Development of Transgenic Tomato Expressing a High Level of Resistance to Cucumber Mosaic Virus Strains of Subgroups I and II

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ABSTRACT

Agrobacterium-mediated transformation was employed to develop transgenic tomato (Lycopersicon esculentum) of line G-80 that contains the coat protein gene of cucumber mosaic virus white leaf (CMV-WL) strain, a member of Cucumovirus subgroup II. Twenty-nine independently transformed G-80 plants were obtained. R0 transgenic plants accumulated detectable levels of CMV-WL coat protein, and 16 of the 19 inoculated plants were resistant to the CMV China strain, a member of subgroup I. Progenies from three R0 transgenic plants were tested for resistance to infection by CMV-WL and CMV-China. These R1 plants showed high levels of resistance to systemic infection by both strains, as virus could not be recovered from asymptomatic inoculated plants. The development of transgenic tomatoes that are resistant to isolates from both subgroups of CMV should have practical significance for controlling this serious disease, especially since natural sources of CMV resistance have not been identified.

Tomato (Lycopersicon esculentum Mill.) is a popular vegetable throughout the world. Its production has increased in the last 70 yr to meet demands for processing and fresh market. Although tomato ranks 16th among the most common vegetable crops in nutritional value, it ranks first in consumption because of its attractive color, flavor, and versatility (21).

Innovative and efficient breeding methods have increased yields and quality of many commercial cultivars, especially those developed for machine harvesting or to be grown in greenhouses and plastic tunnels. Breeding for resistance has also contributed to the control of many diseases caused by bacteria, fungi, and viruses. However, efforts to find and utilize natural resistance genes to cucumber mosaic virus (CMV) have not been successful (23). CMV resistance factors have been located in wild Lycopersicon species, but none has been utilized because of its polygenic nature.

CMV, the type member of the Cucumovirus group, possesses a segmented genome consisting of three single-stranded RNAs and a subgenomic RNA coding for the viral coat protein (CP). CMV can infect several hundred plant species and in nature is spread by 60 or more aphid species in the nonpersistent manner. It is mechanically transmissible and has been reported to be seedborne in 19 plant species, but not in tomato. A large number of strains have been reported and classified; they can be classified into two subgroups, I and II (previously S and WT), according to differences in their serological properties and nucleotide sequence homologies (17). Also, CMV has been found to be associated with a number of satellite RNAs, which can significantly modify the expression of disease symptoms.

The phenomenon of coat protein-mediated protection (CPMP), a form of parasite-derived resistance (22), provides an alternative method for developing tomato that has genetically inheritable resistance to many viruses, including CMV (1). Several reports have shown that transgenic tobacco and cucurbits expressing the CP gene of CMV show resistance to infection (2,6,7,14-16,19, 25). In this report, we show that tomato transformed with the CP gene of the white leaf strain of cucumber mosaic virus (CMV-WL) has high levels of resistance to CMV-WL, a strain that belongs to subgroup II, and to a severe CMV strain that belongs to subgroup I.

MATERIALS AND METHODS
Plant materials. The tomato line Geneva 80 (G-80), developed by R. Providenti, is of early maturity, resistant to tobacco mosaic virus (TMV) (Tm-2/Tm-2), Verticillium wilt, and Phytophthora infestans (race 0) (Ph/Ph).

Plant transformation and regeneration. The disarmed Agrobacterium tumefaciens strain C58Z707 containing CMV-WL CP (15) was used to transform tomato. This strain contained the binary plasmid pGA482GG/cpCMV-WL 5-14, which includes the neomycin phosphotransferase II (NPTII), β-glucuronidase (GUS), and CMV-WL CP gene within its T-DNA region. The cauliflower mosaic virus 35S promoter and termination signals and the CMV-C leader sequence were used for expression of the CP gene (15).

Transformation and regeneration of tomato were done by a modification of the procedure of McCormick et al (12). Tomato seeds were surface-sterilized for 15 min in 5.25% sodium hypochlorite. Seeds were washed three times with sterile distilled water and then plated onto Murashige and Skoog (MS) (13) medium with B5 vitamins, 3% sucrose, and 0.8% agar. The pH of the medium was adjusted to 5.8-6.0 with KOH and autoclaved 20 min at 121 C. Sterilized seeds were grown at 25 ± 2 C under cool-white fluorescent light for 7 days.

The 7-day-old cotyledons were cut transversely into three sections, and the medial cotyledon sections (0.2 × 0.5 cm) were conditioned in a regeneration medium (12) containing MS salts, B5 vitamins, 2.5 mg/L of BA, 1.0 mg/L of IAA, 3% sucrose, and 0.8% agar for 36 hr at 25 C in light. These sections were then soaked in a fresh overnight culture (about 105 to 106 bacteria per milliliter) of A. tumefaciens for 25-30 min. In an alternate method, cotyledons were cut and the medial sections immediately soaked in the A. tumefaciens solution for 15 min. Explants from both treatment methods were then blotted dry and placed onto solidified conditioning media in petri plates for cocultivation with the bacteria. After 2 days, explants were transferred to selection medium (regeneration medium containing 50 mg/L of kanamycin and 500 mg/L of carbenicillin) to select transformed tomato cells. After 3 wk, cotyledon sections with developing callus were transferred to fresh selection medium. Finally, after another 3 wk, young shoots were transferred onto a rooting medium containing MS salts, B5 vitamins, 3% sucrose, 0.8% agar, 50 mg/L of kanamycin, and 500 mg/L of carbenicillin. Plants with roots were transferred to soil and grown in the greenhouse.

GUS assays. These were performed as described by Jefferson (10). The fluoros-
cent methyl umbelliferone product was visualized on a UV light box.

**PCR analysis.** Genomic DNA was isolated from transgenic and nontransformed tomato plants according to the method of Dellaporta et al (3). DNA from each plant, oligonucleotide primers CMV-WL CP 5' (5'-AGCTAACCCTGGAAATCTGGATCTCCCAAT-3'), CMV-WL CP 3' (5'-CCATCATAACGAAGTTTAGCAGGTAC-CTTACGA-3'), NPTII 5' (5'-CCCTCCGGTGATCCAATTAG-3'), and NPT II 3' (5'-CCCGGGGTGGGCGAAGAATCCG-3') were used for the PCR. DNA was amplified by 35 cycles of 1 min at 94 C, 2 min at 60 C, and 3 min at 72 C. Reaction samples were directly loaded and electrophoresed in 1.3% agarose gels for 60 min.

**Kanamycin resistance.** Young leaves of R₀ tomato plants growing in the greenhouse were excised, surface-sterilized in 5.25% sodium hypochlorite for 10 min, washed three times with sterile distilled water, and cut into pieces approximately 2 x 1 cm. The leaf pieces were placed on McCormick et al (12) medium containing 50 mg/L of kanamycin for 4 days in darkness and were then transferred to a lighted room. Leaves of transformed plants developed callus within 2 wk, whereas leaves of nontransformed plants did not form callus.

**Detection of CMV-WL CP gene in transgenic tomato plants.** The CMV-WL CP was detected with antibodies and the ELISA techniques described by Nambara et al (15). ELISA results were read on a micro-ELISA autoreader (MR 200, Dynatech Laboratories, Inc., Chantilly, VA) 1 hr after the substrate was added.

**Inoculation of tomato plants with CMV strains.** Two strains of CMV were used: CMV-WL and CMV-China. The first belongs to subgroup II and is the homologous strain from which the CP gene was derived (8,20). The second, originally recovered from tomatoes grown in southern China, is a severe strain belonging to subgroup I (11). These strains were maintained in tomato G-80 and in tobacco (Nicotiana tabacum L.) cv. Havana 423, which is also resistant to TMV. Seven to 14 days after transplanting, test plants were dusted with conramum and rubbed with CMV-WL or CMV-China at a 1:10 (w/v) dilution of tissue extracts from infected tomato or tobacco. This treatment was repeated after 3-4 days to ensure successful inoculation. Controls were nontransformed plants similarly inoculated with these strains. *Chenopodium quinoa* Willd., a local lesion host, was included to check for latent infection in inoculated plants. Plants were observed daily for symptom development for 4-6 wk after inoculation.

**RESULTS**

**Transformation.** Several critical factors, including tissue culture medium, kanamycin level, and conditioning times for explants, were tested to optimize the transformation of G-80. Initial experiments showed that cotyledon explants produced the best callus when incubated in McCormick et al (12) regeneration medium containing either 2.5 mg/L of BA and 1.0 mg/L of IAA or 2.0 mg/L of BA and 0.5 mg/L of IAA. However, this medium was not optimal for developing shoots from leaf pieces.

The sensitivity of nontransformed tomato cotyledons to kanamycin was examined using regeneration medium containing 0, 25, 50, 75, and 100 mg/L of kanamycin. Normal calli and shoots developed on the medium without kanamycin. With 25 mg/L, a few explants produced calli and shoots, whereas with 50 mg/L or higher, growth of nontransformed explants was inhibited. Consequently, 50 mg/L of kanamycin was chosen for selection of transformed tissue.

When cotyledon sections were incubated in regeneration medium for 36 hr prior to transformation, the rate of transformation greatly increased. In one experiment, 29 of 70 cotyledon sections treated with *A. tumefaciens* developed callus on kanamycin selection medium and shoots were later produced. In a transformation experiment without incubation prior to transformation, only nine of 70 cotyledon sections formed callus.

Generally, callus developed by the third week after explants were placed onto selection medium, and plantlets could be transplanted to the soil 5 mo after initiation of transformation. Fifty putative transformed plants were obtained from cotyledon sections of G-80. Most of these plants appeared morphologically normal, and selected plants produced fruits with viable seeds.

**Analysis of R₀ transgenic plants.** The 50 putative transformed R₀ plants were

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**Fig. 1.** Coat protein gene expression levels and reaction of GUS-positive R₀ transgenic G-80 tomato plants to inoculation by CMV-China. Inoculated plants were rated as resistant (R) or susceptible (S); plants not rated were not inoculated. Plants designated with an asterisk were selfed, and R₁ plants were evaluated for resistance to CMV. OD ELISA values for GUS-negative R₀ plants ranged from 0.034 to 0.129 and averaged 0.084 (data not shown).

**Fig. 2.** PCR analysis of R₀ transgenic G-80 tomato plants. Fragments were amplified with NPTII and CMV-WL primers. Lane 1, DNA standard Phi X 174 RF DNA HaellI fragments; lane 2, nontransformed plant; lanes 3-6, four independent R₀ transgenic plants. The observed PCR-generated products for the NPTII and CMV CP genes were of the expected size.
PCR and tests for calli formation were used as the criterion for determining whether a plant was transformed. Twenty-nine of the G-80 plants were GUS-positive and had an average ELISA reading of 0.188 OD_{405} (range from 0.108 to 0.450), compared with an average ELISA reading of 0.084 (range from 0.034 to 0.129) for GUS-negative R_0 G-80 plants (Fig. 1). These data showed that not all plants that came through the kanamycin selection process were transformed; thus, the GUS test was used to confirm the identity of transgenic R_0 plants.

**Reaction of R_0 transgenic plants to CMV-China.** Nineteen GUS-positive and 13 GUS-negative R_0 G-80 tomato plants were further tested by inoculation with the CMV-China strain. CMV-China was used in favor of the homologous CMV-WL strain because it infected G-80 tomato more consistently and caused more pronounced symptoms than the CMV-WL strain (Fig. 3) and because previous work had shown that tobacco transformed with the CMV-WL CP gene gave good resistance to CMV-China (15). Sixteen of the 19 GUS-positive plants that were inoculated did not develop symptoms (Fig. 1), while all of the 13 GUS-negative plants that were inoculated developed symptoms (data not shown). Plants that became infected developed symptoms within 8–12 days after inoculation, showing prominent stunting, foliar green-yellow mosaic, upward curling, and reduction of the laminae (Fig. 3).

**Reaction of R_1 transgenic tomato plants to CMV-WL and CMV-China.** Three GUS-positive transgenic R_1 G-80 plants (TT1, TT4, TT5) that showed various levels of CMV-WL CP gene expression (Fig. 1) were self-pollinated and the resulting progenies were tested for resistance against the CMV-WL and CMV-China strains. The R_1 seedlings were tested by GUS assays and CMV-WL CP ELISA and subsequently inoculated with one of the CMV strains (Table 1). Fifty-seven of 62 GUS-positive G-80 plants expressed detectable levels of CMV-WL CP, and 56 of 62 of the inoculated R_1 plants did not develop any symptoms (Fig. 3) within 4–6 wk after inoculation with CMV-China. All 50 of 50 of the CMV-China inoculated control plants (GUS-negative progeny of the transgenic R_0 plants and nontransformed G-80 plants that did not go through tissue culture) developed symptoms (Fig. 3) within 8–12 days after inoculation, compared with 85% (44 of 52) of those inoculated with CMV-WL, confirming our previous observations (unpublished) that CMV-WL was not as infectious as CMV-China on tomato line G-80.

Transgenic plants without symptoms did not have latent systemic infection, since inoculation of tissue extracts from noninoculated leaves of these plants did not produce local lesions on C. quinoa, a sensitive local lesion host of both CMV-WL and CMV-China. As expected, tissue extracts from symptomatic plants produced numerous local lesions on C. quinoa.

**DISCUSSION.**

The R_1 transgenic plants derived from the tomato line G-80 (TT5, TT4, TT1; Table 1) and expressing the CMV-WL CP gene are resistant to mechanical inoculation with the homologous CMV-WL, a strain of subgroup II, and to the CMV-China strain belonging to subgroup I. No systemic infection was detected by symptomatology or inoculation.
loration to C. quinoa. This is the first report of the development of transgenic tomato plants expressing the CP gene of CMV that are highly resistant to mechanical inoculation of this virus. Since CMV is one of the major viral pathogens affecting tomato, and since all previous efforts to incorporate a valuable source of resistance have been unsuccessful (23), these transgenic plants may have practical impact for controlling CMV in tomato.

An important feature of these transgenic tomatoes is their resistance to mechanical inoculation with isolates from CMV subgroups I and II, which are segregated on the basis of nucleic acid sequence homology and serology (17). CMV WL belongs to subgroup II but transgenic plants with CMV-WL CP gene were resistant to CMV-China, which belongs to subgroup I. The broad resistance of plants with the CMV-WL CP gene was first observed by Namba et al (15), who compared the resistance of transgenic tobacco with the CMV-WL and CMV-C CP genes. They showed that the former CP gene gave a broader spectrum of resistance than the CMV-C CP gene. Quemada et al (19) also showed that transgenic tobacco with the CMV-C CP gene did not give good resistance against CMV-WL. Recently, Provvidenti and Gonsalves (18) demonstrated that transgenic tomatoes developed from this work have excellent resistance to 10 strains belonging to subgroup I and II and obtained from different regions of the world.

Results of published works on coat protein-mediated protection have generally shown that resistance is in the form of delay of symptom expression or that the percentage of plants becoming infected increases as the virus concentration in the inoculum increases (4,9,24). In contrast, our transgenic tomatoes were resistant even when inoculated with highly infectious tissue extracts at low dilutions, as shown by this study and by Provvidenti and Gonsalves (18). However, the CMV-WL CP gene appears to be effective in some plant species but not in others. For example, transgenic squash expressing the CMV-WL CP gene developed local infection in the inoculated leaves but not systemic infection (B. Xue et al, unpublished; 5). On the other hand, transgenic melons expressing high levels of the CMV-WL gene showed delay of symptom expression, and only a low percentage of the plants did not become infected (6).

The primary objective of this work was to develop transgenic tomato plants that would offer substantial levels of resistance to strains of CMV belonging to both subgroups. We have developed a horticulturally acceptable line that does not become systemically infected after mechanical inoculation. At present, our efforts are directed toward characterizing the inheritance of this CP gene to determine the influence of certain factors (e.g., temperature, satellites, etc.) on resistance and to evaluate the performance of these transgenic plants under field conditions (6,18).

LITERATURE CITED