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Specificity determinants for Cry insecticidal proteins: Insights from their mode of action

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ABSTRACT

Insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) are used as active components of biopesticides and as plant incorporated protectants in transgenic crops. One of the most relevant attributes of these Bt protein-based insecticidal technologies is their high specificity, which assures lack of detrimental effects on non-target insects, vertebrates and the environment. The identification of specificity determinants in Bt insecticidal proteins could guide risk assessment for novel insecticidal proteins currently considered for commercialization. In this work we review the available data on specificity determinants of crystal (Cry) insecticidal proteins as the Bt toxins most well characterized and used in transgenic crops. The multi-step mode of action of the Cry insecticidal proteins allows various factors to potentially affect specificity determination and here we define seven levels that could influence specificity. The relative relevance of each of these determinants on efficacy of transgenic crops producing Cry insecticidal proteins is also discussed.

1. Introduction

Among the insecticidal proteins produced by the bacterium *Bacillus thuringiensis* (Bt), the crystal (or Cry) proteins are the most well studied and produced by currently commercialized transgenic crops (Bt crops). Apart from their efficacy in controlling targeted pest species, Bt crops are also recognized for their environmental safety as a result of their high specificity (Koch et al., 2015). As with other proteins, specificity of Cry toxins is determined by the different steps involved in their mode of action, which are in part reflected in the three dimensional (3D) structure of the proteins. Among the currently >350 holotype Cry toxins, the most common 3D structure in the active toxin form involves three domains (reviewed in Xu et al., 2014). Domain I is composed of seven amphipathic alpha helices organized in a bundle with helix alpha-5 located centrally. Its structural similarity with pore-forming domains of alternative bacterial toxins and currently available experimental evidence supports a role for domain I in insertion in cell membranes. Domain II presents the highest diversity (suggestive of a role in specificity), and is composed of three antiparallel beta sheets arranged in a beta prism, displaying structural similarities with lectins in the jacalin family (Burton et al., 1999; Xu et al., 2014). In these lectins, three loops protruding from the beta prism structure determine specificity for carbohydrate binding (Meagher et al., 2005). Similar protruding loops in domain II have been shown to be involved in determination of binding specificity to host midgut proteins (Dean et al., 1996; Pigott et al., 2008), although their potential role in recognizing glycan moieties has not been experimentally tested. The three dimensional structure of domain III, also composed of beta sheets but arranged in a jelly roll topology, displays morphological similarities with cellulose binding domains of cellulolytic enzymes (Xu et al., 2014), supporting a role in recognizing specific carbohydrate moieties on proteins. In some cases, specific carbohydrate-binding regions in domain III have been detected and shown to be critical in determining specificity, as in the case of the N-acetylgalactosamine (GalNAc) binding pocket in Cry1Ac (Burton et al., 1999; Jurat-Fuentes and Adang, 2004).

As noted above, three dimensional protein structures give clues to specificity determinants. In the case of three domain Cry toxins, their structural features suggest a mode of action (reviewed in Adang et al., 2014) that includes interactions with midgut proteins (domains II and III) and insertion in cell membranes (domain I). However, when examined, there is a lack of direct correlation between structure and activity against specific targets, i.e. the same Cry toxin can be active against taxonomically diverse insects and Cry toxins with diverse binding determinants (domains II and/or III)

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may be active against the same insect (Palma et al., 2014). Consequently, Cry protein structure is generally not predictive of specificity and additional determinants, probably provided by the host, need to be considered.

It is well established that Cry toxins target host midgut cells and that they need to be ingested to reach the midgut epithelium. If the toxin is ingested as a parasporal crystalline body it must undergo solubilization to liberate a protoxin form. This protoxin form has been recently suggested to display toxicity through an alternative pathway (Tabashnik et al., 2015), but given the lack of direct experimental evidence for this process we focus our analysis of specificity determinants on the activated toxin, which is generated after sequential proteolysis of the protoxin form. The resulting activated toxin core must then traverse the peritrophic matrix and bind to receptors on the surface of midgut cells. Interaction between Cry toxin and midgut receptors is considered the main step dictating specificity of the toxin, although there are cases of high affinity binding not being associated with toxicity (Wolfersberger, 1990). While the specific mechanism responsible for enterocyte death by Cry toxins is still a matter of debate (Vachon et al., 2012), it is generally accepted that the toxin forms a pore that kills the cell by osmotic shock. Massive enterocyte death disrupts integrity of the midgut epithelial layer, allowing Bt and potentially other resident gut bacteria to invade the nutrient-rich hemocoel where they proliferate leading to septicemia and death of the insect (Raymond et al., 2010).

The goal of this manuscript is to review available information on the mode of action of Cry toxins that identifies potential specificity determinants of these proteins as relevant models of highly specific insecticidal proteins. For the purpose of this work, we define specificity as the condition of Cry proteins being toxic to a particular insect. We predict that since most Cry proteins produced by transgenic *Bt* crops are soluble, their specificity is not affected by the crystal solubilization step described below. However, all the specificity levels described below and in Fig. 1 would have a significant effect on specificity of *Bt* pesticides.

2. Specificity level I: Exposure to the insecticidal protein

An obvious first step determining specificity is the probability of the particular insecticidal protein encountering a host. The presentation of most of the Cry toxins as insoluble crystals limits their availability to certain hosts, for example sap feeding hemipterans. The poor ability of Bt to colonize various habitats including plant surfaces (Maduell et al., 2008), would also seem to limit the extent to which insects in those environments are exposed to Bt, unless transmission is primarily through insect-to-insect interactions (Milutinovic et al., 2015). Various interactions between Bt and nematodes have also been proposed as a mechanism by which the bacterium and its Cry toxins can be delivered to a susceptible host (Ruan et al., 2015). The specificity of certain Cry toxins (parasporins) towards human cancer cells (Mizuki et al., 2000) is particularly difficult to explain in evolutionary terms. In this case, it is possible that specificity determinants for interaction between the Cry toxins and these tumor cells are actually shared with as yet unidentified targeted gut insect cells.

Another interesting ecological observation is that some Cry toxins present inter-order activity, which has been documented for 6 of the 68 Cry families. Maybe an extreme example is Cry2Aa, which has been described as active against species of Lepidoptera, Diptera, and Hemiptera (van Frankenhuyzen, 2009). In this case, given the distinct ecological niches of each host, one would expect that the toxin contains specificity determinants for each of the orders, as most Cry toxins display activity against species within a single taxonomic order.

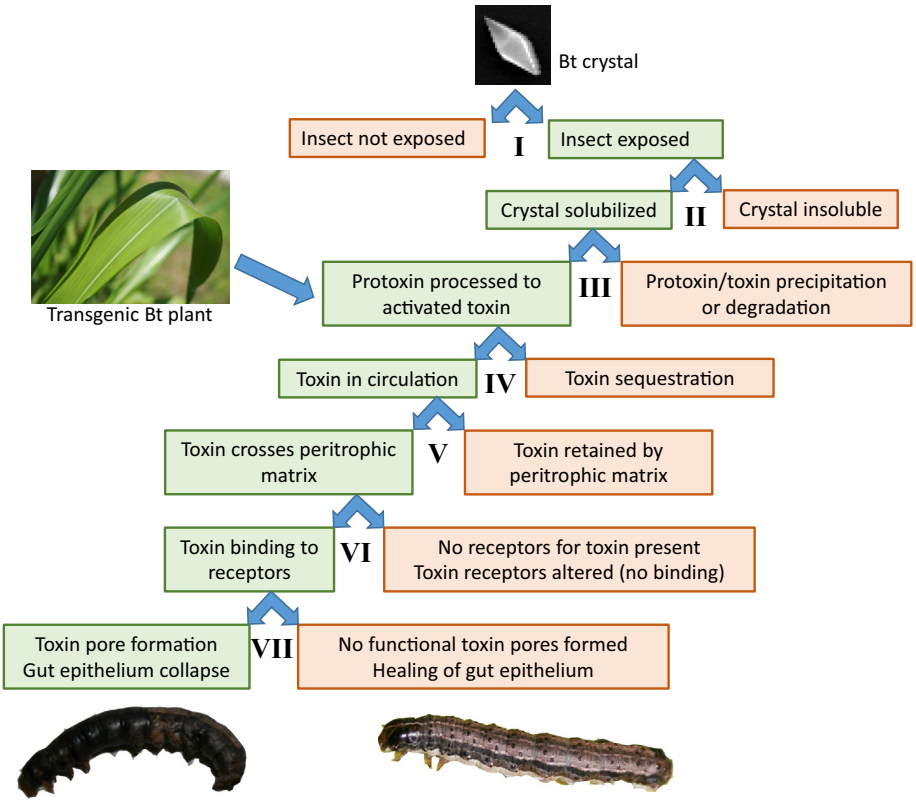

Fig. 1. Dichotomous flow chart detailing seven steps in the mode of action of Cry insecticidal proteins that determine toxin specificity. Each specificity determining step is shown as a dichotomous key in roman numeral. Cry proteins produced by transgenic Bt crops are not subjected to the two first specificity determinants.