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# *Bacillus thuringiensis* resistance in *Plutella* — too many trees?

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*Plutella xylostella* was the first insect for which resistance to *Bacillus thuringiensis* was reported in the field, yet despite many studies on the nature of this resistance phenotype its genetic and molecular basis remains elusive. Many different factors have been proposed as contributing to resistance, although in many cases it has not been possible to establish a causal link. Indeed, there are so many studies published that it has become very difficult to 'see the wood for the trees'. This article will attempt to clarify our current understanding of Bt resistance in *P. xylostella* and consider the criteria that are used when validating a particular model.

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## Introduction

*Plutella xylostella* (the diamondback moth) is a major pest of crucifer crops and was the first insect to be shown to have acquired field-evolved resistance to Cry toxin-containing *Bacillus thuringiensis* (Bt) insecticides [1]. By far, the most common resistance phenotype is known as 'mode 1' in which the insect shows resistance to several Cry1A but not to Cry1C or Cry2A toxins [2], it is this phenotype that will be discussed here. Since the first Bt-resistant insects were identified much research effort has been expended in the search for the underlying genetic, physiological and biochemical mechanisms [3]. For some insects such as *Helicoverpa armigera* mutations in a known receptor for Bt toxins (cadherin), were found to associate with the resistance phenotype [4]. For *Plutella xylostella* biochemical assays have failed to identify a plausible receptor candidate while genetic studies eliminated mutations in various putative receptors such as cadherin [5,6].

## Alternative resistance mechanisms and the casual vs causal problem

Although resistance in *P. xylostella* is normally associated with the loss of binding of the Bt toxin to epithelial cells of the insect gut [7], researchers have also looked at alternative resistance mechanisms. A number of these are discussed below and while each presents a plausible mechanism it is often very difficult to establish a causal link. One study [8] looked at the possible influence of midgut proteases on resistance and on comparing resistant and susceptible populations found that the former had significantly lower levels of both total and trypsin-like proteases. Since Bt toxins require proteolytic cleavage to become active lowering of proteolytic activity could reduce the availability of active toxin and thus reduce the insect's susceptibility. Unfortunately, a causal link could not be made in this case and the very small (two) sample size made it impossible to make a strong association between the observed difference and the resistance phenotype. Another report also considered the role of toxin activation in resistance after observing that a resistant population was more susceptible to pre-activated toxin than to protoxin [9]. Although this finding was consistent with a defect in toxin activation the authors noted that alternative explanations existed — such as preferential sequestration of the protoxin form. In a related paper a resistant population was once again found to be more susceptible to activated toxin, although no defect in toxin activation could actually be established [10]. Another example where an indirect observation could have had several explanations was seen in a paper by Sayyed *et al.* [11] in which it was observed that an esterase inhibitor could synergise the activity of a Cry toxin against a resistant population. Since esterases had previously been implicated in Bt resistance mechanisms [12] it was reasonable to speculate that this observation could indicate an esterase-mediated mechanism, although as with the above examples other explanations — such as an indirect effect of altering the host's physiology — could exist. As well as proteases and esterases, lipids have also been implicated in Bt-resistance [13]. In this study differences were found in the lipid composition of Brush Border Membrane Vesicles between a resistant and susceptible population which the authors speculated could influence the activity of the toxin, despite the fact that no causal link was determined.

## The use of 'omics' studies to investigate resistance mechanisms

In the examples previously discussed the researchers were testing a specific hypothesis concerning the resistance

mechanism. In contrast, the use of transcriptomic or proteomic analyses allow a much broader comparison between susceptible and resistant populations. Ayra-Pardo *et al.* [14•] used suppressive subtractive hybridisation to identify genes that were over-expressed in a resistant population of *P. xylostella* compared to a susceptible control population. Over a hundred genes with differential expression were identified, although few of these had a clear link to Bt pathogenesis. A more extensive screen was undertaken by Lei *et al.* [15] who used RNA-sequencing to compare resistant and susceptible populations. Two different resistant populations were used and in each case around 3000 genes were found to be differentially expressed compared to a susceptible population (the majority being over-expressed). Interestingly of those 3000 differentially expressed genes only around a third were common to both resistant populations. In order to target a subset of molecules that have recently been implicated in a wide range of cellular processes RNA sequencing was also used to compare the distribution of long non-coding RNAs (lncRNAs) between two resistant and a susceptible population [16]. Between 150–200 differentially expressed lncRNAs were found in the two resistant populations of which 59 were common to both. These studies revealed the large number of differences that can be found between susceptible and resistant populations, and although it is tempting to assign roles for these differentially expressed genes in determining resistance, it is likely that many of the differences are unrelated to resistance and simply reflect the different genetic backgrounds of the populations being compared. One way of reducing this variation is to create near isogenic populations of susceptible and resistant insects through continuous backcrossing. Lei *et al.* [17] produced such a pair of *P. xylostella* populations and although no biochemical or transcriptomic comparisons were made between them, genetic mapping studies did confirm that resistance was due to a single, autosomal, recessive locus.

## How many mutations cause resistance in *Plutella*?

Although various reports, such as the one described above with the near-isogenic populations, have found that resistance to Bt is caused by a single, recessive, autosomal locus, other reports suggest a more complex situation. One such paper [10] suggests that resistance was inherited in an incompletely dominant fashion and showed some maternal influence. These differences may represent multiple mutations/mechanisms of resistance but may also reflect significant differences in genetic backgrounds that can confound the analysis of the major resistance-causing mutation(s). A traditional way of comparing the genetic backgrounds of different populations sharing the same phenotype is to perform complementation assays. If two populations containing a recessive resistance mutation in the same gene are crossed, then the offspring should all be resistant. However, if the mutations are in different genes then the offspring would be susceptible. Tabashnik *et al.* [18] performed complementation assays on three resistant populations (PEN, NO-QA and PHI) and found that all three shared a common resistance locus. In 2005 it was reported that complementation tests between an artificial diet adapted derivative of NO-QA (NO-QAGE) and an independently isolated resistant population SC1 demonstrated that the same locus was present in SC1 [19]. Sayyed *et al.* (unpublished data) also indicated that the same locus was present in three strains from Malaysia (SERD4, Kluang and Karak) based on complementation tests between these three and then later between SERD4 and NO-QAGE. In a separate study, a complementation test was undertaken between a resistant population from China (SZBT) and one from the US (Cry1Ac-R) which were also shown to share a resistance locus [20]. Although no link has been made between these latter two populations and the former seven, the intriguing possibility exists that a single worldwide locus is primarily responsible for mode 1 resistance in *P. xylostella*.

## Identification/validation of resistance locus candidates

The transcriptomic studies discussed above have led to many hundreds of genes identified as potentially being involved in Bt resistance. It is unknown whether these differences are primarily due to differences in genetic background or to the putative single resistance mutation, but nonetheless they throw up various candidates for involvement in the resistance mechanism. When faced with such candidate genes it is crucial to validate their involvement. There are various established routes for this validation, to start with though let us consider the hypothesis that the protein cadherin is important. As mentioned above, cadherin is a known receptor for Bt toxins in other insects and mutations in its gene have been found in resistant insects. The observation that a genetically modified form of Cry1A toxin that is believed to by-pass cadherin-based resistance mechanisms [21] could overcome resistance in *P. xylostella* NO-QAGE [22] initially suggested the involvement of this protein. However genetic mapping studies ruled out the possibility that resistance was due to mutations in cadherin in NO-QA [19] and also in Cry1Ac-R [23•]. The latter study proposed that the gene annotated as Px012847 represented the primary cadherin gene and demonstrated by sequencing that this cadherin gene contained no mutations, that transcript levels did not vary significantly and finally that RNAi-mediated suppression of the gene did not affect susceptibility to Bt. In contrast, a more recent study [24•] found that RNAi suppression of the same gene did reduce susceptibility to Cry1Ac and reduced the capacity of the midgut to bind toxin. Thus whether or not cadherin is involved in the mechanism of action of Bt toxins remains unclear. RNA interference is a useful tool with which to validate candidate genes. Of the 134 genes identified by Ayra-Pardo as being over-expressed in a resistant population 3 (a cyclin-dependent kinase 5 regulatory subunit associated