Plant Biotechnology 26, 249–254 (2009)

Original Paper

Agrobacterium-mediated transformation of spinach (*Spinacia oleracea*) with *Bacillus thuringiensis cry1Ac* gene for resistance against two common vegetable pests

Jin Hua Bao[1], Dong Poh Chin[1], Masanobu Fukami[2], Masashi Ugaki[3], Masashi Nomura[1], Masahiro Mii[1]*

[1] Graduate School of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan; [2] Chiba Prefectural Agriculture and Forestry Research Center, Chiba 266-0006, Japan; [3] Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba 277-8562, Japan
* E-mail: miim@faculty.chiba-u.jp Tel & Fax: +81-47-308-8852

Received December 12, 2008; accepted January 13, 2009 (Edited by M. Nakano)

Abstract *Agrobacterium*-mediated genetic transformation was applied to produce transgenic plants of spinach (*Spinacia oleracea*) resistant to 2 pest species, *Trichoplusia ni* and *Autographa nigrisigna*. Leaf segments from *in vitro* spinach plants of cultivar 'Glory' were co-cultivated with *A. tumefaciens* strain EHA105, which harbored the plasmid pBE2111FMB containing a synthetic *cry1Ac* gene encoding an insecticidal crystal protein of *Bacillus thuringiensis*, and neomycin phosphotransferase II and bialaphos resistance genes as selectable marker genes. Kanamycin (Km)-resistant calluses were obtained from co-cultivated leaf segments 1 month after selection on 2.5 g l^{-1} gellan gum-solidified MS medium containing 30 g l^{-1} sucrose, 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg l^{-1} kinetin, 100 mg l^{-1} Km and 20 mg l^{-1} meropenem trihydrate. Regeneration of adventitious shoots from Km-resistant calluses was performed on 2.5 g l^{-1} gellan gum-solidified MS medium containing 30 g l^{-1} sucrose, 0.01 mg l^{-1} 2,4-D, 1 mg l^{-1} kinetin, 1 mg l^{-1} gibberellic acid, 100 mg l^{-1} Km and 10 mg l^{-1} meropenem trihydrate at 14°C. PCR and Southern blot analyses of genomic DNA isolated from T1 plants confirmed the successful integration of T-DNA into the plant genome. Expression of Cry1Ac protein was confirmed in leaves of transgenic plants by Western blot analysis. Insect bioassays against *T. ni* and *A. nigrisigna* performed with T1 plants showed more than 93.3% insect mortality within 1 week. These results suggest that the *cry1Ac* gene was effectively expressed in spinach.

Key words: *Autographa nigrisigna*, Bt gene, insect resistance, transgenic plants, *Trichoplusia ni*.

Spinach (*Spinacia oleracea* L.) is one of the major vegetable crops worldwide and known as a rich source of iron, vitamins and minerals. The major purposes of spinach breeding are to develop cultivars with resistances to various pests and diseases, high temperature resistance, late flowering, and low concentrations of nitrate and oxalate. Although a large number of cultivars have been developed through conventional breeding programs, it is still difficult to produce cultivars with some of such traits, because of the lack of the available germplasm. Thus, it is now expected to apply genetic manipulation to spinach for improvement of this important vegetable crop.

Bt produces a variety of insecticidal crystal proteins, which are encoded by *cry* genes and most widely used as biological insecticides (Höfte and Whiteley 1989). Until now, *cry* genes have been successfully introduced into various agriculturally important crops such as cabbage (Jin et al. 2000), tomato (Mandaokar et al. 2000), tobacco, potato, *Brassica* spp., (Kuvshinov et al. 2001), cotton (Li et al. 2006), etc, and these transgenic plants showed high resistances against lepidopteran insects. It has been known that the expression level of Cry proteins in plants was insufficient when the native or truncated *cry* genes were used due to the AT-rich nucleotide sequences of the genes (Barton et al. 1987; Fischhoff et al. 1987; Vaeck et al. 1987). However, it was found that use of synthetic *cry* genes by substituting the AT-rich regions to ones with more balanced GC contents, resulted in high levels of expression of Cry proteins (Perlak et al. 1991; Fujimoto et al. 1993; Nayak et al. 1997). In the present study, we introduced a synthetic

Abbreviations: *bar*, bialaphos resistance; *Bt*, *Bacillus thuringiensis*; CTAB, cetyltrimethylammonium bromide; 2,4-D, 2,4-dichlorophenoxyacetic acid; DIG, digoxigenin; GA_3, gibberellic acid; IBA, indole-3-butyric acid; Km, kanamycin; MS, Murashige and Skoog; *nptII*, neomycin phosphotransferase II; PAGE, polyacrylamide gel electrophoresis; PGR, plant growth regulator; SDS, sodium dodecyl sulfate.
This article can be found at http://www.jspcmb.jp/

cry1Ac gene into spinach by *Agrobacterium tumefaciens*-mediated transformation, and the transgenic plants were evaluated against the attack of larvae of 2 commonly found destructive insect pests of vegetables, cabbage looper (*Trichoplusia ni* Hübner) and Asiatic common looper (*Autographa nigrisigna* Walker).

Materials and methods

Plant material

In vitro plants of spinach cultivar 'Glory' (Takii Seed and Seedling Co., Kyoto, Japan) were used. Seeds were surface-sterilized successively with 70% ethanol for 3 min and a 1% sodium hypochlorite solution containing a few drops of Tween-20 for 20 min, followed by rinsing 3 times with sterile water. Seeds were then germinated on PGR-free MS medium (Murashige and Skoog 1962) containing 30 g l^{-1} sucrose and solidified with 2.5 g l^{-1} gellan gum (Gelrite; Wako Pure Chemical Industries, Osaka, Japan) under an 8/16 h (light/dark) photoperiod provided by fluorescent lamps (35 μmol m^{-2} s^{-1}) at 20°C.

Plasmid vector and bacterial strain

The binary vector pBE2111FMB was constructed by inserting a *Hin*dIII-digested fragment, which contains a synthetic *cry1Ac* gene (Accession No. AB472847) driven by a strong constitutive promoter cassette El2-35S-Ω from pE2113 (Mitsuhara et al. 1996), into the binary Ti plasmid, pBI121-bar, which contains 2 selectable markers, namely the *nptII* gene under the control of the nopaline synthase promoter and the *bar* gene under the control of the cauliflower mosaic virus 35S promoter (Figure 1).

The synthetic *cry1Ac* gene was produced by modifying the sequences according to Fujimoto et al. (1993) for the former part of the sequence (1-1398) with 7 additional base changes made by Perlak et al. (1991), and according to Nayak et al. (1997) for the latter part of the sequence (1399–) as shown in Figure 1. This vector was introduced into *A. tumefaciens* strain EHA105 (Hood et al. 1993) and used for transformation experiments.

Inoculation and co-cultivation with Agrobacterium

Agrobacterium was grown overnight at 28°C in LB liquid medium (Miller 1972) containing 50 mg l^{-1} Km (kanamycin sulfate; Wako Pure Chemical Industries, Osaka, Japan), 200 mg l^{-1} streptomycin sulfate salt (Sigma-Aldrich, St. Louis, MO, USA) and 10 mg l^{-1} rifampicin (Rifampicin; Wako Pure Chemical Industries, Osaka, Japan). For preparing bacterial inoculation solution, *Agrobacterium* suspension culture was added to 40 ml MS liquid medium containing 30 g l^{-1} sucrose, 0.5 mg l^{-1} 2,4-D, 2 mg l^{-1} kinetin and 100 μM acetosyringone (3′,5′-dimethoxy-4′-hydroxy-acetophenone; Sigma-Aldrich, St. Louis, MO, USA) to make a density of OD_{600}=1.0. For inoculation, leaves were collected from 2 to 3 week-old *in vitro* plants of 'Glory', cut transversely into pieces of *ca.* 5 mm in width, and immersed in the bacterial solution for 10 min. After removing the excess bacterial solution on sterilized filter papers, leaf segments were transferred onto co-cultivation medium consisting of 2.5 g l^{-1} gellan gum-solidified MS medium with 30 g l^{-1} sucrose, 0.5 mg l^{-1} 2,4-D, 2 mg l^{-1} kinetin and 100 μM acetosyringone in Petri dishes (90×20 mm) and maintained for 3 days at 20°C in the dark.

Selection of transgenic cells and plant regeneration

After co-cultivation, the leaf segments were transferred onto the same medium but lacking acetosyringone and containing 20 mg l^{-1} meropenem trihydrate (Meropen; Dainippon Sumitomo Pharma, Osaka, Japan; Ogawa and Mii 2007) for bacterial elimination and 100 mg l^{-1} Km for selection of putative transgenic calluses. They were subcultured every 2 weeks onto fresh medium of the same composition. After 2 months, Km-resistant calluses were isolated from the leaf

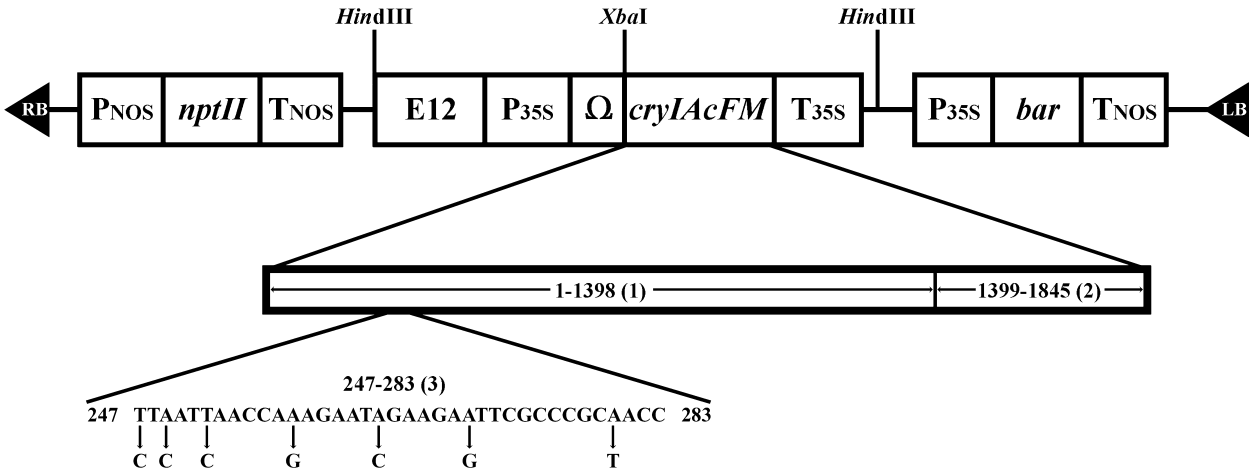

Figure 1. Schematic illustration of T-DNA region used in the transformation experiments. PNOS, nopaline synthase promoter; *nptII*, neomycin phosphotransferase II gene; TNOS, nopaline synthase terminator; El2, 5′-upstream sequence of cauliflower mosaic virus 35S promoter (−419 to −90)×2; P35S, cauliflower mosaic virus 35S promoter; Ω, 5′-untranslated sequence of tobacco mosaic virus; *cry1AcFM*, *cry1Ac* gene; T35S, cauliflower mosaic virus 35S terminator; *bar*, bialaphos resistance gene. The regions of (1) and (2) were synthesized according to Fujimoto et al. (1993) and Nayak et al. (1997), respectively. For the region of (3), sequence of Fujimoto et al. (1993) (upper lane) was substituted according to Perlak et al. (1991) as indicated by arrows.

segments and transferred to regeneration medium, which was 2.5 g l^{-1} gellan gum-solidified MS medium containing 30 g l^{-1} sucrose, 0.01 mg l^{-1} 2,4-D, 1 mg l^{-1} kinetin, 1 mg l^{-1} gibberellic acid, 10 mg l^{-1} meropenem trihydrate and 100 mg l^{-1} Km, and incubated at 14°C under an 8/16 h (light/dark) photoperiod (33 μmol m^{-2} s^{-1}).

For rooting, regenerated shoots were isolated from the calluses, dipped at their basal part into a 20 mg l^{-1} IBA solution for 2 h, and transferred to test tubes (25×100 mm) each containing the mixture of vermiculite and paper pulp blocks (20×20×20 mm) (Florialite; Sanei Co., Gunma, Japan) and 7 ml MS liquid medium (30 g l^{-1} sucrose, PGR-free). After 6 weeks, rooted plants were transferred into pots with soil and kept in a transparent plastic box for acclimatization. The plants successfully acclimatized were then grown in a growth chamber. Throughout root induction and acclimatization process, shoots and plants were kept at 20°C under an 8/16 h (light/dark) photoperiod for inducing vegetative growth since flower bud initiation occurred easily under a long day condition. For inducing flowering, spinach plants were transferred under a 16/8 h (light/dark) photoperiod at 20°C. Since spinach is a dioecious plant, T1 progeny was obtained by crossing the transgenic plants with non-transgenic plants.

DNA isolation and molecular analyses

Total genomic DNA was extracted from leaf tissues of T1 transgenic plants and non-transgenic plants using the CTAB method (Murray and Thompson 1980). For Southern blot analysis, 10 μg of genomic DNA was digested with *Xba*I or *Hin*dIII, which cut a single site or two sites within the T-DNA, respectively, and fractionated on a 0.9% agarose gel. Separated DNA fragments were then transferred to nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co., Billerica, MA, USA). The *cry1Ac* (1.3-kb) probe was generated from the plasmid pBE2111FMB by labeling with DIG using the PCR DIG Probe Synthesis kit (Roche Diagnostics, Mannheim, Germany) and the primer set, 5′-GACGCTGCTGTTGCTG-AAGCCGGA-3′ and 5′-ATGGACAACAACCCCAACATCA-AC-3′. Hybridization and detection were performed according to the instruction manual of the DIG Labeling and Detection System (Roche Diagnostics, Mannheim, Germany).

Detection of the *cry1Ac* and *nptII* genes in T1 plants was also carried out by PCR analysis. Thirty cycles were performed under the following conditions: 94°C for 1 min, 62°C for 1 min and 72°C for 1.5 min. The reaction mixture for PCR amplification contained 20 ng of template DNA, 0.5 μM of each primer, 2 mM of each dNTP, 0.5 U TaKaRa Ex Taq polymerase and 1× Ex Taq buffer (Takara Shuzo, Shiga, Japan) in a 20 μl final volume. Primers used for amplifying a 0.7-kb fragment inside the *nptII* gene were 5′-GAGGCTATTCGGCTATGACTG-3′ and 5′-ATCGGGAGC-GGCGATACCGTA-3′ (Hamill et al. 1991). Primers used for amplifying a 1.3-kb fragment inside the *cry1Ac* gene were the same as described above.

Western blot analysis

Total proteins were extracted from 100 mg of leaf tissues of the T1 transgenic plants and non-transgenic plants. Samples were ground in liquid nitrogen, and 300 ml of 2× sample buffer, which consisted of 2% (v/v) SDS, 10% (v/v) glycerol and 0.1% (v/v) β-mercaptoethanol in 0.5 M Tris-HCl (pH 6.8), was added to each sample. Samples were then boiled for 5 min and centrifuged at 8000×*g* for 5 min. Ten microliters of supernatant from each sample was loaded onto a 12% SDS-PAGE. Proteins were then electro-blotted onto PVDF membrane (Bio-Rad Laboratories, CA, USA). Detection of the Cry1Ac protein was performed using ECL Advance Western Blotting Detection Kit (Amersham Bioscience, Buckinghamshire, UK). Antibodies used were polyclonal antisera raised in rabbit against the Cry1Ac protein (at 1:5000) and goat-anti-rabbit IgG conjugated to horseradish peroxide (HRP) as secondary antibody at 1:100000.

Insect bioassays

T1 transgenic plants and non-transgenic plants were used for bioassays against larvae of 2 common vegetable pests, *T. ni* and *A. nigrisigna*. Leaves were fed to 4th instar larvae and insect mortality was recorded after 7 days. For each insect pest, 25–30 larvae were used.

Results and discussion

Selection of Km-resistant calli and production of transgenic cry1Ac T1 plants

The leaf segments from *in vitro* plants of spinach cultivar 'Glory' were co-cultivated with *A. tumefaciens* EHA105 harboring pBE2111FMB plasmid. After 2 months of culture on selective medium containing 100 mg l^{-1} Km, a total of 22 Km-resistant calluses were obtained from the cut end of 132 leaf segments, while no calluses appeared in the control non-co-cultivated leaf segments. Among these Km-resistant calluses, 6 (27%) showed shoot regeneration after transfer onto regeneration medium and incubated at 14°C.

The adventitious shoots regenerated from the Km-resistant calluses were continuously proliferated by stem cutting under the same culture conditions. However, it is rather difficult to induce an enough root system in these shoots, and thus their acclimatization and transplantation into soil were difficult. Therefore, the regenerated shoots were treated with 20 mg l^{-1} IBA at their base for 2 h, and then transferred to test tubes containing Florialite and liquid PGR-free MS medium. By using this method, most of the shoots rooted within 6 weeks. The regenerated plants were then transferred to pots and successfully acclimatized by keeping in a transparent plastic box for 2–3 weeks. After acclimatization, they grew into flowering stage in a growth chamber by changing the photoperiod from short (8 h) to long day (16 h) conditions. They successfully produced T1 seeds by cross-pollination with non-transgenic female or male plants. T1 seeds were germinated in pots and T1 plants appeared normal in morphology (Figure 2). T1 progenies from a single line of T0 plant were used for further molecular analyses and insect bioassays.

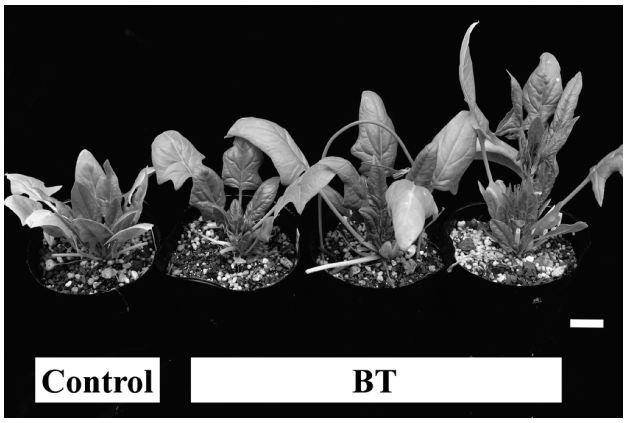

Figure 2. Transgenic spinach T1 plants with *cry1Ac* gene obtained by cross-pollination with non-transgenic plants after 3 months sowing. Germinated plants were grown at 18°C under an 8 h photoperiod. Transgenic T1 plants (BT) showed a similar normal morphology to non-transgenic control plant (Control). Bar=3 cm.

Confirmation of transformation

PCR analysis for the *cry1Ac* and *nptII* genes was performed using DNA extracted from a total of 5 T1 plants derived from a single T0 plant. As shown in Figure 3A, 1.3-kb *cry1Ac* and 0.7-kb *nptII* signals were detected in 4 plants, while 1 plant showed negative result in both *cry1Ac* and *nptII*, revealing that T1 progenies consisted of non-transgenic and transgenic plants due to the segregation of the transgene.

Southern blot analysis was performed to confirm the integration of the *cry1Ac* gene into the plant genome using genomic DNA extracted from leaf tissues of the same T1 plants as used for PCR analysis. The hybridization signals were detected from the genomic DNA of 4 transformants as the result of the PCR analysis. Since *Xba*I cuts single site within T-DNA, the result shown in Figure 3B revealed that these transgenic plants had 2 copies of T-DNA. When the genomic DNA of the T1 plants was digested with *Hin*dIII, which cuts 2 sites within T-DNA, the hybridization signals of the expected size (3.2-kb) were detected. Although the transgenic plants had 2 copies of T-DNA, segregation of the transgenes was not observed in these T1 progenies, suggesting that inserted sites of these 2 copies of T-DNA were closely linked with each other.

Western blot analysis

To determine the expression of Cry1Ac protein in the transgenic plants, total soluble protein was extracted from 3 T1 plants, which had shown positive results in PCR and Southern blot analyses, and 2 non-transgenic control plants, and subjected to Western blotting probed with anti-Cry1Ac antibody. As shown in Figure 4, expression of the 65-kD Cry1Ac protein was confirmed in all the 3 transgenic plants, while no detection of signal in the control plants.

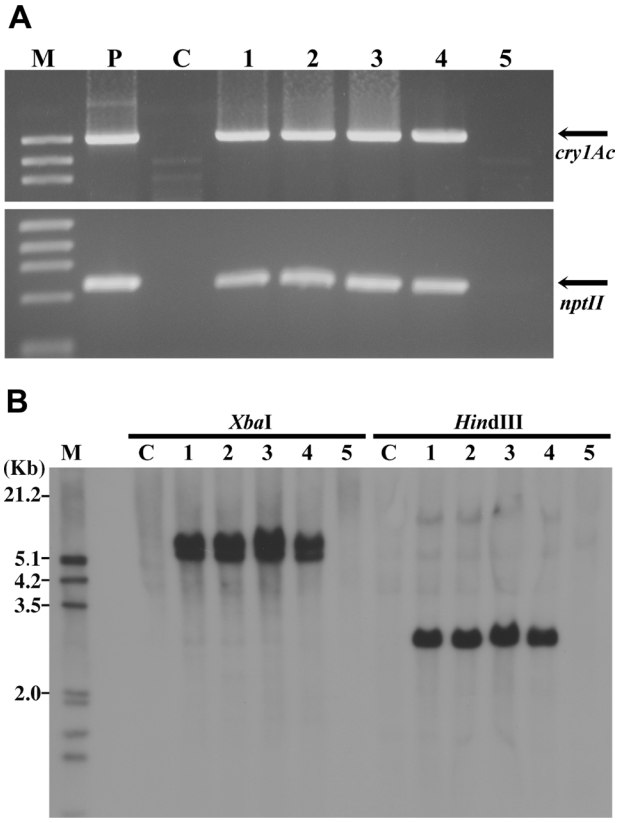

Figure 3. Confirmation of transgenes in T1 plants. (A) PCR analysis for the *cry1Ac* and *nptII* genes. Lane M, molecular size marker (ϕX174/*Hae*III); Lane P, plasmid (pBE2111FMB); Lane C, non-transgenic control plant; Lanes 1–5, transgenic T1 plants. (B) Southern blot analysis of transgenic plants. DNA samples were digested with *Xba*I or *Hin*dIII, fractionated by electrophoresis, transferred to a nylon membrane and hybridized to *cry1Ac* probe. Lane M, molecular size marker (λDNA/*Eco*RI, *Hin*dIII); Lane C, non-transgenic control plant; Lanes 1–5, transgenic T1 plants. Molecular markers are indicated on the left.

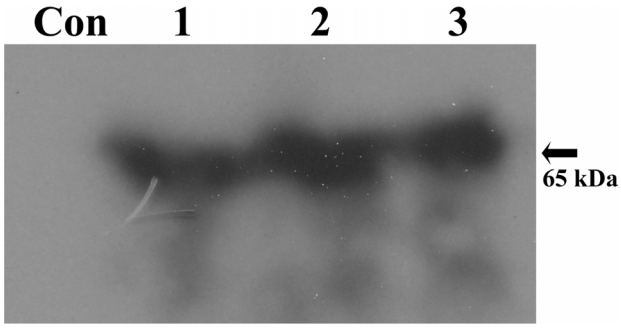

Figure 4. Cry1Ac expression in spinach plants. Western blot analysis using anti-Cry1Ac specific antiserum confirmed the expression of Cry1Ac protein. Lane Con, non-transgenic control plant; Lanes 1–3, transgenic plants.

Insect bioassays

To evaluate the insecticidel effect of the transgenic plants, we used 4th instar larvae of two species, namely, *T. ni* and *A. nigrisigna* for insect bioassays by feeding spinach leaves from transgenic plants expressing Cry1Ac

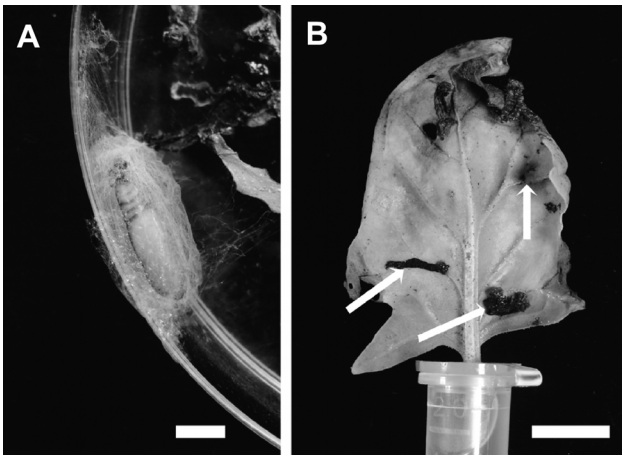

Figure 5. Effect of Cry1Ac toxin on development of *Autographa nigrisigna* larvae. A larva developed into pupal stage when fed with leaves of control non-transgenic plant (A), whereas the larvae fed with leaves of transgenic plants died within one week of feeding (B). Arrows indicated dying larvae. Bar＝10 mm.

Table 1. Bioassays of transgenic plants against *Trichoplusia ni* and *Autographa nigrisigna*

Insects	n	Insect mortality (%)[1)]	
		Control	Cry1Ac
T. ni	25	0	100
A. nigrisigna	30	10	93.3

[1)] Data were recored 7 days after feeding.

and control plants. As a result, only small bites on the leaves of transgenic plants were observed after 1–2 days of feeding. In contrast, larvae showed continuous growth on the control leaves and finally they developed into pupal stage (Figure 5). Larvae mortality was 100% and 93.3% on the leaves of the *cry1Ac* plants in *T. ni* and *A. nigrisigna*, respectively (Table 1). Although some of the larvae of *A. nigrisigna* remained alive after 7 day of feeding on transgenic leaves, all of them completely died within 14 days, which may be due to the difference in resistance against toxicity of Cry1Ac proteins among the individuals tested.

In the present study, we synthesized a *cry1Ac* gene as reported by Perlak et al. (1991), Fujimoto et al. (1993) and Nayak et al. (1997), by modification of the inappropriate coding sequence for plants and used it for transformation of spinach in combination with a high expression promoter cassette based on the report of Mitsuhara et al. (1996). Consequently, we successfully introduced the *cry1Ac* gene into spinach plants, which showed high expression levels of Cry1Ac protein and sufficiently suppressed the growth of relatively advanced age of larvae in two common pests of vegetables, *T. ni* and *A. nigrisigna*. These results suggest that the synthetic *cry1Ac* gene used in this study might be effective for controlling various lepidopteran pests of other vegetables as well as a wide range of food and ornamental crops.

Acknowledgements

We thank Mses. Aoi Hashiyama, Noriko Shintani and Mikiko Takiguchi (Laboratory of Applied Entomology and Zoology, Graduate School of Horticulture, Chiba University, Japan) for providing the larvae of *T. ni* and *A. nigrisigna*.

References

Barton KA, Whiteley HR, Yang NS (1987) *Bacillus thuringiensis* delta-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to *Lepidopteran* insects. *Plant Physiol* 85: 1103–1109

Fischhoff DA, Bowdish KS, Perlak FJ, Marrone PG, McCormick SM, Niedermeyer JG, Dean DA, Kusano-Kretzmer K, Mayer EJ, Rochester DE, Rogers SG, Fraley RT (1987) Insect tolerant transgenic tomato plants. *Bio/Technol* 5: 80–813

Fujimoto H, Itoh K, Yamamoto M, Kyozuka J, Shimamoto K (1993) Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. *Bio/Technol* 11: 1151–1155

Hamill JD, Rounsley S, Spencer A, Todd G, Rhodes MJC (1991) The use of the polymerase chain reaction in plant transformation studies. *Plant Cell Rep* 10: 221–224

Höfte H, Whiteley HR (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Rev* 53: 242–255

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Res* 2: 208–218

Jin RG, Liu YB, Tabashnik BE, Borthakur D (2000) Development of transgenic cabbage (*Brassica oleracea* var. *capitata*) for insectresistance by *Agrobacterium tumefaciens*-mediated transformation. *In Vitro Cell Dev Biol Plant* 36: 231–237

Kuvshinov V, Koivu K, Kanerva A, Pehu E (2001) Transgenic crop plants expressing synthetic *cry9Aa* gene are protected against insect damage. *Plant Sci* 160: 341–353

Li YX, Greenberg SM, Liu TX (2006) Effects of Bt cotton expressing Cry1Ac and Cry2Ab and non-Bt cotton on behavior, survival and development of *Trichoplusia ni* (Lepidoptera: Noctuidae). *Crop Protection* 25: 940–948

Mandaokar AD, Goyal RK, Shukla A, Bisaria S, Bhalla R, Reddy VS, Chaurasia A, Sharma RP, Altosaar I, Kumar PA (2000) Transgenic tomato plants resistant to fruit borer (*Helicoverpa armigera* Hubner). *Crop Protection* 19: 307–312

Miller JH (1972) *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, Ohashi Y (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol* 37: 49–59

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight DNA. *Nucl Acids Res* 8: 4321–4325

Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan NA,

Ghosh M, Sen SK (1997) Transgenic elite indica rice plants expressing CryIAc δ-endotoxinvanda of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). *Proc Natl Acad Sci USA* 94: 2111–2116

Ogawa Y, Mii M (2007) Meropenem and moxalactam: Novel β-lactam antibiotics for efficient *Agrobacterium*-mediated transformation. *Plant Sci* 172: 564–572

Perlak FJ, Fuchs RL, Dean DA, McPherson SL Fischoff DA (1991) Modifications of the coding sequence enhances plant expression of insect control protein genes. *Proc Natl Acad Sci USA* 88: 3324–3328

Vaeck M, Reynaerts A, Höfte H, Jansens S, Beuckeleer M De, Dean C, Zabeau M, Montagu M Van, Leemans J (1987) Transgenic plants protected from insect attack. *Nature* 328: 33–37