Performance of Transgenic Tomatoes Expressing Cucumber Mosaic Virus CP Gene under Epidemic Conditions

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Abstract. Three processing tomato (*Lycopersicon esculentum* Mill.) lines engineered to express the cucumber mosaic virus (CMV) capsid protein (CP) gene were evaluated in the summers of 1995 and 1996 under high levels of naturally occurring CMV disease pressure. One tomato line expressed the capsid protein gene from a subgroup II isolate of CMV (line 11527), whereas two lines (12261 and 12295) expressed the capsid protein genes from a CMV subgroup I and a subgroup II isolate. Evaluation of CMV incidence based on symptomatic plants revealed that only 9% and 8% of the plants in line 11527 were infected in 1995 and 1996, respectively, 5 weeks after being transplanted. None of the plants in line 12261 developed symptoms in 1995, whereas 26% were symptomatic in 1996. There were no symptomatic plants in line 12295 in either the 1995 or the 1996 trial. In contrast to the CMV transgenic lines, 96% and 95% of the susceptible control plants were symptomatic by the 5-week rating period. CMV incidence in the CMV transgenic lines was much higher when infection was based on detection of virus by enzyme-linked immunosorbent assay (ELISA). This was particularly true in the 1996 trial where no less than 97% of the plants within a treatment were determined to be infected. Though a relatively high percentage of the transgenic plants were infected, the amount of CMV that accumulated in these plants was significantly less than in the susceptible controls, which may explain the occurrence of the attenuated symptoms. Despite CMV infection of the transgenic lines in the Alabama field trials, the performance of these lines could be of practical value to growers.

A severe epidemic, caused primarily by cucumber mosaic virus (CMV), has devastated the fresh-market tomato industry in northern counties of Alabama each summer since 1992 (Sikora et al., 1998). In this region, tomatoes are planted as transplants beginning in April and continuing through July, with new settings placed in the field every 2 weeks. A large aphid population appears in the area each year between mid-June and mid-July, which is followed 10 to 14 d later by a severe outbreak of CMV (Sikora et al., 1998). Tomato plants of varying stages of development become infected, although plants infected at earlier stages in their development tend to be more severely affected. All CMV-infected plants have the potential to serve as sources of inoculum for subsequent settings. Unfortunately, there are no fresh-market tomato varieties resistant to infection by CMV. The lack of resistant varieties creates a serious vulnerability to the

fresh-market tomato industry, particularly when threatened by a pathogen such as CMV. This virus poses a serious threat to many cultivated crops worldwide because of its extensive host range and mode of transmission. More than 800 plant species are susceptible to infection by CMV (Palukaitis et al., 1992), including many weed species (Tomlinson, 1987). The ubiquitous presence of CMV in nature is enhanced by its ability to be transmitted from plant-to-plant by at least 75 species of aphid (Palukaitis et al., 1992), and its nonpersistent manner of transmission severely restricts management practices directed at its vector, e.g., application of insecticides to control aphid populations. Moreover, the regimented planting schedules used by the growers in northern counties of Alabama limit their options to manipulate cultural practices in an attempt to avoid large influxes of aphids and delay the onset of CMV infection. Alternative approaches therefore must be evaluated that will allow tomato production to continue under even extreme CMV disease pressure.

Pathogen-mediated resistance has been evaluated extensively as a means to manage viral diseases (for reviews, see Fitchen and Beachy, 1993; Hull and Davies, 1992; Lomonosoff, 1995; Wilson, 1993). The degree and specificity of resistance tends to vary with the type of transgene used, i.e., viral

structural and nonstructural protein genes. Attempts to engineer resistance to CMV using the capsid protein strategy started as early as 1986. Using the native CMV-D capsid protein gene driven by the cauliflower mosaic virus 35S promoter, transgenic tobacco plants were generated that were highly resistant to CMV infection (Cuozzo et al., 1988). Applying the same construct to engineer resistance in tomato, however, was ineffective due to an out-of-frame ATG sequence within the leader sequence. Thus, the CMV capsid protein leader sequence was removed and the cauliflower mosaic virus 35S promoter replaced with the figwort mosaic virus 35S promoter. Transformation with this construct boosted CMV capsid protein expression 20-fold with corresponding high levels of resistance against CMV infection (Kaniewski, unpublished data). Successful pathogen-mediated resistance in tomato against CMV using the capsid protein gene as the transgene has since been demonstrated under greenhouse (Namba et al., 1991; Xue et al., 1994) and field conditions (Fuchs et al., 1996; Gielen et al., 1996; Tomassoli et al., 1995). Here, we report the evaluation of three processing-type tomato lines genetically engineered to express the CMV capsid protein gene. One tomato line was engineered to express the capsid protein gene of a subgroup II isolate of CMV, while two other lines expressed the capsid protein genes of both subgroup I and II CMV isolates. The opportunity to evaluate potential sources of resistance under the naturally occurring severe conditions of the CMV epidemic in North Alabama warranted testing these transgenic lines even though processing tomatoes are not grown in Alabama.

Materials and Methods

Plant material. The tomato lines used in this report, as well as the approaches used for transformation and regeneration, were developed at Monsanto Company and were derived from the processing tomato variety UC82B (Kaniewski et al., 1998). Many lines were initially evaluated for virus resistance. The three transformed tomato lines used in this study were selected by W. Kaniewski and were homozygous R2 progenies. Each line was homozygous for CMV capsid protein and neomycin phosphotransferase II transgenes. All homozygous lines expressed 5 to 20 mg capsid protein per kilogram of fresh-mass leaf tissue. Line 11527 contained the capsid protein gene of CMV-PG, a subgroup II isolate originally isolated from tomato in South Italy (Gallitelli et al., 1991). Lines 12261 and 12295 both contained the capsid protein gene of CMV-PG and CMV-22 (CMV-22 is a subgroup I isolate, supplied by the Instituto Sperimentale per la Patologia Vegetale, Rome).

Experimental design. Two field trials, both in the same field, were carried out in Blount County, a location where tomato production has suffered severe losses due to CMV each summer since 1992 (Sikora et al., 1998). Plants in each treatment were started from seed in

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Todd planter flats (72 wells per flat) and maintained in a greenhouse for 3 weeks, then transplanted to the field 12 June 1995 and 15 June 1996 (hereafter referred to as 1995 and 1996 trials, respectively).

A randomized complete-block design was used for each trial with each treatment (transgenic lines and UC82B) replicated four times with 10 plants per line in each replication. All plants were grown on white plastic mulch in 1995, and in open beds in 1996. Standard cultural practices used by fresh-market tomato growers in northern Alabama were followed (Kovach et al., 1992) except that plants were not staked or tied since they were processing tomato lines.

Visual and serological analyses. Tomato plants in both trials were evaluated for CMV infection visually and by enzyme-linked immunosorbent assay (ELISA). Visual assessment for CMV-induced symptoms was carried out three times (2, 3, and 5 weeks after transplanting) during each trial.

All tomato plants from the 1995 and 1996 trials were tested for CMV infection (i.e., detection of CMV capsid protein antigen, hereafter referred to as CMV CP) by direct double antibody sandwich ELISA at Auburn Univ. In each trial, samples consisting of three young leaves were collected from each tomato plant at ≈ 6 weeks after transplanting. The three leaves were wrapped in a moistened paper towel, placed in a zip-lock freezer bag and transported on ice to the laboratory. All samples were processed the following day. Microtiter plates (Microtest III Flexible Assay Plates; Fisher Scientific, Norcross, Ga.) were coated with anti-CMV immunoglobulin (Ig) at $1 \mu\text{g}\cdot\text{mL}^{-1}$ in 50 mM carbonate buffer, pH 9.6, for at least 12 h at 4°C . Anti-CMV antiserum made to the commelina strain of CMV was purchased from American Type Culture Collection, Rockville, Md. Immunoglobulin was purified by ammonium sulfate precipitation (Harlow and Lane, 1988). Tomato leaf samples were ground using a motorized leaf squeezer to a final dilution of 1 g tissue per 10 mL of phosphate buffered saline containing 0.05% tween 20 (PBS-T), and incubated for at least 12 h at 4°C . Anti-CMV Ig conjugated to alkaline phosphatase (Type VII T; Sigma Chemical Co., St. Louis) was added to microtiter plates at a dilution of 1:850 in PBS-T and allowed to incubate at least 12 h at 4°C . Substrate reactions ($1 \text{ mg}\cdot\text{mL}^{-1}$ p-nitrophenylphosphate diluted in 10% diethanolamine, pH 9.8) were standardized such that samples of purified CMV at $5000 \text{ ng}\cdot\text{mL}^{-1}$ (equivalent to 500 ng of CMV per well) added to each microtiter plate were allowed to achieve an absorbance value of ≈ 1.0 (typically within 60 min). Reactions were read at 405 nm using a Dynatech MR 700 microplate reader (Dynatech Laboratories, Chantilly, Va.). A sample was considered positive for the presence of CMV CP when the ELISA absorbance value was greater than the average ELISA absorbance value of healthy control samples plus three standard deviations ($\approx 98\%$ probability). Healthy samples consisted of greenhouse-grown UC82B plants for the 1995 trial or each

of the transgenic lines for the 1996 trial.

Tomato leaf samples were tested for the presence of CMV isolates belonging to subgroups I or II by direct double antibody sandwich ELISA (Cuozzo et al., 1988). Tomato samples consisting of three young leaves collected from UC82B plants in July 1995 were placed in plastic bags and shipped by express mail to Monsanto Co., Chesterfield, Mo., for analysis. Each sample was tested by ELISA using antiserum to CMV-22 (subgroup I isolate) and CMV-PG (subgroup II isolate). Nunc MaxiSorp microtiter plates (VWR Scientific Products, Batavia, Ill.) were coated with either anti-CMV-22 or -PG IgG at $1 \mu\text{g}\cdot\text{mL}^{-1}$ in 50 mM carbonate buffer, pH 9.6, and incubated at least 12 h at 4°C . Each sample, which consisted of three leaf disks taken from each plant using a number six cork borer ($\approx 50 \text{ mg}$ tissue fresh mass), was ground in an eppendorf tube in 750 μL of PBS-T-O (PBS-T containing ovalbumin at 0.2%), and incubated for at least 12 h at 4°C . Anti-CMV-22 or -PG IgG conjugated to alkaline phosphatase (Type VII, Sigma) were each diluted 1:5000 in PBS-T-O and incubated for at least 12 h at 4°C . Substrate reactions ($1 \text{ mg}\cdot\text{mL}^{-1}$ p-nitrophenylphosphate dissolved in 10% diethanolamine, pH 9.8) were allowed to proceed at room temperature for $\approx 30 \text{ min}$ (a $100 \text{ ng}\cdot\text{mL}^{-1}$ purified CMV standard reacted to 1.5–1.9), at which time reactions were read at 405 nm using a Bio-Rad 3550 microplate reader.

Evaluation of plant performance. The evaluation of the CMV transgenic lines emphasized CMV infection rather than effects on tomato yield. Thus, few data on fruit yield and quality were obtained. Samples of fruit consisting of 10 tomatoes collected randomly from each plot in the 1996 trial were weighed and evaluated for general appearance (i.e., shape, size, and occurrence of discoloration due to infection).

Results and Discussion

Susceptible control UC82B plants exhibited CMV-like symptoms earlier in the season than did plants of the CMV transgenic lines (with the exception of 11527 plants in the 1996 trial) (Table 1), and in each case, symptoms were far more severe in UC82B plants (data not shown). The UC82B plants infected shortly after transplanting (symptoms apparent by the 2-week rating) became severely chlorotic, developed distorted leaves, and were severely stunted. In UC82B plants that became infected at later stages of development (based on the time at which symptoms were first observed, e.g., at the 5-week rating) young leaves became chlorotic and rolled (i.e., outer leaf margins curled upward) or distorted. In contrast to UC82B plants, in those CMV transgenic plants that developed symptoms, the symptoms were mild, e.g., chlorosis of younger leaves with little or no leaf distortion, regardless of when symptoms appeared relative to plant age.

Some UC82B plants became symptomatic by the 2-week rating, while by 5 weeks 96% and 95% of the plants were symptomatic in the 1995 and 1996 trials, respectively (Table 1). Line 11527 plants remained symptomless until after the 3-week rating in the 1995 trial, at which time 9% of the plants had developed symptoms characteristic of CMV infection. In the 1996 trial, 8% of line 11527 plants developed symptoms by the 2-week rating; however, no additional plants in this line became symptomatic. Line 12261 plants were symptomless throughout the 1995 trial while 26% had developed symptoms by the 5-week rating in the 1996 trial. Plants of line 12295 remained symptomless throughout both trials. Note that at the time of the 5-week rating, plants in each of the lines were in the early stages of fruit development. Furthermore, a general rating

Table 1. Cucumber mosaic virus (CMV) infection of transgenic tomato lines expressing the capsid protein gene of CMV in two trials conducted in Blount County, Ala.

Line ^a	Symptomatic plants (%) ^b			ELISA ^c	
	Week: 2	3	5	Plants infected (%)	A ₄₀₅ (SD) ^w
1995					
UC82B	15	69	96	100	0.813 (0.355) a
11527	0	0	9	42	0.187 (0.336) b
12261	0	0	0	68	0.133 (0.186) b
12295	0	0	0	67	0.083 (0.018) b
1996					
UC82B	21	63	95	97	0.505 (0.305) a
11527	8	8	8	100	0.240 (0.392) b
12261	0	0	26	97	0.151 (0.317) c
12295	0	0	0	100	0.101 (0.046) c

^aTransgenic lines include UC82B, a nontransformed susceptible control, and three transgenic lines, 11527 (transgene from CMV-PG), 12261, and 12295 (both containing a transgene from CMV-PG and CMV-22).

^bPlants were evaluated for CMV-like symptoms (stunting, chlorosis, mosaic, and deformation of young leaves) 2, 3, and 5 weeks after being transplanted to the field.

^cELISA (enzyme-linked immunosorbent assay) was used to test tomato leaf samples for CMV capsid protein antigen. Plants were considered positive for CMV infection if the ELISA absorbance value (405 nm) for any given sample was greater than the average ELISA value plus three standard deviations for comparable tissues from noninoculated tomato plants. In 1995, greenhouse-grown UC82B plants served as the healthy control and had a threshold value of 0.034. In 1996, each line was grown in a greenhouse to serve as the respective healthy control with threshold values of 0.041, 0.027, 0.032, and 0.031 for UC82B, 11527, 12261, and 12295, respectively.

^wAverage absorbance values for all plants within a treatment (≈ 40 plants) $\pm$ SD. Mean separation of A₄₀₅ values within years by LSD, $\alpha \leq 0.05$.

for changes in symptom severity and effects on fruit, e.g., discoloration due to virus infection, taken at 7 weeks did not differ from those of the 5-week rating; thus, stresses associated with flowering and fruit set apparently did not induce development of symptoms in nonsymptomatic plants or enhance symptom severity in plants previously expressing symptoms.

In contrast with symptom data, evaluation by ELISA revealed that a large percentage of the plants from each treatment were infected with CMV (Table 1). This was particularly true for plants in the 1996 trial, where no fewer than 97% of the plants among all treatments tested positive for the presence of CMV CP. For plants from each CMV transgenic treatment and UC82B treatment in both trials, the average ELISA absorbance value was positive for the presence of CMV CP, relative to healthy controls. For each of the CMV transgenic lines within each trial, the average ELISA absorbance value was significantly less than the value for UC82B plants ($\alpha \leq 0.05$). Additionally, in the 1996 trial, the average ELISA value for the amount of CMV CP detected in samples from lines 12261 and 12295 was significantly less than that in samples from line 11527 ($\alpha \leq 0.05$). The low ELISA absorbance values observed for these samples do not represent detection of constitutive CMV CP expressed from the transgene; the ELISA system used for these studies did not detect constitutive CMV CP (data not shown). Furthermore, CMV was mechanically transferred to young tobacco plants from tomato samples randomly collected from each treatment (data not shown). Thus, the low ELISA values associated with the CMV transgenic plants are considered accurate, indicating that CMV infection of the transgenic plants did occur, although virus accumulated to a lesser extent than in UC82B plants.

A comparison of symptomatic and serological analysis revealed an association between higher ELISA absorbance values and the presence and severity of symptoms. More severely affected UC82B plants tended to have higher CMV titers than those expressing milder symptoms. Similarly, CMV transgenic plants that were symptomatic in each treatment tended to have higher ELISA absorbance values than nonsymptomatic plants (data not shown).

In July 1995, 39 UC82B plant samples were tested for CMV subgroup identification at Monsanto. Of the samples that tested positive for CMV ($\approx 82\%$), 41% reacted strongly in the CMV subgroup I ELISA system, and 33% reacted in the CMV subgroup II ELISA system, while the remaining samples reacted with both ELISA systems. The occurrence of CMV isolates of both subgroups within a single field has been documented previously (Perry et al., 1993). Gielen et al. (1996) and Xue et al. (1994) observed resistance in CMV transgenic tomato plants against CMV isolates belonging to a different subgroup from which the CMV capsid protein transgene had originated. Identification of CMV isolates belonging to each of the subgroups in UC82B plants indicates that plants in each of the treatments in these

trials were probably inoculated via viruliferous aphids with CMV isolates of each subgroup. If so, plants of line 11527 held up well to infection considering these plants contained only a CMV subgroup II capsid protein transgene. Plants of lines 12261 and 12295, both of which contained CMV subgroup I and II capsid protein genes, had lower virus titers than plants of line 11527.

A qualitative evaluation of fruit for the 1995 and 1996 trials revealed that the CMV transgenic plants produced more fruit of higher quality, i.e., size and shape, than did UC82B plants. The yield and size of fruit from each of the CMV transgenic lines were greater than those from the UC82B line, though no apparent difference was observed between CMV transgenic lines. This was particularly true for severely affected UC82B plants that were stunted. For example, the average mass of 10 tomatoes randomly collected from each plot in the 1996 trial was 40, 54, 60, and 55 grams for treatments UC82B, 11527, 12261, and 12295, respectively.

Tomato plants engineered to express CMV capsid protein (Fuchs et al., 1996; Gielen et al., 1996; Tomassoli et al., 1995) or CMV-associated satellite RNA (Gallitelli et al., 1991; Montasser et al., 1991) have been evaluated under natural conditions. Fuchs et al. (1996) tested CMV capsid protein transgenic plants in the field following both mechanical inoculation and natural infection. Plants infected with CMV were placed in the latter plots to serve as sources of inoculum to be spread by indigenous aphid species. The mechanically inoculated transgenic plants were highly resistant under field conditions. However, in the natural infection plots, only limited spread of CMV occurred, leaving those plants relatively unchallenged (Fuchs et al., 1996). Gielen et al. (1996) also tested CMV capsid protein transgenic plants under natural conditions but with higher levels of CMV disease pressure, using expression of viral-induced symptoms as a basis for evaluating CMV infection. Plants were either fully resistant (no infection) or fully susceptible to CMV infection, with varying percentages of resistant plants within all but one of the treatments.

Our results contrast with those of Fuchs et al. (1996) and Gielen et al. (1996) in several ways. The plants tested in our study were under extremely high levels of natural CMV disease pressure, which, based on disease progress in surrounding fields, occurred throughout the course of each of the trials (Murphy and Sikora, personal observation). The CMV transgenic plants were fairly consistent in their phenotypic response to CMV infection, with most plants remaining symptomless even though many were infected, particularly in the 1996 trial. They also expressed consistency in their resistance to CMV accumulation (based on ELISA). Since some of the UC82B plants had begun to express signs of CMV infection by the 2-week rating period, the reduced accumulation of CMV CP in the CMV transgenic plants was probably not the result of late inoculation. These findings show that, although the CMV transgenic lines 11527,

12261, and 12295 became infected with CMV, they resisted virus accumulation even under high dosages of inoculum. Tomassoli et al. (1995) reported that these same tomato lines were highly resistant to natural infection by CMV in Italy. Our results extend their findings and show that these lines resisted CMV infection in Alabama, which included challenge by CMV isolates of both subgroups; yet the Alabama CMV isolates are clearly distinct from the Italian isolates that are the source of the capsid protein transgenes (Tomassoli et al., 1995). Although a high percentage of the CMV transgenic tomato plants became infected with CMV, their response to infection (i.e., low virus titers, lack of viral-induced symptoms, production of more and larger fruit) clearly indicated their potential commercial value to growers.

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