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Two different *Bacillus thuringiensis* toxin genes confer resistance to beet armyworm (*Spodoptera exigua* Hübner) in transgenic *Bt*-shallots (*Allium cepa* L.)

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Abstract

Agrobacterium-mediated genetic transformation was applied to produce beet armyworm (*Spodoptera exigua* Hübner) resistant tropical shallots (*Allium cepa* L. group Aggregatum). A *cry1Ca* or a *H04* hybrid gene from *Bacillus thuringiensis*, driven by the chrysanthemum ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (Rubisco SSU) promoter, along with the hygromycin phosphotransferase gene (*hpt*) driven by the CaMV 35S promoter, was employed for genetic transformation. An average transformation frequency of 3.68% was obtained from two shallot cultivars, Tropix and Kuning. After transfer of the *in vitro* plants to the greenhouse 69% of the *cry1Ca* and 39% of the *H04* transgenic shallots survived the first half year. After one year of cultivation in the greenhouse the remaining *cry1Ca* and *H04* transgenic plants grew vigorously and had a normal bulb formation, although the *cry1Ca* transgenic plants (and controls) had darker green leaves compared to their *H04* counterparts. Standard PCR, adaptor ligation PCR and Southern analyses confirmed the integration of T-DNA into the shallot genome. Northern blot and ELISA analyses revealed expression of the *cry1Ca* or *H04* gene in the transgenic plants. The amount of Cry1Ca expressed in transgenic plants was higher than the expression levels of H04 (0.39 vs. 0.16% of the total soluble leaf proteins, respectively). There was a good correlation between protein expression and beet armyworm resistance. *Cry1Ca* or *H04* gene expression of at least 0.22 or 0.08% of the total soluble protein in shallot leaves was sufficient to give a complete resistance against beet armyworm. This confirms earlier observations that the H04 toxin is more toxic to *S. exigua* than the Cry1Ca toxin. The results from this study suggest that the *cry1Ca* and *H04* transgenic shallots developed could be used for introducing resistance to beet armyworm in (sub) tropical shallot.

Abbreviations: AL-PCR – adaptor ligation PCR; *Bt* – *Bacillus thuringiensis*; *cry1Ca* – a synthetic gene from *Bacillus thuringiensis*; GUS – β-glucuronidase; *H04* – a hybrid gene from *B. thuringiensis*; *hpt* – hygromycin phosphotransferase gene; LB – T-DNA left border; RB – T-DNA right border; *gusA* – β-glucuronidase gene.

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Introduction

Shallot (*Allium cepa* L. group Aggregatum) is a very important vegetable crop in (sub)tropical zones. However, its cultivation can be severely threatened during the dry season by beet armyworm (*Spodoptera exigua* Hübner). Current methods for controlling *S. exigua* are the use of sex pheromones, pesticides and cultural measures (Takai & Wakamura, 1990, 1996; Wakamura et al., 1990). These practices provide some protection, but are still far from adequate. A problem with spraying chemical agents is that the caterpillars in the hollow leaves of shallot are protected against contact insecticides. Furthermore, chemical control is expensive, causes environmental pollution, and is a threat to the health of farmers and consumers. Moreover, insecticide resistance is reported to occur in *Spodoptera* (Brewer et al., 1990; Brewer & Trumble, 1991a, b; Cheng & Kao, 1993).

In view of this, we started in 1996 a programme aiming at the introduction in shallot of resistance to beet armyworm via a two-way approach, namely marker-assisted breeding (MAB) and genetic transformation. Already early in the programme we found that MAB was not the way forward as no reliable source of resistance could be identified in the germplasm analysed (Zheng et al., 2000). Therefore, most effort in the programme was focussed on genetic transformation. Many protocols for the production of transgenic crops were developed in the last decade, however, for *Allium* species the development of these protocols proved to be difficult. Only recently, reports were published showing that genetic transformation has become possible in onion and shallot (Eady et al., 2000, 2003a, b; Zheng et al., 2001a, b).

The use of genes encoding insecticidal proteins originating from *B. thuringiensis* to confer resistance to various insect pests has been very successful in a number of crops (Schuler et al., 1998; Tu et al., 2000; Perlak et al., 2001; Naimov et al., 2003). The *cry* gene family from *B. thuringiensis* encodes a protein which is active against insect larvae of a subset of species usually belonging to the same order (Schnepf et al., 1998; De Maagd et al., 2001). Cry1 proteins are generally active against lepidopterans (larvae of moths and butterflies). For example, a synthetic *cry1Ca* gene, encoding a *B. thuringiensis* delta-endotoxin, confers resistance to *S. exigua* in alfalfa and tobacco (Strizhov et al., 1996) and overexpression of *Bt cry2Aa2* in chloroplasts resulted in 100% killing of beet armyworm after consuming transgenic leaves (De Cosa et al., 2001). Furthermore the hybrid *Bt* gene *H04,* which encodes a fusion between the N-terminal domains I and II of Cry1Ab and the C-terminal domain III of Cry1Ca, is reported to be highly toxic to *S. exigua* compared to the parental Cry1Ab toxin and significantly more toxic than the Cry1Ca parental toxin (De Maagd et al., 1996, 2000).

In this paper we describe the production of the first transgenic shallot plants harbouring a *H04* or *Cry1Ca* truncated gene, under the control of a chrysanthemum ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (Rubisco SSU) promoter and terminator, which confers a high degree of resistance against beet armyworm.

Materials and methods

Ti plasmid construction for shallot transformation studies

The plasmid pCAMBIA1301, a normal binary vector from the Center for Application of Molecular Biology to International Agriculture (CAMBIA), Canberra, Australia having *hpt* and intron-*gusA* genes in the T-DNA region, was used to make constructs pPB34 and pPB36 (Figure 1A and B). As pCAMBIA1301 has an intron-interrupted *gusA* gene, the expression of *gusA* only occurs in transformed calli and plants rather than in *A. tumefaciens* itself (Ohta et al., 1990). Plasmids pPB34 and pPB36 were constructed as described below. The *H04* gene is a hybrid gene which encodes domains I and II of Cry1Ab and domain III of Cry1Ca (De Maagd et al., 1996) and which was optimized for expression in plants (Carozzi et al., 2002). The *H04* or *cry1Ca* (Strizhov et al., 1996) genes were inserted between the *Nco*I and *Bgl*II sites in plasmid pRBC, flanked by a chrysanthemum ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (Rubisco SSU) promoter and terminator (Outchkourov et al., 2003). The orf's with the flanking promoters and terminators were excised by *Hin*dIII digestion and inserted into the *Hin*dIII

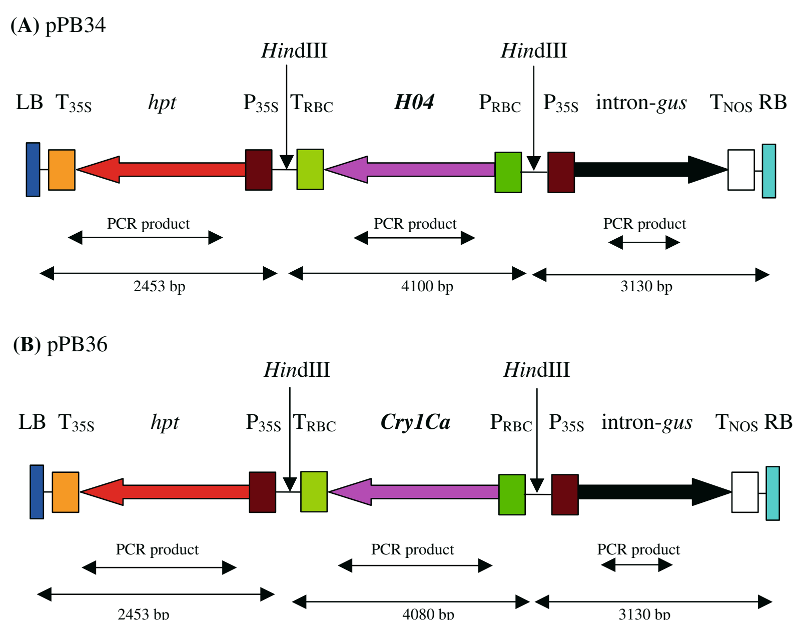

Figure 1. T-DNA region of (A) binary vector pPB34 and (B) binary vector pPB36, showing the size of the T-DNA, the region for PCR amplification, PCR products used as a probe and *Hin*dIII restriction sites used for Southern hybridization. *Abbreviations*: RB: right border; LB: left border; P_{35S} and T_{35S}: CaMV 35S promoter and terminator; T_{NOS}: nopaline synthase terminator; P_{RBC} and T_{RBC}: ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) promoter and terminator; *hpt*: hygromycin phosphotransferase; intron-*gus*: intron interrupted *β*-glucuronidase; *H04*: synthetic *Bt* gene; *cry1Ca*: synthetic *Bt* gene.

site of pCAMBIA1301 resulting in plasmids pPB34 and pPB36 which were introduced into *Agrobacterium* strain AGL0 by electroporation (Mattanovich et al., 1989). *Agrobacterium* strain AGL0 is similar to EHA105 which was used by us in onion and shallot transformation (Zheng et al., 2001a). It is an L, L-succinamopine strain with a C58 chromosome background (Lazo et al., 1991). It contains as virulence helper plasmid pEHA101, originally derived from supervirulent pTiBo542 (Hood et al., 1986, 1993).

Tissue culture and genetic transformation

AGL0(pPB34) and AGL0(pPB36) were streaked out on solidified LB medium with 50 mg/l rifampicin, 50 mg/l kanamycin and grown at 28 °C for 3 days for colonies to appear. A single bacterial colony was collected from a plate and suspended for further culture in LB liquid medium for 2 days. Suspensions were centrifuged at 3000 rpm for 10 min and the *Agrobacterium* pellet was resuspended in liquid callus induction medium with 100 μM acetosyringone at an optical density of 0.5–1 (OD_{600}).

Two shallot (*Allium cepa* L.) cultivars, Tropix and Kuning, were used as a source of mature embryos for transformation. The procedure of isolation of mature zygotic embryos has been described earlier (Zheng et al., 1998, 1999). Briefly, callus induction was carried out on solidified MS medium with 1 mg/l 2,4-D, 0.2 g/l casein hydrolysate, 30 g/l sucrose and 4 g/l gerlite in the dark at 25 °C. Three-week old calli were chopped directly after retrieving them from the *Agrobacterium* suspension. The co-cultivation medium contained callus induction medium with 10 g/l glucose and 100 μM acetosyringone. About 20 calli were examined immediately after the co-cultivation period of 4 days for GUS transient expression. The remaining calli were put on a selective medium. Transient GUS expression detection, resistant callus selection and transformed callus regeneration and rooting conditions were similar as described for onion and shallot earlier (Zheng et al., 2001a). After continuous selection for 1 or 2 months on medium with 400 mg/l cefotaxime and 100 mg/l vancomycin and 50 mg/l hygromycin, putative transformed calli were put on regeneration medium. The

regeneration medium contained MS30 with 1 mg/l kinetin and 50 mg/l hygromycin. Transgenic plantlets were obtained after resistant calli had been on regeneration medium for about 2 months. Regeneration was carried out at an ambient temperature of 25 °C with a 16 h photoperiod (ca. 60 μE m^{-2} s^{-1}; lamps used: Philips, TLD 50W/840HF, and Electronic NG). After 1 month, all material was subcultured. The number of callus lines with shoot regeneration was recorded after 2 months.

The plantlets were transplanted into pots ($8 \times 8 \times 8$ cm^3) in the greenhouse. They were first covered with a plastic cap for 1 week for acclimation they were then grown in the greenhouse at 16/20 °C (night/day) with additional light for 16 h (approximately 100 μE s^{-1} m^{-2}; lamps used: SON-T 400 Watt).

Standard PCR and adaptor ligation PCR analyses

Approximately 0.25 g fresh leaf samples from putative transgenic plants were collected. DNA was isolated from greenhouse-grown plants via a miniprep protocol (van Heusden et al., 2000). DNA concentration was estimated using an Eppendorf Biophotometer. Optimal conditions, such as temperature, DNA concentration and suitable primers for PCR were checked in preliminary experiments. Successful standard PCR was performed using specific primers for *gusA* (forward: 5′GGAATTGA TCAGCG-TTGGTG3′ and reverse: 5′TAGATATCAC ACTCTGTCTG3′), for *hpt* (forward: 5′ATGA-AAAAGCCTGAACTCA3′, and reverse: 5′ACT-GGATTTTGGTTTTAGG3′), for°C for 2 min (1 cycle); 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min (35 cycles); 72 °C annealing extension for 10 min. The reactions were carried out in PTC-200 thermocycler (DNA Engine, MJ Research, INC, USA). Primers for *gusA* amplify a sequence of 710 bp, while *hpt, Cry1Ca* and *H04* primers amplify a sequence of 1.2 kb, 802 and 953 bp, respectively.

DNA was isolated from 1 to 1.5 g frozen leaf tissue with the midi prep DNA-isolation method described by van Heusden et al. (2000) for adaptor ligation PCR (AL-PCR) analysis. The enzyme *Alu*I was selected because it generates a blunt-end fragment which is easily ligated to the adaptor (Zheng et al., 2001b). AL-PCR amplification is targeted to the adaptor sequence specific restriction site with adaptor and to a specific sequence in the left or right genomic DNA flanking border (LB or RB) sequences of the T-DNA. Adaptor design, adaptor primers and T-DNA border sequence primers were the same as described earlier (Zheng et al., 2001b).

Southern blot analysis

Putative transgenic shallot genomic DNA was isolated from 1 to 1.5 g frozen leaf tissue collected from the greenhouse with a midiprep DNA-isolation method as described by van Heusden et al. (2000). The DNA concentration was estimated using an Eppendorf Biophotometer. From each sample 25 μg DNA was digested overnight with 100 units of *Hin*dIII. After digestion, the DNA was loaded on a 1.0% agrose gel with TAE and run at 25 V for overnight electrophoresis (running time about 17 h). DNA was transferred to a nylon Hybond^{TM}-N+ membrane (Amersham Lifescience, UK) by vacuum blotting. One-hundred nanograms DNA of PCR products from *gusA, hpt, cry1Ca* and *H04* were used for random prime labeling, respectively. The RadPrime DNA labeling system (GibcoBRL®, Life Technologies) was used. For pre-hybridisation, hybridisation and blot washing standard procedures were followed. Signals were detected using a Storm 860 Phospho Imager (Amersham Biosciences).

Northern blot analysis

Total RNA isolation from 1 to 2 g frozen leaf tissue from transgenic and control shallots was performed according to the method described by Zhou et al. (1999) with minor modifications. Ten micrograms of RNA was prepared using a modified glyoxal/DMSO method (Sambrook et al., 1989) and run on 1.5% agrose gel made with 15 mM sodium phosphate buffer pH 6.5 and stained with ethidium bromide. RNA was transferred by capillary flow to a nylon membrane (Amersham, Buckinghamshire, UK) using 25 mM phosphate buffer pH 6.5 as transfer solution. As probes, 802 and 953 bp PCR products from *cry1Ca* and *H04* were used, respectively. The product was purified using spin columns (Qiagen, Westburg, the Netherlands) and the probe was

synthesised with the RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA) using [^{32}P] dATP as radioactive label. For loading control RNA, a fragment of 620 bp from the constitutive glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA from shallot cv. Tropix was amplified by RT-PCR using degenerated primers (forward: 5′AGRAA CCCTGARGADATYCCRTGGG3′ and reverse: 5′CCAGCCTTGGCRTCRAARATGCT3′), designed by DNA sequence alignment of several GAPDH from monocot species available in GenBank.

Hybridization was carried out at 60 °C for 3 h with 1 hour pre-hybridization in a buffer containing 10% dextran sulphate, 1% SDS, 1 M sodium chloride and 10 μg/ml herring sperm. Washes were conducted at hybridization temperature twice for 20 min in 2× SSC, 0.1 % SDS and once in 1× SSC, 0.1% SDS. Signals were detected using a Storm 860 Phospho Imager (Amersham Biosciences).

Protein quantification

Sixty milligrams frozen leaf tissue from *cry1Ca* transformants (or 30 mg from *H04* transformants) was ground with 500 μl (or 250 μl for *H04* transformants) of extraction buffer (50 mM NaOH, 20 mM NaS_2O_5, 5 mM EDTA and 10% Polyvinylpolypyrrolidone). Subsequently the extract was neutralized with 80 μl 1 M Tris–HCL, pH 5.5, and centrifuged at 16000*g* for 10 min. The supernatant was transferred into a new Eppendorf tube and centrifuged again at 16000 *g* for 10 min. Protein concentrations in the supernatant were determined by the Bradford method (Bio-Rad Laboratories, Hercules, USA). The amount of Cry protein was estimated using the Cry1C Plate Kit (ENVIROLOGIX Catalog No. AP 003, Portland, USA). The EnviroLogix Cry1C Plate Kit is a ''sandwich'' Enzyme-Linked ImmunoSorbent Assay (ELISA). All calibrators were organized and an illustrative Cry1C standard curve was made based on the optical density (OD). The OD's of the diluted sample extracts to those of the calibrators were compared and the amount of Cry1C from each sample was obtained. Because there is no commercial plate kit available for estimating H04 protein, different kits were tested in advance. We found that the Cry1Ab/Cry1Ac Plate Kit (ENVIROLOGIX Catalog No. AP 003, Portland, USA) was also suitable for measuring the amount of H04 protein. Firstly Bt-toxin (H04) was isolated from *E. coli* based on the standard procedure. Secondly different calibrators from H04 were organized and an illustrative H04 standard curve was made based on the optical density (OD). The OD's of the diluted sample extracts were compared to those of the calibrators and the amount of H04 from each sample was obtained.

Bio-assay for resistance to beet armyworm

The protocol was based on the Zheng et al. (2000) toxic compound bio-assay. In brief four replicates from transgenic shallot and control plants (non-transgenic) were used, with each 3–4 g of fresh leaves on 0.8% sterile water agar in a Petri dish. Five 3-day old larvae were inoculated in each replicate. Four days after inoculation the percentage of surviving larvae per Petri dish was determined. In total three bio-assays were performed on separate dates.

Statistical analyses

The data from the bio-assays for testing for resistance to beet armyworm and the data of the toxic protein content in leaf tissue were analysed using a generalized linear model (McCullagh and Nelder, 1990) based on a binomial distribution using a logit as a link function.

Results

Production and molecular characterization of transgenic shallots

Two shallot cultivars, Tropix and Kuning, were transformed using *Agrobacterium* strain AGL0 carrying plasmids pPB34 and pPB36 with *cry1Ca* and *H04*, respectively (Figure 1). Three-week old calli derived from mature embryos, after cocultivation with *Agrobacterium*, were transferred to Murashige and Skoog (MS) medium containing 50 mg/l hygromycin as a selective agent. Shoots were regenerated from the hygromycin resistant calli on MS medium supplemented with 1 mg/l kinetin and 50 mg/l

hygromycin. In three successive experiments a total of 2092 callus lines from two shallot cultivars, Tropix and Kuning were brought onto selective medium and 77 putative transgenic lines produced shoots, resulting in a mean transformation frequency of 3.68%. After the putative transgenic shoots had developed, they were isolated and put onto rooting medium which consisted of solidified MS medium supplemented with 50 mg/l hygromycin and 30 g/l sucrose without any phytohormones. Later on these individual plants were transferred to the greenhouse and they appeared normal in morphology and bulb formation (Figure 2A, B and C), although the transgenic shallot plants transformed with *H04* had lighter green leaves compared to the *cry1Ca* and control plants. One year after transplanting the *in vitro* transgenic plants to the greenhouse approximately 69% and 39% of the *cry1Ca* and *H04* transgenics respectively survived; all controls survived.

Samples of genomic DNA isolated from hygromycin resistant transgenic plants as well as control plants, were tested for the presence of *gusA, hpt, cry1Ca* or *H04* genes by PCR using appropriate primers. PCR analysis showed the presence of the 710 bp fragment of *gusA*, the 1.2 kb fragment of *hpt,* the 802 bp fragment of *cry1Ca* or the 953 bp fragment of *H04* in the genomes of all the transformants (data not shown). In order to quickly determine whether T-DNA integration had taken place and how many copies of the T-DNA were present in the plant's genome, adaptor ligation PCR (AL-PCR) was carried out. All lines tested gave clear PCR products indicating T-DNA insertion in the shallot genome and it was found that a minimum of 1-3 T-DNA copies per transformant were integrated into the genome from 14 of the 18 transgenic *cry1Ca* lines analysed (data not shown). Different AL-PCR patterns reflect separate transformation events. Therefore, the 14 lines studied represented at least 10 separate transformation events (data not shown).

After AL-PCR analysis, Southern blotting was used to analyse the transgenic plants in more detail. DNA was extracted from young

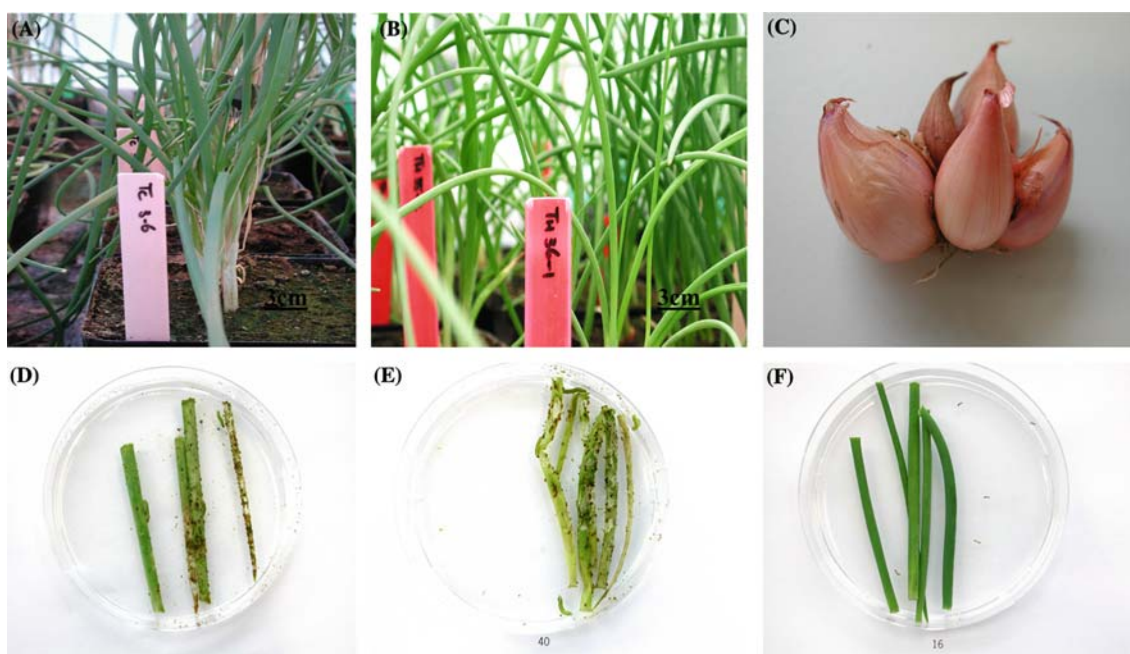

Figure 2. Transgenic shallot plants and leaves in greenhouse and in Petri dishes, respectively. (A) plants transformed with *cry1Ca*; (B) plants transformed with *H04*; (C) mature bulbs from plants transformed with *cry1Ca*; (D) non-transgenic shallot cv. Tropix as control in a bioassay to beet armyworm; (E) susceptible plant transformed with *cry1Ca*; (F) resistant plant transformed with *cry1Ca*.

leaf tissue of individual transgenic plants, digested with *Hin*dIII and hybridized with probes from the PCR products of the *gusA*, *hpt, cry1Ca* or *H04* genes, respectively. DNA from non-transformed plants was used as a negative control and showed no hybridization signals to the probe used (Lane N in Figures 3 and 4, respectively). Because the T-DNA of pPB34 and pPB36 has two *Hin*dIII sites, probing with either the *cry1Ca* or *H04* gene sequence will give one internal fragment harbouring the *cry1Ca* or *H04* gene when the *cry1Ca* or *H04* gene PCR product is used as probe (Figure 1A and B). Probing with either the *gusA* or *hpt* gene sequence will only give an indication of the number of T-DNA copies integrated. In total 25 different lines were identified among the 77 *in vitro* shoots originally regenerated. All seven different lines harbouring the *H04* gene were analysed. These plants had one band, when probed with the *gusA* coding sequence, but they had one or two bands with different sizes when probed with the *hpt* coding sequence (Figure 3). All transgenic plants had one identical band of 4100 bp, when probed with the *H04* coding sequence (Figure 3). This indicates that no truncated integration of the *H04* gene into the shallot genome took place in these seven lines. All 18 different lines harbouring the *cry1Ca* gene were also analysed. All plants had one to three bands with different sizes, when probed with the *gusA* coding sequence, but they had one or two bands when probed with the *hpt* coding sequence (Figure 4). All transgenic plants had one identical band of 4080 bp, when probed with the *cry1Ca* coding sequence (Figure 4). This indicates that no truncated integration of the *cry1Ca* gene into the shallot genome took place.

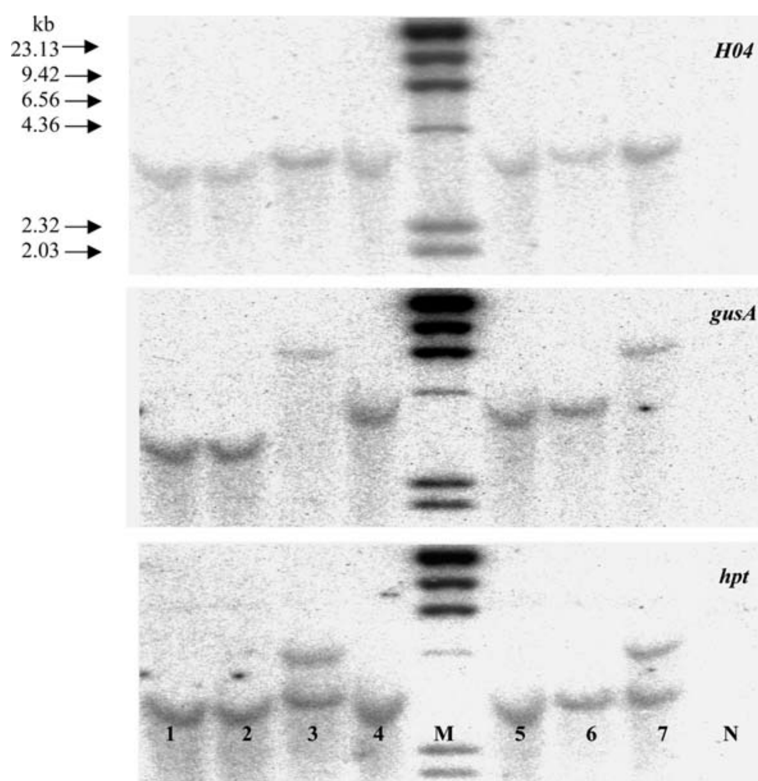

Figure 3. Southern blot analysis of individual shallot transformants harbouring the *H04* gene. DNA from transformants was digested with *Hin*dIII, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to a probe. DNA from a PCR amplification using *H04, gusA and hpt* primers generated 953, 702 and 1200 bp fragments, respectively, which were used as probes. Lane M: λDNA digested with *Hin*dIII; Lane N: untransformed shallot plant as negative control. Lanes 1–7: plants originating from different lines transformed with AGL0 (pPB34).

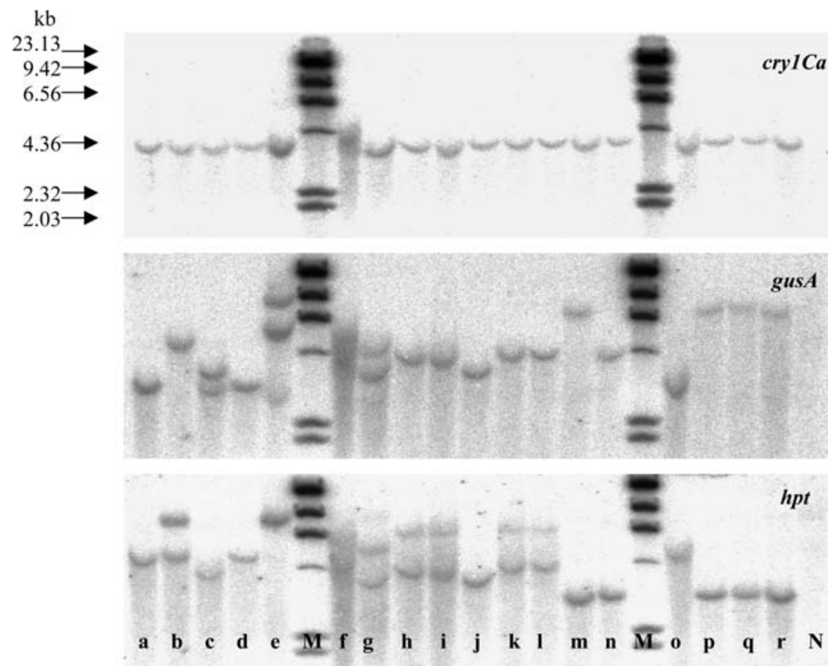

Figure 4. Southern blot analysis of individual shallot transformants harbouring the *Cry1Ca* gene. DNA from transformants was digested with *Hin*dIII, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to a probe. DNA from a PCR amplification using Cry1Ca, *gusA* and *hpt* primers generated 802, 702 and 1200 bp fragments respectively, which were used as probes. Lane M: λDNA digested with *Hin*dIII; Lane N: untransformed shallot plant as negative control. Lanes a–r: plants originating from different lines transformed with AGL0 (pPB36).

Expression of the cry1Ca or *H04* gene in shallots

Northern blot analysis and Enzyme-Linked ImmunoSorbent Assay (ELISA) were carried out to observe the expression of the *cry1Ca* or *H04* gene in transgenic shallot plants. All seven transgenic plants harbouring the *H04* gene were used for northern analysis. In order to compare the amount of RNA loaded, a fragment of 620 bp from the constitutive glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA from shallot cv. Tropix was amplified by RT-PCR as a loading control. All seven plants had a very high expression of the *H04* gene (Figure 5A). Northern analysis of all 18 transgenic plants harbouring the *cry1Ca* gene was also performed. Only one plant (transformant e) had no expression of the *cry1Ca* gene, while the other 17 plants had different levels of expression (Figure 5B).

Leaves of transgenic plants were analysed by ELISA for estimating the amount of Bt protein. The use of the strong green tissue-specific Rubisco promoter of chrysanthemum resulted in high protein levels of Cry1Ca or H04. Highly statistically significant differences were found in protein content among different transgenic *cry1Ca* and *H04* plants ($p < 0.01$). There was as high as 0.39% Cry1Ca protein of the total soluble plant protein in transformant i and as high as 0.16% H04 protein of the total soluble plant protein in transformant 6. In general, the H04 protein content in *H04* transgenic shallot plants was lower than the Cry1Ca protein content in *cry1Ca* plants.

Resistance of transgenic shallots to beet armyworm

To check whether shallot plants transformed with pPB34 or pPB36 were resistant to beet armyworm, five 3-day old beet armyworm (*S. exigua* Hübner) larvae were inoculated on transgenic and non-transgenic leaves as a control in three independent experiments. An average of 13.3% mortality of *S. exigua* was observed on non-transgenic leaves. Statistically significant differences were found in beet armyworm mortality among different *cry1Ca* and *H04* plants ($p < 0.001$). All plants transformed with the *H04*

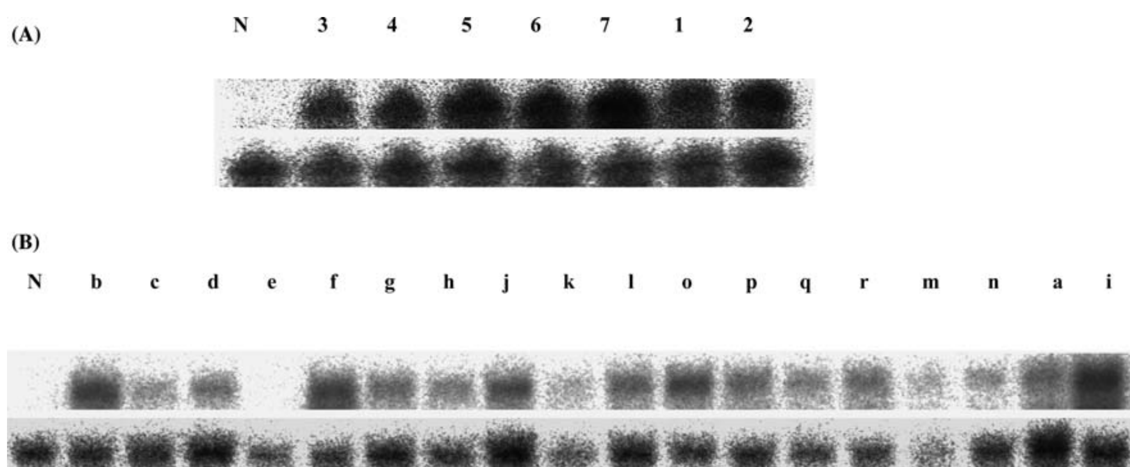

Figure 5. Northern blots for the analysis of gene expression. Lane N: untransformed shallot plant as negative control. Ten micrograms of total RNA was loaded in each lane. Loading control is given by the constitutive shallot GAPDH gene in the lower panels. (A) Different transgenic shallot plants from various lines (1-7) harbouring the *H04* gene. (B) Different transgenic shallot plants from various lines (a-r) harbouring the *cry1Ca* gene.

gene, expressing detectable levels of H04 protein, showed a very high level of resistance to beet armyworm lavae and led to more than 97.8% larvae mortality. No statistically significant differences were found in beet armyworm mortality among different *H04* plants in three independent bio-assays. All plants transformed with the *cry1Ca* gene, expressing detectable levels of Cry1Ca protein, showed a high level of resistance to beet armyworm larvae and resulted in 60 to 100% larvae mortality. Six plants (transformants e, m, n, o, p, q and r) were susceptible to beet armyworm and comparable to the non-transgenic control plant (they had undetectable Cry1Ca protein expression (Figure 2D and E)). Among these six plants, only transformant e had no detectable northern band while the other five plants had different levels of expression of the *cry1Ca* gene with respect to its mRNA level (Figure 5B). Statistically significant differences were found in beet armyworm mortality among different *cry1Ca* plants in the three independent bio-assays.

Plants transformed with the *cry1Ca* and *H04* constructs were analysed in more detail with regard to the correlation between protein expression and beet armyworm resistance. As can be seen in Figure 6, expression of the *cry1Ca* or *H04* gene of at least 0.22 or 0.08%, respectively, of the total soluble protein in shallot leaves is sufficient to give complete resistance against beet armyworm larvae with almost 100% mortality and no visible leaf damage in the bio-assay (Figure 2F). Surprisingly, one plant (transformant 3) which had an undetectable level of H04 protein still resulted in 75.6% larvae mortality (Figure 6). The results obtained confirm earlier observations that the H04 protein is significantly more toxic than the cry1Ca protein (De Maagd et al., 1996, 2000).

Discussion

The present study focuses on an *Agrobacterium*-mediated transfer of *Bt*-genes (*cry1Ca* and *H04*) into two shallot cultivars, namely cvs. Tropix and Kuning, which are highly susceptible to beet armyworm (Zheng et al., 2000). The transgenic plants obtained by this method, and which were cultivated for 1 year in the greenhouse, were found to be vigorous in growth and normal in bulb formation. There were colour differences among leaves of transgenic plants transformed with *cry1Ca* or *H04* as plants with *H04* had brighter leaves than plants transformed with *cry1Ca* and the controls (Figure 2A and B). Another interesting phenomenon was that plants transformed with *cry1Ca* and controls survived more easily the first half year in the greenhouse than plants transformed with *H04* (69 and 100% survival vs. 39% survival). Compared to the Cry1Ca protein, the H04 protein is more toxic to

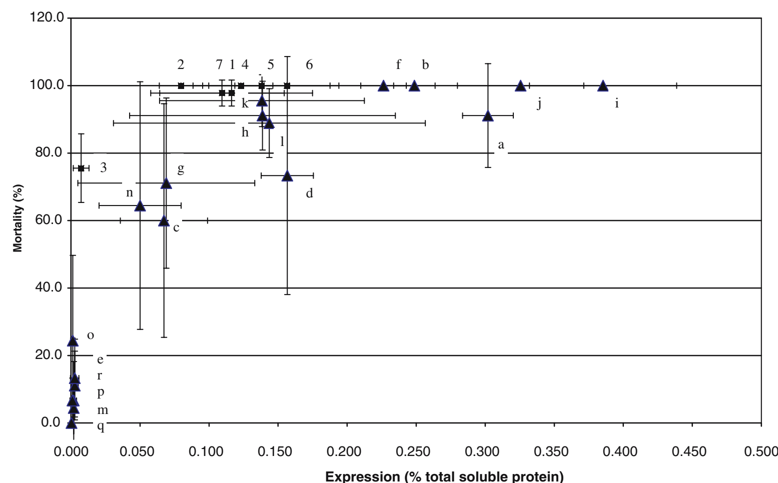

Figure 6. Correlation between *cry1Ca* and *H04* expression (*X*-axis) and *S. exigua* larval mortality (*Y*-axis) in transgenic shallots. Per accession mean Bt toxin expression and mortality for *H04* (×) and *cry1Ca* (▲) and their standard errors (SE; in horizontal and vertical bars) are indicated.

beet armyworm larvae, however, based on our results the *H04* gene seemed also to have a negative effect on plants. This negative effect is even more pronounced in garlic where all transgenic plants harbouring the *H04* gene died after transplanting to the greenhouse, whereas *cry1Ca* transgenic plants survived (Zheng et al., 2004).

The mean transformation frequency of the present transformation experiments was 3.68% and this was considerably higher compared to our previous experiments where a mean transformation frequency of 0.57% was found (Zheng et al., 2001a). Standard PCR, adaptor ligation PCR and Southern analyses of the hygromycin resistant plants confirmed integration of T-DNA into the shallot genome. In this context, we found that the number of bands hybridizing to the *gusA* (RB) and the *hpt* (LB) probes could be different (Figures 3 and 4). In the *H04* and *Cry1Ca* plants where this phenomenon occurred, this finding could be explained by integration of T-DNA into the shallot genome as a head-to-head tandem repeat. Some truncation cannot be excluded here. Using the same basic plasmid pCAMBIA1301 for rice transformation, Sallaud et al. (2003) also found that the number of bands hybridizing to the *gusA* (RB) and the *hpt* (LB) probes were different.

Northern blot and ELISA analyses of RNA and proteins from the transgenic plants respectively confirmed the expression of the *cry1Ca* or *H04* gene in shallot plants (Figures 5 and 6). The variation observed in the amount of *Bt*-gene (*cry1Ca* or *H04*) expressed in different transformants, suggests that the transgene is integrated at different transcriptionally active sites in the shallot genome. In one plant, (transformant e) no band was detected in Northern analysis suggesting interrupted transcription of *cry1Ca* (Figure 5B). The other five plants (transformants m, o, p, q and r) had undetectable levels of Cry1Ca proteins suggesting interrupted translation (Figure 6). All seven plants transformed with the *H04* gene had active transcription of the transgene and all but one (transformant 3) had good levels of H04 protein translation. A strong correlation between insect resistance and protein expression was found in this study. The results demonstrated that the expression of the *Bt*-gene (*cry1Ca* or *H04*) in transgenic shallot plants resulted in a substantial resistance against beet armyworm. In general, the amount of Cry1Ca expressed in transgenic plants was higher than the expression levels of H04 (Figure 6). In our hands, up to 0.39 and 0.16% expression of the Cry1Ca and H04 protein was reached, respectively. The expression of the *H04* and *Cry1Ca* genes to 0.08 and 0.22% of total soluble protein, respectively, or more in shallot leaves is already sufficient to give a complete resistance against beet armyworm. Using an *in vitro* assay on three separate dates, we showed that the *cry1Ca* and *H04* genes functioned satisfactorily as transgenic shallots were not affected by beet armyworm

when they had detectable amounts of the Cry1Ca or H04 protein present (Figure 6). Remarkably, transgenic plants harbouring the *H04* gene, even without a detectable amount of the H04 protein (TC28-1), still proved to be highly resistant to the beet armyworm.

We produced transgenic shallot plants harbouring the *cry1Ca* and *H04* genes which are completely resistant to beet armyworm. The *cry1Ca* and *H04* transgenic plants grew well in the greenhouse, had a normal phenotype and produced bulbs, although the leaves of the *H04* transgenic plants were lighter than their *cry1Ca* counterparts. The transgenic shallot plants developed in this study may form the basis of an improved (sub) tropical shallot cultivation in which beet armyworm can be controlled in a better way then hitherto possible.

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