Coat Protein Transgenic Resistance to Watermelon Mosaic and Zucchini Yellows Mosaic Virus in Squash and Cantaloupe

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ABSTRACT

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Three yellow crookneck squash (*Cucurbita pepo* var. *melopepo*) and five cantaloupe (*Cucumis melo,* Reticulatus group) lines, genetically altered for resistance to zucchini yellow mosaic virus and watermelon mosaic virus, were field tested in 1993 and 1994, respectively. During both years, nontransgenic plants were inoculated with virus before transplanting to provide a high virus threat to the transgenic plants. Before and after transplanting, serological testing (enzyme-linked immunosorbent assay [ELISA]) was used to obtain baseline information on transformed plants and to confirm field virus infection. In both years, plant disease development was rated weekly; yield was assessed in 1993. Disease progression, yield, and end-of-season ELISA indicated a significant reduction in disease incidence in the transgenic lines. Total squash yield did not differ between the transformed and unchanged lines, but the transgenic lines yielded more marketable fruit than did the nontransgenic line.

Cucurbits, including watermelon, cantaloupe, cucumber, and squash, are grown commercially throughout the world. These crops can be completely destroyed when infected by zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), and/or cucumber mosaic virus (CMV) (6,10). These viruses do not always kill the plant but often disrupt the natural processes so severely that the plant produces abnormal fruit or no fruit at all. Control by cultural practices (aphid-repellent mulch, reducing inoculum sources, trap crops) may delay disease progression, but the effect generally is short-lived (12). Control of insect vectors with insecticides also is ineffective, but some limited success has been obtained with the use of mineral oil sprays (15), which interfere with the aphid's ability to acquire and transmit virus particles (1). Traditional breeding techniques to incorporate resistance to these viruses in commercially acceptable cucurbit cultivars has not been successful, except for CMV resistance in cucumber (12).

The virus must uncoat before replication of the viral RNA and subsequent disease progression can occur. Recent evidence has shown that plants expressing a viral coat protein gene are protected against infection by that virus (8,9,11,14). The mechanism of this phenomenon is unknown, but it may involve interference in uncoating of the challenge virus or in virus replication. Transgenic yellow crookneck squash (*Cucurbita pepo* L. var. *melopepo* (L.) Alef. cv. Pavo) and cantaloupe (*Cucumis melo* L., Reticulatus group cvs. Don Luis, Galleon, Hiline, Mission, and parental inbred) containing viral coat protein genes for ZYMV, WMV, and CMV were constructed for expression of the respective coat proteins in plant tissues by Asgrow Seed Co., Kalamazoo, Michigan, according to the procedure of Horsch et al. (5). This study was conducted to test the ability of this engineered cross-protection to prevent natural infection by WMV and ZYMV in yellow crookneck squash and cantaloupe in a field environment.

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MATERIALS AND METHODS

Greenhouse. Transformed and nontransformed squash and cantaloupe of each cultivar were seeded in multicell containers on 4 June 1993 and 13 June 1994, respectively. Two squash constructs (Pavo ZW-B and ZW-H) were engineered (Asgrow) to produce the coat proteins for ZYMV and WMV; the third construct (Pavo CZW) produced the coat protein for CMV as well. All transgenic cantaloupe cultivars produced coat proteins for all three viruses. In a separate greenhouse, 1-week-old nontransformed seedlings (source plants) were inoculated with ZYMV in 1993 and with WMV and ZYMV in 1994 to ensure adequate disease pressure in the field; cotyledons were wounded by abrading and wiped with a buffer solution containing plant sap with one of these viruses (13). All plants were moved to a cold frame for hardening 5 days before transplanting to the field.

Field. Soil type was an Adkins fine sandy loam (coarse-loamy, mixed mesic Xerollic Camborthid). Field plots were prepared by rototilling 1.8-m-wide strips in a fall-planted wheat cover crop, leaving 0.3-m-wide windbreaks between rows. Plots were subsoiled, and metam sodium (560 liters ha^{-1}) was incorporated to a depth of 15 cm with a rototiller. Fertilizer (112N-47P-124K kg ha^{-1}) was broadcast in a 0.7-m band in the center and rototilled to a 15-cm depth. Plots were covered with 0.0254-mm black polyethylene mulch (1.2 m wide), and a single drip irrigation line (0.23 m emitter spacing, 370 liter hr^{-1} 100 m^{-1} at 51.6 Pa) was buried 5 cm in the bed center. Fifty squash and 20 cantaloupe plants per plot were transplanted by hand on 30 June 1993 and 18 July 1994, respectively. Spatial arrangements and populations were as follows: squash, two rows per bed, 0.4 m between rows, 0.92 m between plants (10,350 plants per ha); and cantaloupe, one row per bed, 0.61 m between plants (7,810 plants per ha). Nitrogen sidedress at 28 kg ha^{-1} was fertigated 6 and 10 weeks after transplanting. Recommended commercial production practices were followed, except that insecticides were not applied during the trials to ensure large numbers of insect vectors; substantial numbers of aphids were observed in the plots each year. Source-plant plots (six squash, nine cantaloupe) were situated uniformly among the test plots to serve as inoculum reservoirs. Squash fruit were harvested 10 times at 3-day intervals, beginning 2 August and continuing through 27 August. No effort was made to prevent virus spread within plots during harvest; disposable gloves worn by harvesters were discarded after harvesting each plot to reduce mechanical transmission between plots. Fruit were evaluated visually, counted, and weighed; fruit with abnormal appearance (mosaic, mottling, green streaks, warts, bumps, distortions) were classified as "virus symptomatic." Whole fruit tissue of 10 fruit per plot were combined and prepared according to Lockman (7) for analysis of mineral concentration by gas plasma analyzer (Jarrell-Ash ICAP-9000, Waltham, MA).

Double antibody enzyme-linked immunosorbent assays (ELISA) were performed on nontransformed, transformed, and inoculated nontransformed plants before transplanting to establish baseline reaction values (3). ZYMV (ATCC, PVAS-405) and WMV (Dan Purcifull, University of Florida) were used at a 1:1,000 dilution of both antibody and conjugate. Transmission electron microscopy was used on leaf dip samples to confirm the absence of virus in transformed lines that showed weak positive reactions (≤1.5 times or 3 standard deviations greater than the mean of the healthy control); infected source plants produced strong positive reactions (6 to 15 times the mean of the healthy control) (2). After transplanting, each plant was visually evaluated weekly for virus symptoms; ELISAs were performed on plants to confirm field identification. Squash and cantaloupe plant disease ratings began 4 and 3 weeks after transplanting, respectively. Healthy (0), possibly infected (1), or infected plants (2) were identified. At the conclusion of the production seasons, every third squash plant (1993) or every cantaloupe plant (1994) was sampled for ELISA determination of virus infection.

The experimental design each year was a complete factorial, with three replications. Data for each year were analyzed using the SAS general linear model procedures; the Waller-Duncan test was used to separate means when the F test for squash lines was significant (SAS Institute, Cary, NC). Single degree-of-freedom orthogonal contrasts were performed to compare transformed and nontransformed cantaloupe across cultivars and within cultivars.

RESULTS AND DISCUSSION

Squash. Virus spread quickly from inoculated plants to nontransformed lines in the field. Four weeks after transplanting, some nontransformed control plants exhibited disease symptoms. One week later, the disease symptoms were found throughout the control plots; by 17 August, nearly all nontransformed plants were infected. Analysis of the area under disease progression curve (AUDPC) indicated that the disease progressed rapidly in the control line, moderately in the Pavo CZW line, and slowest in the Pavo ZW-B and ZW-H lines (Table 1).

The percentage of plants that tested positive for ZYMV at the conclusion of the field trial was greatest for the control, intermediate for the CZW line, and least for the ZW-B and ZW-H lines (Table 1). The incidence of WMV was slight; the percentage of plants with positive reactions did not differ between the lines tested.

Fruit symptom development followed the same general pattern. By the first harvest, symptoms were already evident on a small number of fruit (Fig. 1). By the second harvest, the percentage of affected fruit (by weight) in the nontransformed control increased significantly. At the third harvest, fruit infection was highest in the control, intermediate in the CZW, and least in the ZW-B and ZW-H lines; this relationship continued through the last harvest. Total marketable fruit yield from the ZW-B and ZW-H lines was greater than from the CZW and control lines; CZW plants produced significantly more marketable fruit than did the nontransformed controls (Table 2). Few fruit with imperfections were found throughout the harvest period from either ZW-H or ZW-B lines. Total fruit yield and total number of fruit produced were similar in all lines tested.

Fruit concentration of potassium averaged 4.32% in the three transformed lines but was reduced to 3.77% in the control (P = 0.05), probably due to the high incidence of virus infection in the control line, which appeared to reduce plant growth. Concentrations of other mineral elements did not differ among lines tested (data not shown) and were similar to those reported by others for yellow squash (4).

The Pavo CZW was an interesting anomaly. Serological testing before transplanting showed a weak reaction to WMV and ZYMV antisera in about half of these plants (100% reaction expected because transformed plants should produce the viral coat protein). Likewise, in the field, about half of the plants produced fruit with viruslike symptoms. These fruit came from only those plants that failed to cause a weak reaction during serological testing. Apparently, nearly 50% of the plants were not producing the protein and therefore were not protected from infection. Based on this information, the seed producer checked the records and found that the parental cross for this line was between a line that was heterozygous for the transgene and a line that was nontransgenic. The expected 1:1 segregation would result in only half of these plants being resistant to the viruses tested, which is consistent with the results obtained in the field.

Cantaloupe. Spread of both viruses from inoculated plants to nontransformed plants based on symptom development was not evident until 7 weeks after transplanting (Fig. 2). At 8 weeks after transplanting, the control lines had a significantly greater disease rating than did the transgenic lines (P = 0.05); this difference increased through 9 (P = 0.01) and 10 weeks (P = 0.001) after transplanting. More plants were infected than was evident from visual observation of symptom development. At the end of the trial, 60% or more of each nontransformed control line was virus-infected (Table 3). The transformed lines showed little or no virus

Table 1. Effect of genetic transformation of Pavo squash on watermelon mosaic virus (WMV) and zucchini yellow mosaic virus (ZYMV) disease progression and end-of-season plant infection, 1993

Line	AUDPC[z]	Infected (%)	
		WMV	ZYMV
Control	44.2 a	...	100 a
CZW	26.6 b	2.5	45 b
ZW-B	2.1 c	8.3	10 c
ZW-H	0.0 c	0.0	0 c

[z] Means followed by different letters were significantly different at P = 0.05 (Waller-Duncan).

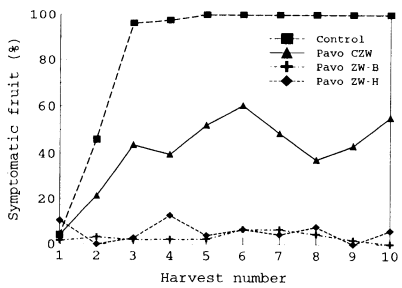

Fig. 1. Virus symptom development in transgenic and nontransgenic yellow crookneck squash fruit.

Table 2. Effect of transgenic virus resistance on Pavo yellow squash production, 1993

Line	Abnormal[y,z] (t ha^{-1})	Marketable (t ha^{-1})	Total		Abnormal (%)
			Weight (t ha^{-1})	Number (1,000/ha)	
Control	23.6 a	7.4 c	31.0	258	84.3 a
CZW	14.2 b	23.9 b	38.1	260	40.6 b
ZW-B	1.8 c	36.0 a	37.8	242	4.7 c
ZW-H	1.5 c	32.0 a	33.4	212	3.7 c

[y] Includes mosaic, mottling, green streaks, warts, bumps, distortions.
[z] Means followed by different letters were significantly different at P = 0.05 (Waller-Duncan).

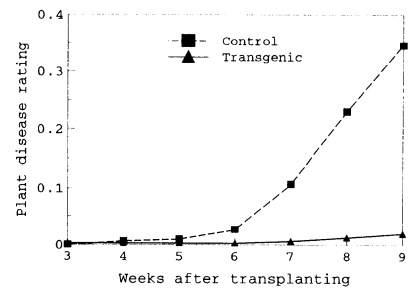

Fig. 2. Watermelon mosaic and zucchini yellows mosaic virus progression in transgenic and nontransgenic cantaloupe plants. Plant rating scale: healthy = 0, possibly infected = 1, and infected = 2.

Table 3. Effect of genetic transformation of cantaloupe on the incidence of watermelon mosaic virus (WMV) and zucchini yellow mosaic virus (ZYMV) in field trials, 1994

	WMV		ZYMV	
Cultivar	**CZW**	**Control**	**CZW**	**Control**
Don Luis	0	15.4 *	13.3	58.3 NS
Galleon	0	1.7 NS	5.0	58.9 NS
Hiline	0	8.3 NS	3.3	60.7 *
Mission	0	6.8 *	3.3	57.7 *
Inbred	0	10.6 NS	0	50.6 *
Average	0	8.5 ***	5.0	57.2 ****

[z] *, ***, **** = comparison of transformed vs. control within cultivar or averaged across cultivars is significant at $P = 0.05$, 0.001, or 0.0001, respectively. NS = not significant.

infection. The difference between the transformed and control lines was highly significant (WMV, $P = 0.001$; ZYMV, $P = 0.0001$); there was no difference between cultivars. As in 1993, ZYMV was more prevalent than WMV.

The use of genetically altered cucurbits resulted in a remarkable disease reduction, even under conditions of high vector numbers and extreme disease pressure. All of the transformed squash and cantaloupe lines had much smaller disease progress curves than the controls. The transgenic squash lines continued to produce significantly greater amounts of marketable fruit than did the nontransformed control until the test was terminated.

This is the first report of field-tested virus resistance with natural transmission in commercially available transgenic crops. Implications for production agriculture include a reduction in the application of pesticides (used to control insect vectors of virus diseases) and an extended growing season with greatly increased yield potential.

LITERATURE CITED

1. Bradley, R. H., Wade, C. V., and Wood, F. A. 1962. Aphid transmission of potato virus Y inhibited by oils. Virology 18:327-328.
2. Christie, S. R., Purcifull, D. E., Crawford, W. E., and Nabila, A. A. 1987. Electron microscopy of negatively stained clarified viral concentrates obtained from small tissue samples, with appendices on negative staining techniques. Agric. Exp. Stn. Bull. 872, Inst. Food Agric. Sci., University of Florida, Gainesville.
3. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses. J. Gen. Virol. 34:475-484.
4. Clough, G. H., Locascio, S. J., and Olson, S. M. 1992. Mineral concentration of yellow squash as affected by irrigation method and fertilization management. J. Am. Soc. Hortic. Sci. 117:725-729.
5. Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231.
6. Lisa, V., and Lecoq, H. 1984. Zucchini yellow mosaic virus. Descriptions of plant viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England.
7. Lockman, R. B. 1980. Review of soil and plant tissue preparation procedures. J. Assoc. Offic. Anal. Chem. 63:766-769.
8. Nelson, R. S., McCormick, S. M., Delannay, X., Dube, P., Layton, J., Anderson, E. J., Kaniewska, M., Proksch, R. K., Horsch, R. B., Rogers, S. G., Fraley, R. T., and Beachy, R. N. 1988. Virus tolerance, plant growth, and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus. Bio/Tech. 6:403-409.
9. Powell-Abel, P., Nelson, R. S., De, B., Hoffman, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus protein gene. Science 232:738-743.
10. Purcifull, D., Hiebert, E., and Edwardson, J. 1984. Watermelon mosaic virus 2. Descriptions of plant viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England.
11. Quemada, H., Gonsalves, D., and Slighton, J. L. 1991. Expression of coat protein gene from cucumber mosaic virus strain C in tobacco: Protection against infections by CMV strains transmitted mechanically or by aphids. Mol. Plant Pathol. 81:794-802.
12. Sherf, A. F., and MacNab, A. A. 1986. Cucurbits. Pages 353-379 in: Vegetable Diseases and Their Control. 2nd ed. John Wiley & Sons, New York.
13. Stevens, W. A. 1983. Virology of flowering plants. Chapman and Hall, New York.
14. Tumer, N. E., O'Connell, K. M., Nelson, R. S., Sanders, P. R., Beachy, R. N., Fraley, R. T., and Shaw, D. M. 1987. Expression of alfalfa mosaic virus coat protein gene confers cross protection in transgenic tobacco and tomato plants. EMBO J. 6:1181-1188.
15. Webb, S. E., and Linda, S. B. 1993. Effect of oil and insecticide on epidemics of potyviruses in watermelon in Florida. Plant Dis. 77:869-874.