The specificity of *Bacillus thuringiensis* crystal proteins

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Proefschrift
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The cover shows a scanning electron micrograph of *B. thuringiensis* bipyramidal protein crystals.

Stellingen

1. Binding aan receptoren is een onvoldoende verklaring voor de specificiteit van Cry eiwitten.

   Haider, M.Z. *et al.* (1986) Eur. J. Biochem. **156:** 500-504
   Wolfersberger, M.G. (1990) Experientia **46:** 475-477

2. Voor de bepaling van de affiniteit van receptoren voor kristaleiwitten gaan Van Rie *et al.* er ten onrechte vanuit dat de binding aan 'brush border membrane vesicles' reversibel is.

   Van Rie, J. *et al.* (1989) Eur. J. Biochem. **186:** 239-247
   Van Rie, J. *et al.* (1990) Appl. Environm. Microbiol. **56:** 1378-1385

3. Bestudering van het mechanisme van resistentie-ontwikkeling in insekten tegen *Bacillus thuringiensis* kristaleiwitten is een voorwaarde voor blijvend succesvolle toepassing van deze eiwitten in de gewasbescherming.

4. De conclusie dat het tweede aminozuur van het manteleiwit van alfalfa mozaïek virus belangrijk is voor 'coat protein mediated resistance' is voorbarig gezien het beperkte aantal geteste transgene planten.

   Tumer, N.E. *et al.* (1991) Proc. Natl. Acad. Sci. USA **88:** 2331-2335

5. In het onderzoek van Hilder *et al.* naar de expressie van het heterologe cowpea trypsine inhibitor gen in transgene tabaksplanten is ten onrechte geen rekening gehouden met de lokale inductie, na verwonding, van de endogene proteïnase inhibitor genen.

   Hilder, V.A. *et al.* (1987) Nature **300:** 160-163

6. De modelplant staat alleen model voor zichzelf.

7. De structurele homologie van infectie-gerelateerde eiwitten uit twee niet verwante plantepathogene schimmels met extracellulaire dierlijke eiwitten waarvoor reeds aangetoond is dat ze binden aan receptoren in de extracellulaire matrix, doet vermoeden dat deze eiwitten in schimmels ten dele een analoge functie hebben.

Xuei, X.L. *et al.* (1992) Gene **110**: 49-55
Pieterse, C.M.J. *et al.* (1992) Neth. J. Plant Pathol. *in press*

8. De variatie in 34-aminozuur-repeats van een avirulentiegenprodukt van *Xanthomonas campestris* pv. *vesicatoria* lijkt een afdoende verklaring te kunnen geven voor de cultivarspecificiteit van dit pathogeen.

Herbers, K. *et al.* (1992) Nature **356**: 172-174

9. De door plantenveredelaars veel gebruikte zinsnede "het pathogeen heeft de resistentie van de plant doorbroken" wekt ten onrechte de indruk dat het pathogeen het resistentiemechanisme van de waardplant onschadelijk maakt.

Stellingen behorend bij het proefschrift *The specificity of Bacillus thuringiensis crystal proteins*.

Wageningen, 19 juni 1992 Guy Honée

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Voorwoord

Dit proefschrift draagt de naam van slechts één persoon, terwijl het tot stand is gekomen door de hulp en inzet van velen. Graag wil ik iedereen bedanken die me de afgelopen jaren met raad en daad heeft bijgestaan. Een aantal wil ik met name noemen.

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An outline of this thesis

Major drawbacks of the use of chemical insecticides for the control of insect pests are their expense, their persistence in and hazardous effects on the environment, and their escalating rates of application because of decreasing effectiveness. Therefore, over the last two decades efforts have increased to investigate possibilities of biological insect control like the use of predators, parasites, insect viruses or feromones. Likewise, increased utilization of the microbial insect pathogen *Bacillus thuringiensis* can be regarded as a form of biological insect control. *B. thuringiensis* is characterized by its ability to produce crystalline inclusions during sporulation which form the major toxic determinant of this bacterium. Most *B. thuringiensis* strains are active against larvae of lepidopteran species (butterflies), but some show toxicity against larvae of dipteran (flies) or coleopteran (beetles) species. Not only strains, but also different crystal proteins occurring in a single crystal may vary in insecticidal spectra. Sprays based on *B. thuringiensis* spore/crystal preparations have been used for over thirty years as biological insecticides. Recently, the cloning of crystal protein genes and their expression in transgenic plants and micro-organisms has provided powerful alternative strategies for the protection of crops against insect damage. These additional potential applications resulted in an increased interest in this bacterium and its crystal proteins in recent years.

This thesis endeavours to contribute to our understanding of the entomocidal activity of *B. thuringiensis*, and more in particular of its crystal proteins. Studies are described to identify crystal proteins with new insecticidal spectra, to gain more insight into the mode of action of crystal proteins, and to examine some of their potential applications.

Chapter 1 reviews the mode of action of the crystal proteins. It presents a survey of histological and physiological changes observed in the insect and *in vitro* cultured cells induced by crystal proteins. In addition, investigations using isolated midgut cells, brush border membrane vesicles and artificial phospholipid vesicles are summarized. Finally, conclusions concerning the molecular basis of the mode of action of both *Lepidoptera* and *Diptera* specific crystal proteins are discussed.

In chapter 2, an analysis of crystal protein genes and their proteins, in particular those occurring in *B. thuringiensis* serotype *entomocidus* 60.5, is presented. The nucleotide sequence of a gene isolated from this bacterial strain, the reference type of *cryIC*, is presented together with its deduced amino acid sequence. Furthermore, several conserved features revealed by an analysis of the amino acid sequences of all crystal proteins are evaluated.

In chapter 3, domain-function studies on the crystal proteins are described using hybrid crystal proteins based on CryIA(b) and CryIC, constructed in order to improve our understanding of the mode of action of crystal proteins, especially of its molecular basis.

Chapter 4 describes the analysis of a translation fusion product of two different insecticidal crystal protein genes. Based on the presented results an alternative strategy for the introduction of more effective insect resistance in transgenic organisms using *B.*

thuringiensis crystal protein genes is proposed.

In chapter 5, the generation of insect resistant transgenic plants using the crystal protein genes *cryIA(b)* and *cryIC* is described.

Finally, in chapter 6, the three-dimensional structure of one of the crystal proteins, which recently became available, is reviewed as well as alternative strategies for *B. thuringiensis* applications to prevent crystal protein resistance in insect populations, all in relation to the results presented in this thesis.

Chapter one

The mode of action of *Bacillus thuringiensis* crystal proteins

Summary

Most strains of the entomopathogenic micro-organism *Bacillus thuringiensis* are active against lepidopteran larvae, whereas other strains are toxic against larvae of dipteran or coleopteran species. Crystals produced by *B. thuringiensis* form the major toxic determinant and may consist of several different crystal proteins. These insecticidal crystal proteins are highly specific with the exception of the broad spectrum *in vitro* cytolytic Cyt proteins produced by *Diptera* specific *B. thuringiensis* strains. The primary target of the crystal proteins is formed by the midgut epithelial cells which swell and burst upon feeding larvae with crystals. Crystal protein toxicity further appears from physiological changes, amongst which an increase of pH and K^+-concentration of the hemolymph appear most prominent.

Upon ingestion by the larvae, the crystals are dissolved in the midgut and the liberated crystal proteins are proteolytically converted releasing toxic fragments encompassing the *N*-terminal part of the crystal proteins. The toxic fragments bind to receptors, presumably glycoproteins, present on the brush border region of the midgut epithelial cells. Following receptor binding pores are formed in the cell membranes disturbing cellular physiology and eventually effecting a total loss of the midgut integrity. The specificity of the crystal proteins is mainly determined by the presence of receptors on the columnar midgut cell membrane, although solubility of the crystals and proteolytic conversion of the crystal proteins also influence insecticidal specificity.

Only dissolved crystal proteins from *Diptera* specific *B. thuringiensis* strains were found to be cytotoxic for a variety of *in vitro* cultured cells. This broad toxicity spectrum can be attributed to Cyt proteins, exclusively present in those strains and constituting a major fraction of the crystals. The proposed mode of action of Cyt proteins on *in vitro* cultured cells resembles the mode of action of other crystal proteins, the difference being the absence of receptor involvement and consequently of cell specificity in the case of Cyt proteins.

The pathogenic effects observed in larvae fed with crystals, result from the contribution of all toxins present. This means that different crystal proteins probably have an additive effect. However, synergism has also been reported for two crystal proteins, whereas the question on the role of Cyt proteins in synergism remains unresolved.

Introduction

Bacillus thuringiensis is the best studied entomopathogenic micro-organism. The bacterium is characterized by its ability to form crystalline inclusions during sporulation, made up of the socalled crystal proteins. The crystal forms the major toxic determinant and may consist of a number of different crystal proteins. During vegetative growth cells produce additional toxins: the α-exotoxin, which is a heat-labile protein, and the ß-

exotoxin which is a heat-stable nucleotide analogue inhibiting RNA synthesis (Lüthy, 1980). In addition, the production of several exoenzymes including lecithinases, chitinases and proteases has been suggested to contribute to entomopathogenic activity (Lüthy, 1980). The exotoxins and exoenzymes display broad spectrum toxic activity, not only against insects but also against vertebrates and micro-organisms. On the contrary, the crystal proteins exhibit highly specific insecticidal activity.

Most *B. thuringiensis* strains are active against *Lepidoptera*, whereas other strains are toxic against dipteran or coleopteran species. Not only different strains, but also different crystal proteins occurring in a single strain vary in insecticidal spectra (reviewed by Höfte and Whiteley, 1989). Because of the confinement of toxicity to insects crystal/spore preparations have been safely used as biological insecticides for over two decades. Recently, transgenic plants (Adang *et al.*, 1987, Barton *et al.*, 1987; Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987; Perlak *et al.*, 1990; McCown *et al.*, 1991; Perlak *et al.*, 1991; Van der Salm and Visser, pers. comm.) and microorganisms (Obukowicz *et al.*, 1986; Chungjatupornchai, 1990; Skφt, 1990; Stock *et al.* 1990; Waalwijk *et al.*, 1991) have been obtained, each producing a single crystal protein in order to control insect attack. In this review, we will focus on the mode of action of *B. thuringiensis* crystal proteins, which has now been elucidated to a considerable extent by histological, physiological and biochemical studies.

Upon ingestion by the larvae, the crystals dissolve in the midgut and the liberated crystal proteins are proteolytically converted releasing toxic fragments encompassing the *N*-terminal part of the crystal proteins. The primary target of the toxic fragments is the larval midgut. For this reason, the morphology and physiology of the midgut will be briefly discussed in the next paragraph, followed by a survey of histopathological changes in the midgut tissue upon ingestion of crystal proteins. Since the toxic fragments also appear to be active against several *in vitro* cultured insect cell lines, which show similar reactions as midgut epithelium cells, the interactions with these cell lines will be compared to the *in vivo* observations. Insect larvae and insect cell lines, as well as isolated midgut cells, isolated brush border membrane vesicles, and artificial phospholipid vesicles were also studied to elucidate the molecular basis of crystal protein toxicity. Potential synergistic effects of different crystal proteins in the pathogenic effects will also be discussed. Since the studies described have been mainly performed with some of the *Lepidoptera* and *Diptera* specific crystal proteins - CryI, and CryIV and Cyt proteins, respectively - we shall restrict our discussion to these proteins classes.

Morphology and physiology of the larval midgut

The midgut morphology and physiology has been extensively reviewed by Dow (1986). The larval midgut is composed of outer longitudinal and circular muscular layers bordered at the interior by a basement lamina and a monolayer of epithelial cells, separated from

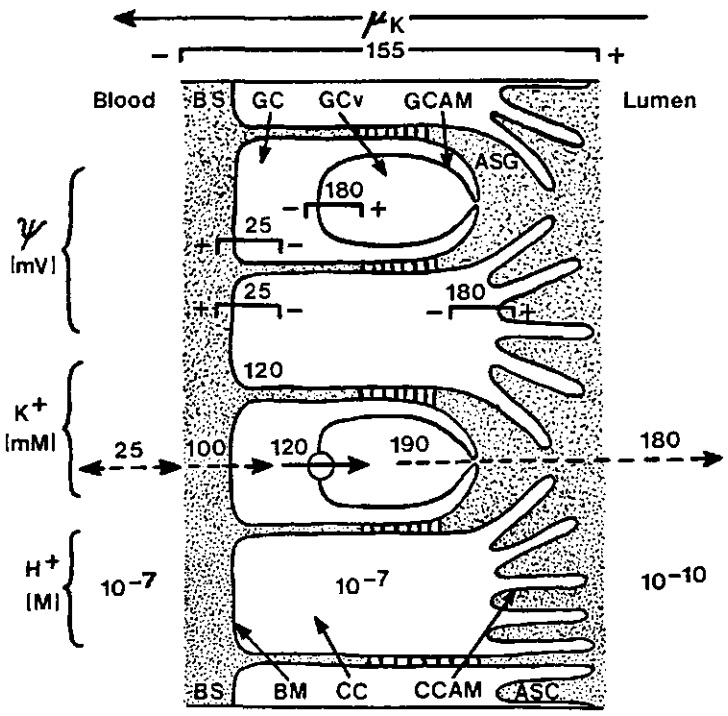

Fig. 1. Model for K^+ homeostasis in lepidopteran midgut (Harvey et al., 1986; with permission of the eds. Samson, R.T., Vlak, J.M., and Peters, D.). GC, goblet cell; GCAM, goblet cell apical membrane; ASG, apical space of goblet cell; GCv, goblet cell cavity; CC, columnar cell; CCAM, columnar cell apical membrane; ASC, apical space of columnar cell; BS, basal space; BM, basal membrane; μ_K, electrochemical gradient; Ψ, transepithelial potential difference.

the food in the gut lumen by a peritrophic membrane. The peritrophic membrane functions like a dialysis membrane with a cutoff value below 100 kDa (Wolfersberger *et al.*, 1986). The epithelial cell layer consists mainly of columnar and goblet cells. The columnar cells have apical microvilli lining the gut lumen, and basal infoldings with mitochondria in between. The columnar cells are involved in the absorption of nutrients. Goblet cells have no function in nutrients absorption, but are responsible for the active transport of K^+ from the hemocoel into the gut lumen. The goblet cells have intracellular cavities, into which numerous thin projections extend each containing a mitochondrion. Two other types of epithelial cells, the regenerative cell and basal-granulated cell, are found occasionally on the basement lamina. The regenerative cells are inactive in feeding larvae but divide during metamorphosis in the prepupa. In addition, these cells can

differentiate into columnar cells and goblet cells during larval moulting or in response to gut injury. The basal-granulated cells are endocrine cells.

The midgut epithelium of lepidopteran and dipteran insects contains an electrogenic K^+ pump which differs from the Na^+-K^+ exchange pump found in gastrointestinal epithelia of vertebrates in that it forces K^+ out of cells instead of into cells (Harvey and Nedergaard, 1964). The molecular basis of this pump is an alkaline K^+-stimulated ATPase which couples ATP hydrolysis to the electrogenic movement of K^+. Its activity is not inhibited by the typical inhibitors of vertebrate (Na^+-K^+) ATPases, ouabaine and vanadate.

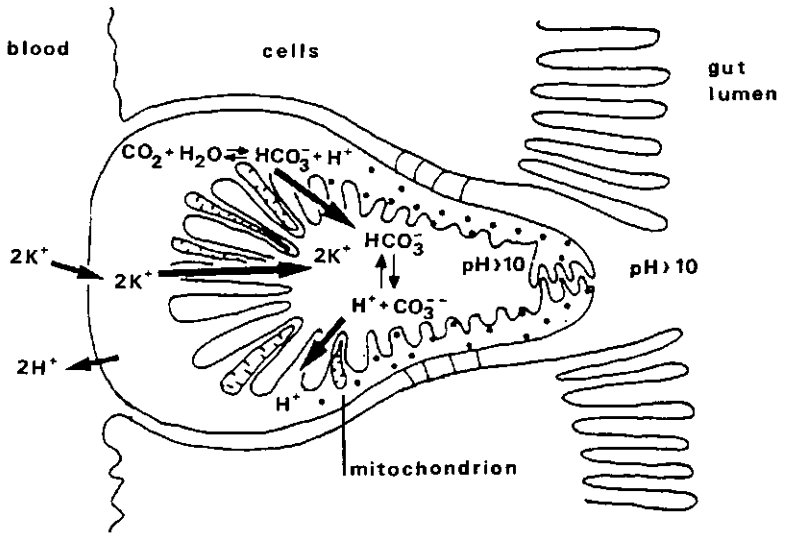

Fig.2. Model for extremely high pH values in the larval midgut, see text (modified after Dow (1984); with permission of The American Physiological Society). The figure shows a scheme of ion fluxes from and into a goblet cell.

In the larval midgut, this unique ATPase activity is located in the goblet cell apical membrane bordering the lumen (Wieczorek *et al.*, 1986). The K^+ pump establishes a K^+ gradient across the midgut epithelium. X-ray microanalysis of *Manduca sexta* larvae revealed K^+-concentrations of 25 mM in the hemolymph, 120 mM in the cytoplasm of goblet and columnar cells, 190 mM in the goblet cavity, and 180 mM in the gut lumen. On the other hand, Na^+ was undetectable at any place and both the goblet cavity and the gut lumen were Cl^- deficient (Figure 1) (Dow *et al.*, 1984). A potential gradient across the apical membrane of the epithelial cells, exceeding 180 mV, is generated by the K^+

pump (Dow *et al.*, 1984). A model has been suggested in which the potential gradient results in a 1000-fold H^+-gradient leading to extremely high pH values in the larval midgut (Dow, 1984). According to this model CO_2, which permeates freely across the cell membranes, is trapped in the goblet cell by carbonic anhydrase via conversion to carbonic acid, which subsequently dissociates to form protons and bicarbonate molecules (Figure 2). The bicarbonate molecules are secreted into the goblet cell cavity and protons are stripped from the transported bicarbonate. The large potential gradient favours the released protons to move back into the cytoplasm. The net result is a trans-epithelial proton gradient rendering an alkaline lumen with pH 10-12.

Studies with isolated brush border membrane vesicles of several lepidopteran insects demonstrated the existence of an amino acid-potassium cotransport for several neutral and basic amino acids *via* the microvilli of columnar cells (Giordana *et al.*, 1982; Giordana *et al.*, 1985; Wolfersberger *et al.*, 1987; Hennigan and Wolfersberger, 1989). It was shown that the uptake of amino acids in brush border membrane vesicles was inhibited by the potassium ionophore valinomycin, suggesting that the amino acid transport is driven by a K^+ gradient. However, amino acid transport was not exclusively coupled to K^+. Replacement of K^+ by Na^+ resulted in a comparable amino acid uptake. In contrast, amino acid uptake could not be stimulated by Rb^+, whereas the uptake of some amino acids in vesicles could be stimulated by Li^+. However, since *in vivo* only a K^+ gradient occurs across the midgut epithelium, amino acid uptake is often interpreted as K^+-dependent. A passive, potassium-independent amino acid exchange mechanism is responsible for further transport of the amino acids from the columnar cells towards the hemolymph (Nedergaard, 1981).

Pathogenesis

Pathology of the lepidopteran and dipteran midgut upon feeding of B. thuringiensis crystal proteins. The elucidation of the mode of action of *B. thuringiensis* crystal proteins started with histopathological studies. Soon after susceptible larvae have ingested *B. thuringiensis* crystals, the epithelial cells of the larval midgut start to vacuolise and slough off into the lumen. Subsequently, the midgut paralyses followed by total paralysis of the insect. A comparable response upon feeding of *B. thuringiensis* crystals has been demonstrated for larvae from various lepidopteran and dipteran species, although minor differences in timing of the response and severity of the pathological effects occur (Sutter and Raun, 1967; Nishiitsutsuji-Uwo and Endo, 1980; Endo and Nishiitsutsuji-Uwo, 1981; Percy and Fast, 1983; Iizuka, 1973).

The first ultrastructural changes in the epithelial cells of the lepidopteran *Bombyx mori* larvae can be observed within 1 minute after ingestion of crystals from serotypes *kurstaki* or *aizawai*. The internal microfilaments of the columnar cells are first disrupted and then disappear resulting in disorganized microvilli. After 5 minutes the microvilli

start to disappear and are replaced by small bulbous extensions. Also, the morphology of organelles changes within a few minutes leading to enlarged mitochondria, swollen nuclei and Golgi complexes and an endoplasmic reticulum with a vacuole-like configuration. Damage to the cytoplasmic structure begins at the apical end and increases in severeness with time moving progressively to the distal end. The small bulbous extensions on the columnar cells increase in size and gradually surround most of the apical membrane. After 30 to 60 minutes the first epithelial cells begin to burst and slip out from the basement lamina.

Within the first minutes after ingestion no structural changes were observed in goblet and regenerative cells. However, by the time the first columnar cells burst, goblet cells show irregularly enlarged basal infoldings caused by the formation of vacuoles of various sizes. The cytoplasmic projections diminish in number and change into vacuole-like structures. Eventually, these cells also begin to burst.

Similar pathological reactions were described for larvae of the dipteran insect species *Simulium vittatum* and *Aedes aegypti* upon feeding with crystals of *B. thuringiensis* serotype *israelensis* (Lacey and Federici, 1979; Lahkim-Tsror *et al.*, 1983). Although all regions of the larval midgut, anterior and posterior, are sensitive for *B. thuringiensis* toxins (Nishiitsutsuij-Uwo and Endo, 1980), the main target in *Lepidoptera* is formed by the anterior of the larval midgut. More columnar cells are located here than in the posterior. In contrast, in *Diptera* columnar cells are more abundant in the posterior of the midgut which thus forms the primary target in these insects (Lacey and Federici 1979; Lakhim-Tsror, *et al.*, 1983). Although the primary target of the crystal proteins is the larval midgut, proteolysed *Lepidoptera* specific crystal proteins were also found toxic upon injection into the hemocoel of lepidopteran larvae (Lecadet and Martouret, 1967; Lilley et *al.*, 1980). How and where crystal proteins administered by this route exert their function remains unknown.

The physiological response upon ingestion of *B. thuringiensis* crystals, slightly varying from species to species, is formed by an increase in the pH and K^+ concentration in the hemolymph, which is apparent as soon as the larva stops eating (Heimpel and Angus, 1959; Pendleton, 1970; Nishiitsutsuji-Uwo and Endo, 1980; Nishiitsutsuji-Uwo and Endo, 1981). In *Bombyx mori* larvae fed with *B. thuringiensis* serotype *aizawai* crystals, the pH rose from 6.5 to 7.1 within 15 minutes (Heimpel and Angus, 1959; Nishiitsutsuji-Uwo and Endo, 1980). Thereafter, the pH increased gradually to 7.7 - 8.1. Simultaneous to the initial increase in pH, the K^+ concentration in the hemolymph increased up to two times. A sudden increase of the hemolymph Na^+ concentration appeared to be an additional, minor effect and was later followed by a decrease. The concentrations of Ca^{2+} or Mg^{2+} remained unchanged. A disappearance of the glycogen granules of the epithelial cells was reported for various lepidopteran species upon ingestion of *B. thuringiensis* crystals (Endo and Nishiitsutsuij-Uwo, 1981). In another report, glucose uptake by the gut was described to increase within 1 minute after feeding *Bombyx mori* larvae *B. thuringiensis* crystals, followed by a cessation of the metabolism

of the gut to occur 10 minutes after crystal administration (Fast and Donaghue, 1971). The observations that glycogen granules of the epithelial cells disappear and glucose uptake by the gut is increased soon after feeding larvae *B. thuringiensis* crystals suggest that initially larvae increase metabolism in order to maintain homeostasis of the gut which is affected by the crystal proteins.

Different defence reactions of insects infected with *B. thuringiensis* have been reported. *Culex pipiens* larvae liberate proteases into the rearing medium that are able to completely degrade dissolved crystal proteins from *B. thuringiensis* serotype *israelensis* with concomitant loss of toxicity (García-Patrone *et al.*, 1986). Rice moth larvae of *Corcyra cephalonica* fed with *B. thuringiensis* serotype *kurstaki* crystals formed new cells, originating from regenerative cells, to replace discharged columnar cells (Chiang *et al.*, 1986[a]). Finally, Sutter and Raun reported that vegetative *B.thuringiensis* bacteria which had broken through the gut epithelium of *Ostrinia nubilalis*, were retained in a cap of cells of unknown origin located on the exterior of the midgut (Sutter and Raun, 1967). Defence reactions as described here might increase crystal protein tolerance to higher threshold levels, or prolong the life-span of the infected larvae. In addition to these defense reactions, an increasing number of reports describe insect populations that have developed true and heritable resistance against crystal proteins.

Germination of *B. thuringiensis* serotype *kurstaki* spores in the gut of susceptible rice moth larvae of *Corcyra cephalonica* starts 40 minutes after inoculation (Chiang *et al.*, 1986[b]). The germination of spores is probably due to a decrease of the pH of the gut fluid that is caused by crystal protein action (Burgerjon and Martouret, 1971).

Summarising, midgut epithelial cells and more in particular columnar cells form the primary target of *B. thuringiensis* crystal proteins. Increases in pH and K^+-concentration of the hemolymph appear to be the most prominent of the induced physiological changes.

Several different crystal proteins may together constitute the ingested crystal. In some cases several additional toxic molecules might occur in the spore/crystal preparations. Addition of ß-exotoxin to a *B. thuringiensis* serotype *kurstaki* crystal/spore preparation increased the toxicity of this preparation (Dubois, 1986). Moreover, synergism has been reported for several *Diptera* specific crystal proteins (discussed later). These findings illustrate the fact that the pathological changes observed may form the result of an intricate interplay of several toxic proteins and/or other factors.

Toxic effects of crystal proteins on in vitro cultured cells. Some but not all *in vitro* cultured lepidopteran cell lines, started from different origin, appeared to be susceptible to the toxic fragments of crystal proteins with *Lepidoptera* specificity, showing responses comparable to those of intoxicated larval midgut epithelium cells *in vivo* (Nishiitsutsuij-Uwo *et al.*, 1980; Knowles and Ellar, 1986). Mammalian cell lines including the human tumour cell line HeLa and Yoshida ascites sarcoma cells showed no response to proteolysed crystal proteins (Nishiitsutsuji-Uwo *et al.*, 1980; Murphy *et al.*, 1976). In all

cases, toxicity tests with *in vitro* cells were performed with high toxin concentrations in the μM range, e.g. at non-physiological concentrations.

In contrast to the limited insecticidal spectrum of *Lepidoptera* specific crystal proteins, dissolved crystal proteins from the mosquitocidal strain *B. thuringiensis* serotype *israelensis* showed toxicity for a variety of *in vitro* cultured cell lines (Thomas and Ellar, 1983[a]; Chilcott, *et al.*, 1985; Gill and Hornung, 1987). Crystals dissolved in an alkaline solution were cytotoxic against dipteran cell lines derived from *Culex tarsalis* and *Aedes albopictus*, as well as against lepidopteran insect cell lines of *Choristoneura fumiferana*, *Spodoptera frugiperda* and *Trichoplusia ni* origin. Moreover, the dissolved crystals from *B. thuringiensis* serotype *israelensis* were also cytotoxic against several mammalian cell lines and exhibited haemolytic activity. Obviously, the *in vitro* toxicity spectrum of dissolved *B. thuringiensis* serotype *israelensis* crystals differs from the *in vivo* toxicity spectrum, a distinction not observed for dissolved crystals of *Lepidoptera* specific *B. thuringiensis* strains.

Cytolytic effects of a specific class of crystal proteins: the cyt proteins. In addition to the cytotoxicity for a variety of *in vitro* cultured cells described above, neurotoxic and myotoxic activity of dissolved *B. thuringiensis* serotype *israelensis* crystals to the neuromuscular system of *Musca domestica in vitro* and to dissected ganglia of the cockroach *Periplaneta americana* and of *Trichoplusia ni* were reported (Cooksey *et al.*, 1969; Chilcott *et al.*, 1984; Singh and Gill, 1985; Singh *et al.*, 1986; Chueng *et al.*, 1987). Moreover, intravenous or subcutaneous administration of dissolved crystal proteins of *B. thuringiensis* serotype *israelensis* to mice resulted in death, though oral uptake had no effect (Thomas and Ellar, 1983[a]). However, neurotoxic or myotoxic effects have never been described for *Lepidoptera* specific crystal proteins. Therefore, it might be postulated that, at least for the *Lepidoptera* specific crystal proteins, total paralysis of the insect larva is effected through a different, possibly indirect, mechanism. Direct neurotoxic effects might be attributed to one of the crystal proteins only occurring in crystals of *Diptera* specific serotypes like *israelensis* and *morrisoni*, but this remains to be proven.

Protein purification of *B. thuringiensis* serotype *israelensis* revealed a 28 kDa protein, designated CytA, which could be proteolytically processed by several proteases generating a 25 kDa protein (Armstrong *et al.* 1985; Gill and Hornung, 1987). This 25 kDa protein showed cytotoxic activity to mosquito and lepidopteran cell cultures, was able to lyse erythrocytes, and appeared lethal for mice upon intraperitoneal injection, covering all the properties unique for the dissolved crystal proteins of serotype *israelensis*. Further confirmation of the cytotoxicity of the 28 kDa CytA protein and its derivatives was obtained after cloning of the encoding gene (Ward *et al.*, 1984; Waalwijk *et al.*, 1985). Purification of crystal proteins from the mosquitocidal strain *B. thuringiensis* serotype *morrisoni* revealed a protein antigenically homologous to CytA (Gill *et al.*, 1987[a]), whereas antigenically distinct proteins with molecular weights of 28 kDa and 25 kDa,

designated CytB, were purified from crystals of the mosquitocidal strains *B. thuringiensis* serotypes *darmstadiensis* and *kyushuensis* (Drobniewski and Ellar, 1989; Knowles *et al.*, 1990). The Cyt proteins showed a broad-spectrum cytolytic activity when dissolved (Earp and Ellar, 1988; Drobniewski and Ellar, 1989; Knowles *et al.*, 1990), and constitute a major fraction of the crystals occurring in the *Diptera* specific strains (Insell and Fitz-James, 1985).

Molecular mode of action

The reported histopathological effects from the administration of entire crystals, either intact, dissolved or processed, did not give insight in the mechanism of action of the toxic fragments at the molecular level, as the studies mentioned only describe final effects as general cell injury and necrosis. Also, these studies were conducted at a time when the complex nature of most crystal proteins was not yet acknowledged. Therefore, the individual contribution of each crystal protein in the *in vivo* and *in vitro* pathogenesis remained obscure. Modern molecular biological and biochemical techniques now make it possible to investigate the mode of action of individual crystal proteins and their specificity at the molecular level.

Dissolving and proteolytic processing. Crystals ingested by a susceptible larva dissolve in the alkaline environment of the larval midgut, thereby releasing crystal proteins. The composition of the midgut lumen is a factor influencing the entomocidal activity of the crystals (Jaquet *et al.*, 1987). For instance, the activity of crystals from several *B. thuringiensis* strains against *H.virescens* larvae was enhanced by prior dissolving *in vitro*, whereas such dissolving had no influence on the activity against *Pieris brassicae* larvae. This showed that solubility is a factor codetermining crystal protein specificity. In addition to the midgut lumen composition, the total crystal composition influences the efficiency of the dissolving process (Aronson *et al.*, 1991), and consequently crystal protein specificity. Intact crystals produced by a derivative strain of *B. thuringiensis* serotype *aizawai*, which had been cured of a 68-kb plasmid containing the *cryIA(b)* gene encoding a well-studied *Lepidoptera* specific crystal protein, were less toxic against larvae of *Manduc sexta*, *Heliothis virescens* and *Plodia interpunctella* than dissolved crystals. On the other hand, no such differences in toxicity were observed between intact crystals and dissolved crystals derived from the original wild type *aizawai* strain. It is not yet clear if these differences were a direct consequence of the absence of the CryIA(b) protein from the crystals or of the loss of other factors encoded by the 68-kb plasmid. In conclusion, solubility is determined by the interaction between the midgut environment and the crystal composition.

Subsequently, the liberated crystal proteins are proteolytically processed by larval midgut proteases and crystal associated proteases, both optimally active in an alkaline environment (Dow, 1986; Thurley *et al.*, 1985). As a result, toxic fragments encompassing the *N*-terminal domain of the crystal proteins are released (reviewed by Höfte and Whiteley, 1989). The size of the toxic fragments derived from the 130 kDa CryI class and CryIV class proteins is between 60 and 78 kDa, whereas the CryIV class proteins of 75 kDa are converted into proteins of smaller molecular weight down to 30 kDa. The 28 kDa Cyt proteins prominently occurring in crystals from *Diptera* specific *B. thuringiensis* strains are processed to 23-25 kDa proteins. Proteolysis resulting in protease resistant toxic fragments can also be carried out *in vitro* by a variety of proteases (reviewed by Whiteley and Schnepf, 1986). Like solubility, proteolysis can effect the activity of the proteins liberated from *B. thuringiensis* crystals. Crystal proteins from *B. thuringiensis* serotype *aizawai* IC1, toxic to larvae of both lepidopteran and dipteran species, retain their toxic activity to both insect species after proteolysis by lepidopteran gut juices. However, toxic fragments obtained by proteolysis with dipteran gut juices appear only toxic to larvae of dipteran species (Haider *et al.*, 1986). A crystal protein Bta IC1, belonging to class CryIA(b), which forms part of the *aizawai* IC1 crystals, was produced by recombinant *Escherichia coli* cells and showed identical characteristics. It was concluded that the observed dual specificity resulted from differential processing of a single crystal protein (Haider *et al.*, 1987).

Dissolving and subsequent proteolytic conversion of a crystal protein constitute the first two steps in the mode of action and both influence insecticidal specificity.

Interaction of the toxic fragments of Cry proteins with midgut cells. No translocation of the toxic fragments from the gut lumen into the hemocoel was observed following proteolysis of the crystal proteins in the midgut (Tojo, 1986). The interaction of the toxic fragments of *B. thuringiensis* serotype *thuringiensis* crystal proteins with membranes of *in vitro* cultured *Pieris brassicae* midgut cells resulted in predominant binding of the toxic fragments onto the brush border region of the columnar midgut cells, as visualised by fluorescence microscopy (Hofmann and Lüthy, 1986).

Quantitative binding studies were carried out with isolated brush border membrane vesicles using iodinated crystal proteins (Hofmann *et al.*, 1988[a]). These *in vitro* binding studies with toxic fragments of several *Lepidoptera* specific crystal proteins and brush border membrane vesicles of different insect species showed a correlation between toxicity and the occurrence on the membrane vesicles of high affinity binding sites (Hofmann *et al.*, 1988[b]; Van Rie *et al.*, 1989; Van Rie *et al.*, 1990). The results suggested that postulated crystal protein receptors on the midgut epithelium cell membrane form a key factor in determining the specificity of the crystal proteins. These studies also suggested that high toxicity might result from high affinity or from the high number of receptors present. In contrast to these findings, Wolfersberger reported binding

affinity of the toxic fragments of CryIA(b) and CryIA(c), which bind to the same high affinity site on brush border membrane vesicles of the gypsy moth *Lymantria dispar*, to be inversely related to toxicity (Wolfersberger, 1990). This controversy found by Wolfersberger has not yet been resolved.

Several studies of the interactions between crystal proteins and brush border membrane vesicles have shown that binding of a toxic fragment is not a simple reversible process (Van Rie, 1989; Van Rie *et al.*, 1990; Honée *et al.*, 1991). Initial reversible binding of the toxic fragment to the receptor becomes irreversible, probably reflecting initiation of pore formation (discussed below). Thus, although receptor binding is an important step in the mechanism of toxic action of the crystal proteins, determining to a large extent the insect specificity, the efficiency of pore formation might also influence the toxic potency of crystal proteins.

Several reports have suggested the involvement of carbohydrates in the interaction between toxic fragments and receptors (Knowles *et al.*, 1984; Knowles and Ellar, 1986; Hofmann *et al.*, 1988[a]). The toxicity of crystal proteins from *B. thuringiensis* serotypes *kurstaki* on Cf1 cells was counteracted by preincubation of the toxic fragments with N-acetylgalactosamine or N-acetylneuraminic acid but not by a range of other monosaccharides (Knowles *et al.*, 1984). Also the lectins, wheat germ agglutinin and soybean agglutinin, which can bind terminally located N-acetylgalactosamine, protected CF1 cells from toxic activity. These results suggested that the toxin recognises as a receptor a membrane glycoconjugate with a terminally located N-acetylgalactosamine residue. In fact, a glycoprotein of 146 kDa has been suggested to be a putative receptor (Knowles and Ellar, 1986). In contrast to these findings, neither toxicity of *B. thuringiensis* serotype *kurstaki* toxic fragments to *Bombyx mori* larvae *in vivo* nor disruption of brush border membrane vesicles *in vitro* were influenced by preincubation of the toxic fragments with *N*-acetylgalactosamine or *N*-acetylneuraminic acid (Tojo, 1986). Also, N-acetylgalactosamine and wheat germ agglutinin appeared not to interfere with *B. thuringiensis* serotype *thuringiensis* toxin binding to *Pieris brassicae* midgut vesicles (Hofmann *et al.*, 1988[a]), although binding was affected by treatment of the vesicles with proteases or a mixture of glycosidases. In conclusion, a glycoprotein probably forms the receptor for toxin binding, although controversy remains on the nature of the sugar residues of the glycoprotein involved in the binding.

Carbohydrate groups have also been suggested to occur on the toxic fragment of the crystal protein (Pfannenstiel *et al.*, 1987). Sugar residues were reported to be covalently bound to intact crystal proteins of *B. thuringiensis* serotype *israelensis*. The sugar groups were supposed to be involved in the binding of the toxins to a lectin-like receptor (Muthukumar and Nickerson, 1987; Pfannenstiel *et al.*, 1990). However, as shown by the same authors, *in vitro* generated, protease resistant toxic fragments did not contain any covalently bound sugar moieties, implying that carbohydrate groups are not essential for toxicity. A peculiarity is the authors' assumption that the crystal proteins would be *N*-

glycosylated whereas *N*-glycosylation is only known to occur in the endoplasmatic reticulum and Golgi system of eukaryotes.

On the site of action of the toxic fragments. The receptor binding described above immobilises the toxic fragments at the midgut epithelium cell membrane. Histopathological and physiological studies showed toxic effects of crystal proteins in the midgut within the first minute after feeding (Fast and Donaghue, 1971; Percy and Fast, 1983). Therefore, it was assumed that the toxic fragment acts at the level of the cell membrane. In agreement with this assumption was the observation that immobilized toxic fragments of *B. thuringiensis* serotype *kurstaki* crystals, bound to Sephadex beads in order to prevent penetration of *Choristoneura fumiferana* CF1 cells, retained their cytotoxic activity (Fast *et al.*, 1978).

Several reports have proposed that the toxicity of the crystal proteins might result from specific inhibition of the enzymes generating the K^+ pump localized in goblet cells. Such an inhibition would explain the decreased K^+ concentration in the goblet cell cavity from isolated midguts of *Manduca sexta* fed dissolved crystal proteins (Gupta *et al.*, 1985). Although toxic fragments were found to inhibit *in vitro* a membrane bound K^+-ATPase isolated from cells of the embryonic *M.sexta* cell line CHE (English and Cantley, 1985) and a purified (Na^+-K^+)-ATPase from dog kidney (English and Cantley, 1986) one should realize that these results were obtained with cells and enzymes not occurring in the larval midgut. Furthermore, the crystal protein fragments used in these studies appeared trypsin sensitive, whereas crystal protein toxins are trypsin resistant. Also, English and Cantley suggested that the toxic fragments inhibited the ATPases by acting on the cytoplasmic side of the cell which, when applied to inhibition of K^+-ATPase localized in the goblet cell, would imply the entry into goblet cells of toxic fragments, a contradiction to many other data. Taken together, this renders the observed inhibition of ATPases as a step in the mechanism of toxic action unlikely. Likewise, the inhibitory effects of the protease resistant CryIA(c) toxic fragment on a membrane associated phosphatase from *Heliothis virescens* midgut cells reported by English and Readdy (1989) do not prove that the inhibition of midgut phosphatases plays a role in *in vivo* toxicity, although the results indicate that inhibition of phosphatase might be a secondary effect of toxin action.

Pore formation. Several experimental data suggest that the toxicity of crystal proteins is based on disturbance of the permeability of the membrane by pore formation. When *Bombyx mori* larvae were fed the potassium ionophore valinomycin they showed symptoms comparable to those produced upon feeding of crystal proteins (Angus, 1968). In addition, the K^+-driven amino acid uptake by brush border membrane vesicles of *Pieris brassicae* was inhibited by addition of toxic fragments from crystal proteins of *B. thuringiensis* subspecies *kurstaki* and *thuringiensis* (Sacchi *et al.*, 1986), whereas the

amino acid uptake driven by a Na^+ gradient and the permeability for H^+ ions appeared unaffected by toxin addition. These data indicate that the toxic fragments act by increasing the K^+ permeability of the membrane through disruption of the functioning of K^+-specific channels. In agreement with this conclusion, Crawford and Harvey (1988) demonstrated that the presence of Ba^{2+} and Ca^{2+} ions, known to specifically block K^+ channels, protected the isolated *Manduca sexta* midgut from disturbance of the transepithelial potential difference induced by *B.thuringiensis* serotype *kurstaki* crystal proteins. A cascade of events starting with successful pore formation and ultimately leading to cell disruption was proposed by Harvey *et al.* (1986). According to their model, leakage of K^+ through the columnar cells decreases the net flux of H^+ ions from the goblet cavity into the goblet cell which leads to an increase of the pH in the goblet cell. The ATP level in the goblet cell cytoplasm then drops because oxidative phosphorylation cannot proceed in an alkaline cytoplasm; this in turn leads to an increase in glucose levels as the cells try to adjust to the lost oxidative phosphorylation. The now ineffective K^+ pump is destroyed as pH rises in the goblet cell, leading to a further reduction of K^+ levels in the goblet cavity. Because of the continuing rise of the intracellular pH, lysosomes then destroy the cell.

An alternative hypothesis attributes toxicity to the formation of non-selective pores. Recent experiments using brush border membrane vesicles from *M.sexta* and CryIA(b) toxic fragments demonstrated a generally increased membrane permeability (Hendrickx *et al.*, 1990). Uptake of amino acids, driven by either a Na^+ or K^+ gradient, was inhibited by CryIA(b) toxic fragments. Such inhibition was not prevented in the presence of Ca^{2+} or Ba^{2+}. In addition, membrane permeability for larger molecules was increased by CryIA(b) toxic fragments, as was inferred from the release of intravesicular *L*-alanine. From these results Hendrickx *et al.* concluded that pores formed by CryIA(b) toxic fragments were not selective for K^+. However, these experiments were similar to those performed by Sacchi and coworkers (1986), who concluded that pores formed by toxic fragments were K^+ selective (described above). The major difference between the experiments performed by the two research groups is the use of different toxin preparations: a proteolysed crystal protein mixture by Sacchi *et al.* and the well defined toxic fragment from the recombinant crystal protein CryIA(b) by Hendrickx *et al.*. The toxic fragment induced pores may in fact be cation-specific, because the permeability of negatively charged ions like Cl^- was shown not to increase (Slatin *et al.*, 1990; English *et al.*, 1991)

Leakage of radiolabeled internal markers, such as ions or nucleotides with different molecular weights, from *in vitro* cultured cells was presented as another argument for toxin induced non-selective pore-formation (Knowles and Ellar, 1987). Since toxin induced release of the internal markers could be delayed or even prevented by the use of osmotic protectants a colloid-lysis model was proposed. According to this model, toxic fragments cause small non-specific pores in the plasma membrane of midgut epithelial cells, with an estimated size of 0.5-1.0 nM, resulting in a net inflow of ions and an

accompanying influx of water. This water influx leads to cell swelling and causes the size and number of lesions in the membrane to increase which eventually results in cell lysis.

Several experiments with artificial phospholipid membranes and toxic fragments of various crystal proteins showed the toxic fragment to be capable of intercalating in lipid bilayers in agreement with the colloid-lysis model (Yunovitz and Yawetz, 1988; Haider and Ellar, 1989; Slatin *et al.*, 1990). However, these studies with *in vitro* insect cell lines or artificial lipid membrane vesicles were performed using toxin concentrations in the μM range. Such a concentration range is several orders of magnitude higher than the concentration required in susceptible midgut tissue for *in vivo* toxic activity, which fall in the nM range. Recently, it was demonstrated that brush border membranes integrated into phospholipid vesicles reduced the concentration of toxin required for permeability disturbance 1000-fold (English *et al.*, 1991). These results suggest that formation of pores is facilitated after binding of the toxic fragments on the membrane receptors present on the midgut epithelial cells.

Recently, using X-ray cristallography, the three-dimensional structure of the toxic fragment of a crystal protein has been elucidated (Li *et al.*, 1991). The toxic fragment comprises three domains of which the first domain consists of one hydrophobic and six amphipathic α-helices. The second domain encompasses three ß-sheets, whereas the third domain consists of a sandwich of two antiparallel ß-sheets. The structure of the domains suggest that the first and second domain may be involved in pore formation and receptor binding, respectively, whereas the third domain might protect the toxic fragment from further degradation during proteolytical processing. The toxic fragment structure fits in the model explaining that toxicity of the toxic fragment is achieved by receptor binding and pore formation.

In conclusion, the mode of action of the toxic fragments of crystal proteins towards columnar cells includes two steps, membrane receptor binding followed by pore formation. Pore formation eventually results in cell death. It is not clear whether the kind of pores formed are K^+-specific, cation-specific or non-specific. The cascade of events proposed by Harvey *et al.* (1986) and the colloid-lysis model of Knowles and Ellar (1987) may both approach reality which includes the disturbance of ion gradients, pH regulation and nutrient uptake, and eventually the disruption of midgut epithelium cells.

Interaction of Cyt proteins with in vitro cultured cells. Cyt protein containing crystal protein mixtures from the *Diptera* specific strains of serotypes *israelensis*, *darmstadiensis* and *morrisoni* appeared toxic *in vitro* to a broad range of cultured cell lines and *in vivo* when injected in mice intravenously or subcutaneously. Preincubation of dissolved crystals of *B. thuringiensis* serotype *israelensis* with the phospholipids phosphatidylcholine, sphingomyeline and phosphatidyl ethanolamine neutralized the toxic effect to mice as well as the cytotoxic effect to cultured cells *in vitro* (Thomas and Ellar, 1983[b]). The cytotoxic activity of crystal proteins of *morrisoni* harbouring CytA and of

purified CytB protein from *B. thuringiensis* subspecies *darmstadiensis* crystals could be similarly inhibited by preincubation of the crystal proteins with phospholipids (Gill *et al.*, 1987[b]; Drobniewski and Ellar, 1989). Because only phospholipids containing unsaturated fatty acids affected cytotoxicity, results suggested that Cyt proteins exclusively bind to phospholipids containing unsaturated fatty acyl residues. In particular, a significant role in toxin binding was postulated for unsaturated acyl residues at the syn-2 position of the phospholipid (Gill *et al.*, 1987[b]). Leakage of radiolabeled markers like ions and uridine from *Aedes aegypti* cells and human erythrocytes upon treatment with the toxic fragments of the CytA and CytB proteins suggested the formation of pores of 0.6-1.0 nm in diameter, comparable to those reported for Cry proteins (Drobniewski and Ellar, 1988). CytA toxin was also able to form pores in artificial planar lipid bilayers (Knowles *et al.*, 1989). The generated pores were permeable to Na^+ and K^+ but not to Cl^-. The presence of divalent cations, Ca^{2+} and Mg^{2+}, reduced channel opening. A non-specific inhibition of cytotoxicity was reported for divalent cations in general, however at supraphysiological concentrations (Drobniewski *et al.*, 1987).

An analysis of the kinetics of the initial stages of CytA protein action in a heterologous system, i.e. Malpighian tubules of the blood-sucking insect *Rhodnius prolixus* (Hemiptera), suggested that pores are formed by several toxin molecules (Maddrell *et al.* 1988; Maddrell *et al.* 1989). Maddrell *et al.* proposed a model that when a critical number of toxin molecules have become associated at a single membrane site, the complex is activated and starts disrupting the cell membrane. It should be realised that this model has to be interpreted with caution because it is based on results obtained from studies in a heterologous system.

Taken together, the data suggest that similar to Cry proteins Cyt proteins form cation-selective pores and act by a mechanism of colloid-osmotic lysis, the difference being the absence of receptor involvement and consequently of cell specificity in the case of Cyt proteins.

Effects of synergism between different crystal proteins

Although Cyt proteins appeared toxic to various cell lines *in vitro* and to mice upon injection, crystals of mosquitocidal *B. thuringiensis* strains showed no *in vivo* toxicity to lepidopteran insect larvae when orally ingested. Several authors purified a 28 kDa protein from *B. thuringiensis* serotype *israelensis* crystals, which appeared hemolytic but not toxic to dipteran insects (Hurley *et al.*, 1985; Held *et al.*, 1986; Visser *et al.*, 1986; Wu and Chang, 1985). In contrast to these findings, others reported that extracts of recombinant *E.coli* cells expressing the CytA protein were hemolytic and slightly toxic to mosquitoes (Mclean and Whiteley, 1987; Ward *et al.*, 1984). However, in these studies, the cloned plasmid DNA fragment also contained a *cryIVD* gene encoding a highly toxic *Diptera* specific crystal protein of 72 kDa, separated 1,5 kb from the *cytA* gene (Donovan

et al., 1988). The *cryIVD* may have been expressed in the recombinant *E. coli* cells and may have influenced the toxicity of the recombinant cell extracts.

Nevertheless, many reports assigned to the CytA protein an important synergistic role in the *in vivo* toxicity. Samples of chromatographically purified crystal proteins of *B. thuringiensis* serotype *israelensis* were far less toxic against *Aedes aegypti* larvae when CytA was absent (Yamamoto *et al.* 1983). Wu and Chang (1985), and Ibarra and Federici (1986) postulated synergism between purified CryIVD and CytA against *A.aegypti* larvae. Synergism between CytA and CryIVA and CryIVB proteins, but not between CytA and CryIVD was also reported by Chilcott and Ellar (1988).

An interpretation of the above mentioned results concerning the contribution of each protein in total toxic activity is not simple, mainly because purified protein samples might have been contaminated with other crystal proteins or recombinant cells might have expressed more genes simultaneously. However, as a general trend it seems that the Cyt proteins by themselves display only little or no *in vivo* toxicity to dipteran insect larvae as well as to other organisms, whereas CytA proteins might exhibit synergistic effects when combined with the mosquitocidal CryIV proteins. This interpretation has recently been questioned again by Delécluse and collaborators. In an elegant study they showed that the CytA protein did not significantly contribute to the mosquitocidal activity (Delécluse *et al.*, 1991). By *in vivo* recombination the *cytA* gene of *B. thuringiensis* serotype *israelensis* was disrupted by insertion of an erythromycin resistance gene. The recombinant strain, containing wild type-like crystals, produced all CryIV crystal proteins but no CytA protein. Upon dissolving, these crystals were not hemolytic but still showed full wild-type toxicity to *Aedes aegypti*, *Culex pipiens* and slightly less to *Anopheles stephensi*. These results suggest that CytA does not play a role in toxicity through synergism with the other crystal proteins. So at present, the question of the role of the Cyt proteins *in vivo* remains unresolved.

Synergistic effects might still play a role for two other *Diptera* specific crystal proteins, CryIVB and CryIVC (Delécluse *et al.*, 1988). Recombinant *E.coli* cells expressing a subcloned *cryIVB* gene were not toxic against the dipteran insect *Culex pipiens*, nor were recombinant *E.coli* cells expressing a subcloned *cryIVC* gene. On the other hand, a mixture of the two cultures increased toxicity to the level observed for a recombinant *E.coli* expressing both genes simultaneously. These results confirm observations of many research groups over several years which imply that synergistic effects must play a role to explain the high activity of the wild-type crystals of mosquitocidal strains compared to the toxicity of individual proteins.

General conclusions

Except for the Cyt proteins, the crystal proteins are highly specific for insects. Moreover, the insecticidal crystal proteins are insect species specific. On the other hand, the

cytolytic Cyt proteins produced by *Diptera* specific *B. thuringiensis* strains are toxic for a variety of *in vitro* cultured cells and for mice upon injection.

Upon ingestion, the crystal proteins (both Cry and Cyt) are liberated from the crystals and proteolytically converted into their respective toxic fragments. The toxic fragments of the Cry proteins bind to receptors, presumably glycoproteins, present on the membrane of the columnar cells of the midgut epithelium. Following receptor binding pores are formed in the cell membranes. Similar to Cry proteins, Cyt proteins form pores in membranes, but unlike Cry proteins Cyt proteins do not interact with receptors. The *in vivo* function of the Cyt proteins remains open to question.

Pores formed in the epithelial cell membrane disturb the midgut ion fluxes. A cascade of events has been suggested according to which the leakage of K^+ results in disturbance of the transepithelial potential difference, which in turn abolishes pH regulation and nutrient uptake and eventually results in cytolysis of the epithelial cells. Alternatively, cell lysis resulting from pore formation may be explained by a net influx of ions in the midgut cells in general and an accompanying inflow of water. Both modes of action result in cell lysis and a total disturbance of the midgut physiology and function. Presumably, changes in the physiological conditions in the midgut and in the hemocoel subsequently account for the paralysis of the midgut muscles and total paralysis observed in larvae fed with crystal proteins.

The pathogenic effects observed in larvae fed with crystals or crystal/spore preparations, result from the contribution of all toxins present. This means that different crystal proteins at least have an additive effect, but synergism between crystal proteins has also been reported. The question on the synergism between Cyt proteins and other crystal proteins remains unresolved.

The molecular mode of action has also been investigated by domain-function studies on crystal proteins that relate the primary protein structure to its properties (recently reviewed by Visser *et al.*, 1992). The domain-function studies have focused on the identification of sequences determining insect specificity. So far, studies on hybrid crystal proteins have resulted in the identification of domains on the protein responsible for insect species specificity. The availability of the three dimensional structure of the toxic fragment will further enable domain-function studies.

Further knowledge concerning the molecular basis of the pathogenesis and the domain-function relationships of the crystal proteins will provide new insights in the mode of action of *B. thuringiensis* crystal proteins which may lead to improved strategies for the application of these insecticidal proteins.

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Chapter two

Classification of *Bacillus thuringiensis* crystal proteins

Summary

Five different crystal protein genes belonging to four gene classes, *cryIA(a)*, *cryIB*, *cryIC*, and *cryID* were isolated from *Bacillus thuringiensis* serotype *entomocidus* 60.5. The crystal proteins encoded by these genes differed in insecticidal spectrum. One gene, BtVI, representing the reference-type of class *cryIC*, encoded a protein with a calculated molecular weight of 134,740 Da. The insecticidal spectrum of CryIC includes *Spodoptera* species, *Mamestra brassicae*, and *Manduca sexta*. The nucleotide sequence of BtVI and its deduced amino acid sequence is presented.

A survey of 25 different *B. thuringiensis* strains revealed that *cryIA* type genes occurred in all strains tested, whereas *cryIB*, *cryIC*, and *cryID* occurred rarely. Furthermore, Southern analysis of DNA from strain *kenyae* 4F1 using fragments of BtVI as a probe revealed an additional gene, BtXI, representing the reference-type of class *cryIE*. Its gene product showed locally high homology to CryIC and was toxic against *Spodoptera* species. Finally, an analysis of the amino acid sequences of all crystal proteins reveals several conserved features.

Introduction

Classification of *Bacillus thuringiensis* strains can be based on immunological properties, especially on flagellar antigens (H antigens) dividing the strains into serotypes (Bonnefoi and de Barjac, 1963). On the other hand, strains of *B. thuringiensis* can be grouped into different pathotypes on the basis of their target insects. Most strains are active against larvae of *Lepidoptera*, but some show toxicity against larvae of dipteran or coleopteran species. The crystalline inclusions which are produced by *B. thuringiensis* during sporulation form the major toxic determinant of this bacterium. These parasporal crystals may each consist of several insecticidal proteins, the socalled crystal proteins (reviewed by Höfte and Whiteley, 1989). Generally, insecticidal spectrum, crystal structure, and crystal protein size pattern are correlated. Bipyramidal crystals consist of crystal proteins of M_r 130,000 -140,000 specifically toxic against *Lepidoptera*, cuboidal crystals contain proteins of M_r 65,000 specifically toxic against *Lepidoptera* and *Diptera*, whereas ovoid shaped crystals are composed of crystal proteins of M_r 27,000, 70,000 and 130,000 specifically toxic against *Diptera*, and rhomboidal shaped crystals comprise crystal proteins of M_r 70,000 specifically toxic against *Coleoptera*.

Most attention has been paid to *Lepidoptera* specific crystal proteins. Until 1986 classification of genes encoding *Lepidoptera* specific crystal proteins was based on the size of the *Hind*III fragment containing the 5' end of the crystal protein genes. The size of the *Hind*III fragment was either 4.5, 5.3 or 6.6 kb. Therefore, three different crystal protein gene-classes had been described, namely the 4.5, 5.3, and 6.6 kb gene-classes (Kronstad and Whiteley, 1986). The coding sequences of these three genes were

determined for genes from the *B. thuringiensis* strains *kurstaki* HD1 Dipel (Schnepf *et al.* 1985), *kurstaki* HD1 (Thorne *et al.*, 1986), *berliner* 1715 (Höfte *et al.*, 1986), and *kurstaki* HD73 (Adang *et al.*, 1985). The three genes and their encoded proteins show a high degree of homology of more than 80%.

Neither the *B. thuringiensis* strains used for the isolation of the above mentioned genes nor the commercially available *B. thuringiensis* spore/crystal formulations, that were mainly based on the serotypes *thuringiensis* and *kurstaki*, were very toxic for several important plague insects. Therefore, extensive screening programs have been carried out by various research groups to search for *B. thuringiensis* strains with new insecticidal spectra. Further analysis of the selected *B. thuringiensis* strains resulted in the identification of new crystal protein gene types which prompted the need for a new gene classification. In 1989, a classification based on sequence homology and insecticidal spectrum of the encoded crystal protein encomprising all then known *B. thuringiensis* crystal protein genes, was proposed by Höfte and Whiteley (1989). An updated version of the classification scheme assorting more than 40 sequences contains 17 crystal protein gene classes and subclasses and 2 broad range cytolytic protein gene classes (table 1). According to this new nomenclature the 4.5 kb, 5.3 kb, and 6.6 kb class genes represent *cryIA* class genes subdivided into *cryIA(a)*, *cryIA(b)*, and *cryIA(c)* respectively.

Our contribution to the new classification resulted from a search for *B. thuringiensis* strains which were very toxic for plague insects of *Spodoptera* species of the Noctuidae family. A screening of more than 900 isolates of *B. thuringiensis* for high toxicity against larvae of the Egyptian cotton leafworm, *Spodoptera littoralis*, and of the beet army worm, *Spodoptera exigua*, resulted in the selection of several strains which belonged to the serotypes *aizawai*, *entomocidus* and *kenyae* (Kalfon and de Barjac, 1985; Visser *et al.*, 1986).

One of the isolated strains, *B. thuringiensis* serotype *entomocidus* 60.5, was used for further analyses (Visser *et al.*, 1988). A genomic library was constructed containing DNA fragments of 10 kb and longer. This library was screened by Southern analysis for restriction fragments containing sequences of the well-conserved 3' end region of the crystal protein genes. Besides the known hybridization patterns belonging to the 4.5 kb, 5.3 kb, and 6.6 kb class genes hybridization patterns were found representing new gene types. Judged by hybridization, these new genes showed no homology with the 5' parts of the 4.5 kb, 5.3 kb and 6.6 kb class genes. Further analysis by restriction site mapping resulted in the identification of five crystal protein genes, named BtIV, BtV, BtVI, BtVII, and BtVIII. Two genes, BtV and BtVI were localized only 3 kb apart. The restriction site map of BtIV and BtVIII were similar to that of the earlier described 4.5 kb gene type. The other three genes, BtV, BtVI and BtVII, showed novel restriction site maps. According to the classification of Höfte and Whiteley (1989) the genes BtIV and BtVIII were classified as *cryIA(a)* genes, whereas BtV, BtVI and BtVII fell into the new classes *cryID*, *cryIC* and *cryIB* respectively (Table 1).

Table 1. *Bacillus thuringiensis* crystal protein gene classes.

Gene type	specificity of the protein[a]	molecular weight of the protein (kDa)
cryIA(a)	L	133.2
cryIA(b)	L	131.0
cryIA(c)	L	133.3
cryIB	L	138.0
cryIC	L	134.8
cryID	L	132.5
cryIE	L	133.2
cryIF	L	133.6
cryIIA	L/D	70.9
cryIIB	L	70.8
cryIIIA	C	73.1
cryIIIB	C	74.2
cryIIIC	C	74.4
cryIVA	D	134.4
cryIVB	D	127.8
cryIVC	D	77.8
cryIVD	D	72.4
cytA	D/cytol.	27.4
cytB	D/cytol.	28.0

[a]: insect specificity: L, Lepidoptera; D, Diptera; C, Coleoptera; cytol., cytolytic.

Here, the insect specificity of the crystal proteins encoded by the crystal protein genes occurring in strain *B. thuringiensis* serotype *entomocidus* 60.5 is described. As will be shown, BtVI encodes a crystal protein toxic against *Spodoptera* species. The nucleotide sequence of BtVI and the deduced amino acid sequence will be presented. The BtVI gene represents the reference-type of the *cryIC* gene class. The occurrence of crystal protein genes in *B. thuringiensis* strains in relation to the insecticidal spectrum of these strains will be discussed. Also, the present state of the classification of crystal protein genes will be reviewed.

Table 2. Toxicity of recombinant *Escherichia coli cells*.

E. coli cells containing gene	50% growth reduction dose[a]			LD_{50}[a]
	Spodoptera exigua	*Spodoptera littoralis*	*Heliothis virescens*	*Pieris brassicae*
BtIV	2.2	15	1.2	1.2
BtVIII	1.8	NT[b]	1.7	0.4
BtV	>50	40	>50	>50
BtVI	1.0	8	25	50
BtVII	8	>50	>50	0.8

Toxicity assays were performed as described previously (Visser *et al.*, 1988). Stationary cultures of recombinant *E. coli* cells were concentrated 50-fold and in a volume of 50 μl directly or after dilution spotted onto a 2 cm^2 of artificial diet (Poitout and Bues, 1974) in a 24 well tissue culture plate. Second instar larvae of either *Spodoptera exigua*, *S. littoralis* or *Heliothis virescens* were fed on this diet (24 per sample dilution) for 6 days after which larval weight was scored. Toxicity studies on larvae of *Pieris brassicae* were done on fresh cabbage leaf discs (2 cm^2), onto which 5 μl samples were applied. Eighteen second instar larvae were each placed on a leaf disc and after 3 days larval death was monitored. [a] 50% growth reduction dose and LD_{50} expressed as cells x $10^{-7}/cm^2$. [b] NT, not tested.

Results

Analysis of BtIV, BtV, BtVI, BtVII, and BtVIII. Expression in *E.coli* cells of the five genes isolated from *B. thuringiensis* serotype *entomocidus* 60.5 was examined by Western analysis (Visser *et al.*, 1988). A polyclonal antiserum directed against solubilized crystals of *B. thuringiensis* serotype *entomocidus* 60.5 was used to identify the crystal proteins encoded by the genes BtIV, BtV, BtVI, BtVII, and BtVIII. Based on the amount of antibodies bound it was concluded that the constitutive expression of the crystal protein genes in *E.coli* under transcriptional control of their own promoter varied remarkably (data not shown). The highest expression level was found for BtVII, but this was accompanied by an extensive degradation. High expression levels were also found for both BtIV and BtVIII, whereas BtVI was poorly expressed. No detectable expression was found for BtV. These correlations must be regarded with caution because of potential differences in antigenic properties of the various crystal proteins. The toxicity of the

E.coli cells producing the different crystal proteins was examined with larvae of *Spodoptera littoralis*, *S.exigua*, *Heliothis virescens*, and *Pieris brassicae* (Table 2). Recombinant *E.coli* cells expressing BtVII were only found to be toxic against *Pieris brassicae*. Recombinant *E.coli* cells expressing BtIV or BtVIII were toxic against *S.exigua*, *H.virescens*, and *P.brassicae*, whereas comparable amounts of *E.coli* cells expressing BtVI were only toxic to *S.exigua* and *S.littoralis*. Since BtVI expression in *E.coli* was considerably lower than BtIV and BtVIII expression, these bioassay findings suggested a high specific toxicity of this gene product towards *Spodoptera* species. The specificity of the BtVI gene product towards *Spodoptera* larvae was confirmed by determining a toxicity ratio (Table 3). The low toxicity ratio illustrated the much higher toxicity of the BtVI gene product for *S.exigua* than for *H. virescens*.

Table 3. Toxicity ratio *Spodoptera exigua/Heliothis virescens*[a].

gene product	ratio
BtIV	1.8
BtVI	0.04
BtVIII	1.1

[a] Toxicity ratio defined as the ratio of recombinant cells needed for 50% growth reduction of *S. exigua* larvae and the number of cells needed for 50% growth reduction of *H. virescens* larvae.

Nucleotide sequence and deduced amino acid sequence of BtVI. In Fig. 1 the nucleotide sequence of BtVI is presented. The sequence differs at two places from the sequence published previously (Honée, *et al.*, 1988). The sequence shows the presence of a large open reading frame coding for a polypeptide with a calculated molecular weight of 134,740 Da.

Downstream the putative trypsin cleavage site at amino acid positions 627 through 628, which represents the proteolytic cleavage site, the amino acid sequence homology with the crystal proteins encoded by *cryIA(a)*, *cryIA(b)*, and *cryIA(c)* was more than 90%, except for the deleted region in the *cryIA(b)* encoded protein. The sequence upstream from the putative trypsin cleavage site, constituting the toxic fragment, are more diverged and show a homology of only 50%. On the basis of the amino acid sequence and insecticidal spectrum of the encoded crystal protein it was concluded that BtVI

characterises a new class of crystal protein genes. BtVI now represents the reference-type of this new gene class, defined as *cryIC* (Höfte and Whiteley, 1989).

The sequences located upstream from the ATG start codon show strong homology to the *cryIA(a)* upstream region including the transcription start sites active in *B. thuringiensis* and *E.coli* (Wong *et al.*, 1983).

```
-226    tctcaaatctcgatgactgcttagtctttttaatactgtctacttgacaggggtaggaacataatcggtcaattttaaatatggggcatatattga

                                                 2 →        Ec →   1 →
-130    tattttataaaatttgttacgtttttgtatttttcataagatgtgtcatatgtattaaatcgtggtaatgaaaaacagtatcaaactatcagaa

-34     ctttggtagtttaataaaaaaacggaggtattttATGGAGGAAAATAATCAAAATCAATGCATACCTTACAATTGTTTAAGTAATCCTGAAGAAGT
                                           M  E  E  N  N  Q  N  Q  C  I  P  Y  N  C  L  S  N  P  E  E  V

63      ACTTTTGGATGGAGAACGGATATCAACTGGTAATTCATCAATTGATATTTCTCTGTCACTTGTTCAGTTTCTGGTATCTAACTTTGTACCAGGGGG
          L  L  D  G  E  R  I  S  T  G  N  S  S  I  D  I  S  L  S  L  V  Q  F  L  V  S  N  F  V  P  G  G

159     AGGATTTTTAGTTGGATTAATAGATTTTGTATGGGGAATAGTTGGCCCTTCTCAATGGGATGCATTTCTAGTACAAATTGAACAATTAATTAATGA
          G  P  L  V  G  L  I  D  F  V  W  G  I  V  G  P  S  Q  W  D  A  F  L  V  Q  I  E  Q  L  I  N  E

255     AAGAATAGCTGAATTTGCTAGGAATGCTGCTATTGCTAATTTAGAAGGATTAGGAAACAATTTCAATATATATGTGGAAGCATTTAAAGAATGGGA
          R  I  A  E  F  A  R  N  A  A  I  A  N  L  E  G  L  G  N  N  F  N  I  Y  V  E  A  F  K  E  W  E

351     AGAAGATCCTAATAATCCAGAAACCAGGACCAGAGTAATTGATCGCTTTCGTATACTTGATGGGCTACTTGAAAGGGACATTCCTTCGTTTCGAAT
          E  D  P  N  N  P  E  T  R  T  R  V  I  D  R  F  R  I  L  D  G  L  L  E  R  D  I  P  S  F  R  I

447     TTCTGGATTTGAAGTACCCCTTTTATCCGTTTATGCTCAAGCGGCCAATCTGCATCTAGCTATATTAAGAGATTCTGTAATTTTTGGAGAAAGATG
          S  G  F  E  V  P  L  L  S  V  Y  A  Q  A  A  N  L  H  L  A  I  L  R  D  S  V  I  F  G  E  R  W

543     GGGATTGACAACGATAAATGTCAATGAAAACTATAATAGACTAATTAGGCATATTGATGAATATGCTGATCACTGTGCAAATACGTATAATCGGGG
          G  L  T  T  I  N  V  N  E  N  Y  N  R  L  I  R  H  I  D  E  Y  A  D  H  C  A  N  T  Y  N  R  G

639     ATTAAATAATTTACCGAAATCTACGTATCAAGATTGGATAACATATAATCGATTACGGAGAGACTTAACATTGACTGTATTAGATATCGCCGCTTT
          L  N  N  L  P  K  S  T  Y  Q  D  W  I  T  Y  N  R  L  R  R  D  L  T  L  T  V  L  D  I  A  A  F

735     CTTTCCAAACTATGACAATAGGAGATATCCAATTCAGCCAGTTGGTCAACTAACAAGGGAAGTTTATACGGACCCATTAATTAATTTTAATCCACA
          F  P  N  Y  D  N  R  R  Y  P  I  Q  P  V  G  Q  L  T  R  E  V  Y  T  D  P  L  I  N  F  N  P  Q

831     GTTACAGTCTGTAGCTCAATTACCTACTTTTAACGTTATGGAGAGCAGCGCAATTAGAAATCCTCATTTATTTGATATATTGAATAATCTTACAAT
          L  Q  S  V  A  Q  L  P  T  F  N  V  M  E  S  S  A  I  R  N  P  H  L  F  D  I  L  N  N  L  T  I

927     CTTTACGGATTGGTTTAGTGTTGGACGCAATTTTTATTGGGGAGGACATCGAGTAATATCTAGCCTTATAGGAGGTGGTAACATAACATCTCCTAT
          F  T  D  W  F  S  V  G  R  N  F  Y  W  G  G  H  R  V  I  S  S  L  I  G  G  G  N  I  T  S  P  I

1023    ATATGGAAGAGAGGCGAACCAGGAGCCTCCAAGATCCTTTACTTTTAATGGACCGGTATTTAGGACTTTATCAAATCCTACTTTACGATTATTACA
          Y  G  R  E  A  N  Q  E  P  P  R  S  F  T  F  N  G  P  V  F  R  T  L  S  N  P  T  L  R  L  L  Q

1119    GCAACCTTGGCCAGCGCCACCATTTAATTTACGTGGTGTTGAAGGAGTAGAATTTTCTACACCTACAAATAGCTTTACGTATCGAGGAAGAGGTAC
          Q  P  W  P  A  P  P  F  N  L  R  G  V  E  G  V  E  F  S  T  P  T  N  S  F  T  Y  R  G  R  G  T

1215    GGTTGATTCTTTAACTGAATTACCGCCTGAGGATAATAGTGTGCCACCTCGCGAAGGATATAGTCATCGTTTATGTCATGCAACTTTTGTTCAAAG
          V  D  S  L  T  E  L  P  P  E  D  N  S  V  P  P  R  E  G  Y  S  H  R  L  C  H  A  T  F  V  Q  R

1311    ATCTGGAACACCTTTTTTAACAACTGGTGTAGTATTTTCTTGGACGCATCGTAGTGCAACTCTTACAAATACAATTGATCCAGAGAGAATTAATCA
          S  G  T  P  F  L  T  T  G  V  V  F  S  W  T  H  R  S  A  T  L  T  N  T  I  D  P  E  R  I  N  Q

1407    AATACCTTTAGTGAAAGGATTTAGAGTTTGGGGGGGCACCTCTGTCATTACAGGACCAGGATTTACAGGAGGGGATATCCTTCGAAGAAATACCTT
          I  P  L  V  K  G  F  R  V  W  G  G  T  S  V  I  T  G  P  G  F  T  G  G  D  I  L  R  R  N  T  F

1503    TGGTGATTTTGTATCTCTACAAGTCAATATTAATTCACCAATTACCCAAAGATACCGTTTAAGATTTCGTTACGCTTCCAGTAGGGATGCACGAGT
          G  D  F  V  S  L  Q  V  N  I  N  S  P  I  T  Q  R  Y  R  L  R  F  R  Y  A  S  S  R  D  A  R  V

1599    TATAGTATTAACAGGAGCGGCATCCACAGGAGTGGGAGGCCAAGTTAGTGTAAATATGCCTCTTCAGAAAACTATGGAAATAGGGGAGAACTTAAC
          I  V  L  T  G  A  A  S  T  G  V  G  G  Q  V  S  V  N  M  P  L  Q  K  T  M  E  I  G  E  N  L  T
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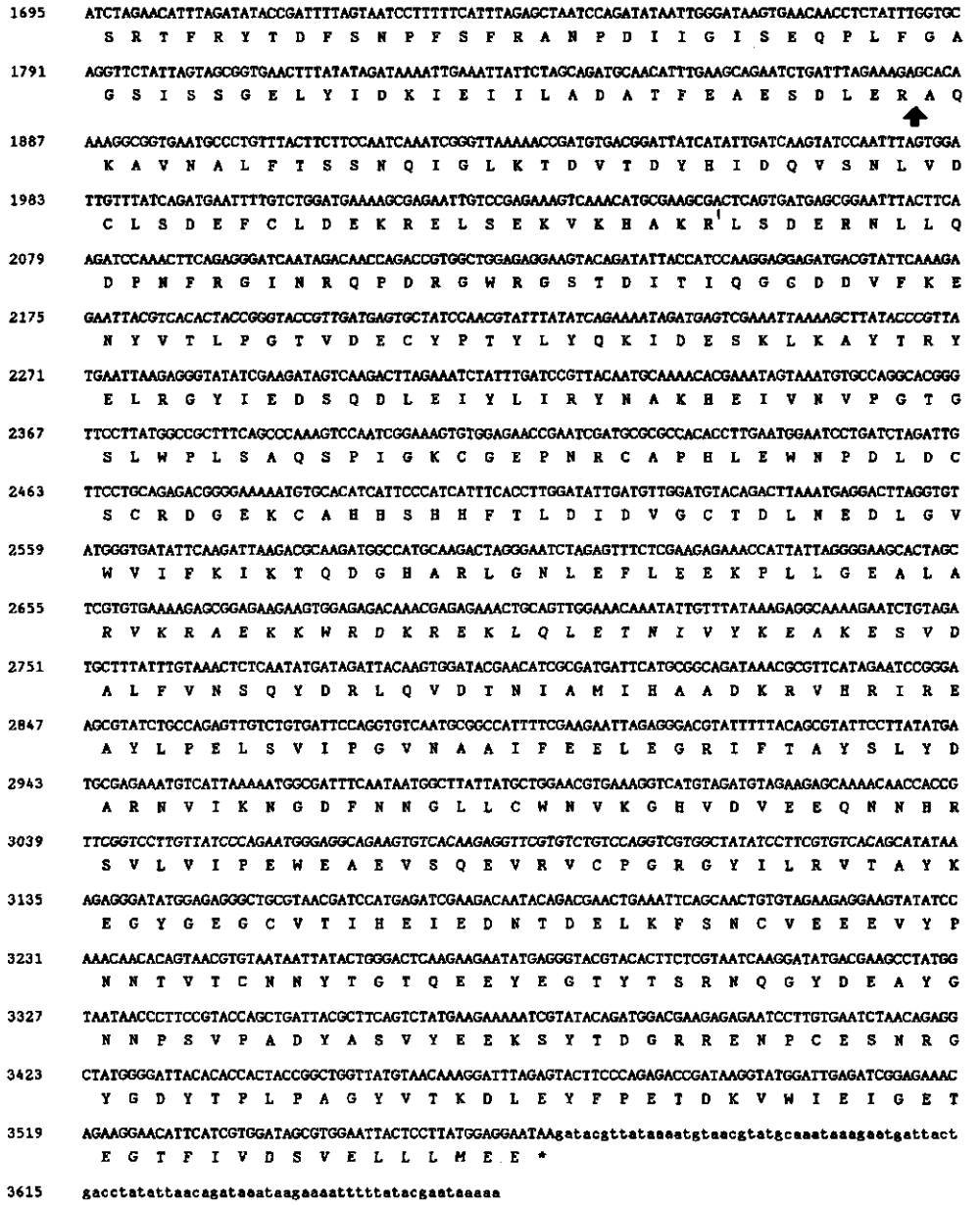

Fig.1. Nucleotide sequence of crystal protein gene BtVI. DNA sequencing was performed on single stranded DNA by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using Sequenase™ (United States Biochemicals) and ^{32}P-dATP (Amersham). Using oligonucleotide primers containing fluorescent dyes the DNA sequence of the fragment upstream from the coding region of the crystal protein gene was automatically analyzed using an Applied Biosystems 370A nucleotide sequence analyzer. The DNA sequence data were analyzed using the PC GENE (Intelligenetics) computer program. The coding

region is represented in uppercase letters. The translation stop codon is indicated by an asterisk. The positions of the putative transcript on start sites for RNA 1 (produced early in sporulation) and RNA 2 (produced at mid-sporulation) in *B. thuringiensis* and the start site in *E. coli*, similar to the positions of the transcription start sites of the cryIA(a) gene of *B. thuringiensis* serotype kurstaki HD1 Dipel (Wong *et al.*, 1983), are indicated by the horizontal arrows 1, 2 and Ec, respectively. The putative trypsin cleavage site at amino acid positions 627 through 628, based on studies determining the minimal toxic fragment of CryIA(b) (Bosch and Martens pers. comm) and on the chrystallography data of CryIIIA (Li *et al.*, 1991), representing the proteolytic cleavage site is indicated by a vertical arrow. The amino acids which belong to the conserved amino acid sequence blocks, as depicted in Fig.2, are underlined. The previously reported nucleotide sequence of BtVI (Honée *et al.*, 1988) should be corrected in the following positions: an Ala codon (GCA) instead of an Arg codon (CGA) is present at position 880 and a sequence of Thr-His (ACGCAT) instead of Thr-Asr (ACCGAT) is present at position 1356.

Occurrence of BtV, BtVI, and BtVII related sequences in other B. thuringiensis strains. Besides BtVI, also BtV and BtVII represent new genes, now classified as *cryID* and *cryIB* type genes, respectively (Höfte and Whiteley, 1989). In order to investigate the occurrence of *cryIB*, *cryIC*, and *cryID* type genes together with the *cryIA* type genes among different *B. thuringiensis* strains total DNA isolated from 25 different *B. thuringiensis* strains was subjected to a Southern analysis (Visser, 1989). The hybridization patterns indicated that all strains tested contained *cryIA* type sequences, whereas *cryIB*, *cryIC*, and *cryID* like sequences occurred rarely. The *cryIC* and *cryID* genes were confined to the serotypes *aizawai* and *entomocidus*. The presence of *cryIC* in the serotypes *aizawai* and *entomocidus* is in accordance with the high toxicity of these serotypes against larvae of *Spodoptera* species. However, many bacterial strains belonging to the serotype *kenyae* were also found to be highly toxic against larvae of *Spodoptera* species (Kalfon and de Barjac, 1985), although these did not contain *cryIC*, as judged by the Southern analysis (Visser, 1989). Southern analysis of total DNA of *B.thuringiensis* serotype *kenyae* 4F1 resulted in the identification of a new gene, BtXI (Visser *et al.*, 1990). Toxicity assays with recombinant *E.coli* cells expressing BtXI, against larvae of *S.exigua*, *H.virescens* and *M.brassicae* showed only insecticidal activity against *S.exigua* larvae at a level comparable to that of the *cryIC* gene product, whereas no toxicity was found against *H.virescens* and *M.brassicae* larvae. According to the nomenclature of Höfte and Whiteley, BtXI represents the reference-type of the new gene class *cryIE*.

Discussion

The isolation and identification of the three new crystal protein genes from *B. thuringiensis* serotype *entomocidus* 60.5 were accomplished using a genomic library containing large inserts and hybridization probes from conserved sequences of the *cryI* coding regions. Two genes, BtV and BtVI were localized only 3 kb apart. Although initially suggested to occur in opposite orientations, the transcription of both genes was later shown to occur in the same direction. A similar organization of *cryIC* and *cryID* was

found in *B. thuringiensis* serotype *entomocidus* 60.1, *aizawai* 7.29 and *aizawai* 7.21 (Sanchis *et al.*, 1988; unpublished results). This suggests a conserved localization of the crystal protein genes *cryIC* and *cryID*. Bio-assay results using recombinant *E.coli* cells showed that the crystal proteins each differed in insect specificity, CryIC being highly toxic against *S.exigua*. In addition, in bio-assays using purified toxic fragments, CryIC was also found to be toxic against larvae of *Mamestra brassicae* and *Manduca sexta* (Höfte and Whiteley 1989, Van Rie *et al.*, 1990).

The BtXI gene from *B. thuringiensis* serotype *kenyae*, classified as *cryIE*, encoded a protein highly toxic to *S.exigua* larvae, whereas no toxicity was found against larvae of *M.brassicae*. Toxicity assays with the purified toxic fragment by Van Rie *et al.* (1990) demonstrated that the *cryIE* gene product is also toxic against *S.littoralis* and *M.sexta* larvae, at a level comparable to that of CryIC.

In conclusion, Southern analyses of genomic DNA by selective hybridisation resulted in the isolation of two genes encoding crystal proteins highly toxic against *Spodoptera* species. Additionally, several other crystal protein genes were isolated encoding proteins each differing in insecticidal spectrum. Obviously, crystal proteins which constitute a crystal together determine the insecticidal spectrum of the *B. thuringiensis* strain. Besides their intrinsic toxic activity, the proportion of each crystal protein in the parasporal crystal will also determine the entomocidal activity of the *B. thuringiensis* strain.

Protein structure and conserved features. Comparison of the crystal protein sequences reveals several characteristic and conserved features. A comparison of the amino acid sequence of CryIE with those of CryIA and CryIC suggests the CryIE sequence to consist of a mosaic of CryIA and CryIC sequences. The *N*-terminal part of the protein from amino acid 60-270 shows a high degree of homology with CryIA up to 79%, whereas the second part of the protein from amino acid 450 to 590 shows high homology with CryIC up to 75%. The *C*-terminal part of the crystal protein not present in the toxic fragment, is highly homologous with the corresponding part of all CryI crystal protein sequences. Similarly, CryIA(b) can be regarded as a mosaic protein consisting of fragments highly homologous with the CryIA(a) and CryIA(c) sequences, respectively (Höfte *et al.*, 1986). Finally, CryID consists of stretches highly homologous with CryIA and CryIC, respectively (Visser *et al.*, 1992). From these observations it might be concluded that the *cryIE*, *cryIA(b)*, and *cryID* genes result from relatively late evolutionary recombination events of the putative parent proteins (Visser *et al.*, 1992).

A comparison of all CryI amino acid sequences shows an extensive homology of 85 to 95% in the *C*-terminal part of the proteins. The *N*-terminal parts encompassing the toxic fragment are more diverged showing a homology of 35 to 85%. The homology within the *N*-terminal half is not randomly distributed. Within the *N*-terminal half six regions containing highly conserved amino acids can be distinguished, as depicted in

Fig.2. These regions, except for block one, are also conserved in the protein sequences of the CryIII and all but one CryIV class proteins. Only in the CryII and CryIVD proteins the homology is restricted to the amino acids of block two and those of the *N*-terminal part of block six. The sequence blocks two, three, five and six, as displayed in Figure 2, represent the first, second, third and fourth sequence block described by Höfte and Whiteley (1989), respectively, whereas the first and fourth sequence blocks represent additional conserved amino acid sequences.

Another conserved feature of the CryI crystal proteins is revealed by a comparison of the hydrophobicity patterns of the toxic fragments. These patterns show that the *N*-terminal parts of the toxic fragments are more hydrophobic than the *C*-terminal parts (results not shown). Furthermore, according to the prediction of Kyte and Doolittle (1982) three hydrophobic domains can be distinguished, as depicted in Figure 2, within the *N*-terminal parts of the toxic fragments. Within the first two conserved hydrophobic domains only the hydrophobic character and not the identity of the amino acids is conserved among different CryI proteins. Hydrophobicity predictions for the CryIII and CryIV classified proteins show similar hydrophobic regions of different amino acid composition. Compared to those of CryI proteins these regions have shifted 30 amino acids towards the C-terminus but the distance between the regions is maintained. This shift in position is only apparent in the protoxin molecules and not in the corresponding toxic fragments due to slightly different proteolytic processing. For both CryIA(b) and CryIC, the toxic fragment obtained after proteolysis with trypsin starts with amino acid residue 29 of the crystal protein sequence (Höfte *et al.*, 1986; Höfte and Whiteley, 1989). Sequence comparison of the CryI proteins suggests a similar proteolysis for all *Lepidoptera* specific crystal proteins, except maybe for CryIB and CryIE. On the other hand, it has been found that for CryIIIA 47 to 57 *N*-terminal amino acids are removed upon proteolysis (Donovan *et al.*, 1988[a]; McPherson *et al.*, 1988). A sequence comparison suggests similar proteolysis for CryIIIB and CryIV sequences. If we assume similar proteolytical processing *in vivo* in the larval midgut, this would imply that the toxic fragment of all crystal proteins starts with a hydrophobic domain at the N-terminus. According to the hydrophobicity prediction by Eisenberg, this *N*-terminal hydrophobic domain may function as a membrane associated α-helix (Eisenberg *et al.*, 1984), suggesting the involvement of this region in pore formation (Schnepf *et al.*, 1985). An alternative main function of this region might be to protect the toxic fragment from proteolytic degradation by rendering the N-terminus less accessible for proteases through its hydrophobic nature. Recently, it was shown that mutations in the two *N*-terminal hydrophobic regions of CryIA(a) resulted in decreased toxicity (Ahmad and Ellar, 1990). The authors suggested the reduced toxicity to be a result of decreased pore formation capacity. However, decreased stability of the mutated proteins might also explain these results.

As mentioned before, the highly conserved *C*-terminal part of the crystal protein is removed during proteolysis generating the toxic fragment. Moreover, deletion studies of

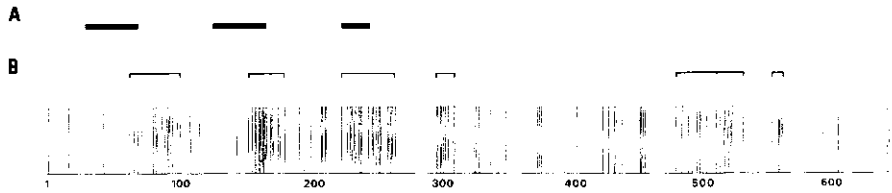

Fig.2. Amino acid sequence comparison of the toxic fragments of *B. thuringiensis* CryI crystal proteins. Gaps that were added for optimal alignment are not indicated. Vertical lines represent conserved amino acids. The six conserved sequence blocks are shown by brackets (B). The positions of the three conserved hydrophobic domains are overlined by solid bars (A).

several genes have shown that the *C*-terminal half is not required for toxicity (Adang *et al.*, 1985; Höfte *et al.*, 1988; Sanchis *et al.*, 1989). Interestingly, although many cysteine residues occur in the crystal proteins the toxic fragments are free of cysteine residues, except for the CryIB and CryIC toxins which contains one and two cysteine residues, respectively. Furthermore, the cysteine residues in the CryI sequences are highly conserved. Two (three for CryIB) are located within the first 28 amino acids which are removed upon processing, whereas all other (11 to 16) are located within the *C*-terminal part of the crystal protein. It has been shown that the cysteine residues of the crystal proteins form interchain disulphide bridges (Couche *et al.*, 1987; Bietlot *et al.*, 1990). These findings suggest a role for the *C*-terminal part of the protoxin in crystal formation in which the conserved cysteine residues allow an assembly of different crystal proteins in the same crystal by the formation of disulphide bridges. Such a structure would also account for the alkali lability of the crystals.

Experimental evidence suggests that, at least for CryIA(c), the *N*-terminal part containing the toxic fragment and the well conserved *C*-terminal part of the crystal protein do not interact and that the protoxin-toxin conversion does not lead to major conformational changes of the toxic fragment (Choma and Kaplan, 1990). Not only the crystal protein but also the toxic fragment appears to be built up from two or three different fragments linked by protease sensitive linkers, as shown for CryIA(b), CryIA(c), and CryIC (Convents *et al.*, 1990; Convents *et al.*, 1991; Choma *et al.*, 1990). Furthermore, a computer analysis of sequence data predicted that the *N*-terminal part of the toxic fragment consists of several α-helices, whereas the *C*-terminal part was predicted to comprise alternating ß-strands and coil structures. In general, the occurrence of regions of highly conserved amino acids or of similar biochemical properties in crystal proteins suggests an overall structural similarity.

Recently, X-ray crystallography data have been obtained for CryIIIA confirming the observations and suggestions above. The CryIIIA protein consists of three structural toxin domains (discussed in Chapter 6). A first domain would run up to residue 290, a second domain would span the segment between residues 290 and 500 and a third domain would

be formed by the C-terminal fragment. The first domain consists of seven α-helices whereas the second domain consists of ß-sheets and the third domain consists of a sandwich of ß-strands. The conserved sequence blocks emerging from the comparison of the crystal protein amino acid sequences form the core of the tertiary structure (Li *et al.*, 1991). Therefore, one might expect the overall three-dimensional structure of the crystal proteins belonging to other classes to be similar to that of CryIIIA.

In conclusion, a similar distribution of conserved amino acid sequences and hydrophobic regions along the polypeptide chain of different *B. thuringiensis* crystal proteins suggests that the three-dimensional structure of crystal proteins will have similar features as the CryIIIA structure which has been determined recently. These conserved features must be functionally significant for crystal formation or for the toxic action of the crystal proteins.

Genetic and evolutionary aspects. Analysis of the genome of *B. thuringiensis* serotype *entomocidus* 60.5 showed that this *B. thuringiensis* strain can contain several crystal protein genes some of which may be (near-) identical (copies) whereas others may only be distantly related. Since the isolation of these genes was achieved using a genomic library constructed from total DNA it is not known whether the genes originate from plasmid or chromosomal DNA. Generally, crystal protein genes are located on large conjugative plasmids with masses larger than 30 MD (Carlton and Gonzalez, 1985). Evidence for this localisation was obtained by transconjugation experiments using Cry^+ *B. thuringiensis* donor strains and Cry^- recipient strains (Gonzalez *et al.*, 1982; Lereclus *et al.*, 1982). Also, crystal protein gene specific probes showed hybridisation with plasmids of several strains (Kronstad *et al.*, 1983). Furthermore, several *cryIA* genes have been cloned from plasmids that were transferred by transconjugation into Cry^- strains as *B. cereus* (Klier *et al.*, 1983, McLinden *et al.*, 1985). Several groups have suggested a chromosomal location of crystal protein genes in strains of serotype *kurstaki* (Held *et al.*, 1982; Kronstad and Whiteley, 1984), serotypes *berliner* 1715 (Kronstad *et al.*, 1983), and *aizawai* 7.29 (Sanchis *et al.*, 1988). However, as some of the authors point out, chromosomal DNA preparations may have been contaminated with fragments of large plasmids (>150 MD), which leaves the existence of chromosomal copies uncertain. Several crystal protein genes appear to be associated with inverted repeats (Kronstad and Whiteley, 1984; Lereclus *et al.*, 1984, Bourgouin *et al.*, 1988). Near *cryIA* crystal protein genes, two transposable elements, Tn4430 (Lereclus *et al.*, 1984) and a configuration containing IS231 copies (Mahillon *et al.*, 1985) have been found. Such findings indicate that crystal protein genes are located on relatively mobile DNA sequences, transposon-like structures and conjugative plasmids. The association of the *cry* genes with mobile DNA might explain the occurrence of multiple copies of a *cry* gene within one bacterial cell. Also, it might explain the occurrence of the same crystal protein genes in genomes of different serotypes. As mentioned before, some crystal protein genes

might have arisen from recombination events between different ancestor genes. An entirely different transfer mechanism is provided by transducing phages occurring in *B. thuringiensis* strains, which have been shown to be involved in crystal protein gene transfer (Reynolds *et al.*, 1988; Walter and Aronson, 1991).

Crystal protein genes are classified on the basis of their primary structure and on the host range of the encoded crystal proteins (Höfte and Whiteley, 1989). Nowadays, the classification consists of 19 different classes and subclasses of which 2 classes, CytA and CytB, represent broad range cytotoxic proteins isolated from *Diptera* specific *B. thuringiensis* strains (Visser *et al.*, 1990; Sick *et al.*, 1989; Gawron-Burke *et al.*, 1990; Knowles *et al.*, 1991). The *Diptera* specific crystal proteins consist of four different Cry classes, CryIVA to CryIVD, next to the Cyt proteins. However, analysis of the crystal protein sequences may lead to a reconsideration of the classification of CryIVD, a 72 kDa protein isolated from *B. thuringiensis* serotype *israelensis* showing *Diptera* activity (Donovan *et al.*, 1988[b]). Sequence comparison reveals that the amino acid sequence of CryIVD is only for 21% homologous to other CryIV subclass proteins. On the other hand, the amino acid sequence of CryIVD shows slightly higher homology (26%) to the CryII proteins which are most diverged from all other crystal proteins. Several characteristics that are shared between CryIVD and CryII proteins emerge. Both in CryII and CryIVD, only the amino acids of the conserved amino acid sequence block two and those of the *N*-terminal part of block six are conserved. Also, the three conserved hydrophobic domains mentioned above are present neither in CryIVD proteins nor in CryII proteins. Therefore, it should be considered to classify CryIVD as a subclass of CryII. The new CryII class would then contain three subclasses of crystal proteins with a molecular weight of 71-72 kDa showing *Lepidoptera* and *Diptera* activity (CryIIA), *Lepidoptera* activity (CryIIB) or *Diptera* activity (CryIIC; CryIVD at present).

Since it has been found that the presence of insects does not correlate with the presence of certain *B. thuringiensis* serotypes in soil samples (Martin and Travers, 1989), the ecological relevance of crystal protein production is still a matter of debate. However, the soil might only represent a sink for *B. thuringiensis* spores that originated from the vegetation. In line with this hypothesis, Smith and Couche (1991) suggested that *B. thuringiensis* should be considered a phylloplane epiphyte having a function in host plant protection. The phylloplane might form the actual ecological niche of *B. thuringiensis* allowing it to be consumed by insects feeding on foliage, to kill the insect *via* the toxin, to sporulate and to replicate. Indeed, high numbers of *B. thuringiensis* cells have been recovered from dead or moribund insects. From this point of view, the phylloplane and, more so, dead insect larvae might form the source whereas the soil forms the sink. In this respect, the presence of several different crystal proteins might render the bacteria more flexible with respect to its insect host, a fact of potentially high ecological relevance.

In order to improve *B. thuringiensis* formulations based on natural spore/crystal preparations ten thousands of isolates have been screened against important insect pests in order to discover new, highly active strains (Kalfon and de Barjac 1985; Visser *et al.*,

1986; Hamal *et al.*, 1991). In an alternative approach, recent reports describe the successful introduction of additional crystal protein genes into *B. thuringiensis* strains with the aim of increasing the insecticidal spectrum (Crickmore *et al.*, 1990). A further potential advantage may be that combinations of toxins act synergistically against certain insect species. The latter has been suggested to occur for two *Diptera* specific crystal proteins, CryIVB and CryIVC (Delécluse *et al.*, 1988), since only mixtures of recombinant *E.coli* cells producing CryIVB or CryIVC appeared to be toxic against *Culex pipiens* larvae. At present, no evidence exists indicating that *Lepidoptera* specific crystal proteins act synergistically. Recently, a synergistic effect has been suggested for the combination of CryIA(a), CryIA(b), and CryIA(c), present in *kurstaki* HD-1 crystals, against *Lymantria dispar* larvae (Franckenhuyzen *et al.*, 1991). However, this was concluded from a comparison of the toxic activity of each separate crystal protein with that of HD-1 crystals but not with purified crystal protein mixtures. Therefore, a possible synergism between these CryI proteins awaits further proof.

Screening of *B. thuringiensis* isolates against insect species of interest has led to the isolation and identification of additional crystal protein genes encoding highly toxic proteins. Although this is a laborious strategy, it has already been proven successful, and in the light of the present big efforts many more additional gene classes and insecticidal spectra can be expected to be described.

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Chapter three

The *C*-terminal domain of the toxic fragment of a *Bacillus thuringiensis* crystal protein determines receptor binding

Summary

The insecticidal crystal proteins of *Bacillus thuringiensis* show a high degree of specificity. *In vitro* binding studies with several crystal proteins demonstrated a correlation between toxicity and binding to receptors of larval midgut epithelial cells. In order to study the domain-function relationships of the toxic fragment, hybrid crystal proteins based on CryIA(b) and CryIC were constructed. Two out of eleven hybrid proteins constructed exhibited insecticidal activity. Both displayed an insecticidal spectrum similar to that of the parental crystal protein from which the *C*-terminal part of the toxic fragment originated. In addition, *in vitro* binding studies directly demonstrated the involvement of the *C*-terminal part of the toxic fragment in receptor binding. These results demonstrate that the *C*-terminal part of the toxic fragment is determining specific receptor binding which in turn determines to a large extent the insect specificity.

Introduction

Crystal proteins of the bacterium *Bacillus thuringiensis* are known for their insecticidal activity. These proteins, produced during sporulation, form crystalline inclusions. Most *B. thuringiensis* isolates exhibit activity against lepidopteran insects (butterflies and moths), whereas other strains are active against dipteran (flies and mosquitoes) or coleopteran (beetles) species. Crystals of most *B. thuringiensis* strains each consist of several crystal proteins differing in insecticidal spectra (Höfte and Whiteley, 1989). Sporulated *B. thuringiensis* preparations have been used as insecticidal agents for over two decades. During the last few years insect resistant transgenic plants (Barton *et al.*, 1987, Fischoff *et al.*, 1987, Vaeck *et al.*, 1987) and microorganisms (Obukowitcz *et al.*, 1986, Waalwijk *et al.*, 1991) have been successfully generated by the introduction of *B. thuringiensis* crystal protein genes.

To some extent the mode of action of the crystal proteins has been elucidated. After oral uptake, the crystals are dissolved in the alkaline environment of the larval midgut. The solubilized crystal proteins of M_r 130-140 kDa (protoxins) are then proteolytically processed by midgut proteases releasing the proteinase-resistant toxin of $M_r \pm 60$ kDa (Choma *et al.*, 1990). Binding of this toxic fragment to receptors of the midgut epithelial cells of susceptible insect larvae disturbs the ion permeability of the cell membranes resulting in swelling and bursting of the cells (Knowles *et al.* 1987, Sacchi *et al.* 1986). As a result of lysis of the midgut epithelial cells, the larvae eventually die.

Several factors together constitute the insect specificity. Firstly, the efficiency of the solubilization of the crystals and the proteolytic activation pattern of the crystal proteins in the larval midgut codetermine the specificity of the crystal proteins (Jaquet *et al.* 1987, Haider *et al.*, 1986). Secondly, the specificity of crystal proteins is, to a large extent, determined by the occurrence of high affinity binding sites on the midgut epithelium cells,

as demonstrated by *in vitro* binding studies on isolated brush border membranes. For several crystal proteins and insect species, the toxicity levels appeared to be positively correlated with the concentrations and/or affinities of such binding sites (Hofmann *et al.*,1988, Van Rie *et al.*, 1989, Van Rie *et al.*, 1990[a]).

In recent studies, hybrid proteins of the closely related crystal proteins CryIA(a)-CryIA(c) (82% homology) and CryIIA-CryIIB (87% homology) have been generated by the exchange of fragments of these crystal proteins differing in insecticidal spectra, and these hybrids have been examined for toxicity. By this strategy, a *Bombyx mori* specificity domain on the CryIA(c) type protein between amino acid residues 332 and 450 and an *Aedes aegypti* specificity domain on the CryIIA type protein between amino acid residues 307 and 382 in the *C*-terminal domain were localized (Ge *et al.*, 1989, Widner and Whiteley, 1990).

Biochemical and biophysical analyses indicated the presence of two structural domains in the toxic fragments of CryIA(b) and CryIC (Convents *et al.* 1990, Convents *et al.* 1991). The *N*-terminal domain of the toxic fragment appeared to consist of several α-helices, whereas the *C*-terminal domain was predicted to occur in a ß-sheet conformation. To gain more insight in the function of these structural domains with respect to the toxic mechanism, we generated hybrid proteins using the rather distantly related crystal proteins CryIC and CryIA(b) and analyzed their toxicity and binding characteristics. Whereas the total amino acid sequences of both proteins show 66% homology, the amino acid sequences of the toxic fragments of both proteins show only 50% homology. These crystal proteins differ considerably in terms of insecticidal spectrum, CryIA(b) being highly toxic against *Heliothis virescens* larvae and CryIC showing high toxicity against larvae of the *Spodoptera* species *S. exigua* and *S. littoralis*. Hybrid proteins were tested for toxicity against *H. virescens* and *S. littoralis*, and binding assays on larval brush border membranes were performed to define the region involved in receptor binding specificity.

Results

Construction of hybrid genes. The cloning of the *cryIA(b)* gene from *B. thuringiensis* serotype *aizawai* 7.21, and the *cryIC* gene from *B. thuringiensis* serotype *entomocidus* 60.5, have been described earlier (Honée *et al.*, 1990). Both genes were expressed constitutively in *Escherichia coli* cells giving rise to CryIA(b) and CryIC proteins of a M_r 130 and 134 kDa, respectively. Hybrid crystal protein genes were constructed by the exchange of homologous fragments between both genes, using restriction sites displayed in Fig.1B. In total, eleven different hybrid crystal protein genes were constructed (Fig.1A) and expressed in *E. coli* under the transcriptional control of either the *cryIA(b)* or the *cryIC* promoter. In Fig. 1 the deduced amino acid sequences of the encoded crystal proteins have been depicted. Within the hybrid crystal proteins BtH1, BtH2, BtH2[b],

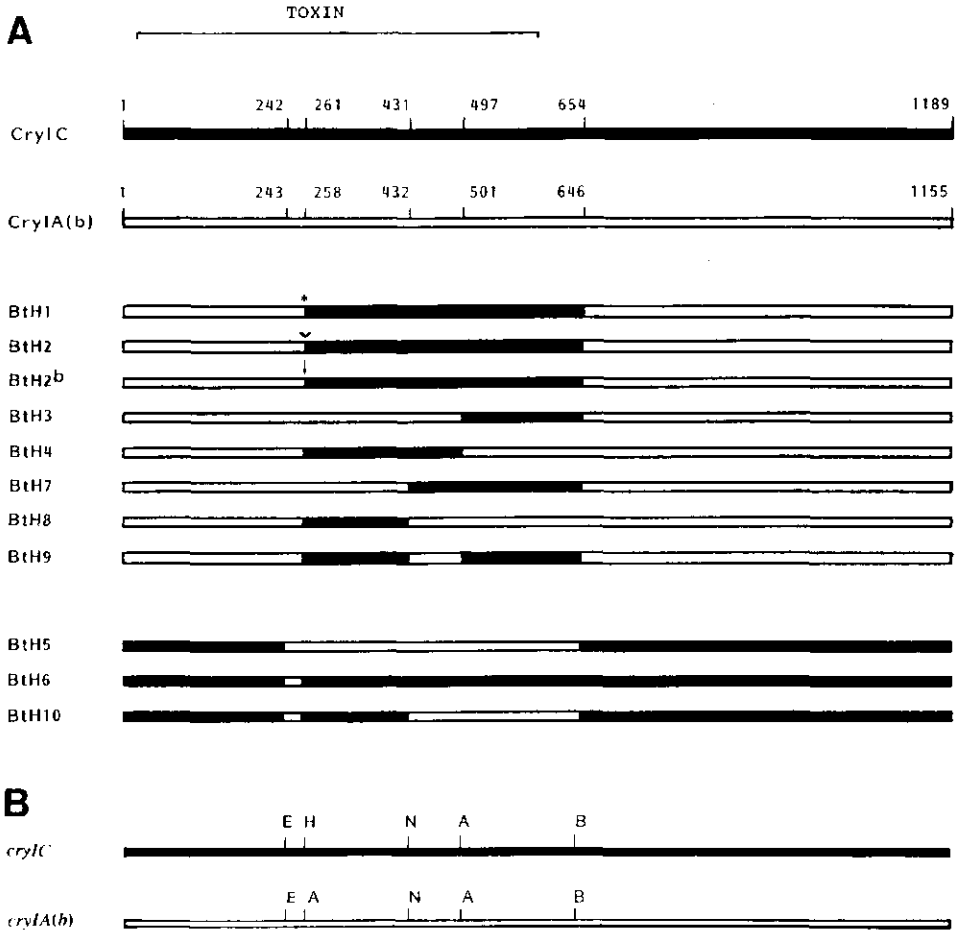

Fig. 1. A. Diagram representing the native crystal proteins CryIA(b) and CryIC and the hybrid proteins generated. Numbers above the depictions of the native proteins represent the amino acid positions of the residues flanking the exchanged fragments of CryIA(b) (open bar) and CryIC (solid bar). The thin line on top of the diagram represents the toxic fragment generated after proteolytic digestion of the crystal protein. BtH1 contains a four amino acid residues deletion at the *N*-terminal CryIA(b)-CryIC junction, indicated by *. In BtH2 these four amino acids, Arg-Thr-Val-Ser, have been reintroduced, indicated by ∨. BtH2^b contained a Thr259Pro substitution, indicated by ↓.
B. Representation of the restriction sites of the *cryIA(b)* (open bar) and *cryIC* (solid bar) genes used in the construction of the hybrid crystal protein genes. Restriction enzyme abbreviations: A, *Asu*II; B, *Bcl*I; E, *Eco*RV; H; *Hind*II; N, *Nla*III.

BtH3, BtH4, BtH7, BtH8, and BtH9 a portion of the CryIA(b) sequence is replaced by homologous CryIC sequences. The CryIA(b) protein sequence runs up to residue 258 in

BtH1, to residue 262 in BtH2, BtH2^b, BtH4, BtH8, and BtH9, to residue 501 in BtH3, and to residue 432 in BtH7. Each of these residues is followed by a CryIC derived fragment. BtH2^b contains a proline residue instead of a threonine residue at amino acid position 259. All these hybrids contain *C*-terminal CryIA(b) sequences from residue 646 onwards, except for BtH4 and BtH8 that contain CryIA(b) type sequences from residue 501 and 432 onwards, respectively. In BtH9, the portion of CryIC running from amino acid 431 to 497 has been replaced by the homologous CryIA(b) fragment from amino acid 432 to 501. The toxic fragment of all hybrids mentioned above, up to residue 600 approximately, thus contained a CryIA(b) derived *N*-terminal part and either a CryIC derived *C*-terminal part (BtH1, BtH2, and BtH2^b) or a *C*-terminal part of mixed origin (BtH3, BtH4, BtH7, BtH8, and BtH9).

In the hybrids BtH5, BtH6 and BtH10 the CryIC protein sequence ends at amino acid residue 242 and is followed a sequence derived from CryIA(b). BtH5, a 'reciprocal' of BtH2, and BtH10 contain *C*-terminal CryIC sequences from residue 654 onwards. In BtH10 a portion of the CryIA(b) derived fragment from position 262 to 431 has been replaced by the homologous CryIC sequence from amino acid residue 262 to 431, resulting in a short *N*-terminal stretch and a large *C*-terminal stretch of CryIA(b) toxin sequences. In BtH6, only 19 amino acids of the CryIC protein sequence from 242 to 261 were replaced by the CryIA(b) type amino acids from 243 to 262, thereby introducing only 8 amino acid changes in the entire hybrid.

Toxicity studies of hybrid proteins. Lysates of recombinant *E. coli* cells producing the above mentioned hybrid crystal proteins were analyzed by Western blotting using a polyclonal antiserum directed against *B. thuringiensis* serotype *aizawai* 7.21 crystals which contain both CryIA(b)- and CryIC-type proteins (Visser *et al.*, 1988). All *E. coli* lysates showed an antigenic polypeptide migrating at a position comparable to that of the lysates containing the parental crystal proteins (results not shown). Simultaneously, these cultures were used to perform toxicity studies against larvae of *H. virescens* and *S. exigua*, as described previously (Visser *et al.*, 1988). Cells expressing CryIA(b) or the hybrid protein BtH5 were toxic against *H. virescens* but not against *S. exigua*, whereas cells expressing CryIC or BtH2 showed toxicity against *S. exigua* but not against *H. virescens* (results not shown). Cells expressing any of the other hybrid proteins were not toxic against any of these insects.

To further examine their properties further, the hybrid proteins were purified from *E. coli* lysates. Western blotting analysis revealed M_r 130 kDa hybrid crystal proteins in all lysates of recombinant *E. coli* cells. However, crystal purification followed by solubilization and tryptic activation of the protoxins resulted in the recovery of stable toxic fragments of M_r 60 kDa only for the parental proteins CryIA(b) and CryIC, and for the approximately 'reciprocal' hybrid proteins BtH2 and BtH5. It had to be concluded that all the other hybrid proteins were unstable during purification of the toxic fragment,

Table 1. Toxicity of toxic fragments of crystal proteins.

Toxin of crystal protein	EC_{50}(ng cm^{-2})[a] against:	
	S. littoralis	*H. virescens*
CryIC	1.42	>125
CryIA(b)	>1350	0.16
BtH2	12.4	>125
BtH5	>1350	8.8

[a]: EC_{50} (concentration giving 50% growth reduction) values are the mean of three different experiments.

including proteins differing by only one residue (BtH2[b] versus BtH2) or a few aminoacids (BtH6 versus CryIC).

To compare the toxicity of the hybrid proteins with that of the wild type proteins, the concentration of purified toxic fragments of CryIA(b), CryIC, BtH2 and BtH5 needed for 50% growth inhibition (EC_{50}) of *S. littoralis* and *H. virescens* larvae was determined (Table 1). As reported before, the crystal proteins CryIA(b) and CryIC appeared highly toxic against *H. virescens* and *S. littoralis*, respectively (Honée *et al.*, 1990). The insecticidal spectra of the hybrid proteins BtH2 and BtH5 were comparable to those of CryIC and CryIA(b), respectively. However, considerable quantitative differences were detected, BtH2 being less toxic against *S. littoralis* than CryIC, and BtH5 being less toxic against *H. virescens* than was CryIA(b).

Binding assays. In conjunction with the toxicity assays, binding studies were performed to investigate the binding characteristics of the BtH2 and BtH5 proteins relative to those of CryIC and CryIA(b). To test the stability of toxins in the presence of the larval brush border membrane vesicle preparations, ^{125}I-labelled toxins of CryIA(b) and BtH5 were incubated with vesicles of *H. virescens*, and ^{125}I-labelled toxins of CryIC and BtH2 were incubated with vesicles of *S. littoralis*. The free and vesicle-bound toxin fractions were separated by centrifugation and analyzed by SDS-PAGE (Fig. 2). No degradation of ^{125}I-labelled CryIC (lane 2 and 3), BtH2 (lane 5 and 6), and CryIA(b) (lane 8 and 9) was observed during incubation with the membrane vesicles. On the other hand, ^{125}I-labelled BtH5 (lane 11 and 12) showed to be unstable during incubation with membrane vesicles of *H. virescens*. For this reason, the binding kinetics of the hybrid crystal protein BtH5

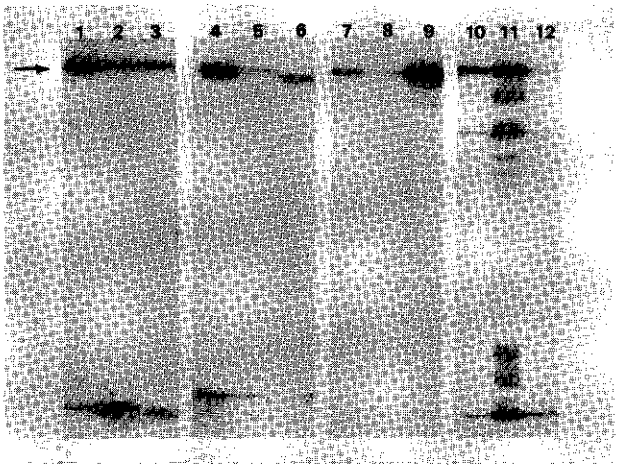

Fig. 2. Autoradiograph of ^{125}I-labelled CryIC (lanes 1, 2 and 3), BtH2 (lanes 4, 5, and 6), CryIA(b) (lanes 7, 8, and 9), and BtH5 (lanes 10, 11, and 12) toxins. Lanes 1, 4, 7, and 10 show the toxin preparations that were not incubated with vesicles. After incubation of the toxins with *S. littoralis* vesicles (lanes 2, 3, 5, and 6) or *H. virescens* vesicles (lanes 8, 9, 11, and 12) free toxins (lanes 2, 5, 8, and 11) and bound toxins (lanes 3, 6, 9, and 12) were separated by centrifugation and analyzed by SDS-polyacrylamide gel electrophoresis (10% acrylamide). The arrow indicates the position of the 60 kD toxin.

could not be studied.

Binding assays were performed with ^{125}I-labelled BtH2 in the presence of varyingconcentrations of either unlabelled BtH2, CryIA(b) or CryIC. Iodinated BtH2 toxin bound significantly to *S. littoralis* vesicles with maximum binding occurring at a vesicle concentration of 100 μg/ml. Competition experiments demonstrated that the binding of labelled BtH2 toxin could be outcompeted by an excess of either unlabelled BtH2 or unlabelled CryIC toxin, but not by unlabelled CryIA(b) toxin (Fig. 3). Inversely, experiments with iodinated CryIC toxin and increasing amounts of unlabelled BtH2 demonstrated that the binding of labelled CryIC toxin could be outcompeted by an excess of unlabelled BtH2 toxin (results not shown). From these observations it was concluded that the CryIC and BtH2 toxins recognize the same binding sites on the *S. littoralis* midgut membranes.

The homologous competition experiments provided data for a quantitative evaluation of BtH2 toxin binding properties to *S. littoralis* vesicles. Binding of BtH2 toxin could be best explained by an one-site model of high affinity sites with an apparent dissociation constant $K_{d\ app.}$ of 10.0 $\pm$ 2.4 nM and a receptor concentration $R_{t\ app.}$ of 16.1 $\pm$ 12.2 picomoles per milligram of vesicle protein. From the heterologous competition

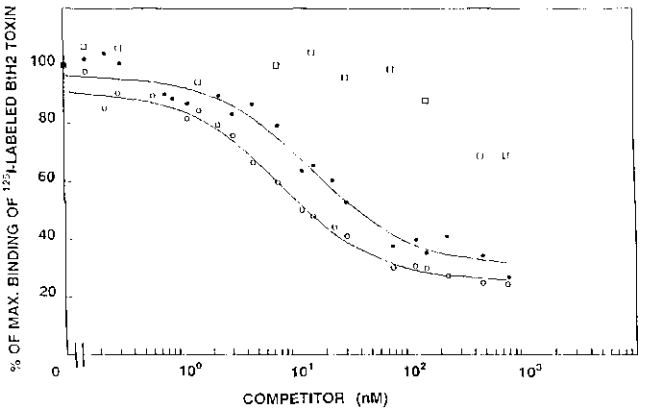

Fig. 3. Binding of ^{125}I-labelled BtH2 toxin on *S. littoralis* brush border membrane vesicles upon incubation of iodinated BtH2 toxin in the presence of increasing concentrations of unlabelled BtH2 toxin (●), unlabelled CryIC toxin (○), or unlabelled CryIA(b) toxin (▫). Binding is expressed as a percentage of the amount bound upon incubation with labelled toxin alone. Non-specific binding was not subtracted. Curves are those predicted by the LIGAND program. Each point is the mean of a duplicate sample.

experiments between labelled BtH2 toxin and unlabelled CryIC toxin, a higher affinity showing a $K_{d\ app.}$ of 5.2 ± 1.1 nM, and a similar receptor concentration $R_{t\ app.}$ of 16.5 ± 10.9 picomoles per milligram of vesicle protein was calculated for CryIC.

The dissociation rate of BtH2 and CryIC toxins bound to *S. littoralis* was examined by addition of excess unlabelled homologous ligand, BtH2 and CryIC toxin respectively, to a incubation mixture that had reached equilibrium (Fig. 4). Neither of the bound toxins dissociated from the *S. littoralis* midgut membrane vesicles at a measurable rate, suggesting that binding of both toxins is a largely irreversible process.

Discussion

Crystal proteins of *Bacillus thuringiensis* show a high degree of insect specificity. In this study, we demonstrate that the *C*-terminal part of the toxic fragment is involved in specific receptor binding and thereby determines the insect specificity.

Toxicity assays with purified toxins of the two 'reciprocal' hybrid crystal proteins, BtH2 and BtH5, established that the *C*-terminal part of the toxic fragment is responsible for the insect specificity. Recent reports describing toxicity patterns of hybrid crystal proteins based upon the closely related crystal proteins CryIA(a) and CryIA(c) (Ge *et al.*,

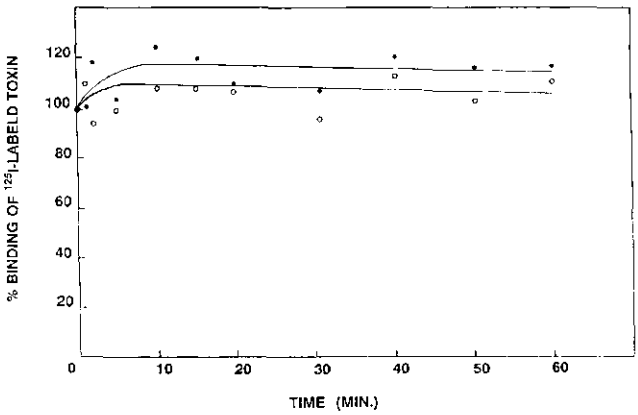

Fig. 4. Dissociation of labelled CryIC (○) and BtH2 (●) toxins from *S. littoralis* vesicles. 90 Minutes after the onset of the association reaction, an excess of the homologous ligands CryIC or BtH2 was added to the incubation mixture. Nonspecific binding was not subtracted.

1989, Caramori *et al.*, 1991) and CryIA(a) and CryIA(b) (Raymond *et al.*, 1990) led to a similar conclusion. Results from this study with much more distantly related toxins suggest that this conclusion may be valid for all *Lepidoptera* specific crystal proteins. Moreover, binding studies with BtH2 in combination with either CryIC or CryIA(b) on *S. littoralis* membrane vesicles demonstrated for the first time that membrane binding specificity is determined by the *C*-terminal part of the toxin molecule.

The results of the competition experiments indicate that binding of the BtH2 toxin and of the CryIC toxin to *S. littoralis* vesicles are best explained by an one-site model. However, in homologous competition experiments CryIC was demonstrated to bind to both high and low affinity binding sites (Van Rie *et al.*, 1990[a]). A possible explanation for this observation may be that the lower affinity of BtH2, used as the ^{125}I-labelled ligand, obscures the presence of the latter type of binding sites. This leaves unexplained the discrepancy in actual dissociation constants for CryIC calculated from the homologous competition experiments ($K_{d\ app.}$=0.2 ± 0.04 nM and $K_{d\ app.}$=13.9 ± 1.3 nM, respectively; Van Rie *et al.*, 1990[a]) and from the heterologous competition experiments ($K_{d\ app.}$= 5.2 ± 1.1 nM; this study). Since differences in toxicity of CryIC and BtH2 against *S. littoralis* larvae can not be explained by the dissociation of receptor bound toxins, the lower toxicity of BtH2 should result from the lower binding affinity of BtH2 or from some further step in the mode of action (e.g. pore formation).

Most *B. thuringiensis* crystal proteins exhibit a similar structure (Choma *et al.*, 1990, Convents *et al.*, 1990, Convents *et al.*, 1991). However, an exchange of any given set of regions between two crystal proteins often does not result in a functional hybrid.

The finding that protein purification from *E. coli* cells, followed by solubilization and proteolytic digestion, resulted in degradation of the hybrid proteins, except for BtH2 and BtH5, demonstrated that the conformational structure of these hybrid proteins is dramatically altered, resulting in unstable proteins. The assumption that such degradation of non-toxic hybrid proteins also occurs *in vivo* in the larval midgut would explain the lack of toxicity of the recombinant *E. coli* cells. Furthermore, the BtH5 toxin, although trypsin-resistant, was found to be degraded during incubation with *H. virescens* vesicles which might well reflect a similar instability of the BtH5 toxin in the larval midgut; this would explain, in part, the low toxicity levels of the BtH5 toxin against *H. virescens* larvae. These results demonstrate that a decreased toxicity may not necessarily result from the replacement of an entire specificity domain but may also be caused by an increased instability of the hybrid protein. Thus, care must be taken in the interpretation of a decrease in toxicity of generated hybrids (Schnepf *et al.*, 1990). Also, since it is known that proteolytic processing may result in different toxins (Haider *et al.* 1986), a given hybrid might exhibit this putative instability in one insect larva but not in the other. In our opinion, these possible drawbacks necessitate the correlation of toxic properties to the known primary and predicted secondary structure of the crystal proteins by measurement of an increased or newly acquired toxicity of hybrid proteins relative to the wild type crystal proteins.

Experimental evidence shows that the toxic fragment consists of two domains (Choma *et al.*, 1990, Convents *et al.*, 1990, Convents *et al.*, 1991). According to the secondary structure prediction of the toxic fragment the *N*-terminal domain consists of four α-helices whereas the ß-sheet conformation is dominant in the *C*-terminal domain of the toxins. Also, the hydrophobicity pattern (Eisenberg *et al.* 1984) predicts that at least one segment of the *N*-terminal domain of the toxic fragment (centring around amino acid position 60 of both CryIA(b) and CryIC) is membrane associated. This leads to the suggestion that the *N*-terminal domain is involved in the actual toxic action, pore formation. Results of the binding studies described in this paper show that the *C*-terminal domain is involved in receptor binding. This suggests that the ß-sheets might form surface structures which interact with membrane receptors.

Like the wild type toxins, the BtH2 toxin probably consists of two structural domains. Indeed, the unfolding patterns for the BtH2 toxin, as obtained by fluorometric measurements (preliminary results), were comparable to those obtained for both the CryIA(b) and CryIC toxic fragments (Convents *et al.*, 1990, Convents *et al.*, 1991). Following the model, the CryIA(b)-CryIC junctions in the hybrid toxic fragments of BtH2 and BtH5 are positioned between the third and fourth α-helix within the *N*-terminal domain, apparently without severely affecting protein function. However, the interactions between the two domains have been altered resulting in a decrease of the binding affinity to CryIC specific receptors of the BtH2 toxin. In general, interdomain interactions might influence the capacity of specific receptor binding, mainly determined by the *C*-terminal toxin domain. Other bacterial toxins like the diphtheria toxin from *Corynebacterium*

diphtheria, the tetanus toxin from *Clostridium tetani* and the exotoxin A from *Pseudomonas aeruginosa* show a three-dimensional structure also made up of fairly autonomous domains, reminiscent of the model proposed for the toxins described here.

Although the major involvement of the *C*-terminal part of the toxin in specificity of membrane binding and insect toxicity has been demonstrated, many questions remain. Considering the large toxin size, it would be of interest to elucidate whether all amino acid residues essential for specific receptor binding are consecutively located on a single peptide stretch, and whether multiple domains, each involved in specific receptor binding, occur in a single toxin molecule. If so, this would enable, in theory, the construction of crystal protein hybrids binding at different midgut membrane receptors of the same insect to improve efficiency of control. In addition, such a strategy might delay the emergence of crystal protein resistance, since recent findings show that resistance can be due to an alteration of the receptor sites on the midgut epithelium (Van Rie *et al.*, 1990[b]). Thus further elucidation of the domain function relationships of *B. thuringiensis* crystal proteins might provide strategies for improving their use as an entomocidal agent.

Addendum

Recent X-ray chrystallography data obtained for the related crystal protein CryIIIA indicate the presence of three structural toxin domains. When these data are applied to the CryI proteins described here, a first domain would run up to residue 260, a second domain involved in recptor binding would span the segment between residues 260-470, and the last domain, perhaps protecting the toxin from proteolytic breakdown, would be formed by the *C*-terminal segment (Li *et al.*, 1991). According to these data the only fully functional hybrid BtH2 contains a junction between CryIA(b) and CryIC sequences at the boundary between the first two structural domains. This might explain its toxicity.

Experimental Procedures

Construction of hybrid crystal protein genes. The *cryIC* gene BtVI, isolated from *B. thuringiensis* serotype *entomocidus* strain 60.5 (Honée *et al.*, 1989), was cloned as a 4,3 kb *Hind*III-*Pst*1 fragment into the Bluescript vector SK$^+$ (Stratagene Inc.). The cryIA(b) gene BtII, isolated from *B. thuringiensis* serotype *aizawai* 7.21, was cloned as a 7 kb *Bam*H1-*Pst*1 fragment into pUC13. These plasmids were designated p60.5G31 and p7.21A, respectively (Honée *et al.*, 1990). The gene encoding BtH1 was made by replacing an 1164 bp *Asu*II-*Bcl*1 fragment of the *cryIA(b)* gene by a 1178 bp *Hind*II-*Bcl*1 *cryIC* fragment. The *Asu*II ends of *cryIA(b)* were partially filled in by the Klenow fragment of *E.coli* polymerase I in the presence of dCTP. Site-Directed Mutagenesis System 2 (Amersham) was used to generate the gene encoding BtH2 by the reintroduction

of the nucleotides (5'-CGAACAGTATCT-3') coding for the amino acids Arg-Thr-Val-Ser present in the CryIA(b) sequence at position 258 to 261. DNA sequence analysis revealed a clone, designated *bth2*^{b}, in which the nucleotide sequence of the introduced nucleotides had been altered, resulting in the amino acid substitution Thr259Pro with respect to the BtH2 amino acid sequence. The hybrid genes *bth3* to *bth10* were made using conserved restriction sites occurring in *cryIA(b)* and *cryIC*. Replacement of the 720 bp *Asu*II fragment or the 471 bp *Asu*II-*Bcl*1 fragment of *bth2* by the homologous 729 bp *Asu*II fragment or the 435 bp *Asu*II-*Bcl*1 fragment of *cryIA(b)* generated *bth3* and *bth4*, respectively. Replacement of the 526 bp *Asu*II-*Nla*II fragment or the 665 bp *Nla*III-*Bcl*1 of *bth2* by the homologous 526 bp *Asu*II-*Nla*III fragment or the 638 bp *Nla*III-*Bcl*1 fragment of *cryIA(b)* generated the genes encoding BtH7 and BtH8, respectively. In *bth9*, the 194 bp *Nla*III-*Asu*II fragment of *bth2* was exchanged for the homologous 203 bp *Nla*III-*Asu*II fragment of *cryIA(b)*. The reciprocal hybrid gene of *bth2*, *bth5*, was obtained by exchanging the 1236 bp *Eco*RV-*Bcl*I fragment of *cryIC*, as in p60.5G31, for the homologous 1209 bp *Eco*RV-*Bcl*I fragment of *cryIA(b)*. To construct *bth6*, the 1164 bp *Asu*II-*Bcl*I fragment of *bth5* was exchanged for the homologous 1190 bp *Asu*II-*Bcl*I fragment of *bth2*. To generate *bth10*, the 665 bp *Nla*III-*Bcl*1 fragment of *bth6* was replaced by the 638 bp *Nla*III-*Bcl*1 fragment of *cryIA(b)*. All hybrid crystal protein genes were expressed in *E.coli* XL-1Blue cells (Stratagene Inc.).

Purification of the crystal protein. The protoxin purification and toxin preparation of the CryIA(b) and CryIC toxins were described previously (Convents *et al.*, 1990, Convents *et al.*, 1991). Crystals isolated from the *E. coli* lysates were solubilized during incubation for 2 hr in 50 mM Na_2CO_3, 10 mM dithiothreitol, pH 10, at 37°C. The protoxin solution was dialysed against 20 mM Tris/HCl, pH 8.0 containing 0.2 M NaCl. Subsequently, the toxic fragment was obtained by trypsin digestion and further purified on a Mono Q 10/10 column connected to a FPLC system (Pharmacia LKB Biotechnologies Inc). The purification of the recombinant BtH2 and BtH5 protoxin and the toxin preparation were as described for CryIA(b) and CryIC except for a slight modification for the BtH5 toxin preparation, which was loaded onto a Mono Q column in 50 Mm Na_2CO_3, pH 10. Protein concentrations were determined spectrophotometrically at 280 nm. Extinction coefficients of $E^{1\%}$= 11.7 cm^{-1} for CryIA(b), $E^{1\%}$= 11.2 cm^{-1} for CryIC, $E^{1\%}$= 13.2 cm^{-1} for BtH2, and E1%= 10.6 cm^{-1} for BtH5 were used calculated from the known amino acid composition.

Toxicity assays. Toxicity assays were performed on first-instar larvae. Diluted samples of purified toxin were applied onto the surface of an artificial diet. *H. virescens* or *S. littoralis* larvae were placed on the diet and allowed to feed for five and six days, respectively. Subsequently, the weight of the larvae was scored and relative growth (EC_{50},

the concentration giving 50% growth reduction) was determined by calculating the ratio between the mean weight of the larvae grown on diet supplemented with toxin and the mean weight of control larvae grown on diet supplemented with an equivalent amount of bovine serum albumin.

Iodination of crystal proteins. Iodination of the toxic fragments of the crystal proteins was performed as described previously (Van Rie *et al.*, 1989, Van Rie *et al.*,1990). CryIA(b) and BtH2 toxins were iodinated by the chloramine-T method, whereas CryIC and BtH5 toxins were iodinated by the iodogen method (Pierce Chemical Company).

Determination of the stability of labelled toxin. Brush border membrane vesicles from fifth instar *H. virescens* and *S. littoralis* larvae were prepared by the differential magnesium precipitation method as described previously. (Wolfersberger *et al.*, 1987). Labelled toxin was incubated with *H. virescens* and *S. littoralis* vesicles under the conditions described in the next paragraph. Free toxin was separated from bound toxin by centrifugation. The pellet was suspended in binding assay buffer and again centrifuged. The final pellet, the first supernatant, and the toxin not incubated with vesicles were separated on a 10% SDS-polyacrylamide gel. The dried gel was exposed to Fuji RX-Safety film for 14 days.

Binding assays. Binding of the crystal proteins to the brush border membrane vesicles was examined as described before (Van Rie *et al.*, 1990). Duplicate samples of ^{125}I-labelled BtH2 toxin (specific activity $1.05*10^6$ Ci/mol as determined by the "sandwich" enzyme-linked immunosorbent assay; Voller *et el.*, 1976) were incubated in the presence of various amounts of unlabelled toxin for 1½ hr at 20°C with 150 μg/ml brush border membrane vesicles of *S. littoralis* in a total volume of 100 μl binding buffer (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.4 with 0.1% BSA). Ultrafiltration through Whatmann GF/F glass fiber filters was used to separate bound toxin from free toxin. The filters were rapidly washed with 5 ml binding buffer and radioactivity was measured with a gamma counter (1275 Minigamma; LKB Instruments, Inc.). Data were analyzed using the LIGAND computer program (Munson and Rodbard, 1980). To study the dissociation process, an excess of unlabelled ligand was added to an incubation mixture that had reached equilibrium. After addition, samples were taken at various time points and binding of labelled toxin was measured as described above.

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Chapter four

A translation fusion product of two different insecticidal crystal protein genes of *Bacillus thuringiensis* exhibits an enlarged insecticidal spectrum

Summary

Two truncated *Bacillus thuringiensis* crystal protein genes, belonging to the classes *cryIA(b)* and *cryIC* respectively, and both coding for insecticidal *N*-terminal fragments of the corresponding crystal proteins, were translationally fused. Expression of the gene fusion in *Escherichia coli* showed a biologically active protein with a toxicity spectrum that overlapped those of the two constituting crystal proteins.

Introduction

Bacillus thuringiensis is a soil bacterium which produces insecticidal crystalline inclusions during sporulation. Most strains are active against larvae of certain members of the order *Lepidoptera*, some show toxicity against larvae of certain dipteran or coleopteran species. The crystals present in *Lepidoptera* specific strains consist predominantly of one or more proteins with M_r 130-140 kDa. These proteins can be processed in the larval midgut into toxic peptides of ca. M_r 60 kDa (for a review, see Höfte and Whiteley, 1989). The toxic fragment is localized in the *N*-terminal half of the protoxin. Deletion studies of several crystal protein genes confirmed that the *C*-terminal half is not required for toxicity of the protein (Adang *et al.*, 1985; Höfte *et al.*, 1988; Sanchis *et al.*, 1988; Schnepf *et al.*, 1985). Analysis of the crystals from several *Lepidoptera* specific *B. thuringiensis* strains showed the occurrence of divergent crystal protein types exhibiting different insecticidal spectra with partial overlap (Höfte *et al.*, 1988; Visser, 1989). Recently it has been suggested that the interaction with high-affinity receptors on the insect midgut epithelium might, to a large extent, determine the host spectra of *B. thuringiensis* crystal proteins (Hoffmann *et al.*, 1988).

Crystal/spore preparations of *B. thuringiensis* have been used as commercial insecticides for many years. Insect-resistant transgenic plants (Barton *et al.*, 1987; Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987) and insecticidal recombinant *Pseudomonas* strains (Obukowitcz *et al.*, 1986) have been successfully engineered by introducing *B. thuringiensis* crystal protein genes. In contrast to the crystal/spore preparations, these transgenic organisms contain only a single crystal protein. This will limit the insecticidal spectrum and may lead to a rapid break-through of the newly acquired insect resistance. The latter possibility is suggested by the findings of McGaughey (1985), who selected a strain of the stored-grain pest *Plodia interpunctella* that was resistant against a specific set of *B. thuringiensis* crystal preparations, and Stone *et al.* (1989), who obtained a line of *Heliothis virescens* showing resistance against a transgenic strain of *Pseudomonas fluorescens*. The simultaneous production of two or more crystal proteins that act independently on the same insect, perhaps through the recognition of different receptors of larval midgut epithelial cells, might prevent or at least delay the appearance of insensitive insect populations. This hypothesis provides a motive for the transfer of more

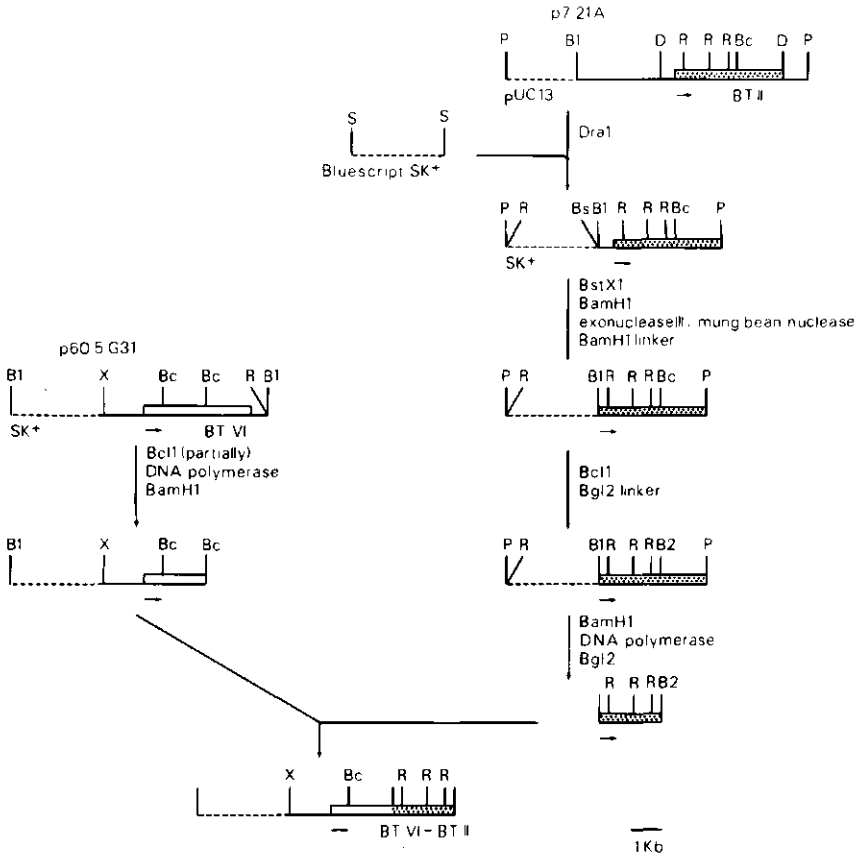

Fig. 1. Strategy for the construction of plasmid p60.5/7.21 containing the translation fusion between the 3'-truncated genes of BTVI and BTII. Restriction enzyme abbreviations: B1, *Bam*HI; B2, *Bgl*II; Bc, *Bcl*I; Bs, *Bst*XI; D, *Dra*I; P, *Pst*I; R, *Eco*RI; S, *Sma*I; X, *Xho*I.

than one *B. thuringiensis* crystal protein gene. Another motive may be the enlargement of the insecticidal spectrum.

However, most transformation vectors available accommodate the transfer of only one gene of interest. For this reason we constructed a tandem *B. thuringiensis* gene producing a fusion protein consisting of the *N*-terminal toxic parts of two different crystal proteins, one of which (gene product of BTII; the gene was classified *cryIA(b)*) was shown to be toxic against *Heliothis virescens* and *Pieris brassicae*, the other (gene product of BTVI; the gene was classified *cryIC*) against *Spodoptera* species and *Mamestra brassicae*.

Results and Discussion

The gene BtII was cloned from *B. thuringiensis* serotype *aizawai* 7.21 (obtained from H. de Barjac, Institute Pasteur, Paris, France) as a *Bam*HI-*Pst*I fragment in pUC13. Cloning of the gene BtVI from *B. thuringiensis* serotype *entomocidus* 60.5 in the Bluescript SK$^+$ vector (Stratagene Inc., La Jolla, Calif.), has been described previously (Visser, 1989). Sequence comparison between BtII and BtVI revealed high homology mainly downstream from the proteolytic cleavage site (Höfte and Whiteley, 1989; Honée *et al.*, 1989). Construction of the gene fusion between the truncated genes of BtII and BtVI, contained in plasmid p60.5/7.21, is outlined in Fig. 1. The 5'-flanking sequences of BtII, present in p7.21A, were deleted up to -1 with respect to the translation start site by using exonuclease III and mung bean nuclease, and a *Bam*HI site was simultaneously created by linker insertion. Translation of the 3' part of the BtII gene was prevented by insertion of a synthetic DNA sequence into the *Bcl*1 site 80 nucleotides downstream from the proteolytic cleavage site. The insertion contained a *Bgl*II site and produced stop codons in all three reading frames (Fischhoff *et al.*, 1987). Subsequently, the 3' part of the BtVI gene, as present in p60.5G31, downstream from the proteolytic cleavage site was replaced by the *Bam*HI-*Bgl*II restriction enzym fragment containing the truncated BtII gene sequence. A complete, continuing reading frame across the BtVI-BtII junction was obtained by filling in the protruding ends of the *Bcl*I and *Bam*HI sites (BtVI and BtII, respectively). The resulting nucleotide sequence of the junction was confirmed by sequence analysis. The nucleotide sequence of this fusion coded for a protein with a calculated molecular weight of 146 kDa.

Overnight cultures of *Escherichia coli* cells harboring recombinant plasmids containing the BtVI-BtII gene fusion, the intact BtII gene, or the intact BtVI gene were analysed by Western immunoblotting with a polyclonal antiserum directed against *B. thuringiensis* serotype *aizawai* 7.21 crystals containing both CryIA- and CryIC-type proteins (Visser, 1989). The *E. coli* lysate containing the plasmid encoding the BtVI-BtII fusion showed an antigenic polypeptide migrating at approximately M_r 130 kDa position, comparable to the migrating position and signal intensity of the lysates containing the BtII and BtVI gene products (Fig. 2). Simultaneously, these cultures were used to perform toxicity studies against larvae of *H. virescens* and *S. exigua* as described previously (Visser, 1989). Cells expressing the BtVI-BtII fusion showed toxicity against both *S. exigua* and *H. virescens*. Toxicity levels against *S. exigua* were comparable to those of cells expressing only the BtVI gene, whereas toxicity levels against *H. virescens* resembled those of cells expressing only the BtII gene (Table 1). From these results it could be concluded that the translation fusion between the two different truncated crystal protein genes coded for an intact and biologically active protein with a toxicity spectrum that overlapped those of the two contributing crystal proteins.

Since protoxins have to be processed by larval midgut proteases into toxic fragments, and *E. coli* cells containing the BtVI-BtII gene fusion produce an intact protein

Table 1. Toxicity of recombinant *E. coli* clones[a]

Clone containing:	Gene	50% Growth reduction dose (10^7 cells/ml[2])	
		S. exigua[b]	*H. virescens*[c]
p7.21A	BtII	68	<0.15
p60.5G31	BtVI	0.36	>150
p60.5/7.21	BtVI-BtII	0.29	0.17

[a] Cells were spotted in a 100 µl volume onto the surface of a solid nutrient medium in a well of 2 cm[2]. One L2 larva was placed in each well. 16-24 larvae were tested in each dilution step (six dilutions). Larval weight was scored after a six days incubation period at 28°C. [b] Mean values of three experiments. [c] Mean values of two experiments.

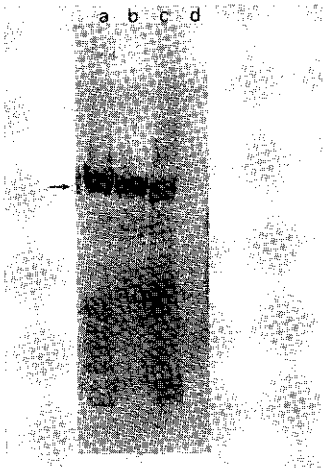

Fig. 2. Detection of crystal proteins in recombinant *Escherichia coli* cells by Western blotting using a 7.5% SDS-polyacrylamide gel and anti-7.21 crystal antiserum. Lane a, p7.21A (BTII); b, p60.5/7.21 (BTVI-BTII fusion); c, p60.5G31 (BTVI); d, Bluescript SK$^+$ (negative control). The arrow indicates the position of the crystal proteins.

and show toxicity against larvae of both insect species, it is most likely that the proteolytic cleavage sites of this fusion are recognised appropriately by the larval midgut proteases of *S. exigua* and *H. virescens*. Similarly, correct processing has been shown previously for translation fusions between a truncated *cryIA(b)* gene of *B. thuringiensis* serotype *berliner* 1715 and the neomycin phosphotransferase II gene (Höfte *et al.*, 1986).

Since the protein encoded by the fusion between the truncated BtVI and BtII genes displayed a broader insecticidal spectrum, these results may provide an attractive strategy for the introduction of insect resistance in plants and insecticidal properties in micro-organisms based on the use of *B. thuringiensis* crystal protein genes, regarding both effectiveness and potential break-through of insect resistance.

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Chapter five

Strategies to increase crystal protein gene expression levels in transgenic plants

Summary

The *Bacillus thuringiensis* genes *cryIA(b)* and *cryIC*, both encoding *Lepidoptera* specific crystal proteins, were introduced in tomato and tobacco plants by *Agrobacterium tumefaciens* mediated transformation. Expression of the crystal protein gene coding regions in the transgenic plants was transcriptionally controlled by either the 35S or the T_R2' promoter. Although expression of the crystal protein genes from the 35S promoter was optimized by the use of a promoter enhancer element and a viral untranslated leader sequence none of the transgenic plants produced crystal proteins at levels above the threshold of insect sensitivity. However, in transgenic plants containing a crystal protein gene with a modified coding region expression was dramatically increased resulting in high insect resistance.

Introduction

A major problem of crop production world-wide is the damage caused by insects. Consequently, great efforts are made in controlling insect pests, mainly depending on the development of chemical insecticides. However, major drawbacks are faced by their expense, their escalating rates of application because of decreasing effectiveness, and their persistence in and hazardous effects on the environment. Therefore, efforts have increased to investigate possibilities of biological insect control like the use of predators, parasites, insect viruses and feromones. In parallel, research of plant breeders and entomologists has focussed on the development of insect resistant crop plants. Success of this latter strategy depends on the identification of resistant genotypes and the ability to breed the involved resistance factors into related cultivars. Nowadays, genetic engineering can be used to introduce insect resistance in plants by transferring genes contributing to insect resistance from non-plant species or other plant species.

A well-characterised source of genes encoding insecticidal proteins is the microbial insect pathogen *Bacillus thuringiensis*. During sporulation the bacterium produces crystals consisting of crystal proteins, the major toxic determinants. Most *B. thuringiensis* strains are active against larvae of lepidopteran species, whereas other strains are toxic against dipteran or coleopteran larvae. A single crystal may be composed of several crystal proteins differing in insecticidal spectra (reviewed by Höfte and Whiteley, 1989). The primary target of the crystal proteins is the larval midgut (Chapter 1).

Sprays of sporulated *B. thuringiensis* bacteria have been safely used as bio-insecticides for many years. However, a practical limitation is the low stability of the crystals in the field and the poor accessibility of some pest insects. In an alternative approach, we and others (Adang *et al.*, 1987, Barton *et al.*, 1987, Fischhoff *et al.*, 1987, Vaeck *et al.* 1987, Perlak *et al.*, 1990; McCown *et al.*, 1991; Perlak *et al.*, 1991) have explored the feasibility of generating insect resistant transgenic plants by transferring

crystal protein genes.

In this report the development of insect resistant transgenic tomato and tobacco plants is described. Two crystal protein genes were used of which one, the *cryIA(b)* classified BTII gene, encodes a crystal protein with a molecular weight of 130.000 Da, and the other, the *cryIC* classified BTVI gene, encodes a crystal protein with a molecular weight of 134.000 Da. Whereas both crystal proteins are toxic against *Manduca sexta* larvae (Van Rie *et al.*, 1990) the insecticidal spectra of both proteins also differ. The CryIA(b) protein is highly toxic against *Heliothis virescens* larvae, whereas the CryIC protein is highly toxic against *Spodoptera* species (Chapter 2, Chapter 3).

We have made several constructs containing genes encoding the toxic fragments of either one or both of the crystal proteins CryIA(b) and CryIC. In some constructs, the *aph*(3')II coding sequence (encoding aminoglycoside phosphotransferase II, NPTII) has been translationally fused to crystal protein gene coding sequences, enabling direct selection for high expression of the gene fusion. All constructs formed part of a binary vector system for *Agrobacterium* mediated transformation of tomato and tobacco plants. In order to obtain high crystal protein gene expression in transgenic plants different transcriptional and translational regulation signals were used. In addition, a construct was developed that contained a modified coding region of *cryIA(b)*. In the modified *cryIA(b)* coding region, the presence of predicted putative transcription termination sequences (Dean *et al.*, 1986) and ATTTA repeats, which have been shown to destabilize transcripts in other systems (Shaw and Kamen, 1986) was reduced. The transgenic plants were tested for gene expression and insect resistance. The results of these experiments are discussed with respect to their implications for the use of *B. thuringiensis* crystal protein genes, and heterologous genes in general, in molecular plant breeding.

Results

Plant transformation constructs. Two different plant transformation vectors, pGH2 and pCPO1, both components of a binary vector system, were used to produce nine different constructs for the transfer of genes encoding the crystal protein CryIA(b) or CryIC in the plants (Fig. 1). In transgenic plants obtained by transformation with pGH2 derivatives the coding region of the gene of interest is transcriptionally controlled by the 35S promoter. In vectors based on pCPO1 the gene of interest can be placed under the transcriptional control of either the 35S, the T_R1' or the T_R2' promoter.

In pTox1, the full-length *cryIA(b)* coding region was placed under the transcriptional control of the 35S promoter of pGH2. Since deletion studies had shown that the *C*-terminal parts of CryI crystal proteins are not essential for toxicity (Höfte *et al.*, 1986, Sanchis *et al.* 1989) and the expression of 3'-end truncated crystal protein genes in transgenic plants is higher than the expression of full length crystal protein genes (Vaeck *et al.*, 1987), the 3'-end truncated *cryIA(b)* coding region was cloned in pGH2 behind the

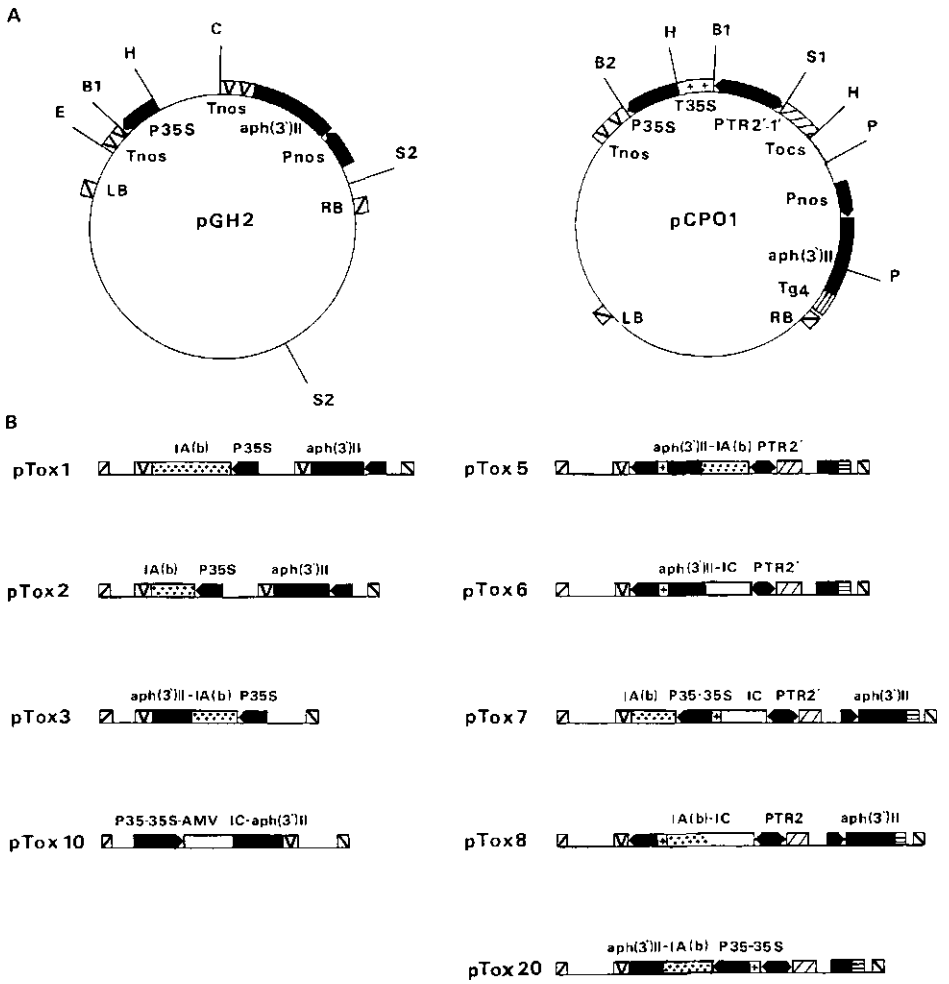

Fig. 1. Physical maps of the transformation vectors pGH2 and pCPO1 (A) and of the T-DNA fragments of their derivatives (B). The constructs pTox1, -2, -3, and -10 were derived from pGH2, pTox5, -6, -7, -8, and -20 were derived from pCPO1. Restriction site abbreviations are as follows: B1, *Bam*HI; B2, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hind*III; P, *Pvu*II; S1, *Sal*I; S2, *Sac*II. Other abbreviations are: LB, left border; RB, right border; aph(3')II, aminoglycoside phosphotransferase II gene encoding NPTII; IA(b), *cryIA(b)*; IC, *cryIC*; P35S, CaMV 35S promoter; P35-35S, CaMV 35S promoter with doubled enhancer region; AMV, Alfalfa Mosaic Virus RNA 4 leader sequence; Pnos, nopaline synthase promoter; PTR2'-1', T_R2' and T_R1' promoters; Tnos, nopaline synthase terminator; Tocs, octopine synthase terminator; T35S, CaMV 35S terminator; Tg4, T-DNA gene 4 terminator.
Open bars represent *cryIC* sequences, dotted bars *cryIA(b)* sequences, filled bars *aph*(3')II sequences.

35S promoter resulting in pTox2. It has been shown that fusion proteins of the *N*-terminal toxic part of CryIA(b) and NPTII exhibit toxicity and NPTII activity comparable to that

of the individual proteins (Höfte *et al.*, 1988). Moreover, a correlation was reported between toxicity and kanamycin resistance of transgenic plants producing these NPTII fusion proteins (Vaeck *et al.*, 1987). Therefore, a gene fusion was constructed encoding an NH_2-terminal 70.000 Da CryIA(b) fragment fused to NPTII. The *cryIA(b)-aph*(3')II fusion was cloned behind the 35S promoter of a pGH2 derivative lacking the *aph*(3')II expression cassette, resulting in pTox3.

Since crystal protein gene expression in transgenic plants obtained with either pTox1, pTox2 or pTox3 was rather low (discussed below) a second set of constructs was developed in order to increase crystal protein gene expression in plants. These constructs were devised using the crystal protein genes *cryIA(b)* and *cryIC*. In contrast to the *cryIA(b)* gene, the *cryIC* gene was adapted by site-directed mutagenesis to include the consensus sequence for translation start sites of eukaryotic genes to provide optimal translation initiation (Joshi, 1987; Kozak, 1987). In pTox7 the 3'-end truncated coding regions of *cryIC* and *cryIA(b)* were cloned in a pCPO1 derivative under the transcriptional control of the T_R2' promoter and 35S promoter containing a doubled 35S enhancer region (Kay *et al.*, 1987, Ow *et al.*, 1987), respectively. In pTox5, the *cryIA(b)-aph*(3')II fusion was cloned behind the T_R2' promoter of a pCPO1 derivative deficient in *aph*(3')II gene expression. A similar *cryIC-aph*(3')II fusion was cloned behind the T_R2' promoter in a pCPO1 derived vector deficient in *aph*(3')II gene expression to produce pTox6, and in a pGH2 derived vector under the transcriptional control of the 35S promoter with a doubled enhancer region (pTox10). Moreover, the coding region in pTox10 was also preceded by a 5' untranslated mRNA leader sequence of Alfalfa Mosaic Virus RNA 4, reported to increase expression levels both *in vivo* and *in vitro* (Jobling and Gehrke, 1987). Finally, a translation fusion between the 3'-end truncated genes of *cryIA(b)* and *cryIC* was constructed (Chapter 4) and placed under the transcriptional control of the T_R2' promoter of pCPO1 (pTox8). Neither of the transgenic plants obtained with the constructs pTox5, pTox6, pTox7, pTox8, and pTox 10 produced high levels of crystal protein (discussed below).

A third type of construct was developed containing a modified *cryIA(b)* coding region. The coding regions of the prokaryotic crystal protein genes contain sequences negatively influencing the expression of these genes in plants (Perlak *et al.*, 1990; Perlak *et al.*, 1991). Therefore, the coding region of *cryIA(b)* was modified including the removal by site-directed mutagenesis of several putative polyadenylation sequences (Dean *et al.*, 1986) around positions 260, 960, 1120, and 1345, and a transcript destabilizing ATTTA repeats (Shaw and Kamen, 1986) around position 500, leaving the encoded amino acid sequence unchanged. Subsequently, the modified 3'-end truncated *cryIA(b)* gene was translationally fused with the *aph*(3')II gene and placed under the transcriptional control of the 35S promoter with a doubled enhancer region in a pCPO1 derived vector resulting in pTox20.

With the exception of the modified *cryIA(b)-aph*(3')II coding region of pTox20, the adapted coding regions of the crystal protein genes were cloned in the expression vector

pINIII (Masui *et al.*, 1984) and analyzed for expression in *E. coli*. Western analysis of the recombinant *E. coli* lysates showed proteins in the expected size ranges (data not shown). Furthermore, recombinant *E. coli* cells producing either the CryIA(b)-NPTII fusion or the CryIC-NPTII fusion were kanamycin resistant and showed toxicity against *H. virescens* and *S. exigua* larvae, respectively, at levels comparable with those of recombinant *E. coli* cells producing the wild type CryIA(b) or CryIC proteins (data not shown). Expression of the *cryIC-cryIA(b)* translation fusion in *E. coli* gave rise to a fusion protein with an insecticidal spectrum overlapping those of both contributing crystal proteins (Chapter 4).

Transgenic plants. Using *Agrobacterium* mediated leaf disk transformation of MsK93 tomato plants 22 transgenic plants were generated with pTox1, 12 plants with pTox2, and 10 plants with pTox3. Although Southern analysis showed the presence of 1-5 copies of T-DNA (results not shown), none of these transgenic plants showed significant toxicity against larvae of the insect species *M. sexta*, *H. virescens*, or *S. exigua*. To study expression levels of the translational *cryIA(b)-aph*(3')II fusion in plants containing pTox3 T-DNA, calli were induced on leaf explants, at different kanamycin levels. Whereas leaves from all plants developed callus at 300 mg/l kanamycin, leaves from only one plant developed callus at 600 mg/l kanamycin. No *cryIA(b)-aph*(3')II fusion transcripts could be detected in Northern blot analysis. These observations were all indicative for rather low expression levels. Therefore, transgenic plants were generated using the constructs pTox5, pTox6, pTox7, pTox8, pTox10, and pTox20 which were developed to increase crystal protein gene expression levels. As depicted in Fig. 1, all contained either different promoter sequences or the alternative *cryIC* gene. The construct pTox20 contained a modified coding region of the 3'-end truncated *cryIA(b)* gene.

To select for high gene expression in an early stage the constructs pTox3, pTox5, and pTox6, containing a translational fusion between a crystal protein gene and the *aph*(3')II gene, were used to transform regenerating protoplasts of *Nicotiana tabacum* cv. Petit Havana SR1 at increasing kanamycin concentrations. The number of calli obtained at various kanamycin concentrations four weeks after transformation was inversely correlated with the kanamycin concentration (Table 1). As expected, such a correlation was not observed for the numbers of calli obtained from a parallel transformation with pTox8, a construct encoding an independently regulated native NPTII. The number of transgenic plants obtained at different kanamycin concentrations is presented in Table 2. With pTox5 or pTox6 constructs, transformation resulted in lower kanamycin resistance levels than with pTox3, suggesting that crystal protein expression levels had not improved.

Leaf explants of eleven transgenic tobacco plants obtained from transformation with pTox3 and selected at kanamycin 500 mg/l were used for callus induction in the presence of kanamycin at a concentration ranging from 200 mg/l to 1000 mg/l. On leaf disks of ten

Table 1. Number of microcalli obtained four weeks after transformation of regenerative protoplasts of *N. tabacum* Petit Havana cv. SR1 at various kanamycin concentrations.

construct	promoter/gene	kanamycin concentration mg/l			
		100	200	500	1000
pTox3	35S/*cryIA(b)-aph*(3')II	>>	>>	5	-
pTox5	T_R2'/*cryIA(b)-aph*(3')II	72	28	-	-
pTox6	T_R2'/*cryIC-aph*(3')II	90	10	-	-
pTox8	T_R2'/*cryIC-cryIA(b)*	27	19	15	-

>>: more than hundred microcalli; -: no microcalli obtained.

plants callus developed at a kanamycin concentration of 500 mg/l whereas on leaf disks of four plants callus growth was observed at a kanamycin concentration of 750 mg/l. A parallel experiment with leaf disks of the four transgenic plants obtained at a selection pressure of 1000 mg/l (Table 2) showed callus growth at maximally 750 mg/l kanamycin. These experiments showed that indeed high kanamycin levels could be used to select for plants showing relatively high kanamycin resistance. Finally, the constructs pTox5, pTox6, pTox7, pTox10 and pTox20 were used for standard leaf disk transformation of *N. tabacum* Petit Havana cv. SR1, and transformed plant cells were regenerated at kanamycin concentrations of 200 mg/l and 500 mg/l, which resulted in 50 independent transgenic plants for most constructs (Table 2).

Insect resistance. Tobacco hornworm larvae (*Manduca sexta*) are susceptible to both CryIC and CryIA(b) toxins although sensitivity to CryIC is less than to CryIA(b) (Van Rie *et al.*, 1990). Therefore, larvae of this species were used to test transgenic tobacco plants for insect resistance. None of the transgenic tobacco plants obtained by transformation with the pTox constructs containing wild-type crystal protein gene coding regions showed either full or partial resistance in three independent bio-assay experiments. No resistance could be monitored against larvae of the species *Heliothis virescens*, sensitive to CryIA(b), and *Spodoptera exigua*, sensitive to CryIC, either. Although the T_R2' promoter has been suggested to be highly active in callus caused by physiological stress (Harpster *et al.*, 1988; Saito *et al.*, 1991), no toxic effects could be

Table 2. Number of transgenic tobacco plants obtained for each construct

construct	promoter/gene	kanamycin concentration mg/l[a]			total
		200	500	1000	
pTox3	35S/*cryIA(b)-aph*(3')II	16\-	35\-	4\NT	55
pTox5	T_R2'/*cryIA(b)-aph*(3')II	29\21	-\1	-\NT	51
pTox6	T_R2'/*cryIC-aph*(3')II	6\11	-\1	-\NT	18
pTox7	3535S/*cryIA(b)* T_R2'/*cryIC*	NT\42	NT\8	NT\NT	50
pTox8	T_R2'/*cryIC-cryIA(b)*	18\-	26\-	-\NT	44
pTox10	3535S-AMVI/*cryIC-aph*(3')II	NT\39	NT\8	NT\NT	47
pTox20	3535S/*cryIA(b)-aph*(3')II[b]	NT\10	NT\NT	NT\NT	10

[a] : number of plants obtained with protoplast transformation\number of plants obtained with leaf disk transformation; -: no transgenic plants obtained; NT: not tested. [b] : translation fusion contains a modified sequence of the *cryIA(b)* coding region, see text.

observed in *M. sexta* larvae that had been fed with callus tissue induced on leaves of tobacco plants transformed with pTox5, pTox6, or pTox7. These results confirmed that expression levels in tobacco plants or plant derived calli obtained with any of these constructs were too low to cause resistance. On the other hand, 7 out of 10 transgenic tobacco plants obtained by transformation with the pTox20 construct containing a modified *cryIA(b)* sequence showed high resistance against *M. sexta* larvae giving 100% mortality after maximally 4 days (Table 3 and Fig. 2). In other words, these results suggest that in the constructs described above, a simple exchange of promoter sequences was insufficient to obtain insect resistance, and that the nucleotide sequence of the *cryIA(b)* coding region is more crucial for the expression levels that can be obtained than the regulation signals controlling its expression.

Discussion

To obtain insect resistant transgenic plants chimeric genes based on the crystal protein genes *cryIA(b)* and *cryIC* were constructed and subsequently transferred by agrobacteria into tomato and tobacco plants. Despite the use of different expression cassettes chimeric genes containing wild type crystal protein sequences were insufficiently expressed, the

Table 3. Insect resistance of tobacco plants transformed with the pTox20 construct.

plant nr.	mortality of *M. sexta* larvae[a]
2001	100%
2005	100%
2010	78%
2012	100%
2013	78%
2016	100%
2017	100%
2018	83%
2014	100%
2015	100%

[a]: mean percentage of mortality of three experiments, scored after 4 days.

Fig. 2. Protection from insect feeding damage in transgenic plants expressing the modified *cryIA(b)* gene. Ten *M. sexta* larvae were applied on leaves of greenhouse plants and allowed to feed for five days. Left, pTox20 transformed tobacco plant 2005. Right, a nontransformed tobacco control plant.

crystal protein level remaining below the threshold of insect resistance. However, expression of a chimeric gene containing a modified coding region of *cryIA(b)* resulted in crystal protein production adequate for protection of transgenic plants against insect attack.

Except for pTox1, our constructs used for transformation contained truncated coding regions of *cryIA(b)* or *cryIC* since it had been shown that expression of the full length coding region in plants was much lower than the expression of a truncated coding region (Vaeck *et al.*, 1979). Furthermore, several of our constructs encoded translation fusion products between the toxic fragment of a crystal protein and the NPTII enzyme. Recombinant *E. coli* cells expressing these translation fusions showed both insecticidal activity and neomycin phosphotransferase activity, in agreement with results obtained by Höfte *et al.* (1988). For this reason it was hoped that regeneration of transformed plant cells at high kanamycin concentrations might select for tobacco transformants with high crystal protein levels. However, calli regenerated at high kanamycin concentrations up to

1000 mg/l resulted in transgenic plants which showed no resistance against *M. sexta* larvae. Furthermore, leaves of several non-resistant plants induced callus growth in the presence of high kanamycin concentrations of 750 mg/l. These results do not agree with those reported by Vaeck *et al.* (1987) who found that for tobacco plants producing translation fusions between a crystal protein gene and *aph*(3')II gene 75% of the plants resistant to kanamycin levels of 1000 mg/l showed insect resistance as well. In conclusion, in our hands fusion proteins containing wild-type crystal protein and NPTII sequences could not be used for the selection of transgenic plants that express levels of the crystal protein sufficiently high to become insecticidal.

The expression cassettes present in our constructs contained either a CaMV 35S promoter or the T_R2' mannopine synthase promoter. Although the T_R2' promoter is root specific (Teeri *et al.*, 1989; Saito *et al.*, 1991), it directs expression in callus as well as in leaf tissue after wounding, which is the effect in bio-assays, at levels similar to or even higher than that of the 35S promoter (Harpster *et al.*, 1988; Teeri *et al.*, 1989). Recently, it was shown that transgenic chrysanthemum callus tissue containing the 3'-truncated coding region of *cryIA(b)* under the transcriptional control of the T_R2' promoter of a pCPO1 derived vector showed high insecticidal activity (Van Wordragen *et al.*, 1991). However, the transgenic callus tissue was obtained using wild-type agrobacteria causing auxin production in the transformed plant cells. High auxin concentrations may be responsible for further elevated expression levels of the crystal protein gene under control of the T_R2' promoter. Such high auxin concentrations are not encountered in calli obtained with disarmed agrobacteria used to transfer the pTox-series (Langridge *et al.*, 1989; Saito, 1991).

Several derivatives of the 35S promoter were used, containing a doubled enhancer region to enhance transcription rates (Kay *et al.*, 1987, Ow *et al.*, 1987), with or without the AMV RNA 4 leader sequence upstream of the coding region of the crystal protein gene to increase the translatability of the transcripts (Jobling and Gehrke, 1987; Gallie *et al.*, 1987[a]; Gallie *et al.*, 1987[b]). In no case did the use of different crystal protein genes, promoters, gene fusions, and/or leader sequences sufficiently increase crystal protein gene expression to cause insect resistance. The variables described above, concerning the use of different crystal protein genes and their derivatives, alone or in combination, and of different promoters, were introduced to allow us the evaluation of the importance of these variables. However, since no insect resistance was measured, such evaluation is not possible.

The development of insect resistant tomato and tobacco plants by transformation with a *cryIA(b)* gene has been reported previously (Barton *et al.*, (1987); Fischhoff, *et al.* (1987); Vaeck, *et al.* (1987). In those cases, 80% to 100% insect mortality was found for 25 to 30% of the obtained transgenic plants challenged with *M.sexta* larvae, whether the gene was placed under the transcriptional control of a 35S promoter or T_R2' promoter. These results are in conflict with those described here, since none of our transgenic plants containing the wild type *cryIA(b)* coding region showed insecticidal activity. The authors

cited above used constructs containing a *cryIA(b)* coding sequence provided with an optimized translation initiation site according to Kozak (1987) and Joshi (1987) whereas the translation start sites of *cryIA(b)* in our constructs were not adapted. Similarly, transformation of tomato plants with a construct identical with pTox20 except for the presence of a wild-type *cryIA(b)* coding region, but containing an optimized translation start site also resulted in several plants exhibiting insecticidal activity (Van der Salm, pers. comm.). Obviously, improvement of the translation initiation site of the *cryIA(b)* gene increases the expression sufficiently to protect transgenic plants against *H.virescens* or *M. sexta* larvae, both insect species that are highly sensitive to CryIA(b).

On the other hand, transgenic plants expressing the *cryIC* gene were not protected from insect attack, although the translation initiation site of the *cryIC* gene in our constructs was adapted to meet the Kozak consensus. This may be explained by the 14-fold lower sensitivity of the test insect *M. sexta* for CryIC as compared to CryIA(b). Alternatively, sequence differences between *cryIA(b)* and *cryIC* might cause even lower expression levels of the *cryIC* gene in plants than those encountered for *cryIA(b)*.

The coding sequences of the *cry* genes are highly A-T rich (63% for *cryIA(b)*) and thereby contain sequences resembling instability elements like the previously mentioned ATTTA motif which as repeats have been shown to destabilize mRNA in other eukaryotic systems (Shaw and Kamen, 1986), and putative polyadenylation signals (Dean *et al.*, 1986). Furthermore, plant intron recognition sequences (Goodall and Filipwicz, 1989) regularly occur in the coding sequence of the *cry* genes. In addition, rare codon usage, a consequence of the high AT%, which may cause ribosomal pausing and impaired translation elongation might also affect mRNA stability (Hoekema *et al.*, 1987). Indeed, transient expression studies of crystal protein genes revealed high instability of the transcripts in which sequences from all parts of the crystal protein gene were involved (Murray *et al.*, 1991). The modifications of the coding region of the 3'-end truncated *cryIA(b)* gene described in this chapter resulted in the removal of several potential plant polyadenylation signals and ATTTA repeats. These modifications dramatically influenced gene expression, as judged from the bio-assay experiments revealing insect resistance in transgenic tobacco plants containing the modified *cryIA(b)* coding region, but not in those expressing the wild-type gene.

Recently, transgenic plants were described containing fully resynthesized 3'-end truncated coding regions of *cryIA(b)* and *cryIA(c)* free of the above mentioned destabilizing elements and rare plant codons (Perlak *et al.*, 1991). Such plants showed up to 100-fold higher levels of crystal protein than plants containing the wild type gene. Expression of the modified genes revealed higher amounts of transcripts than observed for the wild type gene although the increase was not proportional to the increase in protein levels. Some plants expressing a fully resynthesized crystal protein gene contained amounts of transcript comparable to those of plants expressing a partially modified crystal protein gene as described above. However, the plants containing transcripts from a fully resynthesized gene exhibited higher protein amounts if compared to plants containing

partially modified genes. This suggests that some modifications of the *cryIA(b)* gene resulted in an increased mRNA stability whereas others improved the translational efficiency. The region around nucleotide position 260 of *cryIA(b)* was critical to increased gene expression probably because it encompasses three closely spaced putative polyadenylation signals (two AACCAA and one AATTAA sequences).

These findings have important implications for the production of insect resistant plants using crystal protein genes. Moreover, our results improve our understanding of (heterologous) gene expression in general. Additional efforts to improve crystal protein gene expression levels based on these findings will expand our options to protect crop plants from insect destruction.

Experimental procedures

Bacterial strains and plant cultivars. The *Escherichia coli* strains XL1-Blue (Stratagene), JM101 and HB101 were used for transformation and plasmid growth. The *E. coli* strain S17.1 (Simon *et al.*, 1983) was used for biparental plasmid transfer. The non-oncogenic *Agrobacterium tumefaciens* strains LBA4404 (pRAL4404), derived from Ach5 (Hoekema *et al.*, 1983), and GV3101 (pMP90RK), derived from C58 (Koncz and Schell, 1986), were used for transformation of the tomato genotype Msk93 (Koornneef *et al.*, 1986) and the tobacco *Nicotiana tabacum* Petit Havana cv.SR1.

Construction pTox constructs. Two different transformation vectors were used for the transfer of the crystal protein genes, pGH2 and pCPO1. The vector pGH2 is a derivative of pBI121 (Jefferson, 1987) obtained by deletion of the coding region of ß-glucuronidase as a *Sma*1-*Sac*1 fragment from between the 35S promoter and *nos* terminator sequences, leaving unique *Bam*H1 or *Xba*1 cloning sites. The vector pCPO1 is a derivative of pPCV708 (Koncz *et al.*, 1990, Visser *et al.*, manuscript in preparation) in which the CaMV 35S promoter and the T-DNA gene 7 terminator were exchanged for the CaMV 35S promoter and terminator of pRT103 (Töpfer *et al.*, 1987; kindly provided by Töpfer, Cologne). Furthermore, pCPO1 exhibits the dual promoter T_R1'-2', derived from the tumor inducing *Agrobacterium* plasmid pTiAch5 (Velten *et al.*, 1984).

The cloning of the *cryIA(b)* gene BtII from *B. thuringiensis* serotype *aizawai* 7.21 (obtained from H. de Barjac, Institute Pasteur, Paris), the deletion and adaptation of the 5'-flanking sequences and the construction of a truncated *cryIA(b)* gene encompassing the coding region of the toxic fragment have been described previously (Honée *et al.*, 1990).

The translation fusion *cryIA(b)-aph*(3')II encoding the toxic fragment of CryIA(b) and neomycin phosphotransferase II was created by ligating a 2 kb *Bam*H1-*Bcl*1 fragment located at the 5'-end of *cryIA(b)* in front of the *aph*(3')II gene present in pKM109.2 (Reiss *et al.*, 1984). Subsequently, a *Bgl*II site was created by linker insertion at the *Sma*1

site 172 nucleotides downstream of the translation stop codon of the *cryIA(b)-aph*(3')II fusion. As a result, the coding regions of the full length, 3'-end truncated *cryIA(b)* gene and the *cryIA(b)-aph*(3')II fusion were both contained in *Bam*HI-*Bgl*II fragments.

Modification of the nucleotide sequence of *cryIA(b)* was performed as described (van der Salm, in preparation) by site-directed mutagenesis (MutaGene ™phagemid *in vitro* mutagenesis kit, BioRad) based on the method of Kunkel (1985). In total, the nucleotide sequences of 6 regions were changed, underlined symbols indicating actual substitutions. Changes are located around position 1 (tcaccATGG), around position 260 (GTTGATCAACCAGAGGATCGAAGAGTTC), around position 500 (AGCCGCAAACTTGCACTTA), around position 960 (GCACCAGATCATG), around position 1120 (TAACATCGGGATCAACAACCAA), and around position 1345 (TAAGCATCATCAAGA). None of the mutations in the coding sequence resulted in amino acid substitutions. The mutated *cryIA(b)* fragment encompassing the coding sequence of the toxic fragment was translationally fused to the coding region of *aph*(3')II similar to the construction of the wild type *cryIA(b)-aph*(3')II fusion described above.

The cloning of the *cryIC* gene BtVI from *B. thuringiensis* serotype *entomocidus* 60.5 has been described previously (Visser *et al.*, 1988). By site-directed mutagenesis (Site-Directed Mutagenesis System, Amersham), based on the method described by Taylor *et al.* (1985) nucleotides were changed resulting in the underlined sequence 5'-ggatccaaaccATGG-3' upstream of the translation initiation site, containing a *Bam*H1 site and a *Nco*1 site, respectively, and the consensus sequence postulated for plant genes (Joshi, 1987). Furthermore, a *Bgl*II site in the coding region was deleted by changing the thymidine residue at position 1311 into an adenine. Also, nucleotides downstream the translation stop codon were substituted which resulted in the underlined sequence 5'-GAAtaagatct-3', showing a *Bgl*II site. Subsequently, *Bam*HI-*Bgl*II fragments encompassing the 3'-end truncated *cryIC* gene and a *cryIC-aph*(3')II fusion were constructed, as for *cryIA(b)*.

The *Bam*HI-*Bgl*II fragments containing the entire, adapted *cryIA(b)* and *cryIC* genes were cloned in both transformation vectors pGH2 and pCPO1. The constructs pTox1 and pTox2 were derived from pGH2 in which the entire coding region of *cryIA(b)* and the 5'-part of *cryIA(b)*, respectively, were cloned in the unique *Bam*HI site. The *Bam*HI-*Bgl*II fragment containing the *cryIA(b)-aph*(3')II fusion was also cloned in this *Bam*H1 site, in a pGH2 derived vector in which the entire *aph*(3')II cassette was deleted by a *Cla*1-*Sac*II digestion, resulting in pTox3. The pGH2 derived vector missing the *aph*(3')II cassette was also used for the construction of pTox10 by replacing the *Eco*RI-*Hind*III fragment, containing the 35S promoter and *nos* terminator sequences by an *Eco*RI-*Hind*III cassette containing the 35S promoter with doubled enhancer followed by 80 nucleotides representing the Alfalfa Mosaic Virus RNA4 leader sequence, the *cryIC-aph*(3')II fusion and *nos* terminator sequence. The regulation sequences of this cassette were derived from pMOG18 (Sijmons *et al.*, 1990, kindly provided by B. Dekkers, Leiden) in which the *cryIC-aph*(3')II fusion was cloned into the unique *Bam*HI site between the promoter and

terminator sequences. All the other constructs were based on pCPO1.

Construct pTox5 was obtained by cloning the *cryIA(b)-aph*(3')II fusion in the unique *Bam*HI site of a pCPO1 derived vector from which a 550 bp *Pvu*II fragment containing the *nos* promoter and most of the *aph*(3')II coding region had been deleted. This same pCPO1 derivative, no longer allowing *aph*(3')II expression, was also used for the construction of pTox6, obtained by cloning the *cryIC-aph*(3')II fusion in the unique *Bam*HI site between the T_R2' promoter and 35S terminator sequences. In construct pTox7 the 3'-end truncated *cryIA(b)* gene was placed under the transcriptional control of the 35S promoter in a pCPO1 derivative containing a 35S promoter with doubled enhancer region (from position -343 to -90 with respect to the translation start site). In addition, construct pTox7 contained a 3'-end truncated *cryIC* gene which was cloned in the *Bam*HI site between the 2' Tr promoter and 35S terminator sequences. Construct pTox8 contained the *cryIC-cryIA(b)* fusion encoding a translation fusion (Honée *et al.*, 1990), in which the translation start site was changed to include a *Bam*HI site similar to that of the adapted *cryIC* gene and in which at the 3'-end of the fusion a second *Bam*H1 site was created by linker insertion. The resulting 4 kb *Bam*H1 fragment was cloned in the unique *Bam*H1 site of pCPO1 generating pTox8. Construct pTox20 was produced by cloning the translation fusion of the modified *cryIA(b)* gene and *aph*(3')II gene as a *Bam*HI-*Bgl*II fragment in the *Bgl*II site between the 35S promoter containing a doubled 35S enhancer sequence, and the *nos* terminator in a pCPO1 derived vector deficient in the expression of the *aph*(3')II gene.

Plant transformation. The pGH2 based constructs pTox1, pTox2, pTox3, and pTox10 were transferred into *A. tumefaciens* strain LBA4404 (pRAL4404) by a triparental mating using helper plasmid pRK2013 (Ditta *et al.*, 1980). Transconjugants were selected on kanamycin (50 mg/l). The pCPO1 based constructs pTox5, pTox6, pTox7, pTox8, and pTox20 were introduced by biparental mating using *E. coli* strain S17.1 into *A. tumefaciens* strain GV3101, with carbenicillin (100 mg/l) as a selective antibiotic.

Recombinant *A. tumefaciens* bacteria containing either pTox1, pTox2, and pTox3 were used to inoculate leaf disks of tomato plants according to the protocol described by Koornneef *et al.* (1986). Recombinant *A. tumefaciens* containing either pTox5, pTox6, pTox7, pTox10, or pTox20 were used to inoculate leaves of tobacco plants according to the protocol described by Horsch *et al.* (1985). During callus and shoot formation transformants were selected at 200 mg/l and 500 mg/l kanamycin, whereas root formation occurred at kanamycin concentrations of 200 mg/l or 50 mg/l. During regeneration bacterial growth was suppressed with 200 mg/l cefotaxim (Duchefa). Transgenic plantlets free of bacteria were transferred to soil and grown in the greenhouse.

As an alternative to leaf disk transformation, *A. tumefaciens* containing either pTox3, pTox5, pTox6, or pTox8 were used for transformation of regenerating tobacco protoplasts according to the co-cultivation method as described by Márton *et al.* (1979).

Protoplasts (about 10^5/ml) were cultured in 2.5 ml K3 media (0.4 M sucrose as osmoticum, supplemented with 0,1 mg/ml naphtylacetic acid (NAA) and 0.2 mg/l kinetin) for two days in the dark and 1 day at low light (about 500 lux). Regenerating protoplasts were then inoculated with 100-200 bacteria per protoplast and cocultured for 2 or 3 days. Subsequently, 0.5 ml K3 medium containing 0.4 M sucrose with hormones NAA (0.1 mg/l) and kinetin (0.2 mg/l), supplemented with 500 mg/l cefotaxim as antibioticum was added to the plant cell suspension. After one week the regenerating protoplasts were imbedded in 0.6% agarose (Seaplaque), under twofold reduction of the protoplast concentration. The medium containing the embedded protoplasts was refreshed weekly, reducing osmotic pressure (0.1 M sucrose per week). Different kanamycin concentrations ranging from 100, 200, 500, and 1000 mg/l were used for selection immediately after imbedding. Four weeks after transformation the number of microcalli obtained with different constructs at various kanamycin concentrations were counted (Table 1). Resistant calli, first observed after 2-3 weeks, were transferred to MS-medium supplemented with 3% sucrose, kanamycin and 0.1 mg/l naphtaleneacetic (NAA) and 0.5 mg/l benzylaminopurine (BAP). Transgenic shoots were transferred to MS medium containing 3% w/v sucrose and 200 mg/l kanamycin. Transgenic plantlets free of bacteria were transferred to soil and grown in the greenhouse.

Callus induction. Leaves from greenhouse grown transgenic plants were sterilised and sterile leaf disks were cut and transferred to MS medium supplemented with 3% w/v sucrose, 1 mg/l NAA and 0.2 mg/l BAP, the antibiotic cefotaxim (200 mg/l), the fungicide nystatin (20 mg/l), and kanamycin ranging from 100 to 1000 mg/l. After three to four weeks callus growth was scored. Alternatively, calli were used for bio-assays.

Bioassay. Both transgenic plants and calli were tested for insecticidal activity as described by Van Wordragen *et al.* (1991). Six leaf disks from each plant were placed on 1.5% agar supplemented with 0.2% sorbic acid to inhibit fungal growth. One larva of *M. sexta*, *H. virescens* or *S. exigua* was placed on top of each leaf and incubated for 4 to 6 days at 28°C. Each day larval growth and feeding were examined and when necessary fresh leaf material was added. After 4 to 6 days incubation mortality and weight of the living larvae were determined. In each bioassay experiment 5 to 10 untransformed plants were included as controls. The mean larval weight scored on leaves from untransformed plants was taken as 100%.

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Chapter six

General discussion

Bacillus thuringiensis: a rapidly developing field of research. In 1987, at the beginning of the research project reported in this thesis only five classes of crystal protein genes from *B. thuringiensis* had been discribed: three classes containing genes encoding *Lepidoptera* specific crystal proteins, one class containing a gene encoding a *Diptera* specific crystal protein, and one class containing a gene coding for the predominant Cyt protein of *B. thuringiensis* serotype *israelensis* (Kronstad and Whiteley, 1986; Thorne *et al.*, 1986; Waalwijk *et al.*, 1985). In recent years, many more crystal protein genes have been isolated from strains selected in extensive screening programmes carried out to search for *B. thuringiensis* strains with new insecticidal spectra. The number of publications reporting the identification of new crystal proteins and the cloning and sequencing of additional crystal protein genes has rapidly expanded. This led to a new nomenclature and classification based on the primary structure and the insecticidal spectrum of the crystal proteins (Höfte and Whiteley, 1989). Nowadays, the classification scheme consists of nine different *Lepidoptera* specific crystal protein gene classes and subclasses (*cry*I), two *Lepidoptera* and *Diptera* specific crystal protein gene classes (*cry*II), at least three *Coleoptera* specific crystal protein gene classes (*cry*III), and four *Diptera* specific crystal protein gene classes (*cry*IV) (First International Conference on *Bacillus thuringiensis*, Proceedings, July 1991, Oxford). Additional gene types will undoubtedly be discovered in the current intensive screening efforts to isolate crystal proteins with new, specific host ranges.

The structure of crystal proteins and their mechanism of action. Progress has also been made in the understanding of the molecular basis of toxicity and factors that determine the specificity. Domain-function studies on crystal proteins have focused on the identification of sequences determining insect specificity (recently reviewed by Visser *et al.*, 1992). Studies on hybrid crystal proteins consisting of CryIA(b) and CryIC derived segments have shown that the *C*-terminal part of the toxic fragment determines receptor binding, which in turn determines the insect specificity (Chapter 3). Moreover, sequences determining the solubility of the crystal protein, located in the *C*-terminal part of the protoxin, and sequences involved in proteolytic processing of the crystal protein, at least partly located in the *C*-terminal end of the toxic fragment, appeared to influence insect specificity (Visser *et al.*, 1992). The strategies of the domain-function studies performed so far, were based on the analysis of the primary and secondary structure. The latter structure was predicted from chemical studies of the protein conformation and computer evaluations. Recently, X-ray analysis of the crystallized *Coleoptera* specific CryIIIA toxic fragment has made available the tertiary structure (Li *et al.*, 1991) and the different domains in this protein can now be located precisely.

The three-dimensional structure of the CryIIIA toxin shows the presence of three domains with junctions positioned at amino acid residues 290 and 500. The first domain consists of seven α-helices running from amino acid residue 63 to 80, 85 to 99 and 104 to

118, 123 to 153, 160 to 186, 193 to 215, 222 to 255, and 259 to 286. The most hydrophobic fifth α-helix is centrally positioned, the other six amphipathic α-helices, of which the second consists of two pieces, situated around. The second domain, spanning the fragment from amino acid residue 291 to 500, encompasses three ß-sheets which consist of four, four and three ß-strands, respectively. The third domain from amino acid residue 500 onwards, consists of a sandwich of two antiparallel ß-sheets. The sheet that contains the *C*-terminal end is in contact with domain one and is named the inner sheet. The core of the tertiary structure encompassing the domain interfaces is built from the conserved sequence blocks, except for block one, emerging from a homology comparison of the primary structure of the crystal proteins (Chapter 2). Amino acids from the second sequence block form the central, fifth α-helix. Amino acids from the third sequence block form the interface region between the first and second domain, and those of the fifth block the interface between the second and third domain. Amino acid residues of the third sequence block form hydrogen-bonds with amino acids of the third domain as well. Amino acid residues of the fourth sequence block form the linker region between the first and third ß-sheet of the second domain. Amino acid residues of this linker region interact with amino acids from the *C*-terminal part of the fifth conserved sequence block which together with amino acids of block six constitute some of the ß-strands of the inner antiparallel sheet of domain three. The high degree of conservation of internally located residues involved in interdomain interactions implies that homologous proteins are likely to adopt a similar fold. Therefore, the CryIIIA toxin structure can be used as a model to predict the structure of other, homologous crystal proteins.

A distinct function can be attributed to each domain of the toxic fragment. The first domain, containing the α-helices, is presumed to be involved in pore formation. It is suggested that pore formation is initiated by the wedge formed by the sixth and seventh α-helices, and might include a structural rearrangement of the sequences composing the first domain. The ß-sheets of the second domain are presumed to be involved in receptor binding, whereas the third domain, a sandwich of ß-sheets may protect the toxic fragment from further proteolytic breakdown following protoxin-toxin conversion.

When these data are applied to the CryI proteins, this would predict a first domain running up to residue 260, whereas the second domain would span the segment between residues 260 and 470 and the last domain would be formed by the *C*-terminal part from residue 470 onwards. According to these data the putative recombination sites of the crystal proteins that can be regarded as natural hybrids, located around residue 257 and 460 (Chapter 2) coincide with the junctions between the three domains. Furthermore, the specificity domain which determines the toxicity spectrum against lepidopteran insects, positioned between residues 332 and 450 of the CryI proteins on the basis of hybrid protein studies (Visser *et al.*, 1992), is entirely located in the second domain between residues 260 and 470 of the *C*-terminal part of the toxic fragment.

The availability of the three dimensional structure of the toxic fragment will enable more direct strategies for domain-function studies, and will allow a refinement of these

studies down to the level of single amino acid residues. In other words, the model of the tertiary structure allows an analysis of the effects of well chosen amino substitutions on protein conformation, proteolytical processing, receptor binding and pore formation.

Changes in B. thuringiensis applications. Sprays based on spore/crystal mixtures of *B. thuringiensis* have been successfully used for more than two decades as biological insecticides to control agricultural pests. In recent years, alternatives for the application of *B. thuringiensis*, either in the form of spore/crystal mixtures or as spore-free crystal proteins have emerged. Crystal protein genes have been expressed in plants, microorganisms, and insect viruses. The possibility of synthesizing crystal proteins in transgenic plants has already led to the development of the first genetically engineered insect resistant crop plants (Chapter 5). Promising results have been reported for transgenic tomato plants tested for lepidopteran insect resistance under field conditions (Delannay *et al.*, 1989). In addition, the production of *Diptera* specific crystal proteins in cyanobacteria may allow more effective control of dipteran insects acting as vectors for medically important diseases (Chungjatupornchai, 1990). The expression of crystal proteins in plant colonizing bacteria like strains of *Pseudomonas* (Obukowitz *et al.*, 1986, Stock *et al.*, 1990, Waalwijk *et al.*, 1991), in root invading bacteria like *Rhizobium* strains (Skϕt, 1990) or in endophytic bacteria like *Clavibacter xyli* (Taylor and Uratani, 1991) may provide alternative strategies to protect agriculturally important crops from insect damage. Finally, it is studied whether recombinant baculoviruses carrying crystal protein genes are better insecticides to be used against lepidopteran plague insects (Martens *et al.*, 1990, Merrywheather *et al.*, 1990, Pang *et al.*, 1992). Expression of the crystal protein genes in the infected cells of the host insect might cause an earlier death of the insect, and in that way improve the effectiveness of baculoviruses as insecticides. Although *in vitro* cultured insect cell lines infected with recombinant baculoviruses produced insecticidal crystal proteins (Martens *et al.*, 1990, Merryweather *et al.*, 1990, Pang *et al.*, 1992) the *in vivo* virulence was not increased (Merryweather *et al.*, 1990). However, the constructs used in these studies were not yet designed to secrete the involved CryI and CryIV proteins from the infected gut epithelial cells. Such secretion into the midgut is probably a prerequisite for the toxicity of crystal proteins which is dependent on (outside) cell membrane receptor binding and subsequent pore formation. Secretion of the crystal proteins into the midgut might be accomplished by linkage of the crystal proteins to a signal peptide, although up till now such signals are not known to exist. However, the Cyt proteins present in the crystals of *Diptera* specific strains show cytolytic activity without being cell-specific. These proteins are supposed to form pores through binding to phospholipids containing unsaturated fatty acyl residues, a process which is apparently not mediated by receptors (Chapter 1). Therefore, this mode of action suggests that the production of these proteins may appear cytotoxic to the infected cell that produces such proteins by binding to the cell membrane from the inside. In other

words, recombinant baculoviruses carrying *cyt* genes instead of *cry* genes might be more effective in improving viral insecticides. Of course, a thorough assessment of the possible risks of releasing in the environment recombinant viruses producing non-specific toxins, should precede application.

Crystal protein resistance in insect populations. Until three years ago only one report described the isolation of a colony of insects resistant to a particular type of *B. thuringiensis* crystals (McGaughey, 1985). This involved a strain of the Indian meal moth *Plodia interpunctella* isolated from grain bins in which commercial *B. thuringiensis* formulations were used as insecticides. Nowadays, several reports have described development of resistance of insects to crystal proteins in both laboratory populations of *P. interpunctella*, the almond moth *Cadra cautella*, the tobacco budworm *Heliothis virescens*, the gypsy moth *Lymantria dispar*, and the sunflower moth *Homoeosoma electellum* (McGaughey and Beeman, 1988, Stone *et al.*, 1989, Rossiter *et al.*, 1990, Brewer, 1991) and field populations of the diamondback moth *Plutella xylostella* (Tabashnik *et al.*, 1991, Ferré *et al.*, 1991). A general observation of the laboratory selection experiments was that the susceptibility of the insects for the offered crystal proteins decreased in a few generations. Furthermore, resistance was stably inherited, even when selection was discontinued, provided the resistance levels had reached a plateau. If selection was discontinued earlier, resistance declined again. Although these observations were disturbing, a relief to some extent was the notion that at least the acquired resistance of the insects was crystal protein specific (McGaughey and Johnson, 1987, Van Rie *et al.*, 1990, Ferré *et al.*, 1991). These results show that the use of transgenic plants producing crystal proteins must include strategies to prevent or delay the development of insect populations resistant to crystal proteins. Since it was shown that insects more rapidly adapted to insecticidal crystal proteins in a no-choice situation (Gould and Anderson, 1991) these strategies might include: 1) the use of mixtures of plants, some of which produce high doses of crystal proteins, and some of which produce none; 2) time or tissue specific expression of crystal protein genes. The cultivation of plants producing low, sublethal doses of crystal proteins should be discouraged although this might result in weakened insects more prone to become victims of natural enemies (Weseloh *et al.*, 1983). It has been shown that such conditions might enhance selection pressure so that insect populations will timely respond with resistance development (Roush, 1991).

From genetic studies it was concluded that the resistance inherited autosomally as a recessive trait (McGaughey, 1985) or was conferred by several genetic factors (Sims and Stone, 1991). These findings indicate that resistance can evolve from different mechanisms and does not necessarily include a single gene. In addition, for both laboratory- and field selected resistance it was shown that the biochemical mechanism of resistance to *B. thuringiensis* crystal proteins could be caused by a change in the receptors

of the midgut epithelium cells affecting binding of the toxic fragments (Van Rie *et al.*, 1990, Ferré *et al.*, 1991, MacIntosh *et al.*, 1991), whereas the proteolytic activity of the midgut extracts was unchanged and similar to that of susceptible larvae (Johnson *et al.*, 1990). These findings provide information necessary for the development of strategies to delay or even prevent resistance of insects to crystal proteins. From that point of view it seems attractive to use, for instance, transgenic plants that produce different crystal proteins that bind to different receptors, since then simultaneous mutations in at least two receptors will be required to render an insect resistant, which is less likely to occur readily. In this respect, the use of gene constructs that produce fusions of two different crystal proteins might be a valuable alternative strategy as well and even an easier strategy than the use of mixed crystal protein genes (Chapter 4). At the same time, it be will be important to gain more insight in the nature of the resistance and of the genetics of insect resistance to different crystal proteins as this may help in planning further strategies for developing insect resistant plants using crystal proteins.

The long-term benefits of crystal protein application in the pursue of insect control will be highly dependent on the resolution of this now central question, how to circumvent crystal protein resistance in insect populations.

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Summary/Samenvatting

Bacillus thuringiensis is the best studied entomopathogenic micro-organism. The bacterium is characterized by its ability to produce proteinaceous crystalline inclusions during sporulation which form the major toxic determinant. Most *B. thuringiensis* strains are active against larvae of *Lepidoptera*, but some strains are active against larvae of dipteran or coleopteran species. Not only different strains, but also different crystal proteins occurring in a single crystal vary in insecticidal spectra.

The specificity of these crystal proteins is of great interest for both scientific research and applications in insect control programs. This thesis endeavours to contribute to our understanding of the entomocidal activity of *B. thuringiensis* crystal proteins. Studies are described to identify crystal proteins with new insecticidal spectra, to gain more insight into the mode of action of crystal proteins, and to examine their potential applications.

In chapter 1, the mode of action of both the highly specific insecticidal Cry proteins and the broad spectrum *in vitro* cytolytic Cyt proteins only produced by *Diptera* specific *B. thuringiensis* strains is reviewed. The primary target of the crystal proteins is the larval midgut epithelial cells which swell and burst upon challenge. The toxic action is further exhibited by physiological changes in the entire insect larva, amongst which an increase of pH and K^+-concentration of the hemolymph appear most prominent.

Upon ingestion by the larvae, the crystals are dissolved in the midgut and liberated Cry proteins (protoxins) are proteolytically converted, releasing toxic fragments encompassing the *N*-terminal part of these crystal proteins. The toxic fragments bind to receptors, presumably glycoproteins, present on the brush border region of the midgut epithelial cell membranes. The receptors on the columnar midgut cell membrane form a key factor in determining the specificity of the Cry proteins, although the efficiency of solvation of the crystals and the proteolytic conversion of the crystal proteins may also influence insecticidal specificity. Following receptor binding pores are formed in the cell membranes, probably by insertion of the toxic fragments into the membrane, which affect cellular physiology and eventually result in a total disturbance of the midgut integrity. Presumably, changes in the physiological conditions in the midgut and in the hemocoel account for the observed paralysis of the midgut muscles and subsequent total paralysis of the larvae fed with crystal proteins.

Dissolved crystal proteins from *Diptera* specific *B. thuringiensis* strains were found to be cytotoxic for a variety of *in vitro* cultured cells. The broad spectrum toxicity for *in vitro* cultured cell lines could be attributed to Cyt proteins, which constitute a major fraction of the crystals occurring in these strains. The proposed mode of action of Cyt proteins on *in vitro* cultured cells resembles that of the toxic action of Cry proteins, the difference being the absence of receptor involvement and consequently of recognition of specific cell types in the case of Cyt proteins.

The pathogenic effects observed in larvae feeding on crystals, result from the contribution of all toxins present. This means that different crystal proteins may have an

additive effect. Synergism has only been reported for two crystal proteins, whereas the question of a synergistic role for Cyt proteins remains unresolved.

In chapter 2, crystal protein genes and their encoded proteins are analyzed, in particular those occurring in *B. thuringiensis* subspecies *entomocidus* 60.5. Five different crystal protein genes belonging to four gene classes, *cryIA(a)*, *cryIB*, *cryIC*, and *cryID* were isolated from *B. thuringiensis* subspecies *entomocidus* 60.5. The crystal proteins encoded by these genes differ in insecticidal spectrum. One gene, BtVI, representing the reference-type of class *cryIC*, encodes a protein with a calculated molecular weight of 134,740 Da. The insecticidal spectrum of CryIC includes *Spodoptera* species, *Mamestra brassicae*, and *Manduca sexta*. The nucleotide sequence of BtVI and its deduced amino acid sequence are presented.

A survey of 25 different *B. thuringiensis* strains showed that *cryIA* type genes occur in all strains tested, whereas *cryIB*, *cryIC*, and *cryID* occur rarely. Furthermore, Southern analysis of DNA from strain *kenyae* 4F1 using fragments of BtVI as a probe revealed an additional gene, BtXI, representing the reference-type of class *cryIE*. Its gene product showed locally high homology to CryIC and appeared toxic against *Spodoptera* species. Finally, an analysis of the amino acid sequences of all crystal proteins revealed several conserved features which must be functionally significant for crystal formation or for the toxicity of the crystal proteins.

In chapter 3, domain function-studies on the crystal proteins are described with the aim to improve our understanding of the mode of action of crystal proteins, especially of its molecular basis. For this purpose, genes encoding hybrid crystal proteins based on CryIA(b) and CryIC were constructed. Two out of eleven hybrid proteins synthesized exhibited insecticidal activity. Both displayed an insecticidal spectrum similar to that of the parental crystal protein from which the *C*-terminal part of the toxic fragment originated. In addition, *in vitro* binding studies directly demonstrated the involvement of the *C*-terminal part of the toxic fragment in receptor binding. These results showed that the *C*-terminal part of the toxic fragment is determining specific receptor binding, which in turn determines to a large extent insect specificity.

In chapter 4 an alternative strategy for the introduction of insect resistance in transgenic organisms using *B. thuringiensis* crystal protein genes is presented. Two truncated crystal protein genes, belonging to the classes *cryIA(b)* and *cryIC* respectively, and both coding for insecticidal *N*-terminal fragments of the corresponding crystal proteins, were translationally fused. Expression of the gene fusion in *Escherichia coli* showed a biologically active protein with a toxicity spectrum that overlapped those of the two constituting crystal proteins. Introduction of such a gene fusion in transgenic organisms may provide a simple, attractive strategy towards insect resistance in transgenic organisms, that is either more effective or potentially more durable, depending on the gene combination used.

In chapter 5, the route towards insect resistant transgenic plants is described. The *B. thuringiensis* genes *cryIA(b)* and *cryIC* were introduced by *Agrobacterium tumefaciens*

mediated transfer into the genome of tomato and tobacco plants. Expression of the crystal protein gene coding regions in the obtained transgenic plants is transcriptionally controlled by either the CaMV 35S or the T_R2' promoter. Although expression of the chimeric *cryIC* and *cryIA(b)* crystal protein genes from the CaMV 35S promoter was optimized by the use of a promoter enhancer element, in the case of the *cryIC* gene further improved by a viral untranslated leader sequence, none of the transgenic plants produced crystal proteins at levels resulting in considerable insect control. However, in transgenic plants containing a chimeric *cryIA(b)* gene with a modified coding region expression is dramatically increased resulting in high insect resistance. These observations have important implications for insect control based on the use of crystal protein genes in plant systems in particular, and for heterologous gene expression in general.

Finally, in chapter 6 the structure of crystal proteins, which became recently available, is discussed in relation to their mechanism of action. Within the toxic fragment three domains can be distinguished. The first domain, containing α-helices, is presumed to be involved in pore formation, the second domain, containing ß-sheets, is most probably involved in receptor binding, whereas the third domain, containing a sandwich of ß-sheets, may protect the toxic fragment from further proteolytic breakdown following protoxin-toxin conversion. Also, in this final chapter, alternative *B. thuringiensis* based control strategies aimed to minimize the emergence of protein resistance in insect populations are discussed. The use of transgenic organisms that produce different crystal proteins binding to different receptors, either simultaneously or spaced in time, and the addition of a low percentage of non-transgenic, susceptible plants to field crops might well provide such alternatives.

Van alle micro-organismen die pathogeen zijn voor insekten is *Bacillus thuringiensis* het best bestudeerd. Kenmerkend voor deze bacterie is dat deze kristallen vormt tijdens de sporulatie. Deze kristallen zijn de belangrijkste toxische componenten. De meeste *B. thuringiensis* stammen zijn toxische voor larven van *Lepidoptera* (vlinderachtigen), maar sommige stammen zijn daarentegen toxisch zijn voor larven van *Diptera* (tweevleugeligen) of *Coleoptera* (keverachtigen). De kristallen zijn samengesteld uit kristaleiwitten die onderling eveneens verschillen in insektenspecificiteit.
De specificiteit van de kristaleiwitten is zowel interessant voor wetenschappelijke onderzoek als voor de toepassing in insektenbestrijding. Dit proefschrift beoogt een bijdrage te leveren aan de kennis over de toxische eigenschappen van de kristaleiwitten van *B. thuringiensis*. De beschreven experimenten hadden tot doel kristaleiwitten met nieuwe werkingsspectra te identificeren, meer inzicht te verkrijgen in het werkingsmechanisme van de kristaleiwitten en enkele alternatieve toepassingsmogelijkheden van deze eiwitten te bestuderen.

In hoofdstuk 1 is een overzicht gepresenteerd van de huidige staat van kennis met betrekking tot het werkingsmechanisme van de twee te onderscheiden groepen Cry eiwitten en Cyt eiwitten. De Cyt eiwitten worden slechts geproduceerd door *Diptera* specifieke *B. thuringiensis* stammen. Cyt eiwitten zijn toxisch voor een breed spectrum van *in vitro* gekweekte cellijnen.

De kristaleiwitten zijn toxisch voor middendarmepitheelcellen die hierdoor opzwellen en vervolgens lyseren. Deze verschijnselen leiden tot fysiologische veranderingen in het gehele insekt waarbij een verhoging van de pH en de K^+-concentratie in het hemolymfe het meest in het oog springen.

De oraal opgenomen kristallen lossen op in de middendarm van de larve en de vrijgekomen Cry eiwitten worden enzymatisch omgezet in toxische fragmenten. Deze toxische fragmenten worden gevormd door het *N*-terminale deel van de kristaleiwitten. Vervolgens binden de toxische fragmenten aan receptoren, waarschijnlijk glycoproteinen, die gelegen zijn op de borstelzoom van de darmepitheelcellen. De aanwezigheid van deze receptoren bepaalt met name de specificiteit van de Cry eiwitten. Echter, ook de oplosbaarheid van de kristallen en de enzymatische omzetting van de kristaleiwitten in toxische fragmenten hebben invloed op de specificiteit. Receptorbinding wordt tenslotte gevolgd door porievorming waarschijnlijk door insertie van de toxische fragmenten in de celmembraan, met als gevolg een verstoring van de cellulaire fysiologie en uiteindelijk een volledige aantasting van de middendarm. De verlamming van de middendarmspieren en de totale verlamming van de larven als gevolg van kristaleiwitopname worden mogelijk veroorzaakt door de veranderde fysiologische omstandigheden in de middendarm en in het hemocoel.

Een breed spectrum van *in vitro* gekweekte cellijnen blijkt gevoelig te zijn voor opgeloste kristallen van *Diptera* specifieke *B. thuringiensis* isolaten. Deze toxische werking is toe te schrijven aan de zogenaamde Cyt eiwitten, die een groot deel van de *Diptera* specifieke kristallen uitmaken. Het voorgestelde werkingsmechanisme met

betrekking tot de Cyt eiwitten op de *in vitro* cellijnen is vergelijkbaar met dat beschreven voor Cry eiwitten. Een belangrijk verschil echter is dat receptoren geen rol spelen waardoor Cyt eiwitten niet celspecifiek zijn.

De pathogene effecten, zoals die na opname van kristallen in de larven worden waargenomen, zijn het gevolg van de bijdrage van alle eiwitten aanwezig in de kristallen. De kristaleiwitten hebben dus een additief effect. Een synergistisch effect is slechts bij twee kristaleiwitten gevonden. Een synergistische rol van Cyt eiwitten is vooralsnog onbewezen.

In hoofdstuk 2 is de analyse van kristaleiwitten en kristaleiwitgenen, met name die in de *B. thuringiensis* stam *entomocidus* 60.5 voorkomen, beschreven. Uit deze bacterie zijn vijf verschillende kristaleiwitgenen behorend tot de vier klassen *cryIA(a)*, *cryIB*, *cryIC* en *cryID* geïsoleerd waarvan de gecodeerde eiwitten verschillen in insektenspecificiteit. Het BtVI gen, het referentie-type van de *cryIC* klasse, codeert voor een eiwit met een berekend molecuul gewicht van 134.740 Da. Onder het toxisch bereik van CryIC vallen *Spodoptera* soorten, *Mamestra brassicae* en *Manduca sexta*. In dit hoofdstuk zijn de nucleotidevolgorde van BtVI en de afgeleide aminozuurvolgorde gegeven.

Uit een onderzoek van 25 verschillende *B. thuringiensis* stammen bleek dat *cryIA* type genen in alle stammen voorkomen, terwijl *cryIB*, *cryIC* en *cryID* slechts incidenteel voorkomen. Bovendien leverde een Southern analyse van *kenyae* 4F1 DNA met een BtVI fragment als 'probe' een additioneel gen: BtXI, het referentietype van de *cryIE* klasse. De aminozuurvolgorde van CryIE vertoont lokaal sterke homologie met dat van CryIC en net als CryIC is het CryIE eiwit toxisch voor *Spodoptera* larven. Een vergelijking van de aminozuurvolgorde van alle kristaleiwitten leverde een aantal geconserveerde kenmerken op die zeer waarschijnlijk functioneel zijn voor de kristalformatie of voor de toxische activiteit van de kristaleiwitten.

In hoofdstuk 3 zijn domein-functie studies beschreven met als doel ons begrip van de moleculaire basis van het werkingsmechanisme te verbeteren. Hiervoor zijn elf hybride genen, gebaseerd op *cryIA(b)* en *cryIC*, geconstrueerd. Slechts twee eiwitten gecodeerd door deze genen bleken toxisch te zijn. Beide hybride eiwitten bezitten een toxiciteitsspectrum dat vergelijkbaar bleek met dat van het oorspronkelijke kristaleiwit waarvan het *C*-terminale deel van het toxische fragment afkomstig was. Tevens is met behulp van *in vitro* bindingsstudies aangetoond dat het *C*-terminale deel van het toxische fragment betrokken is bij de receptorbinding. Deze resultaten toonden aan dat het *C*-terminale deel van het toxische fragment de insektenspecificiteit van de kristaleiwitten in belangrijke mate bepaalt via de specificiteit in de receptor binding.

In hoofdstuk 4 wordt een alternatieve strategie gepresenteerd voor introductie van insektenresistentie in transgene organismen gebruik makend van kristaleiwitgenen. Twee ingekorte versies van de genen *cryIA(b)* en *cryIC*, beide coderend voor het *N*-terminale fragment van de respectievelijke kristaleiwitten, zijn zodanig gefuseerd, dat expressie leidde tot de produktie van één eiwit dat beide fragmenten bevatte. Expressie van deze zogenaamde translatiefusie in *Escherichia coli* leverde een toxisch eiwit op met een

toxiciteitsspectrum dat overlapte met de spectra van de beide oorspronkelijke eiwitten. Introductie van een dergelijke genfusie in transgene organismen is, met het oog op hetzij de spectrumbreedte hetzij de duurzaamheid van de insektenresistentie, mogelijk een eenvoudige en aantrekkelijk strategie voor het verkrijgen van insektenresistentie in transgene organismen.

In hoofdstuk 5 is de produktie van insektenresistente transgene planten beschreven. De *cryIA(b)* en *cryIC* genen zijn met behulp van *Agrobacterium tumefaciens* overgedragen naar het genoom van zowel tabaks- als tomateplanten. Expressie van beide genen in de transgene planten is gereguleerd door de CaMV 35S promoter of de T_R2' promoter. Ondanks de versterking van de CaMV 35S promoter via verdubbeling van een deel van deze promoter en door het aanbrengen, vooraf aan coderend nucleotidevolgorde van *cryIC*, van een virale, niet vertaalde sequentie bleek geen van de transgene planten voldoende kristaleiwit te produceren voor een meetbaar effect op de insektengroei. In transgene planten met kristaleiwitgenen waarvan de coderende nucleotidevolgorde was gemodificeerd bleek echter de expressie dramatisch te zijn toegenomen resulterend in een hoog niveau van insektenresistentie. Deze waarnemingen hebben belangrijke consequenties voor de toepassing van kristaleiwitgenen in planten gericht op het verkrijgen van resistentie tegen insekten.

Tenslotte is in hoofdstuk 6 de relatie besproken tussen de onlangs bekend geworden structuur van de kristaleiwitten en het werkingsmechanisme van deze eiwitten. Het toxische fragment kan onderverdeeld worden in drie domeinen. Het eerste domein bestaat uit α-helices en wordt verondersteld betrokken te zijn bij de porievorming. Het tweede domein bestaat uit ß-sheets die zeer waarschijnlijk betrokken zijn bij de receptorbinding, en het derde domein bestaat uit een sandwich van ß-sheets waarmee het toxische fragment waarschijnlijk beschermd wordt voor een verdere afbraak na de protoxine-toxine omzetting. Tevens zijn in dit hoofdstuk ook voorgestelde aanpassingen in de toepassing van kristaleiwitten besproken in het licht van het ontstaan van populaties insekten die ongevoelig zijn voor *B. thuringiensis* kristaleiwitten. In dit kader is uiteengezet dat het gebruik van transgene organismen waarin meerdere kristaleiwitten worden geproduceerd, die aan verschillende receptoren in het insekt binden, dan wel het gebruik van een laag percentage niet-transgene gevoelige planten in het veld mogelijk resistentieontwikkeling tegen kristaleiwitten bij insekten voorkomt.

Account

Most of the results presented in this thesis have been published before, or will be published. The contents of the preceding chapters have been based on these publications.

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Curriculum vitae

Guy Honée werd geboren op 15 juni 1960 te Haarlem. In 1977 behaalde hij het HAVO diploma aan de Scholengemeenschap Nijmegen West en vervolgens in 1979 het VWO diploma aan de Nijmeegse Scholengemeenschap. In hetzelfde jaar begon hij aan de studie Biologie aan de toenmalige Landbouwhogeschool te Wageningen alwaar in 1983 het Kandidaatsexamen Biologie, met specialisatie 'Cel', werd gehaald. De studie werd vervolgens voortgezet aan de Katholieke Universiteit te Nijmegen en in 1987 afgesloten met het behalen van het Doctoraalexamen Biologie met als hoofdvak Biochemie (Prof. Dr. J.J.H.H.M. de Pont en Prof. Dr. H.P.J. Bloemers) en bijvakken Moleculaire Biologie (Prof. Dr. R.N.H. Konings) en Immunologie (Prof. Dr. J.H.M. Berden). In mei 1987 werd hij aangesteld als wetenschappelijk onderzoeker bij de Stichting Ital, later het DLO-Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO), waar het in dit proefschrift beschreven onderzoek is uitgevoerd. Vanaf oktober 1991 is hij werkzaam bij de vakgroep Fytopathologie van de Landbouwuniversiteit Wageningen.