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Transgenic Virus Resistance in Crop-Wild *Cucurbita pepo* Does Not Prevent Vertical Transmission of *Zucchini yellow mosaic virus*

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Abstract

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Zucchini yellow mosaic virus (ZYMV) is an economically important pathogen of cucurbits that is transmitted both horizontally and vertically. Although ZYMV is seed-transmitted in *Cucurbita pepo*, the potential for seed transmission in virus-resistant transgenic cultivars is not known. We crossed and backcrossed a transgenic squash cultivar with wild *C. pepo*, and determined whether seed-to-seedling transmission of ZYMV was possible in seeds harvested from transgenic backcrossed *C. pepo*. We then compared these transmission rates to those of non-transgenic (backcrossed and wild) *C. pepo*. The overall seed-to-seedling transmission rate in ZYMV was similar to those found in previous studies (1.37%), with no significant difference between transgenic backcrossed (2.48%) and non-transgenic (1.03%) backcrossed and wild squash. Fewer transgenic backcrossed plants had symptom development (7%) in comparison with all non-transgenic plants (26%) and may be instrumental in preventing yield reduction due to ZYMV. Our study shows that ZYMV is seed transmitted in transgenic backcrossed squash, which may affect the spread of ZYMV via the movement of ZYMV-infected seeds. Deep genome sequencing of the seed-transmitted viral populations revealed that 23% of the variants found in this study were present in other vertically transmitted ZYMV populations, suggesting that these variants may be necessary for seed transmission or are distributed geographically via seeds.

Transgenic crops are currently grown in 27 countries on approximately 175 million ha (James 2013). In the United States alone, the use of transgenic crops has increased dramatically since they were first implemented in 1992 (James 2013), increasing from 1.7 to 125 million ha in 12 years (James 2008). Although the vast majority of transgenic crops confer herbicide or insect resistance and/or tolerance, there are those that provide transgenic protection to viruses. It is thought that resistance, which can be achieved through conventional breeding or trangenesis, is the most effective method to mitigate the effects of viral pathogens in agricultural crops (Fuchs and Gonsalves 2007). Mitigating viral pathogens is important as they can have significant impacts on agricultural crops and can impair plant growth by affecting photosynthesis, metabolism, and resource allocation (Matthews 1991; Radwan et al. 2007; Tecsi et al. 1996), resulting in dramatic losses in crop yields (Oerke 2006; Oerke et al. 1994; Pico et al. 1996).

There are, however, agronomic and ecological risks associated with the use of transgenic crops (Fuchs and Gonsalves 2007; Pilson and Prendeville 2004; Tepfer 2002). Gene flow from cultivars to wild plants is common (Ellstrand 2003), and there are concerns that crop-wild hybridization and introgression of transgenes into wild populations may enhance plant fitness, thereby increasing weediness of wild plants (Fuchs et al. 2004; Fuchs and Gonsalves 2007; Laughlin et al. 2009; NCR 2002; Pilson and Prendeville 2004). Transgenic crops also have the potential to affect nontarget species (Fuchs et al. 2004; Fuchs and Gonsalves 2007; Laughlin et al. 2009; NCR 2002; Pilson and Prendeville 2004). There are additional risks associated with the use of virus-resistant transgenic crops. These include complementation of the viral transgene by an invading virus (Osbourn et al. 1990), which can theoretically lead to the compensation of defects in viral long distance movement (Callaway et al. 2004), the inhibition of gene silencing (Qu et al. 2003; Thomas et al. 2003), and the potential for an expanded host range (Latham and Wilson 2008; Spitsin et al. 1999). When functional structural genes are expressed by the virus, there is also a risk that heteroencapsidation of the incoming viral RNA by the coat protein of a different plant virus can occur. This has been demonstrated in *Cucurbita pepo* where the coat protein of *Watermelon mosaic virus* (WMV) was able to transmit *Zucchini yellow mosaic virus* (ZYMV) at low levels (2%) (Fuchs et al. 1999). An additional risk associated with the use of transgenic crops with virus resistance is the possibility of cryptic seed-to-seedling virus transmission.

Virus-resistant transgenic crops vary in their response to virus infection, including immunity, resistance, or tolerance (Lindbo and Dougherty 1992) (here, immunity is defined as complete resistance that prevents virus replication, in contrast to partial resistance or tolerance, which is defined as reduced virus replication and disease effects), along with virus replication. In particular, there are a number of studies in which transgenic plants have displayed mild symptoms and tested positive for virus infection (Fuchs et al. 2004; Sasu et al. 2009, 2010; Sikora et al. 2006; Tricoli et al. 1995). Since virus-resistant transgenic crops vary in response to virus infection, seed-to-seedling transmission could occur, which may be of both agronomic and ecological consequences if transgenic seeds are distributed without testing.

ZYMV is a single-stranded, positive-sense RNA virus for which natural infection appears to be limited to members of the Cucurbitaceae (e.g., squash, melon, and cucumber) (Desbiez and Lecoq 1997).

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ZYMV symptoms include severe stunting of the plant, distinctive yellow mottling of leaves, and fruit deformities (Desbiez and Lecoq 1997), such that ZYMV is a significant crop pathogen (Blua and Perring 1989). Although ZYMV can be transmitted by seed, it is primarily transmitted horizontally by a variety of aphids (Katis et al. 2006) in a non-persistent manner (Lisa et al. 1981). Seed transmission occurs in approximately 15% of the viruses in the family *Potyviridae* (Simmons and Munkvold 2014), including ZYMV in cultivars (Coutts et al. 2011; Davis and Mizuki 1986; Fletcher et al. 2000; Muller et al. 2006; Robinson et al. 1993; Schrijnwerkers et al. 1991; Tobias and Palkovics 2003), and symptomless seed-to-seedling transmission of ZYMV has previously been found to occur at 1.6% in wild (non-transgenic) *C. pepo* (Simmons et al. 2011). Wild *C. pepo* in North America is thought to be either the progenitor of domestic squash, or an early escape from cultivation (Decker and Wilson 1987; Decker-Walters et al. 2002; Lira et al. 1995). Squash (also *C. pepo*) cultivation occurs within the native range of wild *C. pepo* and cultivated alleles have been found in wild populations (Arriaga et al. 2006; Decker-Walters et al. 1993; Kirkpatrick and Wilson 1988; Wilson et al. 1994). To understand the risk of virus spread from wild-crop hybrid plants with a virus-resistant transgene, we examined seed transmission in virus-resistant, transgenic crop × wild *C. pepo* hybrids. Virus-resistant, transgenic cultivars of yellow crookneck squash was the first such plant deregulated by the United States Department of Agriculture (USDA). Transgenic squash was engineered to express a dual coat protein (CP) gene construct that conferred resistance to WMV and ZYMV (Asgrow, ZW-20) and WMV, ZYMV, and *Cucumber mosaic virus* (CMV; CZW-3) (USDA 1994, 1996).

To evaluate the risk of disease spread associated with seed transmission in crop-wild hybrids, we investigated if ZYMV can be transmitted from ZYMV-infected transgenic backcrossed plants to their seedlings. We examined the seed-to-seedling transmission of ZYMV from seeds harvested from transgenic (*C. pepo* ssp. *ovifera* var. Destiny III) × wild plants (*C. pepo* ssp. *texana* or spp. *ozarkana*). We compared the transmission rate from seeds harvested from non-transgenic *C. pepo* (both wild *C. pepo* ssp. *texana* and ssp. *ozarkana* as well as backcrossed non-transgenic) in the same experimental field. Finally, to assess the genetic variation of ZYMV transmitted to seedlings and to compare this variation to previously sequenced populations of ZYMV, we undertook deep (Illumina) genome sequencing of ZYMV from two infected seedlings, one harvested from a transgenic plant the other from a non-transgenic (wild) plant.

Methods

Backcross generation two squash (BC2). We developed backcross generation two (BC2) squash that segregates for the virus-resistant transgene construct (Fuchs et al. 2004). We collected fruits from wild *C. pepo* from four populations located in the state of Mississippi near the towns of Eagle Lake (referred to as Eagle; *C. pepo* spp. *ozarkana*), Vaiden (*C. pepo* spp. *ozarkana*), Yazoo City (referred to as Yazoo; *C. pepo* spp. *ozarkana*), and Onward (*C. pepo* spp. *texana*). Wild *C. pepo* from Vaiden and Yazoo were each crossed with *C. pepo* ssp. *ovifera* var. Destiny III (Seminis Vegetable Seeds, Inc., Saint Louis, MO) and then backcrossed within population to develop BC2 squash with virus resistance. Destiny III is a variety of virus-resistant transgenic squash with a CWZ-3 transgene construct (hemizygous) that confers resistance to ZYMV, WMV, and CMV and that also possesses a selectable marker, neomycin phosphotransferase II, which confers aminoglycoside antibiotic resistance. Pollen from Destiny III was used to hand pollinate wild *C. pepo* plants to produce F1 seeds. This movement of pollen from virus resistant transgenic squash to wild *C. pepo* simulates the most likely direction of crop-wild hybridization and introgression into wild populations. We germinated F1 plants and identified those with the transgene using PCR (Prendeville et al. 2012; Spencer 2001; Wall et al. 2004) and then backcrossed the transgenic F1 plants to wild *C. pepo* to create a backcross one generation (BC1). BC1 plants are hemizygous for the transgene construct. Again, PCR was used to identify BC1 plants with the transgene, which were backcrossed into wild *C. pepo* to create the BC2 generation. BC2 plants segregated 1:1 for the transgene and each BC2 was tested for the transgene using PCR. To minimize the potential effects of particular genetic backgrounds, individual F1 and BC1 plants were crossed with different individuals. All crosses were completed in greenhouses at the University of Nebraska-Lincoln. To independently verify transgene status, leaf samples from all BC2 plants were sent to GeneSeek Inc. (Lincoln, NE). GeneSeek Inc. conducted PCR using primers to amplify a portion of the transgene construct (Wall et al. 2004) and amplicons were viewed with an infrared fluorescent system (LI-Cor Inc., Lincoln, NE). Similar results were obtained by our laboratory work and GeneSeek Inc.

Common garden experiment. In March 2007, we planted a common garden experiment at the Delta Conservation Demonstration Center in Metcalfe, MS, using BC2 squash segregating for the virus-resistant transgene and wild *C. pepo*. Using a randomized block design, we planted BC2 (i.e., BC2-Vaiden and BC2-Yazoo) and seeds from different wild populations (i.e., Onward, Eagle, and Vaiden). To limit the mechanical transmission of ZYMV, we separated experimental plants by 6 m. However, plants did experience competition from other plant species (non-cucurbits) present in the field. In cultivated squash and other crops in the southeastern United States, aphid transmission of virus is common in summer and fall (Chalfant et al. 1977; Wosula et al. 2013). For this reason, we mechanically inoculated when plants were established (approximately 75 leaves on average) in July. We mechanically inoculated with virus by rubbing two to three new, not yet expanded leaves (at the tip of the vine) with approximately 1 ml of phosphate buffer with celite and homogenized squash leaf tissue infected with ZYMV (ZYMV-CT strain, kindly provided by the Provvidenti lab at Cornell University, Ithaca, NY) on 14 to 15 July. Rows between plants were mowed to provide access to experimental plants.

To assess virus infection, on 10 August 2007 we recorded the presence of visual symptoms of infection such as mosaic patterns and leaf deformities. Then we collected new leaves (at the tip of the vine that had not yet fully expanded) on 7 October and leaf material was dried in individual 50 ml screw cap tubes with desiccant up to the 20 ml mark (Drierite, W. A. Hammond Drierite Co. LTD., Xenia, OH). Dried leaf material (15 to 20 mg) samples in a 96-well plate with a glass bead in each well were sent to Agdia Inc. (Elkhart, IN) to assay for ZYMV using their antigen-coated plate (ACP)-ELISA with an alkaline phosphatase label. We included a positive control for ZYMV and three negative controls to which Agdia was blind. Agdia added a second set of positive controls for ZYMV and two negative controls. Agdia homogenized and analyzed samples for ZYMV by ELISA.

Detection of vertical transmission. To determine if ZYMV seed-to-seedling transmission rates differ due to the presence of the virus-resistant transgene in the maternal plant, we randomly sampled fruits on two BC2 transgenic squash, two BC2 non-transgenic squash, and six wild *C. pepo* plants, all of which tested positive for ZYMV. In total, 49 fruits were collected: 11 from BC2-Vaiden and BC2-Yazoo with the virus-resistant transgene, 11 from BC2-Vaiden and BC2-Yazoo without the virus-resistant transgene, and the remaining 27 from wild *C. pepo* (Eagle, Vaiden, and Onward). Seeds were extracted from fruits, removed from pulp, and dried in a drying oven for 24 h at 55°C. These seeds were planted in flats in a greenhouse at the Pennsylvania State University and 2,026 seedlings emerged. Transgenic maternal plants are hemizygous for the transgene; therefore, approximately 50% of offspring should possess the transgene. The seedlings were not assayed for the virus-resistant transgene, but are referred to as transgenic (T) or non-transgenic (NT) to indicate the genotype of the maternal parent as we were interested in determining the vertical transmission rates of ZYMV in seedlings grown from seeds harvested from transgenic versus non-transgenic plants. At the third true leaf stage, a leaf tissue sample was collected and frozen at –80°C until used for analysis.

Seedlings from each fruit were pooled together and then batched into groups of 10 (or less if there were any remainders) for RNA extraction, cDNA synthesis, and PCR, as described below. Using a calculation for pooled samples based on the binomial model, the percentage of seedlings that tested positive for ZYMV in RT-PCR was estimated (Block et al. 1999; Chiang and Reeves 1962). The percentage of infected seedlings (P) was calculated as $P = 100 \times (1 - (1 - Q)^{1/n})$,

where n is the number of seedlings per pool (10) and Q is the proportion of positive pools. The value Q is the number of positive pools (i.e., groups containing at least one infected seedling) divided by the total number of groups tested. Estimates were made for all seedlings (217 pools), seedlings from transgenic plants (54 pools), seedlings from non-transgenic plants (163 pools), seedlings from each population by transgene type (BC2-Vaiden T, 43 pools; NT, 40 pools; BC2-Yazoo T, 11 pools; NT, 8 pools) and seedlings from each wild population (i.e., Vaiden, 48 pools; Eagle, 47 pools; Onward, 19 pools).

RNA isolation, RT-PCR, and sequencing of seedlings. The methods used for RNA isolation, ZYMV specific RT-PCR and Illumina sequencing of the seedling samples are as previously described (Simmons et al. 2012) with the exceptions that we used the E.Z.N.A. RNA isolation kits (Omega Bio-Tek, Inc. Nocross, GA) to isolate RNA from frozen leaf samples, and the specific primers used for RT-PCR targeted the Helper Component protein and were: forward, TTTTGTCAGGCTCTATTCCCAGT; reverse, GCAACATCCAT CAACGAAGGC. Extracts of two of the samples that tested positive via RT-PCR were prepared for Illumina sequencing following the protocol outlined in Dunham and Friesen (2013) and were submitted for Illumina (deep amplicon) sequencing at the University of Southern California on an Illumina GAIIx; ZYMV from a seedling harvested from a transgenic parent (T; BC2-Vaiden with virus-resistance) and ZYMV from a seedling collected a non-transgenic parent (NT; wild *C. pepo*-Vaiden).

Alignment of raw reads, variant calling, and sequence analysis. Alignments were performed using the Burrows Wheeler aligner (BWA) version 0.6.2 allowing 10 mismatches (Li and Durbin 2009) with the NCBI reference strain of ZYMV (GenBank Accession No. NC_003224.1). BAM to SAM file conversion and filtering was performed with Samtools version 0.1.18 (Li et al. 2009). Varscan (version 2.3.2) (Koboldt et al. 2012) was used to call the minor mutational variants (i.e., those present beneath the consensus). To ensure that any false positives were eliminated, we conservatively only retained variants that occurred at greater than 100× coverage (all reads combined), had a frequency of 1% or greater, had a quality score of 30 or greater, and possessed a minimum of 10 reads (per variant) at a particular nucleotide position. The strand filter was also applied to eliminate any strand bias.

The consensus nucleotide sequences generated here have been submitted to GenBank and assigned accession numbers KJ875864-5.

Statistical analysis. Since ZYMV was detected in seedlings from transgenic backcrossed plants, we compared the presence and absence of ZYMV across all seedlings per plant from plants with the virus-resistant transgene to plants without the transgene (i.e., wild *C. pepo* populations and BC2 squash without the virus-resistance transgene). A generalized linear model was used with a beta error distribution to examine the fixed effects of plant population and transgene, with plant as random effects, on the presence of ZYMV in seedlings (PROC GLIMMIX, SAS 9.4 for Windows, SAS Institute Inc. Cary, NC). To evaluate the fixed effects of population and the presence of the transgene on visual symptoms of virus infection in maternal plants, a generalized linear model with a binomial error distribution was used (PROC GLIMMIX).

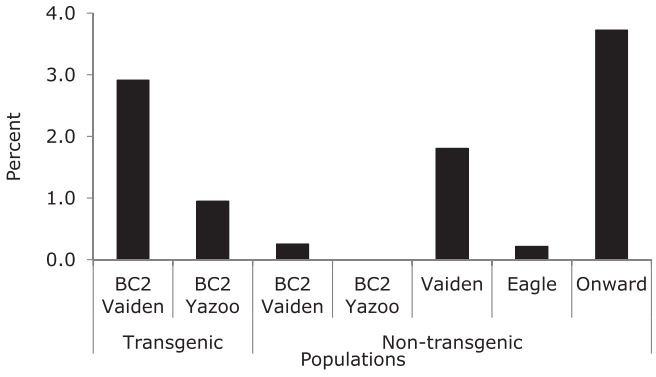

Fig. 1. ZYMV vertical transmission in BC2 crop-wild hybrids and wild populations of *Cucurbita pepo* with the virus-resistant transgene (BC2 Vaiden and BC2 Yazoo) and without the transgene (non-transgenic: BC2 Vaiden, BC2 Yazoo, Vaiden, Eagle, and Onward). There was no statistical difference in ZYMV seed transmission among populations ($F_{4,7}$ = 0.50, P = 0.737) or due to the presence or absence of the virus-resistant transgene ($F_{1,7}$ = 1.03, P = 0.344).

Results

There were 28 groups of 10 seedlings that tested positive for ZYMV out of a total of 217 groups (2,026 seedlings total). This equates to a ZYMV seed transmission rate of 1.37% (Fig. 1). This is in general accordance with the seed transmission rate previously reported from a population non-transgenic wild *C. pepo* in Pennsylvania (1.6%) (Simmons et al. 2011). Of the 28 groups of seedlings that tested positive for ZYMV, 12 groups were from seeds harvested from transgenic plants (T). The ZYMV seed transmission rate for seeds harvested from transgenic plants was 2.48% (12 of 54 groups; 505 seedlings), while the ZYMV seed transmission rate for seeds harvested from all non-transgenic plants (NT) was 1.02% (16 of 164 groups; 1,512 seedlings). The seed-to-seedling transmission rate of ZYMV for seeds harvested from BC2 non-transgenic plants was 0.21% (1 of 48 groups; 441 seedlings) and for wild plants it was 1.38% (15 of 116 groups; 1,071 seedlings). Notably, the frequency of ZYMV seed-to-seedling transmission rate did not differ among plant populations ($F_{4,7}$ = 0.50, P = 0.737). Likewise, there was no effect of the virus-resistant transgene on ZYMV seed-to-seedling transmission rate ($F_{1,7}$ = 1.03, P = 0.344). A comparison of visual symptoms (i.e., mosaic patterns and leaf deformities) among plants inoculated with ZYMV revealed no difference in visual symptoms among populations ($F_{1,65}$ = 2.00, P = 0.162), although there was a difference in visual symptoms due to the transgene with fewer transgenic plants having visual symptoms (7%) than all non-transgenic plants (BC2 and wild 26%; $F_{1,65}$ = 4.17, P = 0.045).

To compare genetic variation between the ZYMV populations sequenced in this study with those that were sequenced previously from Pennsylvania, we performed sequence analysis of two viral populations (one from a transgenic backcrossed plant and the other from a non-transgenic wild plant). The average coverages were 13,115× for the ZYMV sample sequenced from a non-transgenic parent and 240× for the ZYMV sample sequenced from a transgenic parent. The amount of the ZYMV genome sequenced was 99.48% from the seedling harvested from transgenic plants and 98.02% for the seedling harvested from non-transgenic plants. Analyses of these data revealed a total of 53 variants, 32 of which are non-synonymous, 14 synonymous, six harbored frameshifts, and there was a single mutation leading to a stop codon. Twelve of the 53 variants were shared between the seedlings from T and NT parents comprising nine non-synonymous (i.e., 75% of the total) and three synonymous mutations. In total, 26 of the variants were found only in the seedling grown from seed harvested from transgenic plants while 15 were only found in the seedling grown from seed harvested from non-transgenic plants (Table 1). Notably, 29 of the variants described here were also found in ZYMV populations of vertically transmitted samples from Pennsylvania that we sequenced previously (Simmons et al. 2013).

Discussion

The presence of a virus-resistant transgene did not prevent the vertical transmission of ZYMV in BC2 *C. pepo*. In particular, we found no significant difference between the frequency of seed-to-seedling transmission between transgenic and non-transgenic crop × wild *C. pepo*. These results clearly show that seeds from plants with the virus-resistant transgene cannot be assumed to be virus-free; thus, seed quality should be assessed through testing for ZYMV. ZYMV seed transmission occurs in cultivated cucurbits (Fletcher et al. 2000; Schrijnwerkers et al. 1991; Tobias and Palkovics 2003) and conventional-bred ZYMV resistance does not currently exist, such that conventional seeds are tested for ZYMV. However, it is unclear if virus-resistant transgenic squash are tested as growers may falsely assume the lack of visual symptoms indicates complete resistance to ZYMV. Evidently, without testing seeds for ZYMV, vertical transmission

of ZYMV may occur in transgenic virus-resistant squash, which in turn may affect virus epidemiology in crops and adjacent wild plant populations. It is also important to note that that horizontal transmission of ZYMV by aphids can occur from vertically infected plants (Simmons et al. 2011). Although transmission rates were not significantly different in the presence or the absence of the transgene, more extensive research is needed to determine if the presence of the virus-resistant transgene affects virus movement that may facilitate ZYMV seed-to-seedling transmission.

Virus-resistant transgenic crop × wild hybrids are able to transmit ZYMV to their progeny. This unexpected finding indicates that the virus-resistance transgene does not provide complete immunity, but instead confers partial resistance or tolerance (Gaba et al. 2004). Previous field studies of transgenic *C. pepo* × wild *C. pepo* (Fuchs et al. 2004; Laughlin et al. 2009; Sasu et al. 2009, 2010) found that the transgene effectively decreases symptoms of target viruses and increases yield in the face of virus infection. However, several studies have found transgenic plants with mild symptoms that tested positive for ZYMV (Fuchs et al. 2004; Sasu et al. 2009, 2010; Sikora et al. 2006; Tricoli et al. 1995). Together these studies indicate that virus resistance in transgenic squash only confers partial resistance or tolerance to ZYMV infection. Thus, ZYMV infection could occur through maternal vertical transmission to ovules or embryos. Although the use of transgenic plants is instrumental in reducing yield losses to ZYMV (Fuchs et al. 1998), our findings demonstrate that ZYMV is seed transmitted in virus-resistant transgenic squash × wild hybrids.

ZYMV seed-to-seedling transmission has important implications for agriculture (i.e., cultivated squash and its associated seed industries) as well as wild plant populations. For this reason, it is important for growers to monitor virus-resistant transgenic plants for virus and seed transmission since virus infection is not visually apparent. In wild plants, ZYMV may be seed-transmitted from tolerant plants to offspring, which could affect the epidemiology of ZYMV as virus transmission to neighboring non-transgenic plants (wild or crop) via aphids could occur. It has been reported that aphids are preferentially attracted to the volatile organic compounds produced by virus-infected plants, but after probing the plant, the aphids are more likely to depart without feeding (Mauck et al. 2010). Because transgenic plants can become infected with ZYMV, and because aphids are preferentially attracted to ZYMV-infected plants (Salvaudon et al. 2013), it is possible that this may increase the exposure/transmission rate in the host population. The exposure/transmission rate in a virus-resistant transgenic population will likely be greater than zero since virus-resistance transgene construct does not confer complete immunity, yet the exposure/transmission rate will likely be less than conventional plants (Klas et al. 2006). It is unclear how ZYMV seed-to-seedling transmission may affect wild *C. pepo* populations. ZYMV and other viruses reduce fecundity in wild *C. pepo* in comparison with transgenic introgressives (Fuchs et al. 2004; Laughlin et al. 2009; Sasu et al. 2009, 2010), which indicates a selective advantage for the transgene as it introgresses into wild populations. However, ZYMV inoculation did not reduce fecundity in a field experiment with wild populations of *C. pepo* from sites used in this study (Prendeville et al. 2014). Also, ZYMV inoculation affected traits other than fecundity, which resulted in an increase in population growth rate in comparison with no virus inoculation for *C. pepo* from Vaiden, MS (Prendeville et al. 2014).

An assessment of the next generation sequencing data revealed that 29 of the 53 variants observed in this study had been previously documented in nine vertically-transmitted ZYMV populations

Table 1. Nucleotide positions of the variants observed in two samples: ZYMV from one seedling harvested from a transgenic (T) plant and one seedling harvested from a non-transgenic (NT) plant. The first column indicates the viral polyprotein (the nucleotide positions the protein encompasses are noted in bold) and the specific nucleotide position at which the mutation occurred, as well as the type of nucleotide change. The next two columns are the plants in which the variants were found, and the percentage of the reads that occurred for that variant. The penultimate column indicates whether the mutation was S: synonymous, NS: nonsynonymous, FS: a frameshift, or S: a stop, and the last column indicates the amino acid change.

Mutation	NT (%)	T (%)	Type	AA change
P1 (138-1068)				
372 (G→C)		1.12	NS	Arg → Ser
380 (G→A)		1.55	NS	Arg → Gln
390 (G→T)		3.93	NS	Met → ile
395 (A→G)		1.35	NS	Lys → Arg
399 (G→A)		1.35	S	Val
404 (A→G)	1.40	1.66	NS	Lys → Arg
408 (T→C)		1.45	S	Gly
412 (A→G)	1.11	1.55	NS	Ser → Gly
414(T→G)	1.75	2.43	NS	Ser → Arg
425(T→C)		3.81	NS	Val → Ala
428(T→C)		3.98	NS	Leu → Pro
432(C→A)		1.90	S	Arg
452(T→C)		1.16	NS	Val → Ala
541 (G→T)	1.55		NS	Ala → Ser
625 (A→G)		1.17	NS	Arg → Gly
627 (G→)		7.52	FS	
631(A→G)		1.32	NS	Lys → Glu
HC-Pro (1069-2436)				
1071(G→A)	27.81	43.18	S	Ser
1412 (C→T)		8.33	NS	Ser → Phe
1697 (G→A)		4.47	NS	Arg → Lys
P3 (2437-3474)				
2490 (T→C)	19.44		S	Phe
3364 (C→A)	5.10		NS	Gln → Lys
3472 (C→A)	2.73		NS	Gln → Lys
6K1 (3475-3630)				
3555(A→G)	2.45	2.99	S	Arg

(*continued in next column*)

Table 1. (*continued from preceding column*)

Mutation	NT (%)	T (%)	Type	AA change
CI (3631-5532)				
3661 (G→C)	2.69		NS	Asp → His
3708 (T→A)	52.17	24.58	NS	His → Gln
3735 (T→C)	1.98		S	Phe
3742 (T→G)	2.81	2.91	NS	Trp → Gly
3743 (G→A)	2.81	2.92	NS	Trp to Stop
3850 (G→T)	8.23		NS	Ala → Ser
3913 (T→C)	12.71		S	Leu
5193 (G→A)	9.71	24.22	S	Lys
5501 (G→)		8.77	FS	
6K2 (5533-5691)				
5625 (C→)		5.63	FS	
5627 (A→T)		9.46	NS	Tyr → Phe
5628 (T→A)		8.64	Stop	Tyr → stop
5633 (G→)		47.92	FS	
5635 (T→G)	48.45	6.81	NS	Trp → Gly
5641 (G→T)	38.50	6.12	NS	Val → Phe
5643 (C→)		45.04	FS	
5821 (G→A)	1.61		NS	Val → Ile
5888 (T→G)	1.45		NS	Val → Gly
NIa-Vpg (5692-6261)				
6080 (G→A)	1.04		NS	Gly → Asp
NIa-Pro (6262-6990)				
6419 (G→A)	1.27		NS	Gly → Asp
6677 (A→)	1.18		FS	
6753 (A→T)	3.29		S	Ile
Nib (6991-8541)				
7029 (G→A)		1.02	S	Gly
7317 (C→T)		18.94	S	Cys
7320 (A→T)		1.60	S	Arg
7387 (A→G)		2.45	NS	Arg → Gly
7477 (C→T)	1.84	24.74	NS	His → Tyr
7776 (C→T)		5.61	S	Asn
CP (8542-9378)				
8899 (G→A)	1.75		NS	Gly → Arg

(seed-to-seedling) from Pennsylvania (Simmons et al. 2013). As the samples sequenced herein were from a geographically disparate area (they were inoculated with the ZYMV-CT strain), these data are compatible with the notion that seed transmission selects for specific variants. Based on a phylogenetic analysis of the coat protein region, it would appear that although all the American ZYMV isolates appear to share a common ancestor, there do appear to be sequence differences between the Pennsylvania strains and the Connecticut strain (Simmons et al. 2008). Similarly, we previously sequenced 24 horizontally transmitted populations of ZYMV in Pennsylvania (Simmons et al. 2012), and 13 of the variants found in this study were also observed in the ZYMV populations sequenced here. Twelve mutations were found in all three populations; that is, this study, the previously sequenced seed-to-seedling transmitted populations (Simmons et al. 2013), and the horizontally transmitted populations (Simmons et al. 2012). Interestingly, six of the variants found in all three studies are also present as polymorphic sites in the 25 ZYMV full genome sequences on GenBank (3708, 5193, 6753, 7029, 7317, and 7776). What effect, if any, these mutations have on the seed (or horizontal) transmission of this viral pathogen has yet to be determined, and it is possible that these variants are being distributed geographically via seeds or vectors (although vectors are short-lived). Additional viral populations from other regions would need to be investigated to address this question.

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