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ORIGINAL ARTICLE

Pyramiding transgenes for potato tuber moth resistance in potato

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Abstract The feasibility of two strategies for transgene pyramiding using *Agrobacterium*-mediated transformation was investigated to develop a transgenic potato (*Solanum tuberosum* L. cv. Iwa) with resistance to potato tuber moth (PTM) (*Phthorimaea operculella* (Zeller)). 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 resistance (*hpt*) selectable marker gene, into an existing line transgenic for a *cry*9Aa2 gene and a kanamycin resistance (*npt*II) selectable marker gene. Multiplex polymerase chain reaction (PCR) confirmed the presence of the specific selectable marker gene and both *cry* genes in all regenerated lines. The relative steady-state level of the *cry* gene transcripts in leaves was quantified in all regenerated lines by real-time PCR analysis. 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 that was 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, although no lines expressing both *cry* genes exhibited any greater resistance to PTM larvae over that previously observed for the individual genes. It is anticipated that these lines will permit more durable resistance by delaying the opportunities for PTM adaptation to the individual *cry* genes.

Keywords *Agrobacterium*-mediated transformation · *Bacillus thuringiensis* · Gene pyramiding · *cry*1Ac9 · *cry*9Aa2 · *Phthorimaea operculella*

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Introduction

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 and Palacios 1982). Larvae mine leaves and stems, causing transparent tunnels in the leaves, the death of growing points and the weakening of stems. They also mine the tubers in the field as well as in storage, reducing tuber quality (Goldson and Emberson 1985; Trivedi and 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, b), chemical control (Foot 1974a) and cultural practices (Foot 1974b, 1975) 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). The development of potato cultivars that are resistant to PTM could increase the efficacy of cultural and biological methods and reduce the use of insecticides (Arnone et al. 1998), which are environmentally unsafe, 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), although improved tolerance to PTM

has recently been achieved in interspecific hybrids between *Solanum berthaultii* and cultivated potato *Solanum tuberosum* (Malakar-Kuenen and Tingey 2006). However, with the advent of genetic engineering, genes for insect resistance can be transferred into plants to develop insect-resistant plants. Transgenic potatoes that express *cry* genes derived from various strains of the soil bacterium *Bacillus thuringiensis* (*Bt*) have exhibited resistance to PTM (Jansens et al. 1995; Cañedo et al. 1999; Li et al. 1999; Chakrabarti et al. 2000, Douches et al. 2002; Davidson et al. 2002, 2004; Meiyalaghan et al. 2005, 2006a, b; Hagh et al. 2009; Jacobs et al. 2009; Kumar et al. 2010).

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 1997; Gould 1998; Ferré and Van Rie 2002). So far, no insects resistant to *Bt*-transgenic crops have been reported from the field. However, diamondback moth (*Plutella xylostella*) 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 insect-resistant transgenic crops, it is important to implement resistance management strategies simultaneously with their release.

Various strategies can be considered for managing resistance to *Bt*-transgenic crops (Roush 1997; Gould 1998; Brousseau et al. 1999; Shelton et al. 2000). One proposed approach for delaying the development of insect resistance in *Bt*-transgenic 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 in several alternative approaches (Berger 2000; Halpin 2005). The simplest method involves sexual crossing to combine improvements in different traits such as disease resistance, 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 and 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; Barrell and Conner 2006).

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

Materials and methods

Vector construction

All chimeric genes were constructed using standard protocols for DNA manipulations (Sambrook and Russell 2001) and by following manufacturers' recommendations where appropriate. The promoter used for transcriptional control of the *cry* genes was derived from the *S. tuberosum Lhca3* gene (Genbank accession EU234502), which is known to control predominantly light-induced, foliage-specific expression in transgenic potato (Meiyalaghan et al. 2006a). The *Lhca*3-*cry*9Aa2-*ocs* chimeric gene was excised from the primary cloning vector pART7cab9Aa2 (Meiyalaghan et al. 2006a) as a 3.3 kb *Not*I fragment and cloned into pMOA33 (Barrell and Conner 2006; Genbank accession DQ869004) to produce the binary vector pMOA33-1. The *Lhca*3-*cry*1Ac9-*ocs* chimeric gene was excised from the primary cloning vector pART7cab1Ac9 (Meiyalaghan et al. 2006a) as a 3.2 *Not*I fragment. This fragment was blunt-ended using the End-It^{TM} DNA End-Repair Kit (EPICENTRE, Madison, WI, USA) according to the manufacturer's instructions. The binary vectors pMOA33-1 and pMOA34 (Barrell and Conner 2006; Genbank accession DQ869005) 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 the Fast-Link^{TM} DNA Ligation Kit (EPICENTRE), according to the 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 and Willmitzer 1988). Prior to co-cultivation with potato tissue, the *Agrobacterium* cultures harboring the binary vectors were cultured overnight on a shaking table at 28°C in LB broth supplemented with 300 mg/l spectinomycin.

Plant material

Virus-free plants of cultivar Iwa and the existing Iwa transgenic lines I75 and DG4c were multiplied in vitro, as previously described (Meiyalaghan et al. 2006a). The I75 line was transgenic for *35S-cry*1Ac9-*ocs* and *nos-npt*II-*nos* chimeric genes (Davidson et al. 2002), whereas the DG4c line was transgenic for *35S-cry*9Aa2-*ocs* and *nos-npt*II-*nos* chimeric genes (Meiyalaghan et al. 2005). Both I75 and

DG4c produced phenotypically normal plants with high resistance to PTM (Davidson et al. 2002; Meiyalaghan et al. 2005).

Potato transformation protocol

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 as previously described (Meiyalaghan et al. 2006a). To accomplish sequential re-transformation, leaf segments from the DG4c line were transformed with pMOA34-1. This was also performed as previously described (Meiyalaghan et al. 2006a), except that 25 mg/l hygromycin was used as the selective agent for the recovery of transformed cell colonies and shoots and 12.5 mg/l hygromycin was used to screen the recovered plants for root formation under selective pressure.

Screening of putative transformed lines using polymerase chain reaction (PCR)

Genomic DNA was isolated from in vitro shoots of putative transgenic and control plants based on the method described by Bernatzky and Tanksley (1986). DNA was amplified in a PCR containing primers specific for the transgene of interest multiplexed with primers for the endogenous potato actin gene as an internal control (Table 1). PCRs were carried out in a Mastercycler (Eppendorf, Hamburg, Germany). The reactions included 2.5 µl 10× 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 (Advanced Biotechnologies, Epsom, UK), 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.

Insect bioassay using excised leaves from greenhouse-grown plants

All of the putatively transformed lines were transferred to the containment greenhouse as previously described (Conner et al. 1994). Two plants were established in each of three PB5 bags (15 cm × 15 cm × 15 cm black polythene bags) per line, with each PB5 bag treated as a replicate, and the bags were placed in the greenhouse in a randomized 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 previously described (Meiyalaghan et al. 2006a). A growth index (GI) for each larva was calculated as GI = $\log_e$(final weight/mean initial weight). Mean GIs were analysed with analysis of variance, taking into account the between- and within-container variation. The analyses were carried out using GenStat (GenStat Committee 2003), and a probability level of 5% was used to determine significance.

Qualitative reverse transcription (RT)-PCR analyses

Total RNA was isolated from leaves of greenhouse-grown plants using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The

Table 1 PCR primers used for each gene and the expected PCR product size

Target gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Product size (bp)
Primers for qualitative PCR			
*cry*1Ac9	GCCACAGAATAACAACGTGC	GCATACCGTACACGAACTCG	359
*cry*9Aa2	GCATCTAATCGCCGTTCA	CGAATTTGGTCCGGACTT	424
*npt*II	ATGACTGGGCACAACAGACAATCGGCTGCT	CGGGTAGCCAACGCTATGTCCTGATAGCGG	612
hpt	AGCGTCTCCGACCTGATG	TGCCGTCAACCAAGCTCT	784
Actin	GATGGCAGAAGGCGAAGATA	GAGCTGGTCTTTGAAGTCTCG	1069
Primers for quantitative real-time PCR, designed using Primer Express® 2.0 software (Applied Biosystems)			
*cry*1Ac9	GGACAGAATTTGCTTATGGAACCT	CGGTATTTCATCCAGCGAATC	89
*cry*9Aa2	CGACTCAGAATTTGATAGAATTTTAACC	TGGCAAAAGAGGGCAACAA	101
ef1α	ATTGGAAACGGATATGCTCCA	TCCTTACCTGAACGCCTGTCA	101

Primer sequences for the potato actin gene were based on *StPoAc58* (GenBank accession X55749), and the primers for the *elfα* gene were derived from Nicot et al. (2005). All other primer sequences were based on the sequence of the binary vector

RNA samples were treated with DNaseI, Amplification Grade (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's directions. Following DNaseI treatment, the absence of contaminating DNA in the RNA samples was confirmed by PCR amplification as described above, using the actin gene specific primers which 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, *cry*9Aa2 and actin were the same as those used for PCR analysis (Table 1). 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 2× Reaction Mix (a buffer containing 0.4 mM of each dNTP and 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 94°C for 15 s, 60°C for 30 s, 68°C for 90 s (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.

Quantitative real-time PCR amplification from RNA transcripts

For quantitative real-time PCR, cDNA was synthesized from 1 µg of the DNAse-treated RNA using the SuperScript® VILO cDNA Synthesis kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed in triplicate using an Applied Biosystems StepOne™ Plus Real Time PCR instrument (Applied Biosystems, Foster City, CA, USA). PCR reactions contained 2× EXPRESS SYBR® GreenER™ with Premixed ROX (Invitrogen), 200 nM each of the forward and reverse primers (Table 1), 25 ng of template cDNA and water to a volume of 20 µl. The thermal cycling conditions were as follows: 10 min at 95°C to activate DNA polymerase, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Melt curves were generated using StepOne™ Software v2.0 (Applied Biosystems) to confirm that only a single product was analyzed. The threshold value was set automatically by the StepOne™ Software v2.0 in the exponential phase of amplification. The qRT-PCR data for each sample were normalized to the amount of *ef1α* transcript using the same amplification conditions. The relative abundance of each *cry* gene transcript in transgenic plants was determined using the $\Delta\Delta C_t$ method relative to the I75 and DG4c lines, which were used as positive controls that are known to have high resistance to PTM larvae from the expression of the *cry*1Ac9 and *cry*9Aa2 genes, respectively (Davidson et al. 2002; Meiyalaghan et al. 2005).

Results

The binary vectors pMOA33-2 and pMOA34-1 (Fig. 1) were used for *Agrobacterium*-mediated transformation of 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 calli. On the other hand, in hygromycin-supplemented medium, the majority of the cell colonies from DG4c explants were pale green and friable. Following transfer to regeneration medium, most of the hard green cell colonies produced shoots, whereas the friable calli generally failed. Two to 3 weeks after the cell colonies with regenerated shoots had been transferred to potato multiplication medium, 2–4 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 4 lines for the pMOA34-1 construct, labeled as the MV series and the M4c series, respectively).

PCR analysis of regenerated lines

The presence of the *npt*II and *cry* genes in the MV lines and the *hpt* and *cry* genes in the M4c lines was confirmed using

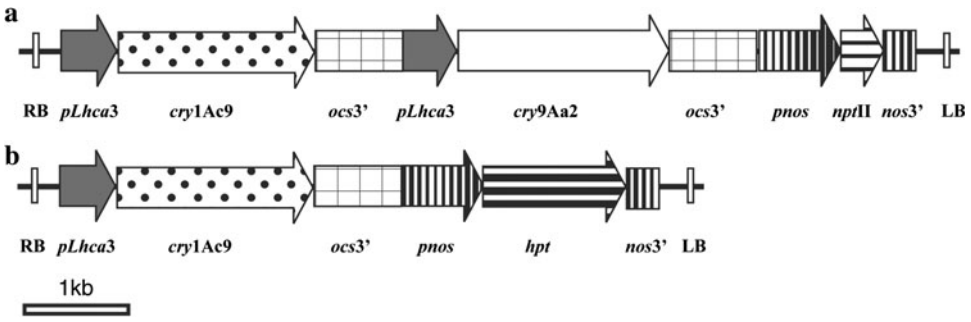

Fig. 1 Schematic representation of the T-DNA regions of the two binary vectors. **a** pMOA33-2 (with an *npt*II selectable marker gene) and **b** pMOA34-1 (with an *hpt* selectable marker gene). *RB* and *LB* denote the right and left T-DNA borders, respectively

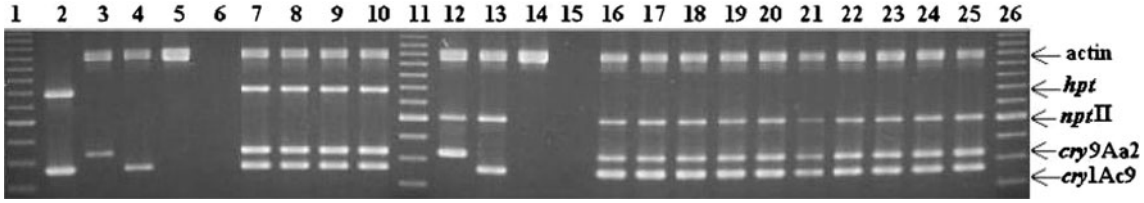

Fig. 2 PCR analysis of the putative gene-pyramiding transgenic potato (*Solanum tuberosum*) cv. "Iwa" lines. *Lanes 2–10* represent a multiplex reaction with the actin primers as an internal control producing a 1069 bp product, the *hpt* primers producing an expected 784 bp product, the *cry*9Aa2 primers producing an expected 424 bp product, and the *cry*1Ac9 primers producing an expected 359 bp product. *Lanes 12–25* represent a multiplex reaction with the actin primers as an internal control producing a 1069 bp product, 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 1*, *11* and *26*: 100 bp molecular ruler 10380-012 (Invitrogen, Carlsbad, CA, USA); *lane 2*: plasmid pMOA34-1 (positive control for *hpt* and *cry*1Ac9); *lanes 3* and *12*: "Iwa" line DG4c that is known to be transgenic for *npt*II and *cry*9Aa2 (positive control, Meiyalaghan et al. 2005); *lanes 4* and *13*: "Iwa" line I75 that is known to be transgenic for *npt*II and *cry*1Ac9 (positive control, Davidson et al. 2002); *lanes 5* and *14*: nontransgenic "Iwa" control; *lanes 6* and *15*: no DNA template control; *lanes 7–10*: lines transformed with pMOA34-1 (M4c#1, M4c#2, M4c#3 and M4c#4, respectively); and *lanes 16–25*: lines transformed with pMOA33-2 (MV1, MV2a, MV2b, MV2c, MV2d, MV2e, MV3a, MV3b, MV3c, MV4, respectively)

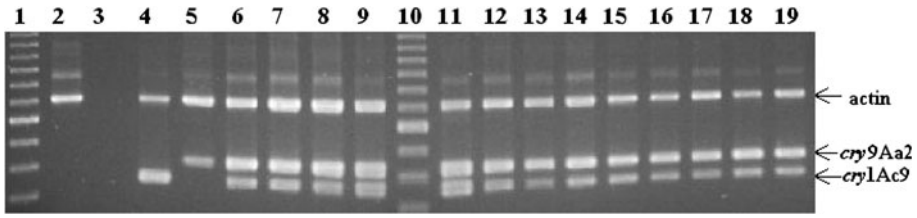

Fig. 3 Reverse transcription (RT)-PCR analysis of transgenic potato lines. *Lanes 2–9* and *11–19* represent a multiplex reaction with the *cry*1Ac9 primers producing an expected 359 bp product, the *cry*9Aa2 primers producing an expected 424 bp product and the actin primers used as an internal control producing an expected 709 bp product. *Lane 2*: nontransgenic "Iwa" control; *lane 3*: no RNA template control; *lane 4*: "Iwa" line I75 that is known to be transgenic for *cry*1Ac9 (positive control, Davidson et al. 2002); *lane 5*: "Iwa" line DG4c that is known to be transgenic for *cry*9Aa2 (positive control, Meiyalaghan et al. 2005); *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, CA, USA)

multiplex PCR with an endogenous actin gene used as an internal positive control. Since the actin product was expected in both transgenic and nontransgenic potato plants, this allowed failed PCR reactions to be conveniently distinguished from a nontransgenic line. All transgenic lines were PCR positive for the selectable marker gene and both *cry* genes (Fig. 2).

Qualitative RT-PCR analysis of 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 used as an internal control (Fig. 3). RT-PCR showed that amplification of the expected 359 and 424 bp fragments had occurred in all of 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 a nonexpressing transgenic line and also provides a baseline for standardising gene expression between transgenic lines. Since the primers flanked two introns in the actin gene (predicted to be 234 and 126 bp for cultivar "Iwa"), 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.

Greenhouse evaluation and insect bioassay

All of the lines were observed to have a phenotypically normal appearance when grown in the greenhouse, as judged by the growth vigor of the plants and the absence of off-type characteristics such as marginal leaf curl, leaf wrinkling, reduced vigor, abnormally small and/or deformed tubers, or a combination of these traits. There were significant differences between lines in larval GI for the populations of the MV and M4c lines, with the major difference being between the nontransgenic control and all of the transgenic lines (Fig. 4). In the MV lines, one line

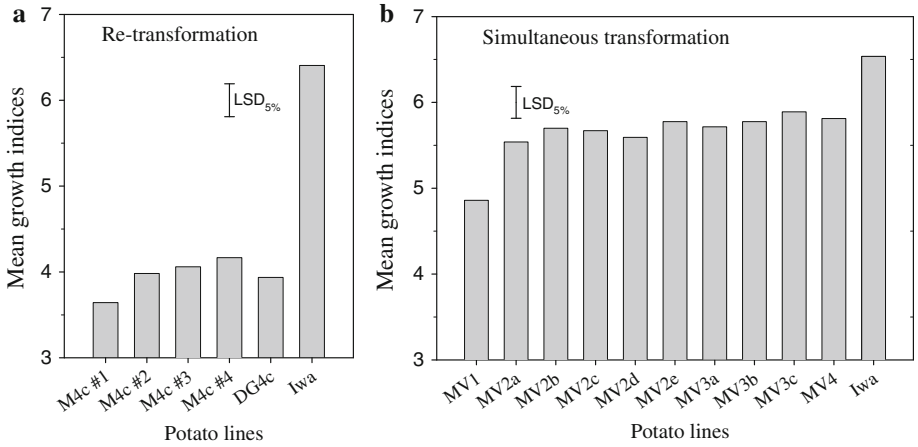

Fig. 4 The mean growth index of surviving PTM larvae reared on leaves of potato plants. **a** Experimental lines developed by re-transformation of line DG4c with the pMOA34-1 vector. **b** Experimental lines developed by simultaneous transformation of cultivar Iwa with the pMOA33-2 vector

(MV#1) had a significantly lower GI than all of 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 a lower larval GI than the DG4c line.

Quantitative real-time PCR amplification of RNA transcripts

The relative steady-state level of the *cry* gene transcripts in leaves of the transgenic lines, as estimated by real-time PCR, is presented in Fig. 5. The *cry*1Ac9 gene was not present in the Iwa control or the DG4c line, so the absence of *cry*1Ac9 transcripts in these lines is expected. Similarly, no *cry*9Aa2 transcripts were detected in the Iwa control and I75, which do not possess this gene. The abundance of *cry* transcripts varied between the various transgenic lines for both the MV series of lines derived from simultaneous transformation and the M4c series of lines derived from sequential transformation. For the *cry*1Ac9 transcripts, the MV series had substantially higher transcript levels than the M4c series. The M4c series exhibited higher transcript abundances of the *cry*9Aa2 gene than the MV series and—surprisingly—the DG4c line from which they were derived.

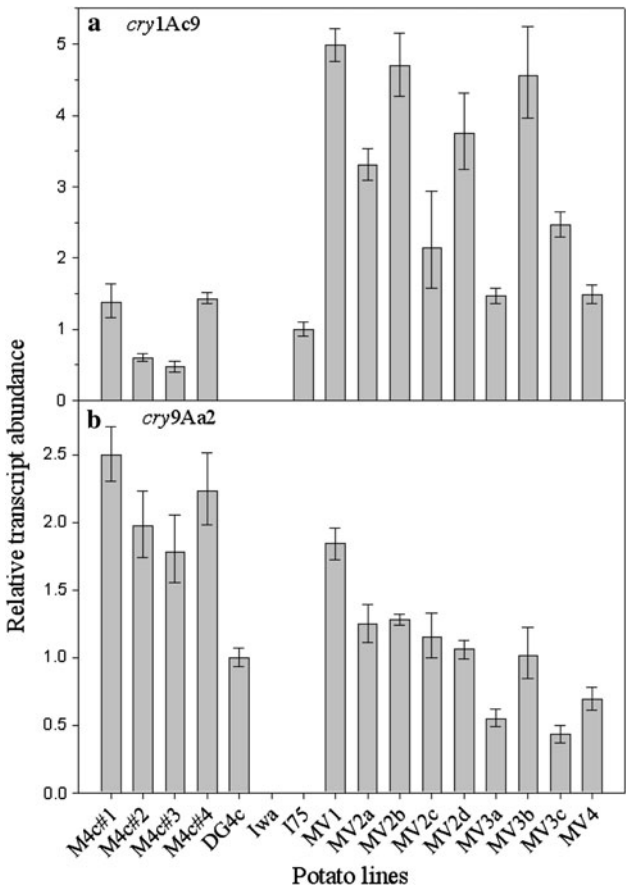

Fig. 5 Quantitative real-time PCR amplification of the *cry* gene transcripts in the experimental lines of cultivar Iwa. The mean relative transcript abundances are shown for: **a** the *cry*1Ac9 gene and **b** the *cry*9Aa2 gene. The I75 and DG4c lines were positive controls with high resistance to PTM larvae based on the expression of the *cry*1Ac9 and *cry*9Aa2 genes, respectively (Davidson et al. 2002; Meiyalaghan et al. 2005). The standard error of each mean is represented by the *vertical line*

Discussion

Agrobacterium-based plasmid vectors allow a wide range of plant species to be transformed by using the T-DNA to introduce foreign genes into the nuclear genome of the plant (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 phenomenon provides an important basis that allows the successful pyramiding of transgenes by sexual crosses or re-transformation.

The simplest approach used to pyramid two transgenes into a single plant line is the 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 and Christey 1994). Transgene pyramiding in potatoes must therefore be achieved by applying 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; Halpin 2005). 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; Barrell and Conner 2006). 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 the *nptII* or the *hpt* gene and both *cry*1Ac9 and *cry*9Aa2 genes in all regenerated lines (Fig. 2), thereby confirming their transgenic status. It also confirmed that both strategies for transgene pyramiding using *Agrobacterium*-mediated gene transfer were successful. RT-PCR analysis on leaf RNA from 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 (Fig. 3), which is equivalent to the genomic fragment minus the two introns. However, a faint product of about 835 bp was also observed in some lines (Fig. 3). 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.

All of the transgenic lines expressing both the *cry*1Ac9 and the *cry*9Aa2 genes exhibited a high level of resistance to PTM larvae, which was evident as a significantly lower mean GI (Fig. 4). However, this level of resistance was no greater than that previously observed from the use of only the *cry*1Ac9 gene (Davidson et al. 2002, 2004, Meiyalaghan et al. 2006a) or only the *cry*9Aa2 gene (Meiyalaghan et al. 2005, 2006a; Jacobs et al. 2009). 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 (Meiyalaghan et al. 2006c). 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 (Meiyalaghan et al. 2006c). It is possible that the development of further transgenic potatoes lines with both the *cry*1Ac9 and *cry*9Aa2 genes might allow the 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 and Christey 1994; Berger 2000; Conner 2007).

The transcript abundances of the *cry* genes in the M4c series and the MV series were determined relative to the I75 and DG4c lines, which were used as positive controls that are known to have high resistance to PTM larvae from the expression of the *cry*1Ac9 and *cry*9Aa2 genes, respectively (Davidson et al. 2002; Meiyalaghan et al. 2005). The majority of the transgenic lines in both the M4c series and the MV series exhibited higher transcript abundances for both *cry* transcripts relative to I75 and DG4c (Fig. 5). The quantitative real-time PCR amplification of the *cry* gene transcripts resulted in two surprising results. Firstly, the transcript abundance of the *cry*1Ac9 gene in the MV series was substantially higher than those for the M4c series or the I75 line. Secondly, when the DG4c line, containing a *cry*9Aa2 gene under the transcriptional control of the 35S promoter (Meiyalaghan et al. 2005), was re-transformed with a *cry*1Ac9 gene under the transcription control of the potato *Lhca3* promoter, the transcript abundance of the original *cry*9Aa2 gene increased in all lines of the M4c series. We cannot explain this result, but the increase in transcript was substantial and consistent for all independently derived transgenic lines. Despite these changes in the transcript levels of the *cry* genes, there was no impact on the phenotypic expression of resistance to PTM larvae in most lines (Fig. 4).

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 of the transgenic lines. This was observed for the MV series and the M4c series, developed 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. 2002, 2004; Meiyalaghan et al. 2006a) or the *cry*9Aa2 gene (Meiyalaghan et al. 2005, 2006a; Jacobs et al. 2009). 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 and 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, the variation in the expression of the two independent genes may modulate the influence on the overall phenotype (Fig. 4). This is evident from the transcript levels observed for each *cry* gene among the MV series (Fig. 5). Despite being controlled by the same promoter, the two *cry* genes delivered by simultaneous transformation exhibited differing transcript abundances among the independently recovered transformed lines. Notably, the one line with the highest transcript levels of both *cry* genes (MV1) had a significantly higher resistance to PTM larvae (Fig. 4).

This study has established that 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 that had previously been 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 those observed for the individual genes, it is anticipated that these lines will permit more durable resistance by delaying the opportunities for PTM adaptation to the individual *cry* genes.

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