Introduction of Delta-endotoxin Gene of *Bacillus thuringiensis* to Chrysanthemum [*Dendranthema* × *grandiflorum* (Ramat.) Kitamura] for Insect Resistance

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We introduced a truncated delta-endotoxin gene, *cryIAb* of *Bacillus thuringiensis* which has a specific biological activity against lepidopteran insects into chrysanthemum [*Dendranthema* × *grandiflorum* (Ramat.) Kitamura]. The chrysanthemum cultivar 'Shuho-no-chikara' was transformed using a disarmed strain of *Agrobacterium tumefaciens*, LBA4404, carrying a binary vector, pIAbT1 that harboured a *cryIAb* gene encoding an insecticidal crystal protein fragment of *B. thuringiensis* var. *kurstaki* HD-1. Leaf discs were co-cultured with *Agrobacterium* and thereafter cultured on the callus induction medium containing G418. A total of 92 shoots were regenerated from 1,760 leaf discs on the regeneration medium (5.2%). The *cryIAb* gene was detected in all the regenerated plantlets by Southern blot analysis. The accumulation of Cry1Ab protein in 20 transformed lines, selected at random, was confirmed by Western blot analysis. The level of accumulation of Cry1Ab protein ranged from 4.5 ng to 40 ng per 50 µg total soluble protein (from 0.009 to 0.08% of the total protein). Insect bioassay was conducted using tobacco budworm (*Helicoverpa armigera*) larvae. On the lines showing a high expression of Cry1Ab (more than 32.5 ng per 50 µg of total soluble protein), a significantly higher feeding inhibition and/or growth inhibition of the insects was observed, compared to those on the non-transformed control plants.

Key Words: chrysanthemum, *Agrobacterium tumefaciens*, transformation, *Bacillus thuringiensis*, insect resistance.

Introduction

Chrysanthemum [*Dendranthema* × *grandiflorum* (Ramat.) Kitamura] was introduced into Japan from China during the Nara Era (A.D.710-794), and is now one of the important ornamental plants characterized by conventional vegetative propagation by stem cutting, adventitious shoot formation from various tissues and calli (Hill 1968, Iizuka *et al.* 1973, Earle

and Langhans 1974, Khalid *et al.* 1989).

Insect damage is one of the serious problems in chrysanthemum cultivation resulting in yield loss and high pesticide cost. For controlling lepidopteran insect pests, synthetic chemical insecticides are applied several times during the plant growing season. The use of chemical insecticides is limited by the cost, pollution of the environment, and development of resistance in insects resulting in a low effectiveness.

Bacillus thuringiensis (*Bt*), a Gram-positive bacterium, produces insecticidal crystal proteins upon sporulation. These proteins called delta-endotoxins or *Bt*-toxins, are highly toxic to lepidopteran (Dulmage 1981), dipteran (Goldberg *et al.* 1977) and coleopteran insects (Krieg *et al.* 1983). *Bt*-toxins are considered to act as 'biorational' control agents due to the lack of toxicity to vertebrates and non-target insects (Parrott *et al.* 1994). Microbial formulations have been used safely for almost 30 years. However, the limited field stability is a major constraint on the use of commercial *Bt*-toxin preparations. Also the time of application is critical because the susceptibility to *Bt*-toxins decreases as larvae mature.

Recently a gene encoding *Bt*-toxin (*Bt*-gene) has been successfully introduced into various crops including tobacco (Vaeck *et al.* 1987, Barton *et al.* 1987), tomato (Fischhoff *et al.* 1987), cotton (Perlak *et al.* 1990), maize (Kozziel *et al.* 1993, Armstrong *et al.* 1995) and rice (Fujimoto *et al.* 1993), resulting in protection against insect attacks. No *Bt*-genes have been introduced into chrysanthemum for pest management because of the difficulty in transformation. Shinoyama *et al.* (1998) have developed a highly susceptible and stable *Agrobacterium*-mediated transformation system for chrysanthemum.

This is the first report on transgenic chrysanthemum plants expressing a gene encoding *Bt*-toxin and on the effect on a lepidopteran pest, *Helicoverpa armigera*.

Materials and Methods

Plant material

The chrysanthemum [*Dendranthema* × *grandiflorum* (Ramat.) Kitamura] cultivar 'Shuho-no-chikara' was used in this experiment. The plants growing in the greenhouse were surface-sterilized in 70% ethanol, and then in a 1% sodium hypochlorite solution for 15 min. They were rinsed 3 times in sterile distilled water. The plants were cultured *in vitro*

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(meristem culture) on Murashige and Skoog (1962) basal medium (MS), containing 3% sucrose and 0.3% Gellan Gum (Pure Chemical Inc., Japan). The medium was adjusted to pH 5.8 prior to autoclaving at 121°C for 15 min. The plants were cultured at 25°C under a 16 h photoperiod using cool-white fluorescent lamps or at 25°C in darkness. The lamps provided a photosynthetic photon flux [PPF (400~700 nm)] of 60 $\mu\text{mol}/\text{m}^2/\text{s}$.

Agrobacterium strain and binary vector

Agrobacterium tumefaciens strain LBA4404 (Ooms *et al.* 1982) harboring a binary vector pIAbT1 was supplied by Dr. S. Asano (University of Hokkaido) (Fig. 1). The vector pIAbT1 has a 1,967 bp fragment containing an active region of the *cryIAb* gene (Kondo *et al.* 1987) with a pair of synthetic linkers of *Bam*HI site and is constructed from pBI121 (Jefferson 1987). The coding region of the gene contains a DNA sequence corresponding to the amino acid positions 29 to 618. The *cryIAb* gene was driven by the CaMV 35S

promoter and the nopaline synthase (Nos) terminator. Kanamycin-resistance gene (*npt II*) was driven by the Nos promoter and Nos terminator.

Plant transformation

The transformation system is shown in Table 1 and Fig. 2 (Shinoyama *et al.* 1998). The leaf segments were cut with a cork-borer. They were immersed for 30 min at room temperature into a suspension containing 5.0% Tween 20 and 50 μM acetosyringon in which *Agrobacterium* had been cultured for 5 h. After immersion, the leaf segments were placed onto a callus induction medium containing 1.0 g/l casamino acid and co-cultured for 3 days at 25°C in the dark.

The leaf segments were transferred to a medium containing 250 mg/l cefotaxime sodium salt for the elimination of *Agrobacterium*, and after 10 days, they were transferred to a selection medium containing 20 mg/l G418 antibiotics for the selection of putative transformed plantlets. The leaf segments which formed green calli were transferred to a regeneration medium for the regeneration of plantlets.

Analysis of Transformed lines

1. Southern blot analysis

Total DNA was extracted from 100 mg of fresh young leaves of the regenerated plantlets or control plantlets by the method of Takagi *et al.* (1993). The leaves were homogenized in liquid nitrogen using a ceramic mortar and a pestle and suspended in 1 ml of HEPES buffer [0.1 M HEPES (pH 8.0), 0.1% PVP, 4% 2-mercaptoethanol]. After centrifugation at 15,000 rpm for 5 min at 4°C, the supernatant was discarded and the pellet was resuspended in a new HEPES

Fig. 1. Structure of Vector pIAbT1, an expression vector pBI121 into which the truncated *cryIAb* gene of *B. thuringiensis* var. *kurstaki* HD-1 was introduced. The coding region of the gene contains the DNA sequence corresponding to amino acid positions 29 to 618.

Table 1. Timetable for transformation of chrysanthemum

Day	Activity
0	Infection with <i>Agrobacterium</i> cultured for 5 h.
3	End of co-culture (72 hr.)
	Transfer to the medium for elimination of <i>Agrobacterium</i> (cef. 250 mg/l)
10	Transfer to selection medium I (cef. 250 mg/l, G418 20 mg/l)
24	Transfer to fresh selection medium I
38	Transfer to fresh selection medium I
52	Transfer to selection medium II (cef. 100 mg/l, G418 20 mg/l)
66	Transfer to fresh selection medium II
80	Transfer of G418-resistant calli to regeneration medium
101	Transfer to fresh regeneration medium
122	Transfer to fresh regeneration medium
	First harvest of shoots
143	Transfer to fresh regeneration medium
	Second and final harvest of shoots
143-180	Rooting and transfer to green house
200 onwards	Clones available for testing

*cef.: cefotaxime sodium salt Media used

Co-culture medium : MS + NAA 1.0 mg/l, BA 0.5 mg/l, Casamino acids 1.0 g/l, Suc.3%, Gel 0.3%

Medium for elimination of *Agrobacterium*: MS + NAA 1.0 mg/l, BA 0.5 mg/l, Suc.3%, Gel 0.3%, cef. 250 mg/l

Selection medium I: MS + NAA 1.0 mg/l, BA 0.5 mg/l, Suc.3%, Gel 0.3%, cef. 250mg/l, G418 20mg/l

Selection medium II: MS + NAA 1.0 mg/l, BA 0.5 mg/l, Suc.3%, Gel 0.3%, cef. 100 mg/l, G418 20 mg/l

Regeneration medium: MS + BA 0.5 mg/l, GA3 0.2 mg/l, Suc.3%, Gel 0.4%, cef. 100 mg/l

Fig. 2. Regeneration of transformed chrysanthemum cv. 'Shuho-no-chikara' with the *cry1Ab* gene of *Bacillus thuringiensis*

- (A) Callus formation from leaf disk 6 weeks after *Agrobacterium* inoculation
- (B) Adventitious shoot formation from transformed calli after 9 weeks of culture on regeneration medium containing G418
- (C) Plantlet growth from adventitious shoot after 3 weeks on rooting medium
- (D) Transformed lines established in the greenhouse

buffer. This procedure was repeated three times to remove polyphenols and polysaccharides. Total DNA was isolated from the pellet by the sodium dodecyl sulfate (SDS) extraction method according to the method of Honda and Hirai (1990).

The DNA digested with *EcoRV* was subjected to electrophoresis and blotted onto a Hybond N+ nylon membrane (Amersham Pharmacia Biotech UK Ltd., England). Southern analysis (Southern 1975) was carried out using a *cry1Ab* gene fragment (250 bp) as a probe (see Fig. 1), with digoxigenin (DIG) labeling and the CDP-star substrate detection system (Roche & Boehringer Mannheim, Germany), according to the supplier's instructions.

2. Western immunoblot analysis

Protein was extracted from 1 g of fresh young leaves of the transformed lines and non-transformed control. The leaves were homogenized in liquid nitrogen using a ceramic mortar and a pestle, and extracted with 1 ml SDS grinding buffer [200 mM HEPES Buffer (pH 8.0), 20 mM EDTA, 100 mM DTT, 200 mM PMSF, 0.2 mg/ml Leupeptin, 50 mg/ml Polyclar VT], and centrifuged at 15,000 rpm for 10 min at room temperature. Protein content of the supernatant was determined by the Bradford method (Bradford 1976). Each gel lane was loaded with a leaf extract corre-

sponding to 50 µg total protein. The proteins were separated on 10.0% SDS-PAGE gels (Laemmli 1970) and blotted onto a PVDF membrane (MICRON SEPARATIONS Inc., USA) using a semidry transfer cell (BioRad, USA). Western blot analysis was performed according to the alkaliphosphatase procedure of Koziel *et al.* (1993). The purification of an antigen, Cry1Ab protein, was modified from the method of Mochizuki (1994). *E. coli* JM109: pIAbT1 was grown at 37°C in Terrific Broth liquid medium containing 0.1 mM IPTG overnight. The suspension was centrifuged at 8,000 rpm for 8 min at 4°C and the pellet was resuspended with A buffer [20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 2 mM PMSF]. Then the samples were centrifuged and resuspended with A buffer containing 0.1 mg/ml lysozyme. They were sonicated for 30 min and centrifuged and resuspended with A buffer containing 0.1 mg/ml lysozyme. This procedure was repeated three times to remove water-soluble protein at pH 7.5. After centrifugation, the pellet was resuspended in B buffer [50 mM Glycin-NaOH (pH 10.0), 10 mM DTT, 2 mM PMSF, 5 mM EDTA] and mixed well for 30 min at room temperature. Then it was dialyzed against distilled water for 3 days and centrifuged at 10,000 rpm for 15 min at 4°C. The proteins were separated on 10.0% SDS-PAGE gels (Laemmli 1970) and Cry1Ab was extracted from the gel at 62 kDa by electroelution.

Cry1Ab was detected immunologically using rabbit polyclonal antibodies against delta-endotoxin from *B. thuringiensis* var. *kurstaki* HD-1. The polyclonal antibodies were prepared by immunizing rabbits with the delta-endotoxin which was produced by *B. thuringiensis* var. *kurstaki* HD-1. The primary antibodies were visualized using goat anti-rabbit immunoglobulins with alkaline phosphatase (BIO-SOURCE International, USA). The density of each band was compared to that of pure Cry1Ab protein (5, 25 and 50 µg) by using a Densitograph AE-6920V-05 (ATTO, Japan).

3. Insect bioassay on transformed lines

The tobacco budworms, *Helioverpa armigera* (Lepidoptera; Noctuidae) were supplied by Mr. T. Fukuda (Kagoshima Prefectural Agricultural Experiment Station). The larvae were reared on an artificial diet (Insecta LF; Nihon Nosan Kogyo, Japan) under 16 h light (60 µmol/m²/s) at 25°C.

Two experiments were performed. The first experiment was performed to examine the degree of resistance of the transformed lines with the *cry1Ab* gene to feeding by *H. armigera* larvae. In the first experiment, on the 5th day after larval feeding, the area of the part of the leaf which could not be fed by the larvae was measured using Leaf Area Measure (Hayashi Denko, Japan). Five first instar larvae of *H. armigera* were allowed to feed on the leaves of the transformed lines. The leaves (1.5 cm length) from each line were detached from the plants, and the petioles were placed in moist cotton. They were put in plastic boxes (φ = 9 cm and 6 cm in depth), with one leaf per box.

The second experiment was performed to examine the effect of the leaves producing the Cry1Ab protein on the growth of the larvae. In the second experiment, leaves (5 cm length) of the transformed lines and non-transformed controls were placed in plastic boxes (φ = 9 cm and 6 cm in depth) containing perlite (about 3 cm in depth) at the bottom. Perlite is necessary for the larvae to become pupae. A third instar larva was put in each box and the culture was continued until the larvae became pupae. When the leaf was fed or wilted, it was replaced by a new leaf. We examined the duration of the larval period (days) from the third instar larvae to pupae, the pupation rate, pupal duration and adult emergence rate. In each experiment, 10 larvae were used for each transformed line and control. Three replicatings were performed.

The average of the respective data was compared by t-test. Percentages of the parameters studies (feeding area, pupation rate and emergence rate) were arcsine-transformed prior to analysis.

Results

1. Regeneration rate

On the selection medium containing 20 mg/l G418, 162 leaf segments among 1,760 formed green calli, with a

callus induction rate of 9.2%, and 92 plantlets were regenerated from the calli on the regeneration medium, corresponding to a 5.2% regeneration rate from initial leaf segments.

2. DNA isolation and analysis

The presence and the copy number of the *cry1Ab* gene in the plantlets were confirmed by Southern blot analysis. One unique band hybridized to the *cry1Ab*-probe was observed in each regenerated plantlet. No hybridization signal was detected in the non-transformed control (Fig. 3). Thus the results indicated that each transformed line harboured one copy of the *cry1Ab* gene.

3. Accumulation of Cry1Ab protein in transformed lines

We selected 20 transformed lines at random. A distinct band could be detected at 56 kDa in all the transformed lines, although pure Cry1Ab protein from *Escherichia coli* JM109: pIAbT1 was detected at 62 kDa. The level of accumulation of the Cry1Ab protein ranged from 4.5 ng to 40 ng per 50 µg total soluble protein (from 0.009 to 0.08% of the total soluble protein) in the transformed lines. No band was detected in the non-transformed controls (Fig. 4).

4. Insect bioassay on transformed lines

The feeding area of the transgenic plants by the *H. armigera* larvae was significantly smaller than that of the non-transgenic controls at 5% level, when the accumulation level of the Cry1Ab protein was higher than 15.5 ng per 50 µg of total protein (Fig. 5, Table 2). The larval duration from the third instar to pupa of the insects fed on the transgenic plants was significantly longer than that of the insects fed on the control plants when the Cry1Ab accumulation level exceeded 32.5 ng per 50 µg of total protein (Table 2). Regarding the pupal duration, the transgenic lines with an expression level of Cry1Ab protein of 32.5 and 36.5 ng per

Fig. 3. Southern blot analysis.

Genomic DNA digested with *EcoRV* and hybridized with the *cry1Ab* specific probe.

Lane 1: pIAbT1

Lane 2: Non-transformed plant 'Shuho-no-chikara'

Lanes 3-10: Bt-transformed lines [Bt(b)-1 - Bt(b)-8]

Fig. 4. Western immunoblot analysis of leaf from chrysanthemum plants expressing the *cry1Ab* gene.

Lanes 1, 2 and 3: 50, 25 and 5 ng of Cry1Ab protein

Lane 4: Non-transformed plant 'Shuho-no-chikara';

Lanes 5-12: Bt-transformed lines (5: Bt(b)-1, 6: Bt(b)-2, 7: Bt(b)-3, 8: Bt(b)-5, 9: Bt(b)-7, 10: Bt(b)-10, 11: Bt(b)-11, 12: Bt(b)-12)

50 µg of soluble total protein was loaded on 4 to 12 lanes.

50 µg total protein (Bt(b)-2 and -12, respectively) were significantly more effective than the controls, while in the transgenic lines accumulating a Cry1Ab level of 40 ng per 50 µg of total protein (Bt(b)-11), the pupal duration was not signifi-

Fig. 5. Bioassay of insect feeding on the chrysanthemum leaf.

The five first instar larvae of *H. armigera* were allowed to feed on leaves of transformed and non-transformed plants.

C: non-transformed plant 'Shuho-no-chikara'

1-12: Bt-transformed lines [Bt(b)-1 - Bt(b)-12]

Table 2. Insect bioassay of transformed lines using *Heliocoverpa armigera*

Lines	Cry1Ab expression (ng/50 µg total protein)	Experiment 1		Experiment 2		
		Feeding leaf area (%)	Larval duration ¹⁾ (days)	Pupation rate (%)	Pupal duration ¹⁾ (days)	Emergence rate (%)
Bt(b)-11	40.0	4.7**	21.6 ± 0.04*	85.6	16.1 ± 0.10	70.0*
Bt(b)-12	36.5	5.7**	21.7 ± 0.05*	87.8	17.5 ± 0.04*	87.5
Bt(b)-2	32.5	5.9**	20.1 ± 0.12*	86.7	17.0 ± 0.05*	81.7
Bt(b)-1	20.0	14.2*	18.8 ± 0.10	92.2	15.2 ± 0.13	78.6
Bt(b)-9	15.5	14.4*	18.0 ± 0.14	94.4	15.5 ± 0.10	84.8
Bt(b)-6	13.5	20.0	17.9 ± 0.04	92.2	16.0 ± 0.06	86.6
Bt(b)-3	12.5	46.2	14.6 ± 0.02	93.3	14.7 ± 0.04	85.7
Bt(b)-4	11.5	44.8	17.7 ± 0.13	95.6	15.9 ± 0.02	86.1
Bt(b)-19	10.5	42.9	17.4 ± 0.10	93.3	15.2 ± 0.13	89.2
Bt(b)-8	9.5	43.5	13.6 ± 0.06	96.7	13.9 ± 0.12	84.8
Bt(b)-18	9.5	46.2	15.8 ± 0.08	88.9	15.3 ± 0.10	92.5
Bt(b)-7	8.5	47.4	13.8 ± 0.10	96.7	15.2 ± 0.03	82.8
Bt(b)-20	8.5	53.2	14.7 ± 0.12	87.8	13.6 ± 0.02	91.3
Bt(b)-5	7.5	63.9	13.1 ± 0.12	87.8	15.1 ± 0.10	90.1
Bt(b)-10	6.5	58.0	12.1 ± 0.10	96.7	13.1 ± 0.12	83.8
Bt(b)-13	6.5	58.9	13.3 ± 0.03	94.4	13.2 ± 0.12	82.3
Bt(b)-14	5.5	54.2	14.5 ± 0.02	93.3	13.7 ± 0.10	78.6
Bt(b)-17	5.5	59.8	14.4 ± 0.05	94.4	13.8 ± 0.03	88.3
Bt(b)-15	4.5	61.0	15.7 ± 0.10	92.2	15.1 ± 0.02	85.6
Bt(b)-16	4.5	62.1	13.0 ± 0.10	96.7	12.8 ± 0.10	85.9
Control	—	62.9	12.0 ± 0.13	94.4	13.3 ± 0.12	85.9

Cry1Ab accumulation was determined using a Densitograph (ATTO co.) compared to pure Cry1Ab protein.

Feeding area was measured with Leaf Area Measure (Hayashi Denko co.) on the 5th day using first instar larvae.

Larval duration, Pupation rate, Pupal duration and Emergence rate were determined by using the data of days or rate of third instar larvae to pupa and pupa to adult.

¹⁾; Average ± SE

*and **: significant at the 5% and 1% levels, respectively

cantly different from that of the control plants. The rate of adult emergence significantly decreased only in the Bt(b)-11 line (accumulation level; 40 ng per 50 µg) (Table 2).

The larval mortality was not significantly different between the transformed lines and the control plants.

Discussion

We have generated transgenic chrysanthemum plants in which the *Bt*-toxin gene was expressed using our stable and highly efficient transformation system of chrysanthemum mediated by *Agrobacterium* (Shinoyama *et al.* 1998).

Bt-toxin genes were classified based on amino acid sequence homologies and insecticidal activities. Padidam (1992) found that the Cry1Ac protein from *B. thuringiensis* was 12 times more toxic than the Cry1Ab protein to *H. armigera*. On the other hand, *Spodoptera exigua*, *S. litura* and *Mamestera brassicae*, which are also important pests of chrysanthemum, were more sensitive to the Cry1Ab protein than to the Cry1Ac protein (Perlak *et al.* 1990, Höfte *et al.* 1988, Asano *et al.* 2000). Therefore, for the protection of chrysanthemum against various lepidopteran pests, *cry1Ab* transgenic plants may be more suitable than *cry1Ac* transgenic plants.

The molecular size of the Cry1Ab protein of the transformed lines was 56 kDa, which was different from that produced by *Escherichia coli* JM109 (62 kDa). The Cry1Ab protein has trypsin cleavage sites located between amino acid positions 28 and 29, and 601 and 602, and the trypsin-resistant polypeptide overlaps with the low activity toxin corresponding to about 60 kDa (Höfte *et al.* 1986). Nagamatsu *et al.* (1984) also isolated a trypsin-resistant toxic core peptide of 58 kDa for the Cry1Ab protein prepared from *B. thuringiensis* var. *dendrolimus*. Fujimoto *et al.* (1993) detected two proteins with a smaller size than that of Cry1Ab, which reacted with the Cry1Ab antibody in leaf extracts of transgenic rice plants and estimated that the size of the smallest protein was 57 kDa. They suggested that these smaller proteins might be derived from shorter mRNAs, or that the Cry1Ab protein may be partially degraded in rice leaf cells. They indicated that the two smaller proteins were biologically active, since the biological activity of Cry1Ab is presented at amino acid positions 29 to 607. In an other case, Perlak *et al.* (1990) reported the existence of a protein with a larger size than that of the trypsin-treated Cry1Ab protein. Thus the size of the Cry1Ab protein was different in each plant. In our case, we used the *cry1Ab* gene corresponding to the amino acid positions 29 to 618. The 56 kDa protein detected in our transgenic chrysanthemum plants is likely to be the protein digested with the endogenous trypsin of chrysanthemum at the second trypsin cleavage site (amino acid positions 601 to 602 from the N-terminal portion) of the 62 kDa Cry1Ab protein (amino acid positions 29 to 618), and the insect toxicity may be conserved.

The *Bt*-gene was poorly expressed in some plants,

because of the rapid degradation of the mRNA (Rocher *et al.* 1998). Some A/T-rich sequences in the *Bt*-gene have been reported to contribute to mRNA instability in plants (Ohme-Takagi *et al.* 1993, Chen and Shyu 1995). The *Bt*-toxin level in plants increased due to the removal of A/T-rich sequences from the *Bt*-gene, up to 100-fold (Perlak *et al.* 1990, 1991). In this case, the maximum expression level of the non-modified *Bt*-gene was 1 ng per 50 µg of total protein. In our case, the maximum expression level of the non-modified *Bt*-gene was 40 ng per 50 µg of total protein in chrysanthemum, which is comparable to the level in transgenic cotton plants with sequence-modified *Bt*-gene (Perlak *et al.* 1990). The reason for such a high expression of the non-modified *Bt*-gene in chrysanthemum remains to be elucidated. Perlak *et al.* (1990) succeeded in protecting cotton plants against the cotton bollworm, *Heliothis* (= *Helicoverpa*) *zea*, using transgenic plants with modified *cry1A(b)* gene in which the expression level of the Cry1Ab protein was up to 0.1% of total soluble protein. *Helicoverpa armigera* is a closely related species to *H. zea*. We succeeded in inhibiting the feeding and delaying the growth of *H. armigera* larvae on the transgenic plants accumulating Cry1Ab at a level of at least 0.07% of total soluble protein. The larval mortality on the transgenic plants was not different from that on the control non-transgenic plants.

Field tests with *Bt*-transgenic tomato plants have indicated higher levels of expression are required to control the agronomically important lepidopteran pests (Delannay *et al.* 1989). In this experiment, the transgenic plants accumulating Cry1Ab at a level of at least 0.07% of total soluble protein, displayed only insect feeding inhibition effects but did not exhibit insecticidal effects. Recently, the inactivation of transgenes (silencing) has been observed in chrysanthemum (Pavingerova *et al.* 1994, Benetka and Pavingerova 1995, Takatsu *et al.* 2000). GUS activity level in transgenic chrysanthemum was 10-fold lower than that of tobacco (Daub *et al.* 1994) and 100-fold lower than that of *Kalanchoe blossfeldiana* (Aida and Shibata 1996). In addition, Wordrangen *et al.* (1992) reported that the expression of the GUS gene driven by the cauliflower mosaic virus (CaMV35S) promoter started slowly in chrysanthemum (5 days after infection) compared to tobacco plants (2 days after infection). Since these facts indicated that the CaMV35S promoter is weaker in chrysanthemum than in tobacco plants, it will be necessary to clone chrysanthemum original promoters to obtain a high expression of *Bt*-genes.

We should modify the coding sequence of the *cry1Ab* gene by removing A/T-rich regions resembling plant introns (Goodall and Filipowicz 1989), potential plant polyadenylation signal sequence (Dean *et al.* 1986), ATTTA sequence (Shaw and Kamen 1986) and codons seldom used in Compositae to increase Cry1Ab protein accumulation in chrysanthemum.

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