Inheritance of Resistance to Cucumber Mosaic Virus in a Transgenic Tomato Line Expressing the Coat Protein Gene of the White Leaf Strain

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In tomato (*Lycopersicon esculentum* Mill.), the coat protein (CP) gene of the white leaf strain of cucumber mosaic virus (CMV-WL) conferred a high level of resistance against American, Asian, European, and Oceanian strains belonging to both serogroups of CMV. An analysis of genetic populations deriving from crosses and backcrosses of a homozygous CMV-resistant tomato line (TT5-007-11) with susceptible cultivars revealed that (1) the high level of resistance is conferred by a single dominant gene to which the symbol *Cmv* is assigned; (2) in grafts between CMV-resistant and -infected plants, the resistant plants developed systemic symptoms, indicating that they are not immune; (3) the CMV resistance is independent of the virus inoculum titer, and it can be effectively used for the production of F_1 commercial hybrids; (4) the two markers, neomycin phosphotransferase II (NPT-II) and β-glucuronidase (GUS), present in transgenic plants are not completely reliable for predicting resistance; and (5) *Cmv* confers resistance to most CMV strains containing satellites (RNA5), but one mutant satellite derived from CMV-WL infected transgenic plants. This is the first report of a satellite that can interfere with the function of a CP gene. The valuable breeding line TT5-007-11 is resistant also to tobacco mosaic virus (*Tm-2²*), *Verticillium* wilt, and *Phytophthora infestans* (Race 0).

For several decades, all attempts to incorporate resistance to cucumber mosaic virus (CMV) in tomato (*Lycopersicon esculentum* Mill.) were without success. Although sources of resistance to this virus were found in some wild tomatoes (*L. cheesmanii, L. hirsutum, L. parviflorum, L. pennellii, L. peruvianum,* and *Solanum lycopersicoides*), the polygenic nature of resistance factors and incompatibility between donors and receivers created great difficulties in exploiting them (Kariyama et al. 1971; Latterot 1980; Phills et al. 1977; Watterson 1993).

In 1973, a simple way to combine DNA from two different organisms and then clone identical copies gave birth to genetic engineering. Ten years later, a practical and efficient way for foreign genes to be introduced and expressed in plant species was reported. This was accomplished by eliminating (disarming) tumor-causing genes from the Ti plasmid of *Agrobacterium tumefaciens* and by inserting foreign genes. Hence, by leaving intact the DNA transfer mechanism, it was possible to incorporate useful genes into a variety of plant cells (Leemans 1993).

For viral diseases, the most common form of biotechnological control involves the insertion of the cDNA of a coat protein (CP) gene into a plant genome (Grumet 1990). The exploitation of this "coat protein mediated protection" or "transgenic cross-protection" has been attempted for several plant species (Beachy et al. 1990). For tomato, strains of CMV causing prominent symptoms were the major targets, but the resulting CP genes have proven to be viral titer dependent, serogroup specific, or only to condition a low level of resistance (Quemada et al. 1991). Consequently, in the past 2 years, our efforts were directed toward the utilization of a versatile CP gene that had derived from CMV-WL, a member of subgroup II of the Cucumovirus Group (Namba et al. 1991). Ordinarily, this strain incites a bright whitish mosaic on tomato leaves (WL = White Leaf), but when its RNA5 satellite is removed by purification or through passage in cucurbits, the virus causes only mild symptoms (Gonsalves et al. 1982). In tobacco, the CMV-WL CP gene provided a broad spectrum of protection against strains of both serotypes of the virus (Namba et al. 1991). Recently, this CP gene was incorporated into tomato and appeared to be as effective as a natural resistance gene (Xue et al. 1994). Thus, the

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purpose of this study was to determine the inheritance of resistance of the CMV-WL CP gene in tomato and assess the influence of factors that may affect the expression of resistance.

Materials and Methods

The transgenic tomato line R_3 TT5-007-11, used in our study, is homozygous for resistance to CMV, tobacco mosaic virus (*Tm-2²*), *Verticillium* wilt, and *Phytophthora infestans,* Race 0. Plants of this line also contained two useful markers, neomycin phosphotransferase II (NPT-II) and β-glucuronidase (GUS) (Xue et al. 1994). For genetic studies, plants of this line were crossed with Geneva 80 (G-80), which was used for transformation (Xue et al. 1994), and Veemore (early maturity with medium size fruits). Plants of F_1, F_2, and reciprocal backcross populations of these crosses were grown in restricted areas in greenhouses, and mechanically inoculated at the two leaf stage with CMV-China, a member of subgroup I of the Cucumovirus Group (Kearney et al. 1990). Inoculum was prepared by triturating CMV-infected G-80 leaves with 0.05 M phosphate buffer (K^+) at pH 8.5 and used at a dilution 1:10. To minimize escapes, all plants were reinoculated on the third and fourth leaves. To determine the effectiveness of the CMV-WL CP gene, at least 16 plants of TT5-007-11 were tested with each of 13 strains of the virus originally collected in Australia (CMV-Y), California (CMV-CA), Egypt (CMV-E), Florida (CMV-FL), France (CMV-F), Hawaii (CMV-H), Japan, (CMV-J), Mexico (CMV-M), New York (CMV-WL, CMV-LSS, and CMV-L2), New Zealand (CMV-NZ), and Taiwan (CMV-TW). Nine of these strains belonged to subgroup I (CMV-J, CMV-CA, CMV-E, CMV-F, CMV-FL, CMV-H, CMV-J, CMV-NZ, and CMV-TW) and four to subgroup II (CMV-WL, CMV-LSS, CMV-L2, and CMV-M) of Cucumovirus. Some of these strains were associated with satellites, thus offering the opportunity to test the role of specific RNA5 on the expression of the CP gene. Healthy plants of TT5-007-11 were also grafted with CMV-infected G-80 plants, to assess the type of resistance by introducing in the healthy phloem a high titer of the virus. To determine its suitability as a parent for the production of commercial F_1 hybrids, additional plants of this line were crossed with those of the cv. Solarita (late maturity and large fruits). The polymerase chain reaction (PCR) technique was used to detect NPT-II, and the GUS assays were performed with the fluorescent methyl umbelliferon product, which was visualized under an ultraviolet light. The CMV-WL CP gene was detected by enzyme-linked immunosorbent assay (ELISA) using the homologous antiserum (Gonsalves et al. 1982; Xue et al. 1994). All plants were kept in a restricted area of an aphid-free greenhouse, held at 25°C–28°C.

Table 1. Inheritance of resistance to cucumber mosaic virus in the transgenic tomato line TT5-007-11 possessing the coat protein gene of CMV-WL strain

Parents and progeny	Observed segregation: Resistant	Observed segregation: Susceptible	Expected ratio	Goodness-of-fit (*P*)
TT5-007-11 (007-11)	56	0		
Geneva 80 (G-80)	0	47		
Veemore	0	58		
F_1 (G-80 × 007-11)	27	0		
F_1 (007-11 × G-80)	35	0		
F_2 (007-11 × G-80)	81	23	3:1	.49
F_1 (007-11 × Veemore)	45	0		
F_1 (Veemore × 007-11)	37	0		
F_2 (Veemore × 007-11)	85	35	3:1	.30
BC (Veemore × 007-11) × Veemore	24	30	1:1	.43
BC (Veemore × 007-11) × 007-11	85	0		

Results

Inheritance

Plants of TT5-007-11 inoculated with CMV-China reacted with a symptomless infection restricted to the inoculated leaves. Recovery tests and ELISA involving all the other leaves and stem established that the virus had failed to move systemically (systemic resistance). Plants of the susceptible parents G-80, and Veemore, after an incubation period of 8–10 days, developed systemic green mottle, leaf reduction, necrotic spotting, and stunting. From the data presented in Table 1, it is evident that all plants of F_1 TT5-007-11 × G-80, G-80 × TT5-007-11, TT5-007-11 × Veemore, Veemore × TT5-007-11 failed to develop systemic infection, confirming that the resistance was dominant regardless of which was the maternal parent. Plants of the F_2 populations deriving from the above crosses segregated in the ratio 3 resistant to 1 susceptible. Further confirmation of the presence of a single dominant factor was obtained from backcross populations. All plants of (TT5-007-11 × G-80) × TT5-007-11, and (TT5-007-11 × Veemore) × TT5-007-11 were systemically resistant. Conversely, plants of the testcross populations (TT5-007-11 × G-80) × G-80, and (TT5-007-11 × Veemore) × Veemore segregated approximately in the ratio 1 resistant to 1 susceptible. Consequently, to the CP gene conferring monogenically dominant resistance to CMV in tomato, the symbol *Cmv* is assigned.

Resistance to CMV Subgroups I and II

When groups of 16 plants of TT5-007-11 were inoculated with each of the nine Cucumovirus strains belonging to subgroup I (CMV-CA, CMV-E, CMV-F, CMV-FL, CMV-H, CMV-J, CMV-NZ, CMV-TW, and CMV-Y) and three to subgroup II (CMV-WL, CMV-L2, and CMV-M) all were found to be systemically free of the virus. ELISA and recovery tests clearly showed that these viruses failed to move systemically in stems and leaves of TT5-007-11. The resistance conferred by *Cmv* did not depend on the virus inoculum titer, since it was able to withstand a minimum dilution of 1 (infected plant sap) to 1 (buffer) with any of the strains listed above.

Type of Resistance Conferred by the CMV CP Gene

Recovery tests and ELISA had confirmed that the *Cmv* gene conferred a high level of systemic resistance in TT5-007-11 plants. However, when 12 of these resistant plants were individually approach-grafted to CMV-infected G-80 plants, within 12 days resistant plants developed systemic mosaic and stunting. Identical results were obtained when a dozen apical portions (6–8 cm) of healthy TT5-007-11 plants were individually splice-grafted on CMV-infected plants. Local lesion tests on *Chenopodium quinoa* and ELISA revealed a very high titer of the virus in leaf and stem tissues of infected TT5-007-11 plants. Thus, these tests demonstrated that by introducing a steady flow of virus into the phloem, resistant transgenic plants succumbed to infection. Thus, resistance was not at the immunity level.

Effect of the RNA5 Satellites on CP Gene

Plants of TT5-007-11 were fully systemically resistant to the following CMV strains possessing specific RNA5-satellites: CMV-WL+RNA5-WL (white leaf), CMV-China+RNA5-N (necrotic leaf), and CMV-Mexico+RNA5-ML (mild leaf). However, a mutant recently derived from CMV-WL infected plants of TT5-007-11, causing severe stunting and drastic reduction of leaf laminae, thereby giving the appearance of shoe-strings (LSS). When CMV-LSS and CMV-WL were individually cultured in cucumber (*Cucumis sativus* L.) or summer

squash (*Cucurbita pepo* L.) the helper CMV replicated normally, but the satellites (RNA5-WL and RNA5-LSS) replicated very slowly and were easily eliminated through a series of transfers in these hosts (unpublished data). Both CMV isolates lacking the satellite RNAs caused on susceptible plants a very mild chlorotic mottling of foliage and no plant stunting. Conversely, plants of TT5-007-11 inoculated with these CMV isolates free of satellites remained symptomless, and recovery tests and ELISA confirmed the absence of viral infection in leaves and stems. Thus, it was evident that by eliminating the RNA5-LSS satellite, the helper CMV failed to infect plants possessing the *Cmv* gene.

Marker Genes for Predicting CMV Resistance

Although the two marker genes, NPT-II and GUS, were useful during the development of CMV-resistant transgenic plants, they were not completely reliable for predicting resistance. Among plants found to be resistant to CMV in F_2 populations, 5%–15% of them were negative for one or both of these markers. Also, in the same F_2 populations, there was a similar percentage of plants which were positive for one or both of these markers, but were susceptible to CMV. Although resistance to the virus followed Mendelian segregation, the markers did not. Thus, the CMV inoculations proved to be more reliable in indicating which plants in segregating populations possessed the *Cmv* gene.

Resistant Line as a Parent of F_1 Hybrids

Plants of TT5-007-11 were crossed with Solarita, a cultivar of late maturity which produces large fruits and has *Verticillium* and *Fusarium* wilt resistance. The resulting (TT5-007-11 × Solarita)F_1 or (Solarita × TT5-007-11)F_1 plants produced large fruits; were resistant to CMV, TMV, *Fusarium* wilt, *Verticillium* wilt, and late blight; and were of intermediate maturity.

Discussion

For the first time, it was possible, through genetic engineering, to incorporate in tomato a gene conferring a high level of resistance to CMV (Xue et al. 1994). This single dominant gene is comparable to a natural resistance gene; thus, it can be easily incorporated in other cultivars using backcrossing or pedigree breeding. An analysis of transgenic resistant tomato plants revealed: (1) The *Cmv* gene offers very effective protection against a number of CMV strains from America, Asia, Europe, and Oceania, which belong to the two known serogroups of the virus. This resistance is independent of the virus inoculum titer, since it can withstand a minimum dilution of 1 (infected plant sap) to 1 (buffer) with any strain of this virus. (2) Preliminary tests have already shown that the homozygous line TT5-007-11 is well suited for the production of F_1 hybrids, because it is homozygous resistant to CMV, TMV, *Verticillium* wilt, and late blight (Race 0). (3) The two marker genes, NPT-II and GUS, associated with the *Cmv* gene are still useful, but they are not completely reliable; hence, during breeding, the screening for resistance must be based upon viral testing. (4) Recovery tests and ELISA confirmed that the *Cmv* gene confers a high level of resistance, but grafts between resistant and CMV-infected plants determined that this resistance is not immunity. (5) Although *Cmv* conferred resistance to strains of CMV possessing three distinct satellites (RNA5-WL, RNA5-N, and RNA5-ML), a mutant (RNA5-LSS), deriving from RNA5-WL, infected transgenic resistant plants.

There is already evidence that a CP gene can be effective in one plant species and partially or totally ineffective in others. Our CMV-WL CP gene appeared to be far less effective when it was introduced into melons (*Cucumis melo* L.) (Gonsalves et al. 1994). Even in tomato, the best results were obtained with our early ripening TMV-resistant cultivar Geneva 80, which was developed several years ago (Xue et al. 1994). This suggests that, in this cultivar, the gene found a stable chromosomal locus, which allows a full expression of its function.

This study has given the first indication that a satellite can interfere with the function of a CP gene. It was evident that *Cmv* gene was able to control the CMV shared by two strains, but of the two associated satellites (RNA5-WL and RNA5-LSS), only one (RNA5-LSS) interfered with the function of the resistance gene. It is already known that in satellites minor nucleotide sequence changes can be responsible for altering host responses (Palukaitis 1988).

The performance of transgenic crops such as the one described in this article has induced private, as well as state institutions to seek deregulation from the USDA, thus treating them as conventional crops. Environmentalists, however, are not entirely convinced that the ecological risks are minimal. One of their major fears is that these "man-assembled" genes will escape from transgenic crops via pollination and become incorporated into wild species. They remind us that in many areas of the world, exotic plant species have become serious pests. In a similar manner, engineered plants may become the sources of exotic traits causing adverse economical consequences. On the contrary, the majority of researchers believe that the long history of plant modification by classic breeding provides no evidence of ecological disasters (Kareiva 1993). For example, the most effective gene for TMV resistance in tomato (*Tm-2²*) derived from interspecific crosses between *L. peruvianum* (donor) and *L. esculentum* (receiver) (Alexander and Cirulli 1966). Although this gene has been extensively incorporated into hundreds of tomato cultivars of worldwide distribution, there is no evidence that it has moved into any of the wild Solanaceae. It is obvious that with natural or "man-assembled" genes, the possibility of interspecific or intergeneric gene-transfer is the same (Provvidenti and Gonsalves 1993).

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