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Article (Accepted Version)

Crickmore, Neil (2016) Bacillus thuringiensis resistance in Plutella – too many trees? Current Opinion in Insect Science, 15. pp. 85-88. ISSN 2214-5753

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***Bacillus thuringiensis* resistance in *Plutella* – too many trees?**

Neil Crickmore

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 hybridization 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 3,000 genes were found to be differentially expressed compared to a susceptible population (the majority being overexpressed). Interestingly of those 3,000 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 protein 1-like 1 (PxCDKAL1), a stromal cell-derived factor 2-like1(PxSDF2L1) and a hatching enzyme-like (PxHEL) astacin metalloproteinase) were chosen for RNAi studies [14]. In all three cases suppression of the genes in the resistant population increased susceptibility to Bt.

ABCC2 and neighbouring genes

In 2010, a new genetic link to Bt resistance was proposed. Gahan et al [25] mapped the resistance phenotype of a population of *Heliothis virescens* to an ATP binding cassette subfamily C2 (ABCC2) transport protein and identified a putative resistance-causing mutation in that gene. The following year Baxter et al mapped the resistance-causing mutation in the *P. xylostella* NO-QAGE strain to a chromosomal region containing the ABCC2 gene [26]. A landmark paper was then published in 2015 when Guo et al [27] performed a detailed analysis of this region using near isogenic Cry1Ac-resistant and susceptible populations of *P. xylostella*. Six genes from this region were studied in detail: ABCC1-5, and a MAP4K gene. No non-synonymous mutations were found in any of these genes that associated with resistance and there were no differences in expression of ABCC4-5 between susceptible and resistant populations. Although ABCC1 expression was increased in the resistant population, backcrossing experiments failed to show an association with resistance. In contrast, the reduced expression of ABCC2-3 observed in the resistant population tightly associated with the resistant phenotype. This linkage was validated by RNAi where suppression of either of these genes reduced susceptibility to Cry1Ac. The gene encoding MAP4K (now annotated as MAP4K4) was found to be upregulated in the resistant population. RNAi silencing of this gene in the resistant population was found to increase susceptibility to toxin. Although not found in the same region, or even the same chromosome, the authors also studied the gene encoding a membrane-bound alkaline phosphatase (PxmALP) – a putative toxin receptor [28]. No mutations were found in the PxmALP gene cloned from the resistant population although expression levels were found to be significantly reduced compared to susceptible larvae. Silencing of PxmALP by RNAi reduced susceptibility to Cry1Ac. In a further attempt to validate the role of PxmALP, the gene was heterologously expressed in Sf9 cells. This

Spodoptera frugiperda cell line is not susceptible to Cry1Ac toxin, however in cells expressing PxmALP toxin binding and increased cytotoxicity were observed – strongly indicating that this protein is a functional receptor for Cry1Ac in *P. xylostella*. In both *Plutella* and other insects, many of the genes such as MAP4K4 found to be constitutively overexpressed in resistant populations, are induced in susceptible populations upon exposure to Bt toxin [29], indicating a possible role in protecting the organism from the effects of the toxin.

Building a model for mode 1 resistance in *Plutella* In recent years a number of different genes have been implicated in the mode 1 resistance mechanism and validated using RNAi and/or heterologous expression. These include genes encoding ALP, ABCC2, ABCC3, MAP4K4, CDKAL1, SDF2L1 and HEL and can be joined by Pxwhite (an ABCG protein) [30]. Although it is entirely possible that different populations of *Plutella* showing a mode 1 resistance phenotype contain different mutations, the complementation data described above tempts us to look for a unified mechanism. The simplest model for resistance is based on a change – either expression levels or conformation – to a molecule directly involved in the toxic mechanism. This could include a receptor but also other molecules such as an activating protease. RNAi suppression of such a candidate gene would be expected to reduce susceptibility whereas heterologous expression should increase susceptibility in the host cell. Several of the validated candidates—namely ALP, ABCC2, ABCC3 and Pxwhite could act in this way. An alternative resistance mechanism would involve a change in the cell's/organism's physiology that better allows it to deal with toxin challenge, for example by repairing damage more effectively. RNAi of these candidate genes would be expected to increase susceptibility – as was seen with CDKAL1, SDF2L1 and HEL. In the above two scenarios mutations have occurred in molecules directly involved in some way with the toxic mechanism and these are summarised in figure 1 (A&B). Mutations could also occur in genes indirectly involved in the intoxication process, eg as part of a signalling pathway. This is shown in figure 1C where the solid circle represents a signalling effector that is involved in a number of pathways —indicated by the arrows. A mutation in this gene, or a regulatory element, could indirectly alter the physiology of the cell by downregulating the expression, or causing the shedding of, a toxin receptor. As a well-established mediator of signaling pathways MAP4K4 is an obvious candidate for the effector molecule in this indirect mechanism. The final scenario to consider is when a mutation elsewhere in the cell is sensed and feeds back to this aforementioned effector molecule either increasing or decreasing its concentration which would then affect toxin susceptibility as above (figure 1D). The ABC transporters could potentially act in this way, changes in their function could alter the physiology of the cell in such a way that mimics a toxin challenge, and results in the constitutive expression of pathways that protect the cell against toxin. If MAP4K4 is acting as a central effector molecule then one would expect that RNAi suppression of its gene would result in expression changes to other genes and indeed Guo et al [27] showed that RNAi resulted in an increased expression of ALP and ABCC2/3. In the above model where ABCC2/3 could indirectly affect susceptibility one would predict that its suppression by RNAi would alter the expression of MAP4K4 and ALP, and mutations in it would feedback to the central effector molecule. However, Guo et al found that RNAi of ABCC2 did not alter the expression of ALP (its effect on MAP4K4 was not measured), supporting that ABCC2 does not have direct regulatory effect on ALP expression.

Conclusions

Despite the wealth of confusing data on mode 1 resistance in *Plutella*, it is conceivable that there is a single mechanism common to many different resistant populations. A mechanism involving the loss of toxin binding capacity and reduced expression of a receptor such as alkaline phosphatase is consistent with available data. Loss of expression of the receptor would appear to be an indirect effect of a mutation associated with a *trans*-regulator, and

MAP4K4 is a strong candidate for this molecule. A mutation that results in over-expression of MAP4K4 may induce many changes in the cell, via regulatory networks, some of which affect the toxin's ability to act. Interfering with components of these networks could influence susceptibility via feedback mechanisms, even if the components being interfered with do not interact with the toxin mechanism directly. The increase in toxin susceptibility following RNAi of candidate genes such as the methylthiotransferase CDKAL1 could be result of this mechanism. The role of the ABCC2/3 transporters remains unclear, there is mounting evidence that homologues are true toxin receptors in other insects [31] and it is plausible that these proteins are toxin receptors in *Plutella*. It may be a coincidence that three closely linked genes (encoding ABCC2/3 and MAP4K4) are all involved in the resistance phenotype, alternatively all three may be involved in some form of regulon whose organisation or role is not understood. If there are multiple toxin receptor proteins in *Plutella* (eg ALP and ABCC2/3) then they could be acting independently, cooperatively, or sequentially as proposed for *Manduca sexta* [3]. A resistance mechanism driven by perturbations in intracellular regulatory networks has the advantage of being able to explain away a wide range of experimental observations as potentially many stimuli could affect a network and influence susceptibility. It does though make it difficult to identify factors directly involved in the resistance mechanism as many of the factors whose expression is altered, and whose suppression can cause changes in susceptibility, may not be directly involved. Even if a putative receptor is expressed in a heterologous host, and results in an increase in susceptibility, one can argue that its expression has indirectly affected the cell's physiology and it is that change that has directly influenced susceptibility. Thus a much better understanding of *Plutella*'s intracellular networks will be required before we can have complete confidence in such a model.

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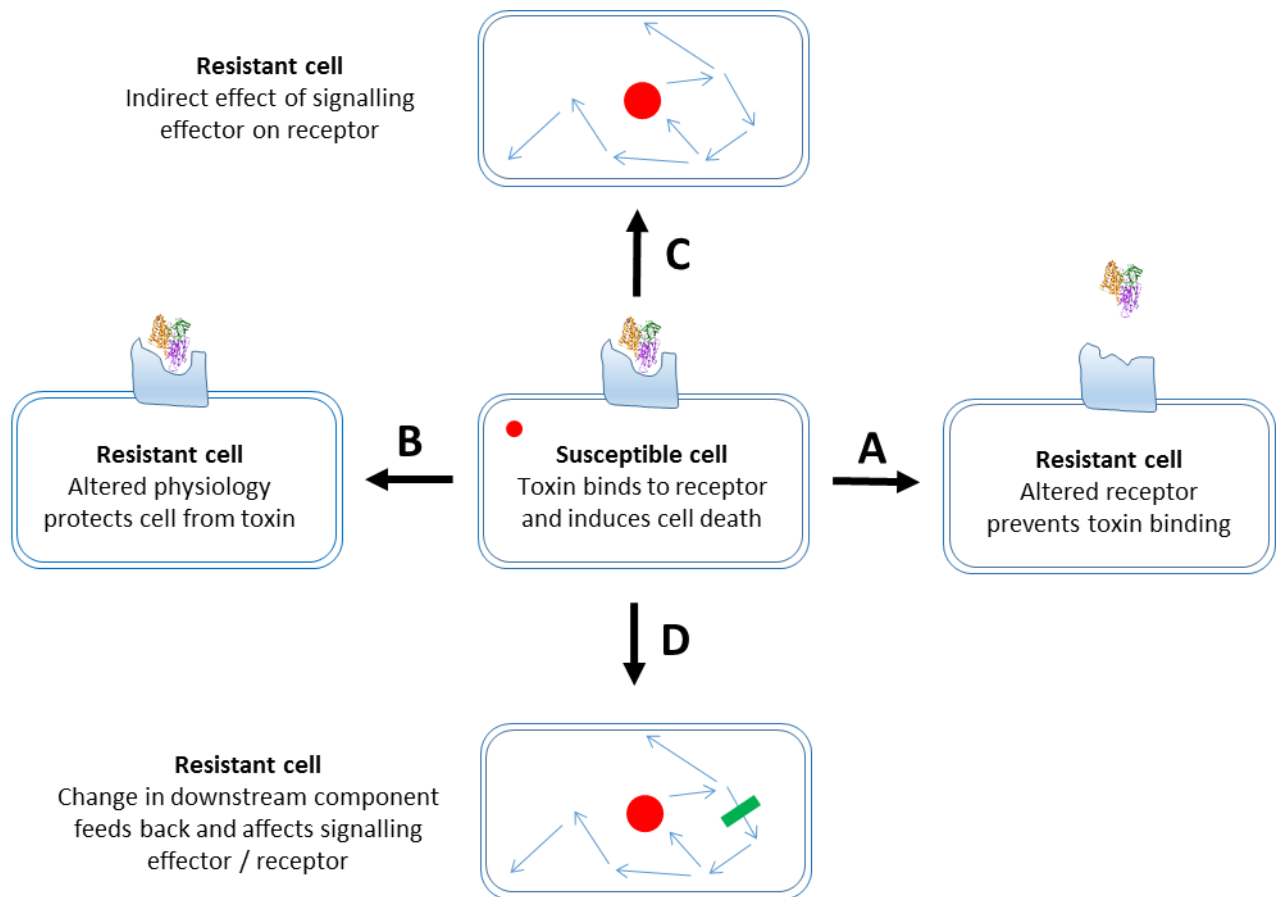


Figure 1. Mechanisms of resistance to Bt toxins. Four potential mechanisms that could affect the ability of the toxin to kill the cell are shown. A: a mutation in the receptor prevents the toxin interacting with the cell. B: mutations in other genes directly affect the toxin's ability to kill the cell. C: altered expression of a signalling effector results in resistance – eg by reducing expression of the receptor. D: altered expression of a downstream component of a signalling pathway feeds back to the aforementioned signalling effector and alters its expression.