Engineering Genetic Resistance against Insects in Japanese Persimmon Using the *cryIA(c)* Gene of *Bacillus thuringiensis*

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Abstract. Japanese persimmon (*Diospyros kaki* L. 'Jiro') was transformed using a disabled strain of *Agrobacterium tumefaciens*, EHA101, carrying the binary plasmid vector, pDU92.710. The T-DNA region of pDU92.710 contained the kanamycin resistance gene (*nptII*), the β -glucuronidase gene (*uidA*), and a synthetic reconstruct of *cryIA(c)* encoding the insecticidal crystal protein fragment of *Bacillus thuringiensis* subsp. *kurstaki* HD-73. Leaf discs made from leaves of shoot cultures were cocultivated with *Agrobacterium* and cultured on a callus-induction medium containing kanamycin and cefotaxime. Among 720 infected leaf discs, 17 putative transformed callus lines showing kanamycin resistance were obtained after 8 weeks of culture. When these were cultured on a regeneration medium containing kanamycin, 15 formed adventitious buds. Of the 15 shoot lines, 11 grew well on a shoot-proliferation medium containing kanamycin, while 4 lines did not grow well. Of the 11 shoot lines, 10 showed GUS activities by fluorometric assay and were subjected to polymerase chain reaction (PCR) and Southern analyses. Except for two lines, all results were consistent with a stable integration of T-DNA into the persimmon genome. The production of CryIA(c) protein in transformed shoot lines was confirmed with Western analysis using anti-CryIA(c) serum. Insect bioassays were conducted with 10 lines showing GUS activity. Many of these lines showed high significant mortality of the test insects, *Plodia interpunctella* Hübner and *Monema flavescens* Walker, when compared to nontransformed controls.

Bacillus thuringiensis, a common soil bacterium, produces crystalline inclusion bodies containing one or more insecticidal crystal proteins (ICPs or δ -endotoxins) (Aronson et al., 1986; Höfte and Whiteley, 1989; Whiteley and Schnepf, 1986). Spore-crystal suspensions have been used as biological insecticides in agriculture and forestry for many years (Höfte and Whiteley, 1989). A major limitation of *B. thuringiensis* formulations is their instability and rapid inactivation under field conditions. Recent advances have enabled the development of insect-resistant transgenic plants that express genes encoding ICPs overcoming this limitation.

The first transformed plants with wild-type genes were obtained in tobacco (*Nicotiana tabacum*) (Vaech et al., 1987) and tomato (*Lycopersicon esculentum*) (Fischhoff et al., 1987). Transgenic plants expressed sufficient quantities of ICPs to protect plants from damage caused by tobacco hornworm (*Manduca sexta*), tobacco budworm (*Heliothis virescens*), or

tomato hornworm (*Manduca quinquemaculata*). These species are extremely susceptible to ICPs compared to other host species. In the case of walnut, the level of expression of a wild-type ICP was

Fig. 1. Binary plasmid vector pDU92.710.

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low and insufficient to control a less-susceptible insect pest species (Dandekar et al., 1994). More recently, synthetic genes encoding specific ICPs have been designed to optimize expression in plants and used to transform several crops, such as cotton (*Gossypium hirsutum*; Perlak et al., 1990), tomato and tobacco (Perlak et al., 1991; Sutton et al., 1992), rice (*Oryza sativa*; Fujimoto et al., 1993), maize (*Zea mays*; Koziel et al., 1993), and potato (*Solanum tuberosum*; Adang et al., 1993). Compared with the wild-type genes, the synthetic genes functioned much better in plants and the levels of expression were increased up to >500 times in some of the transformed plants (Perlak et al., 1991).

In this paper, we describe a successful *Agrobacterium*-mediated transformation of Japanese persimmon (*Diospyros kaki*) with a synthetic *cryIA(c)* gene of *B. thuringiensis*. The product of the gene is a 60-kDa N-terminal fragment of the ICP (ICPF) (Adang et al., 1985) that is toxic to lepidopteran insects. Many of the major persimmon insect pests are lepidopteran, such as persimmon fruit moth (*Stathmopoda masinissa*), persimmon bark borer (*Euzophera batangensis*), persimmon tree borer (*Synanthedon tenuis*), and oriental moth (*Monema flavescens*).

Materials and Methods

VECTOR CONSTRUCTION FOR THE EXPRESSION OF *cryIA(c)* GENE.

The plasmid vector pCGN4072 containing a synthetic construct of *cryIA(c)* was provided by Kevin McBride (Calgene Inc., Davis, Calif.). This plasmid contained a completely resynthesized *cryIA(c)* gene cassette cloned into the binary vector pCGN1578. The synthetic gene cassette contained the active fragment corresponding to the first 591 amino acids from the N-terminal of *cryIA(c)* gene from *B. thuringiensis* subsp. *kurstaki* HD-73 regulated by the *mac* regulatory sequences (Comai et al., 1990). A modified GUS cassette capable of high and stabilized expression in plants (Fisk, 1996) contained in pRTL2. GUS/SV was introduced into this vector. The GUS cassette was cut with *Hind*III, ligated to pCGN4072 (similarly digested with *Hind*III), resulting in the binary vector, pDU92.710 (Fig. 1). pDU92.710 was introduced into a disarmed strain of *A. tumefaciens*, EHA101 (Hood et al., 1986), as previously described (Holsters et al., 1978) and used for persimmon transformation. EHA101 was selected as a host strain because A281, the wild-type strain of EHA101, has been shown to be very virulent on persimmon (Tao et al., 1995).

GROWTH AND VIRULENCE INDUCTION OF AGROBACTERIUM. The bacterial strain, EHA101 (pDU92.710), was inoculated to 10 mL of 523 medium (Kado et al., 1972) with 20 $\mu\text{g}\cdot\text{mL}^{-1}$ gentamicin and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin and grown at 25°C overnight. The bacteria were centrifuged at 1500 g_n for 20 min and diluted and resuspended in low pH (5.2) virulence-induction medium, consisting of MS20 plus 0.1 mM acetosyringone and 1 mM betaine phosphate (James et al., 1993) without antibiotics, to give a final OD₄₂₀ of 0.5. After dilution, bacterial virulence was induced by incubation at 25°C for 5 h with agitation at 200 rpm.

PLANT TRANSFORMATION. Leaf discs (7 mm in diameter) were prepared using a cork borer from leaves of shoot cultures subcultured for 6 weeks. Shoot cultures were derived from dormant buds of 'Jiro' and maintained for >5 years (Tao and Sugiura, 1992a) on Murashige and Skoog (MS) (1962) medium with 5 μM zeatin and 1 μM indole butyric acid (IBA), pH 5.7, and solidified with agar powder for microbiological culture medium (Wako Pure Chemicals C. Td., Tokyo). Growth conditions for the shoot cultures were a 16-h light/8-h dark cycle at 28°C. Light was provided by cool-white fluorescent lamps with a total light intensity of 60 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. Before *Agrobacterium* infection, 48 leaf discs/plate (100 $\times$ 15 mm)

were cultured on one-half-strength MS medium (Sugiura et al., 1986) containing 10 μM zeatin, 10 μM naphthalene acetic acid (NAA), pH 5.7, and 0.8% (m/v) agar for 3 d, with growth conditions as described above. After this preinfection they were inoculated by immersion for 15 min in a low-pH (5.2) virulence-induction medium containing *Agrobacterium* inoculum as described earlier (James et al., 1993). After inoculation, leaf discs were blotted briefly on sterile filter paper to remove excess inoculum, deposited on filter paper (Whatman no. 1), and placed on one-half-strength MS medium containing 10 μM zeatin, 10 μM NAA, pH 5.7, and 0.8% (m/v) agar for cocultivation. Cocultivation was carried out for 3 d at 28°C in the dark. After cocultivation they were transferred to a disinfection medium, which consisted of one-half-strength MS medium with 10 μM zeatin, 10 μM NAA, 500

Fig. 2. Regeneration of transformed persimmon cv. Jiro with the synthetic *cryIA(c)* gene of *Bacillus thuringiensis*. (A) Callus formation from leaf discs after 8 weeks of *Agrobacterium* inoculation. 1) Control uninoculated leaf disc cultured without kanamycin. 2) Control uninoculated leaf disc cultured with kanamycin. 3) Inoculated leaf discs cultured with kanamycin. Arrows indicate putative transformed calli. (B) Adventitious bud formation from transformed calli after 6 weeks of culture on regeneration medium containing kanamycin. (C) Shoot growth from adventitious bud after 6 weeks on shoot medium with kanamycin.

$\mu\text{g}\cdot\text{mL}^{-1}$ cefotaxime, pH 5.7, and 0.8% (m/v) agar. At the disinfection stage, the number of leaf discs cultured on a plate was reduced to 24 and the cultures were grown in the dark at 28 °C. After 3 d, the leaf discs were transferred to a selection medium, which consisted of one-half-strength MS medium with 10 μM zeatin, 10 μM NAA, 500 $\mu\text{g}\cdot\text{mL}^{-1}$ cefotaxime, 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin, pH 5.7, and 0.8% (m/v) agar and cultured for 1 week in the dark at 28 °C. Then they were transferred to the same fresh medium but with kanamycin increased to 100 $\mu\text{g}\cdot\text{mL}^{-1}$ and the number of leaf discs per petri dish was reduced to 12. Leaf discs were further subcultured to the same fresh medium every 2 weeks. The number of leaf discs per petri dish was occasionally reduced to six depending on the growth of leaf discs. Fifty-five days after inoculation with *Agrobacterium*, calli that formed from the cut surfaces of leaf discs were removed and cultured on one-half-strength MS medium with 10 μM zeatin, 1 μM NAA, 250 $\mu\text{g}\cdot\text{mL}^{-1}$ cefotaxime, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin, pH 5.7, and 0.8% (m/v) agar in the dark at 28 °C. At this point, each single callus mass was cultured on an individual petri dish (60 × 15 mm). After 2 and 4 weeks of culture, the calli were transferred to one-half-strength MS medium with 10 μM zeatin, 1 μM indole acetic acid (IAA), 100 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin, pH 5.7, and 0.8% (m/v) agar. Thereafter, calli were maintained in the dark at 28 °C by subculturing to the same fresh medium every 6 weeks. After several subcultures, calli were subdivided and transferred to a differentiation medium consisting of MS medium supplemented with 10 μM zeatin, 0.1 μM IAA, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin, pH 5.7, and 0.8% (m/v) agar to induce adventitious bud formation (Tao et al., 1991). A single callus mass weighing ≈ 200 mg was placed on

the adventitious bud formation medium in a petri dish (60 × 15 mm) with a total of five callus masses initiated for each callus line. Once adventitious buds were formed, a vigorous shoot was chosen per callus line and transferred to a shoot proliferation medium and maintained thereafter with continuous subculturing every 6 weeks. Growth conditions for the shoot cultures were a 16-h light/8-h dark cycle at 28 °C. Light was provided by cool-white fluorescent lamps with a total light intensity of 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. For rooting and acclimatization, basal ends of the shoots were treated with 1.5 mM IBA (50% aqueous ethanol solution) and cultured as previously reported (Tao and Sugiura, 1992a). For rooting, cultures were kept in the dark at 28 °C for the first 10 d, followed by 28 °C under a 12-h photoperiod with a light intensity of 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by cool-white fluorescent lamps. In each experiment, 240 leaf discs were used for inoculation with *Agrobacterium* and 96 were used as noninoculated controls. One-half of the discs used as noninoculated controls was cultured exactly like inoculated explants whereas the other half was cultured without kanamycin. The experiment was replicated three times, and 1008 leaf discs were used.

FLUOROMETRIC DETERMINATION OF GUS ACTIVITY. Fluorometric determination of GUS activity was carried out as previously described (Jefferson, 1987). Leaf discs (8 mm in diameter) were made from unfolded young leaves. A single leaf disc was homogenized with 10 mg of polyvinylpyrrolidone (PVP) in 100 μL of extraction buffer consisting of 50 mM sodium phosphate buffer (pH 7) containing 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 % (m/v) sodium lauryl sarcosine, and 0.1 % (m/v) Triton X-100. The homogenate was centrifuged at 12,000 g_n for 10 min. The superna-

Table 1. Analysis of transformed persimmons with *cryIA(c)* gene of *Bacillus thuringiensis*.

Line ^y	Adventitious bud formation (%) ^x	GUS activity ^w	PCR ^z			No. of T-DNA integrated ^v	Western blotting with <i>cryIA(c)</i> ^u
			<i>cryIA(c)</i>	GUS	<i>npII</i>		
CS	ND ⁱ	—	—	—	—	0	—
CC	100	—	—	—	—	0	—
E1	20	ND	ND	ND	ND	ND	ND
E2	40	++	+	+	+	3	++
E3	40	++	+	+	+	1	++
E4	60	++	+	+	+	1	++
E5	40	++	+	+	+	2	++
E6	60	++	+	+	+	1	++
E7	20	ND	ND	ND	ND	ND	ND
E8	0	ND	ND	ND	ND	ND	ND
E9	40	++	+	+	+	1	+
E10	20	ND	ND	ND	ND	ND	ND
E11	40	+	+	+	+	2	+
E12	80	—	ND	ND	ND	ND	ND
E13	40	ND	ND	ND	ND	ND	ND
E14	20	++	—	+	+	2	—
E15	60	++	+	+	+	2	+
E16	0	ND	ND	ND	ND	ND	ND
E17	80	++	+	+	+	1	++

^zExpected DNA fragments were amplified (+); not amplified (—).

^yCS = nontransformed 'Jiro' shoot line maintained on the shoot medium, CC = nontransformed 'Jiro' regenerated from callus on the regeneration medium without kanamycin, E = transformed Jiro regenerated from callus on the regeneration medium with kanamycin. E1, E7, E10, and E13 were discarded before GUS activity assay because of poor growth. E12 was discarded before PCR because of it showed no GUS activity.

^xAll the data for adventitious bud formation, except for CC, were obtained in the presence of 100 $\text{mg}\cdot\text{L}^{-1}$ kanamycin. For CC, adventitious buds were formed without kanamycin.

^wNo GUS activity (—) and weak (+, 0.3 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ 4-MU, specific activity), and strong (++, 3 to 6 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ 4-MU, specific activity) GUS activities.

^vEstimated from Southern blotting with *cryIA(c)* and *npII* probes.

^uNo bands (—) and faint (+, $\approx 0.005\%$ to 0.01% of the total soluble protein) and intense (++, $>0.02\%$ of the total soluble protein) *CryIA(c)* bands detected.

ⁱNot determined.

tant was either used directly for a qualitative visual determination of GUS activity or it was diluted 200-fold with the extraction buffer for fluorometric quantitative determination of GUS activity. Ten microliters of the supernatant or diluted supernatant was taken and mixed with 50 μ L of 4 mM 4-methyl umbelliferyl-D-glucuronide (MUG) dissolved in the extraction buffer. After incubation at 37 °C for 20 min, 750 μ L of 0.2 M sodium carbonate was added to stop the reaction. The presence of GUS activity was evaluated visually by fluorescence with UV light or quantitatively using a fluorometer as described by Jefferson (1987). GUS activity was expressed as micromoles of 4-methylumbelliferone (4-MU) formed per minute per milligram of protein. Three leaf discs were assayed from each shoot line.

POLYMERASE CHAIN REACTION (PCR) ANALYSIS. Total DNA was isolated from leaves of shoot cultures by the method described by Edwards et al. (1991), and *cryIA(c)*, *uidA*, and *nptII* fragments were identified by PCR. A leaf disc (8 mm in diameter) obtained from shoot culture was crushed with 10 mg of PVP in 400 μ L of 200 mM Tris-HCl buffer (pH 7.5) containing 250 mM NaCl, 25 mM EDTA, and 0.5 % (m/v) SDS. The homogenate was centrifuged at 12,000 g_n for 10 min, and 300 μ L of isopropanol was added to 300 μ L of the supernatant. After incubation for 2 min at room temperature, DNA was recovered by centrifugation and resuspended in 100 μ L of TE buffer (pH 8). About 100 ng of the DNA were amplified in a total volume of 50 μ L PCR reaction mixture by 30 cycles of 1 min at 92 °C, 1 min 30 s at 60 °C, and 2 min at 72 °C. Three sets of oligonucleotide primers were used for the amplification of the fragments of *cryIA(c)* (5'-GCGGA TGGAA GGTTG GAGGA GGTT-3' and 5'-CCCGT TACAA CGACT TGACC CGTTT-3'), *uidA* (5'-GGAAG TGATG GAGCA TCAGG GCGG-3' and 5'-CGGAC GGGTA TCCGG TTCGT TGGC-3'), and *nptII* (5'-ATGAT TGAAC AAGAT GGATT GCACG CA-3' and 5'-GAAGA ACTCG TCAAG AAGGC GATAG A-3'). Reagents and protocols for PCR were from Perkin Elmer. PCR analysis was performed twice for each shoot line.

SOUTHERN ANALYSIS. Total DNA was isolated from leaves of shoot cultures by an SDS method (Dellaporta et al., 1983). One gram of leaf tissue was frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle containing 0.2 g PVP. The tissue was added to 15 mL of extraction buffer (0.1 M Tris-HCl (pH 8) containing 50 mM EDTA, 500 mM NaCl, and 10 mM 2-mercaptoethanol) and 1 mL of 20% (m/v) SDS was added and incubated at 65 °C for 10 min. Then 5 mL of 5 M potassium acetate (pH 5.2) was added, incubated on ice for 20 min, and centrifuged at 12,000 g_n for 20 min. The supernatant was filtered through a layer of Miracloth (Chicopee Mills Inc.) and 10 mL of isopropanol (100%) was added to the supernatant, incubated at -20 °C for 30 min, and centrifuged at 12,000 g_n for 15 min. Pellets were redissolved in 0.7 mL of 50 mM Tris-HCl (pH 8) containing 10 mM EDTA and centrifuged at 10,000 g_n for 10 min to remove debris. Seventy-five microliters of 3 M sodium acetate (pH 5.2) and 500 μ L of isopropanol were added to the supernatant to recover DNA. After centrifugation at 10,000 g_n for 5 min, the DNA pellet was washed twice with 80% ethanol and dissolved in 75 μ L TE buffer. About 8 μ g of DNA was digested with *HindIII*, and Southern blots were prepared as previously described (Dandekar et al., 1988). *CryIA(c)*, *uidA*, and *nptII* probes containing structural sequences digested from plasmid DNA were labeled by random primer incorporation of dUTP linked to DIG-dUTP. Immunological detection of the hybridization was carried out using anti-DIG-alkaline phosphatase conjugate and chemiluminescent substrate Lumi-Phos 530 (Boehringer Mannheim). Chemiluminescence was documented on X-ray films. Reagents and protocols for DNA

labeling and immunological detection of DIG-dUTP were obtained from Boehringer Mannheim. Southern analysis was performed once for each shoot line.

WESTERN BLOTTING. Eight leaf discs (8 mm in diameter) were ground with an ice-cold mortar and pestle with 10 mg of PVP in 200 μ L of sodium phosphate buffer (pH 6.8) containing 150 mM NaCl, 10 mM EDTA, 10 mM DTT, 0.3% (v/v) Tween 20, and 0.05% (v/v) Triton X100. The homogenate was centrifuged at 15,000 g_n for 20 min. Protein content of the supernatant was determined by the Bradford (1976) method. Five parts of the supernatant were mixed with one part of sample buffer, which consisted of 0.35 M Tris-HCl (pH 6.8) containing 10% (m/v) SDS, 36% (v/v) glycerol, 0.6 M DTT, 0.012% (m/v) BPB, boiled for 5 min, and then 25 μ g of total protein of each sample were subjected to electrophoresis on a 12% SDS polyacrylamide gel. After electrophoresis, protein bands were electrophoretically transferred to a nitrocellulose membrane as described by Towbin et al. (1979) for Western blot analysis. *CryIA(c)* protein was detected immunologically using rabbit anti-*CryIA(c)* serum prepared against purified *CryIA(c)* and was provided to us by K. McBride. The primary antibodies were visualized using anti-rabbit serum with alkaline phosphatase (Promega, Protoblot Western Blot AP system). Western blot analysis was performed twice for each shoot line. The amount of protein was estimated from the stained blots using a computerized image analysis software (Alpha Innotech, Calif.).

INSECT FEEDING ASSAY. We first used the Indianmeal moth (IMM), *Plodia interpunctella* Hübner, a pest of stored products worldwide because IMM is quite susceptible to ICPF (Vail et al., 1991) and is readily available in California where host insect species of persimmon do not occur. Then, oriental moth (OM), a natural insect pest of persimmon, was used to evaluate further the shoot lines showing high levels of IMM mortality. For IMM feeding assay, completely unfolded leaves were harvested from 10 independent lines, immediately frozen in liquid N₂, and lyophilized. IMM feeding assays were done by homogenizing lyophilized leaves (20 mg dry mass) for 1 min in 9.8 mL 100 mM sodium carbonate buffer (pH 10) using a Polytron at medium speed. Fifty microliters of this homogenate was applied to the

Fig. 3. PCR amplification of *cryIA(c)* (Lanes b–f), *uidA* (Lanes g–k), and *nptII* (Lanes l–p) fragments. Lane a: DNA size marker. Lanes b, g, and l: Control non-transformed shoot line used for making leaf discs. Lanes c, h, and m: pDU92.710. Lanes d, i, and n: E3. Lanes e, j, and o: E11. Lanes f, k, and p: E14.

surface (1 cm²) of 1-mL agar insect diet (BIOSERV no. 9370) in a 2-mL plastic vial and allowed to air dry for ≈ 1 to 2 h. Each vial was infested with a single first-instar IMM, closed with a plastic snap lid, and held at 27 °C. After 7 d, the test was evaluated for larval mortality and development. This method resulted in exposure of the feeding insects to a level of ICPF ≈ 5 times less than the level occurring in fresh leaves before lyophilization and homogenization. The IMM feeding assay was conducted with lyophilized leaves from the 10 shoot lines showing GUS activity. Two different nontransformed shoot lines were included as controls as well as two blank controls (water only and buffer only). Each replication consisted of 30 IMM larvae for each shoot line and the controls. The assays were replicated twice over time for a total of 60 larvae/line observed, except for the buffer control, which was inadvertently duplicated resulting in a total of 120 larvae tested.

The OM feeding assay involved two transformed persimmon lines, which showed high IMM mortality, and one control (nontransformed) line. Fully unfolded mature leaves from potted plants (≈ 6 months old) were put on a layer of filter paper soaked with distilled water and placed in a petri dish (90 mm in diameter and 20 mm in depth). Ten first-instar OM larvae were introduced into a petri dish and held at 20 °C. After 7 d, the test was evaluated for larval mortality and leaf area consumed by OM larvae. Fifteen leaves were evaluated for each line. The insect mortality and development data were analyzed using chi-square test and ANOVA with Bonferroni means separation option using the Statistical Analysis System (SAS) program (SAS Inst., Cary, N.C.).

Results

CONSTRUCTION OF pDU92.710. A map of pDU92.710 is shown in Fig. 1. The *cryIA(c)* gene sequence is regulated by *mac*, a previously described chimeric plant promoter that combines elements from the CaMV35S and *mas* promoters (Comai et al., 1990). The selectable marker gene encoding kanamycin resistance and the binary plasmid backbone were derived from the previously described vector pCGN1578 (McBride and Summerfelt, 1990). The GUS cassette contained in this vector contains modifications that improve its translation and stability of the GUS protein in plant cells (Fisk, 1996).

TRANSFORMATION OF PERSIMMON. Vigorous calli were induced from the leaf discs cultured on the medium without kanamycin, and all the leaf discs were completely covered with the calli after 8 weeks of culture (Fig. 2A-1), while no callus was formed from the control leaf discs cultured on the medium containing kanamycin (Fig. 2A-2). Of the 720 infected leaf discs, a total of 17 putative transformed callus lines showing kanamycin resistance were obtained (Fig. 2A-3); i.e., five each from replications 1 (E1-5) and 2 (E6-10) and seven from replication 3 (E11-17) (Table 1). All the control calli cultured on the medium without kanamycin formed adventitious buds, whereas only 20% of the control calli formed buds on the medium with kanamycin. No further growth of the adventitious buds induced from the control calli was observed on the shoot medium with kanamycin. Of the 17 putative transformed callus lines, 15 formed adventitious buds on the regeneration medium containing kanamycin, with adventitious bud formation percentages ranging from 20% to 80% (Fig. 2B, Table 1). Of the 15 shoot lines, 11 grew well on the shoot proliferation medium con-

taining kanamycin (Fig. 2C) and were assayed for GUS activity, while, 4 lines did not. Among these, 10 lines showed GUS activity, while nontransformed shoots and one putative transformant showed no GUS activity. GUS activities (4-MU, protein basis) of all lines, except for one (E11), were strong, and ranged from 3.3 to 5.9 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ 4-MU, specific activity. E11 showed a relatively low GUS activity of 0.3 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ 4-MU, specific activity. For further study, we selected the 10 shoot lines having GUS activity (E2, 3, 4, 5, 6, 9, 11, 14, 15, and 17) and two nontransformed control shoot lines. One was the shoot line used to obtain leaf discs (CS), and the other was the shoot line regenerated from the calli induced from the leaf disc (CC) (Table 1).

DNA ANALYSIS. The presence of the T-DNA was first confirmed by PCR. All putative transformed lines, except for E14, showed expected PCR products of 949, 625, and 792 bp, respectively, for *uidA*, *cryIA(c)*, and *nptII* fragments (Fig. 3, Table 1). No amplification was observed for the DNA prepared from nontransformed shoot lines, CS and CC. With E14, the authentic PCR products for *uidA* and *nptII* could be seen, while no PCR amplification occurred for the fragment of *cryIA(c)*. To examine internal T-DNA fragments and to determine the copy number of T-DNA inserted into the persimmon genome, DNA obtained from individual shoot lines was digested with *HindIII*, and Southern analysis was performed using *uidA*, *cryIA(c)*, *nptII* probes and expected fragment sizes are shown in Fig. 4. Hybridization with the *uidA* probe revealed internal 3300-bp fragments. Hybridization of the same blots with the *cryIA(c)* probe revealed the fragments >3974 bp, which were due to hybridization of the *cryIA(c)* sequence adjacent to the right border. Hybridization of the same blots with the *nptII* probe, which also detected sequences adjacent to the left border, was used to further confirm the number of T-DNAs integrated into the persimmon genome. All the data, except for E11 (Fig. 4 lane c) and E14 (Fig. 4 lane d), were consistent with the integration of individual T-DNAs into the persimmon genome (Fig. 4). Based on the

Fig. 4. Southern blot analysis of persimmon genomic DNA digested with *HindIII* showing the pattern of hybridization with three different probes. A physical map of the T-DNA digested with *HindIII* is illustrated below. Lane a: control nontransformed shoot line used for making leaf discs. Lane b: E9. Lane c: E11. Lane d: E14. Lane e: E15. Lane f: E17.

Fig. 5. Western blot of total leaf proteins of persimmon separated on 12% SDS-PAGE and probed with rabbit anti-CryIA(c) antibody. Arrows indicate the position of the CryIA(c) ICPF band. Lane a: Control nontransformed shoot line used for making leaf discs. Lane b: E2. Lane c: E3. Lane d: E9. Lane e: E11. Lane f: E14. Lane g: E17. Lane h: Pure CryIA(c) ICPF 10 ng.

number of the bands hybridized to *cryIA(c)* and *nptII* probes, the number of T-DNA copies incorporated into the persimmon genome was estimated to be between one and three (Table 1). As expected, no hybridization signal was detected in the nontransformed control shoot lines, CS and CC. With E11, the results obtained by the hybridization with the *cryIA(c)* and *nptII* probes indicates the insertion of two T-DNAs in one location, since two bands were detected with the *nptII* probe, while only one band with the *cryIA(c)* probe. Similarly in the case of E14, two bands are observed with the *cryIA(c)* probe and only one band of hybridization with the *nptII* probe. The band intensity in Fig. 4 lanes c and e is higher than those in the other lanes (lanes b, d, and f) obtained with *uidA* probe encoding GUS.

IMMUNODETECTION OF CRYIA(C) PROTEIN. A distinct band could be detected at 60 kDa in all the transformed shoot lines, except for E14 (Fig. 5, Table 1). No reactions at 60 kDa were observed with nontransformed shoot lines (Fig. 5 lane a) and E14 (Fig. 5 lane f). Although the amount of CryIA(c) protein produced varied depend-

ing on the shoot lines, we could estimate that >0.02% of the total soluble protein was CryIA(c) protein in the shoot lines, E2, 3, 4, 5, 6, and 17. Lesser amounts (0.005% to 0.01% of the total soluble protein) could be detected in the other three lines, E9, 11, and 15.

INSECT FEEDING ASSAY. Compared to the nontransformed shoot line, significantly higher IMM mortality was observed with all of the transformed shoot lines (Table 2), except E14, which also produced no detectable band at 60 kDa (Table 1). The shoot lines producing the highest mortality were E2, E3, E5, and particularly E17 (Table 2). These lines were also among those producing intense bands at 60 kDa (indicated by ++, Table 1). Furthermore, these shoot lines and E4, E6, and E9 significantly slowed larval development as indicated by low mean instars after 7 d of feeding (Table 2). As IMM is a nonhost insect of persimmon, we selected two transformed strains (E2 and E17), which showed the highest IMM mortality for testing with a pest species of persimmon. These lines were acclimatized, transferred to pots in the greenhouse, and the leaves from these plants were harvested for bioassay using OM that naturally feeds on persimmon leaves. We observed that the E2 and E17 plants grew as well as nontransformed control plants in the greenhouse (Fig. 6) and showed significantly higher level of OM mortality as compared with the control nontransformed plants (CS). The transformed plants E2 and E17 showed a high level of mortality toward OM larvae, 96.7 ± 1.8 and 98.0 ± 1.9 , respectively, compared to the control (15 ± 3.9). Significant differences could also be observed for the amount of feeding on the leaves (Fig. 6) with the total leaf area consumed by the control at 6.3 ± 1.5 cm² compared to 0.2 cm² for E2 and E17.

Discussion

In this study, we have developed an *Agrobacterium*-mediated transformation system for Japanese persimmon and successfully integrated the *cryIA(c)* gene of *B. thuringiensis* into the persimmon genome. Although transformation with wild-type strains of *A. tumefaciens* (Tao et al., 1995) and *A. rhizogenes* (Tao et al., 1994)

Table 2. Resistance against Indianmeal moth larvae in transformed persimmons with *cryIA(c)* gene of *Bacillus thuringiensis*.

Line ^z	Mortality		No. of live by instar					Mean instar ^y
	(%)	(n)	L1	L2	L3	L4	L5	
Water	6.7 ab ^x	(60)	0	0	0	5	51	4.9 a ^w
Buffer	11.7 ab	(120)	0	0	34	71	1	3.7 b
CS	11.7 ab	(60)	0	7	18	28	0	3.4 b
CC	3.3 a	(60)	0	4	15	39	0	3.6 b
E2	43.3 de	(60)	3	28	3	0	0	2.0 efg
E3	38.3 d	(60)	4	23	10	0	0	2.2 efg
E4	28.3 cd	(60)	2	29	12	0	0	2.2 defg
E5	41.7 de	(60)	4	26	4	1	0	2.1 efg
E6	30.0 cd	(60)	2	31	8	1	0	2.2 efg
E9	28.3 cd	(60)	1	20	21	0	0	2.5 cdefg
E11	33.3 cd	(60)	6	11	12	11	0	2.7 cdef
E14	18.3 bc	(60)	0	9	10	30	0	3.4 b
E15	36.7 d	(60)	3	11	13	11	0	2.8 cde
E17	55.0 e	(60)	16	11	0	0	0	1.4 h

^zWater = water control, CS = nontransformed 'Jiro' shoot line maintained on the shoot medium, CC = nontransformed 'Jiro' shoot line regenerated from callus on the regeneration medium without kanamycin, E = transformed 'Jiro' shoot line regenerated from callus on the regeneration medium with kanamycin.

^yMean instar of live larvae.

^xMean separation within column by χ^2 test ($p \leq 0.05$).

^wMean separation within column by ANOVA with Bonferroni option ($p \leq 0.05$).

Fig. 6. Potted 6-month-old transformed persimmons with the *cryIA(c)* gene of *Bacillus thuringiensis* (E2 and E17) and the control nontransformed plant (CS)(upper photograph). Appearance of the leaves from the CS, E2, and E17 plants after 7 d of feeding with oriental moth larvae (lower photograph).

has been reported in persimmon, this is the first report describing transformation of this fruit species with a disarmed strain of *Agrobacterium* carrying a binary plasmid vector that has the genes of horticultural interest and for selection and screening of transformants. The technique would open the door for the rapid cultivar improvement in Japanese persimmon.

For our leaf disc transformation system, a callus intermediate stage was indispensable to obtain transformants. Earlier work on persimmon has shown that it is difficult to obtain adventitious bud formation directly from leaf discs, and by contrast, a very efficient regeneration system was possible using callus cultures of persimmon (Tamura et al., 1992; Tao et al., 1991; Tao and Sugiura, 1992b). The major drawback of using a callus intermediate is that it takes longer for the regeneration process, and there is a risk of somaclonal variation. The latter will be evaluated when these transgenic plants are introduced into the environment for field testing. The efficiency of the transformants obtained was low, $\approx 1.5\%$ on a per explant basis. This suggests that a higher number of explants needs to be used initially to ensure a significant number of transformed shoots. There might be some possibilities of improving the efficacy of the transformation system by refining the culture methods and conditions during and after *Agrobacterium* infection.

Southern analysis revealed that the number of T-DNA copies integrated into the persimmon genome was one to three (Table 1). Although a negative correlation between the number of T-DNA incorporated and its expression level has been suggested to occur (Flavell, 1994; Hobbs et al., 1990; Palauqui and Vaucheret, 1995), no relation was observed in this study between the number of T-DNA and the amount of *CryIA(c)* protein estimated from Western analysis or from mortality obtained with the insect-feeding assay. Usually the level of expression of foreign genes varies widely among transformants with a single copy of T-DNA, even in a single experiment, due to gene-silencing phenomena and methylation or rearrangements of the integrated T-DNA. The effect of copy number might have been obscured by other effects in a study with small sample size, such as the 10 individual transformants tested in this experiment.

With the IMM feeding assay, significantly higher mortalities compared to controls were observed in all transformed lines,

except for E14, with mortalities ranging from 18.3% to 55.0% (Table 2). There are a few factors that may have contributed to these low mortalities.

- 1) The *CryIA(c)* protein produced might have been degraded during lyophilization or subsequent extraction procedures.
- 2) Some unknown substances in the persimmon leaves might have interacted with *CryIA(c)* protein and reduced its insect-killing activity. The toxicity of ICP has been reported to be altered (both reduced and stimulated) by plant natural products such as chlorogenic acid and polyphenol oxidase (Ludlum et al., 1991), and protease inhibitor (MacIntosh et al., 1990).
- 3) The level of ICPF in the lyophilized leaves was diluted 5-fold during the homogenization process (20 mg dry mass in 9.8 mL buffer) and the amount of *CryIA(c)* protein ingested by the IMM larvae was reduced below effective levels. As the results of IMM assay were obtained with a nonhost insect species we further evaluated two transformed lines using OM, a native insect pest of persimmon to confirm efficacy of our transformed lines. The data strongly indicated that levels of ICPF needed to be effective against native insect pest species were obtained in our transformed Japanese persimmon lines.

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