Molecular Breeding **3:** 279–290, 1997.

Cantaloupe line CZW-30 containing coat protein genes of cucumber mosaic virus, zucchini yellow mosaic virus, and watermelon mosaic virus-2 is resistant to these three viruses in the field

Marc Fuchs[1,*], James R. McFerson[2], David M. Tricoli[3], J. Russell McMaster[3], Rosaline Z. Deng[3], Maury L. Boeshore[3], John F. Reynolds[3], Paul F. Russell[3], Hector D. Quemada[3] & Dennis Gonsalves[1]

[1]*Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456-0462, USA (*author for correspondence; fax: 315-787-2389, e-mail: mf13@cornell.edu);* [2]*Plant Genetic Resources, USDA-ARS, Cornell University, Geneva, NY 14456-0462, USA;* [3]*Experimental Plant Genetics, The Asgrow Seed Company, Kalamazoo, MI 49002, USA*

Received 30 August 1996; accepted in revised form 31 March 1997

Key words: coat protein-mediated protection, *Cucumis melo* L. var. *cantalupensis* Naudin, field evaluation, increased field, transgenic plants, virus resistance

Abstract

Cantaloupe line CZW-30 containing coat protein gene constructs of cucumber mosaic cucumovirus (CMV), zucchini yellow mosaic potyvirus (ZYMV), and watermelon mosaic virus 2 potyvirus (WMV-2) was investigated in the field over two consecutive years for resistance to infections by CMV, ZYMV, and/or WMV-2. Resistance was evaluated under high disease pressure achieved by mechanical inoculations and/or natural challenge inoculations by indigenous aphid vectors. Across five different trials, homozygous plants were highly resistant in that they never developed systemic symptoms as did the nontransformed plants but showed few symptomatic leaves confined close to the vine tips. Hemizygous plants exhibited a significant delay (2–3 weeks) in the onset of disease compared to control plants but had systemic symptoms 9–10 weeks after transplanting to the field. Importantly, ELISA data revealed that transgenic plants reduced the incidence of mixed infections. Only 8% of the homozygous and 33% of the hemizygous plants were infected by two or three viruses while 99% of the nontransformed plants were mixed infected. This performance is of epidemiological significance. In addition, control plants were severely stunted (44% reduction in shoot length) and had poor fruit yield (62% loss) compared to transgenic plants, and most of their fruits (60%) were unmarketable. Remarkably, hemizygous plants yielded 7.4 times more marketable fruits than control plants, thus suggesting a potential commercial performance. This is the first report on extensive field trials designed to assess the resistance to mixed infection by CMV, ZYMV, and WMV-2, and to evaluate the yield of commercial quality cantaloupes that are genetically engineered.

Introduction

Melon (*Cucumis melo* L.), one of the world's major cucurbit crops, is susceptible to numerous viral diseases resulting in economic losses in most production areas [3, 6, 13, 17, 19, 23, 24, 27, 28]. The four most important viruses, cucumber mosaic cucumovirus (CMV), watermelon mosaic virus 2 potyvirus (WMV-2), zucchini yellow mosaic potyvirus (ZYMV) and papaya ringspot potyvirus type w (PRSV-w) are widespread in temperate as well as tropical regions of the world [20], and are readily transmitted by several aphid species in a nonpersistent manner.

WMV-2, ZYMV, and PRSV-w are members of the genus potyvirus in the potyviridae plant virus family. Potyvirus particles are filamentous and about 750 nm long with a monopartite genome composed of a single-stranded RNA species. The coat protein (CP) genes are localized at the extreme 3' end of their respective open reading frames. The CP is expressed by proteolytic

cleavage from the virus-encoded polyprotein [33]. In melons, WMV-2, ZYMV, and PRSV-w induce similar foliar symptoms including systemic chlorosis, mosaic, severe leaf deformation, vein banding, reduced leaf laminae, and plant stunting [38]. WMV-2 and ZYMV are distantly related serologically but have no serological relationship with PRSV-w.

CMV is the type species of the genus cucumovirus in the bromoviridae plant virus family. CMV particles are isometric with a diameter of about 29 nm, and contain a multipartite genome composed of three single-stranded RNA species. The CMV CP gene is located at the extreme 3′ end of RNA 3 but is expressed by subgenomic RNA 4 [25]. In melons, CMV induces systemic mosaic, mottling, prominent downward curling of leaves, chlorosis, reduced leaf laminae, and plant stunting [38].

Coat protein-mediated protection (CPMP) [9, 12, 18] constitutes a major breakthrough to facilitate the incorporation of genetically-based multiple virus resistance in crop plants. Transgenic potato lines with dual resistance to potato virus X potexvirus and potato virus Y potyvirus have been developed [15, 16]. In cucurbits, transgenic squash with resistance to two and three viruses have been obtained. A transgenic F1 hybrid squash containing the ZYMV and WMV-2 CP genes and tested under the code identification ZW-20 showed excellent resistance to mixed infections by both potyviruses [1, 4, 10, 36]. In 1995, this hybrid, marketed as cv. Freedom II, became the first virus-resistant transgenic crop released commercially in the USA. Another transgenic squash line designated CZW-3 and containing the CP genes of CMV, ZYMV, and WMV-2 is resistant to these three viruses [36].

In melon, transgenic lines with single resistance to CMV [11, 37] or ZYMV [8] were developed using CPMP. The first transgenic cantaloupes with multiple virus resistance were developed lately by the Asgrow Seed Company. These plants contain the CP genes of CMV, ZYMV, and WMV-2 and are resistant to these three viruses. Recently, transgenic homozygous and several hemizygous plants were tested in a field environment against ZYMV and WMV-2 [4]. Transgenic plants had a significantly lower visual disease rating than did the controls. No virus was detected in homozygous plants by enzyme-linked immunosorbent assay whereas only a few hemizygous plants were infected by ZYMV (6%) but none by WMV-2. In contrast, 57 and 8.5% of the control plants were infected by ZYMV and WMV-2, respectively. In this study, dual infections by ZYMV and WMV-2 were not investigated and resistance to CMV was not evaluated. Also, yield was not analyzed.

We report here on the evaluation of one transgenic cantaloupe line containing the CP genes of CMV, ZYMV and WMV-2, designated CZW-30, for resistance to infections by CMV, by CMV and ZYMV, and by CMV, ZYMV, and WMV-2. We analyzed in the field the resistance of homozygous and hemizygous plants to natural infections by aphid vectors, monitored infection rates by ELISA, and assessed yield. Transgenic line CZW-30 was highly resistant to mixed infections by the three aphid-borne viruses and yielded significantly more marketable fruits than untransformed controls.

Materials and methods

CP gene constructs and plant material

The CP genes of CMV, ZYMV and WMV-2, and the binary vectors carrying their expression cassettes were previously described [31, 36]. Briefly, the expression cassettes for the ZYMV and WMV-2 CP genes were engineered into one binary vector [36] and the CP gene of CMV was cloned separately into a second binary vector [31]. Expression of each CP gene was driven by the cauliflower mosaic virus (CaMV) 35S promoter, part of the intergenic region of RNA 3 of CMV strain C used as leader sequence, and the nopaline synthase terminator or the CaMV 35S terminator [34, 36]. *Agrobacterium*-mediated transformation was carried out using cotyledonary explants of two proprietary Asgrow western shipper cantaloupe inbreds using a modification of the procedure described by Horsch et al. [14]. Transgenic R_0 plants were recovered by selection on kanamycin and subsequently established in the greenhouse. All transgenic plants and seeds were produced by the Asgrow Seed Company.

Transgenic line CZW-30 was produced by progeny selection of a cross of two transgenic lines designated ZW-102 and C-73-6. Line ZW-102 contained the CP genes of ZYMV strain FL and WMV-2 strain FL [36]. Line C-73-6 contained the CP gene of CMV strain C [31]. These two lines showed high levels of virus resistance in greenhouse and field experiments conducted in 1992 and 1993. Lines ZW-102 and C-73-6 were crossed and their progeny screened for virus resistance. Among the progeny tested, line CZW-30 exhibited high level of virus resistance in the greenhouse and in the field. This latter was developed to

homozygozity and a hemizygous hybrid CZW-30 was subsequently obtained. The presence of the CP genes was verified in resistant plants throughout these experiments by polymerase chain reaction using genomic DNA and appropriate primers.

Transplant production, virus isolates, and mechanical inoculations

Seeds were sown directly into peat pots containing Cornell mix [10]. The homozygous line CZW-30 and untransformed cv. Mission were tested in 1994, whereas the homozygous and hemizygous CZW-30 and untransformed cv. Cristobal were analyzed in 1995. Mission and Cristobal are two proprietary Asgrow hybrids that are susceptible to CMV, ZYMV and WMV-2. Some seedlings were mechanically-inoculated in the greenhouse approximately 12–15 days post-germination when they showed 2–3 expanded leaves. CMV strain Fny [2], ZYMV strain FL [30], and WMV-2 strain Rob [10] or WMV-2 strain NY were used as inocula. These strains of CMV and WMV-2 differed from those used to engineer the CP genes [31, 36] whereas the same strain of ZYMV was used in the CP gene engineering and in the field tests [36].

In 1994, half of the transgenic and half of the untransformed plants were inoculated by rubbing Corundum-dusted cotyledons and expanded leaves with diluted inoculum. This approach was chosen to guarantee resistance data in case natural virus spread by aphid vectors would be unreliable. For inoculations with single viruses, 1:50 dilutions of crude sap from CMV, ZYMV or WMV-2 infected squash cv. President were used. To prepare one inoculum consisting of CMV and ZYMV, and another consisting of CMV, ZYMV and WMV-2, we mixed 1:25 (1:1, v:v) and 1:15 (1:1:1, v:v:v) dilutions, respectively, of crude sap from squash cv. President infected by one of these viruses. Given the efficient natural virus spread in 1994, only untransformed plants were mechanically inoculated in 1995. After inoculation, plantlets were maintained for one week in screenhouses before being transplanted in the fields.

Experimental design and field layout

Field trials were carried out under permits issued by APHIS-USDA. Five isolated field sites, designated A to E, were selected on three different experimental farms at the New York State Agricultural Experiment Station in Geneva, NY. Field A was located in farm 1, fields B and C were located 500 m apart in farm 2, and fields D and E were 250 m apart in farm 3.

In 1994, trials were set in fields A to C. The resistance of homozygous plants was evaluated against CMV in field A, against CMV and ZYMV in field B, and against CMV, ZYMV and WMV-2 in field C. Complete block designs were used with two treatments (mechanically inoculated and uninoculated plants), each treatment representing half of the plant population. Treatments were allocated randomly into 6 blocks. To facilitate uniform distribution of the inoculum throughout each field, additional mechanically inoculated untransformed plants were transplanted at pre-determined locations. These additional virus source plants were infected by CMV in field A, CMV or ZYMV in field B, and CMV, ZYMV or WMV-2 in field C. They represented 10–15% of the total number of plants in each field. Seedlings were transplanted on bare soil 1.2 m apart and 3.6 m between rows. The size of the plots was: 20 m $\times$ 30 m for field A, 22 m $\times$ 30 m for field B, and 28 m $\times$ 45 m for field C. No insecticides were used since one of the objectives was to evaluate resistance to natural infection by aphid vectors.

In 1995, trials were set in replicated fields D and E. The resistance of transgenic homozygous and hemizygous plants was evaluated against aphid inoculations of CMV, ZYMV, and WMV-2 in a complete block design. Plants were allocated randomly into 6 blocks. The size of each plot was 35 m $\times$ 45 m. Mechanically inoculated untransformed plants were planted as border rows consisting of successive groups of 3 plants inoculated either with CMV, ZYMV or WMV-2. These inoculated control plants served as primary virus sources to simulate natural conditions of virus spread by aphid vectors. Rows were covered with black polyethylene mulch (1.8 m wide), and a single drip irrigation line was placed in the bed center. Sevin was applied when needed to control beetle populations, otherwise no insecticides were used.

Data collection on resistance, plant vigor and fruit yield

Disease incidence was assessed by monitoring symptom development every 3–7 days in 1994 and 1995.

Enzyme-linked immunosorbent assay (ELISA) was used to identify the virus(es) causing symptoms and discriminate between single and mixed infections. Leaf samples in positions 1–8 at the apical end of the longest vines of each plant were collected randomly 3–4 times

throughout both growing seasons and stored in ziplock bags at -20 °C until processed. Processing of leaf tissue, γ-globulins, conjugates, ELISA conditions, and data analysis were as previously described [10]. If ELISA results were ambiguous, bioassays were carried out on *Nicotiana benthamiana*, *Phaseolus vulgaris* cv. Black turtle 2, *Chenopodium quinoa* and squash cv. President. *N. benthamiana* is susceptible to WMV-2 and CMV but not to ZYMV, *P. vulgaris* cv. Black turtle 2 is susceptible to WMV-2 but not to CMV and ZYMV. Squash and *C. quinoa* are systemic and local lesion hosts for the three viruses, respectively.

Shoot length of the longest vine was measured for each plant as one of the parameters indicative of growth and vigor. Data was collected once at the end of the trial period in 1994, and at three different dates in 1995.

Fruit yield was measured in 1995 at three different dates by counting the number of fruits and measuring the fresh fruit weight. Also, marketable quality was rated by considering fruit size, shape, and flesh firmness. Fruits were rated as unmarketable if they were misshapen or $<$10 cm in diameter.

Statistical analysis

Data collected on symptom development, ELISA, fruit number, fruit weight, and shoot length were analyzed by ANOVA using SAS [32].

Results

1994 field trials

Resistance to infections by CMV

Transgenic homozygous cantaloupes CZW-30 were evaluated for resistance to CMV in field A. All untransformed plants developed systemic symptoms including mosaic, chlorosis, mottling, prominent downward curling of leaves, reduced leaf laminae, and plant stunting. These symptoms persisted throughout the growing season. Incidence of CMV was noticeably higher early in the season for mechanically inoculated than for uninoculated untransformed plants (data not shown). However, all aphid-inoculated untransformed plants were symptomatic at 48 days after planting (dpp) (Figure 1A).

In contrast, transgenic plants never showed systemic CMV symptoms (Figure 1A). Instead, transgenic plants developed localized CMV symptoms that were clearly distinct and milder than those of untransformed plants. First, localized symptoms were confined to some leaves located close to the vine tips. Second, symptomatic leaves showed a downward curling and mosaic but had no reduction of leaf size, and plants were not stunted. Third, transgenic plants appeared to outgrow the symptoms since most of the newly developed leaves were asymptomatic. Fourth, symptoms were substantially attenuated throughout the season but became more severe at the end of the growing season (68 dpp). The percentage of transgenic plants with localized symptoms increased from 22% at 48 dpp to 56% at 54 dpp, and reached 100% by 68 dpp. Remarkably, transgenic plants reacted similar whether they were mechanically- or aphid-inoculated.

ELISA was used to characterize the incidence of infecting viruses with leaf samples collected 28, 48 and 68 dpp. CMV ELISA-positive untransformed plants increased from 19.5 to 93% by 28 and 68 dpp, respectively (Table 1). Conversely, only 14.5% of the transgenic plants were ELISA-positive for CMV by 68 dpp. Although all transgenic plants displayed some localized CMV symptoms by the end of the growing season, only a few (14.5%) reacted positively for CMV in ELISA. The difference in the percentage of plants exhibiting visual symptoms and the percentage of ELISA positive plants was apparently due to the reaction of the transgenic plants and the sample collection method. Only young leaves at the extremity of the main vines were collected for ELISA, and most of the symptomatic transgenic plants did not exhibit symptoms on these collected leaves. ELISA also revealed a high incidence of potyvirus infection for the untransformed plants since 85 and 23% were positive for WMV-2 and ZYMV, respectively (Table 1). Plants infected by these two potyviruses developed symptoms that were distinct from those caused by CMV. Potyvirus-infected plants showed interveinal chlorosis, blisters, vein banding and severe leaf deformation. These symptoms were observed first on a few plants (5%) by 40 dpp, but on nearly all (95%) by 58 dpp. In contrast, none of the transgenic plants reacted for WMV-2 and ZYMV (Table 1). The presence of PRSV-w was also surveyed by ELISA but it was not detected in field A.

Untransformed plants showed a 34% reduction in shoot length at 68 dpp compared to transgenic plants (104 ± 25 vs 157 ± 29 cm). These differences were significant ($P < 0.01$) (data not shown).

Analysis of variance was used to determine which factor (genotype, treatment, time of data collection) most influenced the differences observed. Results

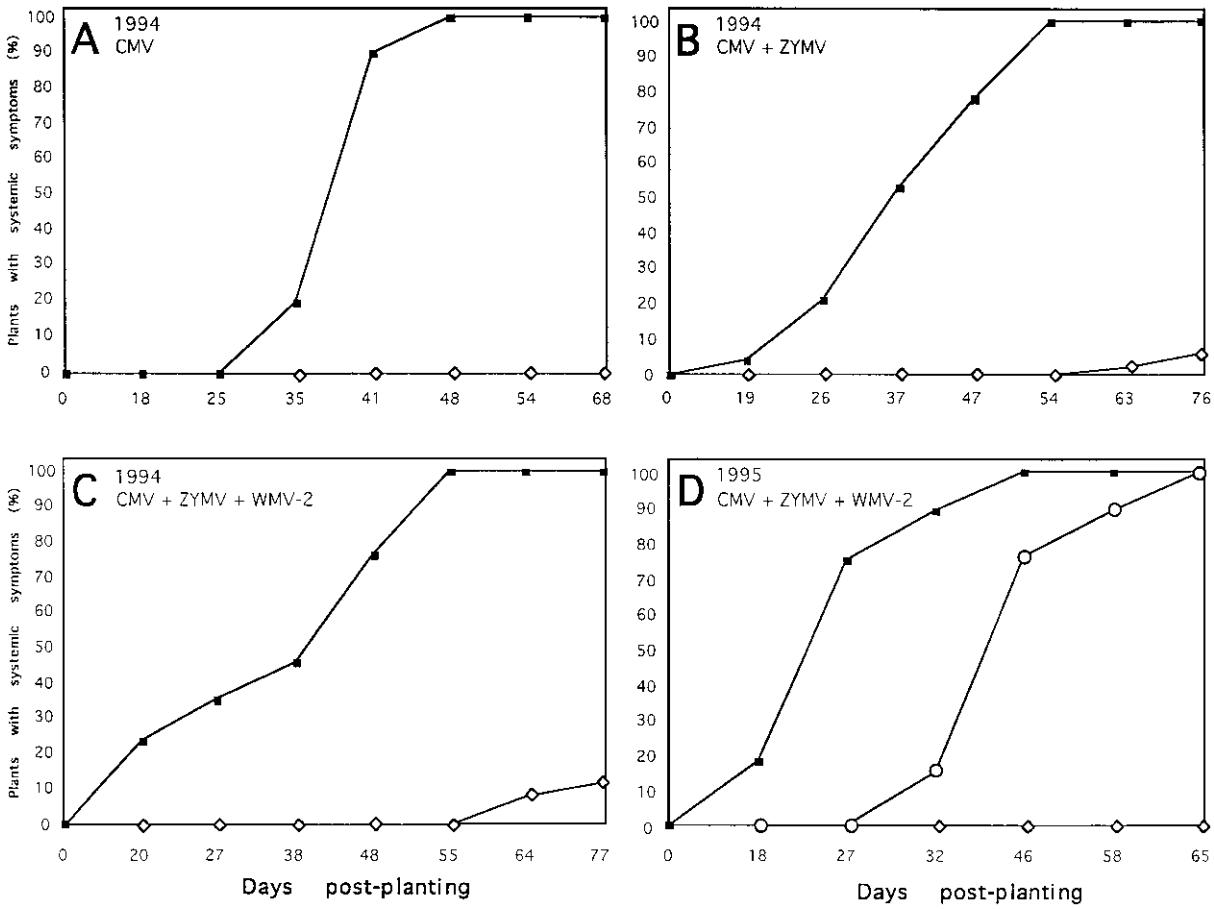

Figure 1. Disease progress curves on homozygous (open diamond) and hemizygous (open circle) cantaloupes CZW-30 containing the CP genes of CMV, ZYMV, and WMV-2 and untransformed cantaloupes (solid rectangle) under field conditions of infection by CMV (**A**), CMV and ZYMV (**B**), CMV, ZYMV and WMV-2 (**C**) in 1994, and CMV, ZYMV and WMV-2 (**D**) in 1995. Data represent the cumulative percentage of plants that showed systemic foliar symptoms (mosaic, chlorosis, mottling, downward curling of leaves, and reduced leaf laminae) due to aphid inoculations.

Table 1. Resistance evaluation of transgenic homozygous cantaloupes CZW-30 containing the CP genes of CMV, ZYMV, and WMV-2 to infections by CMV during the 1994 growing season.

Genotype	Treatment	n	Percent of ELISA-positive plants for CMV[a]			Contaminating viruses[b]	
			Aug. 4 (28 dpp)	Aug. 24 (48 dpp)	Sep.13 (68 dpp)	ZYMV	WMV-2
Transgenic plants							
CZW-30	uninoculated	26	0	0	12	0	0
	inoculated CMV	24	8	8	17	0	0
Untransformed plants							
Mission	uninoculated	20	15	47	90	15	85
	inoculated CMV	46	24	87	96	31	85

[a] Data represent cumulative percentage of ELISA-positive plants for CMV at 28, 48, and 68 days after planting (dpp). Infected plants had OD 405 nm readings at least 8 times higher than control plants (1.2 vs. 0.14) after 30 min substrate hydrolysis. Uninfected transgenic plants had OD 405 nm values of 0.35 after 30 min substrate hydrolysis.
n = number of plants tested. Plants were either uninoculated or mechanically inoculated with CMV-Fny (CMV) on June 23, and transplanted to the field on July 7, 1994.
[b] Percent of ELISA positive plants by 68 dpp. ZYMV, zucchini yellow mosaic virus; WMV-2, watermelon mosaic virus 2.

showed that genotype was the only significant factor accounting for the variations in symptom development, virus incidence, and shoot length among test plants ($P < 0.01$). The inoculation method, e.g. mechanical versus aphid inoculation, and the interaction between genotype and inoculation method were not significant ($P > 0.2$). Also, blocking effects were not significant ($P > 0.3$).

Resistance to infections by CMV and ZYMV

Transgenic homozygous cantaloupes CZW-30 were evaluated for resistance to mixed infections by CMV and ZYMV in field B. Untransformed plants developed systemic CMV-type symptoms similar to those described for their counterparts in field A except for additional severe ZYMV-type leaf deformations, blisters, yellowing and systemic vein clearing. These symptoms persisted throughout the growing season. Virus incidence was significantly higher early in the season for mechanically inoculated than for uninoculated untransformed plants, but all untransformed plants were symptomatic 54 dpp (Figure 1B). In contrast, transgenic plants were highly resistant throughout the growing season since nearly all of them (94%) never developed systemic symptoms. However, a number of transgenic plants (72% by the end of the growing season, e.g. 74 dpp) had non-systemic symptoms and these were clearly distinct from those exhibited by untransformed plants. Symptomatic transgenic plants developed localized symptoms that were identical to those previously described for the transgenic plants in field A (see above) with the addition of discrete yellowing and vein clearing.

ELISA data of leaf samples collected 26, 42, 57, and 74 dpp are summarized in Table 2. All untransformed plants (99%) were positive for CMV and/or ZYMV by 57 dpp, and the majority (75%) was mixed infected by CMV and ZYMV by 74 dpp. The results with transgenic plants were markedly different. Only 38.5% of the latter were infected by CMV and/or ZYMV by 74 dpp, with CMV being dominant (30.5%). Mixed infections by both CMV and ZYMV were observed in only 3% of the transgenic plants. Untransformed plants also reacted in ELISA for WMV-2 (64%) but none of the transgenic plants did (Table 2). ELISA revealed the presence of the nematode-borne tobacco ringspot nepovirus (TRSV) in some transgenic (6.5%) and untransformed (21%) plants (Table 2). TRSV accounted for the few transgenic plants (6%) that showed severe symptoms consisting of chlorotic

Table 2. Resistance evaluation of transgenic homozygous cantaloupes CZW-30 containing the CP genes of CMV, ZYMV, and WMV-2 to mixed infections by CMV and ZYMV during the 1994 growing season.

Genotype	Treatment	n	Percent of ELISA-positive plants[a]												Contaminating viruses[b]	
			Aug. 1 (26 dpp)				Aug. 17 (42 dpp)				Sep. 1 (57 dpp)				Sep. 16 (74 dpp)					
			C	Z	CZ	(+)	C	Z	CZ	(+)	C	Z	CZ	(+)	C	Z	CZ	(+)	WMV-2	TRSV
Transgenic plants																				
CZW-30	uninoculated	30	3	0	0	3	7	0	0	7	7	3	0	10	24	3	3	30	0	3
	inoculated C+Z	30	0	3	0	3	7	3	0	10	14	3	0	17	37	7	3	47	0	10
Untransformed plants																				
Mission	uninoculated	29	0	17	0	17	0	72	0	72	0	45	55	100	0	28	72	100	59	24
	inoculated Z	28	7	78	4	89	0	89	11	100	0	43	57	100	0	11	89	100	71	21
	inoculated C	26	8	31	0	38	0	65	16	81	0	61	35	96	0	35	65	100	62	19

[a] Data represent cumulative percentage of ELISA-positive plants for CMV (C), ZYMV (Z), and CMV+ZYMV (CZ) at 26, 42, 57, and 74 days after planting (dpp). Infected plants had OD 405 nm readings at least 8–12 times higher (0.8 vs. 0.09 for CMV and 1.0 vs. 0.08 for ZYMV) than healthy control plants after 30 min substrate hydrolysis. Noninfected transgenic plants had OD 405 nm values of 0.14 for CMV and 0.1 for ZYMV after 30 min substrate hydrolysis.
n = number of plants tested, (+) total percentage of ELISA-positive plants. Plants were either uninoculated or mechanically inoculated with CMV-Fny (C), ZYMV-FL (Z), or a mixture of CMV+ZYMV (C+Z) on June 23, and transplanted to the field on July 6, 1994.
[b] Percent of ELISA-positive plants by 74 dpp. TRSV = tobacco ringspot virus.

spots and foliar necrosis at the end of the growing season (Figure 1B). PRSV-w was not detected in field B.

By the end of the season (68 dpp), untransformed plants were severely stunted with a 42% reduction in shoot length compared to transgenic plants (76 ± 29 vs 134 ± 35 cm). These differences were significant ($P < 0.01$) (data not shown). Identical to field A, analysis of variance showed that genotype was the only significant factor that accounted for the differences in symptom development, virus incidence and shoot length among plants ($P < 0.01$).

Resistance to infections by CMV, ZYMV, and WMV-2

Transgenic homozygous cantaloupes CZW-30 were evaluated for resistance to CMV, ZYMV, and WMV-2 in field C. Untransformed plants developed systemic symptoms similar to those described for field B due to mixed infections by CMV and the two potyviruses. Although more mechanically inoculated untransformed plants were infected early in the season compared to uninoculated untransformed plants, all untransformed plants were symptomatic by 55 dpp (Figure 1C). In contrast, transgenic plants were highly resistant throughout the growing season since the majority of them never developed systemic symptoms. However, some transgenic plants (81% by the end of the growing season, e.g. 77 dpp) developed non-systemic symptoms and these were clearly distinct from those observed on untransformed plants. Symptomatic transgenic plants displayed the localized reaction previously described for their counterparts in fields A and B.

ELISA was performed with leaf samples collected 26, 41, 58, and 73 dpp. At least one of the target viruses was detected in 39% of the aphid-inoculated untransformed plants at 26 dpp, in 94% at 41 dpp, and in 100% at 58 dpp. In contrast, only 15% of the transgenic plants were ELISA positive at 58 dpp. Table 3 summarizes the cumulative data at 73 dpp. Mixed infections by CMV, ZYMV and WMV-2 were very low in transgenic plants when compared to untransformed plants. Only 5.5% of the latter were infected by CMV and ZYMV while 72% of the untransformed plants had mixed infections (Table 3). PRSV-w was detected in 4.5 and 4% of transgenic and untransformed plants, respectively, and TRSV was found in 31.5 and 26% of transgenic and untransformed plants, respectively (Table 3). PRSV-w and TRSV accounted for the few (10%) transgenic plants that showed severe foliar symptoms starting 64 dpp (Figure 1C).

By the end of the growing season (73 dpp), untransformed plants were severely stunted with a 36% reduction in shoot length compared to transgenic plants (80 ± 21 vs 125 ± 28 cm). These differences were significant ($P < 0.05$) (data not shown). Genotype was the only significant factor accounting for the variations observed among plants ($P < 0.01$), like for the two previous fields.

1995 field trials

Resistance of transgenic homozygous and hemizygous plants to infections by CMV, ZYMV, and WMV-2

Transgenic homozygous and hemizygous cantaloupes CZW-30 were compared for their resistance to mixed infections by CMV, ZYMV, and WMV-2 in replicated fields D and E. Unlike the 1994 field trials, plants were not mechanically inoculated. Instead, border rows of untransformed plants infected with one of the three target viruses served as virus sources. Analysis of symptom development indicated that susceptible control plants displayed similar reactions in both fields D and E with 89 and 100% symptomatic plants by 32 and 46 dpp, respectively (Figure 1D). Symptoms were as previously described for fields A, B and C, except that symptoms caused by non-targeted viruses were not observed. Transgenic plants exhibited resistance in both fields D and E (Figure 2). Homozygous plants never developed systemic symptoms throughout the growing season, but a few plants (3.5 and 7% by 46 and 65 dpp, respectively) showed localized symptoms similar to those described previously for fields A, B, and C. In contrast, hemizygous plants developed systemic symptoms similar to those exhibited by control plants, but they became symptomatic significantly later than the controls. For example, the percentage of symptomatic hemizygous plants was 15% compared to 89% symptomatic control plants at 32 dpp. Consequently, hemizygous plants were substantially larger and more vigorous than control plants (Figure 2). Virus incidence in hemizygous plants increased from 76 to 100% by 46 and 65 dpp, respectively. Symptoms were monitored only up to 65 dpp because transgenic plants were difficult to score individually late in the growing season due to their vigorous growth.

ELISA of leaf samples collected 34, 79, and 102 dpp confirmed that nearly all untransformed control plants (91%) became infected by 34 dpp. At the end of the trial period (102 dpp), all controls were ELISA positive and 99% of them were infected at least

Table 3. Virus incidence in transgenic homozygous CZW-30 and untransformed cantaloupes after mixed infections by CMV, ZYMV, and WMV-2 at 73 days after planting during the 1994 growing season.

Genotype	Treatment	n	Percent of ELISA-positive plants for CMV[a]								Contaminating viruses[b]	
			(+)	CZW	CZ	CW	ZW	C	Z	W	TRSV	PRSV-w
Transgenic plants												
CZW-30	uninoculated	54	39	0	7	0	0	17	15	0	35	7
	inoc. C+Z+W	50	32	0	4	0	0	18	10	0	28	2
Untransformed plants												
Mission	uninoculated	33	100	24	28	0	15	3	30	0	9	3
	inoc. Z	37	100	37	30	0	14	0	19	0	46	0
	inoc. W	41	100	24	25	0	17	0	34	0	20	5
	inoc. C	41	100	32	24	0	17	0	27	0	29	9

[a] Data represent cumulative percentage of ELISA-positive plants for CMV+ZYMV+WMV-2 (CZW), CMV+ZYMV (CZ), ZYMV+WMV-2 (ZW), CMV (C), ZYMV (Z), and WMV-2 (W) on Sep. 16, e.g. 73 days post-planting (dpp). Infected plants had OD 405 nm values at least 11–24 times higher (0.7 vs. 0.06 for CMV, 1.1 vs. 0.06 for ZYMV, and 1.2 vs. 0.05 for WMV-2) than healthy control plants after 30 min substrate hydrolysis. Uninfected transgenic plants had OD 405 nm values of 0.1 for CMV, 0.1 for ZYMV, and 0.05 for WMV-2 after 30 min substrate hydrolysis.
n = number of plants tested, (+) total percent of ELISA positive plants. Plants were either uninoculated or mechanically-inoculated (inoc.) with CMV-Fny (C), ZYMV-FL (Z), or WMV-2-Rob (W), or a mixture of CMV+ZYMV+WMV-2 (C+Z+W) on June 21, and transplanted to the field on July 5, 1994.
[b] Percentage of ELISA-positive plants by 73 dpp. PRSV-w = papaya ringspot virus type w.

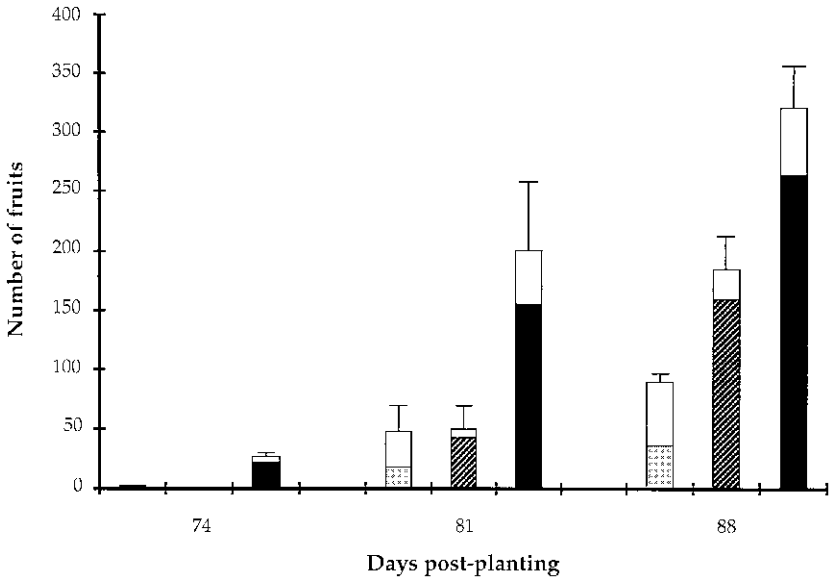

Figure 2. Yield of transgenic CZW-30 and untransformed cantaloupes during the 1995 growing season. Data on total number of fruits (open bars) and marketable fruits (solid bars) were taken for homozygous (stripped bars), hemizygous (black bars), and untransformed plants (stippled bars) at 74, 81, and 88 dpp. Data represent the average of cumulative values for 96 plants of each genotype that were tested in two separate fields. Line on top of bars represent standard deviations.

by two viruses (Table 4). In contrast, only 34% of homozygous plants reacted in ELISA, and only 8% had combinations of viruses by 102 dpp (Table 4). Hemizygous plants had a higher incidence of ELISA positive plants (70% by 102 dpp) than homozygous plants. However, the incidence of mixed infections by two or three viruses was significantly lower (33%) for hemizygous than for control plants (99%) at the end of the growing season (Table 4).

Table 4. Virus incidence in transgenic CZW-30 and untransformed cantaloupes after mixed infections by CMV, ZYMV, and WMV-2 at 102 days after planting during the 1995 growing season.

Genotype	n	Field	Percent of ELISA-positive plants[a]							
			(+)	CZW	CZ	CW	ZW	C	Z	W
Transgenic plants										
Homozygous CZW-30	96	D	32	0	5	0	1	21	1	4
	96	E	36	0	1	3	6	4	6	16
Hemizygous CZW-30	96	D	70	6	14	1	10	16	18	5
	96	E	70	7	6	4	18	9	8	16
Untransformed plants										
Cristobal	96	D	100	21	78	0	0	0	1	0
	96	E	100	72	20	0	7	0	1	0

[a] Data represent cumulative percentage of ELISA-positive plants for CMV+ZYMV+WMV-2 (CZW), CMV+ZYMV (CZ), CMV+WMV-2 (CW), ZYMV+WMV-2 (ZW), CMV (C), ZYMV(Z), and WMV-2 (W) on Sep. 15, e.g. 102 days after planting (dpp). Infected plants had OD 405 nm readings at least 11–18 times higher (1.1 vs. 0.06 for CMV, 0.7 vs. 0.06 for ZYMV, 0.5 vs 0.03 for WMV-2) than control plants after 30 min substrate hydrolysis. Uninfected transgenic plants had OD 405 nm values of 0.06 for CMV, 0.05 for ZYMV, and 0.03 for WMV-2. Infected plants were all aphid-inoculated.
n = number of plants tested in two separate fields designated D and E, (+) total percentage of ELISA-positive plants. Untransformed plants used as primary virus sources were mechanically-inoculated with CMV-Fny, ZYMV-FL or WMV 2-NY on May 31. Seedlings were transplanted to the fields on June 5, 1995.

Shoot length values were averaged for fields D and E. Data confirmed visual observations that transgenic plants were more vigorous and longer than controls (Figure 2). Control plants were severely stunted by 26 dpp with a 33% reduction in shoot length compared to transgenic plants (96 ± 18 vs. 143 ± 21 cm). Growth differences between control and transgenic plants were even more apparent later in the growing season (62 dpp) when controls showed 38 and 49% reductions relative to hemizygous (109 ± 22 vs. 175 ± 25 cm) and homozygous (109 ± 22 vs. 215 vs. 23 cm) plants, respectively. Homozygous and hemizygous plants showed similar growth at 26 and 50 dpp, but homozygous plants had significantly longer vines ($P < 0.01$) at 62 dpp (215 ± 23 vs. 175 ± 25 cm).

Yield of transgenic plants

Figure 3 shows the cumulative number of fruits that were harvested at three different dates. Transgenic plants had significantly higher marketable yield than untransformed controls ($P < 0.01$). Hemizygous plants produced 322 fruits of which 264 (82%) were marketable, and homozygous plants had 185 fruits with 159 (86%) being of marketable quality. Fruits from transgenic plants were uniform in size (diameter 11.5–14 cm) and had good flesh quality (data not shown). Unmarketable fruits were mostly immature at harvest time or had mushy flesh. Control plants showed a significantly lower yield ($P < 0.01$) with only 88 fruits (Figure 3). This value represents a 52 and 73% yield reduction compared to homozygous and hemizygous plants, respectively. Moreover, 53 out of the 88 (60%) fruits were unmarketable because they were small in size or misshapen. Average individual fruit weight was identical for homozygous and hemizygous plants (1.2 kg), but was significantly reduced ($P < 0.01$) by 58% for control plants (0.5 kg). Analysis of variance indicated that genotype was the only significant factor accounting for all the variations observed among plants ($P < 0.01$).

Discussion

Homozygous cantaloupes CZW-30 containing the CP genes of CMV, ZYMV, and WMV-2 showed excellent resistance under field conditions where either one, two or all three aphid-borne viruses were deliberately introduced. Homozygous plants never exhibited systemic symptoms upon infections by CMV, ZYMV and/or WMV-2. However, some plants developed localized symptoms that were clearly distinct from those of control plants because they were substantially milder and confined to a few leaves near the growing point of the plants. In addition, homozygous plants outgrew these symptoms. More importantly, although some homo-

Figure 3. Resistance of cantaloupes CZW-30 to mixed infections by CMV, ZYMV and WMV-2 in field D in 1995. The two right and two left rows contain homozygous and hemizygous plants, respectively, and the two center rows contain control plants that show severe stunting and foliar symptoms. The photograph was taken 53 days after planting.

zygous plants became symptomatic late in the growing season, symptoms were not caused by mixed infections but were primarily due to single infections by one of the three viruses. Our data were highly reproducible over two consecutive years regardless of the experimental design.

The development of transgenic line CZW-30 is especially significant because of its resistance to three of the four most important cantaloupe viruses and its commercially desirable fruit quality. A resistant cantaloupe line with multiple virus resistance has not been previously developed, essentially because of the difficulties in co-transferring and selecting multiple resistance genes by classical breeding [22, 29]. In addition, the numerous genes involved in conferring the resistance, their location on different loci, and their linkage to undesirable traits have made it a more difficult challenge for conventional breeders.

Homozygous line CZW-30 was used as resistant germ plasm to transfer the resistance to CMV, ZYMV, and WMV-2 into hemizygous plants. Seed companies generally prefer hybrids for their superior yield and to protect proprietary rights once the seeds are commercialized. Although hemizygous CZW-30 plants developed systemic symptoms late in the season, they had significantly higher fruit yield than homozygous plants eventhough the latter developed only localized symptoms. Even more striking was the 7.4-fold increase in fruit yield of the hemizygous over the susceptible control plants. Athough the control cultivar was not the exact genotype as the hemizygous plants, the latter clearly outperformed the control plants. Hemizygous CZW-30 plants substantially limited the incidence of CMV, ZYMV and WMV-2 before and during fruit setting, hence marketable fruits were produced. Thus, our data suggest that commercial hybrids originating from the homozygous line CZW-30 should perform well under severe disease incidence of CMV, ZYMV, and WMV-2. Our study expands on the recent report by Clough and Hamm [4] who evaluated homozygous and several hemizygous plants expressing the CP genes of CMV, ZYMV and WMV-2 for resistance

to ZYMV and WMV-2. These authors found that transgenic plants were highly resistant to the two potyviruses. However, they did not investigate the resistance to CMV nor evaluated yield.

Unexpectedly, a differential degree of resistance was observed between homozygous and hemizygous plants. Some homozygous plants developed localized symptoms that were confined to a few leaves close to the extremity of the vines and the symptoms became attenuated as the leaves expanded. In contrast, hemizygous plants showed a delay in the onset of disease but finally developed systemic symptoms comparable to those observed on control plants. A difference in virus incidence was noticed previously between homozygous and hemizygous cantaloupes expressing the three CP genes, however, the authors did not discuss this difference [4]. There have been limited reports on transgenic plants of economic importance that show differential resistance depending on the level of zygosity for the viral transgenes. Recently, Di et al. [7] suggested that homozygous but not hemizygous soybean plants containing a CP precursor of bean pod mottle comovirus were resistant to mechanical inoculations but these authors did not present any definitive evidence. Pang et al. [26] showed that homozygous lettuce plants expressing the nucleocapsid gene of tomato spotted wilt tospovirus were highly resistant to mechanical inoculations while hemizygous plants were moderately resistant. Also, investigations on RNA-mediated protection have indicated that transgenic homozygous tobacco lines expressing the 5′ portion of the potato virus X potexvirus RNA [21] and an antisense gene of the 5′ end of the tobacco mosaic tobamovirus RNA [5] displayed high resistance while hemizygous plants of the same lines exhibited very weak or no resistance. Interestingly, hemizygous squash containing the CP genes of CMV, ZYMV, and WMV-2 have performed well under field conditions [1, 4, 36].

For commercial purposes, the resistance exhibited by transgenic CZW-30 plants against three of the four major aphid-borne viruses affecting cantaloupes in commercial production can still be broadened by incorporating resistance against PRSV-w. Combining host-encoded and virus-derived resistance appears to be the strategy of choice to reach this objective.

Despite the benefits that virus-resistant plants offer to agriculture, concerns have been raised regarding their widespread commercial release. Potential risks include the development of new viruses through transencapsidation and recombination, and the escape of transgenes into free-living crop relatives [35]. The environmental risks due to heteroencapsidation and recombination would appear to be minimal compared to the use of untransformed plants since line CZW-30 is significantly reducing the incidence of mixed infections. This latter performance is of epidemiological significance. However, the introgression of transgenes derived from viral genomes into wild relatives has not been measured. We are currently addressing these environmental issues to assess whether risks outweigh benefits.

Acknowledgments

We are grateful to Sheri Ecker-Day, Dave Hummer, and Vânia Souza for their excellent help, and to Daniel Jarecke, William Boone, Mary Margaret Loman, Pat Cooley (Cornell University, Geneva, NY), Daniel Van Vleet and Douglas Davis (Plant Genetic Resources, Geneva, NY). We wish to thank Dr T. Zitter for providing CMV-Fny, and Dr R. Provvidenti for providing the two WMV-2 isolates. We are indebted to Dr J. Barnard for statistical advice, and to Dr L. M. Yepes for critical reading of the manuscript. This work was partially supported by grants (92-39210-8261 and 95-33120-1878) from USDA's Biotechnology Risk Assessment Research Program awarded to D.G. and M.F.

References

1. Arce-Ochoa JP, Dainello F, Pike LM, Drews D: Field performance comparison of two transgenic summer squash hybrids to their parental hybrid line. Hortscience 30: 492–493 (1995).
2. Banik MT, Zitter TA: Determination of cucumber mosaic virus titer in cantaloupe by ELISA and correlation with aphid transmission. Plant Dis 74: 857–859.
3. Blua MJ, Perring TM: Effect of zucchini yellow mosaic virus on development and yield of cantaloupe (*Cucumis melo*). Plant Dis 73: 317–320 (1989).
4. Clough GH, Hamm PB. Coat protein transgenic resistance to watermelon mosaic and zucchini yellows mosaic virus in squash and cantaloupe. Plant Dis 79: 1107–1109 (1995).
5. de Feyter R, Young M, Schroeder K, Dennis ES, Gerlach W: A ribozyme gene and an antisense gene are equally effective in conferring resistance to tobacco mosaic virus on transgenic tobacco. Mol Gen Genet 250: 329–338 (1996).
6. Delgadillo Sánchez F, Vega Piña A, Garzón Tiznado JA: Identificación y distribución de los virus del melón en el valle de Apatzigan, Michoacán. Rev Mex Fitopatol 5: 17–20 (1987).
7. Di R, Purcell V, Collins GB, Ghabrial SA: Production of transgenic soybean lines expressing the bean pod mottle virus coat protein precursor gene. Plant Cell Rep 15: 746–750 (1996).

8. Fang G, Grumet R: Genetic engineering of potyvirus resistance using constructs derived from the zucchini yellow mosaic virus coat protein gene. Mol Plant-Microbe Inter 6: 358–367 (1993).
9. Fitchen JH, Beachy RN: Genetically engineered protection against viruses in transgenic plants. Annu Rev Microbiol 47: 739–763 (1993).
10. Fuchs M, Gonsalves D: Resistance of transgenic hybrid squash ZW-20 expressing the coat protein genes of zucchini yellow mosaic virus and watermelon mosaic virus 2 to mixed infections by both potyviruses. Bio/technology 13: 1466–1473 (1995).
11. Gonsalves C, Xue B, Yepes M, Fuchs M, Ling K, Namba S, Chee P, Slightom JL, Gonsalves D: Transferring the cucumber mosaic virus-white leaf strain coat protein gene into *Cucumis melo* L., and evaluating transgenic plants for protection against infections. J Am Soc Hort Sci 119: 345–355 (1994).
12. Gonsalves D, Slightom JL: Coat protein-mediated protection: analysis of transgenic plants for resistance in a variety of crops. Sem Virol 4: 397–405 (1993).
13. Grafton-Cardwell EE, Perring TM, Smith RF, Valencia J, Farrar CA: Occurrence of mosaic viruses in melons in the central valley of California. Plant Dis 80: 1092–1097 (1996).
14. Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT: A simple and general method for transferring genes into plants. Science 227: 1229–1231 (1985).
15. Kaniewski, LM, Lawson C, sammons B, Haley L, Hart J, Delannay X, Tumer NE: Field resistance of transgenic Russet Burbank potato to effects of infection by potato virus X and potato virus Y. Bio/Technology 8:750–754 (1990).
16. Lawson C, Kaniewski W, Haley L, Rozman R, Newell C, Sanders P, Tumer NE: Engineering resistance to mixed virus infection in a commercial potato cultivar: resistance to potato virus X and potato virus Y in transgenic Russet Burbank. Bio/technology 8: 127–134 (1990).
17. Lecoq H, Pitrat M: Field experiments on the integrated control of aphid-borne viruses in cantaloupe. In: Plant Virus Epidemiology, pp. 169–176. Blackwell Scientific Publications, Oxford (1983).
18. Lomonossoff GP: Pathogen-derived resistance to plant viruses. Annu Rev Phytopathol 33: 323–343 (1995).
19. Miller ME: Virus diseases of cantaloupes in the lower Rio Grande valley. J Rio Grande Val Hort Soc 42: 19–23 (1989).
20. Milne RG: The economic impact of filamentous plant viruses. In: The Plant Viruses, vol 4, pp. 331–407. Plenum Press, New York (1987).
21. Mueller E, Gilbert J, Davenport G, Brignet G, Baulcombe DC: Homology-dependent resistance: transgenic virus resistance in plants related to homology-dependent gene silencing. Plant J 7: 1001–1013 (1995).
22. Munger MH: Breeding for viral resistance in cucurbits. In: Kyle MM (ed) Resistance to Viral Diseases of Vegetables: Genetics and Breeding, pp. 8–43. Timber Press, Portland, OR (1993).
23. Nameth ST, Dodds JA, Paulus AO, Kishaba A: Zucchini yellow mosaic virus associated with severe diseases of melon and watermelon in southern California desert valleys. Plant Dis 69: 785–788 (1985).
24. Orozco MS, Lopez-A O, Perez-Z O, Delgadillo-S F: Effect of transparent mulch, floating row covers and oil sprays on insect populations, virus diseases and yield of cantaloupe. Biol Agric Hort 10: 229–234 (1994).
25. Palukaitis P, Roosinck MJ, Dietzgen R, Francki RIB: Cucumber mosaic virus. Adv Virus Res 41: 281–348 (1992).
26. Pang SZ, Jan FJ, Carney K, Stout J, Tricoli DM, Quemada HD, Gonsalves D: Post-transcriptional transgene silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. Plant Cell 9: 899–909 (1996).
27. Perring TM, Royalty RN, Farrar CA: Floating row covers for the exclusion of virus vectors and the effect on diseases incidence and yield of cantaloupe. J Econ Entomol 82: 1709–1715 (1989).
28. Perring TM, Farrar CA: Stimulation of growth and yield of virus-infected cantaloupe with pyrethroids. Plant Dis 77: 1077–1080 (1993).
29. Provvidenti R: Resistance to viral disease in cucurbits. In: Kyle Mm (ed) Resistance to Viral Diseases of Vegetables: Genetics and Breeding, pp. 8–43. Timber Press, Portland, OR (1993).
30. Purcifull DE, Adlerz WC, Simone GW, Hiebert E, Christie SR: Serological relationships and partial characterization of zucchini yellow mosaic virus isolated from squash in Florida. Plant Dis 68: 230–233 (1984).
31. Quemada HD, Gonsalves D, Slightom JL: Expression of coat protein gene from cucumber mosaic virus strain C: Protection against infection by CMV strains transmitted mechanically or by aphids. Phytopathology 81: 794–802 (1991).
32. SAS-Institute: SAS/STAT user's guide, release 6.03. SAS Institute, Cary, NC (1991).
33. Shukla DD, Ward CW, Brunt AA: Genome structure, variation, and function. In: The Potyviridae, pp. 74–112. CAB International, Cambridge (1994).
34. Slightom JL: Custom polymerase-chain-reaction engineering of a plant expression vector. Gene 100: 251-255 (1991).
35. Tepfer M: Viral genes and transgenic plants. What are the potential environmental risks?. Bio/technology 11: 1125–1132 (1993).
36. Tricoli DM, Carney KJ, Russell PF, McMaster JR, Groff DW, Hadden KC, Himmel PT, Hubbard JP, Boeshore ML, Reynolds JF, Quemada HD: Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to cucumber mosaic virus, watermelon mosaic virus 2, and/or zucchini yellow mosaic virus. Bio/technology 13: 1458–1465 (1995).
37. Yoshioka K, Hanada K, Harada T, Minobe Y, Oosawa K: Virus resistance in transgenic melon plants that express the cucumber mosaic virus coat protein gene and in their progeny. Jpn J Breed 43: 629–634 (1993).
38. Zitter T, Hopkins DL, Thomas CE: Diseases caused by viruses. In: Compendium of Cucurbit Diseases, pp. 37-45. APS Press, St. Paul (1996).