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Production of transgenic eggplant (*Solanum melongena* L.) resistant to Colorado Potato Beetle (*Leptinotarsa decemlineata* Say)

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Abstract A modified gene of *Bacillus thuringiensis* var. *Tolworthi* (Bt), encoding a coleopteran insect-specific CryIIIB toxin, was transferred via *Agrobacterium tumefaciens* to the female parent of the eggplant commercial F_1 hybrid 'Rimina'. One-hundred and fifty eight transgenic plants were regenerated and tested by PCR and NPTII expression assays. The presence of the CryIIIB toxin in leaf extracts was demonstrated in 57 out of 93 transgenic plants tested by DAS-ELISA assay. High Bt-expressing plants contained a 74-kDa protein cross-reacting with serum anti-CryIIIB toxin. Twenty three out of 44 *S. melongena* plants tested by insect bioassay showed significant insecticidial activity on neonate larvae of Colorado Potato Beetle (CPB). The Bt transgene and the toxic effect on CPB larvae were transmitted to progenies derived by selfing. Thus, transgenic Bt eggplants represent a very effective means of CPB pest control.

Key words *Bacillus thuringiensis*, var. *Tolworthi* · Coleoptera · Transgenic plant · Insect control

Introduction

Bacillus thuringiensis (Bt)-derived Cry genes have been widely used to generate transgenic plants resistant to insects (Fischhoff 1996). The level of toxin expression in Bt-transgenic plants may differ depending on the plant species, age, tissues and organs (Koziel et al. 1993). This can affect the survival of target insects. Thus, insect-plant relationships need to be investigated in each case to evaluate the potential of any field release of Bt-transgenic crops (Boulter 1993). The Colorado Potato Beetle (*Leptinotarsa decemlineata* Say) (CPB) represents the target insect of transgenic potatoes transformed with the CryIIIA gene (Perlak et al. 1993). CPB also feeds on eggplants and is the most important insect pest of this crop in Europe and America. When not properly controlled, this insect causes heavy economic losses (Cotty and Lashomb 1982; Maini et al. 1990; Arpaia et al. 1995).

Eggplants transgenic for wild-type Bt genes have shown that low expression of the transgene did not allow successful insect control (Rotino et al. 1992; Chen et al. 1995). In the present paper we report that transformation of an eggplant commercial F_1 hybrid parent with a mutagenized *B. thuringiensis* Berl. var *Tolworthi* gene (CryIIIB) results in transgene expression levels sufficient for CPB control.

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Materials and methods

Plant material

The female parent of the F_1 eggplant hybrid 'Rimina' (hereafter indicated as *Solanum melongena*), released by the Istituto Sperimentale per l'Oricoltura Sezione di Monsampolo del Tronto, was used for genetic engineering.

Vector

The Bt CryIIIB gene employed was a mutant version of the modified Bt gene reported in Iannacone et al. (1995). The binary plasmid pBinCryIIIB was obtained by subcloning the 35S-ΩTMV-CryIIIB-OCS cassette into the pBin19 plasmid (Bevan 1984), which also contains the selectable marker gene NOS-NPTII-NOS. The

disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) carrying the plasmid pBinCryIIIB was employed in the transformation experiments.

Plant transformation

The procedure for eggplant transformation was essentially as described by Rotino and Gleddie (1990) and Rotino et al. (1992) with modifications. Leaf, cotyledon and hypocotyl explants were precultured for 2 days in MS macro- and micro-nutrients (Murashige and Skoog 1962), Gamborg vitamins (Gamborg et al. 1968), 0.5 gl^{-1} of MES, 20 μM of acetosyringone supplemented with the growth regulators (mgl^{-1}) 0.5 ZEA, 0.3 BAP, 0.2 KIN and 0.1 NAA; media were solidified with 2 gl^{-1} of phytagel (Sigma), pH 5.8. For explant infection, an overnight *A. tumefaciens* liquid culture was centrifuged and the pellet re-suspended at 0.1 OD_{600} density in MS basal medium, 2% glucose, 200 μM of acetosyringone, pH 5.5. The cut edges of the hypocotyls were cut again and all the explants were infected by dipping in the bacterial suspension for 5 min, blotted dry onto sterile filter paper and then placed back in the same plates. After 48 h the explants were transferred to selective medium (described above) without acetosyringone and supplemented with 30 mgl^{-1} of kanamycin and 500 mgl^{-1} of cefotaxime. Shoot-bud differentiation and shoot elongation was achieved by transferring calli with compact green nodules to the same selective medium without NAA. Shoots were rooted and propagated in V3 medium (Chambonnet 1985) without antibiotics. Regenerated plants were labelled according to the original callus (first number) and shoot (second number). Transgenic plantlets were grown in the greenhouse and flower buds were covered with paper bags for self-pollination.

Re-callusing and kanamycin-spraying assays

Leaf-discs from putative transformants were cultured on regeneration medium containing 30 mgl^{-1} of kanamycin to verify their ability to produce callus. Expression of the NPTII marker gene was also monitored just after plantlet acclimatation by spraying with a 300 mgl^{-1} kanamycin solution according to Sunseri et al. (1993).

Polymerase chain reaction

Plant DNA was isolated from young leaves according to Doyle and Doyle (1990). PCR analysis was performed using the primers 5′ATGATTGAACAAGATGGATTGCACGCAGG3′ and 5′GAAGAACTCGTCAAGAAGGCGATA3′, which amplified a 839-bp fragment of the NPTII coding region, and the primers 5′AAGTTCGAAGTTCTGTTCCTTCCA3′ and 5′TAGTCTACAGATCTATGGGTCC3′, which amplified a 1000-bp fragment of the Bt CryIIIB coding region. PCR reactions were performed using 100 ng of template DNA in 50 μl of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% (w/v) gelatine, 200 μM dNTPs, 50 pM of each primer and 1 U Ampli*Taq* polymerase (Perkin Elmer). Amplifications were carried out in a thermocycler (Perkin Elmer) programmed for: one cycle of 5 min at 95°C; 35 cycles of 15 s at 95°C, 1 min at 60°C, 3 min at 72°C; and one final cycle of 10 min at 72°C. PCR products were subjected to electrophoresis in a 1% (w/v) agarose gel containing 0.1 μgml^{-1} of ethidium bromide.

DAS-ELISA and Western blotting

Toxin extraction was carried out by grinding a young leaf in 50 mM of Na_2CO_3, pH 9.5, 100 mM of NaCl, 0.05% Tween 20, 1 mM of phenylmethylsulfonyl fluoride and 1 μM of leupeptin. The ratio w/v was 1:10 for double-antibody ELISA (DAS-ELISA) tests and 1:3 for Western blots. For DAS-ELISA analyses we employed: a monoclonal antibody (1:1000 dilution) against CryIIIB toxin (Grassi et al. 1995) for direct coating of ELISA plates, a rabbit anti-CryIIIB toxin serum (1:6000 dilution), and a peroxidase-conjugate anti-rabbit immunoglobulin G (1:15 000 dilution). In each specific experiment, a transgenic plant was considered positive when its OD_{492} nm value was more than twice that of control plants. The approximate CryIIIB toxin concentration in plant extracts was determined by a standard (CryIIIB toxin produced by *E. coli*) according to the procedure described by Mennella et al. (1995). The total protein content was measured according to Bradford (1976). For Western-blot analyses, the samples were further diluted 1:1.5 in the above mentioned extraction buffer containing 2.5% SDS and 5% β-mercaptoethanol. After centrifugation, the supernatants were boiled for 5 min and 4 μl was analysed by PAGE. Proteins were blotted onto nitrocellulose filter and incubated with rabbit anti-CryIIIB toxin serum (1:2000 dilution) overnight at room temperature. The subsequent steps were as described by the manufacturer (ECL-Amersham). By using these procedures, 93 *S. melongena* plants representing 22 different transformation events were analyzed at least twice for CryIIIB-toxin presence by DAS-ELISA; ten plants were also analyzed by Western blotting.

Insects

Colorado Potato Beetle larvae came from a laboratory colony, reared on potted potato plants at Metapontum Agrobios since 1990. The colony is maintained every year with new individuals obtained from potato and eggplant fields.

Leaf-disc bioassays

To assess the toxicity of transformed plants, 53 transgenic plants were tested in ten different experiments. Leaf-discs were used for in vitro bioassays according to the protocol described in Iannacone et al. (1995). The effect of the treatments was established by an ANOVA completely randomized design. Differences between control and transgenic plants were investigated by using ANOVA-Dunnett's test.

Progeny analysis

Genetic analysis of four transformed T_2 progenies derived by selfing were carried out by spraying with 300 mgl^{-1} of kanamycin solution according to Sunseri et al. (1993). All plants were scored for kanamycin resistance/sensitivity by observing the absence/presence of bleaching sectors in the sprayed leaves. Data were analyzed using chi-square for evaluating segregation ratios of the active NPTII gene. Two resistant and two sensitive randomly chosen plants of the segregating progenies were transferred to pots and bioassayed.

Results

Plant transformation

A high percentage of calli (51%) were obtained from 639 leaf and cotyledon explants compared to the 14% kanamycin-resistant calli produced from 432 hypocotyl segments. However, since hypocotyl-derived calli showed a better morphogenetic response, 70% of the

158 regenerated plants were produced from hypocotyls. Transgenic plants appeared phenotypically normal and set seed upon controlled self-fertilization.

Re-callusing, spraying, Bt and NPTII PCR assays

Seventy five plantlets obtained from 31 independent putative transgenic calli were analyzed. Agreement was observed among the four kinds of assays employed regarding the presence of transgenes and the expression of kanamycin resistance (data not shown). Successful transformation was evident, based on PCR analysis, in 64 plantlets derived from 27 different calli. In a few cases both transformed and untransformed plantlets were regenerated from the same callus. Moreover, five plantlets derived from two calli were positive to the PCR test for the NPTII gene, and yet no amplification product for the Bt gene was detected (examples in Fig. 1, lanes 9 and 10).

DAS-ELISA and Western blotting

Fifty seven (61.3%) *S. melongena* plants were positive by the DAS-ELISA test. On the basis of OD_{492} values compared to results from the *E. coli*-produced CryIIIB toxin, we estimate that approximately 320 ng/ml of toxin were present in extracts of high-expressing transgenic plants. The level of CryIIIB toxin in leaves correlates significantly ($r = 0.906$) with the result of the insect bioassays, indicating that the plants with a high level of CryIIIB toxin were more toxic to CPB larvae (Table 1). In protein extracts of high Bt-expressing transgenic plants a specific immunoreactive polypeptide of approximately 74 000 Da co-migrates with the standard CryIIIB toxin generated in *E. coli* (Fig. 2). Some additional bands of lower mobility were also noted. Tissues from the untransformed control plant did not contain the 74 000 Da polypeptide, although other cross-reacting polypeptides were detected.

Insect bioassays

Larvae feeding on transgenic leaf-discs were mostly dead within 72 h. The few survivors always had a significantly lower body weight compared to control larvae and they rarely reached the second instar. Fifty two percent of the transgenic plants were toxic to neonate CPB larvae. Data from different experiments were pooled (Table 1). Sublethal effects were evident when the larval stage of the survivors is considered. Regular moults were severely hampered in larvae feeding on Bt-expressing leaves; weight reduction was a second evident effect, but was also occasionally observed in some susceptible plants. For example, larvae feeding on *S. melongena* # 1-3 and # 3-10 showed a strong weight difference compared to the control larvae, but reached the second instar as quickly as control larvae. It is concluded that data of larval fresh weight should be considered only in association with other parameters. Two plants showed sublethal effects on larvae (larval growth and moult were severely reduced compared to the control) but did not cause a significantly different mortality with respect to control plants. These partially resistant plants may be of some interest in obtaining transgenic lines exerting low selection pressure against the target insect, with the aim of delaying its possible adaptation to the resistant plants.

Comparison of detection methods

The data of six different tests available for 39 plants provided an evaluation of the NPTII and CryIIIB genes presence (PCR analyses) and activity (leaf-disc, spraying, ELISA and insect assays). Twenty six plants gave a constant response (positive or negative) to all the different tests. Six plants were successfully transformed as evidenced by a positive PCR for the Bt and NPTII genes but did not produce enough protein to be detected in the Western-blot analysis or in the bioassays

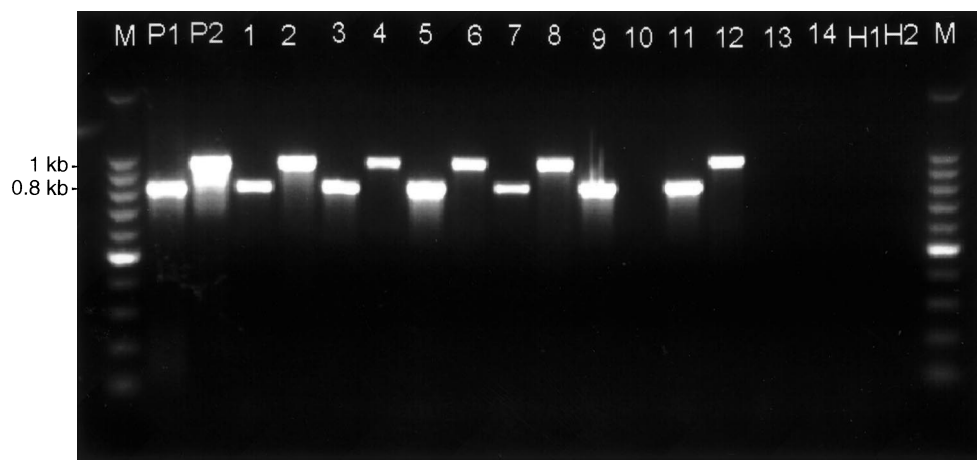

Fig. 1 PCR analysis of DNA from transgenic *S. melongena*. *Odd lanes*, amplification with NPTII gene primers; *even lanes*, amplification with Bt gene primers; *lanes: 1–2*, plant # 7-3; *3–4*, plant # 6-1; *5–6*, plant # 1-2; *7–8*, plant # 3-1; *9–10*, plant # 41-1; *11–12*, plant # 1-1, *13–14*, untransformed control plant. *P1 and P2* positive control plasmids containing the NPTII and Bt CryIIIB gene. *H1 and H2* negative control NPTII and Bt CryIIIB gene amplifications. M_1 molecular-weight marker (100-bp DNA Ladder)

Table 1 Results of insect bioassays and DAS-ELISA immunoassays. Asterisks indicate a significant difference [ANOVA Dunnett *P* (0.05)] from the control in the specific insect bioassay experiment. The O*D* ratio represents the ratio between the adsorbance value at 492 nm of the transgenic plant and that of the untransformed control in the specific DAS-ELISA experiment; nt, not tested

Plant #	Insect bioassays[a]			Toxin concentration DAS-ELISA O*D* ratio (transformed vs control)
	Mortality[b]	Larval weight (% of control)	Larvae reaching the second instars (% of control)	
3-9	100*	–	–	5.0
6-2	100*	–	–	4.7
6-6	100*	–	–	5.6
7-1	100*	–	–	5.3
7-2	100*	–	–	4.4
7-3	100*	–	–	3.7
9-1	100*	–	–	5.4
9-2	100*	–	–	5.6
9-4	100*	–	–	5.6
19-1	100*	–	–	3.6
6-3	95*	21.05*	0*	5.4
8-2	94.74*	8.97*	0*	3.6
9-3	94.74*	14*	0*	4.7
14-2	94.12*	20*	0*	6.3
4-1	90*	26.32*	0*	4.2
6-1	88.23*	15.38*	0*	5.6
6-4	88.23*	30*	0*	5.8
9-5	76.47*	36*	0*	6.5
3-2	64.71*	17.95*	0*	3.9
6-5	50*	28.95*	11.11*	3.8
28-2	16.66	31.15*	38.89*	2.1
1-1	11.76	61.54	50	1.6
1-7	11.76	27.94*	47.06*	2.9
3-4	10.53	> 100	89.47*	1.3
3-6	10	91.7	89.47	1.2
3-5	5.88	> 100	100	1.1
1-5	5.26	72*	66.32*	0.7
15-7	5	> 100	100	nt
1-2	0	81.97	100	2.2
3-1	0	67.8	100	1.1
3-7	0	96.15	100	1.3
3-11	0	63.24	> 100	1.0
3-12	0	61.76	> 100	1.0
5-1	0	74.58	> 100	2.1
5-2	0	> 100	94.74	3.2
5-9	0	> 100	> 100	2.1
41-1	0	73.53	> 100	1.1
2-1	− 5.26	> 100	> 100	1.0
1-3	− 5.80	57.35	100	2.1
13-1	− 5.88	97.44	100	2.4
2-6	− 11.76	89.71	> 100	1.0
3-3	− 11.76	80.88	> 100	1.0
3-8	− 11.76	> 100	> 100	1.2
3-10	− 11.76	54.41	100	1.0
34-1	− 11.76	75	> 100	1.0

[a] Data collected at 72 h
[b] Expressed according to the Abbott's formula

(e.g. plant # 3-1 in: Fig. 1, lanes 7–8; Fig. 2, lane 2; Table 1). Six other plants produced CryIIIB protein as evidenced by DAS-ELISA tests, but the amount of toxin was not sufficient to show a significant effect on CPB neonate larvae. The higher sensitivity of the DAS-ELISA test when compared to the CPB larvae bioassay had already been proven in previous laboratory experiments with CryIIIB toxin expressed in *E. coli* (Mennella et al. 1995). In one case the NPTII gene was present in the plant DNA but the Bt gene was not.

Progeny analysis

Among the T_2 progenies sprayed with kanamycin, the chi-square test showed a 3 KmR : 1 KmS ratio as

expected for a monogenic dominant trait in the progeny derived from plant # 9-3. Two other plants segregated as if two independent loci were involved in kanamycin resistance. All the selfed seed-derived plantlets of *S. melongena* # 6-2 were resistant to kanamycin, suggesting the integration of several T-DNA copies in different chromosomes and/or independent integration in a close allelic position into two homologous chromosomes (Table 2). Insect bioassays conducted on two chlorotic and on two symptomless plants, chosen among the plants of the selfed segregating progenies, showed that in all cases kanamycin resistance co-segregated with the insect resistance trait (data not shown) (Fig. 3).

Among the types of explants tested, hypocotyl segments showed a better capability to regenerate shoots. Since this process is very fast, a second cutting was done to expose fresh-wounded less-differentiated cells to agrobacteria and so reduce the frequency of escapes. The presence of transgenic and non-transgenic plants from the same callus could be due a chimeric origin of some selected calli. An incomplete integration of the Bt gene might explain the fact that, in a few cases, the presence and expression of selectable NPTII gene was noted while both the expected Bt gene fragment (lacking at least one of the annealing sites) and CryIIIB protein were not detected.

Discussion

Bt-expressing transgenic plants of worldwide important plant species such as rice, potato and corn have already reached the market. Valuable vegetable crop species, cultivated on a smaller scale, are also of interest particularly if their transformation and the level of Bt gene expression can be optimized. This article reports on the engineering of transgenic eggplants bearing a mutagenized Bt gene which allows for the production of a level CryIIIB protein sufficient to control CPB. Transformed plants of *S. melongena* showed complete protection from CPB larval attack. Indeed, the transgenic plants were so toxic that individuals surviving after 3 days of feeding on transgenic leaves were rarely visible.

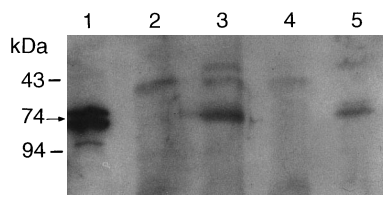

Fig. 2 Western-blot analysis of *S. melongena* plants and *E. coli* extracts. Gel electrophoresis and blotting onto nitrocellulose membrane was performed by a Phast System apparatus (Pharmacia). *Lane 1*, standard Bt-toxin generated in *E. coli*; *lane 2*, plant # 3-1; *lane 3*, plant # 6-2; *lane 4,* untransformed control plant; *lane 5*, plant # 9-2

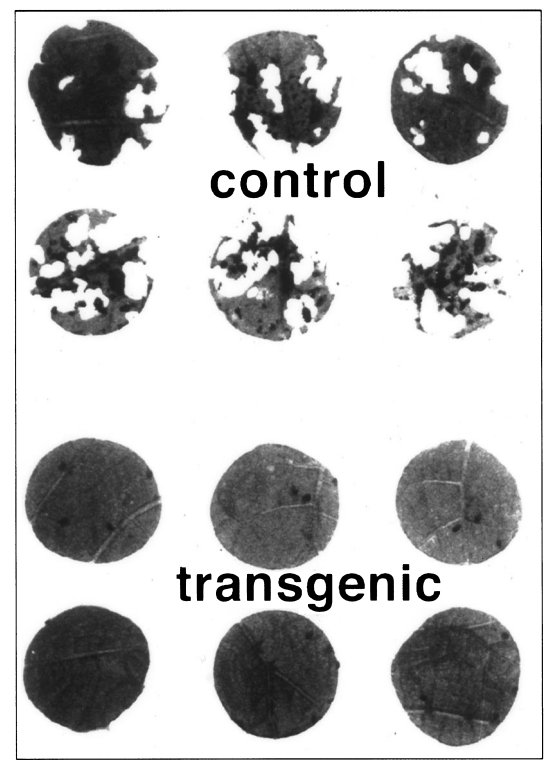

Fig. 3 Insect bioassay carried out on progenies derived from self-pollination of plant # 9-3. The two upper rows show Petri plates containing the larvae eating on leaf-discs cut from plants which showed chlorotic symptoms due to the kanamycin spray treatment. Larvae of the two lower rows were fed with leaf-discs taken from non-chlorotic plants

Table 2 Segregation ratios for kanamycin resistance in four T_2 progenies obtained by selfing (KmR, kanamycin-resistant: KmS, kanamycin-sensitive)

Progeny #	Tested plants	Phenotype		Ratio (KmR/KmS)	χ^2 value	*P* value
		KmR	KmS			
9-3	114	89	25	3:1	0.582	0.40–0.50
5-1	92	88	4	15:1	0.713	0.30–0.40
5-5	100	92	8	15:1	0.709	0.30–0.40
6-2	119	119	0	–	–	–
Control	114	0	114	–	–	–

The results indicate that DAS-ELISA represents a sensitive test having a remarkable specificity to detect a very low amount of CryIIIB toxin expressed in transgenic plants. In the most actively expressing plants, CryIIIB toxin levels ranged from 800 to 1400 ng per g fresh weight and these levels were sufficient to demonstrate that extracts of plants contain a polypeptide of the same size as the CryIIIB toxin. Low amounts of the toxin, still detectable with immunochemical methods, did not prevent CPB larvae from damaging plants. Nevertheless, the high degree of accordance between the DAS-ELISA test and the insect bioassay allows a rapid screening of transgenic eggplants. The identification of eggplant lines with high, medium or low toxic effect on CPB will enlarge the possibility of testing different methods (McGaughey and Whalon 1992) for an effective management of transformed CPB-resistant eggplants.

Gene transmission to the progeny occurred according to the expected Mendelian ratio, except for one case which gave all-resistant plants. The correlation between the absence of bleaching (resistance to kanamycin) in the leaves and the presence of an active Bt gene has been demonstrated based on the insect bioassay.

Experimental field trials with our transgenic eggplant lines are now planned in order to follow protein expression over time and in different tissues.

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