A hybrid *Bacillus thuringiensis* delta-endotoxin gives resistance against a coleopteran and a lepidopteran pest in transgenic potato

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Summary

Expression of *Bacillus thuringiensis* delta-endotoxins has proven to be a successful strategy for obtaining insect resistance in transgenic plants. Drawbacks of expression of a single resistance gene are the limited target spectrum and the potential for rapid adaptation of the pest. Hybrid toxins with a wider target spectrum in combination with existing toxins may be used as tool to mitigate these problems.

In this study, Desiree potato plants were genetically modified to resist attack by insect species belonging to the orders Coleoptera and Lepidoptera, through the insertion of such a hybrid gene, SN19. Transgenic plants were shown to be resistant against Colorado potato beetle larvae and adults, potato tuber moth larvae, and European corn borer larvae. These are the first transgenic plants resistant to pests belonging to two different insect orders. In addition, the target receptor recognition of this hybrid protein is expected to be different from Cry proteins currently in use for these pests. This makes it a useful tool for resistance management strategies.

Keywords: *Bacillus thuringiensis*, Colorado potato beetle, European corn borer, hybrid Cry1 protein, potato tuber moth, transgenic potato plants.

Introduction

The coleopteran Colorado potato beetle (*Leptinotarsa decemlineata* Say; CPB) and the lepidopteran potato tuber moth (*Phthorimaea operculella* Ziller; PTM) are some of the most destructive pests of cultivated potato. Their life cycle, feeding habits and demonstrated ability to develop resistance to chemical insecticides have made the control of CPB and PTM an increasing agricultural problem (Forgash, 1985; Trivedi and Rajagopal, 1992). Another important agricultural pest on potato is the European corn borer (*Ostrinia nubilalis* Hübner, ECB) (Hanzlik et al., 1997). At the present time the control of these insect pests is accomplished primarily by the use of chemical insecticides, through the use of different insecticidal Cry proteins originating from *Bacillus thuringiensis* in sprays, or by the expression of Cry proteins in transgenic plants.

The *cry* gene family is a large, still growing family of homologous genes, with each gene encoding a protein active on insect larvae of a subset of species usually belonging to the same order (Schnepf et al., 1998). Cry1 proteins are generally active against lepidopterans (larvae of moths and butterflies). Cry1Ba also has some activity against coleopterans (beetles), although its toxicity for CPB is much lower than that of Cry3Aa, the most active natural toxin (Bradley et al., 1995). Somewhat higher activity against CPB was reported for Cry1Ia (Tailor et al., 1992). A *cry1Ba/cry1Ia* hybrid gene (SN19) encoding a protein consisting of domains I and III of Cry1Ba and domain II of Cry1Ia, with high activity against CPB was constructed and described earlier by us (Naimov et al., 2001). Both parental proteins, Cry1Ba and Cry1Ia, are highly toxic for European corn borer and potato tuber moth (Van Frankenhuysen and Nystrom, 2002), suggesting that the hybrid protein may also have these properties.

Cry toxins have been expressed in a number of plant species. Expression of one member of this family usually results in resistance against a single pest insect or against a few relatively closely related insect species within one order. Cry3Aa-expressing potatoes with resistance to CPB (Perlak et al., 1993), and Cry1Ia-expressing potatoes with resistance to PTM (Douches et al., 2001) are examples of this.
In this manuscript we describe production of the first transgenic potato plants with leaves protected against attack by a number of insect pests, which are members of two different orders – Coleoptera (CPB) and Lepidoptera (PTM and ECB) – by expression of the previously described SN19 hybrid gene under the control of a chrysanthemum ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (Rubisco SSU) promoter and terminator.

Results

Domain II modification and plant transformation vectors

For the reconstruction of the gene encoding the toxic fragment of SN19, a synthetic and truncated (2090 bp) cry1Ba gene (Desai, 1999), optimized for expression in plants, was used as the scaffold for insertion of the domain II-encoding part of cry1Ba. A sequence analysis of this domain II-encoding part identified a number of potential RNA instability elements and polyadenylation sites (Figure 1A). By a combination of PCR with mutagenic primers and recombination of overlapping fragments via PCR (overlap extension) (Figure 1B) 28 single nucleotides were changed, eliminating putative polyadenylation sites, mRNA instability sequences, and consecutive C + G and A + T stretches. The mosaic SN19 with unmodified domain II-encoding DNA, and SN19 with modified domain II-encoding DNA, were cloned between a promoter and terminator fragment derived from the chrysanthemum Rubisco SSU gene (obtained from N.S. Outchkourov, manuscript in preparation) in the binary transformation vector pBINPLUS. This resulted in transformation vectors pSN32 and pSN48, respectively. These expression cassettes were introduced in potato cultivar ‘Desiree’ by Agrobacterium tumefaciens-mediated transformation. Sixteen and 20 transgenic potato lines per construct, respectively, were obtained and successfully adapted to greenhouse conditions.

Insect resistance of transgenic plants

Leaves of transgenic plants were analysed by immunochemically estimating the expression of the protein, and by testing for resistance to CPB larvae in a single bioassay. As expected, the highest accumulation of the protein of interest was obtained with the fully modified hybrid gene. The use of the strong green tissue-specific promoter of the chrysanthemum Rubisco SSU in combination with a modification of the open reading frame resulted in levels of SN19 protein which were as high as 0.25% of total soluble plant protein. None of the 16 plants transformed with SN32 (unmodified domain II) had detectable Cry protein expression (lower detection limit = 0.025% of total soluble leaf protein), although presence of the SN19 gene could be demonstrated by PCR (results not shown). Only the plants transformed with SN48 (modified domain II) expressing detectable levels of SN19 showed some level of resistance to neonate CPB larvae, with 10 of the 20 transformed lines leading to more than 80% CPB mortality.

Plants transformed with the SN48 construct were analysed in more detail with regard to the correlation between protein expression and CPB resistance. As can be seen in Figure 2, expression of the modified SN19 (construct SN48) gene to 0.2% or more of total soluble protein in potato leaves is sufficient to give complete resistance against CPB larvae with 100% mortality and no visible damage in a leaf feeding assay (Figure 3A,E). Two of the SN48 lines (lines 8 and 11, having on average 0.25% and 0.23% Cry protein expression, respectively) were tested for resistance against adult CPB. In contrast to control leaves, the transgenic leaves were completely undamaged, even after 10 days (Figure 3B,F). After this period, most of the Colorado potato beetles which had been placed on transgenic potato leaves were still alive, but not feeding and smaller in size than control beetles.

The SN48 lines 11 and 16 (having on average 0.23% and 0.25% Cry protein expression, respectively) were tested for resistance against potato tuber moth and European corn borer – both Lepidopteran pests. Leaf infestation with 10 neonate PTM larvae, in three separate experiments resulted in extensive tunnelling by four or five live larvae in control leaves after 4 days (Figure 3C). In contrast, no live PTM larvae could be recovered from the SN19-expressing plants in all three experiments. No signs of tunnelling or other visible damage were recorded in these leaves (Figure 3G). Control leaves infested with 2-day-old ECB larvae in three separate experiments resulted in tunnelling in the leaf stem with subsequent wilting of the leaves. ECB mortality was zero after 3 days in control leaves (Figure 3D). In contrast, SN19-expressing leaves remained healthy, and caused a 100% mortality of the ECB larvae in all three experiments. Only small wounds on the leaf surface, presumably from feeding attempts, could be observed here (Figure 3H).

Discussion

Although current transgenic plants expressing a Cry protein are effectively protected against one or a few relatively related pests, their activity spectrum is limited.

Expression of the SN19 gene in potato was an approach designed to prove our hypothesis that the effective expression of a single hybrid delta-endotoxin gene could provide effective resistance against a coleopteran and a lepidopteran pest simultaneously. Expression of SN19 resulted in complete protection of transgenic potato leaves against CPB larvae and adults, PTM larvae, and ECB larvae. A strong correlation between insect resistance and SN19 expression was found for CPB larvae. In our hands, up to 0.25% expression of SN19 was reached, which was higher than the protein level required for complete plant protection. In agreement with results reported in previous studies (Perlak et al., 1991) we...
found that a significant modification of the *Bacillus thuringiensis* delta-endotoxin encoding hybrid gene *SN19* was necessary for successful expression in plants. Although the optimization of codon usage in the domain II encoding part could possibly enhance expression of the transgene even further (Perlak et al., 1991), in our case the partial modification of domain II seems to be sufficient for expression of the *SN19* hybrid gene in potatoes. We conclude that the expression of *SN19* in transgenic potato plants could provide excellent protection against several major potato pests in the field. Whereas an expanded host range has economic advantages, it may also have disadvantages in the form of increased effects on nontarget and/or beneficial insects. Pre-release testing for these effects would have to be included in the safety assessment of any such crop, as indeed it was for already commercialized insect-resistant transgenic crops.

The relatively low homology of *SN19* with Cry3Aa and Cry1Ab suggests that *SN19* may bind to midgut receptors that are different from those for Cry3Aa in CPB and for Cry1Ab in PTM or ECB, respectively. Indeed, it has been shown that Cry1Ba and Cry1Ab do not compete for the same binding site in ECB (Denolf et al., 1993) and PTM (Escríche et al., 1994). In contrast, the more homologous Cry1Aa, Cry1Ab and Cry1Ac show a high degree of overlap of binding specificities in many insects, including PTM (Escríche et al., 1997) and ECB (Denolf et al., 1993; Hua et al., 2001). Changes in toxin binding sites is the most commonly occurring resistance mechanism against Cry proteins in insects (Ferré and Van Rie, 2002), and occur in Cry3Aa-resistant CPB (Loseva et al., 2002). For this reason ‘pyramiding’ or ‘stacking’ of two genes encoding proteins with different receptor

![Figure 2](image1.png)

**Figure 2** Correlation between delta-endotoxin SN19 expression and CPB larval mortality of transgenic potato plants in independent SN48 transgenic lines expressing the *SN19* hybrid gene with modified domain II-encoding DNA. Only two of the lines with undetectable protein levels are shown. Error bars depict standard deviation from the means of three experiments.

![Figure 3](image2.png)

**Figure 3** Leaf feeding assays comparing control lines (A–D) and SN19-expressing (E–H) line 11 (expressing 0.23% of total soluble protein). A, E: CPB larvae; B, F: CPB adults; C, G: PTM larvae; D, H: ECB larvae.
recognition properties (Roush, 1998), or deploying mixtures of seeds with two different toxins (Caprio, 1998) have been considered as resistance management strategies. Cry7 and Cry8, which have relative low homology with Cry3’s, and which have been shown to be active against CPB (Van Frankenhuysen and Nystrom, 2002) may be alternative ‘second genes’ for resistance management, but in contrast to SN19 their use in transgenic plants has not so far been demonstrated. Although the use of a single hybrid toxin would not have an advantage over the use of a single wild type toxin, SN19 may well play the role of the second toxin for resistance management, simultaneously with Cry1Ab against ECB and PMT and with Cry3Aa against CPB in transgenic potatoes. This would reduce the number of transgenes necessary to be transferred from four to three.

Experimental procedures

Modification of domain II of cry11a

For the modification of DNA motifs with a possible negative effect on gene expression in plants, site directed mutagenesis and recombination via PCR were performed. Eight different primer sets (Eurogentec) containing the desired nucleotide changes, the following modifications of a previously described protocol (Ho et al., 1989) were made: (i) four different DNA fragments were assembled during the first combinatorial PCR, and (ii) the amplified product from the first combinatorial PCR was used as a template for a second combinatorial PCR, and (ii) the amplified product from the first combinatorial PCR was used as a template for a second combinatorial PCR (see Figure 1B). The PCR products were separated on 0.8% agarose gel and purified using a QIAEX II agarose gel extraction kit (Qiagen). For all PCR steps, Pfu-Turbo DNA polymerase (Stratagene) was used. After the final amplification step the product was digested with MunI at bp 890, and Rsrl at bp 1480. The obtained transgenic lines were subsequently multiplied and adapted to greenhouse conditions: 25 °C and a 16 h light/8 h dark cycle.

Insect bioassays

All bioassays were performed on detached fully grown potato leaves stuck in water agar. For the CPB bioassays, 10 neonate larvae were placed on the upper leaf surface. After 2 days, the leaves were replaced by fresh ones, and mortality was scored after 4 days. For adult CPB, four newly emerged insects were placed on leaves for up to 10 days. Potato tuber moth bioassays were performed as described earlier (Mohammed et al., 2000) by placing 10 neonate larvae on the back surface of potato leaves. For testing of European corn borer resistance, 2-day-old larvae were used, grown on an artificial diet at 28 °C. Larvae were allowed to feed on potato leaves for 2 days. Plants transformed with the empty pBINPLUS vector were used as a negative control. All bioassays were performed threefold on separate dates.

Construction of binary vectors

A BamHI–BglII fragment containing the truncated, synthetic cry1Ba gene (2080 bp) (Desai, 1999) was cloned into a BglII-site inserted in the SacI-site of pBluescript SK+ vector. Using the Quick-Change™ Site Directed Mutagenesis Kit (Stratagene), three restriction sites were subsequently introduced: BspHI at base 1, MunI at bp 890, and Rsrl at bp 1480. The resulting pSN23 plasmid was subsequently used for reconstruction of the SN19 hybrid encoding gene by replacement of the domain II-encoding part with the corresponding part of cry1la as a MunI–Rsrll fragment (Naimov et al., 2001) with or without modifications, giving pSN45 and pSN24, respectively. The open reading frames were cut from pSN24 and pSN45 by BspHI and Bgll digestion and ligated into the Ncol–BglII sites of pUCRBC1, between the Chrysanthemum ribulose-1,5-bisphosphate carboxylase/ oxygenase small subunit promoter and terminator (N.S. Ouchkhourov, manuscript in preparation). Resulting expression cassettes RBC-unmodified SN19 (pSN28) and RBC-modified SN19 (pSN46), were cloned in pBINPLUS (van Engelen et al., 1995) using the HindIII and EcoRI sites flanking the cassettes. Resulting binary vectors pSN32 and pSN48, respectively, were subsequently introduced in Agrobacterium tumefaciens strain Agl0 (Lazo et al., 1991) by electroporation (Mersereau et al., 1990). A. tumefaciens mediated potato transformation was performed following the previously described protocol (Lauterslager et al., 2001). The obtained transgenic lines were subsequently multiplied and adapted to greenhouse conditions: 25 °C and a 16 h light/8 h dark cycle.

Protein quantification

Leaf tissue (0.2 g) was ground with 400 μL of extraction buffer (50 mM NaOH, 20 mM Na2SO4, 5 mM EDTA and 10% Polyvinilpoly pyrrolidone), subsequently neutralized with 80 μL 1 M Tris-HCl, pH 5.5., and centrifuged at 16 000 g for 10 min. The supernatant was transferred into a new Eppendorf tube and additionally centrifuged at 16 000 g for 10 min. Protein concentrations in the supernatant were determined by the Bradford method (Bio-Rad Laboratories).
The amount of Cry protein of interest was estimated by dot-blot analysis as follows. Equal amounts of soluble leaf protein (20 μg) were transferred to a nitrocellulose membrane using an S&S Minifold Dot blotter (Schleicher & Schuell). Immunological detection was performed by treating the membrane with blocking solution containing Tris buffered-saline (TBS: 10 mM Tris-HCl, pH 7.6, 150 mM NaCl), 5% (w/v) non-fat dry milk, and 3% (w/v) Bovine serum albumin for 1 h, washed three times with TBST buffer (TBS buffer, with 0.2% Tween-20). 1 : 1000 diluted anti-Cry1Ba serum was applied and the membrane was incubated for 1 h at room temperature. After three washing steps with TBST, alkaline phosphatase conjugated anti-rabbit IgG (Sigma-Aldrich) was added (1 : 1000) and incubated for 1 h. The membranes were washed three times with TBST buffer, and once with carbonate buffer (0.1 M NaHCO₃, 1.0 M MgCl₂, pH 9.8). After 15 min incubation with 50 mL carbonate buffer, the membranes were developed with 0.015% (w/v) 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) and 0.03% (w/v) Nitro Blue Tetrazolium (Sigma-Aldrich) in carbonate buffer. Serial dilutions of trypsin-activated SN19 in phosphate buffered saline (10 mM Na₂HPO₄/KH₂PO₄, 0.8% (w/v) NaCl) added to negative control plant extracts were used for comparison and estimation of SN19 content in leaf tissues.

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