Plant Biotechnology 25, 17–29 (2008)

Original Paper

Environmental risk assessment of genetically modified chrysanthemums containing a modified *cry1Ab* gene from *Bacillus thuringiensis*

Harue Shinoyama[1,*], Atsushi Mochizuki[2], Yukio Nomura[1], Hiroshi Kamada[3]

[1] Fukui Agricultural Experiment Station, Fukui, Fukui 918-8215, Japan; [2] National Institute for Agro-Environmental Sciences, Tsukuba, Ibaraki 305-8604, Japan; [3] Gene Research Center, Graduate School of Life and Environmental Science, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

* E-mail: harue_shinoyama@fklab.fukui.fukui.jp Tel: +81-776-54-5100 Fax: +81-776-54-5106

Received December 21, 2007; accepted January 17, 2008 (Edited by T. Nakamura)

Abstract Insect-resistant transgenic chrysanthemum plants expressing a modified *cry1Ab* gene of *Bacillus thuringiensis* were produced, and an environmental risk assessment of these plants was undertaken in preparation for release their into the field. These plants were examined for molecular profiles, morphological and growth characteristics, cross compatibility, production of allelopathic substances, and influence on soil microbes. The results showed that the insect-resistance trait of the GM chrysanthemum plants is stably integrated in the nuclear genome, and the expression of the *mcbt* gene did not cause significant differences in the morphological characteristics, the production of allelopathic substances, or the effect on soil microorganisms compared to non-GM chrysanthemums. However, because these plants have cross-compatibility with wild related species, they will be forbidden to be cultured in the open field under the Cartagena Protocol on Biosafety. Therefore, we are attempting to introduce a male and/or female sterility trait to GM chrysanthemums with insect resistance to reinforce their biosafety for the practical uses.

Key words: Biosafety, insect resistance, transgenic chrysanthemum.

Progress in biotechnology has made it possible to transfer traits that are unachievable by classical breeding into crops through the introduction of foreign genes from any organism. The genetically modified (GM) crops were first cultured commercially at 1996 and since then area planted with GM crops has been increased consistently. These increases are due to the many benefits of the culture of GM crops, such as reduced production costs and farm labor. However, since GM crops are generated without ordinal crossing and with intra-species gene transfer, there are concerns about their potential impact on people and the environment. Risk assessment must be performed on GM crops before their release into cultivation fields.

The requirements of the risk assessment of GM crops are well documented in the international templates of Article 15 and Annex II of the Cartagena Protocol on Biosafety. However, the actual assessment methods and experimental procedures vary with kinds of the introduced genes, the plant species, and the environment into which the GM crop is to be released.

We previously reported the production of insect-resistant transgenic chrysanthemum (*Chrysanthemum morifolium* Ramat. 'Shuho no chikara') containing an introduced modified delta-endotoxin gene of *Bacillus thuringiensis* (Shinoyama et al. 2003). The primary benefits of this chrysanthemum are reduced insecticide use, improved control of target insect pests, improved yield and cut-flower quality, and reduced production costs, resulting in improved economics for chrysanthemum cultivators.

To assess the environmental risks of insect-resistant transgenic chrysanthemums, here we examined the molecular profiles, morphological and growth characteristics, cross compatibility, production of allelopathic substances, and influence on several strains of soil microbes. This is the first environmental risk assessment study of a combination of insect-resistance gene and chrysanthemums.

Abbreviations: GM, genetically modified; ICP, insecticidal crystal protein; LB, left border; *mas2'-1'*, mannopine synthase-2' and -1'; *nptII*, neomycin phosphotransferase II; *nptIII*, neomycin phosphotransferase III; PMSF, phenylmethylsulfonyl fluoride; PPFD, photosynthetic photon flux density; RB, right border.

This article can be found at http://www.jspcmb.jp/

Materials and methods

Recipient plant materials

The chrysanthemum cultivar 'Yamate-shiro', was used for this experiment and that is independent of day length, produces single white flowers in the mid-August. Shoot tips of plants growing in the greenhouse were surface-sterilized by dipping briefly in 70% ethanol, and then in a 1% sodium hypochlorite solution for 15 min and rinsed three times in sterile distilled water. The shoot tip explants were cultivated *in vitro* (meristem culture) on Murashige and Skoog's basal medium (MS) (Murashige and Skoog 1962) containing 3% sucrose and 0.3% Gellan Gum (Pure Chemical Co., Japan). The medium was adjusted to pH 5.8 prior to autoclaving at 120°C for 15 min. The cultures were put 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 μmol m^{-2} s^{-1}.

Rhizobium strain and binary vector

Rhizobium radiobactor strain EHA105 (provided by Dr. L. S. Melchers, Zeneca Mogen) harboring a binary vector pBIK102*mcbt* was used for this experiment. The pBIK102*mcbt* is the binary Ti plasmid vector produced from the vector pBI121. The length of the plasmid pBIK102*mcbt* is 13.3 kbp. Figure 1 shows a map of the plasmid and the origins of the componens are shown in Table 1.

The DNA sequence of the wild-type *cry1Ab* of *Bacillus thuringiensis* var. *kurstaki* HD-1 was modified based on the method of Perlak et al. (1991) to remove the ATTTA sequences that are known to destabilize eukaryotic mRNA (Shaw and Kamen 1986). The modified *cry1Ab* (*mcbt*) gene, encoding 615 amino acid sequence, was designed for the preferred codon usage of the *Compositae* plants without modification of the amino acid sequence (Shinoyama et al. 2003).

The normal *neomycin phosphotransferase II* (*nptII*) gene (amino acid position 182 is GAG [glutamic acid]; Yenofsky et al. 1990), which encodes a selectable marker enzyme, was also present on the plasmid pBIK102*mcbt* to facilitate the selection of transgenic chrysanthemum cell. The wild-type NptII protein served no other purpose and has no pesticidal properties. The *mcbt* and *nptII* genes were driven by a bidirectional promoter fragment for the *mannopine synthase-2′* and *-1′* (*mas2′-1′*) genes of *Rhizobium radiobacter* (Shinoyama et al. unpubl.). Mannopine synthase is composed of two enzymes, a conjugase encoded by the *mas2′* gene and a reductase encoded by the *mas1′* gene (Ellis et al. 1984). These two genes are located on the T-DNAs of certain octopine-type Ti and Ri plasmids

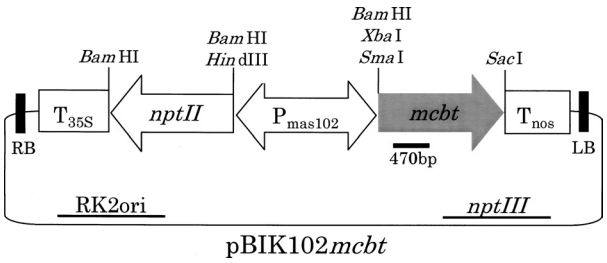

Figure 1. Structure of the vector pBIK102*mcbt*, constructed by inserting the *mcbt* gene into the expression vector pBIK102G. RB, right border; LB, left border; P_{mas102}, bidirectional promoter fragment of the *mannopine synthase*-2′ and *-1′* (*mas2′-1′*) genes; T35S, cauliflower mosaic virus 35S terminator; Tnos, nopaline synthase terminator; *nptII*, neomycin phosphotransferase II gene; *mcbt*, modified *cry1Ab* gene. The probe used for Southern blot analysis of *Xba*I-digested genomic DNA, a 470-bp PCR product, is indicated below the *mcbt* gene.

Table 1. Origin and function of component elements

Component elements	Size (kbp)	Origin and function
		modified *cry1Ab* (*mcbt*) gene expression cassette
P_{mas102}	0.5	It is the dual promoter of *mannopin synthase* (*mas*) gene and is derived from *Rhizobium radiobactor* strain AtC1 (MAFF301276).
mcbt	1.8	It is derived from *Bacillus thuringiensis* var. *kurstaki* HD-1, and encodes insecticidal crystal protein (ICP). The DNA sequence was modified for removal of ATTTA unstable sequences for preferred codon usage of *Compositae* family without any modification of the amino acid sequence of the encoded polypeptide.
Tnos	0.3	It is the 3′ untranslated region of *nopaline synthase* gene derived from pBI121. It terminated transcription and causes 3′ polyadenylation.
		nptII gene expression cassette
P_{mas102}	0.5	Same to above
nptII	0.8	It encodes a wild-type *neomycin phosphotransferase II* (*nptII*) gene [amino acid position 182 is glutamic acid (GAG codon) (Yenofsky et al. 1990)] which confers kanamycin and G418 tolerance, derived from the transposon Tn5.
T35s	0.2	It is the 3′ entranslated region of CaMV 35S RNA gene derived from a binary Ti vector pBI333.
		Others
RB	0.02	It is the right border of the T-DNA derived from pBI121.
LB	0.02	It is the left border of the T-DNA derived from pBI121.
nptIII	1	It encodes *neomycin phosphotransferase III* (*nptIII*) gene which confers amikacin and kanamycin tolerance, derived from the *Escherichia coli*.
RK2ori (*oriV*)	0.6	The origin of replication (*oriV*) from the broad-host-range plasmid RK2.

(Velten et al. 1984; Bouchez et al. 1991). The *mas2′* and *mas1′* promoters used are found in a head-to-head orientation on a 483-bp fragment of pTiAch5 (Velten et al. 1984).

The plasmid pBIK102*mcbt* possesses the amikacin- and kanamycin-tolerance gene (*nptIII*) and the RK2ori sequence (Table 1, Figure 1). The *nptIII* gene, used as a selectable marker for the plasmid, is driven by a bacterial promoter. RK2ori is a replication origin and causes autonomous replication both in *E. coli* and *R. radiobactor*. The *nptIII* gene and RK2ori were located outside of the T-DNA region and were not integrated or transferred into the chrysanthemum genome.

The host range for the autonomous replication origin (RK2ori) of the plasmid pBIK102*mcbt* is limited to *Rhizobium radiobactor*, *E. coli*, and a few gram-positive bacteria. The plasmid pBIK102*mcbt* does not possess infectious characteristics to other bacterial species.

For the production of the gene-modified chrysanthemum (GM chrysanthemum), the plasmid pBIK102*mcbt* was used. In pBIK102*mcbt*, the mcbt gene cassette and the *nptII* gene expression cassette [T_{35S}]-[*nptII*]-[P_{mas102}]-[*mcbt*]-[Tnos] were located between the left (LB) and right border (RB) on the vector. The position and direction of the nucleic acid element in the plasmid vector (Figure 1).

Method of transferring nucleic acid to the recipient organism

The *Rhizobium*-mediated transformation system (Shinoyama et al. 2002) was used to transfer the gene to the recipient chrysanthemum cultivar, 'Yamate-shiro'. After the *R. radiobactor* EHA105 harboring the plasmid pBIK102*mcbt* was produced by triparental mating, and used to infect a leaf segment of the recipient, and the T-DNA region between the RB and LB on pBIK102*mcbt* was transferred into the chrysanthemum genome.

Culture of modified plants

Closed greenhouse

In October 2003, GM and non-GM chrysanthemums were planted in a plastic tray (15×10×5 cm) in vermiculite (Fujimi Industry Co., Japan) and covered with a plastic sheet for acclimation in a culture room. The culture room was held at a temperature of 25°C and a 16-h photoperiod with cool-white fluorescent lamps providing a photosynthetic photon flux density [PPFD, 400–700 nm] of 15 μmol m^{-2} s^{-1}. After 2 weeks, the plantlets were repotted into plastic pots (ϕ=6 cm) and grown in the culture room for 40 days under conditions of 10°C, a 16-h photoperiod, and a PPFD of 15 μmol m^{-2} s^{-1}, for vernalization. The plants were then repotted into plastic pots (ϕ=15 cm) containing soil (Tsuchi-taro, Sumitomo Forestry Co., Japan) and grown in a closed greenhouse beginning in April 2004. The windows of the greenhouse were closed, the temperature was maintained at 25°C with an air conditioner, and the day length was natural. The plants were fertilized every 2 weeks with Hyponex solution (Hyponex Japan Co., Japan).

Special-netted greenhouse

GM- and non-GM chrysanthemum plants were also acclimatized and vernalized in October 2004 and grown in a special special-netted greenhouse beginning in April 2005. The windows of the greenhouse were covered with screens, the plants were grown under natural daylength and temperature conditions, and they were fertilized every 2 weeks with Hyponex solution.

Molecular characterization

According to the methods of Shinoyama et al. (2003), the integration, translation, and translation of the *mcbt* gene in transformed chrysanthemum plants were confirmed by Southern (Southern 1975), Northern and Western blot analysis, respectively.

Insect resistance

The insects used in the bioassay of the transformed plants were tobacco budworm (*Helicoverpa armigerdda*; Lepidoptera) and common cutworm (*Spodoptera litura*; Lepidoptera) collected in Ono, Fukui Prefecture, Japan. The larvae were reared on an artificial diet (Insecta LF; Nihon Nosan Kogyo, Japan) under conditions of 25°C and a 16-h daylength at a PPFD of 60 μmol m^{-2} s^{-1}.

The consumption of GM and non-GM chrysanthemum leaves by the insects and the mortality of the budworms were determined according to Shinoyama et al. (2003). Based on the results, we selected three GM chrysanthemum lines exhibiting high resistance to both *H. armigera* and *S. litura*, high resistance to *H. armigera* and medium resistance to *S. litura*, and low resistance to both *H. armigera* and *S. litura*. These three lines were used for environmental biosafety assessment experiments.

Environmental assessment

Morphological and growth characteristics

GM and non-GM chrysanthemum plants were planted in pots (500×340 mm with a depth of 263 mm; Takii Co., Japan) in May 2004 and 2005. The plantlets were pinched 2 weeks after planting, and two stems were allowed to grow. In August 2004 and 2005, morphological assays of the stem length, leaf number, flower diameter, and days to flowering were conducted in the closed and special-netted greenhouses.

Cross-compatibility

a) Pollen structure

In August 2004 and 2005, pollen was collected from GM and non-GM chrysanthemums that flowered in the closed and special-netted greenhouses, and the pollen morphology was observed using a microscope.

b) In-vitro pollen fertility and longevity

In August 2004, pollen collected from GM and non-GM chrysanthemums that flowered in the closed greenhouse was placed on dry microscope slides and incubated in a temperature-incline incubator (TG-100-A, NK System Co. Ltd., Japan) at 10 to 25°C for 0 to 72 h. To estimate the pollen longevity, the pollen was then stained with acetocarmine solution, placed on pollen germination (PG) medium, and incubated at 25°C for 1 h. The number of stained pollen grains

was then counted under a microscope.

For pollen germination, pollen was cultured on diluted pollen germination (PG) medium in the closed greenhouse at 25°C for 2 h. The PG medium contained 100 mg l^{-1} H_3BPO_4, 300 mg l^{-1} $CaCl_2 \cdot 2H_2O$, 1 mg l^{-1} $CoCl_2 \cdot 6H_2O$, and 30 g l^{-1} sucrose. The PG medium was diluted 1 : 4 with acetoethyl petal solution (Ikeda and Numata 1991), which was prepared as follows. Chrysanthemum petals (20 g) were incubated in acetoethyl solution for 24 h and air-dried. Ten ml of PG medium were added to the residue, which was then incubated at 30°C overnight. Another 10 ml of PG medium were added and the mixture was homogenized with a ceramic rotor. The supernatant was collected and designated acetoethyl petal solution.

c) Pollen dispersal using artificial wind

At the flowering stage in August 2004, when flowers on plants growing in the closed greenhouse showed stigmas protruding from the tubular flower and pollen on the anthers, flowers were placed in front of a fan (F140B, Toshiba Co., Japan) in the closed greenhouse. The distance between the flowers and the fan was 1 m and the wind speed was 3.5 to 7.5 m s^{-1}. Microscope slides coated with egg albumen were placed horizontally behind the flower. After 1 h, the number of trapped pollen grains on the egg albumen-covered slides was counted.

Similarly, in August 2005, flowers from plants grown in the special-netted greenhouse were placed 1.5 m from the windows, with a wind velocity of 1.3 ± 0.2 to 5.1 ± 2.2 m s^{-1}. Microscope slides coated with egg albumen were positioned horizontally behind the flowers. After 1 h, the number of trapped pollen grains on the egg albumen-coated slides was counted.

d) Cross-compatibility with wild Compositae

GM and non-GM chrysanthemums were artificially crossed with wild Compositae species (*C. morifolium* 'Rosanna', 'Kofuku-no-tori' and 'Motte-no-hoka', *C. japonense* Fukui No. 1, *C. pacificum* and *Aster ageratoides* ssp. *avalus*). This experiment was performed in August 2004 in the closed greenhouse and in August 2005 in the special-netted greenhouse. For the pollen parent, pollen was collected from dehiscent anthers of a tubular flower. For the seed parent, immature straw-shaped flowers were removed from the inflorescence, leaving completely mature straw-shaped flowers with protruding stigmas. Ten flowers that had 50 receptive stigmas were used. Pollen was placed on stigmas using a small brush, and each flower was covered with a paper bag. After 2 months, seeds were collected and sown on vermiculite, and the germination rate was calculated.

Production of allelopathic substances in GM chrysanthemum

These experiments were conducted according to the methods of Tabei et al. (1994).

a) Dispersal into air

After vernalization in October 2003, GM and non-GM chrysanthemum plants were cut back to 5 cm and potted in vermiculite. Rooting GM and non-GM chrysanthemum plants were potted in Agripots (Asahi Techno Glass Co. Ltd., Japan) in soil (Tsuchi-taro, Sumitomo Forestry Co., Japan). Lettuce (*Lactuca sativa* 'Great Lakes 366') seeds were surface-sterilized with a brief dip in 70% ethanol, 15 min of incubation in 1% sodium hypochlorite, and three rinses in sterile distilled water. Fifty sterilized lettuce seeds were placed in a plastic dish ($\phi = 3$ cm) on wetted filter paper, and the dishes were set near the chrysanthemum plants in the Agripots. The Agripots were covered and cultured in the culture room (conditions of 25°C and a 16-h photoperiod provided with cool-white fluorescent lamps). After 2 weeks, the germination rates and germinating energy were assessed.

b) Dispersal in soil

As above, GM and non-GM chrysanthemums were potted in soil in Agripots and cultured for 2 weeks in a culture room (conditions of 25°C and a 16-h photoperiod). Surface-sterilized lettuce seeds were placed in a plastic dish, added to the soil water of the Agripots, and cultured for 2 weeks. The germination rate and germinating energy were then assessed.

c) Plant production of allelopathic substances

GM and non-GM chrysanthemums were homogenized in 5 ml of distilled water using a ceramic rotor. Fifty sterilized lettuce seeds were placed in a plastic dish, and 1 ml of homogenized plant solution was added. The dishes were cultured in the culture room (conditions of 25°C and a 16-h photoperiod) for 2 weeks, and the germination rate and germinating energy were assessed.

d) Influence of plant plowing

After cultivation of GM and non-GM chrysanthemums in 2004 in the closed greenhouse, the remaining plants were plowed into the soil, and 50 lettuce seeds were sown in the soil. After 2 weeks, the germination rate and growth of the lettuce were assessed.

Influence on soil microorganisms

Experiments were performed according to the method of Tabei et al. (1994). Soil in which GM and non-GM chrysanthemums was cultured in 2004 was collected at four intervals: before planting, during plant growth, during flowering, and post-harvest. Thirty grams of dry soil were suspended in 270 ml of sterilized phosphate buffer (15 mM, pH 7.0) in a glass flask. The suspension was then diluted from 10^2 to 10^5 with 15 mM phosphate buffer. For detection of bacteria and actinomycetes, 100 ml of suspension were spread on diluted PTYG agar plates (Balkwill and William 1985). The PTYG medium contained 10 g l^{-1} Bacto Peptone, 5 g l^{-1} Bacto Tryptone, 10 g l^{-1} Bacto Yeast Extract, 600 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 70 mg l^{-1} $CaCl_2 \cdot 2H_2O$, and 15 g l^{-1} agar. The PTYG medium was diluted 1 : 20, keeping the agar concentration at 15 g l^{-1}. The plates were incubated at 25°C for 7 days. For detection of fungi, 100 μl of suspension were spread on autoclaved rose bengal medium (Martin 1950; King et al. 1979) consisting of 5 g l^{-1} Bacto Peptone, 10 g l^{-1} glucose, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1 g l^{-1} K_2HPO_4, pH 7.2, 15 g l^{-1} agar, and 25 mg l^{-1} rose bengal. The

plates were incubated at 25°C for 4 days, after which the number of colonies was counted.

Residual Rhizobium

Ten grams of GM and non-GM chrysanthemum plant tissues were homogenized with a ceramic rotor and added to 5 ml of sterilized water, and 100 μl of the homogenized solutions were spread on AB minimal medium plates (Clark and Maaloe 1967) containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin (AB-Km plate). The plates were cultured in a culture room at 28°C in darkness. After 1 week, the colony numbers were counted.

Results

Production of insect-resistant chrysanthemums

G418-resistant calli formed on the 1,455 leaf segments that were cultured, and 320 plantlets regenerated from these calli on regeneration medium. The regeneration frequency was 22.0% based on the initial number of leaf segments.

Molecular characterization

a) Southern blot analysis

The presence and copy number of the *mcbt* gene in the regenerated plants were determined by Southern blot analysis. Twenty-microgram aliquots of genomic DNA from regenerated and non-GM chrysanthemum were digested with *Xba*I, because only one *Xba*I site is present within the T-DNA region. One or more unique bands that hybridized to the *mcbt* probe were detected in each regenerated plant, but no hybridization signal was detected in the non-GM chrysanthemums (Figure 2). These results indicated that all of the regenerated plants harbored one or more copies of the *mcbt* gene.

b) Northern blot analysis

GM chrysanthemums containing the *mcbt* gene and non-GM chrysanthemums were subjected to Northern blot analysis to assess transcription of the gene. A distinct band of 1.8 kbp was detected in all GM chrysanthemums, and no hybridization signal was detected in the non-GM chrysanthemums (Figure 3).

c) Expression of Cry1Ab ICP in GM chrysanthemums

Twenty GM chrysanthemums lines were selected at random for immunoblot analysis. A distinct band of 58 kDa was detected in all of the GM chrysanthemums; pure Cry1Ab ICP from *E. coli* JM109 : pBIK201*mcbt* was detected at 62 kDa. The levels of Cry1Ab ICP in the GM chrysanthemums ranged from 10.0 to 82.0 ng per 50 μg of total soluble protein (0.02 to 0.16% of total soluble protein). No band was detected in non-GM chrysanthemums (Table 2, Figure 4). This result shows that the *mcbt* gene in GM chrysanthemums was stably translated.

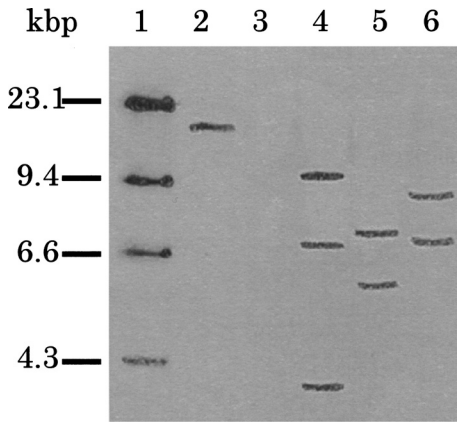

Figure 2. Example of Southern blot analysis. Genomic DNA digested with *XbaI* and hybridized with the *mcbt*-specific probe is shown. Lane 1, λ/*Hind*III marker; lane 2, pBIK102*mcbt*; lanes 3–6, DNA from the non-GM chrysanthemum cultivar 'Yamate-shiro' and the GM chrysanthemums lines mcbt-4, mcbt-8, and mcbt-14, respectively.

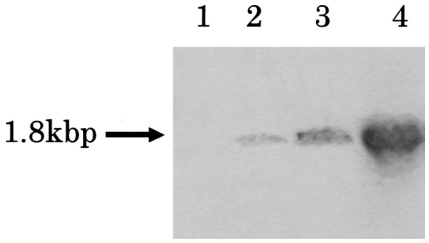

Figure 3. Example of Northern blot analysis. Total RNA was isolated from the leaves of non-GM plants and plants containing the wild-type *cry1Ab* gene and the *mcbt* gene, and approximately 20 μg total RNA were applied to each lane. The probe corresponds to a 470-bp fragment that is specific for the *mcbt* gene. Lanes 1–4, DNA from the non-GM chrysanthemum 'Yamate-shiro' and the GM chrysanthemums lines mcbt-4, mcbt-8, and mcbt-14, respectively.

Insect resistance of GM chrysanthemum

In the GM chrysanthemums lines carrying the *mcbt* gene, the percentage of the leaf area consumed by *H. armigera* and *S. litura* larvae was correlated with the Cry1Ab ICP expression level (R^2=0.833, P<0.0001; R^2=0.852, P<0.0001). In the insect bioassay, all *H. armigera* larvae died during the first instar when they were supplied with leaves of the *mcbt* transformants whose Cry1Ab ICP expression levels exceeded 48.9 ng per 50 μg of total soluble protein. The leaf areas consumed by five larvae were <1% of the total leaf areas. All *S. litura* larvae died during the second instar when provided with leaves of the *mcbt* transformants whose Cry1Ab ICP expression levels exceeded 63.9 ng per 50 μg of total soluble protein. The leaf areas consumed by five larvae were 0.8 to 4.9% of the total leaf areas (Table 2, Figure 5).

The GM chrysanthemums lines that were correlated with high larval mortality and a low percentage of leaf

area consumed harbored either one or two copies of the *mcbt* gene, whereas the GM lines that showed low larval mortality and a high percentage of leaf area consumed harbored three copies of the *mcbt* gene (Table 2).

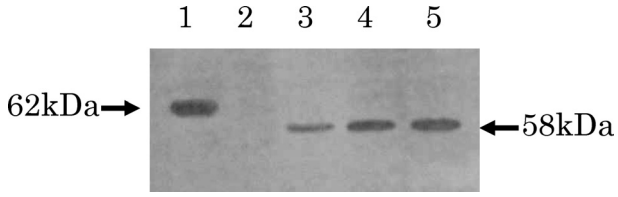

Figure 4. Immunoblot analysis of leaves of chrysanthemum plants expressing the *mcbt* gene. Lane 1, 50 ng of Cry1Ab ICP; lanes 2–5, 25 μg of soluble total proteins from the non-GM chrysanthemum cultivar 'Yamate-shiro' and the GM chrysanthemums lines mcbt-4, mcbt-8, and mcbt-14, respectively.

Three GM chrysanthemums lines were selected for environmental biosafety assessment experiments: No. 14 (high resistance to both *H. armigera* and *S. litura*), No. 8 (high resistance to *H. armigera* and medium resistance to *S. litura*), and No. 4 (low resistance to both *H. armigera* and *S. litura*).

Morphological and growth characteristics

In August 2004 and August 2005, the three selected GM chrysanthemum lines and non-GM chrysanthemums cultivated in the closed and special-netted greenhouses flowered. The stem length, number of leaves, number and diameter of flowers, and days to flowering of the GM chrysanthemums were close to those of the non-GM chrysanthemums (Table 3), and there was no significant difference between GM and non-GM chrysanthemums.

Table 2. Insect bioassay of GM and non-GM chrysamthemus using *Helicoverpa armigera* and *Spodoptera litura* larvae.

Lines	Cry1Ab ICP expression level (ng 50 μg^{-1} of totalprotein)	No. of transgene	Insect bioassay			
			H. armigera		*S. litura*	
			% of leaf area consumed[1)]	Average stage of larvae that died[3)]	% of leaf area consumed[1)]	Average stage of larvae that died[3)]
mcbt-14	82.0	2	0.8a[2)]	L1a[4)]	0.8a[2)]	L2a[4)]
mcbt-8	48.9	2	0.9a	L1a	53.0b	L4b
mcbt-4	10.0	3	94.2b	L3b	95.4c	— c
Control	ns	ns	97.2b	— c	98.3c	— c

Cry1Ab expression level was determined using a Densitograph (ATTO Co.) compared to pure Cry1Ab ICP. Number of introduced genes was detected number of bands by Southern blot analysis. L1, L2, L3, and L4 indicate first larval stage, second larval stage, third larval stage, and fourth larval stage, respectively. 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 larval stage to died larval stage by Tukey-Cramer's HDS.

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

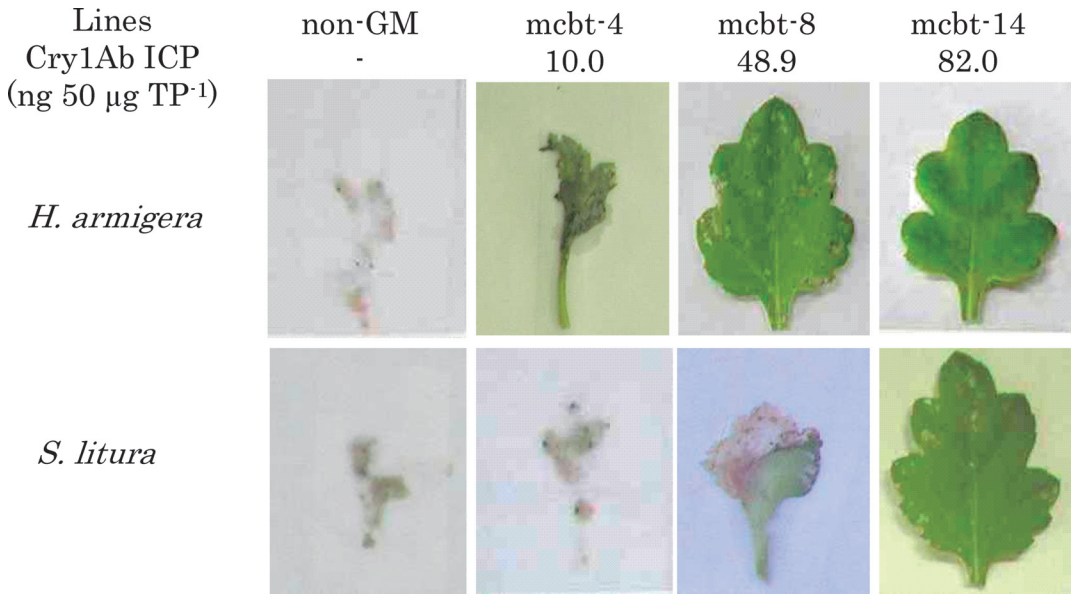

Figure 5. Bioassay of insect feeding on chrysanthemum leaves. "Lines" indicates the names of the transformed lines, and Cry1Ab ICP indicates the Cry1Ab expression level (ng per 50 μg of total protein). ICP, Insecticidal crystal protein; TP, total protein.

Table 3. Morphological and growth characteristics.

Lines	Area[1)]	The day of flowering (day/month)	Total weight (g)	Stem length (cm)	No. of leaves	Flower characteristics	
						Total number	Diameter (mm)
mcbt-4	CH-A	26 Aug. ± 0.3	61.2 ± 0.4	60.2 ± 0.7	42.1 ± 3.1	12.0 ± 0.1	40.2 ± 0.1
mcbt-8	CH-A	24 Aug. ± 0.3	62.4 ± 0.2	68.2 ± 0.9	44.2 ± 2.2	13.0 ± 0.2	40.2 ± 0.2
mcbt-14	CH-A	26 Aug. ± 0.2	64.4 ± 0.2	66.4 ± 0.2	45.2 ± 2.2	13.2 ± 0.2	39.2 ± 0.2
Control	CH-A	26 Aug. ± 0.0	65.2 ± 0.2	65.2 ± 0.5	44.2 ± 2.2	12.0 ± 0.2	39.9 ± 0.2
mcbt-4	CH-B	25 Aug. ± 0.1	62.1 ± 0.3	63.6 ± 0.3	46.2 ± 1.2	12.3 ± 0.2	40.2 ± 0.2
mcbt-8	CH-B	25 Aug. ± 0.2	66.1 ± 0.4	69.2 ± 0.7	50.2 ± 2.2	13.0 ± 0.2	41.2 ± 0.2
mcbt-14	CH-B	26 Aug. ± 0.2	64.2 ± 0.5	65.2 ± 0.1	49.2 ± 2.2	12.0 ± 0.2	40.5 ± 0.2
Control	CH-B	27 Aug. ± 0.1	60.1 ± 0.1	64.2 ± 0.2	48.4 ± 1.2	12.2 ± 0.2	40.2 ± 0.1
mcbt-4	SNH-A	21 Aug. ± 0.1	65.2 ± 0.3	68.2 ± 0.6	50.2 ± 1.2	12.0 ± 0.2	45.2 ± 0.5
mcbt-8	SNH-A	24 Aug. ± 0.3	64.2 ± 0.1	70.2 ± 0.2	52.1 ± 0.9	12.5 ± 0.2	42.3 ± 0.6
mcbt-14	SNH-A	25 Aug. ± 0.2	66.2 ± 0.2	68.0 ± 0.6	53.7 ± 0.9	13.0 ± 0.2	46.7 ± 0.6
Control	SNH-A	23 Aug. ± 0.1	64.3 ± 0.1	70.2 ± 0.7	50.6 ± 0.2	12.2 ± 0.2	44.6 ± 0.5
mcbt-4	SNH-B	25 Aug. ± 0.1	67.2 ± 0.4	69.4 ± 0.2	52.1 ± 0.1	13.1 ± 0.2	46.2 ± 0.9
mcbt-8	SNH-B	26 Aug. ± 0.2	68.2 ± 0.9	69.2 ± 0.1	55.2 ± 0.1	12.7 ± 0.1	45.2 ± 0.2
mcbt-14	SNH-B	24 Aug. ± 0.1	67.2 ± 0.2	70.2 ± 0.2	53.7 ± 0.1	13.1 ± 0.1	47.8 ± 0.2
Control	SNH-B	24 Aug. ± 0.1	64.2 ± 0.2	72.1 ± 0.1	53.2 ± 0.2	12.0 ± 0.2	45.2 ± 0.1

The top of plant was pinching after 2 weeks from planting and 2 stems were grown.
The data means the average ± SE of 10 samples.
[1)] CH, closed greenhouse (2004); SNH, special-netted greenhouse (2005)

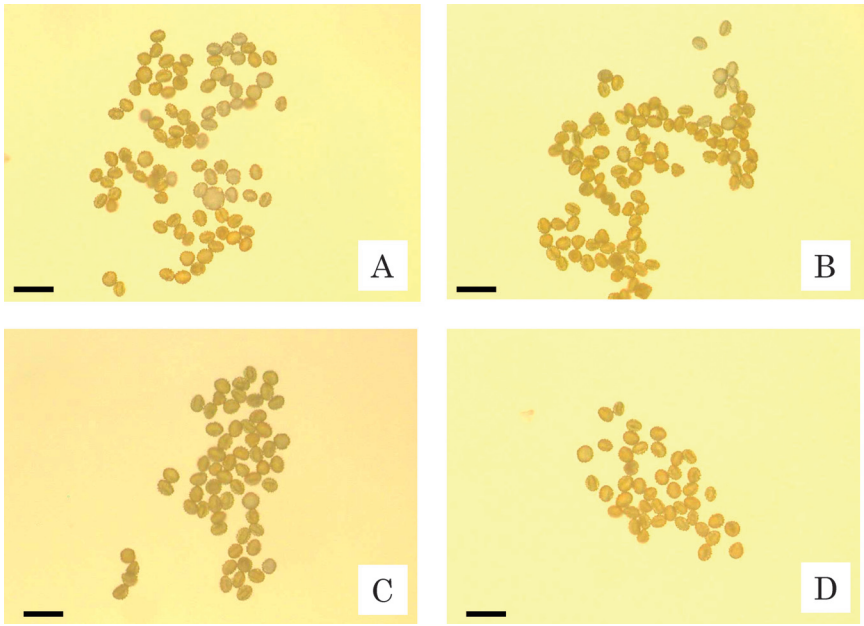

Figure 6. Structures of pollen from GM and non-GM chrysanthemum flowers. (A) Pollen of the non-GM chrysanthemum cultivar 'Yamate-shiro'. (B)–(D) Pollen from the GM chrysanthemum lines mcbt-4, mcbt-8, and mcbt-14, respectively. Bars indicate 100 μm.

The diameters of the flowers in the special-netted greenhouse were slightly larger than those in the closed greenhouse. Therefore, the introduction of the *mcbt* gene did not cause significant differences in the morphological or growth characteristics of GM and non-GM chrysanthemums.

Pollen structure

The pollen of GM and non-GM chrysanthemums, observed using a microscope, was found to be very similar. The pollen grains of all of the lines had diameters of 30 to 40 μm, were yellowish, oval, and had many fine spines (Table 4, Figure 6).

In-vitro pollen fertility and longevity

At all temperatures, the pollen of GM and non-GM chrysanthemums stained well for the first 5 h, with the frequency of acetocarmine staining of the pollen being greater than 60%. However, the frequency of staining decreased with time, notably at higher temperatures. At 25 to 30°C, the staining level fell to <50% after 24 h, to 23.3 to 25.3% after 48 h, and to 0.0% after 72 h. At

Table 4. The pollen longivity *in vitro*.
1. The staning by acetocarmin solution.

Lines	The frequency of staining pollen by the passage time (h)				
	0	24	48	72	80
25–30°C					
mcbt-4	68.2 ± 0.1	40.2 ± 0.1	24.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
mcbt-8	70.2 ± 0.1	44.2 ± 0.1	25.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
mcbt-14	69.2 ± 0.2	48.2 ± 0.1	24.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Control	70.2 ± 0.0	42.4 ± 0.0	23.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20–25°C					
mcbt-4	65.3 ± 0.0	55.2 ± 0.3	37.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
mcbt-8	67.3 ± 0.2	58.2 ± 0.4	33.2 ± 0.1	0.1 ± 0.4	0.0 ± 0.0
mcbt-14	69.2 ± 0.2	52.0 ± 0.4	38.2 ± 0.1	0.1 ± 0.4	0.0 ± 0.0
Control	64.2 ± 0.1	58.0 ± 0.4	32.3 ± 0.2	0.0 ± 0.3	0.0 ± 0.0
15–20°C					
mcbt-4	69.2 ± 0.2	58.2 ± 0.1	40.2 ± 0.5	1.1 ± 0.1	0.0 ± 0.1
mcbt-8	69.6 ± 0.1	62.3 ± 0.4	41.5 ± 0.0	0.6 ± 0.0	0.0 ± 0.0
mcbt-14	67.2 ± 0.1	62.5 ± 0.4	44.6 ± 0.0	0.9 ± 0.0	0.0 ± 0.0
Control	64.5 ± 0.3	61.7 ± 0.2	40.9 ± 0.0	1.1 ± 0.1	0.0 ± 0.1
10°C					
mcbt-4	69.3 ± 0.0	59.2 ± 0.1	55.2 ± 0.1	2.1 ± 0.3	0.0 ± 0.0
mcbt-8	67.6 ± 0.0	60.2 ± 0.0	53.4 ± 0.0	1.4 ± 0.4	0.0 ± 0.0
mcbt-14	69.3 ± 0.0	59.6 ± 0.0	52.5 ± 0.0	1.4 ± 0.4	0.0 ± 0.0
Control	68.2 ± 0	61.7 ± 0.1	50.2 ± 0.1	1.7 ± 0.0	0.0 ± 0.0

The data means the average± SE of 10 samples.

2. The germination ability on the diluted pollen germination medium.

Lines	The frequency of germination of pollen by the passage time (h)				
	0	24	48	72	80
25–30°C					
mcbt-4	10.2 ± 0.1	5.6 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
mcbt-8	11.2 ± 0.1	5.3 ± 0.2	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
mcbt-14	11.2 ± 0.2	5.2 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Control	9.8 ± 0.0	5.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20–25°C					
mcbt-4	11.1 ± 0.2	6.2 ± 0.1	3.7 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
mcbt-8	10.0 ± 0.3	6.5 ± 0.2	3.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
mcbt-14	12.1 ± 0.1	6.2 ± 0.1	4.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Control	9.9 ± 0.0	6.9 ± 0.2	3.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
15–20°C					
mcbt-4	11.2 ± 0.2	7.3 ± 0.1	4.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
mcbt-8	10.0 ± 0.1	7.5 ± 0.2	5.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
mcbt-14	12.0 ± 0.2	7.7 ± 0.3	4.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Control	11.0 ± 0.1	7.2 ± 0.2	4.8 ± 0.0	0.0 ± 0.0	0.0 ± 0.01
10°C					
mcbt-4	12.0 ± 0.1	8.4 ± 0.1	5.4 ± 0.1	0.2 ± 0.0	0.0 ± 0.0
mcbt-8	10.0 ± 0.1	8.2 ± 0.1	5.3 ± 0.2	0.4 ± 0.0	0.0 ± 0.0
mcbt-14	11.0 ± 0.2	8.2 ± 0.3	5.2 ± 0.1	0.5 ± 0.0	0.0 ± 0.0
Control	10.0 ± 0.0	8.1 ± 0.0	5.7 ± 0.0	0.2 ± 0.0	0.0 ± 0.0

The data means the average± SE of 10 samples.

10°C, the staining level decreased to <50% after 48 h, and few pollen grains were stained after 72 h. However, there was no significant difference between GM and non-GM chrysanthemums (Table 4).

The germination ability of the pollen also decreased with time. Germination ceased at 25 to 30°C after 56 h, and at 10°C after 80 h. As the temperature decreased, the pollen longevity increased, but there was no significant difference between GM and non-GM chrysanthemum pollen (Table 4).

Dispersal of pollen by artificial and natural wind

In the closed and special-netted greenhouses, egg albumen-coated microscope slides placed at several different distances from flowers did not accumulate pollen from either GM or non-GM chrysanthemums (Data not shown).

Table 5. Cross compatibility between GM chrysanthemum and wild species.

P-parent / S-parent	*Chrysanthemum morifolium*			*C. japonense*	*C. pacificum*	*A. ageratoides*	Self
	RS	KT	MH	NF1	IG	KG	
Closed greenhouse (2004)							
mcbt-4	16.8	15.5	3.5	21.3	8.6	0.0	0.0
mcbt-8	17.5	14.8	3.8	20.5	8.4	0.0	0.0
mcbt-14	18.4	16.0	4.1	23.5	8.1	0.0	0.0
Control	16.9	16.1	4.8	24.6	9.6	0.0	0.0
Special-netted greenhouse (2005)							
mcbt-4	19.3	16.9	4.6	26.5	10.2	0.0	0.0
mcbt-8	20.6	17.2	5.1	24.7	13.2	0.0	0.0
mcbt-14	18.6	16.4	4.8	28.9	11.7	0.0	0.0
Control	19.4	15.5	5.1	26.4	10.9	0.0	0.0

P-parent / S-parent	*Chrysanthemum morifolium*			
	mcbt-4	mcbt-8	mcbt-14	Control
Closed greenhouse (2004)				
RS	20.5	22.6	20.6	22.6
KT	16.2	15.9	16.2	17.2
MH	5.3	5.2	5.1	4.9
NF1	26.5	25.4	27.4	28.4
IG	10.5	11.2	12.9	11.9
KG	0.0	0.0	0.0	0.0
Special-netted greenhouse (2005)				
RS	24.5	26.4	25.4	27.1
KT	20.6	21.6	22.0	23.4
MH	6.2	5.9	6.1	5.6
NF1	29.4	30.4	28.6	30.2
IG	11.6	12.4	13.4	11.9
KG	0.0	0.0	0.0	0.0

The data means the F_1 seed germination rate (average) of 10 flowers.
P-parent; pollen parent, S-parent; seed parent, RS; Rossanna, KF; Kofuku-no-tori, MH; Motte-no-hoka, NF1; Noji-giku Fukui No. 1, IG; Iso-giku, KG; Kon-giku.

Cross-compatibility with wild species

With GM and non-GM chrysanthemum seed parents, the stigmas of *Chrysanthemum* species such as *C. morifolium* cultivars, *C. japonense*, and *C. pacificum* retracted. The germination rates of F_1 seeds of GM and non-GM chrysanthemums crossed to *C. morifolium* cultivars were 3.5 to 18.4% in the closed greenhouse and 4.6 to 20.6% in the special-netted greenhouse. The germination rates of F_1 seeds of GM and non-GM chrysanthemums crossed to *C. japonense* were 20.5 to 24.6% in the closed greenhouse and 24.7 to 28.9% in the special-netted greenhouse. The germination rates of F_1 seeds of GM and non-GM chrysanthemums crossed to *C. pacificum* were 8.1 to 9.6% in the closed greenhouse and 10.2 to 13.2% in the special-netted greenhouse. The stigmas of *A. ageratoides* ssp. *avalus* did not retract in response to GM or non-GM chrysanthemum pollen, nor to self pollen. In the opposite crossing, with the wild species as the pollen parents, the stigmas of GM and non-GM chrysanthemums retracted upon contact with pollen from *C. morifolium*, *C. japonense*, and *C. pacificum*. The germination rates of F_1 seeds of *C. morifolium* cultivars crossed to GM or non-GM chrysanthemums were 4.9 to 22.6% in the closed greenhouse and 5.6 to 27.1% in the special-netted greenhouse. The germination rates of F_1 seeds of *C. japonense* crossed to GM or non-GM chrysanthemums were 25.4 to 28.4% in the closed greenhouse and 28.6 to 30.2% in the special-netted greenhouse. Finally, the germination rates of F_1 seeds of *C. pacificum* crossed to GM or non-GM chrysanthemums were 10.5 to 12.9% in the closed greenhouse and 11.6 to 13.4% in the special-netted greenhouse. However, neither GM nor non-GM chrysanthemum stigmas retracted upon contact with pollen from *A. ageratoides* ssp. *avalus*.

The germination rates of F_1 seeds in the special-netted greenhouse were higher than those in the closed greenhouse, but there was no significant difference in the germination rates of GM and non-GM chrysanthemum pollen, and the introduction of the *mcbt* gene caused no significant differences in the cross-compatibilities with *Chrysanthemum* species between GM and non-GM chrysanthemums (Table 5).

Production of allelopathic substances by GM chrysanthemums

The results are shown in Table 6. (1) Disposal into air. After 2 weeks, the germination rate and germinating

Table 6. The production of allelopathific substance in GM and non-GM chrysanthemums.

1. Disposal into air

Lines	Growth factor			
	Germination rate (%)	Fresh weight (mg)	Stem length (mm)	Root length (mm)
mcbt-4	99.2 ± 2.4	14.2 ± 1.6	16.2 ± 2.4	24.3 ± 2.1
mcbt-8	98.2 ± 2.2	15.2 ± 1.6	17.2 ± 2.4	25.3 ± 1.3
mcbt14	96.2 ± 3.2	14.2 ± 1.6	16.9 ± 2.4	24.7 ± 1.3
Control	99.3 ± 3.6	14.8 ± 1.4	17.1 ± 2.3	27.2 ± 1.6

2. Disposal in soil

Lines	Growth factor			
	Germination rate (%)	Fresh weight (mg)	Stem length (mm)	Root length (mm)
mcbt-4	99.5 ± 0.2	15.2 ± 1.5	17.0 ± 2.0	22.8 ± 0.5
mcbt-8	97.8 ± 1.5	14.9 ± 0.6	16.9 ± 1.8	23.4 ± 1.4
mcbt14	98.5 ± 0.2	14.8 ± 1.4	16.4 ± 1.0	22.8 ± 0.6
Control	99.7 ± 0.2	15.1 ± 0.8	16.2 ± 1.7	25.4 ± 1.3

3. Plant production of allelopathic substances

Lines	Growth factor			
	Germination rate (%)	Fresh weight (mg)	Stem length (mm)	Root length (mm)
mcbt-4	99.6 ± 0.5	15.2 ± 2.1	16.3 ± 0.3	22.6 ± 0.5
mcbt-8	97.8 ± 0.1	16.4 ± 2.6	16.8 ± 0.5	23.5 ± 1.2
mcbt-14	98.9 ± 1.5	15.8 ± 1.9	16.4 ± 0.6	22.7 ± 0.6
Control	98.7 ± 1.6	15.5 ± 2.2	16.0 ± 0.7	23.4 ± 0.6

4. Influence of plant plowing

Lines	Growth factor			
	Germination rate (%)	Fresh weight (mg)	Stem length (mm)	Root length (mm)
mcbt-4	98.6 ± 0.5	15.6 ± 0.5	16.3 ± 0.4	22.8 ± 2.0
mcbt-8	99.1 ± 0.6	16.1 ± 0.6	15.9 ± 1.6	23.1 ± 0.1
mcbt-14	97.9 ± 0.4	15.7 ± 1.2	16.6 ± 2.0	22.4 ± 2.4
Control	98.4 ± 1.4	15.9 ± 0.4	16.4 ± 1.4	23.2 ± 1.1

The data means the average ± SE of 10 samples.
The lettuce seeds 'Great Lakes 366' were used for these experiments and measured growth factors after 2 weeks.

energy of lettuce seeds were not significantly different in Agripots containing GM and non-GM chrysanthemums. (2) Disposal in soil. After 2 weeks, the germination rate and germinating energy of lettuce seeds were not significantly different in plastic dishes containing GM or non-GM chrysanthemum culture water. (3) Plant production of allelopathic substances. After 2 weeks, the germination rate and germinating energy of lettuce seeds were not significantly different in plastic dishes containing a solution of GM or non-GM chrysanthemum homogenate. (4) Influence of plant plowing. After 2 weeks, the germination rate and germinating energy of lettuce seeds were not significantly different in pots containing soil in which GM or non-GM chrysanthemums had been cultured.

Influence on soil microorganisms

The number of soil bacteria changed throughout the growth period, but there was no significant difference in the number in soil in which GM and non-GM chrysanthemums were grown. Likewise, the number of actinomycetes and other fungi also changed throughout the growth period, but without a significant difference between GM and non-GM chrysanthemums. The number of soil microorganisms did not differ significantly in the closed and special-netted greenhouses (Table 7).

Residual Rhizobium

On an AB-Km plate, colonies of *Rhizobium radiobacter* EHA101 harboring the binary vector pBIK102*mcbt*, appeared 2 days after the start of the incubation. When GM or non-GM chrysanthemum solution was applied on the AB-Km plate, no colonies of *R. radiobacter* or other microorganisms appeared (Figure 7).

Discussion

In Japan, the tobacco budworm and cotton cutworm are very serious pests of chrysanthemums, causing considerable yield losses. To control these lepidopteran insect pests, synthetic chemical insecticides are applied several times during the growing season, comprising about 3% of the total production cost for chrysanthemums.

Table 7. The influence on soil microorganisms.

Lines	Number of fungi (CFU g^{-1} dry weight)				
	Before planting	Early stage	Middle stage	Flowering stage	Post hervest
Closed greenhouse (2004)					
mcbt-4	6.2×10^4	7.2×10^4	8.2×10^4	7.2×10^4	5.2×10^4
mcbt-8	6.7×10^4	8.9×10^4	8.2×10^4	8.8×10^4	4.2×10^4
mcbt-14	9.2×10^4	10.2×10^4	9.2×10^4	8.2×10^4	3.2×10^4
Control	7.2×10^4	8.8×10^4	7.8×10^4	8.4×10^4	5.8×10^4
Special-netted greenhouse (2005)					
mcbt-4	4.2×10^4	5.7×10^4	6.2×10^4	8.8×10^4	4.2×10^4
mcbt-8	3.3×10^4	6.6×10^4	7.7×10^4	9.4×10^4	3.4×10^4
mcbt-14	3.7×10^4	6.2×10^4	6.9×10^4	7.9×10^4	4.3×10^4
Control	5.2×10^4	7.9×10^4	7.3×10^4	8.9×10^4	5.4×10^4

Lines	Number of fungi (CFU g^{-1} dry weight)				
	Before planting	Early stage	Middle stage	Flowering stage	Post hervest
Closed greenhouse (2004)					
mcbt-4	2.4×10^2	4.3×10^2	5.6×10^2	4.3×10^2	4.1×10^2
mcbt-8	3.2×10^2	2.8×10^2	5.1×10^2	4.8×10^2	3.5×10^2
mcbt-14	3.4×10^2	2.8×10^2	4.7×10^2	5.2×10^2	3.3×10^2
Control	3.3×10^2	3.5×10^2	4.7×10^2	5.4×10^2	2.9×10^2
Special-netted greenhouse (2005)					
mcbt-4	3.2×10^2	4.4×10^2	5.4×10^2	4.8×10^2	4.3×10^2
mcbt-8	4.3×10^2	5.4×10^2	5.9×10^2	6.1×10^2	5.7×10^2
mcbt-14	3.4×10^2	4.5×10^2	6.0×10^2	5.7×10^2	3.9×10^2
Control	3.5×10^2	5.7×10^2	6.2×10^2	7.4×10^2	4.7×10^2

Lines	Number of fungi (CFU g^{-1} dry weight)				
	Before planting	Early stage	Middle stage	Flowering stage	Post hervest
Closed greenhouse (2004)					
mcbt-4	2.4×10^2	4.3×10^2	6.2×10^2	8.2×10^2	6.9×10^2
mcbt-8	3.2×10^2	5.3×10^2	8.3×10^2	7.9×10^2	7.2×10^2
mcbt-14	3.2×10^2	5.9×10^2	7.3×10^2	9.3×10^2	5.6×10^2
Control	4.2×10^2	6.2×10^2	5.2×10^2	9.6×10^2	6.2×10^2
Special-netted greenhouse (2005)					
mcbt-4	2.2×10^2	6.4×10^2	9.4×10^2	7.3×10^2	6.8×10^2
mcbt-8	2.4×10^2	5.8×10^2	8.6×10^2	6.6×10^2	7.3×10^2
mcbt-14	3.1×10^2	6.8×10^2	9.7×10^2	7.2×10^2	6.9×10^2
Control	3.6×10^2	6.8×10^2	8.9×10^2	8.0×10^2	8.8×10^2

CFU: The colony formation unit
The data means the average ± SE of 5 samples.

The primary potential benefit of the insect-resistant GM chrysanthemums described in this report is the reduced production cost, mainly based on reduced insecticide applications to control lepidopteran insect pests. Additionally, the GM chrysanthemums have secondary benefits, including reduced labor time, enhanced populations of beneficial insect and wildlife populations, reduced potential runoff of insecticides, and improved safety for cultivators. Since 1996 in the USA, the culture of Bollgard cotton (Monsanto Co.), which contains an introduced *cry1Ac* gene, has resulted in reduced insecticide use, with the application of insecticidal active ingredients cut back by 2.7 million pounds, and reduced insecticide applications, which have decreased by 15 million pounds.

In this study, we performed an environmental assessment of the GM chrysanthemum lines. According to Southern, Northern, and Western blot analyses, the insect-resistance trait in GM chrysanthemums is stably integrated in the chrysanthemum genome. The introduction of the *mcbt* gene caused no significant differences in morphological and growth characteristics, pollen structure, pollen fertility and longevity, or dispersal of pollen between GM and non-GM chrysanthemums. Moreover, there was no significant difference between GM and non-GM chrysanthemums in cross compatibility with wild species, production of allelopathic substances, or influence on soil microorganisms. Finally, no residual *R. radiobacter* was found in the GM chrysanthemums. Thus, we observed no differences between GM and non-GM chrysanthemums.

Recently, concerns have been raised about gene flow from GM plants to wild weeds (Kamada 2001).

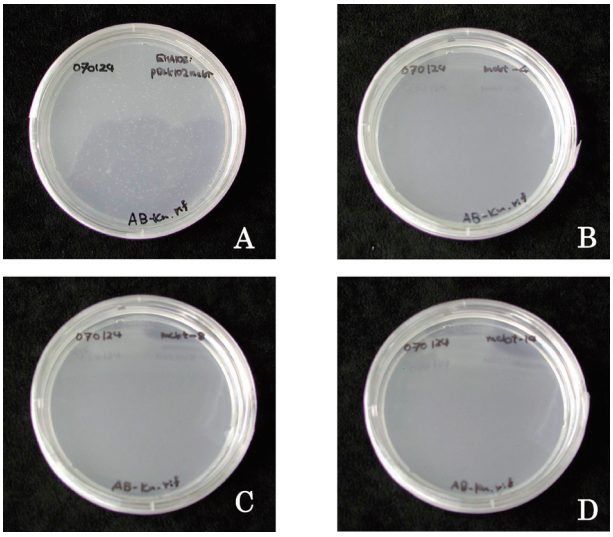

Figure 7. Residual *Rhizobium* on GM chrysanthemum plants. The plants shown are A, the binary vector pBIK102*mcbt*, and B-D, the GM chrysanthemum lines mcbt-4, mcbt-8, and mcbt-14, respectively. Ten grams of GM and non-GM chrysanthemum plant tissues were homogenized with a ceramic rotor and diluted with deionized water, and 100 μl of the solutions were spread on plates of AB medium, which contained 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin. The plates were incubated at 28°C for 7 days.

Chrysanthemums are predominantly self-incompatible plants that can be cross-pollinated by certain insects. Many wild weeds in the family Compositae that are cross-compatible with chrysanthemum cultivars are widespread throughout Japan. It is possible that the pollen of insect-resistant transgenic chrysanthemums could be carried by certain insects and pollinate wild species, resulting in the production of insect-resistant F_1 wild plants. Because this is a risk that could cause the collapse of local ecosystems, it is not permitted to culture GM chrysanthemums in open fields. It would be necessary to introduce a male- and/or female-sterility trait together with insect resistance or other useful traits to prevent foreign gene flow.

Risk-benefit assessments of these GM lines will be necessary. The advantages of transgenic chrysanthemums should be demonstrated in chrysanthemum cultivation in widely dispersed locations. These GM chrysanthemums have the potential to reduce the costs and energy required for lepidopteran insect control, as well as the environmental pollution and the health risks to cultivators due to pesticide use. Therefore, we are attempting to introduce a male- and/or female-sterility trait along with insect resistance into a chrysanthemum in order to put GM chrysanthemums to practical use.

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