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CLONING AND EXPRESSION OF *BACILLUS THURINGIENSIS* INSECTICIDAL PROTEINS IN NEW HOSTS: APPLICATIONS FOR DEVELOPING COUNTRIES

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ABSTRACT

The recent progress in the transformation techniques of prokaryotic and eukaryotic hosts has created new opportunities for the use of *Bacillus thuringiensis (B.t.)* insecticidal crystal proteins. Herei n, we will discuss two approaches which could provide potential applications, especially useful for developing countries.

INTRODUCTION

From all different insecticidal *B.t.* strains, four major pathotypes have been described: strains toxic to Lepidoptera, Lepidoptera/Diptera, Diptera and Coleoptera. The four pathotypes have each a typical crystal protein pattern, as can be demonstrated by SDS-PAGE electrophoresis. Lepidopteran insecticidal proteins are always in the range of 130 kd and a protein of 140 kd. One of the genes [crylA(b)], coding for the 130 kd protein has been cloned and expressed in *E. coli* (Hofte et al., 1986). Several strains from the Lepidopteran pathotypes also contain crystal proteins toxic to both Lepidoptera and Diptera (Yamamoto and McLaughlin, 1981). Two genes encoding these 65 KD proteins, formerly designated as P2 proteins, have been described by Widner and Whiteley (1989). In crystals of *B.t. israelensis* belonging to the Diptera palhotype, there are at least four different proteins, ranging from 130 kd to 25 kd (Ibarra and Federici, 1986). The CrylVB protein has been expressed in *E. coli* (Chungjatupomcai et al., 1988). The crystals of the Coleoptera pathotype, exemplified by *B.t. tenebrionis*, contain one major protein of 66 kd (Krieg et al., 1983). Cloning of the toxin gene in *E. coli* results in the expression of a 72 kd and a 66 kd protein (Hofte et al., 1987). Apparently, the 72 kd protein is a precursor which is processed by *B.t.* before the assembly of the crystal.

Even within the group of Lepidoptera toxins, different types can be identified on the basis of the molecular weight of the protoxins and the toxins, and by their immunological reaction with a panel of monoclonal antibodies (Hofte et al., 1988). There is a good correlation between a certain crystal protein type and its toxicity for some Lepidoptera species (Hofte and Whiteley, 1989).

So, there is a substantial variety of *B.t.* insecticidal crystal proteins which are very specific for certain insects. Therefore, it is essential to select the insecticidal protein with the appropriate toxicity, in order to engineer resistance in a particular crop against its major insect pests.

MATERIALS AND METHODS

The idea to engineer insect resistance in plants by transformation with a *B.t.* gene was first tested with tobacco (Vaeck et al., 1987). We selected a *B.t.* toxin, CrylA(b) which is active against Lepidoptera such as the tobacco homworm *(Manduca sexta)* and the tobacco budworm *(Heliothis virescens)*, both pests on tobacco. CryIA(b) is a protoxin and is processed in the insect gut into a 60 KD protein, which retains full toxicity.

The smallest toxic fragment of CryIA(b) was mapped between amino-acid positions 29 and 607, at the amino-terminal half of the protein (Höfte et al., 1986). Nonessential sequences could be eliminated from the *cryIA(b)* gene, and we could use truncated versions of the gene.

We used the *Agrobacterium*-mediated T-DNA transfer to express *B.t.* toxin genes in tobacco plants. The vectors (pGSH160 and pGSH150) derived from Ti-plasmid of *Agrobacterium*, contain the T-DNA borders which mediate the transfer and the insertion of the foreign DNA in the genome, and contain the TR promoter. In such vectors, we inserted the entire coding sequences of the *cryIA(b)* gene as well as truncated genes.

The idea was then to use a neomycin phosphotransferase (NPTII) selection system in order to increase the probability of obtaining high levels of toxin expression in plants (Herrera-Estrella et al., 1983). NPTII is a selectable marker, which makes transformed plants resistant to kanamycin. First, we expected that the expression of the neomycin phosphotransferase gene (*neo*) controlled by the TR 1′ promoter would be correlated with the expression of the toxin gene directed from the TR 2′ promoter, since both promoters proved to be coordinated (Velten et al., 1984). Second, since foreign polypeptides can be fused to the amino-terminus of the neomycin phosphotransferase (NPTII) without disrupting its enzymatic activity, we anticipated that translational *bt:neo* fusions would code for fusion proteins expressing NPTII activity (Höfte et al., 1988b). And by selecting for high kanamycin resistance, one would also select for plants with a high level of B.t. toxin. So, some of the T-DNA constructs contained a chimaeric neomycin phosphotransferase gene, along with the entire *cryIA(b)* coding sequence or truncated *cryIA(b)* genes, while other constructs included translational fusions between fragments of *cryIA(b)* genes and other construts included translational fusions between fragments of *cryIA(b)* and the *neo* gene.

RESULTS AND DISCUSSION

Transgenic tobacco plants were obtained by leaf disk infection with *Agrobacterium* containing the different T-DNA constructs. From all transformation experiments shoots were selected with different doses of kanamycin, confirming that fusion genes indeed confer NPTII activity on transformed plants. In ELISA with a mixture of monoclonal antibodies against the amino-terminus of *CryIA(b)*, Bt protein was detected in leaves of transgenic tobacco plants. Plants transformed with truncated *cryIA(b)* gene or the *bt:neo* fusions contain about ten times more Bt protein than those transformed with the complete *cryIA(b)* coding sequence. On the average plants transformed with the shorter fusion express higher levels of Bt proteins (up to 42 ng/ml total protein). Moreover, there was a clear correlation between Bt expression levels and insect toxicity. Toxicity was tested with leaves of transformed plants, fed to *Manduca sexta* larvae and mortality was monitored after tree, four and six days of feeding. There was no toxic activity in plants with the complete *cryIA(b)* sequence, while there was clear toxicity in many of the plants containing the fusion proteins. High toxicity was observed in about one quarter of the plants expressing the long fusion, and two thirds of the plants with the short fusion. Insecticidal activity was also recorded in most of the plants containing the truncated *cry* gene. These *cry* genes give rise to expression levels that are highly insecticidal in transgenic tobacco. The insecticidal trait is stably inherited and several rounds of field tests by Rohm and Haas Co. showed protection of transformed tobacco against feeding damage by both *Manduca sexta* and *Heliothis virescens*. The transformation experiments with tobacco have been repeated with tomato and potato, resulting in tomato and potato plants resistant to *M. sexta*.

The results with tobacco, tomato and potato indicate that conferring insect resistance to plants is perfectly feasible and that commercial applications are within reach. There are still a number of improvements that can be made, especially concerning transformation efficiency, plant regeneration, gene expression levels and regulation of expression. Besides, one could also broaden the insecticidal spectrum by screening for new *B.t.* toxins, or by engineering *B.t.* toxins with enhanced and/or modified activity.

Another application of *B.t.* toxin transformation is exemplified with mosquitoes and blue-green algae. There are a number of commercial formulations of *B.t. israelensis (B.t.i.)* which are used in biological control of mosquito and black fly larvae. However, there are several problems with this approach. *Bacillus thuringiensis*, in its present formulations, is unstable in the environment, does not propagate and tends to settle on the bottom of the pond, out of reach of the aquatic mosquito and black fly larvae. If mosquitocidal activity could be conferred to the food source of the aquatic larvae, most of these problems should be overcome. Since blue-green algae constitute an important food base for mosquito larvae, we tried to confer insect toxicity by inserting a *Bt* delta-endotoxin gene in the genome of *Synechocystis* 6803.

We cloned the *cryIVB* gene, coding for a 130 kd crystal protein with specific mosquitocidal activity, from *B.t. israelensis* (Chungjatupornchai et al., 1988). In *E. coli* CryIVB is expressed as a protein of 130 kd and is present as a precipitate. The precipitated CryIVB has an LC50 of 5ng/ml, wich in our bioassay is identical to the toxicity of intact *B.t.i* native crystals. The toxic fragment of CryIVB was localized by deletion mapping, and as was the case with the Lepidoptera toxins, the toxic fragment proved to be in the amino-terminal part of *cryIVB*, between the 5′ end and up to bp 2332. So, again we had the option to use either the complete coding sequence, or to use the toxic fragment of the gene.

The donor plasmid (pKW1188) was a shuttle vector with an ampicillin resistance gene, for selection in *E. coli*, and a kanamycin resistance gene for selection in the transformed cyanobacteria. The vector also contained the Lambda phage PL promoter and fragments of *Synechocystis* 6803 DNA, allowing homologous recombination. The CryIVB was inserted either as a full length sequence (pBTI1188), or as a truncated toxic fragment which was fused to the 5′ end of the *neo* gene (pBIK1188). Both constructs were used to transform *Synechocystis* 6803 cells, which were subsequently selected on kanamycin.

The *cryIVB* gene is expressed as a protein of 130 kd, recognized by anti-CryIVB antibodies. The *bt:neo* fusion is expression as a protein of 110 kd, which reacts with anti-NPTII antibodies and anti-CryIVB antibodies. Some of the transformed *Synechocystis* 6803 cells are toxic to *Aedes aegypti* larvae, but there is still need to improve on the level of toxicity. Lowered toxicity is most likely related to the rath weak PL promoter and we are testing different promoters to boost gene expression and to obtain blue-green algae highly toxic to Diptera such as mosquitoes and black files.

CONCLUSION

The examples in this communication indicate that it is possible to transfer insecticidal activity directly into the insect's food source, thereby eliminating the manufacturing, transportation and repeated application of the insecticide. This genetic engineering technology can be used to protect important crops from insect pests, and can also be used to control insects which present a serious health threat.

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