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EFFECTIVE IRM WITH THE NOVEL INSECTICIDAL PROTEIN VIP3A

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Abstract

The Vip3A protein that is expressed in Syngenta's VipCot™ cotton varieties has been shown to provide excellent control of a range of insect pests of US cotton. By combining the effectiveness and distinct mode of action of Vip3A with the proven efficacy and performance of Cry1Ab, it is possible to develop a stacked product with outstanding insect control characteristics and exceptional benefits from an IRM perspective. The complementary actions of these two proteins thus give especially good control of *Helicoverpa zea, Heliothis virescens, Pectinophora gossypiella* and a number of other species. Syngenta is currently conducting studies to identify the component events for this stack. Vip3A is structurally and functionally distinct from the Cry endotoxins expressed in all other stacked cotton varieties. A range of studies has been conducted to confirm the absence of cross-resistance between Vip3A and Cry1A toxins. *In vitro* competition binding studies and ligand binding studies show that Vip3A binds to specific receptors that are distinct from those for Cry toxins. Dose-mortality assays with Cry1Ac-resistant and susceptible strains have also demonstrated the absence of cross-resistance between Vip3A and Cry1Ac has also been demonstrated for both Heliothine species. Resistance risk assessment modelling has been undertaken to show that the deployment of Vip3A can delay, at least to some degree, the development of resistance to cotton varieties expressing Cry toxins, and that the risk of resistance developing to Vip3A is very low.

Introduction

In the last few years, a number of cotton varieties expressing the novel protein, Vip3A, have been proven to provide exceptionally good control of a range of US cotton insect pests (Cook et al., 2004; Cloud et al., 2004; Mascarenhas et al. 2003; Mascarenhas 2004; Burd et al., 2005; Jackson et al., 2005; Luttrell et al., 2005; Leonard et al., 2005). Despite the effectiveness of these single-gene varieties, it has been shown that by combining the activity and distinct mode of action of the novel Vip3A with the proven efficacy and performance of Cry1Ab, it is possible to develop a stacked product with outstanding insect control characteristics and exceptional benefits from an IRM perspective. We are currently evaluating various events as candidates for this stack and conducting a range of studies with these events in order to expedite registration of a Vip3A x Cry1Ab stacked cotton variety at the earliest opportunity.

Stacked varieties expressing two toxins are generally considered to provide greater durability from an IRM perspective than single gene varieties (Caprio, 1998; Roush, 1998; Zhao et al., 2003). However, the significant IRM benefits that accompany deployment of a stack are highly dependent on a clear lack of cross-resistance between the components of that stack. Syngenta's Vip3A x Cry1Ab VipCot™ stacked variety is entirely unique in that it expresses two quite unrelated proteins and, unlike all other stacked insect control varieties, it does not express two Cry toxins. Because the risk of cross-resistance between Vip3A and Cry toxins may be much more remote than any Cry-Cry mixture, we believe the Vip3A x Cry1Ab stack has the potential for superior durability from an IRM perspective. Accordingly, a range of structural and functional properties clearly distinguish the Vip3A protein that is expressed in the VipCot™ stack from the crystalline δ-endotoxins of Bt that are expressed by currently available insect control cotton varieties in the US: Bollgard® (Cry1Ac), Bollgard II® (Cry1Ac + Cry2Ab) and WideStrike® (Cry1Ac + Cry1F) (Estruch et al., 1996; Lee et al., 2003, 2006). Moreover, using an entirely novel protein such as Vip3A as an insect control tactic has marked benefits in more general terms since it has the potential to lower

overall selection pressures from the widely used Cry toxins. Hence, the novel properties of Vip3A, the absence of cross-resistance between Vip3A and Cry toxins, and the initial positive findings from risk assessment modelling all contribute to the superior IRM properties of the Syngenta stack. These features are briefly reviewed below.

The Syngenta Vip3A x Cry1Ab stack components and their activity

The Vip3A protein expressed in Syngenta's VipCot™ cotton is derived from the soil bacterium *Bacillus thuringiensis*. It represents the first of a new, recently discovered class of insecticidal proteins, the Vegetative Insecticidal Proteins (Estruch et al., 1996). It is highly selective and effective against both of the two key Heliothine cotton pest species *Helicoverpa zea* (Cotton bollworm) (CBW) and *Heliothis virescens* (Tobacco budworm) (TBW) with somewhat greater activity versus the former. Vip3A also has excellent activity against a range of other cotton pest species including *Spodoptera frugiperda* (Fall armyworm), *Spodoptera exigua* (Beet armyworm), *Agrotis epsilon* (Black cutworm) as well as good activity against a number of other species including *Pectinophora gossypiella* (Pink bollworm) and *Trichoplusia ni* (Cabbage Looper) and *Pseudoplusia includens* (Soybean looper) (Estruch et al., 1996; Yu et al., 1997; Cook et al., 2004; Cloud et al., 2004; Mascarenhas et al. 2003; Mascarenhas 2004; Burd et al., 2005; Jackson et al., 2005; Luttrell et al., 2005; Leonard et al., 2005).

The second component of the Syngenta stack is the conventional Cry toxin, Cry1Ab. Cry 1Ab provides excellent activity versus *H. virescens* and *P. gossypiella*, and very good activity versus *H. zea*. We believe the complementary actions of these two components provide an outstanding spectrum of excellent activity versus the key pests of US cotton, as well as delivering especially favourable IRM properties.

The overall insecticidal activity of a stack of two proteins is in part dependent on the independent activity of the two individual components and thus we are assessing the high dose status of both against key pests. Moreover, such information is essential for realistic risk assessment modelling. High dose expression of Cry1Ab events versus *H. virescens* has been shown using two of the US-EPA SAP's proscribed methodologies (one lab, one field). Similarly, a high dose lab method has been used to preliminarily confirm high dose expression of these Cry1Ab events against both *H. zea* and *P. gossypiella*; additional studies are in progress. All of this research is described in detail in the accompanying paper by O'Reilly et al., (2006). Additional Cry1Ab events are being examined, and we are also evaluating the high dose status of potential Vip3A components of the stack. These studies follow previous similar work with the Vip3A events, Cot202 and Cot203, that were shown to be high dose for both Heliothine species (O'Reilly et al., 2005; Mascarenhas et al., 2005). In a similar manner, work will be undertaken to assess the Vip3A x Cry1Ab stack for functional high dose.

Novelty of Vip3A

Sequence and Structural Properties of Vip3A

One of the most important features of the Vip3A protein is that it shares no sequence homology with the known Bt δ-endotoxin genes or other toxin genes (Estruch et al., 1996). This complete lack of sequence homology between the Cry toxins and Vip3A together with the very marked predicted structural differences between Vip3A and the Cry toxins strongly supports the contention that cross-resistance between Vip3A and Cry1Ac, Cry1F or Cry2Ab2 is very unlikely.

Pore formation

After binding, the activated toxin is inserted into the midgut membrane forming pores, which markedly disrupt the ionic balance across the midgut membrane and lead to eventual insect death. The pores formed when the 62kDa proteolytically activated fragment of Vip3A binds to specific sites on the epithelial cells of the brush border membrane of the midgut of target insects have unique structural and functional properties that differ radically from those formed as a result of Cry toxin action (Estruch et al., 1996; Lee et al., 2003). In voltage clamp assays with isolated midguts from the susceptible species, *Manduca sexta*, activated Vip3A (62kDa) showed pore-forming activity, while full-length Vip3A (88kDa) did not form pores (Lee et al., 2003). Vip3A forms stable channels, which are voltage independent and highly cation selective; however, they differ considerably in their principal conductance state and cation specificity from Cry1A toxins. These findings provide further evidence that target site cross-resistance between Vip3A and Cry toxins is highly unlikely.

***In-vitro* binding studies**

One of the key elements in the mode of action of Bt toxins is their binding to specific receptors on the surface of the midgut membrane. In general, biological activity and binding to receptors is positively correlated. Thus, in binding studies with susceptible and resistant insect strains, a significant reduction of receptor binding was observed in the resistant strains (Van Rie et al., 1990) indicating that receptor binding is a critical element in the development of resistance. This mechanism is much more common in laboratory-selected Cry-toxin resistant insect strains and indeed, to date, it is the only principal mechanism to have evolved from selection by formulated microbial Bt products in the field (in *P. xylostella*) (Tabashnik et al., 1997; Wright et al., 1997). To maximize the expected IRM benefits of deploying a stacked cotton variety expressing more than one Bt toxin, it is essential that there is no cross-resistance between the components of the stack. In this regard, it is paramount that there must be a complete absence of any interaction between the stack components at the receptor binding level.

Recent published studies on the mode of action of Vip3A have clearly demonstrated that it has unique receptor binding properties, which are markedly different from those of Cry1A toxins (Lee et al., 2003). Competition assays using BBMV from *M. sexta* showed that Vip3A and Cry1Ab did not inhibit binding for each other, indicating they recognize different receptors. Binding assays to known Cry1A receptors provided more direct evidence. Accordingly, it was shown that Vip3A did not bind to aminopeptidase N (120kDa) or cadherin (>210Kda). In addition, unique Vip3A (80kDa and 100kDa) binding proteins, that are distinct from Cry1A binding proteins, were identified in *Manduca sexta* BBMV (Lee et al., 2003). These findings strongly support the view that Vip3A possesses unique binding properties and a distinct mode of action.

More recently, this work has been extended to assess the possibility of cross-resistance between Vip3A and Cry1Ac or Cry2Ab2 in *H. virescens* and *H. zea*, the two key target pests for VipCot (Chen and Lee, 2005; Lee et al., 2006). This has been conducted using a series of *in vitro* binding studies using BBMV competition assays and BBMV ligand blotting assays, and is summarised below.

In vitro competition binding assays

Competition binding assays with *H. virescens* and *H. zea* BBMV have demonstrated the binding site relationships among Vip3A, Cry1Ac, and Cry2Ab2 toxins. Biotin-labelled Vip3A was shown to competetively bind to the BBMV of both species; excess unlabeled Vip3A significantly reduced this signal as expected. In contrast, no competitive binding was observed between Vip3A and Cry1Ac or between Vip3A and Cry2Ab2; in each case excess cold Cry toxin did not inhibit the binding of labelled Vip3A. These findings indicate that Cry1Ac and Cry2Ab2 do not bind to the Vip3A receptor. The binding of Cry2Ab2 was non-specific (labeled Cry2Ab2 toxin could not be chased by increasing concentration of unlabeled Cry2Ab2 toxin) indicating that Cry2Ab does not exhibit specific binding kinetics with BBMV. This non-specific binding of the Cry2Ab2 was not inhibited by the addition of unlabeled Vip3A indicating that Vip3A does not bind to the Cry2Ab2 binding sites. In summary, the BBMV competition binding assays demonstrated in that both *H. zea* and *H. virescens* Vip3A does not share binding sites with either Cry1Ac or Cry2Ab2 toxins.

BBMV ligand blotting assays

Using BBMV ligand blotting techniques, several putative Cry1Ac toxin-binding proteins (receptors) (80kDa, 120kDa, 150kDa, 170kDa, >210kDa) have been identified (Lee et al., 1995). Likewise, putative toxin binding proteins on BBMV from both *H. virescens* and *H. zea* have been identified by ligand blotting assays , and similar patterns have been observed. In *H. virescens* two major peptides were identified as Cry1Ac binding proteins (120kDa and 150-170kDa). Biotin labelled Vip3A toxin does not show any binding to these peptides. Whilst excess amounts of unlabeled Cry1Ac competed for the binding of labelled Cry1Ac to these peptides (competitive and specific binding), excess amounts of unlabelled Vip3A did not inhibit Cry1Ac binding, confirming that Vip3A did not bind to the Cry1Ac binding proteins. In *H. zea* somewhat more complex binding patterns were observed. Three major peptides were identified as Cry1Ac binding proteins (120kDa, 150kDa, and >210kDa). As in *H. virescens*, Vip3A does not show binding to any of these peptides. While excess amount of unlabeled Cry1Ac toxin competed off the binding of labelled Cry1Ac to these peptides (competitive and specific binding), excess amount of cold Vip3A did not inhibit Cry1Ac binding, confirming that Vip3A does not recognize any of these Cry1Ac receptors.

In summary, the *in vitro* binding studies outlined above (Lee et al., 2006) demonstrate that Vip3A has distinct binding sites from Cry1A and Cry2A toxins. Moreover, lack of binding of Vip3A to the known Cry1A receptors strongly supports the premise that the potential for target site cross-resistance between Vip3A and Cry toxins is negligible, and thus provides a sound basis for developing a durable stack of Vip3A + Cry1Ab which has highly desirable properties from an IRM perspective.

***In vivo* Cross-resistance studies**

In-vivo cross-resistance studies

To further investigate the possibility of cross-resistance between Vip3A and Cry toxins a series of bioassays was conducted at North Carolina State University (NCSU) to investigate the impact of Vip3A toxin on the mortality and growth of *H. virescens* strains that had different mechanisms and levels of resistance to Cry1Ac and other toxins. This work is described more fully in the accompanying paper by Jackson et al. (2006). The resistant strains of *H. virescens* used were YHD2, CXC, and KCBhyb. These strains were developed at NCSU (Gould et al., 1992; Jurat-Fuentes et al., 2003), and compared to a susceptible YDK strain. Resistance to Cry1Ac in the YHD2 strain was so strong that no LC_{50} values or fiducial limits could be calculated, whereas the resistance to Cry1Ac in the KCBhyb and CXC strains varied between 52- and 424-fold. In contrast, similar dose-mortality assays with Vip3A indicated that all the Cry1Ac-resistant and susceptible strains were equally susceptible to this toxin and that no cross-resistance exists between Cry1Ac and Vip3A in this species. At moderate doses of Cry1Ac, growth of YHD2 strain insects was similar to susceptible insects on control diet whereas the growth ratios of CXC and KCBhyb insects were more significantly reduced, reflecting the more moderate level of resistance observed. In contrast, growth of larvae on Vip3A was somewhat variable; however at doses of Vip3A that were equitoxic to those on Cry1Ac, all growth rates declined markedly with increasing toxin dose, indicting a marked lack of cross-resistance.

Further studies were conducted to assess the ability of the YDK, CXC and KCBhyb strains of *H. virescens* to survive on Vip3A-expressing leaves. In preliminary assays, mortality of KCBhyb and CXC insects was much lower than that of YDK on leaf tissues of a Cry1Ac expressing cotton line as expected for these Cry1Ac-resistant strains, and this was reflected in changes in larval weight. In contrast, mortality of the strains varied very little from controls when placed on leaves of the Vip3A-expressing varieties (Cot102, Cot203). Insect weights likewise varied very little. KCBhyb and CXC insects consumed either equal or lesser amounts of non-Bt, Cot102 and Cot203 leaf tissue compared to that of YDK insects (Jackson et al., 2006). All of these findings emphasize the lack of cross-resistance between Vip3A and Cry1Ac in *H. virescens*. Further dose-mortality assays with *H. zea* are being conducted in a similar manner to the studies described above. Initial mortality results and growth observations suggest that there is no cross-resistance between a Cry1Ac-resistant strain (HZ02) and a susceptible strain (XYZ), although these findings have yet to be verified by replication.

Resistance Risk Assessment Modelling

A number of simulation models have previously provided insight into the possibility of resistance in target insects associated with the deployment of transgenic insect-control crop varieties. These models in part guided the development of the high dose + refuge strategy that is mandated for IRM for insect-control cotton varieties. However different models produce different results, depending on the manner in which they were obtained. Such deterministic models will for any given set of input parameters, often produce the same results. To determine the sensitivity of the results of such models to variations in the input parameters it has been usual to run a series of scenarios representing best- to worst-case scenarios. Unfortunately, it is not possible to assign any degree of probability to such scenarios, and hence the relative risks of resistance are uncertain.

At Mississippi State University an alternative risk assessment approach has been used in which the uncertainties in each input parameter are formally specified as a defined distribution with minimum, maximum and most likely values. In this approach, a large number of model realizations are run with randomly drawn parameters from the specified distributions in place of running a specific number of fixed scenarios. The results can be arranged as a probability distribution that is a direct measure of the impact of that parameter uncertainty on the assessment of resistance risk.

Accordingly, the risk of resistance to Vip3A arising in *H. zea* has been assessed using a model developed by Parker and Caprio (Parker, 2000), which incorporates 43 different habitats including early and late season wild hosts, Bt- and non-Bt corn, generic Bt- and non-Bt cotton, Vip3A cotton, soybean, and sorghum. On the basis of the studies described above, cross-resistance between Vip3A and generic Bt-cotton was assumed to be zero. The outcome of this study is described very briefly below.

When Vip3A was included in the system, there was an increased risk of finding Vip3A resistance alleles, but the overall rate of increase was still small. Indeed, in close to 50% of the cases the resistance allele frequency decreased. Even after 15 years, in less than 1% of simulations had the resistance allele increased in frequency more than 10-fold, suggesting that the risk of resistance in this modelled system is particularly low. In addition, when Vip3A was included in the system there was a clear shift towards a delay in the rate of resistance to generic Bt-cotton. Increasing the area of Vip3A cotton delayed this resistance even further. Such a finding suggests that from the perspective of the generic Bt-event, the introduction of Vip3A cotton should be seen as a positive resistance management strategy, with broad industry benefits. The work is now being extended to assess the risk and benefits of the Vip3A x Cry1Ab stack.

Summary

By combining the effectiveness and distinct mode of action of Vip3A with the proven efficacy and performance of Cry1Ab, it is possible to develop a stacked product with outstanding insect control characteristics and exceptional benefits from an IRM perspective. Syngenta is currently proceeding with studies to identify the events for this stack. The complementary actions of these two proteins give especially good control of *H. zea, H. virescens, P. gossypiella* and a number of other species. Vip3A has a unique structure that distinguishes it from the Cry toxins expressed in all other insect-control cotton varieties and the protein differs functionally from the Cry toxins. A range of *in vitro* competition binding studies and ligand binding studies show that Vip3A binds to specific receptors that are distinct from those for Cry toxins, indicating a lack of cross-resistance at the target site. An absence of cross-resistance between Vip3A and Cry1Ac has also been demonstrated for both Heliothine species using dose-mortality assays with Cry1Ac-resistant and susceptible strains. These studies also show that Cry1Ac-resistant strains of *H. virescens* are unable to survive on cotton varieties expressing Vip3A. Resistance risk assessment modelling has been undertaken to show that the deployment of Vip3A can delay the development of resistance to cotton varieties expressing Cry toxins and that the risk of resistance developing to Vip3A is very low. Togther these features indicate that the Vip3A x Cry1Ab stack that Syngenta is developing will provide outstanding control of cotton insect pests and have excellent properties from an IRM perspective.

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