The molecular biology of tolerance to *Bacillus thuringiensis* endotoxin in *Helicoverpa armigera*: a novel mechanism and its genetic transmission

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Abstract

Resistance in insect pests against the endotoxin of *Bacillus thuringiensis* (Berliner) (Bt) is a major threat to the usefulness of this biopesticide both used as traditional formulations and in transgenic crops. A crucial requirement for the development of successful resistance management strategies is a molecular understanding of the nature and inheritance of resistance mechanisms. This information can be used to design management strategies that will delay or counteract Bt resistance.

Most known Bt resistance mechanisms in field or laboratory-selected populations are recessive mutations based on receptor inactivation. However, there are occasional examples of dominant or semi-dominant mechanisms with unusual characteristics. For example, Bt-resistance was selected in a *H. armigera* population by feeding the toxin with artificial diet in the laboratory. The resulting resistance was based on a single gene locus with a semi-dominant trait. This population was backcrossed with a susceptible strain to get nearly isogenic resistant and susceptible lines. After four crosses and selection for resistance, the resulting ISOC4 strain was analysed for Cry1Ac binding to BBMVs. This assay showed differences in binding to an unknown protein, while none of the known aminopeptidase N genes showed any mutation that could be responsible for the lack of toxin binding. Moreover, exposure to Bt toxin imposed a fitness cost, which is likely to derive from pleiotropic effects of the resistance gene rather than effects from other gene loci. This suggested that resistance was probably not based exclusively on the inactivation of one of the major receptor genes but on effects from (an) other unknown gene(s), which also affected developmental functions.

A melanization reaction occurs in the hemolymph and gut lumen of the ISOC4 strain, which may be associated with an elevated immune status in the resistant strain. Further analysis showed that melanization in cell-free hemolymph (plasma) and gut was significantly higher in the ISOC4 than in the susceptible ANGR strain. This is associated with a higher coagulation reaction in the ISOC4 strain, which precluded conventional protein analysis using SDS-PAGE. To study the immune induction in this strain and the effects of an elevated immune status on Bt resistance, sub-lethal doses of the Bt–toxin, Cry1Ac, were fed with the artificial diet. Under these conditions the effects of immune induction were studied in ANGR larvae. It was found that the Bt feeding resulted in the induction of an 85 kDa hemolymph protein.

Analysis of the native p85 was performed using a coagulation bioassay of hemolymph plasma. This showed that p85 specifically interacts with Cry1Ac and other oligomeric lectins, suggesting that p85 is an immune-related protein. To determine the identity of p85, an in-gel protein digest, or and peptide sequence analysis were performed to determine approprate primers for PCR amplification of the gene. The DNA product of the PCR reaction was sequenced and showed strong similarity to hexamerin from *H. virescens.* Antibodies created against a recombinant peptide from the *H. armigera* hexamerin were obtained in rabbits and recognised p85 on Western blots. Using the anti-hexamerin antibodies, hexamerin was found in the gut lumen in addition to the hemolymph. In protein extracts from ISOC4 gut preparations, multiple bands of hexamerin are detected, which may be caused by proteolytic digestion or post-translational modifications. This suggests that hexamerin has a dual function. Apart from its metabolic function as a storage protein, it also takes part in coagulation reactions, which is consistent with similar findings in *Drosophila melanogaster*.

Since hexamerin may have been affected in the ISOC4 strain by post-translational modifications, altered glycosylation in the resistant strain may directly or indirectly occur as a result of the elevated immune-status. Anti-glycosyltransferase antibodies from nematodes were examined for possible cross-reactivity to insect glycosyltransferase (Gly-T). In Western blots from gut extracts the anti-glycosyltransferase antibodies reacted with both ISOC4 and ANGR extracts, but binding was stronger with the ANGR extracts. In addition, Gly-T activity was examined in BBMVs from the gut and found to be different in ISOC4 and ANGR. Since Gly-T can transfer sugars onto proteins or lipids, it may modify hexamerin or associated lipids thereby changing hexamerin aggregation in the gut lumen. Since altered coagulation is a dominant trait, the observation that resistant *H. armigera* had a reduced Gly-T activity, is compatible with a change in post-translational modification of gut proteins and lipids in the ISOC4 strain.

If the tolerance to Bt toxin is caused by a transient induction of the immune system, the effect should not be visible in the next generation. However, since hexamerin is a storage protein and incorporated into oocytes during oogenesis, it is possible for a modified hexamerin to stimulate the immune system in the embryo, or directly sequester the toxin in the embryonic gut by a coagulation reaction, where it may protect the emerging neonates against the toxin. If this is correct, the neonates will only be protected if they derived from a resistant mother, whereas the offspring from reciprocal crosses will be susceptible. To test this assumption, bioassays were performed on the progeny of the two crosses and survival of neonates examined. When resistant females were mated with susceptible males, the offspring showed higher tolerance to Cry1Ac compared to offspring from the reciprocal cross. Furthermore, the tolerance was not sex-linked. This suggests that the Bt tolerance in ISOC4 is transmitted via a maternal effect when *H. armigera* larvae were subjected to relatively low dosage of Cry1Ac toxin. However, the results from the two reciprocal crosses also showed that embryonic traits (nuclear genes) might also contribute to the Bt resistance.

When Western blots of gut protein extracts were developed with alkaline phosphatase reagents in the absence of the antibodies, a stained band was detected in ANGR extracts, which was significantly reduced in ISOC4 extracts. This chance observation suggests that a gut alkaline phosphatase, which retains enzymatic activity after SDS-PAGE, is significantly reduced in the resistant strain. Microsequencing of this band revealed four peptides, which all have similarity to a membrane-bound *Bombyx mori* alkaline phosphatase. Since alkaline phosphatase proteins are known bind to Cry1Ac, a genetic alteration in the ISOC4 strain may account for the recessive trait of Bt resistance at high dosage, which did not show a maternal effect. The role of the alkaline phosphatase protein in the cause of resistance in the ISOC4 strain remains to be elucidated. The corresponding gene may carry a mutation and thus represent the recessive gene locus assumed to be responsible for the phenotype. Alternatively, gene expression may be affected by another unknown gene as a result of the elevated immune status in the ISOC4 strain.

In summary, the observed alterations in the ISOC4 strain are compatible with a complex phenotype consisting of two components, a recessive mutation in an unknown gene, which could either be a Bt-receptor such as alkaline phosphatase and a dominant trait that appears to operate through an elevated immune status.