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INSECT RESISTANCE MANAGEMENT FOR VIPCOTTM

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Abstract

Syngenta's VipCot™ cotton events, Cot202 and Cot203 express the novel insecticidal protein Vip3A. Both events provide outstanding control of *Heliothis virescens* (TBW) and *Helicoverpa zea* (CBW), as well as a range of secondary pests of cotton. Cot202 and Cot203 meet the requirements for an IRM strategy based on high dose plus the use of structured refuges. The strategy proposed includes the use of 20% external sprayed, 5% external unsprayed, 5% embedded and community refuge options. Using two of the five methods endorsed by the US-EPA, both Cot202 and Cot203 have been shown to express a high dose of Vip3A for the control of both Heliothine species. Modelling studies are underway to validate this approach and preliminary findings indicate that resistance to Vip3A will not occur within 20 years. Moreover, these preliminary data also indicate that deployment of VipCot can significantly delay resistance to Cry1Ac in CBW. Vip3A has structural and functional properties that are very distinct from those of the Cry toxins. These features together with *in vitro* receptor binding studies indicate that cross-resistance between Vip3A and Cry toxins is highly unlikely. *In vivo* assays using Cry toxin-resistant strains of TBW and CBW also indicate that that cross-resistance between Cry toxins and Vip3A is unlikely. A comprehensive product stewardship program is being developed to support the proposed IRM strategy to accompany deployment of VipCot™ and togther these approaches will ensure the durability of this valuable new insect management tool for cotton.

Introduction

The Vip3A protein that is expressed in Syngenta's VipCot™ cotton varieties is derived from the soil bacterium *Bacillus thuringiensis* and it represents a new, recently discovered class of insecticidal proteins, the Vegetative Insecticidal Proteins (Estruch et al., 1996). It is highly selective and extremely effective against both of the two key Heliothine cotton pest species *Heliothis virescens* (Tobacco budworm) (TBW) and *Helicoverpa zea* (Cotton bollworm) (CBW) and has good activity against a range of other cotton pest species including *Pectinophora gossypiella* (Pink bollworm), *Spodoptera frugiperda* (Fall armyworm), *Spodoptera exigua* (Beet armyworm), *Agrotis epsilon* (Black cutworm) as well as good activity against a number of other species including *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).

Syngenta has developed a number of varieties expressing Vip3A including Events Cot102, Cot 202 and Cot 203. The events Cot202 and Cot203 show particularly outstanding activity against both TBW and CBW (Mascarenhas et al., 2003; Mascarenhas, 2004; Burd et al., 2005; Jackson et al. 2005; Leonard et al., 2005; Luttrell et al., 2005; Mahaffey et al., 2005), which together are responsible for a majority of the damage to cotton across the US cotton; belt. VipCot™ thus provides levels of cotton pest insect control comparable with the best commercialised insect-control transgenic cotton varieties currently available in the US, including the two-gene constructs. VipCot™ achieves this without the necessity of expressing a second protein.

Vip3A is characterised by a range of properties that very clearly distinguish it from the crystalline δ-endotoxins of Bt that are expressed by the other insect control cotton varieties that are available to US cotton growers: Bollgard®

(Cry1Ac), Bollgard II® (Cry1Ac + Cry2Ab) and WideStrike® (Cry1Ac + Cry1F) (Yu et al., 1997; Lee et al., 2003; Chen and Lee, 2005). This novelty implies that cross-resistance between Vip3A and the Cry toxins is highly unlikely, and experimentation that demonstrates the validity of this statement is discussed below.

The successful deployment of VipCot™ will thus provide growers with a powerful and novel pest management tool that gives outstanding control of key pest insects. VipCot will provide growers with a new choice for cotton pest insect control and its use will introduce a toxin diversity into the current Cry toxin-dominated marketplace for the first time. This will help reduce the overall level of selection by any one toxin at a macro level, and help sustain all these technologies.

Because of the clear environmental, agronomic, economic and resistance management benefits that use of VipCot™ will bring, it is essential that once this technology is deployed, its continued utility is protected through the implementation of an appropriate and effective insect resistance management (IRM) strategy. Moreover, any IRM plan for VipCot™ must fit into the current multiple variety landscape. We have developed an IRM plan for VipCot™ that does this, and this is briefly outlined below together with some of the studies being used to support this approach. Detailed justification for the IRM strategy especially with respect to the biology of TBW and CBW is developed elsewhere.

Proposed IRM strategy for VipCot

Growers are burdened with the responsibility of undertaking the IRM strategies that are required of them, and it is essential for technology providers to develop IRM plans that encourage understanding, implementation and compliance. As the cotton pest management marketplace becomes ever more complex, with an increasing number of transgenic offerings, there is a clear premium on compatibility with existing technologies. The IRM strategy that Syngenta proposes to support VipCot™ use in the US cotton belt is therefore similar to that currently used in cotton with current transgenic insect-control cotton varieties. It is based on the high dose + refuge strategy that is already well-understood (Alstad and Andow, 1995; US EPA, 1998, 2001; Gould, 1998; Matten and Reynolds, 2003; Roush, 1997; Tabashnik 1990) and familiar to cotton growers. This approach will assist in understanding, promote compliance and avoid the marketplace complexities that might well arise were use of cotton varieties with differing IRM strategies permitted. Moreover, such an approach provides growers with maximum choice and flexibility and it maximises the benefits of the novel Vip3A technology.

Refuge requirements

Growers will be permitted to select a number of refuge options. The definition of these refuge requirements has been driven by a number of key considerations. Importantly, use of the options defined below complements other technologies, provides a degree of uniformity for cotton lepidoptera-control and avoids marketplace confusion.

The object of this strategy is to maximise the likelihood that any rare homozygous (RR) resistant insects that survive on VipCot™ will mate with the abundant susceptible (SS) insects emerging from the refuge fields (Alstad and Andow, 1995; US EPA, 1998, 2001; Gould, 1998; Roush, 1997). The organisation and maintenance of these refuges is designed to optimise the balance between effectiveness and convenience. The resultant heterozygote (RS) individuals will be killed by the high dose expressed in VipCot, thus preventing the spread of resistance alleles in the population. Refuge options include:

1 An external, unsprayed refuge of non-lepidoptera control cotton equivalent in area to a minimum of 5% of the associated VipCot™ acres. The size of the refuge must be at least 150 feet wide, but preferably 300 feet wide. This refuge must not be treated with sterile insects or pheromones or insecticides labelled for the control of TBW, CBW or PBW. However, the refuge may be treated with acephate or methyl parathion at rates that will not control TBW or CBW as is allowed for existing insect-control cotton varieties. This non-insect control refuge must be placed within at least 0.5 linear miles of the associated VipCot™ fields, and preferably adjacent to, or within 0.25 miles.

2 An external, sprayed refuge of non-lepidoptera control cotton equivalent in area to a minimum of 20% of the associated VipCot™ acres. This refuge must be placed within at least one linear mile of the associated VipCot™ field(s), and preferable 0.5 miles or closer. The refuge may be treated with sterile insects or pheromones or insecticides (excluding foliar Bt products) labelled for the control of TBW, CBW or PBW

3 An embedded refuge of non-lepidoptera control cotton equivalent in area to a minimum of 5% of the associated VipCot™ acres. This refuge type must be embedded as a contiguous block surrounded on all sides by VipCot™ plants, and not at one edge of the VipCot™ field. For very large fields, multiple embedded refuge blocks may be used. For small or irregularly shaped fields neighbouring fields farmed by the same grower may be grouped into blocks to represent a larger field unit, provided the block exists within one square mile of the VipCot™ cotton, and the block is at least 150 feet wide, but preferably 300 feet wide. Within the larger field unit, one of the smaller fields planted to non-lepidopteran control cotton can be used as the embedded refuge. The embedded refuges must be managed in an identical manner to VipCot™. The embedded refuge may thus be treated with sterile insects or pheromones or insecticides (excluding foliar Bt products) labelled for the control of TBW, CBW or PBW.

4 A community refuge plan that utilizes external 20% sprayed or external 5% unsprayed refuges. This option is organized in a manner that allows multiple growers to contribute to the overall required refuge acres. It cannot be used for embedded or in-field options. It must meet the requirements of the 5% unsprayed or 20% sprayed options outlined above, or an appropriate combination of the two options, in a manner which provides the necessary overall refuge requirements to support the IRM strategy.

There are several key general requirements. Refuges must conform to specific requirements in terms of cotton variety, shape, placement, proximity and management. Refuge fields must not be planted with other transgenic cotton varieties used to control lepidoptera since neither may act as a refuge for the other.

High dose

The EPA has adopted a definition of high dose for a Bt Plant Incorporated Protectant (PIP) that is 25 times that which is sufficient to kill all neonates (US EPA, 2001). This is based on the fact that empirical data on resistance of lepidoptera to Bt Cry toxins has shown that heterozygotes are rarely greater than 25-fold resistant unless the inheritance of resistance is dominant (US EPA, 2001). A high dose product is therefore expected to cause at least 95% mortality of the most tolerant heterozygotes in the field, i.e. that functional dominance is likely <0.05. As a Bt PIP it is not unreasonable to assume that the characteristics of any resistance that might develop to Vip3A may be similar to that known for other Bt PIPs, and expression of a high dose at this level would provide a key component of an effective IRM strategy for VipCot.

Five imperfect methods to demonstrate high dose have been devised by the EPA's Scientific Advisory Panel (US EPA, 1998, 2001). A Bt PIP can be considered to provide high dose if verified by at least two of these methods. For both TBW and CBW, two methods have been used to demonstrate that Vip3A is expressed at high dose in the lead events Cot202 and Cot203. The findings of these field and laboratory studies are described in accompanying papers at this conference (Mascarenhas et al., 2005; O'Reilly et al., 2005). For TBW and CBW 25-fold dilutions of lyophilized Cot202 or Cot203 tissue in artificial diet (EPA SAP method #1) have been shown to give >95% mortality and for CBW a similar >95% mortality of has been demonstrated for larvae that are well in excess of 25-fold less susceptible than neonates (EPA SAP method #5) (O'Reilly et al., 2005). An artificial infestation field trial method (EPA SAP method #4) has also been used to show that Cot202 and Cot203 are high dose for TBW (Mascarenhas et al., 2005). Taken togther these results clearly demonstrate that both Cot202 and Cot203 express a high dose of Vip3A versus both TBW and CBW.

Significantly, VipCot is the only single-gene insect-control cotton that expresses a high dose of insecticidal protein with respect to both TBW and CBW. To achieve this high dose status for both pests, and the IRM benefits associated with this, VipCot does not required the additional expression of a second protein.

Novelty of Vip3A and lack of cross-resistance

The significant IRM benefits that accompany deployment of VipCot result from the novelty of Vip3A, and a lack of any cross-resistance between Vip3A and the Cry toxins expressed in all currently registered insect-control transgenic cotton varieties. The Vip3A protein that is expressed in VipCot is characterised by a range of structural and functional properties that very clearly distinguish it from the crystalline δ-endotoxins of Bt that are expressed by the other insect control cotton varieties that are available to US cotton growers: Bollgard® (Cry1Ac), Bollgard II® (Cry1Ac + Cry2Ab) and WideStrike® (Cry1Ac + Cry1F). It has no sequence similarity to the Cry toxins, and it has a predicted protein structure that is entirely dissimilar to that of the Cry toxins. The pores formed as a result of the binding of the 62kDa proteolytically activated fragment to specific sites on the epithelial cells of the brush border

membrane of the midgut of target insects, as a result of Vip3A action, have structural and functional properties that differ radically from those formed as a result of Cry toxin action (Lee et al., 2003).

In vitro competition binding studies and ligand binding studies with *H. virescens* and *H. zea* have been used to demonstrate that Cry1Ac and Cry2Ab2 do not bind to specific Vip3A receptors. These studies demonstrate that cross-resistance at the target site between Vip3A and the currently most widely used Cry δ-endotoxins is highly unlikely. The research is described in detail in the accompanying paper by Chen and Lee (2005).

In vivo cross-resistance studies are being conducted in the laboratories of Dr JR Bradley, Dr J Van Duyn and Dr Fred Gould at North Carolina State University, and preliminary findings are presented at this conference (Marcus et al., 2005). The YHD2 strain of *H. virescens* (Gould et al., 1995) was shown to be highly resistant to Cry1Ac (LC50 ~2000µg/ml), but was shown to have no resistance to Vip3A in diet treated bioassays, and was not resistant to leaf tissues form Vip3A-expressing cotton plants. Likewise, the CXC strain of *H. virescens* (Jurat-Fuentes, 2003) that is resistant to a broader range of Cry toxins (LC50 for Cry1Ac 211µg/ml) was susceptible to Vip3A protein in diet assays and susceptible to leaf tissue from Vip3A-expressing cotton plants. The KCBhyb strain of *H. virescens* (Jurat-Fuentes, 2003) is also more broadly resistant to a number of Cry toxins (LC50 for Cry1Ac 137µg/ml) and in leaf assays it performed comparably to control YDK strain with regard to tissue consumption and larval weight but growth ratios in these preliminary studies were somewhat higher and further studies are required to evaluate this (Marcus et al., 2005).

Similar studies have shown that a field collected XYZ strain of *H. zea* that was selected in the laboratory for resistance to Cry1Ac (LC50 ~1000µg/ml) was susceptible to leaf tissue from plants expressing Vip3A and was also susceptible to Vip3A protein in diet treatment assays. Further studies are planned to confirm these findings.

Taken together, the properties of Vip3A, the *in vitro* binding studies and the *in vivo* bioassay studies outlined above suggest that in both TBW and CBW cross-resistance between Cry toxins and Vip3A is highly unlikely. Should resistance to Cry1Ac evolve, Vip3A should be unaffected. Likewise, in the unlikely event that resistance to Vip3A should arise, susceptibility to Cry toxins would be unaffected.

Predictive modelling

VipCot™ will provide growers with a powerful and novel pest management tool that gives outstanding control of key pest insects. By introducing a new choice for cotton pest insect control, its use will introduce a toxin diversity into the current Cry toxin-dominated marketplace for the first time. It is believed that this will help reduce the overall level of selection by any one toxin at a macro level, and help sustain all these technologies. Predictive modelling studies are being conducted by Dr Mike Caprio at Mississippi State University and are being used to validate this approach and the high dose and refuge strategy that is proposed for VipCot™. These studies examine not only the evolution of resistance to Vip3A but also seek to understand the impact of Vip3A deployment in a matrix of insect-control cotton varieties. Preliminary findings have shown that the frequency of alleles for resistance to Vip3A does not increase significantly within 20 years. Moreover, similar preliminary findings indicate that the introduction of Vip3A expressing varieties can significantly delay resistance to Cry1Ac in CBW. These modelling studies will be further developed in the future to help optimise the use of multiple insecticidal proteins.

Product stewardship

To preserve the benefits to growers of VipCot™, Syngenta is committed to implementing an aggressive stewardship program that will maintain the long-term efficacy of VipCot™ cotton by reducing the potential for pests to develop resistance to the Vip3A protein. The various elements of this program include: 1 an ongoing comprehensive, multi-faceted education program including collaboration with other bodies to promote educational initiatives. 2 Use of Grower Agreements to reinforce grower understanding and compliance. 3 A compliance assurance program that is designed to a) evaluate the extent to which growers of VipCot™ are complying with the IRM requirements, and b) take actions reasonable needed to assure that growers who have not complied with the IRM requirements are brought back into compliance with those requirements. 4 Monitoring for changes in pest susceptibility to Vip3A. 5 A requirement for growers and seed distributors to contact Syngenta or a local authorized dealer if incidents of unexpected levels of damage by lepidoptera occur during use of VipCot™ cotton. 6 Reporting of all instances of confirmed TBW or CBW resistance to VipCot™ to the EPA within 30 days. 7 Defined immediate mitigation

measures in the event that resistance to VipCot™ is confirmed. All of these activities will be developed as VipCot™ goes forward to the market.

In preparation for routine monitoring of susceptibility to Vip3A, initial bioassay method devlopment work is underway. Baseline studies are being conducted with University co-operators: For TBW and CBW, studies are being performed by Drs Randy Luttrell and Ibrahim Ali at the University of Arkansas. Syngenta believes that there is considerable scope for future co-operation and cost saving with other technology providers with regard to monitoring activities.

Summary

Syngenta's insect-control cotton events Cot202 and Cot203 have outstanding activity against the two key Heliothine pests of cotton, *Heliothis virescens* and *Heliothis zea*, and provide excellent control of a range of other lepidoptera species infesting cotton. Both events express a high dose of Vip3A for the control of both Heliothine species and both are amenable to the implementation of an IRM strategy based on high dose plus structured refuges. The refuge requirements proposed for VipCot™ are entirely similar to those used with current commercialised insect-control cotton varieties. Syngenta believes that this approach avoids marketplace complexity and favours grower understanding and compliance. The introduction of VipCot™ into the marketplace provides a novel toxin and brings considerable IRM benefits. Initial modelling studies indicate that the introduction of VipCot™ into a matrix of current varieties may delay the development of resistance to Cry toxins. The novel features of VipCot™ and the findings of practical *in vitro and in vivo* cross-resistance studies suggest that cross-resistance between Cry toxins and Vip3A is highly unlikely. In order to preserve these benefits and support the IRM strategy Syngenta is committed to a comprehensive product stewardship programme. In summary, we believe VipCot™ provides growers with an exciting new pest insect management tool with unique properties and considerable IRM benefits.

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