Ann. appl. Biol. (2001), **138**:281-292
Printed in Great Britain

Minor modifications to the *cry*1Ac9 nucleotide sequence are sufficient to generate transgenic plants resistant to *Phthorimaea operculella*

By L L BEUNING*, D S MITRA, N P MARKWICK and A P GLEAVE

The Horticulture and Food Research Institute of New Zealand Ltd, Mt Albert Research Centre, Private Bag 92-169, Auckland, New Zealand

(*Accepted 15 February 2001; Received 7 September 2000*)

Summary

Minor modifications were made sequentially to the nucleotide sequence of truncated *cry*1Ac9 to produce *cry*1Ac9^{A} (one nucleotide change) and then *cry*1Ac9^{B} (seven nucleotide changes). The derivative genes under the control of the CaMV 35S promoter were transformed into *Nicotiana tabacum* in order to determine whether these modified genes conferred resistance on the resulting transgenic tobacco plants to larvae of the potato tuber moth (*Phthorimaea operculella*). Over two trials with PTM larvae on the transgenic plants expressing the *cry*1Ac9^{B} gene, lower larval growth, development and survival was evident for most of the lines compared to the control plants. In the second trial, for four of these lines (7, 25, 26 and 28) larval growth rates were very low (0.28, 0.3, 0.42 and 0.28, respectively) compared to the control growth rate (4.18) and leaf damage was minimal. Northern analysis and RT-PCR analysis showed that higher levels of *cry*1Ac9 mRNA were present in the transgenic tobacco lines containing *cry*1Ac9^{B} than in the tobacco lines containing *cry*1Ac9^{A}. These results suggest that certain minor modifications to the nucleotide sequence of *cry*1Ac9 are sufficient to improve the stability of its mRNA when expressed in tobacco and that this increase in steady state mRNA is sufficient to confer significant resistance to PTM larvae.

Key words: *Bacillus thuringiensis*, *cry*1Ac9, sequence modification, transgenic tobacco, potato tuber moth

Introduction

Bacillus thuringiensis (Bt) is a commonly occurring soil bacterium, characterised by the production of proteinaceous crystals during sporulation (reviewed by Andrews *et al.*, 1987). The crystals are composed of 1-5 proteins (Cry proteins) that have highly potent and specific insecticidal activity.

Bt formulations have been in use for many years as an effective alternative to synthetic insecticides for the control of various insect pests. However, more recently, the focus on the Cry proteins has been on the production of transgenic plants expressing *cry* genes (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987; Barton, Whiteley & Yang, 1987; reviewed by Peferoen, 1997; Schuler, Poppy, Kerry & Denholm, 1998; de Maagd, Bosch & Stiekema, 1999). Such an approach is regarded as an additional tool for the control of crop pests and can lead to a reduction in the use of broad-spectrum insecticidal sprays.

Native *cry* genes, however, are very poorly expressed in transgenic plants and, consequently, such plants show minimal resistance to insect pests (Delannay *et al.*, 1989). Nucleotide sequence modifications are needed to remove destabilising elements, mRNA processing signals and rare codons in order to sufficiently stabilise the *cry* mRNA transcripts and improve translation in plants. These modifications are necessary so that adequate levels of the Cry proteins are produced to give effective and prolonged protection from insect pests (Perlak *et al.*, 1991). This approach has enabled the commercial development of transgenic crops expressing *cry* genes (James, 2000).

Potato tuber moth (PTM, *Phthorimaea operculella* Zeller) is a major insect pest of members of the Solanaceae in warm-temperate and tropical regions. Preferred hosts are potato and tobacco (Varela & Bernays, 1988; Fenemore, 1988). In the potato, PTM larvae damage both the leaves and the tubers. In the case of the potato leaf, the larvae feed on the mesophyll, forming transparent blisters in the leaves. Sometimes the larvae burrow into the petiole and continue into the stem. With the potato tuber, larvae usually enter through the "eyes" from eggs laid nearby and make slender tunnels throughout the tuber. High levels of tuber infestation occur in the field during summer and stored tubers can suffer severe damage all year round (Foot, 1984; Varela & Bernays, 1988). As part of a programme to improve the potato's resistance to PTM infestation, this

*Corresponding Author E-mail: lbeuning@hortresearch.co.nz

research tests the susceptibility of PTM larvae to transgenic tobacco expressing *cry*1Ac9. This work contributes to a collaborative research effort to produce transgenic potatoes expressing one or more *cry* genes (Gleave, Hedges, Broadwell & Wigley, 1998).

Previously we isolated a gene encoding a 133kDa insecticidal crystal protein from *Bacillus thuringiensis* var. *kurstaki* isolate DSIR 732 (Gleave, Hedges, Broadwell & Wigley, 1992). Sequence comparisons between this gene and the sequences of other *cry* genes revealed that the gene was a member of the *cry*1Ac class of *cry* genes. This class consists of *cry* genes that are active against lepidopteran larvae (Whiteley & Schnepf, 1986). Cry proteins are classified according to their molecular weight and insecticidal spectrum (Höfte & Whiteley, 1989), but more recently have been reclassified based on amino acid homology (Crickmore *et al.*, 1998). The *cry*1Ac gene (now reclassified as *cry*1Ac9) was expressed in *Escherichia coli* and extracts were obtained and shown to be insecticidally active against larvae of the lepidopteran potato tuber moth (*Phthorimaea operculella*, Zeller) (Gleave *et al.*, 1992).

The insecticidal activity of the Cry1Ac protein is located within the N-terminal 611 amino acids. Fischhoff *et al.* (1987) and Vaeck *et al.* (1987) showed that a truncation of the *cry* gene encoding 5' amino acids improved insecticidal activity in comparison to the entire coding sequence when expressed transgenically. Perlak *et al.* (1991) then demonstrated that modifications to the coding sequence of *cry*1Ab and *cry*1Ac resulted in the enhanced expression of insecticidally active protein. However, they found that not all the nucleotide changes resulted in an increase in expression of the corresponding Cry protein and that changes in one specific region (designated oligonucleotide B) were responsible for a significant increase in expression. We report here on the modification of a truncated version of the *cry*1Ac9 nucleotide sequence that incorporates the changes of oligonucleotide B, the introduction of the modified gene into tobacco and the subsequent analysis of the expression and insecticidal activity of the modified *cry*1Ac9 transgene.

Materials and Methods

Modification of cry*1Ac9*

The region of the *cry*1Ac9 gene encoding the N-terminal 615 amino acids was PCR-amplified from pPOM4 (Gleave *et al.*, 1992) using primers 13051/1 (5'-GCCGGGTACCAACCATGGCTAACAATC CGAACATCA-3') and 13051/2 (5'-GCCGAAGCTT TATTATTCAGCCTCGAGTGT-3') that anneal to the *cry*1Ac9 sequence (Genbank Accession No. U89872) at nucleotides 388-409 and 2218-2234, respectively (Fig. 1). This truncated version of the *cry*1Ac9 gene is herein referred to as *cry*1Ac9^{A}. Primer 13051/1 introduces a *Nco*I site around the translation initiation codon (ATG) and a translation initiation consensus sequence immediately 5' of this codon (Lütcke *et al.*, 1987). Utilising the *Kpn*I site (underlined) in primer 13051/1 and the *Hin*dIII site (underlined) in primer 13051/2, the 1.9-kb PCR product was cloned into pART7 (Gleave, 1992) placing *cry*1Ac9^{A} under the transcriptional control of the 35S promoter and generating pART7732. The 35S-*cry*1Ac9^{A}-ocs 3' cartridge was then cloned as a 3.9-kb *Not*I fragment into the binary vector pART27 (Gleave, 1992), generating pART27*cry*1Ac9^{A} (Fig. 2).

To further modify *cry*1Ac9 to produce *cry*1Ac9^{B}, PCR amplification was used to introduce nucleotide changes into the 5' end of *cry*1Ac9^{A}. Primer 13051/3 (5'-GCCGTCTAGAAATGGCTTGATTCCTAGC GAACTCTTCGATTCTCTGGTTGATGAGCTGTT CAATTTGTAC-3') was designed to anneal to the *cry*1Ac9^{A} sequence at nucleotides 684-619 and introduce seven nucleotide changes into the coding sequence at positions 634 (T-C), 636 (A-C), 639 (T-C), 646 (A-G), 651 (A-C), 657 (A-G) and 668 (C-T). These changes remove six potential polyadenylation signals (AATTAA, AACCAA, AAGAAT, AATAGA, AAGAA and AACCA), while not altering the amino acid sequence of the Cry1Ac9 protein. PCR amplification was carried out on pART7732 using primers 13051/1 and 13051/3 (Fig. 1). Utilising the *Xba*I site (underlined) in primer 13051/3, the 0.3-kb PCR product was cloned as a *Kpn*I-*Xba*I fragment into pSP72 (Promega) and the nucleotide sequence was determined to ensure all the intended changes had been incorporated. The 1.6-kb *Xba*I-*Hin*dIII fragment from pART7732, encoding the 3' end of the *cry*1Ac9^{A} gene, was then cloned downstream of the 0.3-kb PCR product, generating pSP*cry*1Ac9^{B}. The 1.9-kb *cry*1Ac9^{B} gene was cloned as a *Kpn*I-*Hin*dIII fragment into pART7 placing *cry*1Ac9^{B} under the transcriptional control of the 35S promoter and the expression cartridge was then cloned as a 3.9-kb *Not*I fragment into the binary vector pART27 (Gleave, 1992), generating pART27*cry*1Ac9^{B} (Fig. 2).

Tobacco transformation

The two binary derivatives, pART27*cry*1Ac9^{A} and pART27*cry*1Ac9^{B}, were electroporated into *Agrobacterium tumefaciens* strain LBA4404. Tobacco (*Nicotiana tabacum* cv. Samsun) leaf discs were cocultivated with *Agrobacterium tumefaciens* containing either pART27*cry*1Ac9^{A} or pART27*cry*1Ac9^{B} and placed on regeneration medium (MS salts, plus vitamins and sucrose, 1.0 mg litre^{-1} BA and 0.1 mg litre^{-1} NAA, 100 mg ml^{-1}

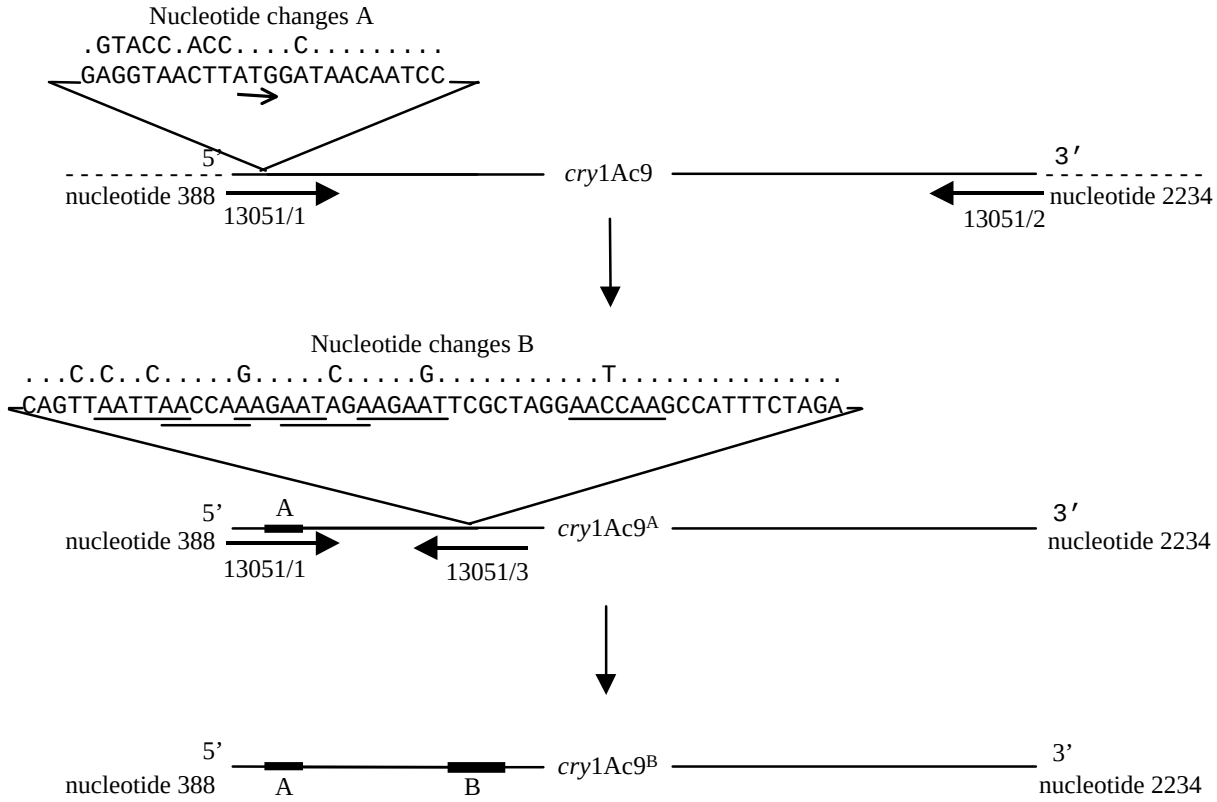

Fig. 1. PCR strategy for modifying the nucleotide sequence of *cry*1Ac9. PCR primers 13051/1 and 13051/3 were used to introduce Nucleotide changes A and Nucleotide changes B, respectively, into a truncated version of *cry*1Ac9, as described in Materials and Methods. Construct *cry*1Ac9^{A} was obtained by modifying *cry*1Ac9 and construct *cry*1Ac9^{B} was obtained by modifying *cry*1Ac9^{A}. ➜ = **ATG** initiation codon. Underlined sequences = potential polyadenylation (AATAAA-like) signals. → or ← = the direction of PCR amplification. Unchanged nucleotides are indicated by a "**.**".

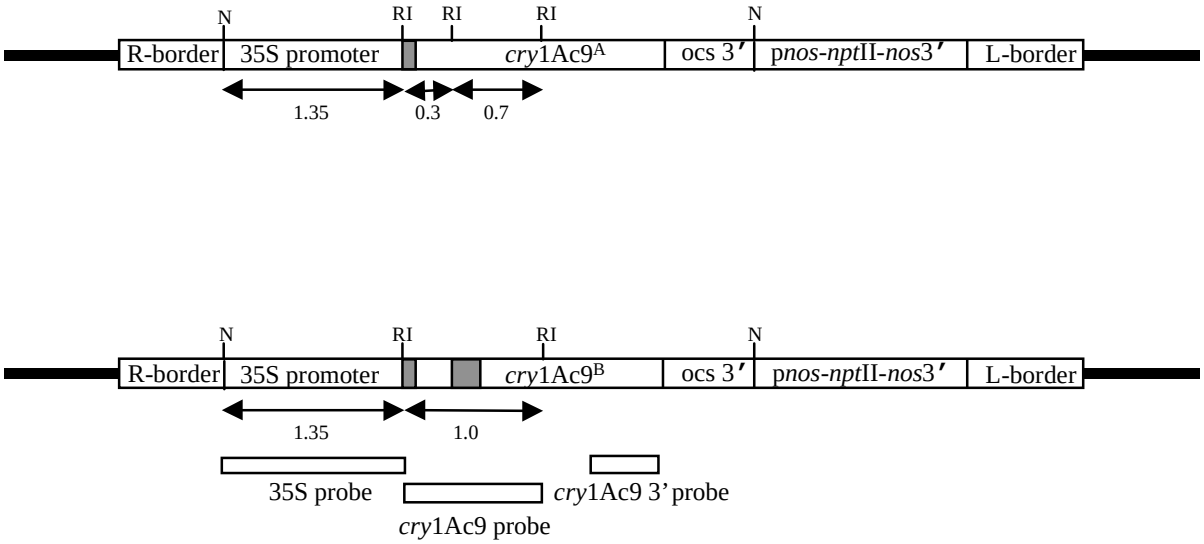

Fig. 2. The T-DNA organisational structure of the *cry*1Ac9^{A} and *cry*1Ac9^{B} constructs is shown. The blocks labelled 35S probe and *cry*1Ac9 probe indicate the size and location of the DNA sequences used for Southern hybridisation. The block labelled *cry*1Ac9 3' probe indicates the sequences used for northern hybridisation and for RT-PCR analysis. N, *Not*I; RI, *Eco*RI: 35S, cauliflower mosaic virus 35S promoter; ocs 3', octopine synthase transcriptional terminator; R-border, right border of T-DNA; L-border, left border of T-DNA; p*nos-npt*II-*nos*3', nopaline synthase promoter-neomycin phosphotransferase II-nopaline synthase transcriptional terminator.

Cefotaxime) for 2 days. The leaf discs were placed on regeneration medium containing 100 mg litre^{-1} kanamycin for 3-4 wk. Transformed shoots were then transferred to regeneration medium containing 100 mg litre^{-1} kanamycin. Twenty-four independent transgenic tobacco lines containing *cry*1Ac9^{A} and 27 independent transgenic tobacco lines containing *cry*1Ac9^{B} were obtained. Each independent line was multiplied by subculturing on to maintenance medium (MS salts, plus vitamins and sucrose, 100 mg ml^{-1} Cefotaxime) containing 100 mg litre^{-1} kanamycin to obtain sufficient plants for analysis. Resulting plantlets were potted in pumice mix and transferred to the glasshouse.

Insect bioassays

Excised leaf bioassays

Detached leaf pieces from transgenic tobacco lines containing *cry*1Ac9^{A} or *cry*1Ac9^{B} or from control transgenic tobacco lines containing pART27*gus* were infested with neonate PTM larvae in an insect feeding trial. Each leaf piece (approximately 3 cm square) was infested with five neonate larvae (hatched within the previous 24-h period) and then placed into a 75 ml specimen container. Leaf pieces were replaced every 2-3 days for a period of 14 days. For each plant line, two leaf pieces infested with PTM were set up (as above) on two occasions, so that in total, 20 larvae were tested against each line. The feeding trials were conducted under a 16:8 hour light:dark cycle at 25°C ± 2°C. Larval survival was recorded at 2, 7 and 14 days and pupation at 14 days. Larvae were not weighed in this preliminary bioassay.

Whole plant bioassays

The transgenic tobacco lines containing either *cry*1Ac9^{A} or *cry*1Ac9^{B} that ranged from the most to the least effective in the "Excised Leaf Bioassays" were chosen for whole plant insect feeding trials. Ten lines each of the transgenic plants and five control plants were infested with neonate PTM larvae as follows. Five larvae were weighed and placed on a small (2.5 cm diameter) glass microfibre filter disc that was then placed on a leaf of each plant. Larvae were observed until they had moved off the filter disc and were replaced if unable to make the transition to the leaf.

The infested plants were placed individually in large vented cylindrical clear acetate containers (20 cm diameter × 30 cm). After 9 days damaged leaves were removed and photographed. Live larvae were then removed from the leaves and weighed. The larvae lose weight as they begin to lay down fatty tissue in preparation for pupation. Therefore, only the period of exponential growth (9 days) was used for the calculation of growth rate (see below). The trial was replicated with one different clonal plant from each line being tested in each replicate.

Statistical analyses

Survival and pupation data involving percentages were first transformed using arcsin ($\sqrt{x}$) and then compared by analysis of variance, followed by two-sample tests (Minitab® for Windows Release 12.1, 1998). Growth rates were calculated for individual larvae, using the formula: Growth rate = $\log_e$ (final weight/initial weight). The initial weights used were the mean for five larvae. The weight of individual neonate larvae does not register on a 5-place balance. Mean growth rates for each plant line in the whole plant bioassay trials were then calculated and compared by one-way ANOVA, followed by two-sample tests (Minitab® for Windows Release 12.1, 1998) to detail differences between lines.

Southern analysis

Genomic DNA was extracted from tobacco leaves using a CTAB method (Doyle & Doyle, 1990). The genomic DNA (10 μg) was digested with *Eco*RI, electrophoresed through 0.7% agarose (BRL) and transferred to Hybond N^{+} (Amersham) nylon membrane in 0.4M NaOH. Prehybridisation at 65°C (5 h) in 10 ml 0.5 M Na phosphate, pH 7.2, 0.7 g SDS, 0.001 M EDTA, 200 μg denatured salmon sperm DNA was followed by hybridisation at 65°C (16 h) with two denatured radioactive DNA probes. One DNA probe consisted of an *Eco*RI fragment encoding the first 1000 bp of truncated *cry*1Ac9 (Fig. 2, *cry*1Ac9 probe) to verify the presence of the *cry* gene. The other probe consisted of a *Not*I/*Xho*I fragment encoding the 1.35 kb 35S promoter (Fig. 2, 35S probe) to determine the copy number of T-DNA inserts. Both probes were labelled with [α-^{32}P]dCTP using Megaprime (Amersham). Following hybridisation membranes were washed in 50 ml 0.3 M NaCl, 30 mM sodium citrate at room temperature for 10 min twice. They were then washed in 50 ml 0.15 M NaCl, 15 mM sodium citrate, 50 mg SDS at 65°C for 15 min and finally in 50 ml 15 mM NaCl, 1.5 mM sodium citrate, 50 mg SDS at 65°C for 15 min. The membranes were then exposed to X-ray film (Agfa).

Northern analysis

Total RNA was extracted from tobacco leaves with Trizol Reagent (Gibco BRL), following the manufacturer's procedures. RNA (20 μg) was electrophoresed through a 1% agarose-formaldehyde gel (GibcoBRL) and transferred to Hybond N^{+} (Amersham) nylon membrane in 0.05 M NaOH. Membranes were neutralised in 1.0 M NaCl, 0.5 M Tris-HCl, pH 7.0. Prehybridisation at 65°C (2 h) in 20 ml 0.1 M NaCl, 0.2 g SDS, 2 g dextran sulphate, 500 μg salmon sperm DNA was followed by hybridisation at 65°C (16 h) with a denatured

radioactive DNA probe. The DNA probe was a 270 bp PCR fragment homologous to the 3' end of truncated *cry*1Ac9 (Fig. 2, *cry*1Ac9 3' probe) that was amplified with the primers 1Aca (5' GATATCGAGTTCGTGTACGG 3') and 1Acb (5' TTCAGCCTCGAGTGTTGCAG 3') and labelled with [α-^{32}P]dCTP using the RTS Radprime DNA Labeling System (Gibco BRL). Hybridisation of a [α^{32}P]dCTP-labelled *Bam*HI/*Eco*RI restriction fragment encoding *Arabidopsis thaliana* actin (Nairn, Winesett & Ferl, 1988) was used to check RNA loadings and efficiency of transfer. Following hybridisation membranes were washed in 50 ml 0.3 M NaCl, 30 mM sodium citrate at room temperature for 5 min twice. They were then washed in 50 ml 0.3 M NaCl, 30 mM sodium citrate, 0.5 g SDS at 65°C for 30 min twice and finally in 50 ml 15 mM NaCl, 1.5 mM sodium citrate at room temperature for 30 min. Hybridisation signals were visualised by exposure to X-ray film (X-Omat, Kodak) or by scanning on a Storm 840 phospho-imaging system (Molecular Dynamics) and analysed using ImageQuant software.

RT-PCR analysis

Total RNA extracted from tobacco leaves was treated with DNase I (Gibco BRL), according to the manufacturer's directions. RT-PCR was performed on the DNase I-digested RNA (1 μg) using the Titan™ One Tube RT-PCR System (Boehringer Mannheim). The PCR primers used, 1Aca and 1Acb, were those used for northern analysis, amplifying a 270 bp DNA fragment homologous to the 3' end of truncated *cry*1Ac9. The resulting PCR products were electro-phoresed through an agarose gel (1 g 100 ml^{-1}), ethidium bromide-stained and photographed on an ultra-violet transilluminator (UVP). For Southern analysis, the PCR products were then transferred to Hybond N^{+} (Amersham) nylon membrane in 0.4 M NaOH. Prehybridisation, hybridisation with the [α-^{32}P]dCTP-labelled 270 bp PCR fragment and visualisation of hybridisation signals were as for northern analysis.

As a check on the amount of RNA used for RT-PCR amplification, for each sample an equal amount of RNA was electrophoresed through a 1 g 100 ml^{-1} agarose-formaldehyde gel, transferred to Hybond N^{+} (Amersham) nylon membrane in 0.4 M NaOH and hybridised with [α-^{32}P]dCTP-labelled crab apple 18S rRNA (Simon &Weeden, 1992). Prehybridisation, hybridisation and visualisation of hybridisation signals were as for northern analysis.

Results

Excised leaf bioassays

In preliminary insect-feeding trials on leaf pieces excised from the transgenic tobacco plants, survival of the PTM larvae after 14 days (Table 1) was almost identical on both the control plants and the tobacco lines containing *cry*1Ac9^{A}. Construct *cry*1Ac9^{A} had only nucleotide changes that improved initiation of translation (Lütcke *et al.*, 1987). However, by comparison larval survival appeared to be reduced (10%) on the tobacco lines containing *cry*1Ac9^{B} ($F = 2.96$, $P = 0.06$, df = 2, 26). The effect of the plant line on larval survival was significant among the tobacco plants containing *cry*1Ac9^{B} ($F = 2.29$, $P = 0.002$, df = 26, 81), but not among tobacco lines containing *cry*1Ac9^{A} ($F = 0.65$, $P = 0.866$, df = 21, 66) or among control lines ($F = 1.17$, $P = 0.323$, df = 15, 48). Larvae that survived on the plants containing *cry*1Ac9^{B} ranged from two tiny individuals from all replicates of line 25 to many large healthy larvae on all replicates of lines 4, 11 and 24. In contrast, survivors on the tobacco lines containing *cry*1Ac9^{A} and on the control plants were observed to be more uniform in size and development. Photographs of larvae taken from some of the tobacco lines containing *cry*1Ac9^{B} clearly show their small size compared to larvae found on the control plants (Fig. 4A), indicating the extent to which some of the lines containing this construct reduced larval growth.

With larval pupation, there was a major effect due to the presence of *cry*1Ac9^{B} in the transgenic tobacco ($F = 52.38$, $P < 0.001$, df = 2, 64). Only 1.2% of the survivors on the transgenic tobacco lines containing this construct had pupated by day 14 (Table 1) and these were all found on only one of the tobacco lines (line 24). This line was subsequently found not to contain the *cry*1Ac9^{B} gene (see later). In comparison, 17% of the larvae on the transgenic tobacco lines containing *cry*1Ac9^{A} and 29.3% of the larvae on the control tobacco plants had pupated, and many large larvae on both sets of plants were about to pupate. These percentages were also significantly different ($t = 2.54$, $P = 0.016$, df = 32).

Whole plant bioassays

For larvae on the control tobacco plants there were no significant differences in growth rates between

Table 1. *Survival and pupation (mean and standard error) of potato tuber moth larvae after feeding for 14 days on control tobacco plants or on transgenic tobacco leaves transformed with either* cry*1Ac9^{A} or* cry*1Ac9^{B}*

*cry*1Ac9 construct	No. plant lines	% larval survival	% larvae pupated
Controls	16	74.7 (± 2.5)^{a}	29.3 (± 4.4)^{x}
*cry*1Ac9^{A}	24	74.2 (± 2.1)^{a}	17.0 (± 2.7)^{y}
*cry*1Ac9^{B}	27	64.6 (± 3.8)^{b}	1.2 (± 1.2)^{z}

Data arcsin$\sqrt{x}$ transformed. ^{a,b} $P < 0.05$, ^{x,z} ^{y,z} $P < 0.001$ {Minitab, two sample *t*-test}

individual plants or between replicates and so these were combined to give a mean larval growth rate for all control plants. In the case of the *cry*1Ac9^{A} and *cry*1Ac9^{B} -transformed tobacco plants, larval growth rates within lines showed no significant differences between replicates (except *cry*1Ac9^{B} lines 7, 26 and 28 where there were no survivors in one replicate), and so replicates were combined (Fig. 3A and B).

Mean growth rates for larvae on the *cry*1Ac9^{A}-containing lines were not significantly different from the combined larval growth rate for the control plants ($F = 1.38$, $P = 0.195$, df = 10, 131). However, mean larval growth rates on the *cry*1Ac9^{B}-containing lines were significantly lower than those for both the control plants and the *cry*1Ac9^{A}-containing lines ($F = 14.96$, $P < 0.001$, df = 20, 197). Within this overall picture, though, larvae on individual tobacco lines containing *cry*1Ac9^{B} showed significant variation in growth rates ($F = 5.49$, $P < 0.001$, df = 8, 57) (Fig. 3A). Furthermore, tobacco lines inducing the lowest larval growth rates also showed the lowest

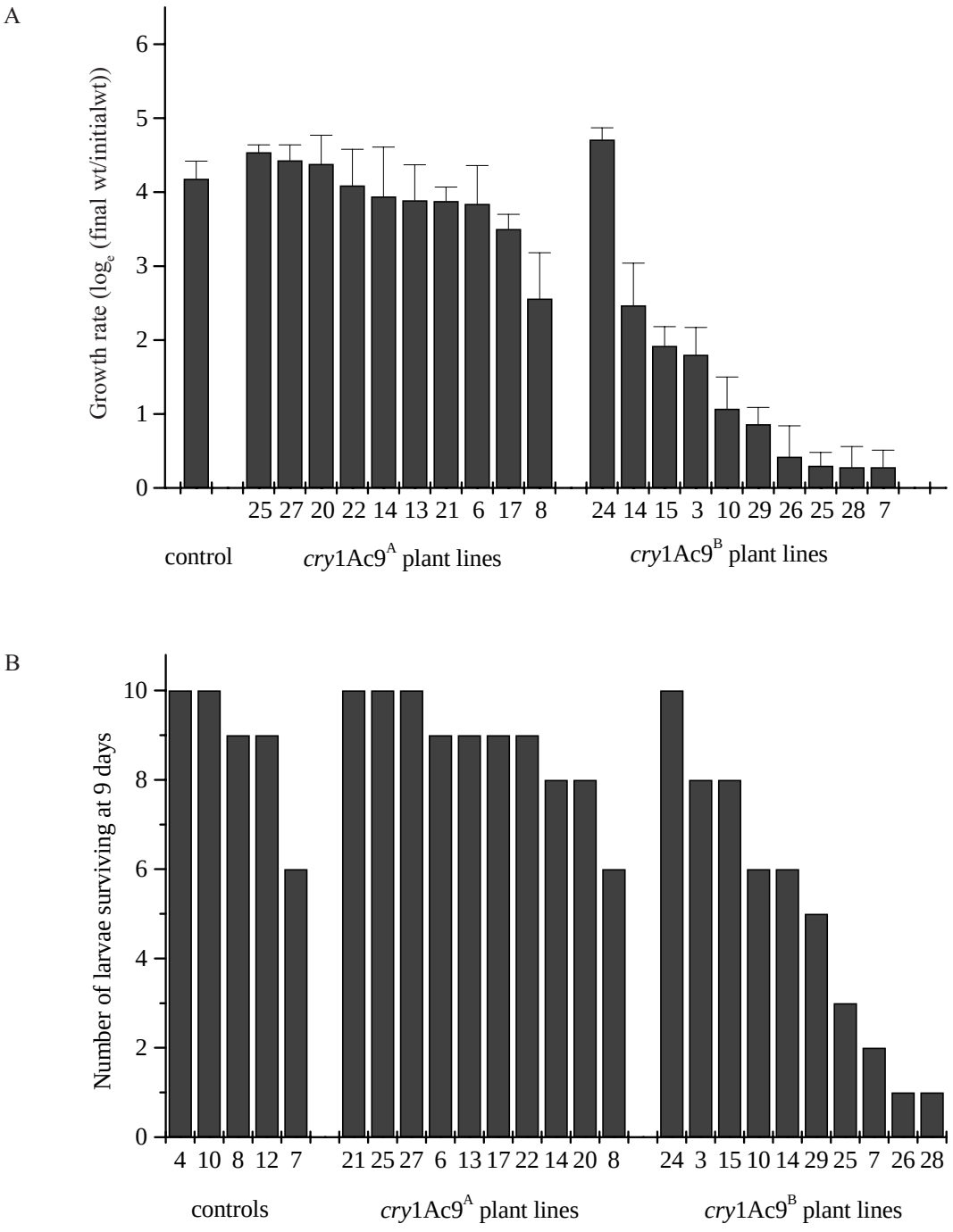

Fig. 3. Growth rate and survival of potato tuber moth larvae on transgenic tobacco plants containing *cry*1Ac9^{A} and *cry*1Ac9^{B}.

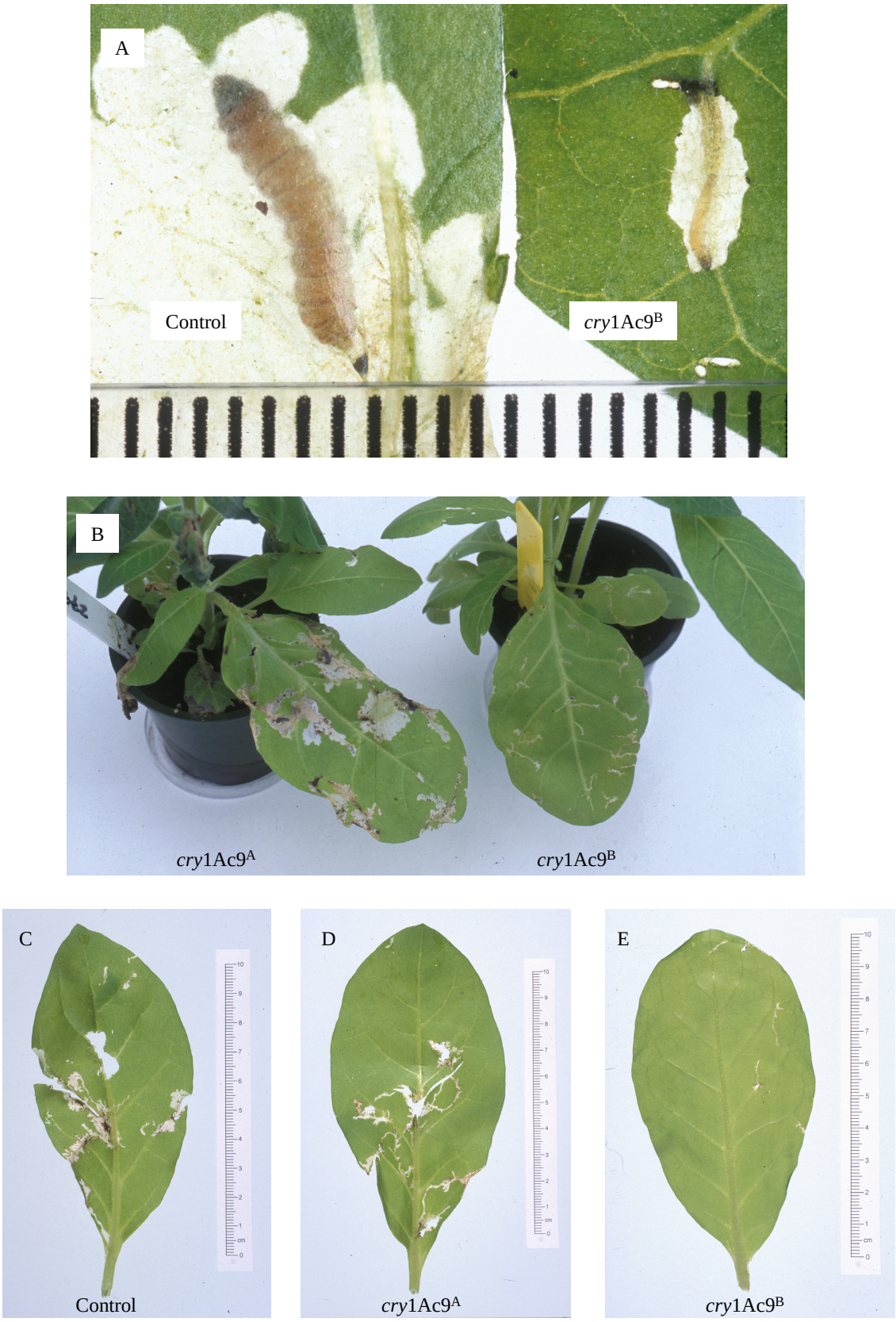

Fig. 4. PTM larval feeding trials on transgenic tobacco plants containing *cry*1Ac9^{A} and *cry*1Ac9^{B}. A. PTM larval size after feeding on control tobacco plants (left) and on transgenic tobacco plants containing *cry*1Ac9^{B} (right) for 9 days. Scale: 1 division = 1 mm. B. Extent of damage to whole transgenic tobacco plants containing *cry*1Ac9^{A} (left) and *cry*1Ac9^{B} (right) after infestation with PTM larvae. C.-E. Extent of damage to transgenic tobacco leaves from the whole plant bioassays after infestation with 5 PTM larvae. C. Control (left), D. *cry*1Ac9^{A} (centre) and E. *cry*1Ac9^{B} (right).

larval survival (Fig. 3B). Four of the *cry*1Ac9^{B}-containing lines (7, 25, 26 and 28) showed only 10-30% larval survival over the 14-day trial, while five lines (3, 10, 14, 15 and 29) showed 50-80% larval survival. One line (24) showed no mortality or growth rate reduction. However, subsequent Southern analysis indicated that this line did not contain the *cry*1Ac9^{B} gene.

After 9 days of infestation with PTM larvae the amount of leaf damage varied considerably between plants (Fig. 4B). Transgenic tobacco lines containing *cry*1Ac9^{B} had minimal leaf damage consisting of small thread-like mines where larvae had started and abandoned feeding sites (Fig. 4E). In contrast, both control tobacco plants and transgenic tobacco lines containing *cry*1Ac9^{A} exhibited extensive leaf and stem damage caused by larvae that generally stayed within and expanded their first mine to form a large window or blister in the leaf (Fig. 4C and D).

The extent of damage to the tobacco lines correlated with both larval growth rate and survival. For example, on transgenic tobacco line 28, transformed with *cry*1Ac9^{B}, where only one larva survived (Fig 3B), its growth rate was the lowest recorded at 0.279 (Fig 3A) and the leaf damage sustained consisted of only tiny thread-like mines (Fig. 4E).

Molecular analysis

The presence and copy number of the *cry*1Ac9 transgene in the transgenic tobacco lines were determined by Southern analysis. With one exception, all transgenic tobacco lines transformed with *cry*1Ac9^{B} had a 1 kb hybridisation band that verified the presence of *cry*1Ac9^{B} (Fig. 5). The exception was line 24 in which no hybridisation band was detected, suggesting that this line did not contain *cry*1Ac9^{B}. In the case of the tobacco lines transformed with *cry*1Ac9^{A}, because of the presence of an additional *Eco*RI site within the 1kb *Eco*R1 fragment of this construct (Fig. 2), all these lines had two hybridisation bands, 0.7kb and 0.3kb (Fig. 5). The 1.35kb 35S fragment that was included in the same hybridisation showed that for each tobacco line the number of copies of the transgene ranged from one to seven (Fig. 5).

The expression of the *cry*1Ac9 transgene in five of the transgenic tobacco plants was examined by northern analysis (Fig. 6A). For the two tobacco lines transformed with *cry*1Ac9^{A} (21 and 22) there was no detectable *cry* mRNA present. However, for the tobacco lines transformed with *cry*1Ac9^{B} (25,

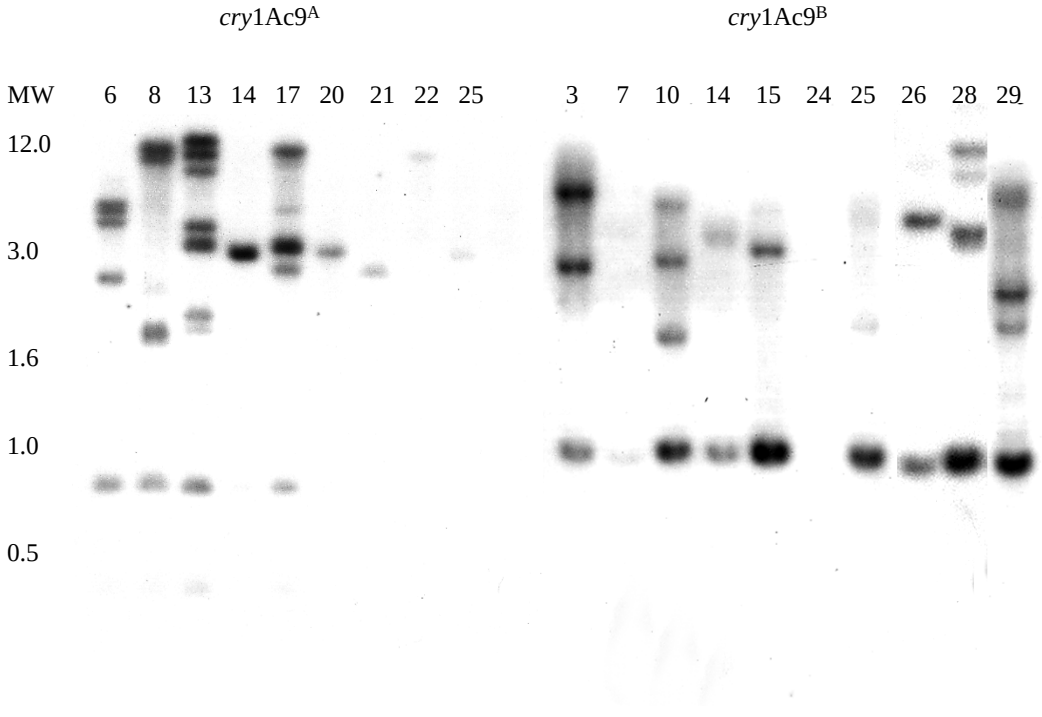

Fig. 5. Southern analysis of the transgenic tobacco lines containing *cry*1Ac9^{A} and *cry*1Ac9^{B}. Probes used for hybridisation were a 1.35 kb DNA fragment corresponding to the 35S promoter sequence and a 1.0 kb DNA fragment corresponding to the 5' end of the *cry*1Ac9^{B} sequence. Genomic DNA extracted from each transgenic tobacco line was digested with *Eco*RI. For the *cry*1Ac9^{B}-containing plants the transgene is visualised as a 1.0 kb band, however, because of an internal *Eco*RI site in *cry*1Ac9^{A} the transgene is visualised as two bands (0.7 kb and 0.3 kb) in the *cry*1Ac9^{A}-containing plants. Numbers above the lanes refer to the plant lines analysed. MW, molecular weight markers (kb).

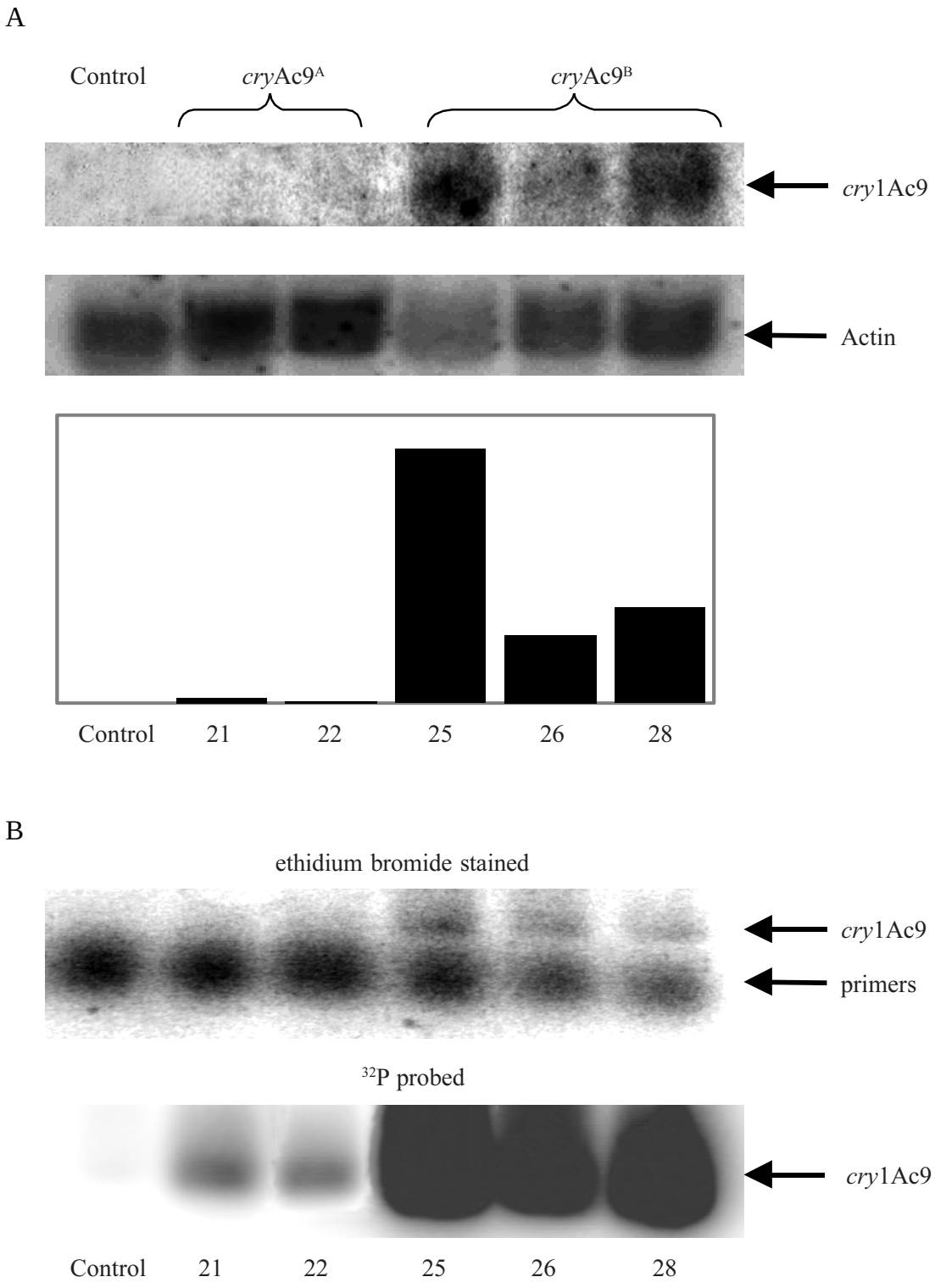

Fig. 6. Northern and RT-PCR analysis of the transgenic tobacco lines containing *cry*1Ac9^{A} and *cry*1Ac9^{B}. A. Northern analysis using as a probe a 270bp PCR fragment homologous to the 3' end of truncated *cry*1Ac9. For each plant line, the level of *cry*1Ac9 mRNA was adjusted for loading differences by hybridisation with an actin fragment from which a histogram of *cry*1Ac9 mRNA levels was plotted. B. RT-PCR analysis using primers that amplified a 270 bp fragment at the 3' end of truncated *cry*1Ac9. The ethidium bromide-stained gel was then analysed by a Southern using the 270 bp PCR fragment as a probe. Numbers along the bottom of A. and B. refer to the plant lines. Control, control tobacco plant.

26 and 28) a faint band corresponding to full-length mRNA was detectable. These three *cry*1Ac9^{B} lines also showed good resistance to PTM infestation in the bioassays, unlike the two *cry*1Ac9^{A} lines (see Fig. 3).

Because of the low levels of *cry* mRNA detected by northern analysis, these results were confirmed by RT-PCR (Fig. 6B). When visualised by electrophoresis and ethidium bromide staining, only the transgenic tobacco lines containing *cry*1Ac9^{B} (25, 26 and 28) contained a DNA fragment of the predicted size (270 bp). However, Southern analysis of the PCR products showed that amplification of the 270 bp fragment had occurred in all five *cry*1Ac9-transformed tobacco lines but was present at much higher levels in *cry*1Ac9^{B} lines 25, 26 and 28. This result suggests that there were higher levels of stable *cry*1Ac9 mRNA in the transgenic tobacco lines containing the *cry*1Ac9^{B} than the transgenic tobacco lines containing *cry*1Ac9^{A}. No band of hybridisation was visible in the control tobacco line.

Discussion

Minor modifications were made to the nucleotide sequence of *cry*1Ac9 to produce *cry*1Ac9^{B}. Before introducing these modifications, several nucleotide changes were made to improve the context of the *cry*1Ac9 translation initiation codon, generating *cry*1Ac9^{A}. Seven nucleotides in the *cry*1Ac9^{A} sequence were then altered to remove a cluster of six potential polyadenylation (AATAAA-like) signals, generating *cry*1Ac9^{B}. Transgenic tobacco containing *cry*1Ac9^{B} were then shown to have increased resistance to infestation by PTM larvae.

Preliminary insect-feeding trials using excised leaf pieces revealed that no PTM larvae had pupated after 14 days on the tobacco plants containing *cry*1Ac9^{B}. On some lines, the PTM larvae present were so small that it is unlikely that they would have survived through to pupation. There was also a significant difference in percentage pupation between PTM larvae feeding on the tobacco plants containing *cry*1Ac9^{A} and those on the control tobacco plants, suggesting that the plants containing *cry*1Ac9^{A} may have expressed the gene at a very low level sufficient to slow larval development.

Based on these results, tobacco lines were selected for further insect-feeding trials using whole plants. In the whole plant bioassays, larval growth rates at 9 days showed no significant differences between larvae on the control plants and those on the plants containing *cry*1Ac9^{A}. However, larvae on the plants containing *cry*1Ac9^{B} showed significantly lower growth rates and survival than those on either the control plants or the plants containing *cry*1Ac9^{A}.

Within both groups of transgenic plants (*cry*1Ac9^{A} and *cry*1Ac9^{B}), however, plant line effects were observed. These were more obvious between plant lines containing *cry*1Ac9^{B} but some variation did occur between plant lines containing *cry*1Ac9^{A}. For example (Fig. 3A), one line containing *cry*1Ac9^{A} (line 8) had the same mean larval growth rate as the least effective line containing *cry*1Ac9^{B} (line 14). This supports the suggestion above that, in some lines, plants containing *cry*1Ac9^{A} may have expressed the gene at a very low level sufficient to slow larval development. Plant line effects on larval survival were also observed. On plants containing *cry*1Ac9^{B} larval survival ranged from eight down to only one survivor in two lines (26 and 28). These two lines together with lines 7 and 25 also had the lowest growth rates.

Northern analysis revealed that a faint band of *cry*1Ac mRNA was detectable in the transgenic tobacco lines containing *cry*1Ac9^{B}, but was not detectable in either the control lines or the lines containing *cry*1Ac9^{A}. However, because of the low level of *cry*1Ac9 mRNA detected, transcriptional expression of the gene in the transgenic tobacco lines was confirmed by RT-PCR analysis. When visualised by agarose gel electrophoresis and ethidium bromide staining, an amplified fragment of the expected size was observed in only those lines containing *cry*1Ac9^{B}. Southern analysis of the gel confirmed this result, but also showed that a very low level of amplification had occurred in the lines containing the *cry*1Ac9^{A} gene. This latter result supports the earlier suggestion from the preliminary insect-feeding trials that a very low level of stable expression of the mRNA for *cry*1Ac9^{A} may be sufficient to slightly slow the developmental time of the PTM larvae.

It is possible that the improved expression of the *cry* gene in the tobacco plants containing *cry*1Ac9^{B} may be the result of better transcription of this construct in comparison to the tobacco plants containing *cry*1Ac9^{A}. However, by using nuclear run-on analysis we have shown previously that the sequence modifications to another *cry* gene, *cry*9Aa2^{T} (Gleave *et al.*, 1998), did not appear to increase the rate at which transcription was initiated. Similar rates of transcription were present in transgenic tobacco plants containing either unmodified or modified *cry*9Aa2^{T}.

Therefore, together these results suggest that the minor modifications made to the nucleotide sequence of *cry*1Ac9 to produce *cry*1Ac9^{B} resulted in improved expression of the gene in tobacco to a level sufficient to severely reduce PMT larval growth and survival. However, there was considerable variation between plant lines in the degree of insect resistance conferred on these plants. Four lines (7, 25, 26 and 28) showed severely retarded growth and the few tiny larvae alive after 9 days were unlikely to have survived to pupation. Other lines (3, 10, 14,

15 and 29) had higher growth rates and more survivors, of which some, despite their delayed development, may have survived, at least to pupation. This variation was subsequently shown by Southern analysis to be irrespective of the copy number of the transgene and is presumably due to chromosomal positional effects.

The seven nucleotide modifications to the *cry*1Ac9 sequence appear to improve the stability of the mRNA, leading to higher steady state mRNA levels in transgenic tobacco, resulting in increased resistance of these plants to infestation by PTM larvae.

Acknowledgements

This work was supported by the New Zealand Foundation for Science, Research and Technology Contract CO6412. The authors would also like to thank S Marshall and L Burgess for their critical comments on the manuscript.

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