Tolerance to Citrus mosaic virus in Transgenic Trifoliolate Orange Lines Harboring Capsid Polyprotein Gene

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ABSTRACT

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Trifoliolate orange plants (*Poncirus trifoliata*) were transformed with a binary vector containing the capsid polyprotein (pCP) gene of Citrus mosaic virus (CiMV) via *Agrobacterium tumefaciens* LBA4404. Transformation was performed on the epicotyl segments obtained from a young seedling that was grown in the dark. Southern blot hybridization analysis showed that the transgene was stable in the transgenic lines after regeneration and propagation by grafting. Transgenic lines were screened for tolerance to CiMV by mechanical inoculation. Infection was monitored 30, 60, 90, and 120 days after inoculation by reverse transcription-polymerase chain reaction. The transgenic line 24 had the lowest infection rate (7.1%) at 60 days after inoculation, in contrast to that of nontransgenic plants (65.1%). The response of other lines to inoculation ranged from susceptibility to moderate tolerance.

Additional keywords: satsuma dwarf, nepovirus, soilborne disease

Citrus mosaic virus (CiMV) is a serious production constraint on citrus in Japan (12,22,23). The infected trees grow poorly and often develop ringspot symptoms on the fruit, which drastically reduces the commercial value. CiMV is closely related to Satsuma dwarf virus (12–14,22), which is a tentative member of the genus *Nepovirus* in the family *Comoviridae* (24). CiMV has a bipartite, positive-strand RNA genome consisting of RNA1 (7.0 kb) and RNA2 (5.4 kb), which are separately encapsidated in polyhedral particles about 28 nm in diameter (11). Both genomic RNAs are polyadenylated at the 3' termini (11). Unlike definitive members of the genus *Nepovirus*, CiMV has two coat proteins (CP). The nucleotide sequences of the 3' regions of RNA1 and RNA2 of CiMV isolate Ci-968 have been reported (11,12). Both RNAs encode a polyprotein, and CPs are located at the C-terminus of the polyprotein encoded by RNA2 (11). The vector has not been identified (23). However, local natural spread, apparently through

soil, has been observed in many citrus orchards, and once an orchard is infested, the disease may become permanently established (12). The persistent, soilborne nature of this virus prompted us to develop a tolerant rootstock.

The CP-mediated tolerance, which had been initially reported in transgenic tobacco that accumulated CP of *Tobacco mosaic virus* (1), has been demonstrated in many other plants (7). Tolerance against viruses like comoviruses, which have two capsid proteins, has been achieved by expressing the polyprotein that contains the two capsid proteins (5,18,20).

In this paper, we report our attempt to obtain tolerance to CiMV by expressing the CP polyprotein (pCP) in trifoliolate orange (*Poncirus trifoliata*), which is a major rootstock in citriculture in Japan.

MATERIALS AND METHODS

Construction of the binary plasmid vector (pBCiCP) and plant transformation. The plasmid CIK116, which contained cDNA for the 3' region of CiMV RNA2, had been constructed previously (11) and was used as the source of the pCP gene. The pCP gene consists of a set of larger and smaller CP genes. The DNA fragment of the pCP gene was amplified from the plasmid CIK116 using a set of forward (CCPFW1, 5'-cctctctagacaaatggg attacagaat-3') and reverse (CCPRV1, 5'-tgtgagctcagctaaaggtccggtgg-3') primers. The forward and reverse primers contain the *Xba*I and *Sac*I restriction sites at their termini (underlined), respectively. The

forward primer contained the initiation codon for the expression of pCP. The resulting DNA fragment was ligated into the plasmid pUC19 to produce the plasmid pCCP1. An expression vector was constructed by replacing the β -glucuronidase (GUS) gene in the binary vector pBE2113-GUS (16) with the cloned pCP sequence (Fig. 1). The resulting plasmid was designated pBCiCP and was introduced into *Agrobacterium tumefaciens* strain LBA4404. The plasmid pBCiCP had no viral protease, and the transgene was supposed to express a polyprotein without cleavage into larger and smaller CPs in plants.

Plant transformation, screening, and propagation. Transformation and regeneration of trifoliolate orange plants were conducted as previously reported (15). The regenerated shoots on a selection medium containing Kanamycin (200 μ g/ml) were screened for the presence of transgene by polymerase chain reaction (PCR) amplification using a primer set of CCPF72 (5'-tctctctatgcattggtcttact-3') and CCPRV926 (5'-tgagagttagtgatcagcgttg-3') after extraction with cetyltrimethylammonium bromide (CTAB), as reported previously (17). Amplification was carried out for 40 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 90 s, after the initial denaturation at 94°C for 4 min. The plants, which were positive in PCR screening, were grafted to etiolated sour orange seedlings and subsequently side-grafted to rough lemon rootstock to promote vigorous growth. After the second grafting, the transformants were grown in a closed-system greenhouse, which was maintained at a constant 25°C.

Mechanical inoculation with CiMV. Etrog citron cuttings infected with CiMV isolate Ci-968K (11) were used as the inoculum source. Five to 10 plants of each transgenic and nontransgenic trifoliolate orange were inoculated mechanically by either stem-slashing (8) or conventional grafting. For stem-slash inoculation, the young, tender leaves of the CiMV-infected Etrog citron were macerated and diluted 1:5 (wt/vol) in neutral phosphate buffer (0.1 M) containing 0.1% (vol/vol) 2-mercaptoethanol. The stem of the trifoliolate orange was slashed 50 times with a knife that was immersed in the extract after every 10 cuttings. For graft inoculation, blind buds were excised from a CiMV-

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infected Etrog citron plant and inserted into the stem of the trifoliate orange.

Enzyme-linked immunosorbent assay (ELISA) detection. Tender, immature leaves from transgenic and nontransgenic trifoliate orange were assayed by double-antibody sandwich ELISA (DAS-ELISA) (4) using CiMV polyclonal antibody, which had been raised against an isolate Ci-968K and used routinely in our labora-

tory (13). Typically, two samples were taken from a tree, and two wells in the ELISA plate were used for each extract. Leaves were macerated in 1:10 (wt/vol) volumes of citrate buffer (pH 6.5) containing 0.1% 2-mercaptoacetic acid, and each well of the plate was filled with 200 μ l of the extract. IgG (1 mg/ml) for coating ELISA plate and alkaline phosphate-IgG conjugates, which had been conjugated

with IgG of 0.5 mg/ml, were diluted 1:1,000 and 1:500, respectively. Etrog citron leaves infected with CiMV isolate Ci-968K and healthy leaves were used for positive and negative controls, respectively.

Southern blot hybridization analysis. Total DNA was extracted from mature leaves with CTAB (17). After ethanol precipitation, extracted DNA was dissolved with an aliquot of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), then adjusted to 500 ng/ μ l by determining the concentration with a DyNA quant 200 fluorometer (Amersham Bioscience, Buckinghamshire, UK). Total DNA (5 μ g), digested with the indicated restriction enzymes (Fig. 2), was separated on 1.0% agarose gel, then transferred onto Hybond-N+ nylon membrane (Amersham Bioscience) by capillary action. Following transfer, the DNA was covalently linked to the filter by UV exposure. The pCP DNA fragment was labeled with 11-fluorescein by a gene image random prime labeling kit (Amersham Bioscience) according to manufacturer's instructions. The filters were hybridized with the 11-fluorescein labeled pCP DNA fragment in a mixture (5 $\times$ SSC, 10% formamide, 0.1% sodium dodecyl sulfate [SDS], 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, and 10% nonfat dry milk) at 50°C. After successive washing of the filter with 0.1 $\times$ SSC, 0.1% SDS at 50°C and 60°C, the hybridization signal was detected by disodium-2-chloro-5-(4-methoxy)spiro[1,2-dioxetane-3,2'-(5'-

Fig. 1. Restriction map of the binary plasmid vector. pBE2113-GUS, a recombinant binary transformation vector (16); El2, two tandem repeats of the upstream sequence of *Cauliflower mosaic virus* (CaMV) 35S promoter; P35S, upstream sequence of CaMV 35S promoter; Ω , 5'-untranslated sequence of *Tobacco mosaic virus*; GUS, β -glucuronidase gene; Tnos, polyadenylation signal of the gene for nopaline synthase in the Ti plasmid (16); pBCiCP, a recombinant binary transformation vector prepared in this study; CiMVpCP, LCP, and SCP represent genes for coat protein polyprotein, large coat protein, and small coat protein of Citrus mosaic virus.

Fig. 2. Southern blot hybridization analysis of DNA from lines of trifoliate oranges, transformed with coat protein polyprotein gene of Citrus mosaic virus (CiMV). Total DNA from the transgenic lines was double-digested with *HindIII* and *XbaI* in A, and with *SphI* and *EcoRI* in B, and probed with the CiMV pCP gene probe. Lane numbers correspond to the transgenic lines of trifoliate orange, and W indicates DNA from nontransformed wild-type trifoliate orange.

chloro)-tricyclo[3.3.1.1]decan]-4-yl) phenyl phosphate (CDP-star) containing ECL system (ECL, Amersham Bioscience).

Reverse transcription (RT)-PCR for monitoring CiMV after challenge inoculation. After transgenic plants were mechanically inoculated with CiMV, the infection was monitored by RT-PCR for up to 120 days after inoculation (dai). Since both forward and reverse primers were designed to be specific to RNA1, the RT-PCR product was considered to be amplified from viral RNA, not from transcript of pCP transgenes, which originated from RNA2.

Total RNA was isolated from 0.5 g of young tender leaf tissue as reported previously (2,3), and the final RNA pellet was dissolved in 100 μ l of TE buffer. First strand cDNA was synthesized from 1 μ l of RNA sample added to 20 μ l of the reverse transcription mixture containing 1 $\times$ first strand buffer (10 mM Tris-HCL, 50 mM KCl, 2.5 mM MgCl₂, pH 8.3), 1 mM of each dNTP, 20 units of RNase inhibitor, 50 units of MuLV reverse transcriptase, and 1 μ M of reverse primer (5'-gttgcaagcagggcatctgtctac-3'), which is complementary to the 3' region of CiMV RNA1. The reaction mixture was incubated at 42°C for 30 min and added to a PCR reaction mixture to a final volume of 100 μ l, containing 1 $\times$ PCR buffer (10 mM Tris-HCL, 50 mM KCl, 2.5 mM MgCl₂, pH 8.3), 200 μ M of each dNTP, 2.5 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), and 1 μ M each of reverse and forward primer (5'-gaagaggttgattctgaagcgg-3'). The amplifications were performed in a DNA Thermal Cycler 9600 (Applied Biosystems) for 40 cycles under the following conditions: 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C.

RESULTS

Integration of the CiMV CP genes in trifoliate orange. After a typical transformation experiment on trifoliate orange, eight of 144 epicotyl segments were resistant to Kanamycin, and transgene was detected by PCR from five of the eight lines regenerated on Kanamycin-containing medium. Transformation was conducted several times, and 32 candidates were established. After grafting, the established transgenic lines grew vigorously in a closed greenhouse, and they were about 10 mm in diameter just above the graft-union in 15 months. Southern blot hybridization after digestion of the plant genomic DNA with *Hind*III and *Xba*I, which cuts upstream region of the transgene (Fig. 1), is shown in Figure 2A. Under these conditions, the number of the bands should correspond to the copies of transgene. The results indicated that one to three copies of pCP gene were present in the plant genome except for lines 27, 45, 46, 48, and 97. Transgenes were not detected from six transformant candidates (lines 27, 45, 46,

47, 48, and 97) by Southern blot hybridization (Fig. 2A), suggesting false-positive results in the PCR assay on these lines. After excluding lines that have no transgene, a second Southern hybridization test was conducted to estimate the size of the transgene. To do this, the genomic DNA of the transgenic lines were digested with *Sph*I and *Eco*RI to cut both ends of the transgene (Fig. 1). The results showed that the blot of expected size (4.6 kbp) was present in the transgenic lines except for lines 3, 28, and 63 (Fig. 2B). The transgenic lines 24, 25, and 26 were considered to be identical, judging from the similarity of the hybridization bands (Fig. 2A) and their origin from the same regenerated shoot. Furthermore, we concluded that the transgenic lines 3 and 28 were identical, and likewise for the transgenic lines 31 and 32. Finally, 22 transgenic lines listed in Table 1 were established and used for further analysis. The expression level of the transgenes in these lines was estimated by DAS-ELISA using anti-CiMV antiserum, and summarized in Table 1. Since the ELISA was conducted on the transgenic plants before inoculation with CiMV, the values are interpreted as the expression level of pCP transgene. The ELISA values for the established transgenic plants after subtraction of background ranged from 0.006 to 0.960 A_{405nm} , suggesting different levels of expression of pCP gene among the transgenic lines (Table 1).

Response to virus inoculation. The results of stem-slash inoculations of the

transgenic trifoliate orange plants are summarized in Table 1. In a typical inoculation test, five replications were used for each transgenic line, while 10 seedlings of trifoliate orange, which were as old as transformed lines, were inoculated as controls.

Differences in response to CiMV inoculation between transformed and nontransformed plants were most apparent at 60 dai (Table 1). Using RT-PCR amplification, CiMV was detected in only one of 14 plants (7.1%) in the transgenic line 24, in contrast to 28 of 43 nontransgenic plants (65.1%). Also, fewer than 2 of 5 or 10 plants (20% or less) were infected in transgenic lines 31, 80, and 83 at 60 dai. Establishing a threshold of 20% at 60 dai, these lines were considered tolerant to CiMV infection. Differences between transgenic line 24 and nontransgenic trifoliate orange were apparent up to 120 dai, while transgenic line 80, which showed difference at 60 dai, were all infected at 90 dai. CiMV was detected at high frequencies in transgenic lines 74, 91, 93, and 95, and these lines were considered susceptible. On the basis of low infection rates, line 24 was considered the most tolerant, in spite of lower level expression of pCP, as estimated by ELISA. Overall, tolerance to CiMV infection was not correlated with level of pCP expression (Table 1).

When graft-inoculation was tried, all of the tested transgenic lines and nontransgenic controls were infected at 60 dai, as determined by RT-PCR (data not shown).

Table 1. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) of transgenic trifoliate orange lines and response to inoculation with Citrus mosaic virus (CiMV) at 60, 90, and 120 days after inoculation (dai)

Line	DAS-ELISA values ^y			
	(405 nm)	60 dai	90 dai	120 dai
Control	0.017	28/43 ^z	34/43	42/43
24	0.006	1/14	3/14	4/14
28	0.021	4/10	7/10	10/10
30	0.080	2/5	4/5	4/5
31	0.960	0/5	2/5	2/5
35	0.952	4/10	7/10	7/10
39	0.641	3/9	4/9	8/9
51	0.281	2/5	3/5	5/5
56	0.933	2/5	4/5	5/5
63	0.060	2/5	4/5	5/5
68	0.557	2/5	5/5	5/5
74	0.504	4/5	4/5	5/5
80	0.521	0/5	5/5	5/5
83	0.810	1/10	5/10	6/10
87	0.814	2/10	4/10	6/10
89	0.059	1/5	4/5	4/5
90	0.704	2/5	5/5	5/5
91	0.332	3/5	3/5	5/5
92	NT	3/3	3/3	3/3
93	0.092	3/5	5/5	NT
95	0.580	3/5	3/5	5/5
96	0.951	1/2	2/2	2/2
98	0.945	2/5	5/5	5/5

^y DAS-ELISA values are the average of optical density readings at four wells after subtraction of background. DAS-ELISA was conducted on the transgenic plants before inoculation with CiMV, and the values are interpreted as the expression level of the coat protein polypeptide transgene.

^z Number of reverse-transcription polymerase chain reaction positive plants/number of inoculated plants.

DISCUSSION

The efficiency of transformation in citrus is low, and few agriculturally important genes have been transferred. To our knowledge, the transfer of a viral gene to citrus has been successfully conducted using the CP gene of *Citrus tristeza virus* (CTV; 6,9). Satsuma mandarin, a major citrus in Japan, Korea, and some parts of China, is tolerant to CTV and susceptible to CiMV. The results presented in this paper suggested that the pCP gene of CiMV was successfully transformed into trifoliate orange rootstock. The inoculated epicotyl was transformed at a frequency of 2.1 to 4.6% (data not shown). The stability of the transgene was shown, for most of the lines, by Southern hybridization analysis on the plants that were propagated by grafting after transformation and regeneration. In lines 3, 28, and 63, the transgene was larger than the expected size. We speculate that the transgene is partially dimers in these transgenic lines.

Lines 24, 31, 80, and 83 of trifoliate orange transformed with pCP of CiMV exhibited tolerance. There was little correlation between tolerance and the level of transgene expression. In this study, the expression of transgene was estimated only by ELISA. Our trial to detect pCP by Western blotting failed (data not shown). It was not clear that intact pCP accumulates in lines that showed high values in ELISA. A lack of correlation between tolerance and the transgene protein level has been observed in reports on genetically engineered tolerance to RNA viruses (19,21). In some of these cases, the tolerance has been referred to as RNA-mediated (19). The tolerance mechanism to CiMV in transgenic trifoliate orange lines needs to be confirmed by further experiments.

We are now planning to plant the most promising transgenic lines in a CiMV-infested field, and to evaluate their tolerance to CiMV infection under natural conditions.

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