Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*

Shogo Atsumi1,2, Kazuhisa Miyamoto3,1, Kimiko Yamamoto5, Junko Narukawa6, Sawako Kawai7, Hideki Sezutsu5, Isao Kobayashi8, Keiro Uchino9, Toshiki Tamura4, Kazuei Mitaka10, Keiko Kadono-Okuda11, Sanae Wada12, Kohzo Kanda13, Marian R. Goldsmith14, and Hiroaki Noda15

1Division of Insect Sciences, 2Agrogeneomics Research Center, and 3Genetically Modified Organism Research Center, National Institute of Agrobiological Sciences, Tsukuba 305-8634, Japan; 4Faculty of Agriculture, Saga University, Saga 840-8502, Japan; and 5Biological Sciences Department, University of Rhode Island, Kingston, RI 02881

**AUTHOR SUMMARY**

Toxins produced by *Bacillus thuringiensis* (Bt) are widely used for controlling insect pests as insecticidal constituents in agricultural chemicals and transgenic crops. The increasing use of Bt insecticides and widespread cultivation of Bt crops have raised concerns for the potential of accelerated development of Bt resistance in field populations (1). Despite the broad use of Bt toxin and the discovery of molecules involved in Bt resistance in agricultural pests, such as the tobacco budworm *Heliothis virescens*, the diamondback moth *Plutella xylostella*, and the pink bollworm *Pectinophora gossypella*, its mode of action is not fully understood (2).

Inbred strains of the domesticated silkworm *Bombyx mori*, in which this bacterial pathogen was first reported, show various levels of susceptibility to Bt toxin. Taking advantage of recent advances in genome databases and high-density genetic maps for map-based (i.e., positional) cloning (3), together with transgenic techniques for the study of gene function, we initiated cloning of a silkworm gene conferring resistance to Bt toxin Cry1Ab. In these studies, we used two strains differing substantially in their response to Bt insecticides and the broad use of Bt toxin and the development of Bt resistance in agricultural chemicals and transgenic crops. The increasing use of Bt insecticides and widespread cultivation of Bt crops have raised concerns for the potential of accelerated development of Bt resistance in field populations (1). Despite the broad use of Bt toxin and the discovery of molecules involved in Bt resistance in agricultural pests, such as the tobacco budworm *Heliothis virescens*, the diamondback moth *Plutella xylostella*, and the pink bollworm *Pectinophora gossypella*, its mode of action is not fully understood (2).

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**Fig. P1.** Positional cloning scheme for a gene from silkworm conferring resistance to Cry1Ab. Two strains, resistant C2 (blue) and susceptible Rin (yellow), were crossed, and silkworms surviving after Bt toxin screening in the BC1 generation were used for chromosome linkage analysis and positional cloning. The susceptibility allele of the candidate gene for Bt resistance (an ABC transporter from the Rin strain) was introduced into a resistant strain (w1-pnd) that is routinely used for transgenesis. Transformed strains carrying the susceptibility allele showed susceptibility by toxin screening; susceptibility could be transferred to previously resistant silkworms. A single amino acid deletion/insertion in the second outer loop of the predicted ABC transporter gene was found to discriminate between susceptible and resistant strains.


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1S.A. and K. Miyamoto contributed equally to this work.

2Present address: Ishihara Sangyo Kaisha, Central Research Institute, Kusatsu 525-0025, Japan.

3To whom correspondence should be addressed. E-mail: hnada@affrc.go.jp.

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these were subsequently removed as a result of incorrect assignments in the database. Of the four remaining candidate genes, two genes were not expressed in the midgut. Given that resistance is presumed to involve proteins with active functions in the gut, we focused on the two remaining genes expressed in this tissue, and examined their complete coding sequences in C2 and Rin strains. One of these genes was identical between the two strains, excluding it from further study; however, many nucleotides were different between C2 and Rin strains in the second gene, BGIBMGA007792-93, which we annotated as an ATP-binding cassette (ABC) family C transporter. Upon examining six additional resistant and nine susceptible strains for the sequence polymorphisms of this gene, we found that only a single, common amino acid (tyrosine) insertion/deletion located in the second outer loop of a predicted 12-transmembrane structure (Fig. P1) was able to distinguish resistant from susceptible strains, including C2 and Rin. Based on the known function of ABC transporter proteins, we determined that BGIBMGA007792-93 was a plausible candidate for Bt resistance.

To verify the role of BGIBMGA007792-93 in the Bt toxin response, we introduced the ABC transporter gene from the susceptible strain Rin into resistant strain w1-pnd, which is routinely used in silkworm transgenesis. Silkworm transformation was performed based on the GAL4-UAS (upstream activation sequence) system in the silkworm, which is routinely used in the fruit fly. The transgenic silkworms are expected to possess endogenous resistant ABC transporter genes in both sister chromosomes, together with transformed susceptible gene(s) from Rin. They (second- and fourth-instar larvae) were highly susceptible to Bt toxin, consistent with the dominant trait of the susceptible gene. We confirmed expression of the transgene and endogenous genes by real-time reverse transcriptase-PCR. Therefore, this study demonstrates that the germline introduction of a functional form of a gene associated with susceptibility to Bt toxin can alter the resistance phenotype of an insect.

In line with our findings, ABC transporter genes have also been linked to toxin resistance in other species. For example, an orthologue of the gene mapped in this study was recently implicated in Cry1Ac resistance in *H. virescens* (4) and two other lepidopteran pests (5); nucleotide deletions in ABC transporter genes were reported in two of these species. It is interesting to note that binding of Cry1Ac to a preparative form of midgut membrane was abolished in homozygous mutants of *H. virescens* (4); however, we found no difference in Cry1Ab binding to the midgut membrane between susceptible and resistant silkworms, suggesting differences in the mechanisms underlying the response to the two classes of toxin or in *B. mori*. An ABC transporter (subfamily C) homologous to the *Bombyx* gene is known to function in human multidrug resistance, suggesting that the ABC transporter in *B. mori* might confer resistance via a detoxification mechanism. Detoxification is expected to act like a dominant trait, in that a heterozygote bearing only a single copy of the functioning gene should still be able to inactivate or excrete some toxin. However, in four lepidopteran species, Bt resistance is a recessive trait that requires two nonfunctioning gene copies. A more plausible mechanism for its mode of action is that the ABC transporter acts in the midgut in conjunction with a toxin receptor, such as a cadherin-like protein or aminopeptidase N for binding to the midgut membrane or insertion of the toxin into the cell. Involvement of a single amino acid difference in this gene for Bt resistance will provide tools for critical functional studies of the transporter in the mechanism of Bt action. Primary importance of the mutation of the ABC transporter for Bt resistance can also facilitate field monitoring for Bt resistance level of lepidopteran pests.