearance as the parent cultivar, they can be considered isogenic lines. The use of such isogenic lines is an important factor when evaluating responses to transgene pyramiding, since it eliminates other potential interacting factors that may interfere with the intended comparison (Conner and Christey 1994). Although isogenic transgenic potato plants expressing a *cry*1Ca5 gene developed (Chapter 3), all the recovered lines induced 100% mortality of PTM larvae. Unfortunately, no appropriate lines expressing this gene and exhibiting moderate resistance were available for the present study.

In the present study, the use of plant material expressing *cry* genes was considered to provide a more realistic food source than an artificial diet supplemented with *Bt* toxins, since it better reflects transgenic crops. Although PTM larvae mine both leaves and tubers (Foot, 1979) foliage was used in this study since larvae can be more easily recovered from mined leaves with minimal or no injury compared to those recovered from mines in tubers

The bioassay involved alternating the daily feeding of larvae between two isogenic lines of each pair to ensure that the larvae fed on leaf material from each line. This was to avoid the possibility that larvae may feed preferentially on one line if leaves from each line had been provided simultaneously. Since environmental conditions, such as heat or water stress, have been suggested as factors influencing transgene expression (Broer, 1996), the pairs of plants were established in a same bag (Figure

4.1) to reduce any possible physiological and morphological variations that may occur if the plants had been grown in separate bags.

The experimental design involved transferring larvae between leaves each day. Repeated handling of small fragile neonate larvae can result in injury or death. Consequently, 5 day old larvae were used instead. Neonate larvae were fed non-transgenic Iwa leaves for the first five days, then used to initiate the bioassay. A previous study found no age-specific differences in PTM larval growth when larvae of different ages (0, 3, 5 or 7 days old) were fed transgenic potato foliage (Davidson *et al.*, 2005a). The bioassay was terminated after four days and the final weight for each surviving larva was recorded when they were 9 days old. This was because larval growth is exponential in the first nine days, after which larvae begin to lay down fatty tissue in preparation for pupation, resulting in weight loss (Beuning *et al.*, 2001).

The mean GI for PTM larvae reported in this study for each transgenic line (DG4c, Iwa75 and M211) and the non-transgenic controls differ from those reported in original studies (Davidson *et al.*, 2002a; Meiyalaghan *et al.*, 2005a; Chapter 3). In the present study, 5-day-old larvae were exposed to transgenic leaves only for 4 days, while neonate larvae were fed transgenic foliage for at least 9 days in the original studies. Consequently larval GIs were calculated over a shorter period in the present study (Figure 4.2). Also, previous studies did not involve the daily transfer of larvae to fresh leaves. Therefore, a comparison of daily larval growth between the current study and previous studies is not appropriate. In a previous study no age-specific effects on PTM larval growth were found when the susceptibility of larvae of different ages to transgenic foliage was examined (Davidson *et al.*, 2005a). Furthermore, the daily growth of larvae was similar for a given isogenic potato line regardless of the initial age of larvae (0, 3, 5 or 7 day old), when they were introduced to transgenic foliage (Davidson *et al.*, 2005a).

The pair-wise combinations involving transgenic and control plants were relatively unimportant in the evaluation of the effects of expressing two dissimilar insect

resistance genes on insect larval growth. However, such treatments are an important control to verify the reliability of the experimental approach involving the alternate daily feeding of larvae on excised leaves. In all experiments of the present study, the GI of PTM larvae when alternately fed on transgenic and non transgenic potato plants was generally intermediate between continuous feeding on transgenic or non-transgenic plants (Figure 4.2). The effect of plants expressing different Cry proteins were additive in their inhibition of larval growth compared to the effects of from the expression of single *cry* genes. Alternating larval feeding on transgenic and non-transgenic leaves gave expected outcome where larval growth was higher on transgenic/non-transgenic pairs compared to transgenic/transgenic pairs of a single *cry* gene.

In conclusion, this study has demonstrated an effective experimental approach to simulate pyramiding of insect-resistant transgenes in clonal crops, which enabled to identify potential combinations of *cry* genes for future use in a pyramid strategy. This experimental approach involved alternating of the daily feeding of PTM larvae between pairs of isogenic potato plants expressing different *cry* gene. In this manner we have simulated the pyramiding of pairwise combinations of *cry*1Ac9, *cry*9Aa2 and *cry*1Ba1 genes in potato. This allowed the interaction of pairs of these three *cry* genes on the growth rate of PTM larvae to be evaluated. The larval growth responses to all the *cry* gene combinations were largely consistent with additive impacts, although results from the combination of the *cry*1Ac9 and *cry*9Aa2 genes were suggestive of slight synergistic effects. The pyramiding of *cry*1Ac9, *cry*9Aa2 and *cry*1Ba1 genes in potato could therefore provide more effective resistance to PTM than using each *cry* gene individually.

Chapter 5. Pyramiding transgenes for potato tuber moth resistance in potato

5.1 Introduction

The potato tuber moth (PTM), *Phthorimaea operculella* (Zeller), is one of the most important insect pests of potato (*Solanum tuberosum* L.) in warm-temperate, subtropical, and tropical regions (Radcliffe, 1982; Raman & Palacious, 1982). Larvae mine leaves and stems causing transparent tunnels in the leaves, death of growing points and weakening of stems. They also mine the tubers in the field as well as in storage, reducing tuber quality (Trivedi & Rajagopal, 1992). The damaged areas can also provide an infection point for pathogens (Plaisted *et al.*, 1994).

Strategies such as biological control (Kroschel *et al.*, 1996a & 1996b), chemical control (Foot, 1974a) and cultural practices (Foot, 1974b & 1975b) are commonly used to manage PTM. Integrating the use of these management strategies can help to manage this pest effectively in the field as well as in storage (Hanafi, 1999). However, the development of potato cultivars resistant to PTM could increase the efficacy of cultural and biological methods and reduce the use of insecticides (Arnone *et al.*, 1998), which can be environmentally damaging, costly and can be ineffective when larvae exist in mined tunnels of leaves and tubers.

The development of PTM-resistant cultivars through traditional breeding methods has not been successful (Arnone *et al.*, 1998). With the advent of genetic engineering, genes for insect resistance can be transferred to develop resistant plants. Transgenic potatoes that express *cry* genes derived from various strains of the soil bacterium *Bt*, have been proved to be resistant to the PTM (Jansens *et al.* 1995; Cañedo *et al.*, 1999; Li *et al.*, 1999; Chakrabarti *et al.*, 2000; Douches *et al.*, 2002; Davidson *et al.*, 2002a; Meiyalaghan *et al.*, 2005a).

The potential for the evolution of resistance to transgenic plants by the target pest raises concerns about the effectiveness of *Bt*-transgenic crops (Tabashnik, 1994;

Roush, 1997b; Gould, 1998; Ferré and van Rie 2002). So far, no insects resistant to *Bt*-transgenic crop have been reported from the field. However, the diamondback moth has evolved resistance to *Bt*-sprays in the field (Tabashnik *et al.*, 1990; Liu *et al.*, 1996; Ferré and van Rie, 2002). To increase the usefulness and effectiveness of the transgenic crops it is important to implement resistance management strategies simultaneous with the release of transgenic crops.

Various strategies can be considered for managing resistance to *Bt*-transgenic crops (Roush *et al.*, 1997b; Gould, 1998; Shelton *et al.*, 2000; Brousseau *et al.*, 1999). One approach proposed to delay the development of insect resistance in *Bt*-crops involves the use of mixtures of different *Bt*-toxins (Gould, 1998; Ferré and van Rie, 2002; Zhao *et al.*, 2003 & 2005).

The stable introduction of more than one useful transgene into plant genomes, termed "transgene pyramiding", can be achieved by several strategies (Berger, 2000). The simplest approach involves sexual crossing to combine improvements in different traits such as disease and pest resistance and yield etc. (Cao *et al.*, 2001; Datta *et al.*, 2002; Samis *et al.*, 2002). However, in clonal crops such as potato it is impossible to maintain the genetic integrity of a cultivar by combining traits through sexual hybridization (Conner & Christey, 1994). For such clonal crops alternative selection systems can be valuable to pyramid transgenes into the same cultivar by successive transformation events (Barrell *et al.*, 2002).

This Chapter describes the feasibility of two strategies for transgene pyramiding using *Agrobacterium*-mediated transformation for development of durable resistance in potato to PTM. The first approach involved the simultaneous introduction of *cry*1Ac9 and *cry*9Aa2 genes, using a kanamycin (*npt*II) selectable marker gene. The second approach involved the sequential introduction (re-transformation) of *cry*1Ac9 gene, using a hygromycin *(hpt)* selectable marker gene, into an existing line transgenic for a *cry*9Aa2 gene and a *npt*II marker gene.

5.2 Material and methods

5.2.1 Vector construction

All chimeric genes were constructed using standard protocols for DNA manipulations (Sambrook & Russell, 2001) and following manufacturers' recommendations where appropriate. The *Lhca*3-*cry*9Aa2-*ocs* chimeric gene was excised from the primary cloning vector pART7cabG14 (Meiyalaghan *et al.*, 2005a) as a 3.3 kb *Not*I fragment and cloned into pMOA33 (Figure 5.1) to produce the binary vector pMOA33-1. The *Lhca*3-*cry*1Ac9-*ocs* chimeric gene was excised from the primary cloning vector pART7cab1Ac9 (Chapter 2) as a 3.2 *Not*I fragment. This fragment was blunt-ended using the End-It™ DNA End-Repair Kit (EPICENTRE, Madison, Wisconsin) according to manufacturer's instructions. The binary vector pMOA33-1 and binary vector pMOA34 (Figure 5.1) were cleaved using *Stu*I, which generated blunt-end fragments, and independently ligated with the blunt-ended *Lhca*3-*cry*1Ac9-*ocs*3' fragment to produce the binary vectors pMOA33-2 and pMOA34-1 respectively. The blunt-end ligations were performed using Fast-Link™ DNA Ligation Kit (EPICENTRE, Madison, Wisconsin), according to manufacturer's instructions.

The binary vectors pMOA33-2 and pMOA34-1 were individually transferred to the disarmed *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.*, 1993) using the freeze-thaw method (Höfgen & Willmitzer, 1988). Prior to co-cultivation with potato tissue, the *Agrobacterium* cultures harboring the binary vectors were cultured overnight at 28°C, on a shaking table, in LB broth supplemented with 300 mg L^{-1} spectinomycin.

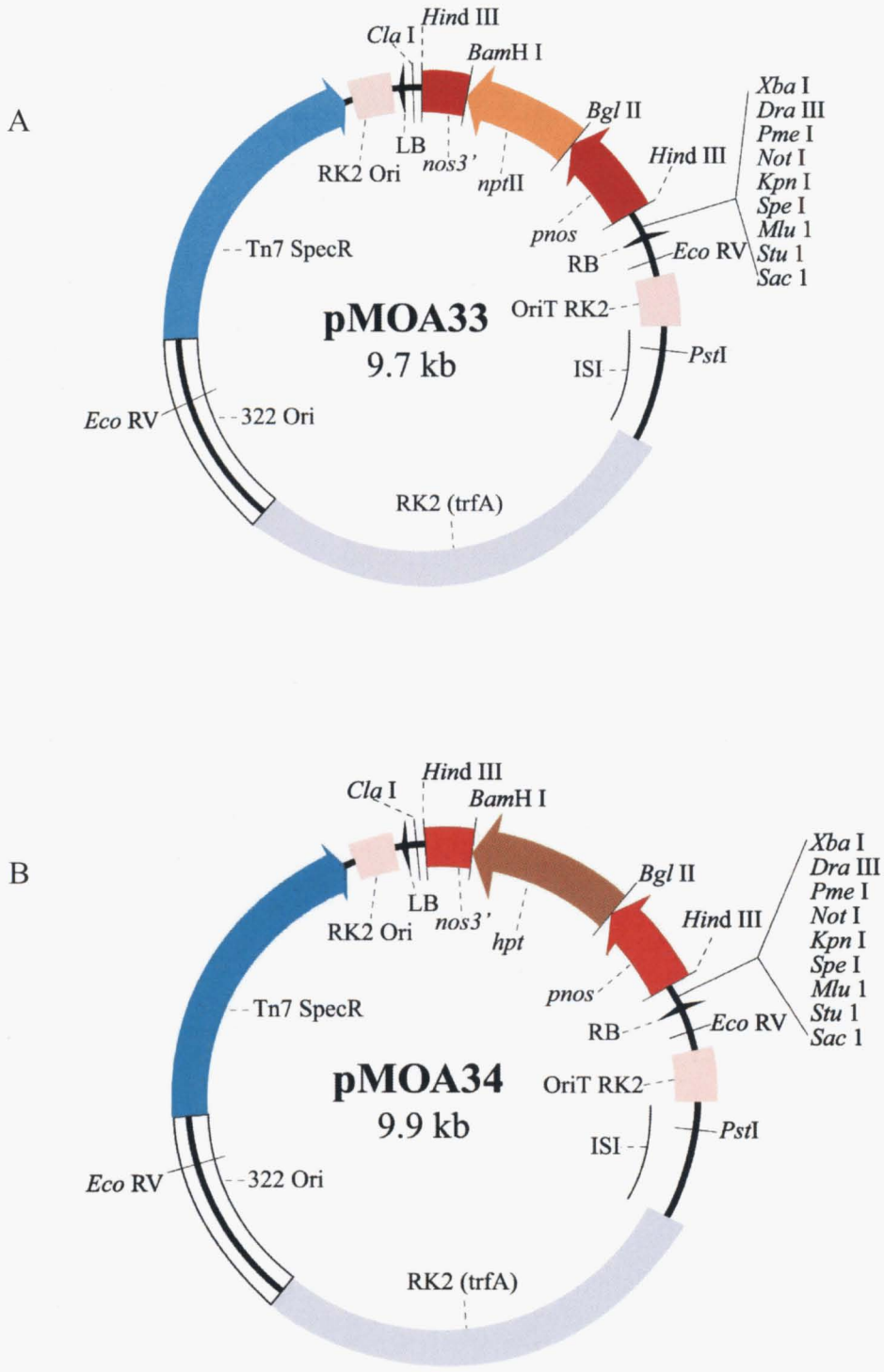


Figure 5.1 Schematic representation of the two binary vectors.

(A) The binary vectors pMOA33 (with a *npt*II selectable marker gene) and (B) pMOA34 (with a *hpt* selectable marker gene). Source: Philippa Barrell, Crop and Food Research, Lincoln, New Zealand.

5.2.2 Potato transformation protocol

Virus-free plants of cultivar Iwa and the existing transgenic line DG4c (Meiyalaghan *et al.*, 2005a) were multiplied *in vitro* as described in Chapter 2. For simultaneous transformation with both *cry* genes, leaves from the *in vitro* Iwa plants were co-cultivated with *Agrobacterium* containing pMOA33-2 and subjected to kanamycin selection (see Chapter 2). To accomplish sequential re-transformation, leaf segments from the DG4c line transgenic for *35S-cry*9Aa2-*ocs* and *nos-npt*II-*nos* chimeric genes (Meiyalaghan *et al.*, 2005a) were transformed with pMOA34-1. This was also performed as described in Chapter 2, except that 25 mg L^{-1} hygromycin was used as the selective agent for the recovery of transformed cell colonies and shoots and 12.5 mg L^{-1} hygromycin was used to screen the recovered plants for root formation under selective pressure.

5.2.3 Screening of putative transformed lines using PCR

This was performed as described in Chapter 2 with primers specific for the transgene of interest multiplexed with primers for the endogenous potato actin gene as an internal control (Table 5.1).

Table 5.1 Primers for PCR of each gene and expected product size.

Target gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size (bp)
*cry*1Ac9	GCCACAGAATAACAACGTGC	GCATACCGTACACGAACTCG	359
*cry*9Aa2	GCATCTAATCGCCGTTCA	CGAATTTGGTCCGGACTT	424
*npt*II	ATGACTGGGCACAACAGACAATCGGCTGCT	CGGGTAGCCAACGCTATGTCCTGATAGCGG	612
hpt	AGCGTCTCCGACCTGATG	TGCCGTCAACCAAGCTCT	784
Actin	GATGGCAGAAGGCGAAGATA	GAGCTGGTCTTTGAAGTCTCG	1069

5.2.4 Insect bioassay using excised leaves from greenhouse-grown plants

All of the putatively transformed lines were transferred to the containment greenhouse using the method described in Conner *et al.* (1994). Two plants were established in each of three PB5 bags (15 cm x 15 cm x 15 cm black polythene bags) per line, with each PB5 bag treated as a replicate, and the bags placed in the greenhouse in a randomised block design. The greenhouse conditions provided heating below 15°C and ventilation above 22°C. Day length was supplemented to 16 h when needed with 500 W metal halide vapour bulbs, and relative humidity was maintained above 60%.

Insect bioassays using excised leaves were performed as described in Chapter 2. Mean growth indices (GI) were analysed with analysis of variance, taking into account the between- and within-pottle variation. The analyses were carried out using GenStat (GenStat Committee, 2003), and a probability level of 5% was used to determine significance.

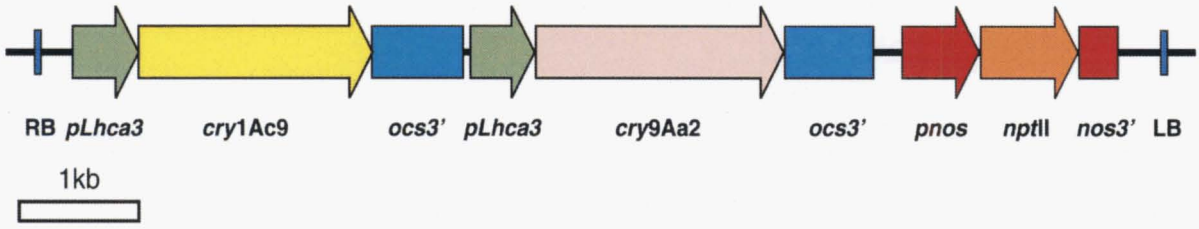
5.2.5 Reverse transcription (RT)-PCR analyses

This was performed as described in Chapter 2 The RT-PCR primers used for *cry*1Ac9, *cry*9Aa2 and actin were the same as those used for PCR analysis (Table 5.1)..

5.3 Results

The final T-DNA structure of the pMOA33-2 and pMOA34-1 binary vectors is outlined in Figure 5.2. These binary vectors were used for *Agrobacterium*-mediated transformation the potato cultivar Iwa and the DG4c transgenic line respectively. Small cell colonies developed along the cut leaf edges and/or leaf surfaces after 3-4 weeks on callus induction medium. In kanamycin-supplemented medium, most of the cell colonies from Iwa explants were green and grew as hard, compact callus. Whereas, in hygromycin supplemented medium, majority of cell colonies from DG4c explants were pale-green and friable. Following transfer to regeneration medium most, hard green cell colonies produced shoots, whereas the friable calli generally failed. Two to three weeks after the cell colonies were transferred with regenerated shoots to potato multiplication medium, two-four fully grown shoots developed from each original cell colony. In both kanamycin and hygromycin selection media, single healthy shoots excised from each shoot clump readily formed roots within a week. A total of 14 independently derived, putative transgenic potato lines were established (10 lines for the pMOA33-2 construct and four lines for the pMOA34-1 construct, labeled as MV series and M4c series respectively).

(A)



(B)

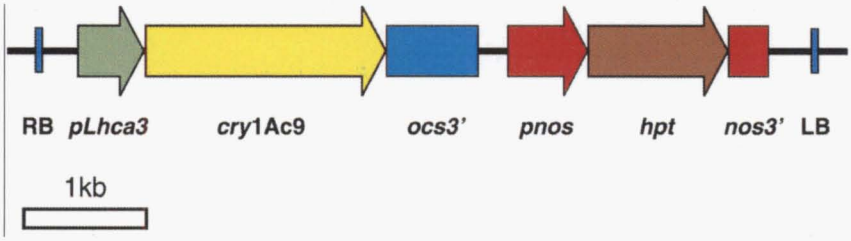


Figure 5.2 Schematic representation of the T-DNA regions of the two binary vectors.

(A) The T-DNA region of pMOA33-2. (B) The T-DNA region of pMOA34-1. RB, LB indicate the right and left T-DNA borders respectively.

5.3.1 PCR analysis of regenerated lines

The presence of the *npt*II and *cry* genes in MV lines, *hpt* and *cry* genes in M4c lines was confirmed using multiplex PCR with an endogenous actin gene as an internal positive control. Since the actin product was expected in both transgenic and non-transgenic potato plants, this allows failed PCR reactions to be conveniently distinguished from a non-transgenic line. All transgenic lines were PCR positive for the selectable marker gene and both *cry* genes (Figure 5.3).

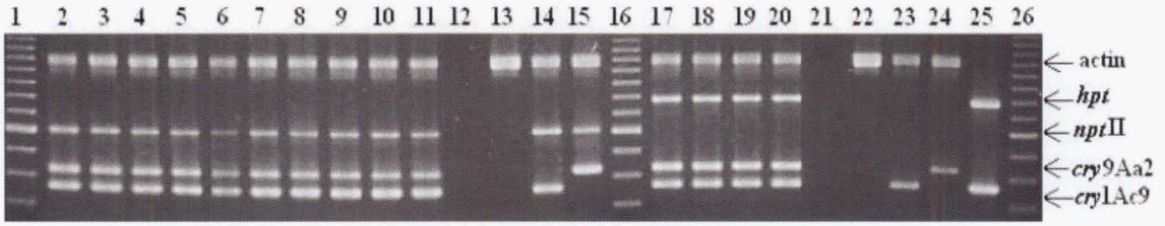


Figure 5.3 PCR analysis of putative gene-pyramiding transgenic potato (*Solanum tuberosum*) cv. 'Iwa' lines.

Lanes 2-15 represent a multiplex reaction with the actin primers as an internal control producing product 1069 bp, the *npt*II primers producing an expected 612 bp product, the *cry*9Aa2 primers producing an expected 424 bp product and the *cry*1Ac9 primers producing an expected 359 bp product. Lanes 17-25 represent a multiplex reaction with the actin primers as an internal control producing product 1069 bp, the *hpt* primers producing an expected 784 bp product, the *cry*9Aa2 primers producing an expected 424 bp product and the *cry*1Ac9 primers producing an expected 359 bp product. Lanes 2-11, lines transformed with pMOA33-2 (MV1, MV2a, MV2b, MV2c, MV2d, MV2e, MV3a, MV3b, MV3c, MV4 respectively); lanes 12 and 21, no DNA template control; lanes 13 and 22, non-transgenic 'Iwa' control; lanes 14 and 23, 'Iwa' line I52 known to be transgenic for *npt*II and *cry*1Ac9 (positive control, ex Davidson *et al.*, 2002a); lanes 15 and 24, 'Iwa' line DG4c known to be transgenic for *npt*II and *cry*9Aa2 (positive control, ex Meiyalaghan *et al.*, 2005a); Lanes 17-20, lines transformed with pMOA34-1 (M4c#1, M4c#2, M4c#3 and M4c#4 respectively); lane 25, plasmid pMOA34-1 (positive control for *hpt* and *cry*1Ac9); lanes 1, 16 and 26, 100 bp molecular ruler 10380-012 (Invitrogen, Carlsbad, California).

5.3.2 Greenhouse evaluation and insect bioassay

All the lines were observed to have a phenotypically normal appearance when grown in the greenhouse. There were significant differences between lines in larval GI for the populations of MV and M4c lines, with the major difference being between the non-transgenic control and all the transgenic lines (Figure 5.4). In the MV lines, one line (MV#1) had a significantly lower GI than all the other lines. However, in the M4c series all transgenic lines showed a similar GI, although the results suggest that one line (M4c#1) had lower larval GI than DG4c line.

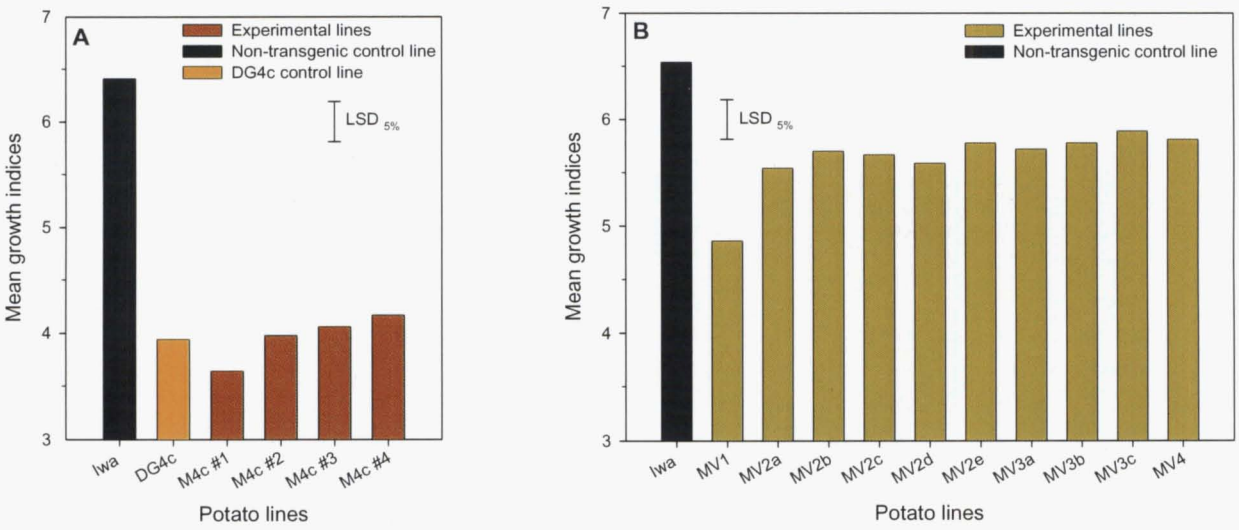


Figure 5.4 The mean growth index of surviving PTM larvae reared on leaves of potato plants.

(A) Experimental lines developed by re-transformation of line DG4c with the pMOA34-1 vector. (B) Experimental lines developed by simultaneous transformation of cultivar Iwa with the pMOA33-2 vector.

5.3.3 RT-PCR analysis transgenic lines

The expression of the *cry* genes in leaves of transgenic lines was determined by multiplex RT-PCR analysis with an endogenous actin gene as an internal control (Figure 5.5). RT-PCR showed that amplification of the expected 359 and 424 bp fragments had occurred in all the transgenic lines for the *cry*1Ac9 and *cry*9Aa2 genes respectively. The use of the endogenous actin gene as an internal control allows failed reactions to be conveniently distinguished from non-expressing transgenic line and also provides a baseline for standardising gene expression between transgenic lines. Since the primers flank two introns in the actin gene, it also provides a convenient check for DNA contamination of the RNA samples i.e. amplification of an 1069 bp fragment would indicate the presence of the intron sequences, and therefore represents DNA contamination. Once the introns have been spliced out, the expected RT-PCR product is 709 bp. RT-PCR analysis using actin gene primers produced the expected 709 bp product in all samples. However, RT-PCR actin products also showed a faint 835 bp fragment in some samples.

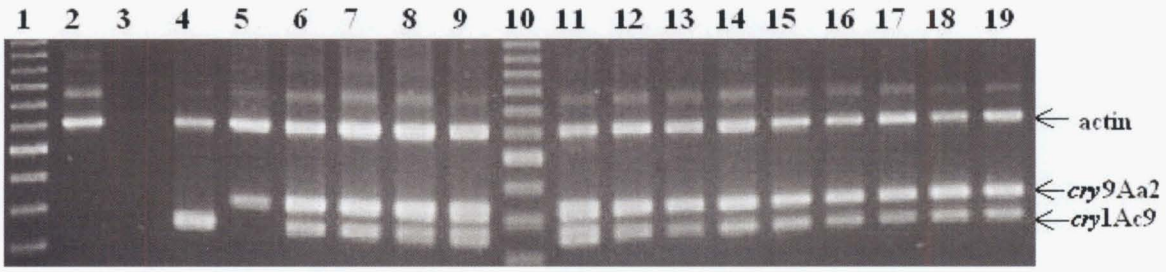


Figure 5.5 Reverse transcription (RT)-PCR analysis of transgenic potato lines.

Lanes 2-9 and 11-19 represent a multiplex reaction with the *cry*1Ac9 primers producing an expected 359 bp product, the *cry*9Aa2 primers producing an expected 424 bp product and the actin primers as an internal control producing an expected 709 bp product. Lane 2, non-transgenic 'Iwa' control; lane 3, no RNA template control; lane 4, 'Iwa' line I52 known to be transgenic for *cry*1Ac9 (positive control, ex Davidson *et al.*, 2002a); lane 5, 'Iwa' line DG4c known to be transgenic for *cry*9Aa2 (positive control, ex Meiyalaghan *et al.*, 2005a); lanes 6-9, lines M4c#1, M4c#2, M4c#3 and M4c#4 respectively; lanes 11-19, lines MV1, MV2a, MV2b, MV2c, MV2d, MV2e, MV3a, MV3b, MV3c and MV4 respectively; lanes 1 and 10, 100 bp molecular ruler 10380-012 (Invitrogen, Carlsbad, California).

5.4 Discussion

Agrobacterium-based plasmid vectors allow the transformation of a wide range of plant species by using the T-DNA to introduce foreign genes into the nuclear genome of plants (Hellens *et al.*, 2000). During transformation, independent T-DNA insertions appear to occur at random throughout the genomes of plants, including potatoes (Jacobs *et al.*, 1995; El-Kharbotly *et al.*, 1996). This phenomena provides an important basis to allow the successful pyramiding of transgenes by sexual crosses or re-transformation.

The simplest approach to pyramid two transgenes into a single plant line is via sexual hybridisation of transgenic plants. This has been achieved in broccoli by crossing *cry*1Ac- and *cry*1C- transgenic plants (Cao *et al.*, 2002). However, sexual hybridization to pyramid transgenes is inappropriate in clonal crops such as potatoes. Since potatoes are autotetraploids with tetrasomic inheritance, high heterozygosity and severe inbreeding depression, the genetic integrity of cultivars is instantly lost upon self or cross pollination (Conner & Christey, 1994). Transgene pyramiding in potatoes must therefore be achieved by either a simultaneous transformation strategy with multiple genes at the same time, or a sequential re-transformation strategy into an existing transgenic line using a different selectable marker gene. A disadvantage of the re-transformation approach is the requirement for a second selectable marker gene. This may raise additional biosafety and intellectual property complications (Berger, 2000). Furthermore, the *npt*II gene for kanamycin resistance is the preferred selectable marker for potato transformation, whereas the alternatives required for re-transformation are less efficient (Barrell *et al.*, 2002). This is evident by the recovery of more independently derived transgenic lines from the simultaneous transformation strategy relative to the re-transformation strategy in the present study.

Analysis of all putative transgenic lines using multiplex PCR established the presence of either *npt*II or *hpt* gene and both *cry*1Ac9 and *cry*9Aa2 genes in all regenerated lines (Figure 5.3), thereby confirming their transgenic status. It also

confirms success from using both strategies for transgene-pyramiding using *Agrobacterium*-mediated gene transfer. RT-PCR analysis of leaf RNA of transgenic lines confirmed the transcriptional expression of the *cry*1Ac9 and *cry*9Aa2 genes in the foliage of transgenic plants. The internal control using the actin primers produced the expected 709 bp product as a bright band (Figure 5.5) which is equivalent to the genomic fragment minus the two introns. However, a faint 835 bp product was also observed in some lines (Figure 5.5). This is consistent with previous results (Chapter 2) when the latter RT-PCR product retained the 126 bp intron.

All the transgenic lines expressing both the *cry*1Ac9 and *cry*9Aa2 genes exhibited a high level of resistance to PTM larvae. However, this level of resistance was no greater than that previously observed from the use of only the *cry*1Ac9 gene (Davidson *et al.*, 2002a & 2004a; Chapter 2) or only the *cry*9Aa2 gene (Takla, 2001; Meiyalaghan *et al.*, 2005a; Chapter 2). The failure to observe an enhanced level of resistance to PTM larvae is surprising given the previous experimental results from simulating the pyramiding of several *cry* genes (Chapter 4). When alternating the daily feeding of PTM larvae between pairs of potato plants, combinations of *cry* genes were largely consistent with additive impacts on PTM larvae. The results from the combination of the *cry*1Ac9 and *cry*9Aa2 genes were even suggestive of slight synergistic effects (Chapter 4). It is possible that the development of further transgenic potatoes lines with both the *cry*1Ac9 and *cry*9Aa2 genes might allow recovery of some lines with higher expression of both genes and the expected enhanced resistance to PTM larvae. It is well known that many independently derived transgenic events need to be screened to obtain one that exhibits acceptable expression of the transgenic trait (Conner & Christey, 1994; Berger, 2000).

Although improved resistance to PTM larvae was not readily apparent from pyramiding the *cry*1Ac9 and *cry*9Aa2 genes in potato, one notable effect was the similar level of resistance in all the transgenic lines. This was observed for both the MV series and the M4c series development by simultaneous and sequential

transformation strategies respectively. Considerable variation in transgene expression among independently derived insect-resistant transgenic plants has been commonly reported in other studies (e.g. Peferoen *et al.*, 1990; van Rie *et al.*, 1994; Beuning *et al.*, 2001), including Iwa potato lines transgenic for either the *cry*1Ac9 gene (Davidson *et al.*, 2002a & 2004a) or the *cry*9Aa2 gene (Takla, 2001; Meiyalaghan *et al.*, 2005a; Chapter 2). Such variation is usually attributed to unpredictable levels of transgene expression as a consequence of position effects resulting from differences in the integration site of the transgenes within the plant genome and/or differences in T-DNA copy number (Conner & Christey, 1994). When two different transgenes contributing to the same phenotype are expressed in plants, such as the *cry*1Ac9 and *cry*9Aa2 genes for PTM resistance in potato, it is possible that variations in the expression of the two independent genes has a modulating effect in the overall phenotype.

This study has established that the transgene-pyramiding can be successfully accomplished in potatoes for PTM resistance by both simultaneous and sequential transformation strategies using *Agrobacterium*-mediated gene transfer. Furthermore, re-transformation was identified as a flexible approach to effectively pyramid genes for PTM resistance in potato, since it allowed the second gene to be added to a line previously identified as having a high level of resistance. Although none of the lines expressing both *cry* genes exhibited any greater resistance to PTM larvae over that observed for the individual genes, it is anticipated that these lines will have a more durable resistance by delaying the opportunities for PTM adaptation to the individual *cry* genes.

Chapter 6. Concluding discussion

6.1 Overview of thesis

In transgenic potato it is often desirable to couple high-level expression in foliage with no expression in the edible tubers, especially for resistance to pests that primarily infest foliage. To accomplish this, the use of a light inducible *Lhca*3 promoter for transcriptional control of *cry*1Ac9 and *cry*9Aa2 genes for resistance to PTM was investigated in Chapter 2. Thirty-five and thirty-one independently derived transgenic lines of potato cultivar Iwa were regenerated for the *cry*1Ac9 and *cry*9Aa2 genes respectively. Significantly inhibited larval growth of PTM on excised greenhouse-grown leaves was observed in 51% of the *cry*1Ac9-trangenic lines and 84% of the *cry*9Aa2-transgenic lines (Figures 2.4 & 2.5). RT-PCR analysis identified several transgenic lines with high levels of *cry* gene mRNA in leaves and no to low levels in tubers (Figure 2.6). Southern and ELISA analyses on eight selected *cry*1Ac9-transgenic lines revealed that they contained 2 to 9 copies of the *cry*1Ac9 gene (Figure 2.7) and the amount of Cry protein in leaves was less than 60 ng g^{-1} of fresh leaf tissue. Southern analysis for four selected *cry*9Aa2-transgenic lines revealed that they contained 2 to 6 copies of the *cry*9Aa2 gene (Figure 2.7). This study has established that the expression of either the *cry*1Ac9 gene or the *cry*9Aa2 gene in transgenic potato plants offers protection against PTM larval damage in foliage when expressed under the transcriptional control of a *Lhca3* light-inducible promoter. Several transgenic lines were identified with high *cry* gene expression, high resistance to PTM larvae in the foliage, and no or minimal *cry* gene expression in tubers.

It is important to assemble a suite of genes capable of effecting PTM control to allow future evaluation of different approaches toward deployment of PTM resistance management strategies. The effectiveness of two further *cry* genes for PTM resistance in transgenic potato was demonstrated in Chapter 3. *cry*1Ca5 and *cry*1Ba1 genes under transcriptional control of a CaMV 35S promoter were transferred to Iwa potato using *Agrobacterium*-mediated transformation. Thirty-six

and thirty-eight independently derived transgenic lines were regenerated with the *cry*1Ca5 and *cry*1Ba1 genes respectively. Multiplex-PCR confirmed the presence of the *npt*II selectable marker gene and specific *cry* gene in all regenerated lines (Figure 3.2). In greenhouse trials, approximately 90% of each transgenic population produced phenotypically normal plants. Over 90% of the *cry*1Ca5-transgenic lines gave 100 % larval mortality of PTM on excised greenhouse-grown leaf and tuber bioassays. In contrast, only 40-50% of the *cry*1Ba1-transgenic lines gave 50 to 100% of larval mortality of PTM using the same bioassays, although all *cry*1Ba1-transgenic lines, except one, significantly inhibited larval growth (Figure 3.3). Southern blot analysis for eight selected lines transgenic for *cry*1Ca5 gene revealed that they contained 1 to 6 copies of the *cry* gene (Figure 3.5). ELISA determined the amount of Cry1Ca5 protein in these lines to range from 0.26 to 8.42 µg per g of leaf tissue and from 0.23 – 1.02 µg per g of tuber tissue (Table 3.3). No relationship was apparent between *cry*1Ca5 gene copy number and amount of Cry protein expressed in the leaves or tubers. Southern blot analysis for seven selected lines transgenic for the *cry*1Ba1 gene revealed that they contained 2 to 8 copies of the *cry* gene (Figure 3.5). The *cry*1Ca5 and *cry*1Ba1 genes therefore offer additional sources of resistance to pyramid with other effective *cry* genes. Alternatively, they could be deployed in mixtures of transgenic lines with different *cry* genes, either in space or over time, to prevent or delay the development of PTM resistance.

Plants that express two dissimilar *Bt* toxins (gene pyramiding or gene stacking) have the potential to delay the appearance of insect resistance more effectively than single-toxin transgenic plants (Gould *et al.*, 1994; Gould, 2003; Zhao *et al.*, 2003 & 2005). Transgenic potato lines expressing several different single *cry* genes have been developed with resistance to potato tuber moth. Since potatoes are a clonal crop with tetrasomic inheritance, high heterozygosity and severe inbreeding depression, the genetic integrity of cultivars is instantly lost upon self or cross pollination (Conner & Christey, 1994). Consequently, sexual crossing to pyramid transgenes is inappropriate in potatoes. Re-transformation strategies are therefore

required, in which case it is important to first investigate how the expression of two dissimilar *Bt* toxins interact to confer insect resistance in transgenic plants.

An effective experimental approach to simulate transgene pyramiding in clonal crops was investigated in Chapter 4. The experimental design involved alternating the daily feeding of PTM larvae between all six possible pair-wise combinations of three isogenic lines. These included two transgenic lines expressing different *cry* genes and the non-transgenic control (Figure 4.1). In this manner the pyramiding of pairwise combinations of *cry*1Ac9, *cry*9Aa2, and *cry*1Ba1 genes in potato was simulated and allowed an evaluation of how pairs of these three *cry* genes interact to influence the growth rate of PTM larvae. The results show that all combinations of the three *cry* genes were largely consistent with additive impacts on PTM larvae, although results from the combination of the *cry*1Ac9 and *cry*9Aa2 genes were suggestive of slight synergistic effects (Figure 4.2). Pyramiding the *cry*1Ac9, *cry*9Aa2 and *cry*1Ba1 genes in potato should therefore provide a more durable resistance strategy to control PTM.

The feasibility of two strategies for transgene pyramiding using *Agrobacterium*-mediated transformation was investigated in Chapter 5 to develop transgenic potato with resistance to PTM. In the first approach, *cry*1Ac9 and *cry*9Aa2 genes were introduced simultaneously using a kanamycin (*npt*II) selectable marker gene. The second approach involved the sequential introduction (re-transformation) of a *cry*1Ac9 gene, using a hygromycin *(hpt)* selectable marker gene, into an existing line transgenic for a *cry*9Aa2 gene and a *npt*II marker gene. Multiplex-PCR confirmed the presence of the specific selectable marker gene and both *cry* genes in all regenerated lines (Figure 5.3). RT-PCR analysis revealed that expression of both *cry* genes in all these transgenic lines (Figure 5.5). Re-transformation proved to be a flexible approach to effectively pyramid genes for PTM resistance in potato, since it allowed the second gene to be added to a line previously identified as having a high level of resistance. Larval growth of PTM was significantly inhibited on excised greenhouse-grown leaves in all transgenic lines (Figure 5.4), although no lines expressing both *cry* genes exhibited any greater resistance to PTM larvae over

that observed for the individual genes. It is anticipated that these lines will have a more durable resistance by delaying the opportunities for PTM adaptation to the individual *cry* genes.

6.2 Contribution of this thesis to developing transgenic potatoes

Large populations of transgenic lines were developed and discussed in this thesis (Chapter 2 and Chapter 3). Considerable variation in the level of PTM resistance among the independently derived transgenic potato lines was observed within the same experiment (Figures 2.3, 2.4 and 3.3). Such variation is usually attributed to unpredictable levels of transgene expression as a consequence of position effects resulting from differences in the integration site of the transgenes within the plant genome (Peach & Velten, 1991; Conner & Christey, 1994) and/or differences in T-DNA copy number (Hobbs *et al.*, 1993). Therefore, this thesis reinforces the need to recover for large populations of independently derived transgenic events to recover one that exhibits acceptable expression of the transgenic trait.

This research also illustrated the use of the endogenous potato actin gene as a novel internal control for molecular screening techniques such as multiplex PCR and multiplex RT-PCR. In PCR this provides a very useful check to allow failed reactions to be conveniently distinguished from a non-transgenic line (Figures 2.2, 3.2 & 5.2). For RT-PCR it also provided an approximate baseline for standardising gene expression between transgenic lines and a convenient check for DNA contamination of the RNA samples (Figures 2.5 & 5.4). For the latter example it is necessary for the PCR primers to span an intron so that a smaller product is expected from RNA samples than DNA samples. Therefore, RT-PCR products of RNA samples only contain an intron when the RNA samples are contaminated with DNA.

Although being available for some time, the use of light-inducible promoters to drive genes of interest is not necessarily common place. But, this research has investigated the value of the potato *Lhca3* promoter, known to be light-inducible, as a potential foliage-specific promoter for the transcriptional control of *cry* gene

expression in potatoes. This research also established that the expression of either the *cry*1Ac9 gene or the *cry*9Aa2 gene in transgenic potato plants offers protection against PTM larval damage in foliage when expressed under the transcriptional control of a *Lhca3* light-inducible promoter. The *Lhca*3 gene encoding the apoprotein 2 (type III chlorophyll *a/b* binding protein) of the light harvesting complex of Photosystem I and expresses mostly in the green parts, especially in foliage (Nap *et al.*, 1993). Consequently, *cry* genes under the transcriptional control of the *Lhca*3 promoter are not expected to be expressed in the edible tubers. The recovery of some transgenic lines with a high level of resistance to PTM in foliage and no or minimal *cry* genes expression in the tubers was demonstrated in Chapter 2. Therefore, this research has made an effort to minimise some of the concerns held by the general public about acceptance of transgenic crops.

To date, several *cry* genes have been demonstrated to be highly effective at controlling PTM, with up to 100% mortality, when expressed in potato plants (Table 1.3). Furthermore, biotin-binding proteins such as avidin and streptavidin also gave 100% PTM larval mortality when expressed in tobacco (Markwick *et al.*, 2003) and potato (Meiyalaghan *et al.*, 2005b) plants. However, transgenic potato lines accumulating avidin or streptavidin also showed a range of off-type characteristics due to some phytotoxic effects from transgene expression (Meiyalaghan *et al.*, 2005b). This research has identified modified *cry*1Ba1 and *cry*1Ca5 genes to be two of the most effective transgenes to confer resistance to PTM when expressed in potato.

To pyramid transgenes in clonal crops requires either simultaneous transformation or re-transformation approaches. In this case, it is important to first investigate how the expression of two dissimilar *cry* genes might interact when expressed in transgenic plants, since *Bt* toxins are known to interact with antagonistic (Del Rincón-Castro *et al.*, 1999; Lee *et al.*, 1996), additive (Poncet *et al.*, 1995) and synergistic effects (Tabashnik, 1992; Poncet *et al.*, 1995; Lee *et al.*, 1996; Liu *et al.* 1998). This research has illustrated an experimental approach to simulate *cry* gene pyramiding in clonal crops. The use of plant material in this approach provides a

more realistic food source than an artificial diet, since it better reflects transgenic crops. This represents the first attempt to simulate transgene pyramiding in this manner.

Douches *et al.* (1998) attempted gene pyramiding for resistance to PTM by transformation of potatoes with some natural resistance. In this case, a codon-modified *cry*5 gene was transferred to two genotypes each possessing different natural host plant resistance factors, either leaf leptines or glandular trichomes. In contrast, the present research has demonstrated that the feasibility of two strategies for *Agrobacterium*-mediated transgene pyramiding in potato to confer resistance to PTM. However, transgene pyramided-plants by sequential- or simultaneous-transformation did not enhance any phenotypic resistance to PTM even though they expressed two *cry* genes. Matzke *et al.* (1989) reported that in a double transformation of tobacco, genes of the first constructs were suppressed by the second transformation. Similarly in potato, products of the *npt*II and GUS gene in the first construct were reduced by varying degrees in double transformation (Park *et al.*, 1995). Matzke *et al.* (1995) described that *cis*-inactivation and *trans*-inactivation involve an interaction between a homologous "target" gene in the linked and unlinked transgene complexes respectively that causes methylation. More studies on these phenomenon are required to allow the design of effective constructs for gene pyramiding.

6.3 Directions for further research

6.3.1 Development of potatoes transgenic for *cry*1Ba1 and *cry*1Ca5 genes under a light-inducible *Lhca*3 promoter

Modified *cry*1Ba1 and *cry*1Ca5 genes under 35S promoter in transgenic potatoes gave up to 100% PTM mortality (Chapter 3). The transfer of these *cry* genes under *Lhca*3 promoter to develop transgenic potatoes would be more valuable. It will also facilitate to further investigation of the use of a *Lhca*3 promoter.

6.3.2 Simulating further transgene pyramiding

Transgenic potatoes with protease inhibitors (Christeller *et al.*, 1995) and biotin-binding proteins (Meiyalaghan *et al.*, 2005b) have been developed and evaluated for PTM control. Protease inhibitors irreversibly bind to protease enzymes in the insect gut and interfere with protein digestion, thereby preventing normal growth and development (Ryan, 1990). Biotin-binding proteins, such as avidin and streptavidin, cause a lethal vitamin deficiency in insects when ingested at low doses (Morgan *et al.*, 1993; Markwick *et al.*, 2001). Whereas, the insecticidal proteins from *cry* genes bind to insect-specific receptors on the brush border epithelial surface of mid-gut cells and insert into the cell membranes, forming leakage pores that result in cell death by lysis (Ellar, 1994). Further, several *cry* genes have been demonstrated to be highly effective at controlling PTM when expressed in potato plants (Table 1.3, Chapter 2 & Chapter 3). Simulating transgene pyramiding using these potato plants with genes conferring different modes of action on PTM larvae will be useful to identify valuable combinations of transgenes for pyramiding into potato.

6.3.3 Develop transgenes-pyramided potato plants

Theoretical models (Gould 2003) and experimental data (Zhao *et al.* 2003) indicate that plants containing two dissimilar *Bt* genes have the potential to significantly delay the evolution of insect resistance compared with single-gene *Bt* crops. Therefore, developing pyramided potato plants with additional *cry* genes and other genes such as protease inhibitors and biotin-binding proteins is important. Strategies described in Chapter 5 can be used to develop transgenes-pyramided potato plants.

6.3.4 Evaluation of transgenic potatoes in field conditions

The transgenic lines developed and described in this thesis were not evaluated for PTM resistance under field conditions. The future field testing of these transgenic potato lines is essential for the following reasons:

- To confirm the *cry* genes perform under field conditions. This has been evaluated for potatoes transgenic for *cry*1Ac9 gene under *35S* promoter (Davidson *et al.* 2002b, 2004b, 2005b), but not tested for the other *cry* genes used in this study (*cry*9Aa2, *cry*1Ba1 and *cry*1Ca5 genes) or transgenes under control of the *Lhca*3 promoter. This is important since transgene expression can vary with environmental conditions (Broer, 1996).

- To identify elite lines with good transgene expression in the field as well as retaining all the characteristics and performance of the parental cultivar. Off-types due to somaclonal variation can sometimes only be exhibited under field conditions i.e. plants in greenhouse may look good performance, but when they are in the field they can appear very different and malformed with low yield (Conner *et al.* 1994, Davidson *et al.* 2002b).

- Sufficient quantities of tubers produced from numerous sites and field trials are necessary for complete evaluation of processing characteristics, nutritional content, and assessment of glycoalkaloid level. These data will be essential for future application to relevant authorities that may involve the release of transgenic potato tubers into the food chain.

- It is important that this elite genetic material is incorporated into long term traditional breeding programmes as soon as practically possible.

- The use of these lines in conjunction with other forms of integrated pest management need to be investigated in order to assess the best ways in which to maximise the control of pest and disease resistance in potato crops.

- It is essential to understand how PTM interacts with these plants in large scale field trials, so that management strategies can be designed to minimise the opportunity for PTM to "overcome" the resistance mechanism in these transgenic plants.

Therefore, it is important that ongoing research evaluates the transgenic lines developed in this thesis under field conditions.

6.3.5 Investigations on PTM evolving resistance to transgenic potatoes

The potential for the evolution of resistance to transgenic plants by the target pest raises concerns about the effectiveness of *Bt* transgenic crops (Tabashnik, 1994; Roush, 1997b; Gould, 1998; Ferré & van Rie, 2002). The method of rearing successive generations of PTM on tubers, described by Davidson *et al.* (2005b) could be used to develop a resistant population. It may then be possible to determine various characteristics of resistance such as inheritance, stability, mechanism(s) and cross-resistance to other *Bt cry* toxins. Information on the characteristics of resistance development in PTM is important for determining optimal management strategies.

Resistance management strategies proposed to prevent the development of PTM resistance to the transgenic potatoes are outlined in Table 1.4. Of these approaches, the use of the high dose or moderate dose strategy could be applied to the transgenic plants developed in this study. The high dose strategy could be used in conjunction with spatial and/or temporal refuges, while the moderate dose could be used in combination with augmenting natural mortality factors, in particular natural enemies (predators and parasitoids). Both must be used in conjunction with cultural practices, such as increased seed depth (cultivar dependent), mounding and maintaining the soil cover over tubers, grading tubers immediately after harvest to remove infested ones, and using screens on storage or cool storage facilities for harvested tubers. These cultural practises minimise the pest's exposure to the Cry protein. Such theoretical considerations need to be verified using the material developed in this thesis, including the use of large-scale field trials.

Another possible strategy worth investigating involves the use of mixtures or rotations of transgenic lines expressing the different Cry proteins. This approach should only be investigated once no cross-resistance between the four Cry proteins is verified.

6.3.6 Investigations on the ecological impacts on non-target organisms

Transgenic potato lines developed in this study may impact on other non-target organisms. For example, GM crops engineered to produce proteins with toxicity to specific pests could have other downstream effects on higher trophic levels. If aphids feed on potatoes engineered for resistance to tuber moth, it is important to confirm that beneficial ladybirds feeding on the aphids are not harmed. Therefore, further studies will need to evaluate the impact of transgenic potato plants on secondary non-target pests.

Future field trials will also provide an excellent opportunity to investigate the fate of *Bt cry* toxin exudates in the soil and their potential effects on the soil micro flora, and micro- and macro-fauna. Large-scale field trials will also provide the chance to investigate non-target insect community composition and their abundance in PTM-resistant transgenic potato crops compared with crops using conventional methods for managing PTM populations. Such studies are essential for full risk assessment of transgenic crops prior to applying to regulatory bodies for release into agricultural environments.

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