

## Full Length Research Paper

# Yield and Disease Resistance of Transgenic Summer Squash in Commercial-Scale Trials

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Fruit yield of transgenic crookneck summer squash ZW-20 resistant to Zucchini yellow mosaic virus (ZYMV) and Watermelon mosaic virus (WMV) and of a susceptible nontransgenic lineage of the same genotype was compared over two consecutive years. Field trials relied on small-scale plantings that reflected commercial settings under conditions of severe disease pressure by ZYMV and WMV with infection achieved via aphid-mediated inoculation from virus source border plants. Across all trials, all transgenic plants were highly resistant to ZYMV and WMV, and the majority (79%, 331 of 421) produced 3 to 9 fruits per plant. In contrast, all control plants had severe systemic symptoms and the majority (80%, 336 of 421) produced 0 to 4 fruits per plant. In addition, all fruits of transgenic squash ZW-20H and ZW-20B were of marketable quality whereas most fruits of nontransgenic controls (96%, 947 of 989) were unmarketable. Differences in fruit number ( $P = 0.0001$ ) and fruit weight ( $P = 0.0001$ ) between transgenic and conventional squash plants were significant but not between ZW-20H and ZW-20B plants ( $P = 0.933$  and  $P = 0.964$ , respectively). This is the first report on a comparative analysis of fruit yield of transgenic versus conventional summer squash under conditions approaching commercial plantings in which high infection rates of ZYMV and WMV were achieved via indigenous aphid populations.

**Key words:** Fruit yield, transgenic, high disease pressure, summer squash, virus-resistant

## INTRODUCTION

Virus-resistant transgenic summer squash (*Cucurbita pepo* spp. *ovifera* var. *ovifera*) have been successfully developed (Tricoli et al., 1995) and commercialized in United States (Gaba et al., 2004; Johnson et al., 2007). Transgenic squash line ZW-20 was deregulated in 1994 (Medley, 1994). This was the first disease-resistant transgenic crop to receive exemption status in the United States. Plants of transgenic squash ZW-20 express the coat protein (CP) gene of Zucchini yellow mosaic virus (ZYMV) and Watermelon mosaic virus (WMV) and are

highly resistant to single and mixed infection by ZYMV and/or WMV, as shown by multiple field trials in different locations (Arce-Ochoa et al., 1995; Clough and Hamm, 1995; Fuchs and Gonsalves, 1995; Fuchs et al., 1998; Klas et al., 2006; Schultheis and Walters, 1998; Tricoli et al., 1995). Transgenic plants restrict ZYMV and WMV to chlorotic dots or blotches primarily in lower leaves when subjected to infection by these two viruses (Fuchs and Gonsalves, 1995; Fuchs et al., 1998; Klas et al., 2006). In contrast, nontransgenic squash develop severe systemic foliar symptoms consisting of mosaic, deformation, shoestringing and stunted growth following infection by ZYMV and WMV.

Two types of plants were identified during the development of transgenic line ZW-20 (Tricoli et al., 1995).

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Some ZW-20 plants remained asymptomatic or developed chlorotic dots upon infection by ZYMV or WMV and contained the H insert of the WMV CP gene. These plants were labeled ZW-20H (Tricoli et al., 1995). Other ZW-20 plants remained asymptomatic or developed chlorotic dots or blotches upon infection by ZYMV or WMV and lacked the H insert of the WMV CP gene. These plants were labeled ZW-20B (Tricoli et al., 1995). A spatiotemporal analysis indicated that, despite localized mild symptoms, transgenic ZW-20H and, to a lesser extent, ZW-20B do not readily serve as virus source for secondary spread of ZYMV and WMV (Klas et al., 2006). Plants of transgenic squash ZW-20, whether ZW-20H or ZW-20B, produce similar yield of marketable fruits as conventional summer squash under conditions of low, if any, disease pressure (Clough and Hamm, 1995). In contrast, under conditions of severe disease pressure by ZYMV and WMV, transgenic squash ZW-20 yield significantly more marketable fruits than conventional controls (Arce-Ochoa et al., 1995; Clough and Hamm, 1995; Fuchs and Gonsalves, 1995; Schultheis and Walters, 1998; Tricoli et al., 1995).

Most of field experiments designed to compare the fruit yield of virus-resistant transgenic and virus-susceptible conventional summer squash were carried out on complete randomized block designs with mixtures of different genotypes (Arce-Ochoa et al., 1995; Fuchs and Gonsalves, 1995; Fuchs et al., 1998; Schultheis and Walters, 1998; Tricoli et al., 1995). This approach provides valuable insight into the performance of test plants but does not reflect conditions of commercial settings for which plantings of a given genotype are uniform. Furthermore, comparative yield analyses have often been carried out under conditions of high disease pressure achieved by mechanically inoculating a few test plants in the greenhouse prior to transplanting and establishing them uniformly among test plants. These virus-infected plants served as primary inoculum source from which secondary infection takes place through aphid-mediated spread. This approach achieved 44 to 100% infection in nontransgenic plants (Clough and Hamm, 1995; Fuchs and Gonsalves, 1995; Fuchs et al., 1998) but did not reflect natural conditions of virus infection. Indeed, aphids essentially transmit viruses to plants in commercial fields from outside sources and subsequently spread them within fields. Some resistance screening experiments relied exclusively also on indigenous aphid populations to spread viruses, with no mechanically inoculated plants as primary virus source. These conditions achieved a 53% infection rate in conventional plants (Arce-Ochoa et al., 1995). Other field experiments reproduced commercial conditions with no virus source plants and insecticides applied to control aphid populations; infection rates reached 65% in nontransgenic controls (Schultheis and Walters, 1998).

To date, no information is available on the comparative fruit yield analysis of transgenic versus conventional squash under field conditions approaching commercial

settings for which a 100% virus infection rate is achieved in susceptible plants via aphid inoculation from virus source plants located outside of experimental plantings. In this paper, we present a comparative analysis of fruit production of transgenic summer squash ZW-20 and conventional summer squash in small-scale field settings simulating commercial fields with high virus infection rates achieved through aphid-mediated inoculation

## MATERIALS AND METHODS

### Plant materials

Transgenic crookneck summer squash line ZW -20 was developed by *Agrobacterium tumefaciens*-mediated transformation. It contains the CP genes of ZYMV and WMV (Tricoli et al., 1995). Transgenic hybrids ZW -20H and ZW -20B, resulting from crosses of transgenic plants, which were homozygous for the two CP transgenes, and an untransformed parent, were used in this study. The commercial conventional cultivar Pavo, which is susceptible to ZYMV and WMV, and has the same genetic background as the two transgenic hybrids, was used as control.

### Field layout

Six squash field plots were established over two consecutive years at the Crittenden farm, Cornell University, New York State Agricultural Experiment Station in Geneva, NY (Klas et al., 2006). In 1994, two field plots were planted under permits issued by APHIS-USDA (U.S. Department of Agriculture's - Animal and Plant Health Inspection Service), one with transgenic ZW -20B plants and one with nontransgenic control plants. The two field plots were established 20 m apart with 8 rows, 2 m apart, and 27 plants per row at a 1 m within-row distance. In 1995, four field plots were planted, two with transgenic plants (one with ZW -20H and one with ZW -20B) and two with nontransgenic control plants (Control 1 and Control 2).

The four field plots were established 20 m apart and consisted of 10 rows with 20 plants each. The between- and within-row distances were the same as in 1994. Permits were not required in 1995 because transgenic line ZW -20 was deregulated in December of 1994. The six field plots were surrounded by a single row of nontransgenic controls, which were mechanically inoculated with ZYMV or WMV prior to transplanting (Fuchs and Gonsalves, 1995). Mechanically inoculated plants were transplanted in alternating groups of four ZYMV-infected and four WMV-infected plants. Infected plants were used as primary virus inoculum for aphid-vectored infections. No insecticide was used throughout the two growing seasons for efficient virus spread by indigenous aphid populations.

### Disease progress

Virus incidence was monitored by visual observation of symptoms every 3 to 4 days and by double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) using immunoglobulins specific to ZYMV and WMV that were produced in our laboratory. Tissue from leaves in positions 3 to 5 at the apical end of each test plant was sampled three times during the growing seasons and tested in DAS-ELISA (Fuchs and Gonsalves, 1995). Samples were considered positive if their optical density reading at 405 nm ( $OD_{405nm}$ ) were at least twice the values of the healthy nontransgenic controls (Klas et al., 2006).



**Figure 1.** Resistance of transgenic crookneck summer squash ZW-20 to infection by ZYMV and WMV, and fruit production. (A) Squash ZW -20H or ZW -20B (second to fourth row from the left) surrounded by mechanically-inoculated conventional squash (left row and first plant of the second row), (B) Marketable fruit production of summer squash ZW -20H or ZW -20B, (C) Close-up of a marketable fruit of a transgenic (left) and of a five unmarketable fruits of conventional summer squash (right), and (D) Unmarketable fruit production of conventional summer squash.

### Fruit yield and data analyses

In 1994, fruit yield was assessed three times, irrespective of fruit size. In 1995, fruit yield was determined nine times at two to three-day intervals from August 7 to August 25. In both years, fruits of each test plant were visually rated for marketability, counted, and weighed. Fruit marketability was scored based on shape, size (12-18 cm) and absence of virus symptoms. Cull fruits were distorted, too small in size at maturity, and showed partial or complete discoloration, and green streaks or dots.

The fruit production frequency distribution was composed for each genotype in both growing seasons. Also, the cumulative number of fruits was plotted against time for each genotype. Data on 1995 fruit number and weight were summarized and subjected to paired *t*-test using the SPSS 8.0 software package for assessing significant differences between pairs of transgenic (ZW-20B and ZW-20H) and conventional (Control 1 and Control 2) squash plant genotypes.

## RESULTS

### Resistance to ZYMV and WMV in transgenic summer squash

The reaction of transgenic and conventional summer squash plants to infection by ZYMV and WMV in 1994 and 1995 was reported previously (Klas et al., 2006). Briefly, transgenic squash ZW-20H and ZW-20B were

highly resistant in both growing seasons (Figure 1A). Plants of both transgenic lines were either asymptomatic or developed mild symptoms that consisted of chlorotic dots or blotches mainly on old leaves. ZYMV and WMV could be detected by DAS-ELISA in symptomatic but not in asymptomatic leaf tissue. At the end of the two growing seasons, some transgenic plants reacted positively to ZYMV (9-36%), WMV (2-17%), or ZYMV and WMV (4-12%) (Klas et al., 2006). In contrast, conventional squash were highly susceptible to infection by ZYMV and WMV, and showed severe systemic symptoms. The two target viruses were readily detected in leaves of nontransgenic plants by DAS-ELISA. At the end of the two growing seasons, 95-100% of the control plants reacted positively to ZYMV and WMV, and the rate of mixed infection by ZYMV and WMV was high (57-86%) (Klas et al., 2006).

### Total fruit production and frequency distribution:

In 1994, fruits were harvested at 38, 65, and 82 days post-planting (dpp) because the major emphasis of the work was on spatio-temporal virus spread rather than fruit production. Therefore, most fruits were oversized (25 to 30 cm), in particular for the two last harvest dates.

**Table 1.** Cumulative fruit yield of transgenic ZW -20H and ZW -20B, and nontransgenic squash over two consecutive years.

Year	Genotype <sup>c</sup>	Total fruits <sup>a</sup>			Marketable fruits <sup>b</sup>		
		N <sup>d</sup>	Number	Weight (kg)	Number	Total weight (kg)	Weight/fruit (kg) <sup>e</sup>
1994	ZW-20B	143	567	313.2	567	313.2	na
	Control	140	389	121.8	42	26.4	na
1995	ZW-20H	139	790	150.3	790	150.3	0.19
	ZW-20B	139	798	149.5	798	149.5	0.19
	Control 1	139	443	62.3	0	0	0
	Control 2	142	217	30.4	0	0	0

<sup>a</sup>Fruits were harvested three times in 1994 and nine times at 2-3 days intervals in 1995. Fruits of each test plant were counted and weighed.

<sup>b</sup>Marketable fruits were asymptomatic and 12-18 cm in size. <sup>c</sup>Transgenic ZW -20B and ZW -20H express the CP gene of ZYMV and WMV. Crookneck summer squash cv. Pavo was used as control. <sup>d</sup>Number of plants tested per genotype. <sup>e</sup>na: not application because fruits were not harvested at 2-3 days interval according to agricultural practice

Nevertheless, transgenic ZW-20B produced 1.5 times more fruits than nontransgenic controls ( $P = 0.006$ ) (Table 1). In 1995, fruits were harvested at regular time intervals, e.g. 31, 33, 35, 38, 40, 42, 45, 47, and 49 dpp, and transgenic ZW-20 plants produced 1.8-3.7 times more fruits than controls (Table 1). Differences between transgenic and conventional squash were significant ( $P = 0.0001$ ) but not between transgenic ZW-20H and ZW-20B ( $P = 0.933$ ) (Table 1).

Analysis of the frequency distribution of fruit production in 1994 indicated that the majority of transgenic ZW-20B plants (85%, 121 of 143) produced 3 to 6 fruits while most nontransgenic controls produced only 1 to 4 fruits (91%, 127 of 140) (Figure 2A). In 1995, most transgenic ZW-20H (80%, 111 of 139) and ZW-20B (71%, 99 of 139) plants produced 3 to 9 and 4 to 9 fruits, respectively (Figure 2B). A few plants of both transgenic genotypes even had 11 to 13 fruits. In contrast, most nontransgenic plants produced 1 to 4 fruits in Control 1 (68%, 95 of 139) and 0 to 2 fruits in Control 2 (80%, 114 of 142) fields (Figure 2B).

### Fruit weight, size and marketability

In 1994, transgenic ZW-20B plants yielded 2.6 times more than conventional squash plants in terms of fruit weight. In 1995, transgenic ZW-20H and ZW-20B yielded 2.4-5 times more than controls (Table 1). Differences between transgenic ZW-20H and nontransgenic plants in Control 1 ( $P = 0.05$ ) and Control 2 ( $P = 0.0016$ ) were significant. Similarly, differences between transgenic ZW-20B and nontransgenic plants in Control 1 ( $P = 0.001$ ) and Control 2 ( $P = 0.0001$ ) were significant. Differences between transgenic ZW-20H and transgenic ZW-20B were not significant ( $P = 0.964$ ), confirming an equivalent performance of these two genotypes.

In terms of fruit size, some fruits of transgenic ZW-20B (33%, 62 of 190) had a marketable size (12-18 cm) at the

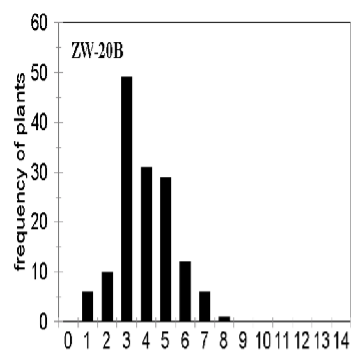
first harvest (38 dpp) but not (0%, 0 of 268) at the second harvest (65 dpp) dates in 1994. Fruits of the latter harvest date were oversized. In contrast, only 23% (42 of 185) of the fruits of conventional plants reached a marketable size at the first harvest but none (0%, 0 of 186) at the second harvest. In 1995, when harvest was done at 2-3 days intervals according to commercial practice, fruits of transgenic ZW-20H and ZW-20B plants were of marketable size (Figure 1B) with an average weight of 0.19 kg (Table 1). In addition, all transgenic fruits were asymptomatic and marketable (Figure 3). In contrast, only a few fruits of conventional plants (21%, 93 of 443 in Control 1 and 16%, 35 of 217 in Control 2) reached marketable size at maturity with an average weight of 0.14 kg (Table 1). However, none of them was marketable (Figure 3) because they were symptomatic (Figure 1C and 1D). Therefore, in contrast to the 1994 season, no marketable fruit was harvested from nontransgenic plants in 1995. This result reflected a difference in infection pressure between both years with 99% of the control plants being severely symptomatic already at 25 dpp in 1995 and only 40% of them at 30 dpp in 1994 (Klas et al. 2006).

### DISCUSSION

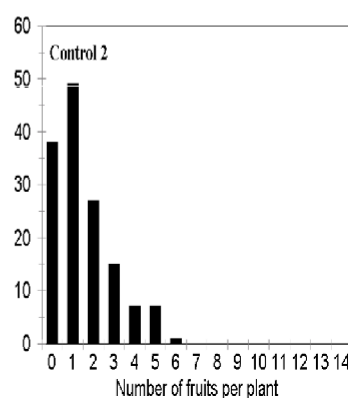
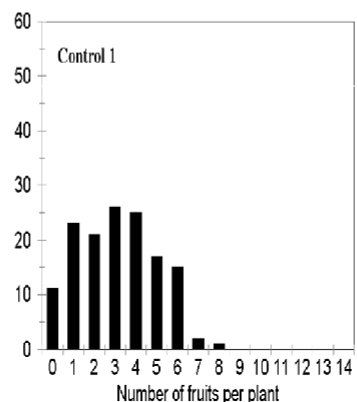
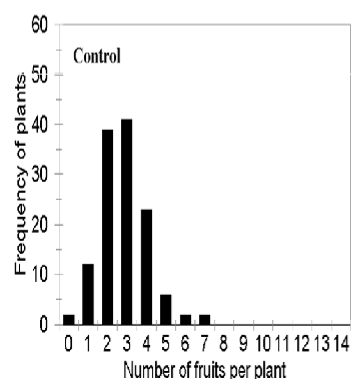
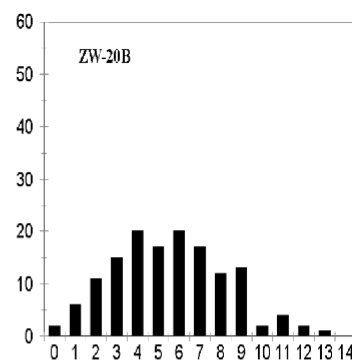
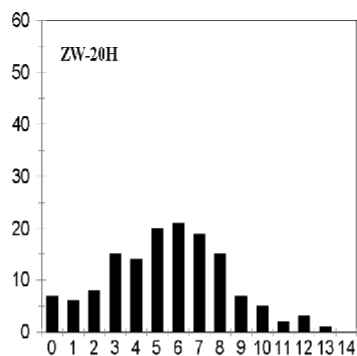
The cumulative production of marketable fruits of transgenic summer squash ZW-20 was significantly higher as compared to conventional summer squash of the same genotype in small-scale field settings simulating commercial plantings that were established under conditions of high disease pressure by ZYMV and WMV achieved via aphid-mediated inoculation. These findings achieved, for the first time, under simulated commercial conditions confirm previous reports on the performance of transgenic squash ZW-20 (Arce-Ochoa et al., 1995; Clough and Hamm, 1995; Fuchs and Gonsalves, 1995; Fuchs et al., 1998; Schultheis and Walters, 1998; Tricoli et al.,



## A. 1994



## B. 1995



**Figure 2.** Frequency distribution of the number of fruits per plant for (A) transgenic ZW -20B and nontransgenic controls in 1994, and (B) transgenic ZW -20H, transgenic ZW -20B, and nontransgenic controls in 1995.

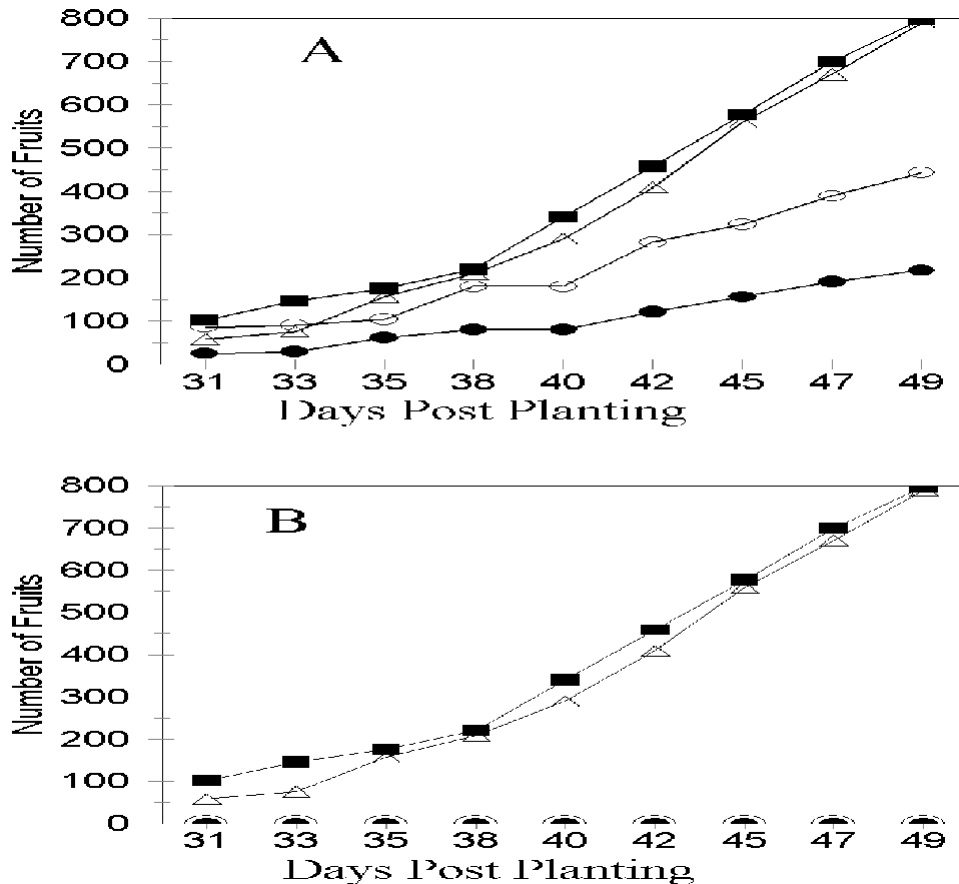
1995).

Earlier studies on the performance of virus-resistant transgenic summer squash relied on small-scale field experiments in which different genotypes were mixed (Tricoli et al., 1995; Fuchs and Gonsalves, 1995; Fuchs et al., 1998). Therefore, they did not reflect commercial settings. Also, some of the earlier studies were based on conditions of low, if any, disease pressure (Arce-Ochoa et al., 1995; Schultheis and Walters, 1998). Eventually high disease pressure was achieved by mechanical or aphid-mediated inoculation (Clough and Hamm, 1995; Fuchs and Gonsalves, 1995; Fuchs et al., 1998; Tricoli et al., 1995) but none of the earlier studies relied on conditions of severe disease incidence exclusively achieved through virus infection via indigenous aphid populations from primary virus source plants located outside of the experimental fields. Therefore, to the best of our knowledge, our study is the first to report on a comparative yield assessment of virus-resistant transgenic and virus-susceptible conventional summer squash in settings that simulate commercial fields and natural conditions of virus infection with no insecticides to control

indigenous aphid populations. These conditions are important for a good appreciation of the practical horticultural performance of virus-resistant transgenic squash plants.

In our study, a 95-100% infection rate was achieved in control plants. In spite of this very high disease pressure of ZYMV and WMV, fruits of transgenic squash ZW-20 exhibited no symptoms and maintained a marketable value, confirming previous findings (Clough and Hamm, 1995; Fuchs and Gonsalves, 1995; Tricoli et al., 1995). In addition, transgenic ZW-20B plants had a 2.4 to 8-fold increase in weight of marketable fruits compared to controls. Transgenic ZW-20H was also superior to conventional plants in terms of marketable fruit production.

In 2008, squash cultivars were grown on approximately 18,130 hectares in the United States. The seven major squash producing states were Florida (19% of U.S. output), Michigan (15%), California (13%), Georgia (12%), New York (9%), New Jersey (8%) and North Carolina (8%), producing 83% of the nation's squash crop. Yield losses of summer squash to aphid-transmitted viruses are often ranging from 20 to 80% and estimated



**Figure 3.** Cumulative number of (A) total and (B) marketable fruits of transgenic squash ZW -20H (- -) and ZW -20B (- -) plants, and nontransgenic squash (Control 1 -O- and Control 2 - -) plants in 1995.

to \$2.6 million in Georgia in 1997 (Gianessi et al., 2002). Traditionally, control of aphid-borne viruses in squash is attempted by cultural practices, including delayed transplanting relative to aphid vector flights, use of film mulch to repel aphid vectors, and application of stylet oil in combination with insecticides to reduce aphid vector populations (Blancard et al., 2004; Gaba et al., 2004; Perring et al., 1999; Zitter et al., 1996). In Georgia, it is estimated that 10 applications of stylet oil and insecticides are made routinely per acre to control aphids and, hence, limit virus transmission (Gianessi et al., 2002). The selection and use of squash cultivars is also a critical component for virus disease management. A number of squash cultivars with virus resistance have been developed successfully through conventional breeding (Munger, 1983; Provvidenti, 1993). However, cultivars with a high degree of resistance to multiple viruses have not been successfully developed yet through conventional breeding approaches (Gaba et al., 2004; Tricoli et al., 1995). This is mainly due to genetic incompatibility between donors and recipients of resistance genes, and linkage of virus resistance genes to undesirable traits. The adoption of virus-resistant transgenic summer

squash cultivars is steadily increasing since 1996. In 2006, the adoption rate was estimated to 24% (3,025 hectares) in five of the seven major producing states with an average rate of 70% in New Jersey, 20% in Florida and Georgia, 7% in North Carolina and 5% in Michigan (Johnson et al., 2007). The first virus-resistant transgenic squash line, denoted ZW-20, and cultivars derived thereof were deregulated in the United States in 1994 (Medley, 1994). Transgenic line ZW-20H was used as one of the parents to develop commercial F1 summer squash cultivars, including crookneck cvs. Freedom II (Tricoli et al., 1995) and Prelude II, straightneck cv. Patriot II, and zucchini cvs. Independence II and Declaration II. In addition to transgenic squash ZW-20, another transgenic line, denoted CZW-3, was developed (Tricoli et al., 1995). Transgenic line CZW-3 expresses the CP genes of CMV, ZYMV, and WMV, and is highly resistant to single or mixed infections by these three aphid-borne viruses (Tricoli et al., 1995; Fuchs et al., 1998; Schultheis and Walters, 1998). Transgenic line CZW-3 and cultivars derived thereof were deregulated in 1996 (Acord, 1996) and commercialized thereafter as semi-crookneck cv. Destiny III, green-stem straightneck

cv. Conqueror III, straightneck cv. Liberator III, and zucchini cvs. Judgement III and Justice III.

As shown in our study, the use of virus-resistant transgenic summer squash not only prevents crop failure due to severe virus incidence but also provides sustained production even under conditions of severe virus infection. Furthermore, lessons from the cultivation of virus-resistant transgenic summer squash over more than a decade in the United States and extensive safety assessment studies have provided compelling evidence that the technology is an efficient, safe and sustainable approach to control devastating virus diseases (Fuchs and Gonsalves, 2007).

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