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ORIGINAL ARTICLE

Cry1Ac-mediated resistance to tomato leaf miner (*Tuta absoluta*) in tomato

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Abstract Tomato leaf miner (*Tuta absoluta*) is a major pest of tomato in regions with hot climates such as South America and the Mediterranean. This insect feeds on almost every part of the plant and forms galleries while feeding on the plant's inner tissues. Thus, it can cause plant death and is costly and difficult to control with chemical sprays. In this study, we transferred a modified *Bacillus thuringiensis cry1Ac* gene to tomato plants via *Agrobacterium tumefaciens* mediated transformation. Introduction of the *cry1Ac* gene to the tomato genome was confirmed with PCR and Southern blot analysis in 12 independent events. Insertion sites of the transgene in the tomato genome were determined with TAIL-PCR (thermal asymmetric interlaced polymerase chain reaction) for four selected transgenic lines. *Cry1Ac* gene expression was verified at both the transcriptional and translational levels, with RT-qPCR and Western blot analyses, respectively. Expression of the Cry1Ac protein in tomato resulted in *T. absoluta* mortality rates of 38–100% depending on transgenic line. In addition, gallery formation was reduced in 57–100% of the transgenic plants. Moreover, it was found that a single copy of the gene in the hemizygous condition is sufficient to confer tolerance to leaf miner. This is the first reported development of tomato plants resistant to *T. absoluta*. These transgenic plants are promising for development of commercial tomato cultivars resistant to leaf miner, which will limit the use of environmentally harmful chemicals for control of this pest.

Keywords *Agrobacterium tumefaciens* · *Bacillus thuringiensis* · Bt · Genetic transformation · *Solanum lycopersicum* · Tomato pinworm

Introduction

Tomato (*Solanum lycopersicum*) is one of the world's most important horticultural crops. As a result, both in vitro and in planta methods have been developed to genetically modify tomato (Gerszberg et al. 2015; Shah et al. 2015; Sharada et al. 2017) for tolerance to abiotic and biotic stresses. Abiotic stress factors that limit tomato production and quality include cold, heat, drought and salt with significant recent progress made toward engineering tolerance to these environmental challenges (for e.g., Al-Abdallat et al. 2015; Metwali et al. 2015; Shah et al. 2015). In addition, sustainable production of tomato is limited by biotic stresses caused by pathogens and insects. Genetic transformation has also been used to develop resistance to some of these factors in tomato (reviewed in Gerszberg et al. 2015); however, there are still many diseases and pests that require effective control methods.

Tuta absoluta, tomato leaf miner has been an important pest in South America for many years with yield losses as high as 100% (Maluf et al. 2010). Tomato leaf miner

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was first reported in Europe (Spain) in 2006 (Urbaneja et al. 2012). Since then, *T. absoluta* has quickly spread throughout the Mediterranean coastal countries of Europe and Africa (Ponti et al. 2015). Spread is thought to have occurred by agricultural trade (Desneux et al. 2011) despite the fact that *T. absoluta* is a key quarantine pest (EPPO 2005). *T. absoluta* larvae feed at any stage of plant development and eat apical buds, leaves, stems and fruits thus causing reductions in yield and fruit quality (Desneux et al. 2010). Tomato is the primary host of *T. absoluta*, however, the insect can also feed on other solanaceous crops including eggplant, potato, pepper and tobacco (Desneux et al. 2010).

Management of *T. absoluta* has primarily relied on chemical control including organophosphate and pyrethroid pesticides (Desneux et al. 2010). Heavy use of pesticides can result in pesticide-resistant strains which have been reported in South America and Europe (Lietti et al. 2005; Roditakis et al. 2015; Silva et al. 2011; Siqueira et al. 2000). Bt pesticide, derived from *Bacillus thuringiensis*, is an alternative to chemical preparations. Native Bt strains and commercially available preparations have been shown to be highly effective for control of tomato leaf miner with no need for additional pesticide use (González-Cabrera et al. 2010). Moreover, Bt was shown to have no negative effects on a parasitoid of *T. absoluta* which is used for biological control (Riquelme et al. 2006). Bt-based formulations reduced *T. absoluta* damage up to 90%; however, they had low persistence on plants and had to be applied weekly (González-Cabrera et al. 2010).

Although several biotechnological approaches to achieving insect resistance in plants have been described, the most widespread and well-tested is the use of *cry* genes from *B. thuringiensis* (Ferré et al. 2008). *Cry* genes encode insecticidal crystal proteins which selectively bind to insect midgut receptors and insert into the gut cell membrane. This causes formation of pores in the membrane and eventual cell lysis and insect death (Knowles and Dow 1993). Different Cry proteins are known to have varying toxicities on different taxa of insects such as Lepidoptera, Coleoptera, Hymenoptera, Diptera and also nematodes (Bravo et al. 2005). Because of the broad spectrum toxic effect of *B. thuringiensis* Cry proteins, crop plants expressing *cry* genes have been commercially available since the 1990s and are widely used for control of insect pests in field crops (James 2007). Bt crops have received wide acceptance and now account for more than one-third of all genetically modified crops grown worldwide (Ferré et al. 2008). However, the use of *cry* genes to control insects has been less popular in vegetable crops than in field crops. Bt tomato lines have also been developed which carry resistance to tomato fruit borer, Colorado potato beetle, and root-knot nematodes (Koul et al. 2014; Kumar and Kumar 2004; Li et al. 2007; Mandaokar et al. 2000; Saker et al. 2011). However, Bt tomato cultivars are not yet commercially available. To our knowledge, the only reported use of introduced Bt Cry proteins to control *T. absoluta* was a Bt potato line developed by CIP (International Potato Center). In that work, a line expressing *cry1Ab* exhibited tolerance to both tuber moth and *T. absoluta* in field trials (Canedo et al. 1997). Given this report, we hypothesized that transgenic tomato plants expressing a Cry protein gene may exhibit resistance to tomato leaf miner. Thus, we transferred *cry1Ac* into tomato cultivar Moneymaker and confirmed the transgenic plants' resistance to *T. absoluta* using both leaf and whole plant bioassays.

Materials and methods

Vector constructs

A synthetic *cry1Ac* gene was obtained from Dr. I. Altosaar, University of Ottawa, Ontario, Canada (Cheng et al. 1998). The gene construct contained the *cry1Ac* gene driven by a double *CaMV 35S* promoter with a *nos* terminator (Suppl. Fig. 1). The construct was in pRD400, a binary vector that contains a kanamycin selectable marker gene (*nptII*). This vector was introduced into *A. tumefaciens* strain LBA4404 via heat shock transformation (Cheng et al. 1998).

Tomato transformation

Transformation of tomato cv. *Moneymaker* was carried out as described by Frary and van Eck (2004). Briefly cotyledons of 1-week old tomato seedling grown in 1/2 MS0 (1/2× MS salts; 100 mg/L myoinositol, 2 mg/L thiamine–HCl, 0.5 mg/L pyridoxine–HCl, 0.5 mg/L nicotinic acid, 1% sucrose, and 0.8% agar, pH 5.8) were cut into two pieces and dipped in a suspension of *A. tumefaciens* transformed with the pRD400 plasmid containing the *cry1Ac* gene construct. Cotyledon explants were co-cultivated with *A. tumefaciens* for 48 h and then transferred to selective regeneration media for shoot formation (1× MS salts, 100 mg/L myoinositol, 1× Nitsch vitamins, 2% sucrose, 0.52% agargel, 300 mg/L timentin, 50 mg/L kanamycin, 2 mg/L zeatin, pH 6.0). Selective regeneration medium was renewed every 3 weeks until shoots were large enough to transfer to selective rooting media (1× MS salts, 1× Nitsch vitamins, 3% sucrose, 0.8% bacto-agar, 300 mg/L timentin, 50 mg/L kanamycin, pH 6.0). Only one shoot per cotyledon explant was retained to ensure that all candidate transgenic plants represented independent transformation events. After rooting, T_0 plantlets were transferred to soil and gradually acclimated to normal atmospheric conditions as described

in Frary and van Eck (2004). T_0 plants were grown and self-pollinated in the greenhouse to produce T_1 seeds.

Molecular characterization of T_0 plants

Transformation of plants with the *cry* T-DNA was initially confirmed by PCR analysis on T_0 and T_1 putative transformants and non-transformed control *Moneymaker* plants. Genomic DNA was extracted from young leaves of greenhouse-grown plants using a CTAB method (Stewart and Via 1993). Polymerase chain reactions were performed using *cry1Ac* specific *1Ac-1* primers and *nptII* specific primers. The 25 µL PCR reactions contained: 2.5 µL 10× PCR buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 100 ng template DNA and 1 U Taq DNA polymerase. The reaction started with incubation for 5 min at 94 °C followed by 30 cycles of 60 s at 94 °C, 60 s at 58 °C, 60 s at 72 °C and final extension of 5 min at 72 °C. The amplification products were run on 0.8% agarose gels. The *cry1Ac* plasmid construct was used as positive control in PCR experiments.

Further confirmation of transformation was obtained from Southern hybridization analysis. Genomic DNA was extracted from leaves of approximately 1-month old tomato plants grown in the greenhouse. A total of 25 µg genomic DNA from each PCR-verified T_0 plant was digested with *Eco*RI restriction enzyme which cuts once within the T-DNA region (Suppl. Fig. 1). Restriction products were separated in 0.8% agarose gel. The gels were blotted to positively charged nylon membrane (Roche, Germany) via capillary transfer method. Digoxigenin (DIG) labelled probes were prepared using *cry1Ac* specific primer (*1Ac-1*) using the PCR DIG probe synthesis kit (Roche, Germany). Hybridization, wash and immuno-detection were carried out according to the manufacturer's instructions with chromogenic NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) used as substrate [DIG High Prime DNA labelling and detection starter kit I (Roche, Germany)].

TAIL-PCR determination of *Cry1Ac* transgene insertion site

Cry1Ac transgene insertion sites in tomato genome were determined using thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) in four selected transgenic lines (T_1-18, T_1-20, T_1-22, T_1-25). Five arbitrary primers (ad1, ad2, ad3, ad20 and w4) (Yang et al. 2013) were tested with three Cry1Ac gene-specific sequential nested primers for amplifying the 5′ upstream (Cry1Ac51, Cry1Ac52, Cry1Ac53) and 3′ downstream regions (Cry1Ac31, Cry1Ac32, Cry1Ac33). A three step sequential PCR method was used for TAIL-PCR. The primary PCR amplification was carried out using 1 µg genomic DNA (obtained by CTAB method) as template. The secondary PCR amplification was performed using a 10-fold dilution of primary PCR product. The product of this amplification was diluted in the same way and used for tertiary PCR amplification. The PCR mixture and the amplification conditions were in accordance with Pillai et al. (2008). The two largest bands produced by tertiary TAIL PCR for both 5′ upstream and 3′ downstream regions were extracted from agarose gel and cloned into TA cloning vector (pTZ57R/T, Thermo Scientific) and subsequently sequenced for four tested transgenic lines. The sequence analyses for the *Cry1Ac* gene 5′ and 3′ proximal regions were performed via blast analysis against the tomato genome database (https://solgenomics.net/tools/blast/).

Expression analyses

mRNA expression levels of the *cry1Ac* gene were determined by RT-qPCR experiments. Primers specific to *cry1Ac* were designed (*1Ac*-2, Suppl. Table 1) using Primer3 software (Rozen and Skaletsky 1999). The *cac* gene (clathrin adaptor complex medium subunit/endocytic pathway gene) was selected as the reference gene (primers given in Suppl. Table 1) as it was shown to be stably expressed in tomato leaf tissues (Expósito-Rodríguez et al. 2008). Total RNA was extracted from T_1 plants from four independent transgenic T_0 plants using the RNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA samples were treated with DNase (NEB, UK) and total RNA concentration was quantified using Nanodrop Spectrophotometer (MultiskanGO Microplate Spectrophotometer, Thermo Scientific, USA). Total RNA (1 µg) was used for cDNA synthesis via Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) using anchored oligo$(dT)_{18}$ primers, according to the manufacturer's instructions. RT-qPCR reactions were performed using LightCycler 480 SYBR Green master mix (Roche, Germany) according to the manufacturer's instructions with 1:10 diluted cDNA samples as template. PCR reactions (10 µl) were performed with a LightCycler 480 (Roche, Germany) with a program containing pre-incubation, amplification, melting curve and cooling steps according to the manufacturer's instructions. Experiments were carried out with three biological and two technical replicates for each sample and relative expression levels were calculated using the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001). The ΔCt value in the formula was calculated by subtracting the Ct value of the target gene *cry1Ac* from the Ct value of the reference gene, *cac*. Relative expression levels were calculated as fold change proportional to the T_1 individual having the lowest expression level (T_1-20-5, a progeny of T_0-20).

Immunodetection of total protein extracts was carried out with the leaves of approximately 1-month old greenhouse grown plants using 50 mM MES/KOH buffer (40 mM KCl, 2 mM $CaCl_2$, 0.02 M PMSF, pH 6). After protein extraction, total protein concentration was determined according to Bradford protein assay (Bradford 1976). Total protein (100 µg) from each sample was boiled for 5 min with 2× sample loading dye (50 mM Tris–HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and loaded on a 12% SDS–PAGE gel. Separated proteins were blotted to 0.2 mm pore size immunoblot PVDF membrane (Bio-Rad, USA) using a Mini TransblotCell (Bio-Rad, USA) at 30 V overnight in transfer buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). Blotted membrane was blocked with TBST buffer (137 mM NaCl, 2.7 mM KCl, 19 mM Tris base, 0.1% Tween-20, pH 7.4) containing 10% fat-free milk protein. After blocking, the membrane was incubated with primary antibody (Anti-*Bacillus thuringiensis* CRY1Ab toxin antibody ab51586, Abcam, USA) diluted 1:2500 in TBST buffer with 5% fat-free milk protein. The membrane was washed with TBST buffer three times and incubated with a 1:10,000 dilution of secondary antibody (Anti-rabbit IgG, HRP-linked Antibody 7074, Cell Signalling Technology, UK). Finally, the Luminata Crescendo Western HRP substrate (Millipore, USA) was added and the membrane was visualized in a Biorad Versa DOC (Bio-Rad, USA) chemiluminescence detection system.

Insect bioassays

Both detached leaf and whole plant insect bioassays were conducted using four genotypes. *T. absoluta* larvae were fed on *Moneymaker* tomato leaves for approximately 30 days until adult *T. absoluta* were obtained. Adult moths were then placed in insect cages with tomato plants and damp cotton pads to provide egg laying surfaces. Transgenic tomato plants were grown for approximately 1 month in the greenhouse. Detached leaves were placed in petri dishes containing 1.5% agar which helped the leaves to preserve their freshness for 10 days. For each genotype, 2–3 leaves were used for each test and 20 *T. absoluta* eggs (4–5 days old) were placed on each leaf using a soft brush. After transfer of the eggs, the petri dishes were covered with stretch film and 20–30 holes were made in the film to provide ventilation for the eggs. Larvae emerged from eggs 1 or 2 days after transfer. The number of living larvae was recorded after 8–10 days. Larvae growth and feeding characters were investigated for each tomato genotype under a stereo microscope.

For whole plant assays, 4–5 day-old *T. absoluta* eggs (30) were transferred to three or four leaves of tomato plants that were 1 month old. 8–10 days after larvae emergence, the number of the living larvae was counted and the growth and feeding habits of the larvae were investigated as in the detached leaf bioassay.

Correlation analyses were carried out between relative *cry1Ac* gene mRNA expression levels and observed traits in leaf bioassays using R software Hmics package with Kendall rank correlation method.

Results

Development of transgenic tomato lines and confirmation of *cry1Ac* integration

Agrobacterium tumefaciens strain LBA 4404 transformed with pRD400 plasmid containing the *cry1Ac* gene in its T-DNA (Suppl. Fig. 1) was transformed into tomato cotyledon explants. The *Moneymaker* cultivar was used in transformation studies and 25 independent candidate transgenic plants were regenerated. Of these 25 T_0 plants, 12 were verified to contain the *cry1Ac* gene via PCR amplification using *cry1Ac* specific primers (*1Ac*-1) (Suppl. Fig. 2). Southern blot analysis was carried out on the 12 T_0 plants for verification of transgene integration and determination of copy number. *Eco*RI was used for DNA digestion as it cuts the T-DNA once (Suppl. Fig. 1). Southern hybridization with *cry1Ac* gene specific DNA probes indicated that three plants contained single copies of the transgene (T_0-19, T_0-20, T_0-26) while the remaining nine contained two copies (data not shown).

Four of the transgenic T_0 plants were selected for further experiments based on gene copy number. One of the plants (T_0-20) had a single insertion while the other plants had two insertions (T_0-18, T_0-22, T_0-25). These T_0 plants were self-pollinated to generate T_1 seeds which were germinated in 1/2 MS0 medium containing 50 mg/L kanamycin and transferred into soil 3 weeks after germination. DNA was extracted from the kanamycin resistant T_1 plants and PCR amplifications were carried out using *cry1Ac* (*1Ac-1* and *1Ac-2*) and *nptII* gene specific (*nptII*) primers. All of the T_1 seeds that germinated on selective medium were confirmed to be transgenic. Southern analysis also confirmed stable transmission of the T-DNA to the T_1 generation (data not shown).

The locations of the *Cry1Ac* transgene integrations in the tomato genome were identified in the four selected transgenic lines (T_1-18, T_1-20, T_1-22, T_1-25) using TAIL-PCR for determination of the 5′ and 3′ flanking regions. Three step sequential nested PCR was carried out and the two longest fragments of the tertiary TAIL-PCR reaction were cloned and sequenced. The resulting DNA sequences were searched against the tomato genome and MIPS tomato repeat collection databases using Blast (https://

solgenomics.net/tools/blast/). The MIPS database includes repetitive elements such as transposons, micro- and mini-satellites as well as high copy number genes like histones (Spannagl et al. 2007). For transgenic line T_1-20, the isolated TAIL PCR fragments mapped to tomato chromosome 4 (99% match of 997 bp fragment) (Suppl. Table 2), confirming the single copy results observed in Southern blot analysis. For transgenic line T_1-22, the isolated TAIL-PCR fragments mapped to tomato chromosomes 5 (99% match of a 317 bp fragment) and 6 (99% match of 1068 bp). Thus, the results indicate insertion of two copies of the transgene, consistent with Southern blot analysis. For transgenic line T_1-18 there was no match against tomato chromosomes, however, the isolated fragments matched the MIPS tomato repeat library, with a match to k_29 (mips:kail02.01.02.10 SINE|rptmsk SINE|tigr TERT04) (97% match to a 133 bp fragment) and camv (|Waksman|02.01 Retroelement|rptmsk -|tigr TERT) (93% match of a 312 bp fragment) (Suppl. Table 2). The fact that two matches were found is consistent with Southern blot analysis which indicated that two copies were inserted in the tomato genome. TAIL-PCR fragments for transgenic line T_1-18 also matched the MIPS tomato repeat library, with a match to k_29 (mips:kail02.01.02.10 SINE|rptmsk SINE|tigr TERT04) (97% match to a 133 bp fragment) and camv (|Waksman|02.01 Retroelement|rptmsk -|tigr TERT) (96% match of a 334 bp fragment), thus, confirming that this plant contained two copies of the transgene.

Expression of *cry1Ac* at the mRNA and protein levels

RT-qPCR experiments were performed for analysis of mRNA expression levels in transgenic T_1 plants from the four independent T_0 plants described above. As expected, non-transgenic *Moneymaker* tomato plants did not show *cry1Ac* transcription. T_1 progenies of T_0-20 had the lowest *cry1Ac* transcript levels. The expression level of the lowest *cry1Ac* expressing individual (T_1-20-5) was set as the standard (given a value of 1) and the expression levels of the other plants were compared to this individual (Fig. 1). T_1 progenies of T_0-25 had the highest *cry1Ac* expression in leaf tissue with expression levels 160- to 750-fold higher than those of the standard. The T_1 progenies of T_0-18 had expression levels between 160- and 200-fold the standard while T_0-22 progenies' mRNA expression levels were between 27- and 120-fold. Thus, the expression level of the transgene in the plant with single copy insertion (T_0-20) was significantly lower than the plants with two copies in their genomes In order to verify that transgenic plants were expressing Bt crystal protein, Western blot analysis was carried out on selected T_1 plants. Thus, the T_1 progenies of the transgenic plants with *Cry1Ac* expression were found to contain the Cry1Ac protein (Suppl. Fig. 3).

Bioassays for *T. absoluta* tolerance

T_1 plants from the four independent T_0 plants were subjected to *T. absoluta* bioassay using two methods. In the first method, 20 eggs were placed on leaves of control and transgenic T_1 plants in petri dishes (Fig. 2a). Larvae feeding and vigor were evaluated 8–10 days after hatching. Non-transgenic *Moneymaker* leaves were full of galleries made by *T. absoluta* larvae and all the larvae were well-fed (Fig. 2b). In contrast, larvae did much less feeding on leaf mesophyll tissue of transgenic T_1 plants. In most of the transgenic plants, in particular the progenies of T_0-22,

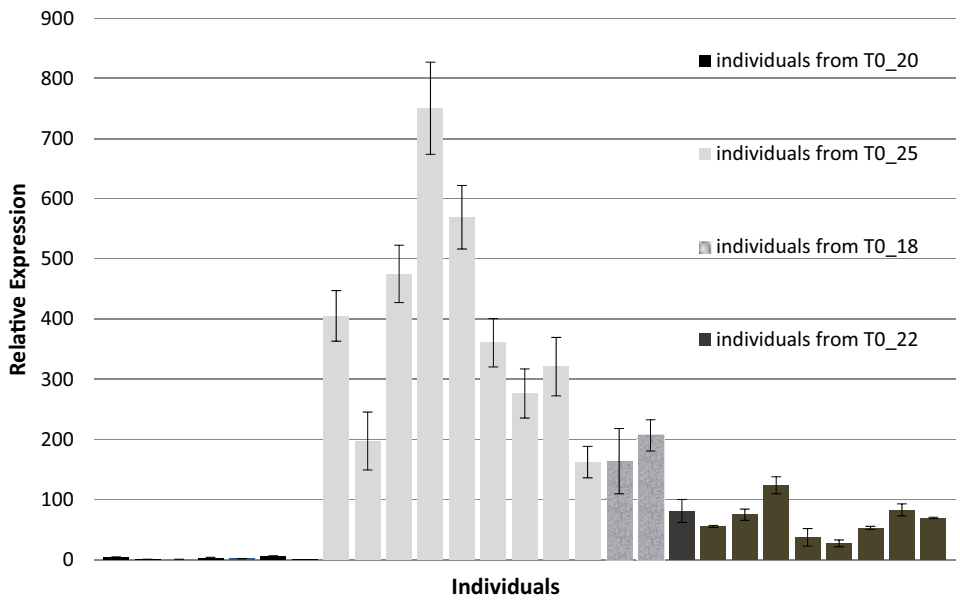

Fig. 1 Detection of *Cry1Ac* gene expression with RT-qPCR in 26 T_1 transgenic seedlings resulting from self-pollination of four T_0 lines. Results are shown as the relative expression levels normalized to the lowest *Cry1Ac* expressing T_1 plant (T_1-20-5) which was set as 1. Data are given as means ± SE of two technical replicates per plant

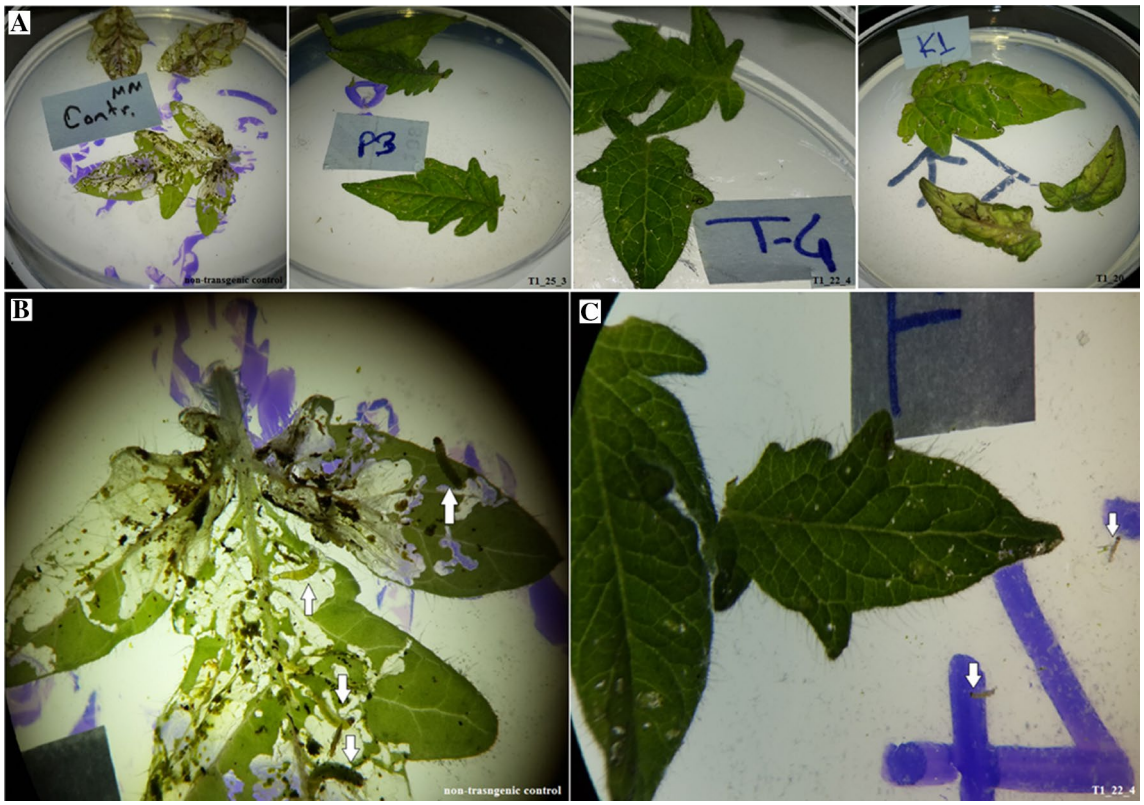

Fig. 2 Insect bioassays with *T. absoluta* in non-transgenic *Moneymaker* plants and transgenic T_1 plants from three different T_0 transgenic lines. Photos were taken 8 days after larvae hatched. **a** Non-transgenic and transgenic tomato leaves explants artificially infested with *T. absoluta* eggs in petri dishes. **b** Well-fed, vigorous larvae (*arrows*) feeding on non-transgenic *Moneymaker* plant. **c** Underdeveloped, dead larvae (*arrows*) after feeding on transgenic T_1 plant leaves

T_0-25 and T_0-18, gallery formation was rare and only small scars caused by initial feeding of larvae were observed (Fig. 2c). Average larvae mortality rate was 75% in transgenic lines with mortality rates ranging from 38 to 100% for the different lines (Table 1). Larvae mortality rate was identified to be significantly correlated with relative level of *Cry1Ac* mRNA expression ($r=0.5$, $p=0.005$, Kendall rank correlation test). Surviving larvae were scored in terms of health, size and mobility from 3 (largest and most mobile larvae) to 0 (no surviving larvae) and the frequencies of the highly observed scores were calculated. The surviving larvae were smaller and less mobile on transgenic lines

Table 1 Detached leaf bioassay via artificial infestation of non-transgenic control *Moneymaker* and transgenic T_1 tomato leaf explants with *T. absoluta* larvae

Name of individual	No. of gene copy	Mortality rate %[a]	Bite scars[b]	Gallery formation[c]	Larvae vigor[d]
Moneymaker	0	0 (12.7 ± 0.3)	5 (100%)	3 (100%)	3 (100%)
T1-20	1	38 (3.7 ± 2.1)	3 (71%)	2 (57%)	3 (57%)
T1-25	2	95 (0.7 ± 0.4)	1 (67%)	0 (89%)	0 (78%)
T1-18	2	67 (4 ± 1.0)	2 (50%)	1 (100%)	0 (100%)
T1-22	2	100 (0 ± 0)	1 (90%)	0 (90%)	0 (100%)

[a]Viability and mortality of insects were recorded at 8 days after larvae emergence. Number of living larvae ± SE is given in parenthesis

[b]Bite scars caused by feeding of larvae were scored up to 5 for the most scars and 1 for the least scars. Frequencies of the most common group are given in parenthesis

[c]Gallery formation caused by larvae feeding in the leaves was scored up to 3 for the most and widest galleries and 0 for no gallery formation. Frequencies of the most common groups are given in parenthesis

[d]Larvae vigor was scored as 3 for the best grown, most mobile larvae and 0 for larvae that were dead by the end of the experiment. Frequencies of the most common group are given in parenthesis

compared to those fed on non-transgenic control leaves. The non-transgenic plants had the largest and most mobile larvae with a score of 3 for 100% of the tested plants. The scores for the transgenic plants were most often 0 with only line T_1-20 having a score of 3 indicating feeding similar to the non-transgenic control (Table 1). Gallery formation and bite scars were inversely correlated with *Cry1Ac* mRNA expression ($r=-0.58$, $p=0.005$ and $r=-0.54$, $p=0.007$, respectively, Kendall rank correlation test).

Similar results were obtained from whole plant tests. For these experiments, 30 *T. absoluta* eggs were placed on leaves of the tomato plants and damage caused by feeding and larvae vigor was monitored 8–10 days after larvae emerged. Leaves of the non-transgenic *Moneymaker* tomato plants were heavily damaged by feeding of the larvae. However, little damage was caused by larvae feeding in transgenic plants with small scars or only a few, small galleries observed in different transgenic individuals. Average number of the surviving larvae varied from 1.1 to 6 in transgenic lines and was 15 for non-transgenic controls (Table 2). Larvae vigor was scored as described for detached leaf tests. The non-transgenic plants had the largest and most mobile larvae whereas the transgenic plants showed limited feeding (Table 2).

Discussion

Bt *cry* genes are widely used in crop genetic engineering and the resulting plants, including cotton and maize, comprise a huge portion of total transgenic crop production (Sanahuja et al. 2011). Bt crops are used for eco-friendly control of insect pests on plants and have been reported to be safe to vertebrates and natural enemies of insect pests (Ferré et al. 2008). *T. absoluta* is a major insect pest of tomato limiting the yield and quality of tomato production in both open fields and greenhouses especially in South America and the Mediterranean. Control of *T. absoluta* based on chemical sprays is costly and can be harmful to the environment and human health. Moreover, such measures are ineffective for *T. absoluta* because its larvae feed within the galleries they form in the mesophyll tissue of leaves (Desneux et al. 2010). The insect can be controlled with commercial Bt formulations containing different strains of *B. thuringiensis* (ex: Dipel® *B. thuringiensis kurstaki* strain expressing Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cry2Ab proteins, Valent Bioscience). However, control requires frequent spraying and is not consistent (González-Cabrera et al. 2010). In previous studies, *cry* genes were over-expressed in tomato plants and were identified to confer tolerance to several different insect pests including *Helicoverpa armigera, Heliothis zea, Spodoptera litura*, and *Manduca sexta* (Delannay et al. 1989; Fischhoff et al. 1987; Koul et al. 2014; Kumar and Kumar 2004; Mandaokar et al. 2000; Saker et al. 2011). However, these plants were not tested for their resistance to *T. absoluta*. Thus, our study is the first report of the development of transgenic tomato lines specifically for resistance to *T. absoluta*.

The transgenic tomato plants over-expressing the *Cry1Ac* gene were evaluated for their resistance to *T. absoluta* via artificial infestation of detached leaves and whole plants with *T. absoluta* larvae. The damage caused by *T. absoluta* feeding in leaf tissues of transgenic plants carrying one or two copies of the transgene was significantly reduced. Gallery formation in leaves caused by larvae feeding was restricted compared to the non-transgenic individuals, in which the leaf tissue was almost completely consumed by the larvae. Damage was limited to small scars in the leaves of the transgenic plants which were caused by the initial feeding attempts of the larvae. Moreover, the viability of the larvae was low in

Table 2 Whole plant bioassay via artificial infestation of non-transgenic control *Moneymaker* and transgenic T_1 plants with *T. absoluta* larvae

Name of individual	No. of gene copy	No. of alive[a]	Bite scars[b]	Gallery formation[c]	Larvae vigor[d]
Moneymaker	0	15	3	3	3
T1-20	1	4.4 ± 1.3	3 (80%)	2 (80%)	2 (60%)
T1-25	2	1.8 ± 0.5	1 (50%)	0 (50%)	1 (40%)
T1-18	2	6 ± 1.0	2 (100%)	2 (100%)	1 (50%)
T1-22	2	1.1 ± 0.3	1 (80%)	1 (80%)	0 (50%)

[a]Viability and mortality of insects were scored at 8 days after the hatch of larvae. Number of living larvae ± SE is given

[b]Bite scars caused by feeding of larvae were scored up to 5 for the most scars and 1 for the least scars. Frequencies of the most common group are given in parenthesis

[c]Gallery formation caused by larvae feeding in the leaves was scored up to 3 for the most and widest galleries and 0 for no gallery formation. Frequencies of the most common groups are given in parenthesis

[d]Larvae vigor was scored as 3 for the best grown, most mobile larvae and 0 for larvae that were dead by the end of the experiment. Frequencies of the most common group are given in parenthesis

transgenic plants. In addition, the surviving larvae were smaller and less mobile compared to those fed on non-transgenic *Moneymaker* plants. Thus, there was a clear difference in the *T. absoluta* resistance of non-transgenic *Moneymaker* and transgenic plants.

Variability was seen in the degree of tolerance of different T_1 lines. We observed significant differences directly related to copy number which could also be due to insertion site effects. The variability in tolerance was associated with the observed differences in mRNA expression levels of the lines. We also mapped the transgene insertion sites in the tomato genome with TAIL-PCR and we identified the chromosomal locations of the transgenes in two of the transgenic lines. The other two transgenic plants were found to have insertions in transposable elements in the tomato genome and, thus, could not be mapped to specific locations. The three transgenic lines with the highest *cry1Ac* mRNA expression had two copies of the transgene and had the least damage caused by larvae feeding. These plants also had fewer surviving larvae compared to the low expressing line with a single copy of the transgene. Thus, we observed a significant correlation between relative mRNA expression of the *cry1Ac* gene and larvae mortality rate. There were also inverse correlations between relative mRNA expression of the *cry1Ac* gene and both gallery formation and bite scars. Because T_1 transgenic lines were used, we analyzed a mixed population of hemizygous and homozygous individuals. Despite the differences in gene copy number and zygosity, we observed similar degrees of tolerance within the lines which indicates that a single copy of the gene in the hemizygous condition is sufficient to confer tolerance to this pest.

In conclusion, we developed transgenic tomato plants over-expressing the *cry1Ac* gene and confirmed that these lines are resistant to *T. absoluta*, one of the most damaging insect pests of tomato. The transgenic lines developed in this work can be used in breeding programs for the introgression of insect resistance into commercially important tomato standard and hybrid varieties. The lines should also be tested for their resistance to other important tomato pests for which the Bt toxin is effective.

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Author contributions HS transformed plants, performed molecular analyses and drafted paper with AF; FD and NM designed and performed insect bioassays; SD and AF devised and oversaw experimental work.

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