# INSECT RESISTANCE IN TRANSGENIC PLANTS EXPRESSING *Bacillus thuringiensis* TOXIN GENS[1]

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## ABSTRACT

A crystal protein gene (bt2) has been cloned from plasmid DNA of *Bacillus thuringiensis (B.t.) berliner* 1715 and directs the synthesis of a 130 kd protein (Bt2) in *E. coli* which is toxic to larvae of *Pieris brassicae* and *Manduca sexta*. Treatment of the Bt2 protein with trypsin or chymotrypsin yields a 60 kd protease resistant fragment which is fully toxic towards insect larvae "in vivo" and insect cell lines "in vitro". The minimal portion of the Bt2 protein required for toxicity has been mapped by deletion analysis and coincides with the 60 kd protease resistant Bt2-fragment. Tobacco plant cells have been transformed with chimeric toxin genes using a Ti plasmid vector. Transformed plants express a functional toxin and exhibit resistance against insect larvae.

## INTRODUCTION

*Bacillus thuringiensis (B.t.)* is a gram positive bacterium which produces endogenous crystals upon sporulation. The crystals are composed of protein and are specifically toxic against

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certain insect larvae, mainly lepidopteran and dipteran species [DULMAGE *et al.*, 1980]. Upon ingestion by larvae the crystals disolve in the alkaline conditions of the insect midgut and release proteins of molecular weight (M.W.) 130 - 160 kd [HUBER *et al.*, 1981]. These high M.W. protoxins are proteolytically processed by midgut proteases to yield smaller toxic fragments [LILLEY *et al.*, 1980]. Most crystal protein genes have been localised on large plasmids [GONZALEZ *et al.*, 1982; KRONSTAD *et al.*, 1983]. Some genes have recenlty been cloned and functional toxin was expressed in *E. coli* [SCHNEPF *et al.*, 1981; KLIER *et al.*, 1982; SHIBANO *et al.*, 1985; ADANG *et al.*, 1985].

*B.t.* toxins are highly specific in their activity and therefore represent a perfectly safe insecticide and an interesting alternativ for chemical control agents. Commercial preparations based on *B.t.* have already been used for several years as a biological insecticide, however only with limited success, mainly due to high production costs and instability of *B.t.* in the field. We have used the *Agrobacterium* vector system to transfer and express the *B.t.* toxin gene in tobacco plants. We present data on the resistance of those plants against insect larvae.

## RESULTS

### Cloning of a B. t. gene encoding a 130 kd crystal protein exhibiting insect toxicity.

Since most crystal protein genes in *B. thuringiensis* strains are plasmid-born [KRONSTAD *et al.*, 1983], we used a purified plasmid preparation from strain *B. thuringiensis* berlineer 1715 for the cloning of crystal protein genes. A library was constructed by clining size fractionated Sau 3A digested plasmid DNA into an *E. coli* expression vector. Using a colony immunoblot assay with rabbit anti-crystal protein serum, 4 clones containing overlapping DNA fragments were isolated. A 7.5 kb BamHI-PstI fragment containing the toxin gene was subcloned into pUC8, generating plasmid pGI502.

Total cell extract of *E. coli* K514 (pGI502) was analysed on SDS-PAGE. An intense protein band, with apparant M.W. of 130 kd, was visible and was not present in K514 containing the pUC8 plasmid without insert. This protein, termed Bt2, comigrated in SDS-PAGE with one of the major crystal proteins of *B.t.* berliner. Bt2 protein represented between 5 and 10% of the total protein content in K514 (pGI502) and was present as a precipitate in *E. coli*.

The relationship between Bt2 and *B.t.* crystal proteins was confirmed by its immunological properties and biological activity. In Western blotting, Bt2 reacted strongly with a rabbit

anti-Bt berliner crystal serum and in ELISA, 8 out of 16 monoclonal antibodies, generated against *B.t.* berliner crystal proteins, reacted with the purified Bt2. The toxic activity of Bt2 protein was assayed on *Pieris brassicae* and *Manduca sexta* larvae. Purified preparations of Bt2 showed toxicity levels comparable to those of solubilized crystals from *B.t.* berliner (Table 1).

TABLE 1 - Insect toxicity of B.T. crystal proteins

| | P. brassicae (LD50) ng/larva | M. sexta (LD50) ng/cm$^2$) |
|---|---|---|
| Bt berliner cry | 15 | N.T. |
| Bt berliner cry (solubilized) | 0.6 (±0.3) | 7.5 |
| Bt2 protein | 1.6 (±1.3) | 6 |
| Bt2 protein/trypsin | 1.5 | 5 |

## Nucleotide sequence of the gene and N-terminal amino acid sequence of the crystal protein

The *bt2* toxin gene was localized by deletion mapping on a 4343 bp HpaI-PstI fragment. The sequence of this fragment showed one large open reading frame encoding a protein of 1155 amino acids with a predicted molecular weight of 130533 d. This agrees well with the molecular weight of Bt2 determined in SDS-PAGE. The amino acid sequence of the N-terminal end of this purified protein was determined by gas-phase sequencing to be X - Asp - Asn - Asn - Pro - Asn - Ile - Asn - Glu - X - Ile - Pro - Tyr - Asn - Leu - X - Asn - Pro. This sequence was identical to the N-terminal amino acid sequence deduced from the nucleotid sequence.

## Generation of a 60 kd toxic polypeptide through proteolytic degradation of the 130 kd Bt2 protein.

The delta-endotoxins of *B.t.* are generally believed to be protoxins which, upon ingestion by insects, are degraded by insect gut proteases into smaller active toxin(s) [KLIER *et. al.*, 1982]. We therefore investigated whether smaller toxic polypeptides could be generated from Bt2 by proteolytic cleavage. Purified Bt2 protein was digested with either trypsin or chymotrypsin and at defined time intervals, aliquots were analysed on SDS-PAGE. The results demonstrated that the 130 kd Bt2 protein is rapidly degraded by trypsin or chymotrypsin (after 10 min. at 37°C) yielding a predominant polypeptide of 60 kd.

This 60 kd polypeptide was relatively resistant to further degradation by both enzymes over a 2 h period, indicating that it constitutes a protease resistant fragment within the Bt2 protein. The 60 kd polypeptide generated by trypsin cleavage was purified by gel filtration and its insect toxicity was determined. It was equally toxic to *P. brassicae* and *M. sexta* larvae as intact 130 kd Bt2 (Tabel 1). The 60 kd protein starts at the isoleucine at position 29 in the N-terminal half of the molecule.

Using insect cell lines it was deduced that the 60 kd trypsin fragment represent an active toxin molecule which is specifically toxic against the cell lines derived from Lepidoptera species and most toxic towards a Diptera cell line. In contrast, the intact 130 kd Bt2 protein was not toxic towards all four cell lines.

## Genetic mapping of the minimal gene fragment ecoding an active toxin

Different deletions at the 5' or the 3' end of the *bt2* gene were constructed and expressed in *E. coli*. We analysed the produced polypeptides to delineate the minimal Bt2 fragment exhibiting insect toxicity (table 2).

TABLE 2 - Toxicity of cell extracts of NF1 strains containing different plasmids expressed as % mortality after 4 days; 50 3rd instar *P. brassicae* were used per dilution of extract.

| Type of construct | *E. coli* clone | Position 3' end Bt2 sequence | 1 | 1/10 |
|---|---|---|---|---|
| Deletions | pLB16 | 2170 | 100 | 100 |
| | pLB820 | 2031 | 100 | 100 |
| | pLB822 | 1911 | 100 | 100 |
| | pLB828 | 1830 | 100 | 100 |
| | pLB826 | 1827 | 100 | 100 |
| | pLB884 | 1821 | 100 | 100 |
| | pLB879 | 1821 | 98 | 50 |
| | pLB834 | 1798 | 0 | 2 |
| | pLB950 | 1731 | 6 | 0 |
| | pLB876 | 1695 | 2 | 0 |
| | pLB12 | 1692 | 0 | 0 |
| Bt2-lacZ fusions | pBZ12 | 1821 | 100 | 74 |
| | pBZ13 | 1798 | 4 | 0 |

To delineate the 5' end of the gene fragment encoding an active toxin, we constructed a 5' deletion in the Bt2 gene up to the ClaI site at the 110th bp, removing the first 36 codons of the *bt2* gene.

The experimental data show that the minimal toxic fragment of the Bt2 protein is a 60 kd polypetide delineated by positions 29 and 37 at the N-terminus and amino acid 601 and 607 at the C-terminus.

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     Trypsin cleavage
     site
     GLY GLU ARG ILE GLU THR GLY TYR THR PRO ILE ASP ILE
...'GGA'GAA'AGA'ATA'GAA'ACT'GGT'TAC'ACC'CCA'ATC'GAT'ATT'...
        80          90              100          110
                                                pRB210

            600                   605
     TYR ILE ASP ARG ILE GLU PHE VAL PRO ALA GLU VAL
...'TAT'ATA'GAT'CGA'ATT'GAA'TTT'GTT'CCG'GCA'GAA'GTA'
           1800          1810        1820
         pLB834                              pLB879
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FIGURE 1. Mapping of the 5' and 3' end for the minimal toxic fragment.

## Transfer and expression of chimeric B.t. genes in tobacco plants.

Recent progress in plants genetic engineering techniques made it possible to transfer foreign DNA into many plant species using vectors derived from the Ti-plasmid of *Agrobacterium tumefaciens*. Using such vectors, several genes of bacterial origin mostly antibiotic resitance genes have been expressed in transgenic plants using plant specific promotor sequences. The latter have been obtained from the Ti-plasmid T-DNA genes [HERRERA-ESTRELLA *et al.*, 1983; VELTEN *et al.*, 1984], from plant viruses [ODELL *et al.*, 1985] or from nuclear plant genes [HERRERA-ESTRELLA 1984; SIMPSON *et al.*, 1985; KREUZALLER *et al.*, 1986]. We have used Ti-plasmid derives vectors (DEBLAERE *et al.*, 1984) to transfer *bt2* gene fragments into *N. tabacum* plants using a leaf disc transformation procedure (HORSCH *et al.*, 1985). NPTII was used as a selectable marker. Leaf discs have been grown for 2 x 3 weeks in a selective medium containing 50-200 mg/ℓ of Km. Afterwards shoots were removed from the leaf disc, grown in non selective conditions and retested for resistance in a callus induction assay on different concentrations of Km (50-500 mg/ℓ). Plants showing high Km resistance were retained for turther tests.

Transformed tobacco plants containing a chimeric *bt2* gene were analysed for production of Bt2 related polypeptides in an

ELISA assay using a rabbit anti-Bt crystal serum, as well as monoclonal antibodies generated against the Bt2 protein.

Variable levels of Bt, ranging from 10 to 100 ng per g of plant tissue were recorded in independent transformants.

Insect resistance of transformed tobacco plants.

We evaluated the toxic activity exhibited by transformed tobacco plants on insect larvae. Twenty first instar larvae of *M. sexta* were placed on leaf discs of 4 cm diameter in petri-dishes. Fresh leaf material was given each day, growth rate and mortality of the larvae feeding on the leaves were scored over a 6 day period. Larvae feeding of Bt positive plants rapidly showed marked growth inhibition, which retarded their transition to the second larval stage. Most importantly, significant mortality among the larvae became apparent at day 3 and reached 100% at day 6 for several of the independent transformed plants tested (Table 3).

Table 3 - Mortality rates in *M. sexta* larvae feeding on transformed tobacco plants.

| | % Mortality | | |
|---|---|---|---|
| | Day 3 | Day 4 | Day 6 |
| Bt + plants | | | |
| 1 | 90 | 100 | 100 |
| 2 | 10 | 45 | 65 |
| 3 | 80 | 95 | 100 |
| 4 | 5 | 60 | 95 |
| 5 | 5 | 35 | 85 |
| 6 | 85 | 90 | 100 |
| 7 | 65 | 75 | 95 |
| Control plants | | | |
| 8 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 |
| 10 | 0 | 0 | 5 |
| 11 | 5 | 5 | 5 |

In contrast, larvae growing on untransformed control plants developed normally and their mortality rate was only 0-5%. Thus, the effect on insect larvae of *B. t.* toxin expressed in plant cells was comparable to the effect of *B. t.* toxin of bacterial origin, namely growth inhibition in the initial stage followed by death.

Independant transformants was also variable (Table 3). However for one and the same transformant the degree of insect resistance was highly reproducible when tested in independant experiments over a period of time.

In a next set of experiments, we investigated whether the levels of *B.t.* toxin produced in transformed plants would protect them from significant insect damage. Fifteen freshly hatched larvae were placed on the leaves of 40 cm high tobacco plants in the greenhouse. On control plants, considerable damage was obvious after 4-7 days and they were completey consumed after 10-15 days. In contrast, all larvae died within 4 days and they were completely consumed after 10-15 days. In contrast, all larvae died within 4 days when feeding on plants expressing high levels of Bt2 protein, and leaf damage was restricted to holes of a few $mm^2$ caused by each insect.

## Conclusion and prospects.

A functional analysis has been performed on Bt2, a cloned *Bacillus thuringiensis* crystal protein. The Bt2 protein is highly toxic to larvae of *Pieris brassicae* and *Manduca sexta*. This 130 kd polypetide is a protoxin which is not toxic in an *in vitro* assay on insect cell lines. However, treatment of Bt2 with proteolytic enzymes results in a 60 kd protease resistant and fully toxic polypeptide.

We have precisely determined, through genetic mapping, the minimal fragment of Bt2 which still exhibits full toxicity. This fragment was found to coincide with the 60 kd polypeptide generated by proteolytic degradation and located in the $NH_2$-terminal half of the protein. According to our data, it is very unlikely that toxic fragments, smaller than this 60 kd can be obtained. A detailed analysis to identify the functional domains of Bt2 is presently in progress, using monoclonal antibodies specific for well defined regions on the molecule.

The feasibility of using plants genetic engineering techniques to obtain insect resistant plants is clearly exemplified here. Fully normal plants were obtained, expressing sufficiently high levels of *B.t.* toxin to give resistance against insect damage under greenhouse conditions. Further experiments will be directed to fully evaluate this new approach under natural conditions of stress caused by insect pests and to investigate the inheritance of the newly acquired defense mechanisms.

A large number of natural *B.t.* strains have been isolated, covering a wide spectrum of insecticidal activities and directed against Lepidoptera, Coleoptera and Diptera species.

Analysis of the molecular basis of the specificity will allow to design new types of toxins with the desired activity against agriculturally important insect pests. This technology, in combination with new developments in the field of plant engineering will enable us to engineer genetically modified varieties of commercially important crops, expressing resistance agains major insect pests.

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## LITERATURE CITED

ADANG, M.J.; STAVER, M.J.; ROCHELEAN, T.A.; LEIGHTON, J.; BARKER, R.F.; THOMPSON, D.V. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstake* HD-73 and their toxicity to *Manduca sexta*. *Gene 36:* 289, 1985.

DEBLAERE, R.; BYTEBIER, B.; DE GREVE, H.; DEBOECK, F.; SCHELL, J.; VAN MONTAGU, M.; LEEMANS, J. Efficient octopine Ti plasmid-derives vectors for *Agrobacterium*-mediated gene transfer to plants. *Nucl. Acid. Res. 13*:4777, 1985.

DULMAGE, H.T. Insecticidal activity of isolates of *Bacillus* thuringiensis and their potencial for pest control. *In:* Burges, H.D. ed. *Microbial control of pests and plant diesease 1979-1980*. London, Academic Press, 1980. p. 193.

HERRERA-ESTRELLA, L.; DE BLOCK, M.; MESSENS, E.; HERNALSTEENS, J.P.; VAN MONTAGU, M.; SCHELL, J. Chimeric genes as dominant selectable markers in plant cells. *Embo. J. 2:*987, 1983.

HERRERA-ESTRELLA, L.; VAN DEN BROECK, G.; MAENHAUT, R.; VAN MONTAGU, M.; SCHELL, J.; TIMKO, M.; CASHMORE, A. Light-inducible and chloroplast-associated expression of a chimaeric gene introduced into *Nicotiana tabacum* using a Ti plasmid vector. *Nature 310:*115, 1983.

HORSCH, R.B.; FRY, J.E.; HOFFMAN, N.L.; EICHHOLTZ, D.; ROGERS, S.G.; FRALEY, R.T. A simple and general method for transferring genes into plants. *Science 227*:1229, 1985.

HUBER, H.E.; LUTHY, P.; EBERSHOLD, H.R. The subunits of the parasporal crystal of *Bacillus thuringiensis:* size, linkage and toxicity. *Arch. Microbiol. 129:*14, 1981.

GONZALEZ, J.M.; BROWN, B.J.; CARLTON, B.C. Transfer of *Bacillus thuringiensis* plasmids coding for delta-entotoxin among strains of *B. thuringiensis* and *B. cerem. Proc. natn. Acad. Sci. U.S.A. 79:*6951, 1982.

KAULEN, H.; SCHELL, J.; KREUZALER, F. Light-induced expression of the chimeric chalcone synthase-NPTII gene in tobacco cells. *Embo. J. 5:* 1, 1986.

KLIER, A.; FARGETTE, F.; RIBIER, J.; RAPOPORT, G. Cloning and expression of the crystal protein genes from *Bacillus thuringiensis* strain *berliner* 1715. *Embo. J. 1:* 791, 1982.

KRONSTAD, J.W.; SCHNEPF, H.E.; WHITELEY, H.R. Diversity of locations for *Bacillus thuringiensis* crystal protein genes. *Bact. 154:*419, 1983.

LILLEY, M.; RUFFEL, R.N.; SOMMERVILLE, H.J. Purification of the insecticidal toxin in crystals of *Bacillus thuringiensis. Gen. Microbiol. 118:* 1, 1980.

ODELL, J.T.; NAGY, F.; NAM-HAI, C. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature 313:* 810, 1985.

SCHNEPF, H.E. & WHITELEY, H.R. Cloning and expression of the *Bacillus thuringiensis* crystal protein in *Escherichia coli. PNAS 78:* 2893, 1981.

SHIBANO, Y.; YAGAMATA, A.; NAKAMARA, N.; IIZUKA, T.; SUGISAKI, H.; TAKANAMI, M. Nucleotide sequence coding for the insecticidal fragment of the *Bacillus thuringiensis* crystal protein. *Gene 34:* 243, 1985.

SIMPSON, J.; TIMKO, M.; CASHMORE, A.R.; SCHELL, J.; VAN MONTAGU, M.; HERRERA-ESTRELLA, L. Light-inducible and tissue-specific expression of a chimaeric gene under control of the 5'-flanking sequence of a pea chlorophyll a/b-binding protein gene. *Embo. J. 11:* 2723, 1985.

THOMAS, W.E. & ELLAR, D.J. *Bacillus thuringiensis* var. *israeliensis* crystal delta-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo. Cell. Sci. 60:* 181, 1983.

VELTEN, J.; VELTEN, L.; HAIN, R.; SCHELL, J. Isolation of a dual plant promoter fragment from the Ti plasmid of *Agrobacterium tumafaciens. Embo. J. 3:* 2723, 1984.