Theor Appl Genet (2011) 123:729–740
DOI 10.1007/s00122-011-1622-8

ORIGINAL PAPER

Responses of maize (*Zea mays* L.) near isogenic lines carrying *Wsm1*, *Wsm2,* and *Wsm3* to three viruses in the Potyviridae

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Received: 21 November 2010 / Accepted: 14 May 2011 / Published online: 12 June 2011

Abstract Genes on chromosomes six (*Wsm1*), three (*Wsm2*) and ten (*Wsm3*) in the maize (*Zea mays* L.) inbred line Pa405 control resistance to *Wheat streak mosaic virus* (WSMV), and the same or closely linked genes control resistance to *Maize dwarf mosaic virus* (MDMV) and *Sugarcane mosaic virus* (SCMV). Near isogenic lines (NIL) carrying one or two of the genes were developed by introgressing regions of the respective chromosomes into the susceptible line Oh28 and tested for their responses to WSMV, MDMV, and SCMV in the field and greenhouse. F_1 progeny from NIL × Oh28 were also tested. *Wsm1*, or closely linked genes, provided resistance to all three viruses, as determined by symptom incidence and severity. *Wsm2* and *Wsm3* provided resistance to WSMV. *Wsm2* and/or *Wsm3* provided no resistance to MDMV, but significantly increased resistance in plants with one *Wsm1* allele. NIL carrying *Wsm1*, *Wsm2,* or *Wsm3* had similar SCMV resistance in the field, but NIL with *Wsm2* and *Wsm3* were not resistant in the greenhouse. Addition of *Wsm2* to *Wsm1* increased SCMV resistance in the field. For all viruses, symptom incidence was higher in the greenhouse than in the field, and relative disease severity was higher in the greenhouse for WSMV and MDMV. An Italian MDMV isolate and the Ohio SCMV infected the *Wsm1* NIL, while the Ohio MDMV and Seehausen SCMV isolates did not. Our results indicate that the three genes, or closely linked loci, provide virus resistance. Resistance conferred by the three genes is influenced by interactions among the genes, the virus species, the virus isolate, and the environment.

Communicated by T. Luebberstedt.

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Introduction

The genetic basis for maize resistance to viruses in the family Potyviridae has been well investigated (reviewed in Redinbaugh and Pratt 2008). The two most important maize-infecting potyviruses are *Maize dwarf mosaic virus* (MDMV) and *Sugarcane mosaic virus* (SCMV; previously called MDMV-B). MDMV causes disease in North America, Europe, and Australia, while SCMV is found in all areas where maize is grown. Disease caused by these viruses is controlled primarily with resistant hybrids and through control of virus reservoirs such as Johnsongrass (*Sorghum halepense*). Resistance to another member of the Potyviridae, the tritimovirus *Wheat streak mosaic virus* (WSMV), has also been studied (McMullen et al. 1994). WSMV is not considered to be a major pathogen of maize, as field resistance to this virus is conferred by a number of alleles at any of three dominant resistance genes, making susceptible germplasm rare. However, WSMV infections produce a bright mosaic on susceptible maize, and resistance is well characterized, making the WSMV—maize system a good model for studying virus resistance mechanisms.

The maize inbred line Pa405 is highly resistant to infection by MDMV, SCMV, and WSMV. MDMV resistance is controlled by a single gene, *Mdm1*, on the short arm of chromosome 6 (McMullen and Louie 1989). WSMV resistance in Pa405 is controlled by three independent dominant genes: *Wsm1* on the short arm of chromosome 6, *Wsm2* near the centromere on chromosome 3, and *Wsm3* on the long arm of chromosome 10 (McMullen et al. 1994). Although resistance to SCMV has not been mapped in Pa405, the line is highly resistant to this virus (Jones et al. 2007; Mikel et al. 1984). However, SCMV resistance in several European inbred lines was shown to be controlled by two genes: *Scmv1* on chromosome 6 and *Scmv2* on chromosome 3 (Melchinger et al. 1998; Dussle et al. 2000; Xia et al. 1999).

Resistance to viruses in general, and potyviruses in particular, is clustered in the maize genome (Redinbaugh and Pratt 2008). *Mdm1, Scmv1,* and *Wsm1* all co-segregate with the marker(s) *umc85* and/or *bnl6.29*, and it is not yet known whether these are three very closely linked genes or a single gene that acts pleiotropically (Dussle et al. 2003; Simcox et al. 1995; Xu et al. 1999; Yuan et al. 2003). Similarly, *Scmv2,* and *Wsm2* map to the interval flanked by *umc92* and *umc102* near the centromere of chromosome 3 in European lines (Dussle et al. 2000, 2003; Xia et al. 1999), Pa405 (McMullen et al. 1994), and B73 (Marçon et al. 1999). *Wsm3* is in a similar genomic location to a minor QTL on chromosome 10 for SCMV resistance (Xia et al. 1999), and this region was implicated in MDMV resistance for a number of inbred lines, including Pa405 (Jones et al. 2007). Resistance to potyviruses has been identified in a variety of maize germplasm (Brewbaker et al. 1991; Kuntze et al. 1997; Louie et al. 1990; Scott and Louie 1996). Although resistance maps to similar locations in U.S. and European germplasm, potyvirus resistance apparently arose more than once, because the markers *umc85* and *bnl6.26* that border *Mdm1, Scmv1, and Wsm1* have different banding patterns between and among European, US and tropical lines (Jones et al. 2007; Xu et al. 2000).

Near isogenic lines (NIL) carrying the *Scmv1* and/or *Scmv2* genomic regions from the highly resistant European inbred FAP1360A in a virus susceptible background from the inbred line F7 were tested for their responses to virus inoculation (Lübberstedt et al. 2006; Xing et al. 2006). The $F7^{RR/RR}$ NIL, which is homozygous for both the *Scmv1* and *Scmv2*, was similar to the resistant parent in its response to inoculation with a SCMV isolate from Germany (SCMV-Seehausen), the MDMV MD isolate (Israel), the Ohio WSMV isolate, and a *Zea mosaic virus* isolate from Israel (ZeMV). NIL homozygous for only one of the two genes were nearly as susceptible to SCMV, MDMV, and ZeMV as F7, but those with at least one allele of each gene had intermediate resistance. Recently, these lines have been used to develop fine mapping populations, and the genomic region containing *Scmv2* has been narrowed to a ca. 1.3 Mb region (Ingvardsen et al. 2010).

Potyvirus species have differential ability to infect resistant maize germplasm, with ZeMV and WSMV being less virulent than MDMV and SCMV (Redinbaugh and Pratt 2008; Xing et al. 2006). However, very little information on the abilities of different potyvirus isolates to infect defined maize germplasm is available. Earlier studies indicated some differential ability of Ohio MDMV isolates to infect the maize inbred lines (Louie and Knoke 1975), but until very recently no potyvirus isolates that break resistance derived from FAP1360A or Pa405 were described. A newly identified highly pathogenic isolate of MDMV was isolated from maize from Italy (MDMV-It; Uzarowska et al. 2009). This isolate is infectious on the $F7^{RR/RR}$ line, but does not infect FAP1360A or Pa405.

To characterize the contributions of the Pa405 chromosomal regions encoding *Wsm1*, *Wsm2,* and *Wsm3* to virus resistance, NIL carrying one or two of the three chromosomal segments in a susceptible background were developed and tested for their responses to inoculation with MDMV, SCMV, and WSMV in the field and greenhouse. The responses of a subset of these lines to virulent and less virulent pairs of MDMV and SCMV isolates were also tested.

Materials and methods

Materials

Ohio isolates of MDMV (MDMV-OH), SCMV (SCMV-OH) and WSMV were maintained by serial passage on susceptible maize (*Zea mays* L.) as previously described (Jones et al. 2007; McMullen and Louie 1991). A highly virulent isolate of MDMV (MDMV-It; Uzarowska et al. 2009) and the SCMV-Seehausen isolate (SCMV-See; Fuchs and Gruntzig 1995) were provided by Stephan Hentrup (Danish Institute of Agricultural Sciences, Research Centre Flakkebjerg, Slagelse, Denmark) and maintained by serial inoculation as above. Maize inbred lines Oh28 and Pa405 were maintained at the Ohio Agricultural Research and Development Center, Wooster, OH.

Germplasm development

NIL were developed from the BC_1-F_1 progeny of the backcross of Pa405 (donor) × Oh28 (recurrent parent). Initially, BC_1-F_1 families were inoculated with WSMV in the greenhouse and resistant plants from families that segregated 1:1 for resistance and susceptibility were

selected. For lines carrying *Wsm1*, resistant plants were asymptomatic; for those carrying *Wsm2*, resistant plants had delayed and limited symptoms (DL); and, for lines carrying *Wsm3*, resistant plants developed delayed and dispersed spots and rings (DDSR). BC_6 populations were developed by screening each generation with WSMV and selecting for the resistant phenotypes. NIL were selected by self-pollinating the BC_6F_1 plants and screening the progeny with restriction fragment length polymorphism (RFLP) markers on chromsome 6 (umc85) for *Wsm1*, chromosome 3 (umc102) for *Wsm2,* and chromosome 10 (umc44) for *Wsm3*, to identify plants that were homozygous for the respective resistance locus. NIL homozygous for *Wsm1*, *Wsm2*, or *Wsm3* will be referred to as $Oh28^{SS/RR/SS}$, $Oh28^{RR/SS/SS}$, and $Oh28^{SS/SS/RR}$ (Table 1), respectively. In analogy to the nomenclature used by Xing et al. (2006) for NIL carrying *Scmv1* and *Scmv2* from FAP1360 in an F7 background, Oh28 refers to the susceptible backcross parent, and the xx/xx/xx superscripts indicate the presence of alleles from the susceptible (S) or resistant (R) parent at loci on chromosome 3 (*Wsm2*), chromosome 6 (*Wsm1*), and chromosome10 (*Wsm3*), respectively. Lines homozygous for two resistance genes ($Oh28^{RR/RR/SS}$, $Oh28^{SS/RR/RR}$, and $Oh28^{RR/SS/RR}$) were developed by crossing the respective NIL and selecting plants from F_2 populations that were homozygous for both resistance loci with the SSR markers umc2196 (*Wsm1*), umc2002 (*Wsm2*), and umc1506 (*Wsm3*). Heterozygous lines were produced by crossing the NIL to Oh28. The genotypes of all NIL used experimentally were assessed using umc2196, umc2002, and umc1506, which are closely linked to *Wsm1*, *Wsm2,* and *Wsm3*, respectively.

Table 1 Genotypes of maize lines

Line[a]	Genotype[b]
Oh28	Oh28
Pa405	Pa405
Wsm1 NIL	$Oh28^{SS/RR/SS}$
Wsm2 NIL	$Oh28^{RR/SS/SS}$
Wsm3 NIL	$Oh28^{SS/SS/RR}$
Wsm1 × Oh28 F_1	$Oh28^{SS/RS/SS}$
Wsm2 × Oh28 F_1	$Oh28^{RS/SS/SS}$
Wsm3 × Oh28 F_1	$Oh28^{SS/SS/RS}$
Wsm1 Wsm2 NIL	$Oh28^{RR/RR/SS}$
Wsm1 Wsm3 NIL	$Oh28^{SS/RR/RR}$
Wsm2 Wsm3 NIL[c]	$Oh28^{RR/SS/RR}$
Wsm1 Wsm2 × Oh28 F_1	$Oh28^{RS/RS/SS}$
Wsm1 Wsm3 × Oh28 F_1	$Oh28^{SS/RS/RS}$
Wsm2 Wsm3 × Oh28 F_1	$Oh28^{RS/SS/RS}$

The designations and genotypes of the near isogenic and F_1 lines used in this study

[a] NIL, near isogenic line. F_1 lines were made by crossing the indicated NIL with Oh28

[b] The designations are based on those used by Xing et al. (2006). Oh28 indicates the genetic background with the xx/xx/xx superscript indicating the presence of an allele from the susceptible (S) or resistant (R) parent at the locus on chromosome 3 (*Wsm2*)/6 (*Wsm1*)/10 (*Wsm3*), respectively

[c] This genotype had poor germination in the field, resulting in stands insufficient to be included in all experiments

Response of NIL to MDMV-OH, SCMV-OH, and WSMV in the field and greenhouse

Field experiments were planted between May 10 and May 20 in Wooster, OH. MDMV-OH resistance was evaluated in 2007, 2008, and 2009; SCMV-OH in 2007, 2008, 2009, 2010; and WSMV in 2009. For each virus, plots consisted of 25 seeds planted in a 6-m row spaced at 0.76 m for each genotype and were replicated three times in a complete randomized block design. Inoculum was produced, and plots were inoculated four times at two-day intervals beginning at V2 using a Paasche model H3 airbrush (Paasche Airbrush Co., Chicago, IL) as previously described (Louie et al. 1983). Plants were scored for the appearance of systemic mosaic and limited symptoms beginning 2 weeks after the first inoculation and continuing at weekly intervals until no further increase in symptom development was seen.

The responses of NIL to inoculation with MDMV-OH, SCMV-OH, and WSMV in the greenhouse were conducted as previously described (Jones et al. 2007). MDMV-OH resistance was evaluated in 2004, 2006, and 2010; SCMV-OH in 2004 and 2006; and WSMV in 2004, 2006, and 2010 (Table 2). Each experiment consisted of three replications of 12 seed rows of each genotype planted into 30 × 46 × 7 cm flats of autoclaved greenhouse soil in a complete randomized block design. Plants were inoculated four times at 2-day intervals beginning at the V2 stage as previously described (Louie 1986). Individual plants were scored for the appearance and type of symptoms beginning 10–12 days after the first inoculation (dpi) and continuing at weekly intervals for 3 weeks.

Response of NIL to inoculation with Ohio and European MDMV and SCMV isolates

These experiments were carried out under USDA, APHIS permit P526P-09-00694, which allows inoculated plants to be grown only in growth chambers. Seeds of the indicated maize lines were planted five seeds per pot and grown in a growth chamber with a 12 h light/12 h dark (24 C) photoperiod. At 6 to 8 days after planting, when seedlings had developed at least three leaves, ten plants per line were inoculated once by leaf rub inoculation with MDMV-OH, MDMV-It, SCMV-OH, or SCMV-See. Plants were scored

Table 2 Infection of the virus susceptible inbred line, Oh28, in field and greenhouse experiments

Date	Field/GH[a]	% Infection[b]								
		MDMV-OH			SCMV-OH			WSMV		
	Rep—	1	2	3	1	2	3	1	2	3
2007	Field	100	100	79	100	100	79			
2008	Field	93	87	97	75	72	86			
2009	Field	88	94	95	81	100	75	97	92	78
2010	Field				100	73	100			
Winter 2004	GH	100	100	100	95	100	100	100	100	100
Winter 2006-07	GH	100	100	100	100	100	100	100	100	100
Spring 2010	GH	100	100	100				100	100	100

The percentage of symptomatic plants for the susceptible control line (Oh28) is shown for individual experiments by year, environment, and replicate. Plants were inoculated with the Ohio isolates of *Maize dwarf mosaic virus* (MDMV-OH), *Sugarcane mosaic virus* (SCMV-OH) or *Wheat streak mosaic virus* (WSMV)

[a] Experiments were carried in the field or greenhouse (GH)

[b] Data shown are the percentage of symptomatic plants for line Oh28 in each replication (*Rep*)

twice for symptom appearance at 5–8 dpi and 10–13 dpi (data not shown). At 10–12 dpi, tissue from the youngest non-inoculated leaves was tested for the presence of virus using a tissue blot immunoassay (TBIA) (S.A. Tolin, personal communication). Briefly, leaf samples were applied to nitrocellulose membranes by rubbing with a plastic microfuge tube pestle. Membranes were dried, then blocked, and decolorized for 1 h in PBS (20 mM Na-phosphate, pH 7.4, 0.15 M NaCl) containing 5% non-fat dry milk (NFDM, Carnation, Nestlé USA, Glendale, CA) and 5% Triton X-100. Membranes were then incubated with antisera raised against MDMV-OH or SCMV-OH diluted 1:3,000 and 1:800, respectively, with PBS-T (1× PBS, 0.05% Tween-20) containing 1% NFDM. After washing with PBS-T, membranes were incubated for 1 h with a 1:20,000 dilution of goat anti-rabbit IgG-alkaline phosphatase (Sigma, St. Louis) in PBS-T plus 1% NDFM, and washed again with PBS-T. Alkaline phosphatase activity was visualized using the BioRad (Hercules, CA) AP Colorimetric Kit per the manufacturer's instructions. The primary antibody used was directed against the inoculated virus. In addition to samples from test plants, each membrane carried samples from plants known to be infected with MDMV-OH and SCMV-OH, and uninoculated plants of each maize genotype. Three independent experiments with a single replication of each treatment (virus–line combination) were carried out.

Data analysis

For greenhouse and field experiments, the percentage of plants with any symptom type on the last rating date was calculated for each plot. Percentages of plants with systemic mosaic or limited symptoms were also determined and used to calculate mean plot disease severity scores using a simplified rating system (asymptomatic plant, 0; plant with limited symptoms, 1; and plant with systemic mosaic symptoms, 2) (Jones et al. 2007). Areas under the disease-progress curve (AUDPC) scores were determined using the mean plot disease score at each rating date (Shaner and Finney 1977). For growth chamber experiments, the percentage of TBIA positive plants was calculated for each genotype. Analysis of variance (ANOVA) and means separations were calculated using SAS PROC GLM (SAS Version 9.1; SAS Institute, Cary, NC). Spearman rank correlations of the genotypes were calculated using SAS PROC CORR.

Results

Development of near isogenic lines

Lines carrying *Wsm1, Wsm2,* or *Wsm3* were developed from BC_1-F_1 progeny of the backcross of Pa405 (donor) and Oh28 (recurrent parent). Families having markers linked to the *Wsm1* locus segregated 1:1 for systemic mosaic (Fig. 1a) and symptomless phenotypes after inoculation with WSMV. Inoculated plants from families with markers linked to *Wsm2* segregated for plants with systemic mosaic and delayed, limited symptoms (DL; Fig. 1b). DL symptoms developed later than systemic mosaic symptoms and were limited to sectors defined by leaf veins. Symptoms spread longitudinally, following leaf veins, but did not spread laterally, creating streaks of mosaic symptoms bordered by healthy tissue. Broader, but still restricted, streaks of mosaic symptoms developed on emerging leaves and eventually coalesced into a systemic

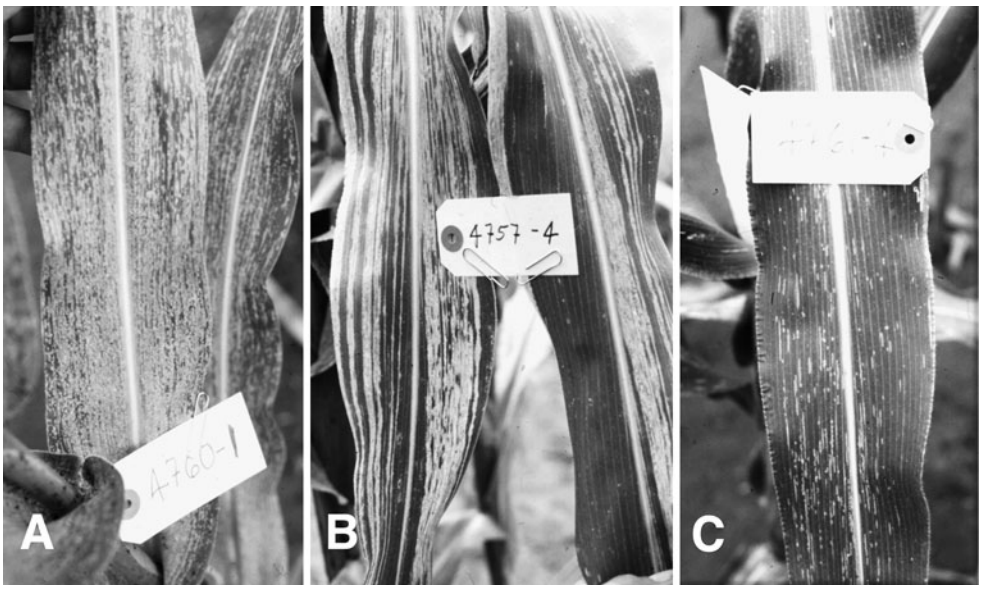

Fig. 1 Symptoms associated with WSMV infection of maize. **a** Systemic mosaic on Oh28. **b** Delayed limited (DL) symptoms on a *Wsm2* NIL × Oh28 F_1 plant. **c** Delayed, dispersed spots and rings (DDSR) on a *Wsm3* × Oh28 F_1 plant

mosaic. Families with markers linked to *Wsm3* segregated for plants that developed a systemic mosaic and delayed, dispersed spots, and rings (DDSR; Fig. 1c). DDSR symptoms began as isolated chlorotic flecks that developed along leaf veins into green islands with chlorotic borders. DDSR symptoms spread and eventually coalesced into a systemic mosaic at later scoring dates. Homozygous NIL were selected from resistant BC_6F_2 plants using linked markers. The *Wsm1* NIL ($Oh28^{SS/RR/SS}$; see Table 1 for a summary of genotype annotations) carries a ca. 29 cM region from the short arm of Pa405 chromosome 6 bound by the markers umc85 and umc1887. The *Wsm2* NIL ($Oh28^{RR/SS/SS}$) carries a ca. 19 cM region from near the centromere of Pa405 chromosome 3 bound by the markers bnlg1063 and umc2263. The *Wsm3* NIL ($Oh28^{SS/SS/RR}$) carries a ca. 25 cM region of Pa405 from chromosome 10 bound by the markers umc1506 and umc1084. Marker-assisted selection was used to identify lines homozygous for multiple *Wsm* genes, and heterozygous lines were created by crossing NIL to the susceptible inbred Oh28. The $Oh28^{RR/SS/RR}$ NIL had very poor germination, resulting in insufficient stands to be included in all experiments.

The dates of field and greenhouse experiments to characterize the responses of the germplasm to WSMV, MDMV-OH, and SCMV-OH are outlined in Table 2. The resistant parent Pa405 and susceptible parent Oh28 were included as controls in each experimental replicate. In all cases, Oh28 plants developed systemic mosaic symptoms, with infection ranging from 72 to 100% (Table 2). Pa405 was strongly resistant to all three viruses in the field and greenhouse: a single Pa405 plant was scored as symptomatic after inoculation with SCMV-OH in the greenhouse in 2009, and two field-grown Pa405 plants showed transient symptoms after inoculation with WSMV in 2005, but otherwise no symptoms were observed in the resistant parent. It is likely that symptoms in Pa405 were associated with a veinal chlorosis this line occasionally exhibits in stress environments rather than virus infection. The consistently high percentage infection of Oh28 (Table 1) indicated the multiple inoculation protocols were effective for virus transmission. For all three viruses in all NIL, symptoms remained the same or increased in intensity with time, and no recovery phenotype was seen (data not shown).

Field and greenhouse inoculation of maize lines with WSMV

The data presented are for field inoculation of maize lines with WSMV in 2009 (Table 3); however, similar results were obtained in experiments with single replications in 2004, 2005, and 2006. There was no difference in mean % infection among replications in 2009 (data not shown). About 80% of Oh28 plants, and a very few plants carrying a single *Wsm2* or *Wsm3* allele from Pa405 became symptomatic (Table 3). No other lines became symptomatic under field conditions. AUDPC scores were not calculated for this experiment due to the very low infection rates.

In the greenhouse, plants that carried one or two *Wsm1* ($Oh28^{SS/RR/SS}$ and $Oh28^{SS/RS/SS}$) alleles did not develop symptoms (Table 3). A small percentage of plants with *Wsm2* alleles ($Oh28^{RR/SS/SS}$ and $Oh28^{RS/SS/SS}$) developed symptoms, but these symptoms were limited and developed later than in Oh28, as indicated by AUDPC scores similar

Table 3 Response of near isogenic lines to inoculation with *Wheat streak mosaic virus* (WSMV) in the field and greenhouse

Genotype[a]	Field	Greenhouse	
	% Inf.[b]	% Inf.	AUDPC
Oh28	80.2a	100.0a	24.0a
Pa405	0.0b	0.0f	0.0e
$Oh28^{SS/RR/SS}$	0.0b	0.0f	0.0e
$Oh28^{RR/SS/SS}$	0.0b	4.0ef	0.5de
$Oh28^{SS/SS/RR}$	0.0b	76.9c	7.5c
$Oh28^{SS/RS/SS}$	0.4b	0.0f	0.0e
$Oh28^{RS/SS/SS}$	0.0b	8.8de	1.0de
$Oh28^{SS/SS/RS}$	1.0b	88.9b	14.9b
$Oh28^{RR/RR/SS}$	0.0b	0.2f	0.1e
$Oh28^{SS/RR/RR}$	0.0	2.0ef	0.1e
$Oh28^{RS/RS/SS}$	0.0b	0.0f	0.0e
$Oh28^{SS/RS/RS}$	0.0b	0.0f	0.0e
$Oh28^{RS/SS/RS}$	0.0b	4.5ef	0.3e

AUDPC area under the disease progress curve

[a] Genotypes at the three WSMV resistance loci are as outlined in Table 1

[b] The percentage of inoculated plants with any symptom at the last rating date. Field data are the mean of three replications done in 2009, and greenhouse data are the mean of nine replications in three experiments as outlined in Table 2. Values followed by the same letter within a column are not significantly different ($p > 0.05$)

to Pa405. Lines with *Wsm3* alleles ($Oh28^{SS/SS/RR}$ and $Oh28^{SS/SS/RS}$) had low infection rates, and initial symptoms were DDSR resulting in AUDPC scores lower than Oh28. Similar percentages of $Oh28^{SS/SS/RR}$ and $Oh28^{SS/SS/RS}$ plants developed symptoms, but AUDPC scores were higher for the heterozygous plants, indicating a dosage effect. The high level of WSMV resistance in plants with *Wsm1* and *Wsm2* alleles prevented identification of any effects of combining alleles from different genes. The percentages of symptomatic plants and AUDPC scores differed among the three greenhouse experiments, with a significant interaction detected between year and line.

Field and greenhouse inoculation of maize lines with the MDMV-OH

In experiments to test the response of the NIL to MDMV in the field, mean infection and AUDPC were similar among replications within years. AUDPC scores varied among years, with a significant genotype × year interaction detected for the $Oh28^{RS/RS/SS}$ line in 2007 and 2009; however, the relative rankings of AUDPC scores were similar among years ($p < 0.0001$). Plants carrying *Wsm1* alleles ($Oh28^{SS/RR/SS}$ and $Oh28^{SS/RS/SS}$) had lower incidence and severity of MDMV infection than Oh28 (Table 4). More $Oh28^{SS/RR/SS}$ plants developed symptoms than Pa405 plants, but symptoms were limited and delayed in appearance as indicated by the similar AUDPC scores. MDMV infection and severity in $Oh28^{SS/RS/SS}$ plants was intermediate between Oh28 and $Oh28^{SS/RR/SS}$, indicating a dosage effect. Plants with *Wsm2* alleles ($Oh28^{RR/SS/SS}$ and $Oh28^{RS/SS/SS}$) or one *Wsm3* allele ($Oh28^{SS/SS/RS}$) were similar to the susceptible parent. Symptom incidence and severity was intermediate for plants with two *Wsm3* alleles ($Oh28^{SS/SS/RR}$). $Oh28^{RR/RR/SS}$ and $Oh28^{RS/RS/SS}$ had lower incidence than $Oh28^{SS/RR/SS}$ and $Oh28^{SS/RS/SS}$, respectively, primarily due to a lower frequency of DL symptoms (data not shown). $Oh28^{SS/RS/RS}$ plants were substantially more resistant than $Oh28^{SS/RS/SS}$ plants, with disease incidence and severity in $Oh28^{SS/RS/RS}$ plants being similar to Pa405. This effect could not be seen in the homozygous lines, as both $Oh28^{SS/RR/SS}$ and $Oh28^{SS/RR/RR}$ plants had AUDPC scores similar to the resistant parent.

Relative to field experiments, there was higher incidence of both systemic and DL symptoms in the greenhouse for all but completely susceptible NIL (Table 4). In the greenhouse, mean infection and AUDPC scores in MDMV-OH inoculated plants varied significantly among replications in 2004, but not in 2006 or 2010 (data not shown). Infection and AUDPC also varied among years, with a significant genotype × year interaction detected (data not shown). However, the relative ranking of lines did not vary among years in greenhouse experiments, or between greenhouse and field experiments ($p < 0.05$). The infection rate for the $Oh28^{SS/RS/RS}$ was lower than that for $Oh28^{SS/RR/RR}$ line in the field, but this effect was reversed in the greenhouse. However, infection development was delayed and symptoms were limited for both lines as indicated by low AUDPC scores.

Field and greenhouse inoculation of maize lines with the SCMV-OH

Field experiments to evaluate resistance to SCMV-OH had significant differences among years for percent infection and AUDPC, with each being lowest in 2008 and highest in 2007 (data not shown). Symptom incidence in the susceptible parent Oh28 was also lowest (76%) in 2008 and highest (97%) in 2007 (Table 2). Significant genotype by environment and genotype by year interactions were observed (data not shown); however, relative AUDPC scores rankings for genotypes were significantly correlated between years in the greenhouse ($p < 0.1$) and field ($p < 0.01$). In the field, lines with alleles from a single *Wsm* locus had symptom incidence similar to Oh28, but had slightly lower AUDPC scores than the susceptible parent (Table 5). $Oh28^{RR/RR/SS}$ plants had lower symptom incidence and severity than plants with alleles from a single *Wsm* locus, and disease severity was also reduced in

Table 4 Response of near isogenic lines to inoculation with the Ohio isolate of *Maize dwarf mosaic virus* (MDMV-OH)

Genotype[a]	Field		Greenhouse	
	% Inf.[b]	AUDPC	% Inf.	AUDPC
Oh28	92.6a	44.0a	100.0a	26.2ab
Pa405	0.0f	0.0e	0.0e	0.0g
$Oh28^{SS/RR/SS}$	16.4e	2.7e	35.6c	1.9fg
$Oh28^{RR/SS/SS}$	91.1ab	45.0a	83.2b	21.3c
$Oh28^{SS/SS/RR}$	83.9b	36.1b	97.1a	25.8ab
$Oh28^{SS/RS/SS}$	72.4c	21.2c	98.1a	16.3d
$Oh28^{RS/SS/SS}$	90.7ab	43.8a	99.5a	27.0a
$Oh28^{SS/SS/RS}$	90.7ab	41.6a	97.2a	26.3ab
$Oh28^{RR/RR/SS}$	3.3f	1.3e	19.7d	2.3fg
$Oh28^{SS/RR/RR}$	13.2e	3.2e	26.6cd	3.1f
$Oh28^{RR/SS/RR}$			98.2a	23.1bc
$Oh28^{RS/RS/SS}$	53.6d	12.7d	76.0b	6.9e
$Oh28^{SS/RS/RS}$	3.6f	0.6e	79.5b	8.0e
$Oh28^{RS/SS/RS}$	92.6a	41.3a	100.0a	25.6ab

AUDPC area under the disease progress curve

[a] Genotypes at the three WSMV resistance loci are as outlined in Table 1

[b] The percentage of inoculated plants with any symptom at the last rating date. The data presented are the mean of the replications outlined in Table 2. Values followed by the same letter within a column are not significantly different ($p > 0.05$)

Table 5 Response of near isogenic lines to inoculation with the Ohio isolate of *Sugarcane mosaic virus* (SCMV-OH)

Genotype[a]	Field		Greenhouse	
	% Inf.[b]	AUDPC	% Inf	AUDPC
Oh28	84.2ab	40.8a	98.7a	28.5ab
Pa405	0.0g	0.1e	1.5f	0.0e
$Oh28^{SS/RR/SS}$	81.3abc	34.9b	76.5d	9.1cd
$Oh28^{RR/SS/SS}$	77.2bcde	35.6b	96.9a	27.9ab
$Oh28^{SS/SS/RR}$	78.1bcd	36.1b	99.4a	28.6a
$Oh28^{SS/RS/SS}$	80.2abcd	35.4b	99.4a	27.5ab
$Oh28^{RS/SS/SS}$	77.5bcd	34.4b	93.3abc	27.5ab
$Oh28^{SS/SS/RS}$	80.4abcd	36.1b	99.5a	29.3a
$Oh28^{RR/RR/SS}$	48.2f	18.1d	61.9e	6.9d
$Oh28^{SS/RR/RR}$	88.4a	33.2bc	82.7bcd	11.6c
$Oh28^{RR/SS/RR}$			97.0a	26.7ab
$Oh28^{RS/RS/SS}$	75.5cde	30.1c	80.1cd	9.8cd
$Oh28^{SS/RS/RS}$	72.9de	29.9c	93.4abc	25.1b
$Oh28^{RS/SS/RS}$	68.6ef	29.6c	95.6ab	27.9ab

AUDPC area under the disease progress curve

[a] Genotypes at the three WSMV resistance loci are as outlined in Table 1

[b] The percentage of inoculated plants with any symptom at the last rating date. Data presented are the mean of the replications outlined in Table 2. Values followed by the same letter within a column are not significantly different ($p > 0.05$)

$Oh28^{SS/RR/RR}$ plants. The heterozygous line, $Oh28^{RS/RS/SS}$ line had a somewhat lower AUDPC score than $Oh28^{RS/SS/SS}$ or $Oh28^{SS/RS/SS}$.

In the greenhouse, no difference in symptom incidence for plants inoculated with SCMV-OH was observed between experiments done in 2004 and 2006, but there was a small difference in AUDPC between years. Rankings of genotypes were similar between years (data not shown). $Oh28^{SS/RR/SS}$ had lower symptom incidence and severity than Oh28 after inoculation with SCMV-OH, but these

values were higher than in Pa405 (Table 5). Oh28$^{SS/RS/SS}$ was similar to Oh28. Lines with *Wsm2* and *Wsm3* alleles, alone or in combination also had similar symptom incidence and severity to Oh28. However, Oh28$^{RR/RR/SS}$ had somewhat lower symptom incidence than Oh28$^{SS/RR/SS}$, and Oh28$^{RS/RS/SS}$ had substantially lower AUDPC than Oh28$^{SS/RS/SS}$. In contrast, plants with both *Wsm1* and *Wsm3* alleles had similar resistance levels to those with *Wsm1* alleles alone (Oh28$^{SS/RR/RR}$ vs. Oh28$^{SS/RR/SS}$ and Oh28$^{SS/RS/RS}$ vs. Oh28$^{SS/RS/SS}$).

Differential responses of maize lines to inoculation with two MDMV and two SCMV isolates

The responses of selected lines to inoculation with Ohio and European isolates of MDMV and SCMV were examined in a growth chamber. In this experiment, the presence of virus in systemic leaves was determined using TBIA (Table 6), but results for symptom appearance were very similar (data not shown). The susceptible control, Oh28, and line Oh28$^{RR/SS/SS}$ were similarly highly susceptible to all four virus isolates. No infected Pa405 plants were found after inoculation with any of the virus isolates, and Oh28$^{RR/RR/SS}$ had no infection or a low incidence infection that was not different from Pa405. However, the MDMV-OH and MDMV-It isolates differentially infected Oh28$^{SS/RR/SS}$, with MDMV-OH infection being similar to the resistant control and MDMV-It infection being similar to the susceptible control. The SCMV-OH and SCMV-See. isolates also differentially infected Oh28$^{SS/RR/SS}$, with SCMV-See. infection being similar to the resistant control and SCMV-OH infection being similar to the susceptible control. The differential infection of Oh28$^{SS/RR/SS}$ by the four virus isolates is significant ($p < 0.05$, LSD), with MDMV-It and SCMV-OH infecting significantly more plants than MDMV-OH and SCMV-See. In a fourth experiment, done under slightly different conditions, nine of nine Oh28$^{SS/RR/SS}$ plants were TBIA positive for MDMV-It and none of ten Oh28$^{SS/RR/SS}$ became infected with SCMV-See., further confirming the results in Table 6. These results indicate differential infection of the NIL carrying *Wsm1* with isolates of two different potyviruses.

Discussion

Our results indicate the responses of the NIL and their F_1 progeny from a cross with Oh28 to virus inoculation depended on the complement of resistance alleles in the line, the virus species and isolate, and the environment. Pa405 was used as the resistance source for NIL development, because the genes conferring resistance to WSMV and MDMV were previously mapped (McMullen and Louie 1989; McMullen et al. 1994) and because it is recognized as being among the most MDMV- and SCMV-resistant maize inbred lines (Findley et al. 1976; Louie et al. 1990; Jones et al. 2007). Oh28 was chosen as the susceptible parent for NIL development, because previous mapping studies indicated there are no resistance loci in this line (McMullen and Louie 1989; McMullen et al. 1994). Further, F_2 progeny of resistant inbred × Oh28 crosses had higher mean AUDPC scores after MDMV inoculation than crosses using *yM14* or K55 as the susceptible parent, indicating the line has a very weak background for MDMV resistance (Jones et al. 2007). The high degree of Oh28 susceptibility was confirmed by the nearly 100% infection of Oh28 with all three viruses under greenhouse conditions, and very high rates of infection in the field.

The responses of the NIL to WSMV inoculation in the field were consistent with *Wsm1*, *Wsm2*, and *Wsm3* acting as dominant genes for WSMV resistance (McMullen et al. 1994), as no or a very few symptoms developed in plants

Table 6 Response of near isogenic lines grown in growth chambers to inoculation with European and Ohio isolates of *Maize dwarf mosaic virus* and *Sugarcane mosaic virus*

Entry[a]	MDMV-OH[b]	MDMV-It	SCMV-OH	SCMV-See.
Oh28	100a[c, d]	97a	92a	100a
Pa405	0b	0b	0b	0b
Oh28$^{SS/RR/SS}$	0b	62a	60a	0b
Oh28$^{RR/SS/SS}$	100a	100a	94a	100a
Oh28$^{RR/RR/SS}$	0b	12b	12b	0b

[a] Genotypes at the three WSMV resistance loci are as outlined in Table 1

[b] Plants were inoculated with MDMV isolates from Ohio (MDMV-OH) or Italy (MDMV-It), and SCMV isolates from Ohio (SCMV-OH) or Germany (SCMV-See.)

[c] Plants were assayed for the presence of virus using a tissue blot immunoassay. Data are the mean percentage of infected plants for three independent experiments

[d] Values within a column followed by the same letter are not significantly different (LSD, $p < 0.05$)

carrying a single resistance allele. The relative strength the three genes could be detected in the greenhouse, where no symptoms developed on plants of lines carrying *Wsm1* ($Oh28^{SS/RR/SS}$ and $Oh28^{SS/RS/SS}$), delayed and limited symptoms occurred on a few plants of lines carrying *Wsm2* ($Oh28^{RR/SS/SS}$ and $Oh28^{RS/SS/SS}$), and DDSR symptoms appeared with shorter delay on a high percentage of plants with *Wsm3* alleles ($Oh28^{SS/SS/RR}$ and $Oh28^{SS/SS/RS}$) (Table 3). DL and DDSR symptoms were previously noted in WSMV-inoculated plants from Pa405 × Oh28 F_2 populations (McMullen et al. 1994). These symptoms also are seen in F_2 plants in the field usually developing more than 30 dpi (Redinbaugh et al. unpublished results). Both *Wsm1* and *Wsm2* acted as dominant genes in the greenhouse, but a dosage effect was seen for *Wsm3* alleles in the greenhouse with a higher incidence and severity of symptoms in heterozygous plants. Interactions among the three genes could not be detected in WSMV-inoculated plants due to the high resistance of plants carrying *Wsm1* and *Wsm2*. On the other hand, it is now possible using NIL carrying individual *Wsm* genes to examine the mechanisms of virus resistance for each gene. For example, the spread of a GFP labeled WSMV (Tatineni et al. 2011) could be used to identify cells and tissues expressing the resistance response, or differential responses of the transcriptome after virus inoculation could be tracked.

Genes at all three loci affected the response of the NIL to MDMV. The *Mdm1* gene for MDMV resistance co-localizes with *Wsm1* in Pa405 (McMullen and Louie 1989; McMullen et al. 1994; Redinbaugh and Pratt 2008), and NIL carrying two *Mdm1* alleles ($Oh28^{SS/RR/SS}$) were highly resistant to MDMV-OH in the field and greenhouse, as expected. Although they were highly resistant to MDMV-OH, some $Oh28^{SS/RR/SS}$ plants developed delayed and limited symptoms. Plants with one *Mdm1* allele ($Oh28^{SS/RS/SS}$) developed limited symptoms, and resistance was intermediate between the resistant and susceptible parents (Table 4), indicating an allele dosage effect. Limited symptom development in MDMV-inoculated segregating Pa405-based populations was noted previously and proposed to be linked to the susceptible parent genotype, genotype by environment interactions or developmental factors (McMullen and Louie 1989; Mikel et al. 1984; Roane et al. 1983). Although alleles from Pa405 on chromosomes 3 and 10 did not, by themselves or together, provide any resistance to MDMV, lines that had *Wsm2* or *Wsm3* in combination with *Mdm1* had lower symptom incidence ($Oh28^{RR/RR/SS}$ vs. $Oh28^{SS/RR/SS}$), or lower incidence and severity ($Oh28^{RS/RS/SS}$ and $Oh28^{SS/RS/RS}$) than lines with *Mdm1* alone. This epistatic interaction of *Wsm2* and *Wsm3* with *Wsm1*/*Mdm1* could not be detected in previous studies, even though 'minor genes' affecting MDMV resistance were previously proposed (Louie et al. 1991; Findley et al. 1984), and a locus on chromosome 10 for MDMV resistance was detected in bulked segregant analysis (Jones et al. 2007). The epistatic enhancement of *Mdm1* suggests that *Wsm2* and *Wsm3* (or linked loci) may act together with *Mdm1* to enhance resistance, as has been proposed for at least two of the three *RTM* genes conferring resistance to *Tobacco etch virus* in *Arabidopsis* (Cosson et al. 2010).

Pa405 resistance to SCMV-OH resistance was previously associated with several genes (Mikel et al. 1981), at least one of which is linked to MDMV resistance on chromosome 6 (Mikel et al. 1981; Louie et al. 1991). Our results show that the *Wsm1* (or closely linked) locus from Pa405 ($Oh28^{SS/RR/SS}$) is not sufficient for resistance to SCMV-OH in the field. However, addition of *Wsm2* alleles in $Oh28^{RR/RR/SS}$ lowered both symptom incidence and severity. This is similar to the European inbred line, FAP1360A, for which QTL on both chromosomes 3 (*Scmv2*) and 6 (*Scmv1*) are required for resistance (Lübberstedt et al. 2006; Melchinger et al. 1998; Xia et al. 1999; Xing et al. 2006). In the greenhouse, the *Wsm1* locus from Pa405 provided more resistance than in the field. Here, addition of *Wsm2* or *Wsm3* alleles did not significantly improve resistance, suggesting an environmental effect on the activity of these two genes.

Our results indicate that genes in all the three chromosomal regions play a role in WSMV and MDMV-OH resistance. The requirement for *Wsm2* alleles for SCMV-OH resistance explains, at least in part, the previously noted increased aggression of SCMV-OH relative to MDMV-OH (Jones et al. 2007). While no epistatic or other effect of *Wsm3* alleles on SCMV-OH resistance was found in this study, it might be possible to detect such an effect by comparing an NIL with all three resistance loci ($Oh28^{RR/RR/RR}$) and the $Oh28^{RR/RR/SS}$ NIL. However, for reasons that are not currently clear, we have not yet been able to identify plants homozygous for the three resistance genes in F_2 plants derived from a cross of $Oh28^{RR/RR/SS}$ and $Oh28^{SS/SS/RR}$. It is interesting that the effects *Wsm2* and *Wsm3* alleles could only be observed directly in WSMV inoculated plants. For the other two viruses, the resistance activity of these genes could only be detected in the presence of *Wsm1*. If the hypothesis that *Wsm1*, *Wsm2,* and *Wsm3* act pleiotropically to give resistance to multiple viruses is correct, then nature of the resistance conferred by the individual genes can be characterized using WSMV.

There was an interaction between the maize genotype and the infectivity of virus species and isolates. WSMV clearly had the least ability to infect the NIL examined in this study, and the MDMV-OH was not as aggressive as the SCMV-OH. WSMV is in the virus genus *Tritimovirus* within family Potyviridae and can infect only a few maize lines (Louie 1999; Redinbaugh and Pratt 2008). The ability

of tritimoviruses to infect maize is generally limited; e.g., *Oat necrotic mottle virus* infects the highly virus-susceptible maize inbred (SDp2), but not other maize inbred lines (Rabenstein et al. 2002). MDMV and SCMV are in the genus *Potyvirus* and have less than 50% sequence identity with WSMV, as is typical of viruses in different genera of the Potyviridae (Adams et al. 2005). Differences in the abilities of potyvirus isolates to infect maize are primarily related to the virus species (MDMV vs. SCMV), but Uzarowska et al. (2009) reported that MDMV-It infected the $F7^{RR/RR}$ NIL, in contrast to previous studies indicating this line was resistant to MDMV MD (Lübberstedt et al. 2006; Xing et al. 2006). In the Pa405-derived NIL, we also observed differences in the response to inoculation with different isolates of MDMV and SCMV (Table 6). The *Wsm1* NIL ($Oh28^{SS/RR/SS}$) had high resistance to MDMV-OH and SCMV-See., but behaved similarly to Oh28 when inoculated with the MDMV-It or SCMV-OH. The inability of the SCMV-See. isolate to infect $Oh28^{SS/RR/SS}$ indicates the Pa405 alleles at *Wsm1* are stronger than those from FAP1360A, which do not by themselves provide resistance to SCMV-See. In addition, the $Oh^{RR/RR/SS}$ NIL, which is equivalent to $F7^{RR/RR}$, was resistant to the MDMV-It under the growth chamber conditions used for these experiments, suggesting the combination of resistance loci from Pa405 chromosomes 3 and 6 is stronger than the homologous loci from FAP1360A. Differences among inbred lines for MDMV and SCMV resistance have been described, and there are several inbred lines that appear to have stronger resistance to these viruses than Pa405 (Jones et al. 2007). The differential responses of NIL to isolates of two different virus species provide a unique opportunity to identify viral determinants of the resistance response (avirulence genes) in maize.

The differences in the rate of symptom appearance and morphology and the differential expression of resistance in the field and greenhouse indicate that virus resistance is regulated developmentally and environmentally, as well as by the genetics of the host and pathogen. Distinctive DL and DDSR symptoms developed on lines with *Wsm2* and *Wsm3* resistance alleles, and limited symptoms were seen on some lines after inoculation with MDMV or SCMV. Although the limited symptoms generally occurred later in the rating cycle, plants exhibiting these symptoms would eventually develop systemic mosaic symptoms, indicating a developmental breakdown in resistance. Similarly, resistance to *Rice yellow mottle virus* conferred by two QTL associated with reduced viral titer is characterized by delays in symptom appearance that eventually break down (Ioannidou et al. 2000; Ioannidou et al. 2003), and *Rsv4*-based resistance to the potyvirus *Soybean mosaic virus* is characterized by a 'late susceptible' phenotype (Ma et al. 2002). The 'stripes' of DL symptoms in *Wsm2* leaves, with continuous but laterally contained virus distribution, are reminiscent of the primarily longitudinal cell-to-cell spread of SCMV (MDMV-B) in inoculated Pa405 leaves (Lei and Agrios 1986). Relative to Oh28, AUDPC scores for most lines were similar in the field and greenhouse, suggesting limited environmental effects on resistance gene expression (data not shown). However, lines with *Wsm3* inoculated with MDMV or WSMV were more resistant in the field than in the greenhouse. In contrast, lines carrying two alleles of *Wsm1* were more resistant to SCMV in the greenhouse than in the field, suggesting gene- and virus-specific environmental effects on resistance. The environment x genotype interaction may be related to differential expression of resistance genes in under different conditions of light, temperature, etc., or could be related to different developmental patterns for plants grown in the field and greenhouse.

The *Arabidopsis*—potyvirus pathosystem has interesting parallels to the maize—potyvirus system. In both systems, multiple dominant interacting resistance genes are involved. In addition, the resistance genes are effective against multiple potyviruses, and viruses replicate and move locally, but not systemically, in resistant plants. *Arabidopsis* plants carrying the *RTM1*, *RTM2,* and *RTM3* genes are resistant to three potyvirus species: TEV, *Lettuce mosaic virus* (LMV), and *Plum pox virus* (PPV) (Decroocq et al. 2006, 2009). The genomes of these three potyviruses have 50–55% sequence identity, compared with about 70% identity between MDMV and SCMV. Changes in the N-terminus of the LMV and PPV coat protein are associated with breaking resistance (Decroocq et al. 2009), and the TEV coat protein is required for long-distance virus movement (Dolja et al. 1995). The N-terminus of potyviral coat proteins, including those for LMV, PPV, TEV, MDMV, SCMV, and WSMV, has a relatively low level of conservation within and among species, including LMV, PPV, and TEV (Adams et al. 2005). *RTM1* encodes a jaclin repeat protein, which has been reported to confer resistance against bacteria, fungi, and insects (Chisholm et al. 2000), and *RTM2* encodes a small heat-shock protein (Whitham et al. 2000). Expression of both proteins is highly phloem specific (Chisholm et al. 2001). *RTM3* was recently isolated, and encodes a protein with meprin and TRAF-containing domains (Cosson et al. 2010). Although homologs of the *RTM* proteins are present in maize, none of the homologs are in the BAC contigs carrying *Scmv1* and *Scmv2*. In yeast-two-hybrid assays, the *RTM1* protein interacts with itself and *RTM3*, but neither interacts with *RTM2* (Chisholm et al. 2001; Cosson et al. 2010), and none of the three proteins interacts with the PPV coat protein. Although all three *RTM* genes are dominant, a knockout in any of the three *RTM* genes causes *Arabidopsis* to become susceptible to TEV, PPV, and LMV (Decroocq et al. 2006;

Cosson et al. 2010). Although it is not yet possible to create *Wsm*-specific knockouts in maize, the *Wsm* genes can act either independently (for WSMV resistance), epistatically (for MDMV resistance) or in concert (for SCMV resistance). Thus, it appears there may be some important differences in the mechanisms for virus resistance associated with these two pathosystems.

In summary, resistance to potyviruses in maize NIL carrying *Wsm1*, *Wsm2,* and/or *Wsm3* indicated that all three loci provide resistance to WSMV, but that *Wsm1* or closely linked genes are essential for resistance to MDMV and SCMV. Nontheless, addition of alleles of *Wsm2* and *Wsm3* affect resistance to these two viruses. In addition to maize genotype and virus species, resistance was influenced by virus isolate, with two isolates MDMV and two of SCMV having differential ability to infect $Oh28^{SS/RR/SS}$. Virus-specific genotype by environment effects on resistance were also observed, with MDMV and WSMV resistance being stronger in the field environment and SCMV resistance being stronger in greenhouse environments. The NIL developed for this study provide research tools for further characterization of maize resistance to this important group of viruses, as well as potential well-defined sources of potyvirus resistance for breeding specialty maize (e.g., sweet corn and popcorn).

Acknowledgments We thank Jane C. Todd and Jean Vacha for excellent technical assistance, and Tom Helmuth, Chelsea Miko, Morgan O'Rourke, Nick Graf and Kristen Willie for assistance with field and greenhouse work. We also thank Stephan Hentrup (Danish Institute of Agricultural Sciences) and Thomas Lübberstedt (Iowa State University) for assistance with obtaining the MDMV-It and SCMV-See isolates, and for helpful discussions.

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