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The Identification and Development of Proteins for Control of Insects in Genetically Modified Crops

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Genetically modified plants that produce insecticidal proteins have recently been added to the arsenal of tools available for controlling specific insect pests of several major crops (Armstrong et al., 1995; Barton et al., 1987; Fischhoff et al., 1987; Koziel et al., 1993; Perlak et al., 1990, 1993; Vaeck et al., 1987). These plants were engineered for insect protection by the introduction of genes encoding d-endotoxin insecticidal proteins from the bacterium *Bacillus thuringiensis* (*Bt*). These proteins are highly potent and specific natural insecticides, some of which constitute the active components of microbial-based formulations that have been widely used for decades as environmentally benign alternatives to chemical insecticide sprays. The cultivation of plants transformed with genes encoding *Bt* proteins or other insecticidally active proteins offers significant agricultural and environmental advantages over the use of traditional pesticide treatments. The agricultural benefits include the ability to control insect pests that have become resistant to chemical pesticides, the ability to control more effectively pests that are marginally exposed to aerial sprays, persistence in the field for season-long control of targeted pests, lack of toxicity to beneficial insects, and a compatibility with traditional integrated pest management strategies. These benefits can translate into new opportunities for farmers to manage their crops with greater flexibility, efficiency, and economy. The potential environmental benefits of this new tech-

nology include a general lack of toxicity to nontarget organisms, including fish, birds, wildlife, and humans, and a reduction in the number of chemical pesticide applications required for management of pest populations.

New insecticidal proteins with novel activities must be discovered and deployed if genetic engineering for insect control is to become a sustainable and widespread technology. First, new proteins with different spectra of insecticidal activity will be essential for providing control of pests that are not susceptible to currently known *Bt* proteins. Second, access to a battery of proteins with different mechanisms of insecticidal activity will reduce the occurrence of pest resistance or allow us to manage it more effectively. The simultaneous expression of multiple insecticidal proteins should significantly reduce the frequency at which resistant insects arise, thereby prolonging the useful lifespan of transgenic insect control products.

There are two basic strategies available for identifying new insect control proteins and each has been used with some degree of success. The first method is directed screening in which new members of already established classes of insect control proteins, such as the *Bt* proteins, are isolated and characterized with respect to their insect control properties. The second method, and one that is potentially more powerful for discovering truly novel insect control proteins, is that of randomly screening large numbers of unique, unknown protein sources for insecticidal activities. In this paper we describe a general approach for random screening that has been used in our laboratories to identify novel insecticidal proteins. We also review progress we have made toward developing one of our new protein leads, cholesterol oxidase, as a new tool for

controlling the cotton boll weevil [*Anthonomus grandis grandis* (Bohemian)].

STRATEGIES FOR DISCOVERING NEW INSECT CONTROL PROTEINS

Directed screening

Bt d-endotoxins are the most potent class of insecticidal proteins known. These proteins bind to specific proteins in the insect midgut epithelial membrane. After binding, they insert into the membrane to form pores that allow the breakdown of the midgut permeability barrier and lead to membrane disruption and midgut lysis (Knowles and Dow, 1993; Knowles, 1994). The *Bt* proteins that are currently expressed in commercial crops are from the CryI and CryIII classes of d-endotoxins. Proteins from these classes are known to be effective in controlling agronomically important lepidopteran and coleopteran pests. Large public and private *Bt* culture collections exist that are comprised of thousands of *Bt* strains that have yet to be thoroughly classified and characterized with regard to their insect activity spectra and d-endotoxin content (Feitelson et al., 1992; Lambert and Peferoen, 1992). The continued study and evaluation of *Bt* strains has been successful in finding d-endotoxins with useful characteristics and activities against additional insect species. For example, d-endotoxins belonging to the CryII class have also been found to have activity against some major lepidopteran crop pests. These CryII proteins originate from a well-known strain of *Bt*, HD-1, that produces previously characterized CryI proteins (Yamamoto and McLaughlin, 1981). The CryII proteins are structurally distinct from the CryI proteins (Donovan et al., 1988; Widner and Whiteley, 1989) and are

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also distinct with regard to their binding to insect midgut membranes and the physiological characteristics of the membrane pores they form (English et al., 1994). CryII proteins may have sufficiently different modes of action that they will be useful in managing resistance of some lepidopterans to CryI proteins. Given the large number of *Bt* strains already available for further screening, there is a high probability that new *Bt* proteins will be found that have biological activity against agriculturally important pests that are insensitive to currently known CryI, CryII, or CryIII proteins.

Protein identification efforts have also focused on the directed screening of other classes of plant proteins known to have insect control properties, such as proteinase inhibitors, lectins, and amylase inhibitors (Boulter, 1993). However, while these proteins can retard insect growth and development, they lack the potency required to cause the acute insect mortality achieved with *Bt* proteins. Commercially acceptable levels of insect control in transgenic plants using these less potent proteins will be more difficult to achieve.

Random screening

In a high-throughput random screen, samples can be derived from virtually any source—plant tissues, microbial fermentation broths, or even commercial protein preparations. Protein samples are added individually or as pooled samples to an artificial diet which is then fed to the insect species of interest. Samples that confer lethal or developmental effects on one or more target insects are identified and subjected to further analysis. A key criterion for purification and biochemical characterization of the active component is the demonstration that it is proteinaceous in nature. Characteristics such as heat lability, approximate molecular size, proteinase digestibility, and ammonium sulfate precipitability are used to determine if the active component is proteinaceous. These attributes should be considered collectively when deciding whether or not to advance a new lead since some proteins may exhibit qualities such as heat stability or protease resistance. The insecticidal protein can then be isolated from the original milieu using bioactivity as a guide for purification. Three parameters are then used in assessing the potential value of a newly identified insecticidal protein: 1) the protein's identity or function; 2) the protein's potency against target insects; 3) the protein's general mode of insecticidal activity.

The first objective is to determine the protein's identity. Partial amino acid sequencing of the purified protein followed by homology searches of protein sequence databases can provide useful structural and functional information. Determination of an enzymatic function can provide an easy assay for following the protein during purification and for identifying duplicate activities among a large number of bioactive samples. Determining the protein's function at an early stage can also be used to identify and discontinue leads that may

present broad spectrum toxicology issues. Finally, primary structure information also allows the construction of oligonucleotide probes that are necessary to identify and clone the protein's gene.

The second objective is to determine the potency of the protein's biological activity. Potency is measured by determining concentrations required in artificial diet insect bioassays to confer specific levels of mortality or developmental stunting. The potency must be of sufficient magnitude that commercial levels of insect control can be achieved upon expression in transgenic plants. Comparison of the potency of a new protein with the activity of *Bt* endotoxins provides a good estimation of whether or not the protein under investigation is potent enough to have practical applications. *Bt* proteins generally have potent biological activity in the low parts-per-million (ppm) concentration range. For example, the *Bt* endotoxin LC₅₀ (concentration required for 50% mortality) for tobacco budworm is ≈ 1 ppm in diet (MacIntosh et al., 1990) and tobacco budworm is effectively controlled in transgenic crops that express this protein. Purcell et al. (1994a) found that several proteinases are active against a broad spectrum of insects, but only at concentrations in the hundreds to thousands ppm range, far below the potency benchmark set by *Bt* proteins. In contrast, we discovered that cholesterol oxidase has activity against boll weevil larvae in the low ppm range (Purcell et al., 1993), a potency that compares favorably with that of *Bt* proteins against other insects.

The third parameter used to judge the value of a potentially insecticidal protein is the general mode of action: that is, the mechanism through which the protein causes the activity observed in the insect diet bioassays. The nature of this mechanism has important implications for the successful adaptation of the protein to transgenic plants. Three modes of action can be envisioned: 1) direct postingestive; 2) preingestive toxic molecule production; 3) preingestive dietary depletion. In the case of the direct postingestive mechanism, the protein is toxic to the insect upon ingestion, requiring no alterations to dietary components to exert its biological effect. The *Bt* endotoxins are classic examples of proteins that function by a direct postingestive mechanism (English and Slatin, 1992; Gill et al., 1992). The successful application of a protein with a direct mode of action should be limited only by the ability to express the protein at sufficient levels in the parts of the plant consumed by the insect pest. The preingestive mechanisms present additional complexities within the context of expression in a transgenic plant. For the toxic molecule mechanism, a constituent of the diet is metabolized by the protein to yield a product that is toxic to the insect. Effective insect control in plants in this case depends upon the presence of sufficient quantities of substrate in the relevant plant tissues so that an efficacious concentration of the toxic product is generated. An example of an insect control protein in this class is lipoxygenase that appears to be active by producing lipid oxidation

products from endogenous substrates such as linoleic acid (Mohri et al., 1990; Shukle and Murdock, 1983). For the dietary depletion mechanism, a constituent of the diet that is required by the insect is depleted to the extent that the insect is nutritionally deprived during feeding. An example of an insect control protein in this class is polyphenol oxidase that can significantly reduce the protein quality of insect diet, reducing larval growth (Felton et al., 1992). Purcell et al. (1994b) identified two classes of proteins active against green peach aphid that function in diet bioassays by depleting sucrose, an essential dietary component (Mittler et al., 1970). These proteins, invertase and hexosyl transferase, were active in diet bioassays at low ppm concentrations but were not advanced further. Since proteins operating by preingestive mechanisms involve either depleting essential plant metabolites or producing broadly toxic products, they could cause perturbations of plant metabolism or development that would result in agronomically unacceptable phenotypes.

DISCOVERY AND CHARACTERIZATION OF CHOLESTEROL OXIDASE AS AN INSECTICIDAL PROTEIN

The cotton boll weevil as a target for insecticidal proteins

The boll weevil has been an agronomically important pest of cultivated cotton in the United States since the insect first migrated to Texas from Mexico at the end of the nineteenth century. The weevils are highly destructive pests that are difficult to control by traditional strategies using chemical insecticides. Part of this difficulty is due to the insect's natural history. Adult weevils feed primarily on pollen obtained after puncturing developing flower buds, or squares. The squares are loosely enclosed in leafy bracts that can shield feeding adults from aerial sprays. Ovipositing females lay eggs in square punctures, with each weevil laying as many as 300 eggs during her life, usually one egg per square. Development of the larvae occurs entirely within the square, where the larvae feed on reproductive tissues and cause abscission of the square. Thus, boll weevil larvae are very well protected from exposure to chemical sprays. After pupation, adults emerge from the fallen squares and begin a new generation. Under optimal environmental conditions the insects may complete as many as seven to eight generations per growing season. Expression of an appropriate insecticidal protein in cotton plants would be an effective way to control this prolific and devastating pest. The boll weevil exhibits no significant level of sensitivity to known *Bt* insecticidal proteins.

Identification of cholesterol oxidase for boll weevil control

In a random screen of more than 10,000 filtrates from microbial fermentations, Purcell et al. (1993) found two *Streptomyces* culture

filtrates that killed neonate boll weevil larvae in artificial diet feeding studies. These activities met the criteria described for proteinaceous molecules. Subsequent purification from one culture, A19249, identified the presence of a major protein of M_r 52,500 that was correlated with the boll weevil mortality. Amino acid sequencing of the purified protein showed close homology to a cholesterol oxidase from another *Streptomyces* species (Ishizaki et al., 1989) and its identity was confirmed by demonstrating the oxidation of cholesterol in an in vitro enzymatic assay. Cholesterol oxidase is produced by a phylogenetically diverse group of microorganisms and usually occurs as a secreted protein. The enzyme catalyzes the oxidation of a variety of 3-hydroxysteroids to ketosteroids and hydrogen peroxide.

Potency

Titration of the purified cholesterol oxidase in a series of bioassays using boll weevil larvae showed that the LC_{50} for neonate larvae ranged from 6.0 ppm in 6-day bioassays to 1.5 ppm in 16-day bioassays (Greenplate et al., 1995). Second instar larvae were equally susceptible to the enzyme in 16-day bioassays. These results showed that cholesterol oxidase is active against boll weevil larvae within the same concentration range as *Bt* proteins against lepidopterans. We concluded that the potency of cholesterol oxidase against boll weevil was adequate for achieving insecticidal levels in transgenic plants and that its further development was warranted pending completion of the general mode of action studies. The possibility that cholesterol oxidase was more active against the insects from the laboratory-reared colony used in the bioassays than against insects from field populations was eliminated by demonstrating that both wild-type and laboratory-reared boll weevil larvae were equally susceptible to cholesterol oxidase (Greenplate et al., 1995).

Spectrum of activity

The insecticidal activity of cholesterol oxidase was discovered as a result of its potent activity against boll weevil larvae. Rescreening with purified cholesterol oxidase of a spectrum of insects within the orders Coleoptera, Lepidoptera, Diptera, Orthoptera, and Homoptera revealed biological activity against other pests (Purcell et al., 1993). While the boll weevil remained the most sensitive insect to cholesterol oxidase, at dietary concentrations of 100 ppm several lepidopterans were also adversely affected by the protein. This activity was characterized by reductions in larval weight, ranging from 30% to 86% depending on the species, during 6-day feeding studies (Corbin et al., 1996). The lack of acute mortality observed with these other insects and the high concentration of cholesterol oxidase required to achieve developmental retardation confirms that cholesterol oxidase is not as potent against the lepidopteran pests tested as it is against boll weevil.

Mode of action

As discussed above, an ideal insect control protein is toxic to an insect as a result of postingestive activity. However, the two alternative modes of action were possible for cholesterol oxidase: 1) production of a toxic molecule upon oxidation of cholesterol in the diet or 2) depletion of dietary sterol, a strict nutritional requirement for insects (Dadd, 1985; Earle et al., 1967). We determined if cholesterol oxidase activity against boll weevil was due to effects of the enzyme on the diet. The two products of cholesterol oxidation, cholestenone and hydrogen peroxide, showed no toxicity when ingested by boll weevil larvae (Purcell et al., 1993). Fractionation of the diet after pretreatment with cholesterol oxidase into high and low molecular weight components showed that small molecules potentially generated by dietary cholesterol oxidation had no measurable insecticidal effects. These results strongly suggested that toxic molecule production in the diet was not the mode of action of this protein. Pretreatment of the diet with cholesterol oxidase, followed by heat inactivation of the enzyme, also did not compromise boll weevil survival in feeding assays, suggesting that depletion of dietary cholesterol was not responsible for the effect (Purcell et al., 1993). These results clearly indicated that the enzyme's insecticidal activity was due to a postingestive process, by acting directly on the boll weevil midgut membrane.

It was also critical to demonstrate that cholesterol oxidase had insecticidal activity when it was ingested with cotton tissue because the physiology of the insect could be different when nourished by artificial diet rather than its natural host plant, or the protein's activity could be inhibited by components of the host plant tissues. We measured the bioactivity against boll weevil larvae after addition of the enzyme to three different cotton-derived assay media (seed, embryos, leaves) and to cotton callus. In each case where all the nutritional material was comprised solely of cotton tissue, strong bioactivity was retained (Purcell et al., 1993). The demonstration that the bioactivity was independent of the composition of the diet suggested that expression in transgenic plants would confer the desired biological activity.

Further evidence for a direct mode of action came from light microscopy examination of midguts from larvae that had ingested diet containing cholesterol oxidase. Disruption of the epithelial cell layers was observed in midguts of larvae that had ingested low doses of cholesterol oxidase and complete lysis of the cells was observed for those that ingested higher doses (Purcell et al., 1993). The concentrations of cholesterol oxidase used in this experiment resulted in larval mortality in parallel bioassays. Similar morphological effects on insect midgut epithelia were observed when *Bt* proteins were fed to susceptible insects (English and Slatin, 1992; Gill et al., 1992), indicating the significance of the midgut as a particularly vulnerable target for insect con-

trol proteins (Federici, 1993). Cholesterol oxidase can oxidize cholesterol in some biological membranes (Crockett and Hazel, 1995; Lange, 1992; Lange et al., 1980; Pal et al., 1980; Patzer et al., 1978; Thurnhofer et al., 1986), and phospholipid, cholesterol, and protein composition of the membranes determine this susceptibility (Gronberg and Slotte, 1990; Lange, 1992; Lange et al., 1980, 1984; Pal et al., 1980; Patzer et al., 1978). Membrane cholesterol oxidation can also affect the structure and function of some membranes (El Yandozi, 1993; Kutryk et al., 1991; Linder et al., 1989; Wood et al., 1995). We have demonstrated that boll weevil larvae are acutely sensitive to ingested cholesterol oxidase and that ingestion leads to lysis of the midgut epithelium. We are currently investigating the biochemical mechanism by which cholesterol oxidase causes midgut membrane disruption and the biochemical basis for the differences in susceptibility observed between boll weevil and other insect species.

Cholesterol oxidase was not acutely toxic when ingested by newly emerged boll weevil adults. However, Greenplate et al. (1995) observed a severe reduction in the fecundity of adult females that had ingested diet containing 50 ppm cholesterol oxidase: an 83% reduction in the number of eggs laid and a 97% reduction in larval survival. Females that ingested cholesterol oxidase had poorly developed ovaries. The decreased rate of oviposition and the reduction in size of ovaries are similar to the effects seen with *B. thuringiensis* CryIIIA protein on adult Colorado potato beetle (Perlak et al., 1993). Reductions in fecundity were also noted when *B. thuringiensis* CryI proteins were fed to adult *Heliothis virescens* (Ali and Watson, 1982). Reduced oogenesis may result from a compromised nutritional state caused by sublethal perturbations of the adult midgut epithelia.

Cloning and expression of cholesterol oxidase

The three parameters that we assess in deciding whether or not to advance an insecticidal protein to gene cloning and expression are its identity, potency, and mode of action. Cholesterol oxidase has potent bioactivity against boll weevil larvae and it acts postingestively, presumably by oxidation of cholesterol in the insect's midgut epithelial membrane (Greenplate et al., 1995; Purcell et al., 1993). Based on these results, cholesterol oxidase was advanced to the next phase of our insect control program. Degenerate oligonucleotide hybridization probes were synthesized based upon partial amino acid sequence information and used to clone the gene from *Streptomyces* sp. strain A19249. The cloned gene was sequenced and expressed in *Escherichia coli* and plant protoplasts to yield enzymatically active cholesterol oxidase (Corbin et al., 1994). The recombinant protein was purified from *E. coli* and shown to have enzymatic, immunological, and insecticidal properties that were indistinguishable from the enzyme isolated from A19249 (Corbin et al., 1994), indi-

cating that we had cloned the gene encoding the bioactive enzyme. Experimentation in a model crop system such as *Nicotiana tabacum* (tobacco) is useful for demonstrating the feasibility of expressing a new insecticidal protein in *planta*. We expressed the cholesterol oxidase gene from *Streptomyces* A19249 in transgenic tobacco and demonstrated insecticidal activity of this tissue against boll weevil larvae (D.R. Corbin, unpublished results). We are currently characterizing the expression and biological activity of cholesterol oxidase produced in transgenic cotton plants.

CONCLUSIONS

As transgenic crop technology for insect control becomes more widely implemented, proteins with new insect specificities and different modes of action will need to be identified. We have demonstrated that novel insecticidal proteins can be found by random screening of microbial cultures. Using this approach, we identified cholesterol oxidase as a novel and potent insecticidal protein. Expression of cholesterol oxidase in transgenic cotton plants may be an effective method for maintaining boll weevil populations below economic thresholds via two mechanisms: reducing the insect's fecundity and reducing larval survival. Cotton plants genetically modified to produce cholesterol oxidase may provide an environmentally safe and highly effective tool for controlling this devastating pest.

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