HYBRID BT TOXINS FOR COLORADO POTATO BEETLE CINTROL

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ABSTRACT. Cry1 delta-endotoxins of *Bacillus thuringiensis* are generally active against lepidopteran insects, but Cry1Ba and Cry1Ia have additional, though low activity against coleopterans such as Colorado Potato Beetle. Here we report the construction of Cry1Ba/Cry1Ia-hybrid toxins, which have increased activity against this insect species.

Desiree potato plants were genetically modified to resist insect attack by insect species belonging to the orders Coleoptera and Lepidoptera, through 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.

KEY WORDS. transgenic potato plants, hybrid Cry1 protein, Bacillus thuringiensis, Colorado potato beetle, potato tuber moth, European corn borer

INTRODUCTION

Bacillus thuringiensis (Bt) is a gram-positive bacterium, which produces insecticidal crystal proteins during sporulation. The crystal (Cry) proteins form a large family of homologous, but different proteins with unique specificities. Each protein is active against only one or a few insect species (for review see: (4))

Proteins of the Cry3 (Herrnstadt, Gilroy et al. 1987; McPherson, Jurnak et al. 1987), Cry7 (Lambert, Hofte et al. 1992) and Cry8 (Sato, Takeuchi et al. 1994)

classes are active against insects of the order Coleoptera (beetles and weevils). Cry3Aa is the most active natural protein for the important potato pest, *Leptinotarsa decemlineata* (Say) (Colorado Potato Beetle; CPB). Cry1 proteins are generally active against lepidopterans. However, Cry1Ba and Cry1Ia have been shown to also have activity against coleopterans, although their toxicity for CPB is much lower than that of Cry3Aa (Bradley, Harkey et al. 1995), (Tailor, Tippett et al. 1992).

Cry proteins are formed as protoxins, which are activated by proteases of the insect gut. This involves cleavage of an N-terminal peptide, and in the larger Cry1 protoxins (but not in Cry3), of a C-terminal extension. Structure determination by X-ray cristallography has shown that the activated toxins of different, and probably most Cry proteins, share a common three-domain structure (Grochulski, Masson et al. 1995), (Li, Carroll et al. 1991). The N-terminal domain I is considered to be inserting into the target membrane, and forming part of the pores that eventually kills the target insect's gut epithelial cells. Both domain II and the C-terminal domain III are more variable, and have been shown to be main determinants of activity against specific insects. Although it is not yet clear how these domains may individually or acting together determine specificity, there is strong evidence that both can be involved in binding to receptors (Burton, Ellar et al. 1999), (de Maagd, Bakker et al. 1999), (de Maagd, van der Klei et al. 1996), (Dean 1984), (Lee, Young et al. 1995). Exchange of domain III between toxins, for example by *in vivo* recombination of their encoding genes may alter specificity of a toxin. Additionally, it may also result in a hybrid toxin with toxicity for certain insects, which is higher than that of its parent toxins (de Maagd, Kwa et al. 1996). So far, this latter phenomenon has only been described for lepidopteran insects. In this paper we show not only that combination of parts of Cry1Ba and Cry1Ia resulted in hybrids with increased activity against CPB, which is a coleopteran, but also that SN19 hybrid gene can be efficiently used for insect control in transgenic potato plants. Our results clearly demonstrates that SN 19 hybrid still coffers resistance against important lepidopteran pests such as potato tuber moth (*Phthorimaea operculella* Ziller; PTM) and European corn borer (*Ostrinia nubilalis* Hübner, ECB).

MATERIAL AND METHODS

Expression vectors. All used Cry protein expression vectors are based on pBD12, a derivative of pKK233-2 (1). Cry1Ba expression vector pMH19 has been described earlier (de Maagd, Bakker et al. 1999). Cry1Ia expression vector pBD172 contains the full cry1Ia gene with the SpeI-site (nucleotide 2180) fused to the SpeI-site in the polylinker in pBD12, which is derived from pBluescript SK$^+$. For Cry3Aa expression, the cry3Aa gene was given a NcoI-site at its start by site directed mutagenesis. A NcoI-XmnI-fragment (nucleotides 1-1935) was combined with a XmnI-BglII-linker, restoring the full coding region, and was used to replace the cry1Ab gene in expression vector pBD1400 (de Maagd, Bakker et al. 1999), giving Cry3Aa expression vector pMH10.

Modification of domain II of *cry1Ia*. For 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 mutations were used (Fig. 3A). In order to introduce multiple nucleotide changes, the following modifications of the previously described protocol were made: 1) Four different DNA fragments were assembled during the first combinatorial PCR and 2) The amplified product from the first combinatorial PCR was used as a template for a second round of primary PCRs, followed by a second combinatorial PCR (see Fig. 3B). 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* and *RsrII* and used for replacement of the corresponding part of SN23 (see below), resulting in SN45, and sequenced in both directions.

Construction of binary vectors. A *BamHI-BglII* fragment containing the truncated, synthetic *cry1Ba* gene (2080 bp) (Desai, 1999) was cloned into a *Bgl*II-site inserted in the *Sac*I-site of pBlueskript SK$^+$ vector. Using the Quick-Change^{TM} Site Directed Mutagenesis Kit (Stratagene), three restriction sites were subsequently introduced: *BspHI* at base 1, *MunI* at bp 890, *and RsrII* at bp 1480. The resulting plasmid pSN23 was subsequently used for reconstruction of the SN19 hybrid encoding gene by replacement of the domain II-encoding part with the corresponding part of *cry1Ia* as a *MunI-RsrII* 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 *BglII* digestion and ligated into the *NcoI-BglII* sites of pUCRBC1, between the Chrysanthemum ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit promoter and terminator. Resulting expression cassettes RBC-unmodified *SN19* (pSN28) and RBC-modified *SN19* (pSN46), were cloned in pBINPLUS using the *HindIII* and *EcoRI* sites flanking the cassettes. Resulting binary vectors pSN32 and pSN48, respectively, were subsequently introduced in *Agrobacterium tumefaciens* strain Agl0 by electroporation. *A. tumefaciens* mediated potato transformation was performed following the protocol described previously. The obtained transgenic lines were subsequently multiplied and adapted to greenhouse conditions: 25°C and a 16h-light/8h-dark-cycle.

Protein isolation and insect bioassays. For large-scale production, all parental and hybrid protoxins were expressed in *E. coli* strain XL-1, extracted and solubilized as described earlier (1). Solubilized protoxins were dialyzed overnight against 25 mM NaHCO$_3$, 100 mM NaCl, pH10. Protein concentrations were estimated by SDS-PAGE. To test toxicity to CPB, leaflets of greenhouse grown potato cultivar Desiree plants were dipped in toxin dilutions in water containing 0.01% Tween-20. After drying of the leaves to the air they were transferred to petri dishes and 10 neonate CPB larvae were placed on each leaf. After incubation for two days at room temperature, the leaves were replaced by fresh leaves dipped in identical protoxin dilutions. Mortality was scored after 4 days. Cry3Aa was taken as positive control and for comparison. LC$_{50}$'s (concentrations giving 50% mortality) and 95% fiducial limits were determined by Probit analysis of results from three or

more independent experiments, using the PoloPC computer program (Russel, Robertson et al. 1977).

All bioassays with transgenic plants were performed on detached fully-grown potato leaves stuck in water agar. For CPB bioassays ten neonate larvae were placed on the upper leaf surface. After two days leaves were replaced by fresh ones, and mortality was scored after 4 days. For adult CPB, 4 newly emerged insects were placed on leaves for up to 10 days. Potato tuber moth bioassays were performed as described earlier (Mohammed, Johnson et al. 1996) by placing of ten neonate larvae on the back surface of potato leaves. For testing of European corn borer resistance two day-old larvae, grown on artificial diet at 28^0C, were used. Larvae were allowed to feed on potato leaves for two days. Plants transformed with the empty pBINPLUS vector were used as negative control. All bioassays were performed three times on separate dates.

Protein isolation from plant material and protein quantification. Leaf tissue (0.2 g) was ground with 400 µl extraction buffer (50mM NaOH, 20mM NaS_2O_5, 5 mM EDTA, and 10% Polyvinylpoly-pyrrolidone), subsequently neutralized with 80 µl 1M Tris-HCl, pH 5.5, and centrifuged at 14,000 rpm for 10 min. The supernatant was transferred into a new eppendorf tube and additionally centrifuged at 14,000 rpm for 10 min. Protein concentrations in 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 a S&S Minifold Dot blotter (Schleicher & Schuell). The immunological detection was performed by treating the membrane with blocking solution containing Tris buffered-saline (TBS: 10 mM TrisHCl, pH7.6, 150 mM NaCl), 5% (w/v) non-fat dry milk, and 3% (w/v) Bovine serum albumin for 1h, 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 1h at room temperature. After three washing steps with TBST, alkaline phosphatase conjugated anti-rabbit IgG (Sigma-Aldrich) was added (1:1000) and incubated for 1h. The membranes were washed three times with TBST buffer, and once with carbonate buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8). After 15 minutes 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 buffed saline (10 mM Na_2HPO_4/KH_2PO_4, 0.8% (w/v) NaCl) added to negative control plant extracts were used for comparison and estimation of SN19 content in leaf tissues.

RESULTS

Cry1Ia/Cry1Ba and Cry1Ba/Cry1Ia-hybrids. In order to be able to directly exchange the domain III encoding regions between *cry1Ba* and *cry1Ia*, a new common restriction enzyme recognition site was made in both genes by site directed mutagenesis. Complementary mutagenic oligonucleotide pairs were used to create

unique *Rsr*II-sites at positions 1464 and 1488 of *cry1Ba* (pMH19) and *cry1Ia* (pBD172), respectively, using the QuickChange™ kit (Stratagene) without changing the encoded amino acid sequences (Fig. 1). These unique restriction sites allowed swapping of *Nco*I-*Rsr*II-fragments between pSN17 (*cry1Ba*) and pSN18 (*cry1Ia*). This resulted in plasmids pSN15 and pSN16, encoding hybrids with domain compositions 1Ia/1Ia/1Ba and 1Ba/1Ba/1Ia-hybrid, respectively (Fig 2). pSN19, encoding a hybrid with domain composition 1Ba/1Ia/1Ba, was made by replacing a *Nco*I-*Mun*I (nucleotide 1-896) fragment encoding domain I of Cry1Ia in pSN15 by the corresponding fragment encoding domain I of Cry1Ba (nucleotide 1-869) derived from pSN16 (Fig. 3B).

In vitro **toxicity assays.** In order to test insecticidal utilities of newly constructed hybrid proteins all of them listed above were tested against neonate Colorado potato beetle larvae. Cry1Ba/Cry1Ia-hybrid protein SN16 had very low toxicity against CPB, and not enough protoxin was purified to determine a reliable LC_{50}. Surprisingly, Cry1Ia/Cry1Ba-hybrid SN15 was more toxic than both the two parent proteins. When the size differences are taken into consideration, SN15 was approximately 2.5 times more toxic than Cry1Ia, and 7.5 times more toxic than Cry1Ba, on a per mol basis (fig.2). This indicates that domains I or II of Cry1Ia, or both, are important determinants of the higher activity of Cry1Ia compared to that of Cry1Ba. However, the combination with domain III and the protoxin-specific C-terminal part of Cry1Ba renders the resulting hybrid even more toxic than Cry1Ia. Therefore as was earlier reported for Cry1 activity against lepidopterans, also for coleopterans hybrid toxins can have improved activity. The additional domain I substitution giving a mosaic 1Ba/1Ia/1Ba-hybrid (SN19) increased the toxicity even further, resulting in activity approaching that of Cry3Aa against Colorado Potato Beetle.

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 scaffold for insertion of the domain II-encoding part of *cry1Ia*. Sequence analysis of this domain II-encoding part identified a number of potential RNA instability elements and polyadenylation sites (Fig. 3A). By combination of PCR with mutagenic primers and recombination of overlapping fragments via PCR (overlap extension) (Fig. 3B) twenty-eight 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. 16 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 analyzed by imunochemically estimating the expression of the protein, and by testing for resistance to CPB larvae in a single bioassay. As expected the highest accumulation of protein of interest was obtained with the fully modified hybrid gene. Use of a strong green tissue-specific promoter as that of the chrysanthemum Rubisco SSU in combination with modification of the open reading frame resulted in levels of SN19 protein 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 the 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 then 80% CPB mortality.

Plants transformed with construct SN48 were analyzed in more detail with regard to the correlation between protein expression and CPB resistance. As can be seen in Fig. 4, expression of the modified *SN19* (construct SN48) gene to 0.2% of total soluble protein or higher in potato leaves is sufficient to give complete resistance against CPB larvae with 100% mortality and no visible damage in a leaf feeding assay (Fig. 5A, B). 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 ten days (Fig. 5C, D). After this period most of the Colorado potato beetles, placed on transgenic potato leaves, were still alive, but not feeding and smaller in size then 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 tunneling by 4 or 5 live larvae in control leaves after 4 days (Fig. 5E). In contrast no live PTM larvae could be recovered from the SN19-expressing plants in all three experiments. No signs of tunneling or other visible damage were recorded in these leaves (Fig. 5F). Control leaves infested with 2 day-old ECB larvae in three separate experiments resulted in tunneling in the leaf stem with subsequent wilting of the leaves. ECB mortality was zero after three days in control leaves (Fig. 5G). In contrast, SN19-expressing leaves remained healthy and caused 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 (Fig. 5H).

DISCUSION

So far new combinations of the receptor binding domains II and III resulted in increased activity of hybrids for several lepidopterans (6, 10). The molecular mechanisms underlying this effect are not well understood, although studies of Cry1Ac binding to a putative receptor, *Lymantria dispar* aminopeptidase N suggested that domain II and domain III confer two separate steps in binding to this protein in a

two-step model, and that one step may be rate-limiting for that binding. Following this line of reasoning, one could speculate that both Cry1Ba and Cry1Ia bind to the same receptor in CPB, but that different steps are rate-limiting for the two toxins. Hence, the proper combination of domains II and III may optimize both binding steps and thus increase activity. Furthermore, the combination of these domains with domain I of Cry1Ba is more active. Since Cry1Ba and SN19 both have extended protoxin-specific C-terminal extensions compared to Cry1Ia, a role of this extension in the higher toxicity of SN19 cannot yet be excluded. This however would be contrary to the findings of Lambert *et al.* for Cry7Aa, which was found to be active against CPB only after solubilization and activation (Lambert, Hofte et al. 1992). In this study we have tested solubilized protoxins, while it was shown earlier for Cry1Ba that solubilization prior to testing was necessary for high activity against CPB (Bradley, Harkey et al. 1995). This may be caused by the relatively low gut pH (6-7) in CPB, compared to that in lepidopterans, inhibiting solubilization of the crystalline protoxin. Whether this would prevent effective application of SN19 in a crystalline form, and whether SN19 may be an alternative for Cry3Aa in CPB-resistant transgenic plants is subject of our further studies

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 to prove our hypothesis that an 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 is higher than the protein level required for complete plant protection. In agreement with results reported in previous studies 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 the codon usage in the domain II encoding part could possibly enhance the expression of the transgene even further 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 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 non-target 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 as well as with Cry1Ab suggests 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 (Escriche, Tabashnik et al. 1995). In contrast, the more homologous

Cry1Aa, Cry1Ab, and Cry1Ac show a high degree of overlap of binding specificities in many insects, including PTM (Escriche, Ferre et al. 1997), and ECB (Denolf, Hendrickx et al. 1997), (Hua, Masson et al. 2001). Changes in toxin binding sites is the most commonly occurring resistance mechanism against Cry proteins in insects (Ferre and Van Rie 2002), and occur in Cry3Aa-resistant CPB (Loseva, Ibrahim et al. 2002). For this reason „pyramiding „or „stacking" of two genes encoding proteins with different receptor recognition properties or deploying mixtures of seeds with two different toxins are 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 may be alternative „second genes" for resistance management, but in contrast to SN19 their utility in transgenic plants has not been demonstrated so far. 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 PTM and with Cry3Aa against CPB in transgenic potatoes. This would reduce the number of necessary transgenes to be transferred from four to three.

ACKNOWLEDGMENTS

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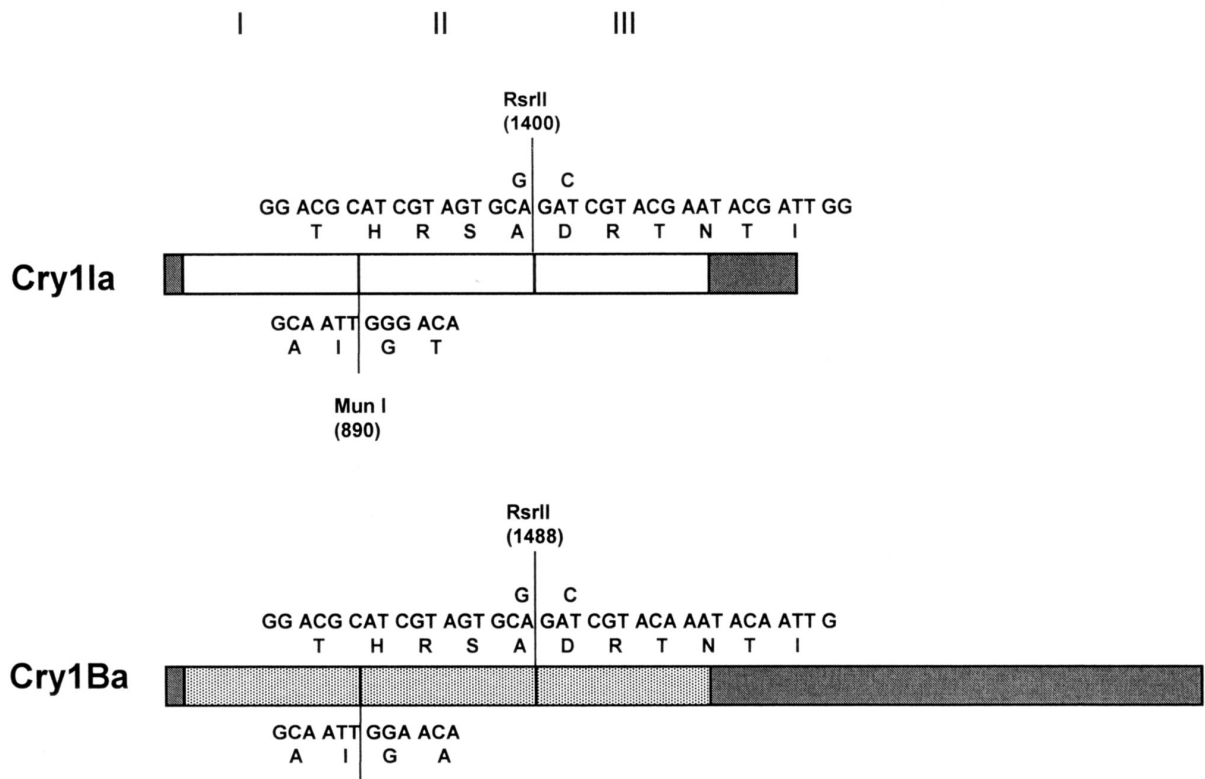
FIGURE

Fig. 1. A. *Oligonucleotides used to create RsrII-sites in the cry1Ba and cry1IAa genes by mutagenesis, and the amino acids encoded by the respective parts of the genes. The two mutated nucleotides of the wild type genes are shown respectively above and below the oligonucleotide sequences.* **B.** *localization of the common MunI site in cry1Ba and cry1Ia genes and the amino acids encoded by the respective parts of the genes.*
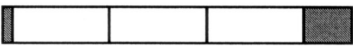
Fig. 2. *Domain compostion and insecticidal activity of Cry1Ia/cry1Ba hybrids and their parental protoxins. [1] Bioassays were performed with solubilized protoxins on potato leaves with neonate CPB. Concentrations are expressed as μg/ml in dipping solution. 95% fiducial limits are shown in parentheses. ND= not determined*
[2] Toxicity relative to that of Cry3Aa on a per mol basis: (MW of protein/MW of Cry3Aa) x (LC$_{50}$ of Cry3Aa/LC$_{50}$ of protein) x 100.
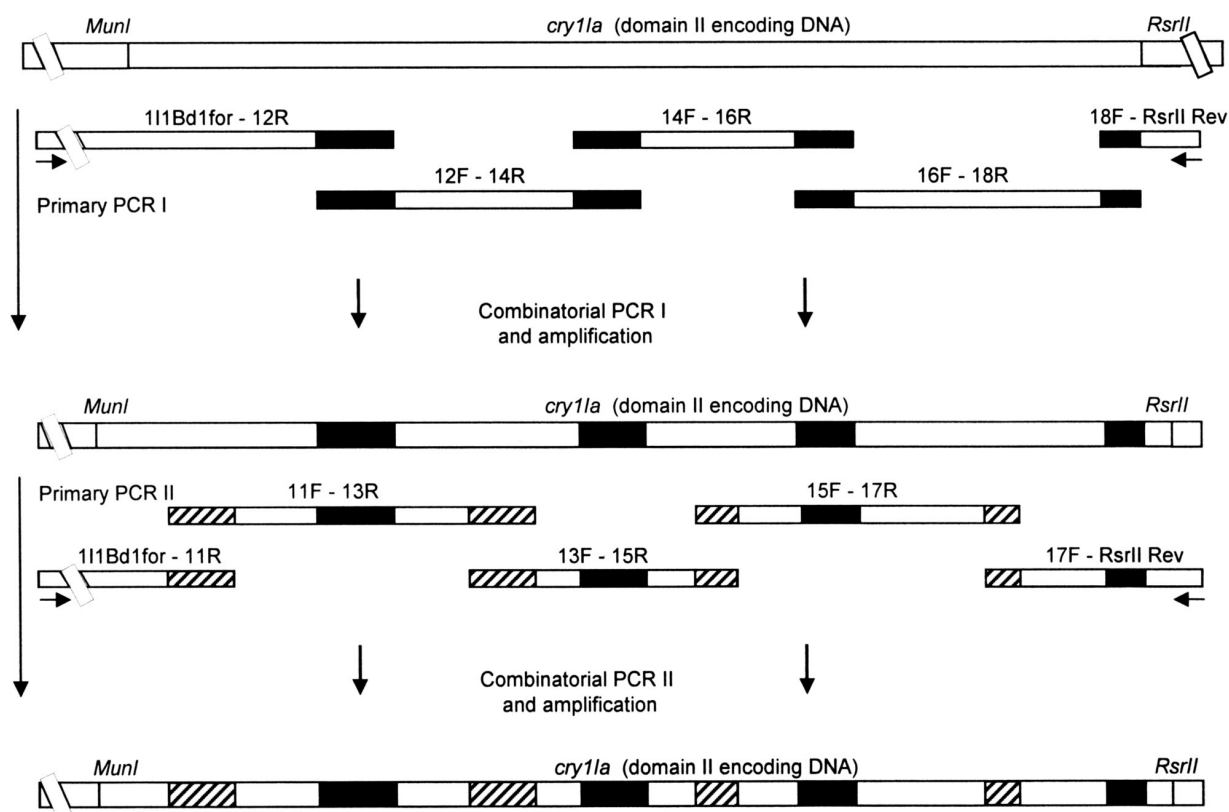
Fig. 3. *Modification of domain II of the SN19 hybrid gene. A. Sequence of the cry1Ia domain II-encoding fragment and (below) the primers used for mutagenesis. Only the nucleotides to be altered are shown in the primer sequence. B. Mutagenesis and recombination via PCR. In separate amplifications four fragments of the SN19 gene were amplified using mutagenic primers. Denatured fragments annealing in the overlapping primer regions were extended. This resulted in a full-length product, which was amplified using the two outside primers (1B1Id1for, 1IRsRIIRev), and subsequently used as template for the second round of mutagenic PCR reactions.*
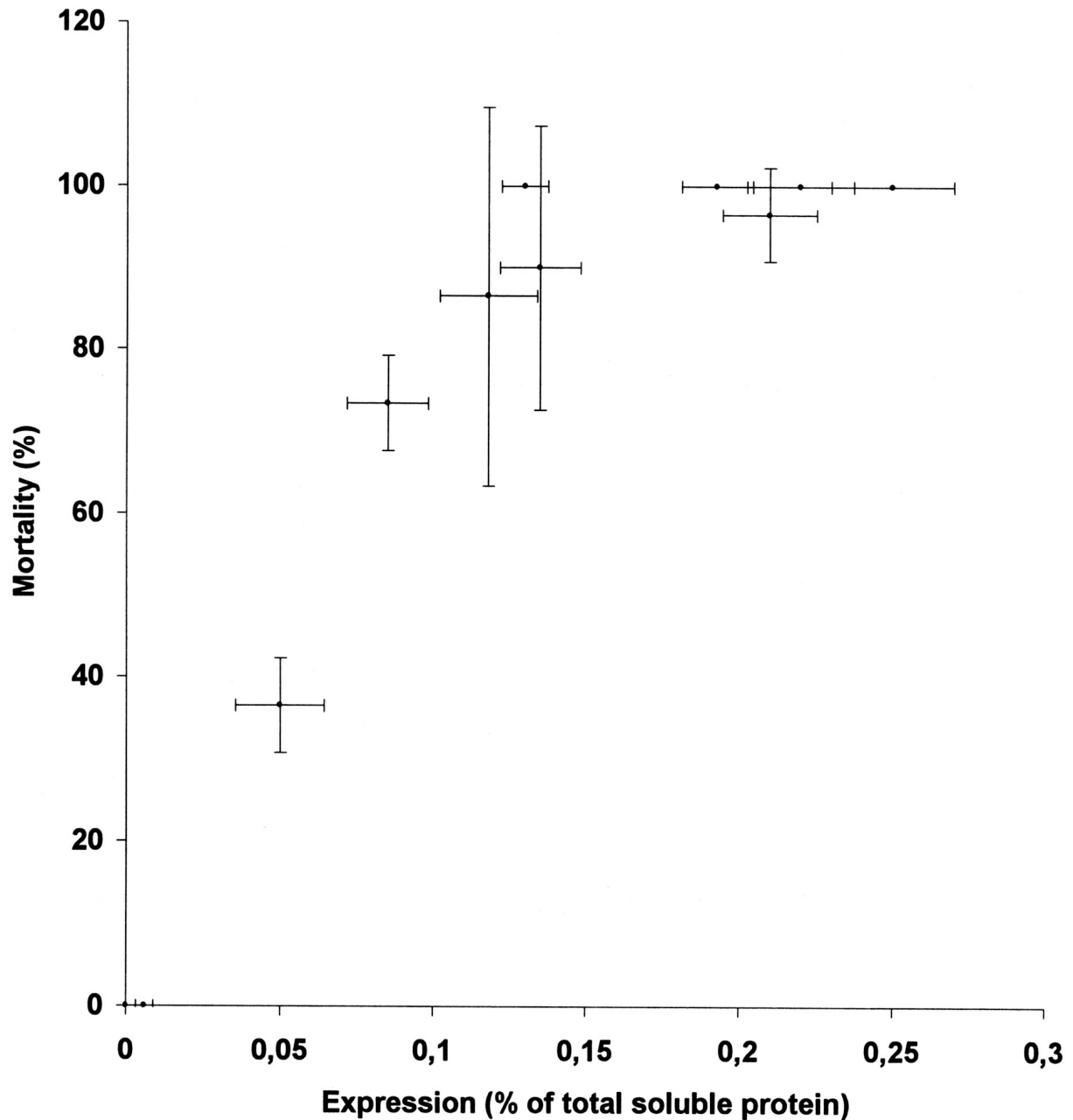
Fig. 4. *Correlation between delta-endotoxin SN19 expression and CPB resistance of transgenic potato plants in independent SN48 transgenic lines expressing the SN19 hybrid gene with modified domain II-encoding DNA. Only two of the lines with undetectable protein levels are shown. Error bars depict standard deviation from the means of three experiments.*
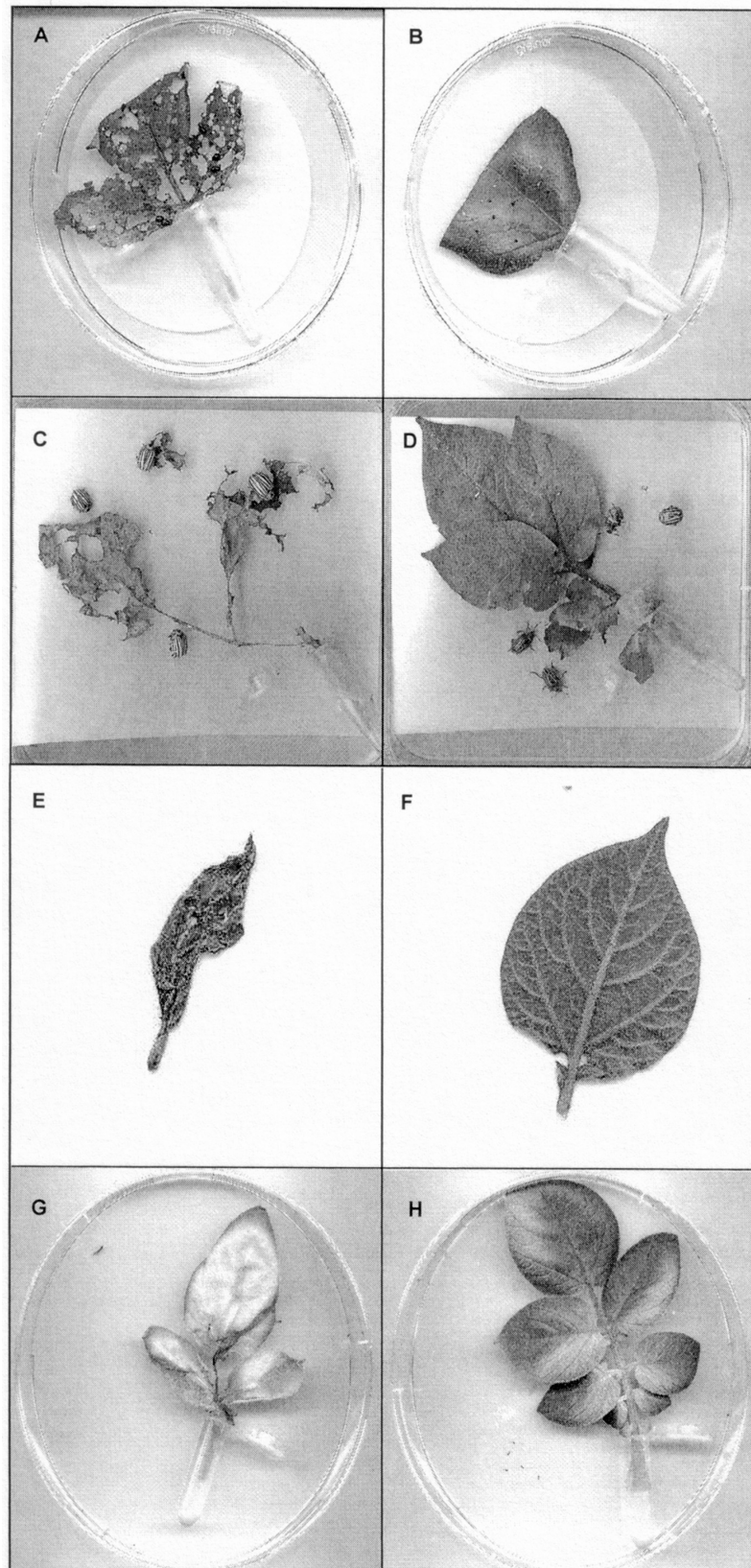
Fig. 5. *Leaf feeding assays comparing control lines (A, C, E, G) and SN19-expressing (B, D, F, H) line 11 (expressing 0.23% of total soluble protein). A, B: CPB larvae; C, D: CPB adults; E, F: PTM larvae; G, H: ECB larvae.*