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Bacillus thuringiensis Delta-Endotoxin Cry1 Hybrid Proteins with Increased Activity against the Colorado Potato Beetle

SAMIR NAIMOV,[1] MIEKE WEEMEN-HENDRIKS,[2] STEFAN DUKIANDJIEV,[1] AND RUUD A. DE MAAGD[2]*

Business Unit Cell Cybernetics, Plant Research International B.V., 6700 AA Wageningen, The Netherlands,[2] and Department of Plant Physiology and Molecular Biology, University of Plovdiv, Plovdiv, Bulgaria[1]

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

Bacillus thuringiensis is a gram-positive bacterium which produces insecticidal crystal (Cry) proteins during sporulation. The crystal 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 reviews, see references 6 and 20).

Proteins of the Cry3 (12, 17), Cry7 (14), and Cry8 (19) 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 activities against coleopterans, although their toxicity for CPB is much lower than that of Cry3Aa (2, 21).

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 crystallography has shown that the activated toxins of different, and probably most, Cry proteins share a common three-domain structure (11, 16). N-terminal domain I is thought to insert into the target membrane and form part of the pores that eventually kill the target insect's gut epithelial cells (20). Both domain II and C-terminal domain III are more varied and have been shown to be main determinants of activity against specific insects (1, 4, 9, 10). Although it is not yet clear how these domains may individually or collectively determine specificity, there is strong evidence that both can be involved in binding to receptors (3, 4, 5, 8, 15). Exchange of domain III between toxins, for example, by in vivo recombination of their genes, may alter the specificity of a toxin (1). Additionally, it may result in a hybrid toxin with a toxicity for certain insects that is higher than that of its parent toxins (7). So far, the latter phenomenon has been described only for lepidopteran insects. In this paper, we show that combination of parts of Cry1Ba and Cry1Ia results in hybrids with increased activities against CPB, which is a coleopteran.

Expression vectors. All used Cry protein expression vectors are based on pBD12, a derivative of pKK233-2 (1). The Cry1Ba expression vector pMH19 has been described previously (9). The Cry1Ia expression vector pBD172 contains the full *cry1Ia* gene with the *Spe*I site (nucleotide 2180) fused to the *Spe*I site in the polylinker in pBD12, which is derived from pBluescript SK(+). For Cry3Aa expression, the *cry3Aa* gene was given an *Nco*I site at its start by site-directed mutagenesis. An *Nco*I-*Xmn*I fragment (nucleotides 1 to 1935) was combined with an *Xmn*I-*Bgl*II linker, restoring the full coding region, and was used to replace the *cry1Ab* gene in the expression vector pBD1400 (7), giving the Cry3Aa expression vector pMH10.

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 a QuickChange kit (Stratagene) without changing the encoded amino acid sequences (Fig. 1A). Alignment of the Cry1Ba and Cry1Ia amino acid sequences with that of Cry1Aa, for which the three-dimensional structure has been established (11), shows that this region in Cry1Aa is located in a conserved region between the last beta strand of domain II (β11) and the following beta strand of domain III (β12). 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 the domain compositions 1Ia/1Ia/1Ba and 1Ba/1Ba/1Ia, respectively (Fig. 2). pSN19, encoding a hybrid with the domain composition 1Ba/1Ia/1Ba, was made by replacing an *Nco*I-*Mun*I (nucleotides 1 to 896) fragment encoding domain I of Cry1Ia in pSN15 by the corresponding fragment encoding domain I of Cry1Ba (nucleotides 1 to 869) derived from pSN16 (Fig. 1B). Alignment with the amino acid sequence and structure of Cry1Aa (11) shows that this region of Cry1Aa is located between beta strand β1a, which directly follows domain I but is structurally a part of domain III, and the first beta strand of domain II,

* Corresponding author. Mailing address: Business Unit Cell Cybernetics, Plant Research International B.V., P.O. Box 16, 6700 AA Wageningen, The Netherlands. Phone: 31(317) 477128. Fax: 31(317) 418094. E-mail: R.A.deMaagd@plant.wag-ur.nl.

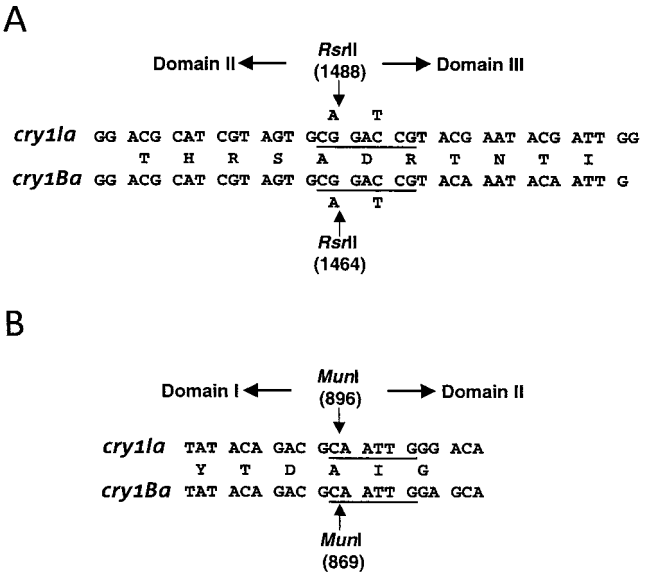

FIG. 1. (A) Oligonucleotides used to create *Rsr*II sites in the *cry1Ba* and *cry1Ia* genes by mutagenesis and the amino acids encoded by the respective parts of the genes. The two mutated nucleotides of the wild-type genes are shown above and below the oligonucleotide sequences. (B) Localization of the common *Mun*I site in *cry1Ba* and *cry1Ia* genes and the amino acids encoded by the respective parts of the genes.

β1b. Therefore, this step not only changes the entire domain I into that of Cry1Ba but also completes domain III of Cry1Ba, which in SN16 contains the area homologous to strand β1a derived from Cry1Ia.

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 previously (1). Solubilized protoxins were dialyzed overnight against 25 mM $NaHCO_3$–100 mM NaCl, pH 10. Protein concentrations were estimated in duplicate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a standard curve of bovine serum albumin. To test toxicity to CPB, leaflets of greenhouse-grown potato cultivar Desiree plants were dipped in protoxin dilutions in water containing 0.01% Tween 20. After air drying of the leaves, they were transferred to petri dishes and 10 neonate CPB larvae were placed on each leaf. After incubation for 2 days at room temperature, the leaves were replaced by fresh leaves dipped in identical protoxin dilutions. Mortality was scored after 4 days. Cry3Aa was analyzed as a positive control and for comparison. Fifty percent lethal concentrations (LC_{50}s) and 95% fiducial limits were determined by Probit analysis of results from three or more independent experiments using the PoloPC computer program (18). Results of bioassays are shown in Fig. 2.

Cry1Ba/Cry1Ia hybrid protein SN16 had very low toxicity against CPB, and not enough protoxin was purified to determine a reliable LC_{50}. Surprisingly, the Cry1Ia/Cry1Ba hybrid SN15 was more toxic than both of the parent proteins. When the size differences are taken into consideration, SN15 is potentially 2.5 times more toxic than Cry1Ia and 7.5 times more toxic than Cry1Ba on a per-mole basis. This finding indicates that domain I or II of Cry1Ia or both domains are important determinants of the higher activity of Cry1Ia relative 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. We therefore conclude that, also for coleopterans, hybrid toxins resulting from domain swapping may have improved properties, as was shown earlier to be the case for lepidopterans. The additional domain I substitution giving a mosaic 1Ba/1Ia/1Ba hybrid (SN19) increased toxicity even further, resulting in activity approaching that of Cry3Aa against CPB. This result not only identifies Cry1Ia domain II, and not domain I, as its most important determinant for activity against CPB but also emphasizes the point that new domain combinations may result in higher activity.

New combinations of the receptor binding domains II and III resulted in increased activities of hybrids for several lepidopterans (7, 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 (13). Following this line of reasoning, one could speculate that both Cry1Ba and Cry1Ia bind to the same receptor in CPBs but that different steps are rate limiting for the 2 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 both Cry1Ba and SN19 have extended protoxin-specific C-termini compared to that of Cry1Ia, the possibility of a role of these extensions 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

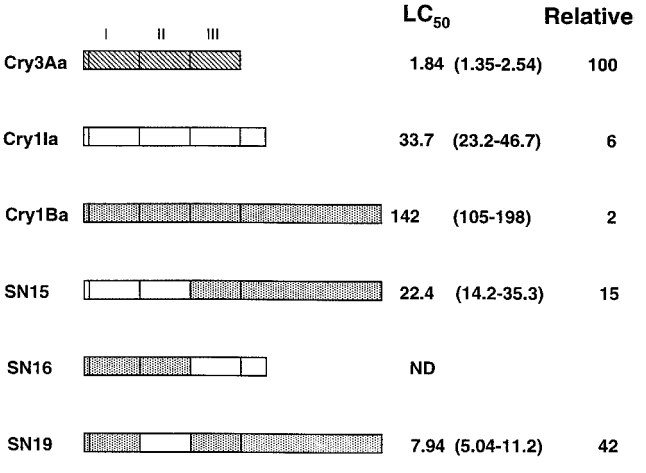

FIG. 2. Domain compositions and insecticidal activities of Cry1Ia/Cry1Ba hybrids and their parental protoxins. Bioassays were performed with solubilized protoxins on potato leaves with neonate CPBs. Concentrations (LC_{50}s) are expressed as micrograms per milliliter of dipping solution. Ninety-five percent fiducial limits are shown in parentheses. ND, not determined. The column of data labeled "Relative" gives toxicities relative to that of Cry3Aa on a per-mole basis: (molecular weight of protein/molecular weight of Cry3Aa) × (LC_{50} of Cry3Aa/LC_{50} of protein) × 100.

against CPB only after solubilization and activation (14). 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 (2). This need may be caused by the relatively low gut pH (pH 6 to 7) in CPB, compared to that in lepidopterans, which inhibits solubilization of the crystalline protoxin. Whether this inhibition would prevent effective application of SN19 in a crystalline form and whether SN19 may be an alternative for Cry3Aa in CPB-resistant transgenic plants are the subjects of our further studies.

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