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Cry9Ca1 Toxin, a *Bacillus thuringiensis* Insecticidal Crystal Protein with High Activity against the Spruce Budworm (*Choristoneura fumiferana*)

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The Cry9Ca1 toxin from *Bacillus thuringiensis* was significantly more toxic to spruce budworm (*Choristoneura fumiferana*) than the Cry1Ab6, Cry1Ba1, Cry1Ca2, Cry1Da1, Cry1Ea1, and Cry1Fa2 toxins. It displayed high activity against silkworm (*Bombyx mori*) but was not toxic to black army cutworm (*Actebia fennica*) or gypsy moth (*Lymantria dispar*). The Cry9Ca1 is the most effective spruce budworm toxin known to date and may offer promise for control and resistance management of that species.

Insecticides derived from the soil bacterium *Bacillus thuringiensis* are gaining worldwide importance as environmentally desirable alternatives to synthetic chemicals for control of pests in agriculture, forestry, and public health (3). In North America, commercial products based on the HD-1 strain of *Bacillus thuringiensis* subsp. *kurstaki* are widely used to control defoliating forest lepidopterans, in particular the spruce budworm (*Choristoneura fumiferana*) (14). The HD-1 strain produces several insecticidal crystal proteins (ICPs) from the *cry1A* and *cry2A* gene families, as per revised nomenclature (1, 1a). Proteins from these families are active against a wide variety of larval lepidopterans, including many species with worldwide agronomic importance. Although these proteins are toxic to spruce budworms (16, 17), the ongoing discovery of new toxin genes (4) offers a prospect of identifying more effective ICPs.

A novel crystal protein designated Cry9Ca1 was recently reported (10). This ICP, previously named CryIH, showed particular promise for insect control because of its broad activity spectrum against larval lepidopterans, including several members of the Noctuidae family, and its specific receptor-binding characteristics. Here, we report its toxicity to spruce budworm and two other economically important forest pests, gypsy moth (*Lymantria dispar*) and black army cutworm (*Actebia fennica*), and to a species of general interest, the Chinese silkworm (*Bombyx mori*).

The toxicity of Cry9Ca1 was compared with the toxicities of Cry1Ab6, -1Ba1, -1Ca2, -1Da1, and -1Ea1, ICPs that were tested previously against the same species (16, 17). We also tested Cry1Fa2 (11), formerly CryIF, which was not included in the earlier assays. The *cry1Ab6* toxin gene was isolated from *B. thuringiensis* subsp. *kurstaki* NRD-12 (Biotechnology Research Institute, Montreal, Canada) (7), *cry1Ba1* from *Bacillus thuringiensis* subsp. *entomocidus* HD-110 (Plant Genetic Systems, Ghent, Belgium) (8), *cry1Ca2* from *Bacillus thuringiensis* subsp. *aizawai* HD-133 (Biotechnology Research Institute) (10a), *cry1Da1* from *B. thuringiensis* subsp. *aizawai* HD-68 (Plant Genetic Systems) (9), *cry1Ea1* from *Bacillus thuringiensis* subsp. *darmstadiensis* (Plant Genetic Systems) (19), and *cry1Fa2* from *B. thuringiensis* subsp. *aizawai* PS81I (Mycogen, San Diego, Calif.) (11). The *cry9Ca1* gene had been modified to eliminate the trypsin cleavage site at position 164 (Arg→Lys substitution) (10). The Cry1Fa2 toxin protein was produced in *Pseudomonas fluorescens* (Mycogen formulation MYX837-936) by the CellCap encapsulation process (5). All other toxins were obtained by expression of cloned genes in *Escherichia coli* as described previously (17).

Insect assays were conducted with activated toxins obtained by digestion of the ICPs with gut juice from silkworm according to the procedure of Gringorten et al. (6). The rationale for using silkworm gut juice was discussed previously (16). The concentration of activated toxin in the final solutions was estimated by electrophoresis and scanning densitometry of Coomassie blue-stained sodium dodecyl sulfate–12% polyacrylamide gels (17) and was checked periodically for stability during storage. The relative amounts of activated toxin as a proportion of the total protein content were as follows: 0.775 for Cry1Ab6, 0.478 for Cry1Ba1, 0.599 for Cry1Ca2, 0.659 for Cry1Da1, 0.246 for Cry1Ea1, 0.529 for Cry1Fa2, and 0.234 for Cry9Ca1.

The insecticidal activity of the toxin preparations was determined by force-feeding larvae of spruce budworm (day 1 sixth instars), gypsy moth (early fourth instars), black army cutworm (early fifth instars), and silkworm (day 1 fourth instars) obtained from the Canadian Forest Service rearing facility in Sault Ste. Marie. The force-feeding technique and general test conditions were described previously (16). We also compared the toxicities of Cry1Ab and Cry9Ca1 to fifth-instar spruce budworm, for which a droplet-imbibing technique (18) was used because fifth instars are too small to force-feed. Both techniques yield the same results (18). Six twofold dilutions of each toxin solution were tested in three to five replicates of 10 to 20 larvae per dilution. Effective dose estimates were obtained by probit analysis (13) of percent mortality observed after 48 h in the silkworm assays (50% lethal dose) or of percent failure to produce frass after 72 h, a measure of feeding inhibition, in all other assays (50% frass failure dose [FFD_{50}]) (15). Control response (larvae dosed with the gut juice-buffer solution, $n = 10$ to 20 per replicate) was <2%.

The Cry9Ca1 toxin was highly active against the spruce budworm, with an FFD_{50} of <10 ng per sixth instar in the force-feeding assays (Table 1). Cry9Ca1 was three- to fourfold more toxic at both the 50 and 95% response levels than Cry1Ab6,

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TABLE 1. Toxicity of activated Cry9Ca1 to sixth-instar spruce budworm in comparison with Cry1 proteins in force-feeding assays

Toxin	n^a	Slope[b] (mean ± SE)	FFD_{50}[c]	95% Fiducial limit		FFD_{95}[c]
				Lower	Upper	
Cry1Ab6	350	1.8 ± 0.17	20.4	16.0	25.3	165
Cry1Ba1	350	2.2 ± 0.20	29.7	24.3	35.8	165
Cry1Ca2	290	2.0 ± 0.29	142.9	112.7	184.7	937
Cry1Da1	280	2.1 ± 0.23	143.6	104.2	197.5	873
Cry1Ea1	340	1.9 ± 0.20	78.7	62.5	98.1	558
Cry1Fa2	250	2.5 ± 0.30	180.5	130.6	248.2	788
Cry9Ca1	310	2.1 ± 0.22	7.5	6.1	9.2	43

[a] Number of larvae tested.
[b] Estimated slope of the probit regression line.
[c] Nanograms of toxin protein per larva.

which until now was the most effective toxin known (16, 17). Similar results were obtained for fifth instars with the droplet-imbibing method (Table 2). Fifth instars were two to three times more susceptible than sixth instars (Tables 1 and 2), which is in agreement with earlier observations on stadial susceptibility to crystal-spore suspensions of commercial products (18). The Cry1Fa2 toxin was approximately ninefold less active than Cry1Ab6 at the FFD_{50} level (Table 1), which makes it the least effective budworm toxin of the Cry1 proteins tested so far.

The Cry9Ca1 toxin was not active against gypsy moth, as it failed to elicit a feeding-inhibitory response at the highest concentration tested (Table 3). The Cry1Fa2 toxin also exhibited very low toxicity to this species (Table 3). Both proteins were highly toxic to silkworm, with 50% lethal dose estimates in the range of 10 to 20 ng per larva. Neither toxin elicited a feeding-inhibitory response in larvae of black army cutworm at the highest concentrations tested.

It must be noted that in our previous assays we observed a higher potency of the Cry1Ca2 and Cry1Ea1 toxins against spruce budworm (FFD_{50}s, 23.6 and 27.1 ng per larva, respectively) than in this study but activities of Cry1Ab6 and Cry1Da1 similar to those obtained here (16, 17). In contrast, the response of gypsy moth, silkworm, and black army cutworm to the Cry1 toxins (Table 3) was consistent with results from previous assays (17). This suggests that an insect laboratory colony can display considerable variation over time, not just in level of susceptibility, as has been well documented (e.g., reference 12), but also in susceptibility spectrum, i.e., in its response to different toxins. High susceptibility of spruce budworm to Cry9Ca1 appears to be consistent over time, as demonstrated by the similarity of response between the force-feeding and droplet-imbibing assays, which were conducted about 1 year apart.

The Cry9Ca1 is unique for its activity against several members of the Noctuidae, including *Spodoptera exigua*, *Spodoptera littoralis*, *Mamestra brassicae*, *Heliothis virescens*, and *Agrotis segetum* (10). Other noctuids, such as *Helicoverpa armigera* and *Spodoptera frugiperda*, were not susceptible. The black army cutworm is another noctuid that does not respond to Cry9Ca1. Results of this study confirm earlier observations that the black army cutworm is not susceptible to any of the Cry1 toxins tested to date, which makes it one of the most refractory members of this family.

Since Cry9Ca1 binds to different receptors than do other ICPs that are currently used in conventional sprays or transgenic plants (10) and resistance to ICPs often involves a binding-site modification (20), this toxin is expected to become a valuable tool for management of insect resistance. The *cry9Ca1* toxin gene has already been used to engineer corn that is resistant to European cornborer and other pests (10). Considering its high toxicity to spruce budworm, it might also be a good candidate for engineering budworm resistance in conifers in place of or in combination with Cry1Ab, which is currently being used (2).

TABLE 2. Toxicity of Cry9Ca1 and Cry1Ab6 to fifth-instar spruce budworm in droplet-imbibing assays

Toxin	n^a	Slope[b] (mean ± SE)	FFD_{50}[c]	95% Fiducial limit		FFD_{95}[c]
				Lower	Upper	
Cry1Ab6	292	1.8 ± 0.20	8.5	5.2	13.3	72
Cry9Ca1	288	1.9 ± 0.24	2.4	1.7	3.0	18

[a] Number of larvae tested.
[b] Estimated slope of the probit regression line.
[c] Nanograms of toxin protein per larva.

TABLE 3. Toxicity of Cry9Ca1 and Cry1 proteins to gypsy moth, silkworm, and black army cutworm in force-feeding assays

Insect species	Toxin	n^a	Slope[b] ± SE	ED_{50}[c]	95% Fiducial limit		ED_{95}[c]
					Lower	Upper	
L. dispar	Cry1Ab6	300	2.1 ± 0.21	46.8	34.7	62.8	263
	Cry1Ca2	300	2.8 ± 0.26	644.8	495.6	840.3	2,505
	Cry1Da1	300	2.8 ± 0.26	88.1	62.8	125.6	336
	Cry1Fa2	360	2.1 ± 0.19	1,300	1,095	1,561	7,842
	Cry9Ca1	40		>615[d]			
B. mori	Cry1Ca2	140	3.0 ± 0.43	30.3	20.2	47.0	104
	Cry1Da1	160	2.7 ± 0.35	7.4	5.9	9.1	29
	Cry1Ea1	170	3.6 ± 0.47	6.6	4.8	8.9	19
	Cry1Fa2	160	3.0 ± 0.41	19.6	10.9	30.8	68
	Cry9Ca1	180	2.2 ± 0.28	13.4	8.1	23.4	74
A. fennica	Cry1Ab6	10		>2,269[d]			
	Cry1Ba1	10		>630[d]			
	Cry1Ca2	10		>5,803[d]			
	Cry1Da1	10		>4,452[d]			
	Cry1Ea1	10		>868[d]			
	Cry1Fa2	10		>4,691[d]			
	Cry9Ca1	10		>615[d]			

[a] Number of larvae tested.
[b] Estimated slope of the probit regression line.
[c] Fifty or ninety-five percent effective dose, expressed as lethal dose (*B. mori*) or frass failure dose (all others) in nanograms of toxin protein per larva.
[d] No feeding-inhibitory response at indicated dose.

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