Insect Resistance in Transgenic Chrysanthemum [*Dendranthema × grandiflorum* (Ramat.) Kitamura] by the Introduction of a Modified δ -endotoxin Gene of *Bacillus thuringiensis*

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We introduced a modified delta-endotoxin gene, modified *cry1Ab* (*mcbt*) of *Bacillus thuringiensis*, which displays 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*, EHA101, carrying a binary vector, pIG121*mcbt* that harboured a *mcbt* gene encoding an insecticidal crystal protein (ICP) 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 317 shoots were regenerated from 3,618 leaf discs on the regeneration medium (8.8%). The *mcbt* gene was detected in all the regenerated plantlets by Southern blot analysis. The accumulation of Cry1Ab ICP in 20 transformed lines, selected at random, was confirmed by Western blot analysis. The level of accumulation of Cry1Ab ICP ranged from 10.5 ng to 80 ng per 50 μ g total soluble protein (from 0.021 to 0.16% of the total protein). Insect bioassay was conducted using tobacco budworm (*Helicoverpa armigera*) larvae and all the larvae of *H. armigera* tested died during the first instar on the leaves of the *mcbt*-transformed lines whose expression level of Cry1Ab ICP exceeded 47.6 ng per 50 μ g compared to the wild type *cry1Ab*-transformed lines. Significantly higher feeding inhibition and/or growth inhibition of the insects was observed, compared to those on the non-transformed control plants.

Key Words: *Helicoverpa armigera*, chrysanthemum, transformation, modified *cry1Ab* gene, insect resistance.

Introduction

Insect damage is a serious problem in chrysanthemum [*Dendranthema × grandiflorum* (Ramat.) Kitamura], result-

ing in yield loss and high pesticide cost. Lepidopteran insects are responsible for a substantial proportion of the loss, particularly 3 species, *Helicoverpa armigera*, *Mamestra brassicae* and *Trichoplusia intermixta*. The introduction of an insect resistance gene such as delta-endotoxin gene of *Bacillus thuringiensis* (*Bt* gene) into chrysanthemum provides an alternative technology that may be able to avoid the problems associated with field application of commercial insecticide formulations.

We introduced the *cry1Ab* gene, one of the *Bt* genes, into chrysanthemum for conferring an insect resistance. However, the expression of the insecticidal crystal protein (ICP) encoded by the *cry1Ab* gene in chrysanthemum was low, compared to that of many other heterologous genes. These transgenic chrysanthemums showed a low resistance to the tobacco budworm (*Helicoverpa armigera*).

For effective control of major insect pests, the expression level of the *Bt* genes should be enhanced. To increase the expression level of the *Bt* genes in transgenic plants, *Bt* genes have been extensively modified, fused to highly efficient promoters and introduced into tobacco (Perlak *et al.* 1991, Sutton *et al.* 1992), tomato (Perlak *et al.* 1991), potato (Adang *et al.* 1993), cotton (Perlak *et al.* 1990), rice (Fujimoto *et al.* 1993) and maize (Kozziel *et al.* 1993). To achieve a high expression of these prokaryotic genes in higher plants, modification of the coding sequence, such as removal of ATTTA sequences and use of the preferred codons, was undertaken. Truncation of the coding sequence of such synthetic *Bt* genes further increased the expression levels.

The low level of expression of the *Bt* gene in plants is due to mRNA instability (Ohme-Takagi *et al.* 1993, Chen and Shyu 1995), which mainly results from the presence of ATTTA sequences (Murray *et al.* 1991, Van Aarssen *et al.* 1995). Modification of the coding sequences successfully enhanced plant expression of *Bt* genes (Perlak *et al.* 1990, 1991, Sutton *et al.* 1992, Adang *et al.* 1993, Kozziel *et al.* 1993).

In this paper, we modified the coding sequence of the *cry1Ab* gene by removing ATTTA sequences (Dean *et al.* 1986, Shaw and Kamen 1986, Goodall and Filipowicz 1989) and by using the preferred codons of *Compositae* family to increase the *cry1Ab* expression in chrysanthemum. The modified *cry1Ab* gene was highly expressed in the transgenic chrysanthemum, and the resultant transformants were

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significantly more resistant to the lepidopteran pest *H. armigera*. This is the first report on transgenic chrysanthemum in which high levels of Cry1Ab ICP were expressed and which exhibited a high resistance to *H. armigera*.

Materials and Methods

Modification of the coding sequence of *cry1Ab* gene

The coding sequence of the wild type *cry1Ab* gene encoding amino acids at positions 1 to 615 originating from *Bacillus thuringiensis* var. *kurstaki* HD-1 was used for this modification based on the method of Perlak *et al.* (1991). The DNA sequence was designed to maintain the amino acid sequence and remove ATTTA sequences (ATT, TAA, ATAT, TATA and ATTTA) which are known to destabilize eukaryotic mRNA (Shaw and Kamen 1986). Moreover, the *cry1Ab* codons were substituted for preferred codons of *Compositae* family without any modification of the amino acid composition of the encoded polypeptide.

For the codon use of *Compositae* family, we refer to the codon use tabulated from GenBank (CUTG) (World Wide Web. address: www.dna.affrc.go.jp/~nakamura/CUTG.html). The available data were as follows: *Lactuca sativa*, 7 coding sequences (2,899 codons); *Helianthus annuus*, 61 coding sequences (21,200 codons); *Bidens pilosa*, 1 coding sequences (150 codons) and *Callistephus chinensis*, 4 coding sequences (1,477 codons). We selected the most frequent codons.

We surveyed the restriction enzyme sites of the sequence. Since the 3 sites of *Bam*HI in *cry1Ab* gene were interrupted to synthesize of the modified fragments, they were deleted. The *Eco*RI site was newly assigned to 411 bp to change the nucleotides without changing the amino acids.

The modified *cry1Ab* gene (designated as *mcbt*, 1,845 bp) was separated into 4 fragments at the individual restriction enzyme sites as follows: *Eco*RI site was assigned to 411 bp, *Nco*I site was assigned to 1,019 bp, and *Kpn*I site was assigned to 1,565 bp. The *Bam*HI site was linked to 5' upstream of the start codon (first ATG) and the *Sac*I site was linked to 3' downstream of the termination codon. For each fragment, 2 to 4 synthetic oligonucleotides about 80 bases long were synthesized. They partially overlapped with about 20 bases. Polymerase chain reaction (Saiki *et al.* 1988) was sequentially carried out with pairs of oligonucleotides to synthesize the complete fragment without templates (Fujimoto *et al.* 1993). After the synthesis, each fragment was digested with the respective restriction enzymes, cloned to pKF3/TH2 *E. coli* and sequenced. The fragment which corresponded to the *mcbt* sequence was cloned to pKF3 and digested with *Xba*I and *Sac*I. This fragment was substituted for binary vector pIG121-Hm (Ohta *et al.* 1990, Hiei *et al.* 1994) at the position of the β -D-glucuronidase (*gus*) gene (*Xba*I to *Sac*I site). We designated this vector as 'pIG121*mcbt*'. The *mcbt* gene was driven by the CaMV 35S promoter and the nopaline synthase (Nos) terminator.

Plant materials

The chrysanthemum [*Dendranthema* $\times$ *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* (meristem culture) on Murashige and Skoog (1962) basal medium (MS), containing 3% sucrose and 0.3% Gellan Gum (Pure Chemical, Inc., Kyoto). 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 infection conditions

We used the *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.* 1986). The pIG121*mcbt* vector (Fig. 1) was introduced into *Agrobacterium* EHA101 by the method of triparental mating. The *Agrobacterium* had been cultured in a liquid LB medium on BIO-SHAKER BR-15 (TAITEC Co., Japan) at 28°C for 5 h.

Plant transformation and analysis of transformed lines

Plant transformation

For the transformation system, the method of Shinoyama *et al.* (2002) was used.

Analysis of transformed lines

Southern blot analysis: Total DNA was extracted from 100 mg of fresh young leaves of the regenerated 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 buffer. This procedure was repeated 3 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 15 μg DNA digested with *Xba*I was subjected to electrophoresis and blotted onto a positively charged Nylon membrane (Roche & Boehringer Mannheim, Germany). Southern blot analysis (Southern 1975) was carried out using a modified *cry1Ab* gene fragment (470 bp) as a probe (see Fig. 1), and a digoxigenin (DIG) labeling and detection kit (Roche & Boehringer Mannheim, Germany), according to the manufacturer's instructions.

Northern blot analysis: Total RNA was extracted from 150 mg 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

RNA was extracted using Quiagen RNeasy Plant Total RNA Kit (Quiagen, USA). The 20 µg RNA was fractionated by formaldehyde gel electrophoresis through 0.8% agarose and ethidium bromide in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). Equal loading was observed by examining the gel containing ethidium bromide with an ultraviolet light. The gel was then blotted onto a positively charged Nylon membrane (Roche & Boehringer Mannheim, Germany). Pre-hybridization, hybridization and detection conditions were the same as those described above for Southern blot analysis (Southern 1975).

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 corresponding to 25 µg total protein. The proteins were separated on 10.0% SDS-PAGE gels (Laemmli 1970) and blotted onto a PVDF membrane (MICRON SEPARATIONS Inc.) using a semidry transfer cell (BioRad). Western immunoblot analysis was performed according to the alkaline phosphatase procedure of Kozziel *et al.* (1993). The purification of an antigen, Cry1Ab ICP, was modified from the method of Mochizuki (1994). *E. coli* JM109:pIG121*mcbt* 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 3 times to remove water-soluble protein at pH 7.5. After centrifugation, the pellet was resuspended in B buffer [50 mM Glycine-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 ICP was extracted from the gel at 62 kDa by electroelution. Cry1Ab ICP 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 (BIOSOURCE International, Co., USA). The density of each band was compared to that of pure Cry1Ab ICP (5, 25 and 50 µg) by using a Densitograph AE-6920V-05 (ATTO Co., Japan).

Insect bioassay on transformed lines: The tobacco budworms, *Helicoverpa 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 conducted. The first experiment was conducted to examine the consumption of the leaves of the *mcbt*-transformed lines by the insects. The leaves (5 cm length) from each line were detached from the plants, and the petioles were placed in moist cotton. The leaves were put in plastic boxes (φ=9 cm and 6 cm in depth), with one leaf per box. Five first instar larvae of *H. armigera* were allowed to feed on the leaves of the *mcbt*- and wild type *cry1Ab*-transformed lines and non-transformed control. The leaf area was measured at 5 days after experiment with Leaf Area Measure (Hayashi Denko Co., Japan). This experiment was performed with 3 replications.

In the second experiment, the mortality of the budworms of the larval, pupal and adult stages was determined. Leaves (5 cm length) of the *mcbt*- and wild type *cry1Ab*-transformed lines and non-transformed control were placed in plastic boxes (φ=9 cm and 6 cm in depth) containing perlite (about 3 cm in depth) at the bottom where the larvae pupated. A first 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. In each experiment, we used 50 larvae and observed the number of dead larvae at each larval stage.

Statistical analysis: For the first experiment conducted to examine the consumption of the leaves of the *mcbt*- and wild type *cry1Ab*-transformed lines and non-transformed control by the insects, Fisher's protected LSD was used. The percentages of the leaf area consumed by the insects were arcsine-transformed prior to analysis. For the second experiment on the survival pattern, survival time analysis by Tukey-Cramer's HSD was used.

Results

Sequence modification of *cry1Ab* gene

The design of the modified *cry1Ab* (*mcbt*) gene was almost complete elimination of the ATTTA sequences, and substitution of a preferred codon of the *Compositae* family. This resulted in a remarkable increase of the G + C nucleotide content of the gene. Of 615 codons, 442 were altered, resulting in the increase in the G + C nucleotide content from 36.9% to 54.9% (Fig. 1).

Regeneration rate

On the selection medium containing 20 mg/l G418, 411 leaf segments among 3,618 formed green calli, with a callus induction rate of 11.4%, and 317 plantlets were regenerated from the calli on the regeneration medium, corresponding to a 8.8% regeneration rate from initial leaf segments.

DNA analysis

The presence and the copy number of the *mcbt* gene in the plantlets were confirmed by Southern blot analysis. The genomic DNA of all the regenerants was digested by *Xba*I, because only one *Xba*I site is present in the T-DNA region. One to 3 unique bands hybridized to the *mcbt*-probe were observed in each regenerated plantlet. No hybridization signal was detected in the non-transformed control (Fig. 2). Thus the results indicated that each transformed line harbored one or multiple copies of the *mcbt* gene.

RNA analysis

The transformed lines containing the *mcbt* gene and the wild type *cry1Ab* gene, and the non-transformed control were analyzed on Northern blots for *mcbt* and wild type *cry1Ab* mRNA transcription. The expected size of both the *mcbt* and wild type *cry1Ab* mRNA was 1.8 kbp. No degradation of the mRNA was detected in the *mcbt*-transformed lines, while degradation of the mRNA was detected in the wild type *cry1Ab*-transformed lines. No hybridization signal was detected in the non-transformed control (Fig. 3).

Expression of Cry1Ab ICP in transformed lines

We selected 20 transformed lines at random. A distinct band could be detected at 58 kDa in all the *mcbt*-transformed lines and at 56 kDa in the wild type *cry1Ab*-transformed lines, although pure Cry1Ab ICP from *E. coli* JM109:pIG121 *mcbt* was detected at 62 kDa. The expression level of the Cry1Ab ICP ranged from 10.5 ng to 80 ng per 50 μ g total soluble protein (from 0.021 to 0.16% of the total soluble protein) in the transformed lines. No band was detected in the non-transformed control (Table 1 and Fig. 4).

Fig. 2. Example of Southern blot analysis. Genomic DNA digested with *Xba*I and hybridized with the *mcbt* specific probe. Lane1: pIG121*mcbt* Lane2: non-transformed plant 'Shuho-no-chikara' Lane3–9: *mcbt*-transformed lines

Fig. 3. Example of Northern blot analysis. Total RNA was isolated from the leaves of the control plants and plants with the wild type *cry1Ab* gene and modified *cry1Ab* (*mcbt*) gene, and approximately 20 μ g total RNA was applied in each lane. The probe corresponds to the 470 bp specific fragment of the *mcbt* gene. Lane1: pIG121*mcbt* Lane2: non-transformed plant 'Shuho-no-chikara' Lane3–4: wild type *cry1Ab*-transformed lines Lane5–7: *mcbt*-transformed lines

Insect resistance of transformed lines

The percentage of the leaf area of the transgenic plants carrying the *mcbt* gene consumed by the *H. armigera* larvae was correlated with the expression level of Cry1Ab ICP ($R^2 = 0.854$, $P < 0.0001$). The percentage of the leaf area consumed by the insects was significantly lower than that of the non-transformed control when the expression level exceeded 9 ng per 50 μ g (5% level by Fisher's protected LSD). The survival pattern from the first instar larvae to the adults of *H. armigera* on the *mcbt*-transformed lines was significantly different from that of the non-transformed control when the Cry1Ab ICP expression level exceeded 12.3 ng per 50 μ g (5% level by Tukey-Cramer's HDS). All the larvae of *H. armigera* tested had died during the first instar on the *mcbt*-transformed lines whose expression level of Cry1Ab ICP exceeded 47.6 ng per 50 μ g (Table 1 and Fig. 5).

The plants with a high larval mortality and a low percentage of leaf area consumed, harbored one to 2 copies of the *mcbt* gene, while the plants with a low larval mortality and a high percentage of leaf area consumed harbored 3 copies of the *mcbt* gene (Table 1).

Discussion

In our previous paper, the maximum expression level of the wild type *cry1Ab* gene was 40 ng per 50 μ g of total protein in chrysanthemum. In this case, the transgenic plants accumulating Cry1Ab ICP at a level of at least 0.07% of total soluble protein displayed only insect feeding inhibition effects but did not exhibit insecticidal effects, presumably

Table 1. Insect bioassay of transformed lines using *Helicoverpa armigera*

Lines	Cry1Ab ICP expression level (ng/50 µg total protein)	No. of bands detected by Southern blot analysis	Experiment 1	Experiment 2						
			Percentage of leaf area consumed by the insects (%) ¹⁾	No. of larvae that survived				No. of pupae	No. of adults that emerged	Results of statistical analysis ³⁾
				L1	L2	L3	L4			
mcBt(b)-1	80.0	2	0.8 a ²⁾	50	0	—	—	—	—	a ⁴⁾
mcBt(b)-15	75.0	1	0.8 a	50	0	—	—	—	—	a
mcBt(b)-10	65.0	1	1.0 a	50	0	—	—	—	—	a
mcBt(b)-12	55.0	2	1.9 b	50	0	—	—	—	—	a
mcBt(b)-17	50.2	2	5.2 b	50	0	—	—	—	—	a
mcBt(b)-5	47.6	1	6.2 ce	50	0	—	—	—	—	a
mcBt(b)-13	47.0	1	6.6 ce	50	14	4	2	2	0	ab
mcBt(b)-3	46.6	2	6.9 ce	50	8	2	0	—	—	ab
mcBt(b)-4	45.6	2	7.2 ce	50	15	6	3	3	0	ab
mcBt(b)-18	45.6	1	7.4 ce	50	16	9	6	3	0	ab
mcBt(b)-7	43.2	1	7.8 ce	50	16	9	7	2	0	ab
mcBt(b)-19	43.2	2	7.9 ce	50	19	13	7	6	1	abc
mcBt(b)-9	41.2	2	8.8 cde	50	27	21	11	10	2	abc
mcBt(b)-20	40.6	1	10.2 de	50	34	19	11	8	2	c
mcBt(b)-6	30.6	3	51.3 f	50	44	38	23	15	4	d
mcBt(b)-8	29.6	3	50.2 f	50	42	39	21	18	11	d
mcBt(b)-11	12.3	3	49.1 f	50	41	37	19	18	13	d
mcBt(b)-2	10.6	3	87.0 g	50	50	48	46	46	41	e
mcBt(b)-16	10.0	3	70.9 g	50	47	47	47	47	42	e
mcBt(b)-14	9.0	3	92.1 h	50	50	48	48	47	41	e
Bt(b)-11	40.0	1	10.3 de	50	48	42	42	42	26	e
Bt(b)-12	36.6	1	12.1 i	50	47	46	43	43	32	e
Control	—	—	91.1 h	50	48	46	43	43	35	e

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

Leaf area before and 5-day after experiment were measured with Leaf Area Measure (Hayashi Denko Co.) on the 5th day using first instar larvae.

¹⁾ (leaf area 5-day after experiment)/(leaf area before experiment) × 100.

²⁾ the data followed by the same letter are not significantly different at the 5% by Fisher's protected LSD.

³⁾ the results of statistical analysis of the larval survival pattern from the first instar larvae to the adults by Tukey-Cramer's HDS.

⁴⁾ the same letter in the column is not significantly different at the 5% by Tukey-Cramer's HDS.

L1, L2, L3, and L4; indicate first larval stage, second larval stage, third larval stage and fourth (final) larval stage, respectively.

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

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

Lane 4: non-transformed plant 'Shuho-no-chikara'

Lane 5–6: wild type *cry1Ab*-transformed lines

Lane 7–12: *mcbt*-transformed lines

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

due to the low accumulation level of Cry1Ab ICP in the transgenic chrysanthemum.

In the present experiment, we modified the *cry1Ab* gene (*mcbt*) to a preferred codon of the *Compositae* family which did not alter the amino acid sequence and we removed ATTTA sequences. We succeeded in increasing the expression level of Cry1Ab ICP up to 80 ng per 50 µg and in improving the resistance to *H. armigera*. In the Northern blot analysis, the mRNA of all the transformed lines for the *mcbt* gene was detected and mRNA was not degraded. On the other hand, the mRNA of the transformed line for the wild type *cry1Ab* gene was not detected and was degraded. It is possible that the incomplete functioning of ATTTA sequences led to processing without polyadenylation, causing instability in the transgenic line with the wild type *cry1Ab* gene. The highest expression level of this *mcbt* gene was 0.16% of total soluble protein.

The molecular size of the Cry1Ab ICP of the *mcbt*-transformed lines was 58 kDa and the size of the wild type *cry1Ab* was 56 kDa, which were different from that of *E. coli*

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'

1–2: wild type *cry1Ab*-transformed lines

3–8: *mcBt*-transformed lines

Lines; indicate the name of the transformed lines,

Cry1Ab ICP; indicates the Cry1Ab expression level (ng/50 μ g total protein)

TP; total protein

JM109 (62 kDa). In Cry1Ab ICP, trypsin cleavage sites are located between the amino acid at positions 28 and 29, and 601 and 602 from the N-terminal part (Höfte *et al.* 1986). The *mcBt* gene corresponded to the amino acids at positions 1 to 615. The 58 kDa protein obtained from the *mcBt* gene was digested with endogenous trypsin from chrysanthemum at 2 trypsin cleavage sites (amino acids at positions 28 and 29, and 601 and 602) of the 62 kDa Cry1Ab ICP (amino acids at positions 1 to 615). The wild type *cry1Ab* gene corresponded to the amino acid positions 29 to 618. According to the Northern blot analysis, the sequence of the 56 kDa protein obtained from the wild type *cry1Ab* gene might have been interrupted at the ATTTA sequences of 1.6 kb (Murray *et al.* 1991) and not digested at the second trypsin cleavage site of the amino acids at positions 601 and 602.

Survival pattern of *H. armigera* reared on the *mcBt*-transformed lines was significantly different from that of the insects reared on the non-transformed control, when the expression level of Cry1Ab ICP in the *mcBt*-transformed lines exceeded 12.3 ng per 50 μ g. Survival pattern of the insects reared on the wild type *cry1Ab*-transformed lines was not significantly different from that of the insects reared on a non-transformed control, even though the expression level of Cry1Ab in the wild type *cry1Ab*-transformed line was 40 ng per 50 μ g. The molecular size of the Cry1Ab ICP of the

mcBt-transformed lines was 58 kDa, while that of the wild type *cry1Ab*-transformed lines was 56 kDa. Nagamatsu *et al.* (1984) isolated a trypsin-resistant toxic core peptide of 58 kDa, suggesting that the 56 kDa protein might not display a sufficient insecticidal activity.

The transformation frequency was different in this experiment (8.8%) from that in a previous experiment (5.2%), presumably due to the difference in the infection ability between *Agrobacterium*, EHA101 and *Agrobacterium* LBA4404. Moreover, when EHA101 was used for infection, one to 3 copies of the transgene were introduced into the plant genome, while in the case of LBA4404, only one copy was introduced into the plant genome. Transgene inactivation (silencing) has been reported in several plant species and some models have been proposed for transcriptional and post-transcriptional silencing (Vaucheret *et al.* 1998). Transgene can undergo transcriptional silencing when single or multiple copies are integrated into a locus located next to silent hypermethylated genomic sequences (position effect), and it has also been suggested that the large discrepancy between the DNA composition of the transgene and that of the surrounding genomic sequences leads to the specific methylation and inactivation of a foreign sequence. On the other hand, in the case of post-transcriptional silencing, mRNA does not accumulate even though transcription occurs, and it

has been suggested that this phenomenon is mainly due to the overproduction and specific degradation of transgene mRNA (Dean *et al.* 1986, Shaw and Kamen 1986, Goodall and Filipowicz 1989). In the present experiment, some of the transgenic chrysanthemums which did not show a weak or an absence of insect resistance harbored 3 copies of the *mcbl* gene. In this case, specific degradation of mRNA should not occur, since we modified the *cryIAb* gene. Therefore, it was suggested that the large discrepancy between the DNA composition of the transgene and that of the surrounding genomic sequences led to the specific methylation and inactivation of a foreign sequence.

This is the first report of transgenic chrysanthemums in which high levels of Cry1Ab ICP were expressed and which exhibited a high resistance to *H. armigera*. The cultivation of such plants may be able to reduce insect control cost and/or working hours. In the future, a safety assessment of these insect-resistant transgenic chrysanthemums should be carried out.

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