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Temperature-Dependent *Wsm1* and *Wsm2* Gene-Specific Blockage of Viral Long-Distance Transport Provides Resistance to *Wheat streak mosaic virus* and *Triticum mosaic virus* in Wheat

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Wheat streak mosaic virus (WSMV) and *Triticum mosaic virus* (TriMV) are economically important viral pathogens of wheat. Wheat cvs. Mace, carrying the *Wsm1* gene, is resistant to WSMV and TriMV, and Snowmass, with *Wsm2*, is resistant to WSMV. Viral resistance in both cultivars is temperature sensitive and is effective at 18°C or below but not at higher temperatures. The underlying mechanisms of viral resistance of *Wsm1* and *Wsm2*, nonallelic single dominant genes, are not known. In this study, we found that fluorescent protein–tagged WSMV and TriMV elicited foci that were approximately similar in number and size at 18 and 24°C, on inoculated leaves of resistant and susceptible wheat cultivars. These data suggest that resistant wheat cultivars at 18°C facilitated efficient cell-to-cell movement. Additionally, WSMV and TriMV efficiently replicated in inoculated leaves of resistant wheat cultivars at 18°C but failed to establish systemic infection, suggesting that *Wsm1*- and *Wsm2*-mediated resistance debilitated viral long-distance transport. Furthermore, we found that neither virus was able to enter the leaf sheaths of inoculated leaves or crowns of resistant wheat cultivars at 18°C but both were able to do so at 24°C. Thus, wheat cvs. Mace and Snowmass provide resistance at the long-distance movement stage by specifically blocking virus entry into the vasculature. Taken together, these data suggest that both *Wsm1* and *Wsm2* genes similarly confer virus resistance by temperature-dependent impairment of viral long-distance movement.

Viruses contain a relatively small genetic coding capacity and, hence, must successfully interact with plant machinery for replication, movement, and spread. In the process of these interactions, sometimes viruses induce disease in plants. In turn, plants have developed diverse mechanisms to combat pathogens, including viruses (Mandadi and Scholthof 2013). One of several

mechanisms employed by plants to defend against viral infections is dominant resistance (*R*) genes encoding nucleotide binding site leucine-rich repeat proteins (NB-LRR) (de Ronde et al. 2014; Fraser 1990). The *R* gene–mediated resistance operates on the basis of the gene-for-gene model, in which a host *R* gene–encoded protein will recognize a pathogen-derived avirulence factor (Jones and Dangl 2006). The *R* gene response in the majority of pathosystems results in expression of a hypersensitive response (HR) or extreme resistance (ER) (Bendahmane et al. 1999; Hajimorad et al. 2005, 2006; Moffett 2009). In both HR and ER responses, pathogens are restricted to the site of entry, thus preventing systemic spread. Additionally, several unusual *R* genes differ from the classical NB-LRR class *R* gene–mediated HR or ER mode of action. The nonconventional *R* genes include *RTM1*, *RTM2*, and *RTM3* genes in *Arabidopsis* against *Tobacco etch virus* (TEV) (Chisholm et al. 2001; Cosson et al. 2010; Whitham et al. 2000), *Scmv2* in maize against *Sugarcane mosaic virus* (Ingvardsen et al. 2010), *Rsv4* in soybean against *Soybean mosaic virus* (SMV) (Saghai Maroof et al. 2010), and *Tm-1* in tomato against *Tomato mosaic virus* (ToMV) (Ishibashi et al. 2007). The above *R* genes provide resistance by restricting viral replication or cell-to-cell and long-distance movement or a combination thereof (Chisholm et al. 2001; Kang et al. 2005a; Khatabi et al. 2012; Mahajan et al. 1998).

Candidate gene approaches have identified a large number of natural recessive *R* genes against several plant viruses (Revers and Nicaise 2014; Truniger and Aranda 2009; Wang and Krishnaswamy 2012). Most of these recessive genes encode the eukaryotic translation initiation factor 4E (eIF4E) or its isomers, with mutation of a few amino acids that prevent physical interaction with the virus-encoded proteins without being lethal to plants. Though recessive resistance appears to be more frequent for potyviruses, it has also been observed for other viruses such as bymo-, cucumo-, ipomo-, sobemo-, carmo-, and waikiviruses (Wang and Krishnaswamy 2012). In potyviruses, interactions between host translation initiation factors and virus-encoded VPg are crucial for infection of plants (Kang et al 2005b; Wittmann et al. 1997; Yeam et al. 2007). A few amino acid mutations within eIF4E and eIF(iso)4E provided resistance to potyvirus infection in a range of plants, primarily due to debilitating interactions between translation initiation factors and VPg (Revers and Nicaise 2014; Wang and Krishnaswamy 2012; Wittmann et al. 1997).

Successful infection of a plant by a virus requires a series of interactions between host and viral factors for replication,

suppression of host defense, and movement (Harries and Ding 2011; Heinlein 2015; Nelson and Citovsky 2005; Voinnet et al. 1999). Systemic spread of viruses throughout plants occurs mainly through successive cycles of cell-to-cell and long-distance movement. Virus movement in plants is an active process mediated by virus-encoded movement proteins through interactions with host factors (Benitez-Alfonso et al. 2010; Harries and Ding 2011; Waigmann et al. 2004; Wang et al. 1998; Xoconostle-Cázares et al. 2000). Interrupting these interactions by silencing or mutation of host factors has been postulated for recessively inherited 'passive resistance' in plants (Fraser 1990).

Wheat streak mosaic virus (WSMV) and *Triticum mosaic virus* (TriMV) are the type species of genera *Tritimovirus* (Stenger et al. 1998) and *Poacevirus* (Fellers et al. 2009; Tatineni et al. 2009), respectively, in the family *Potyviridae*. Both viruses are transmitted mechanically by rub-inoculation at approximately 100% and are efficiently transmitted by the wheat curl mite *Aceria tosichella* Keifer (McMechan et al. 2014; Seifers et al. 2009; Slykhuis 1955). WSMV is an economically important wheat virus in the Great Plains region of the United States, with estimated annual yield losses of 2 to 5% with 100% losses in localized infections (Brakke 1987; French and Stenger 2004). TriMV, a recently discovered virus, is widespread in the Great Plains region (Burrows et al. 2009; Byamukama et al. 2013; Seifers et al. 2008). The impact of TriMV on yield loss is not known; however, WSMV and TriMV interact synergistically in coinfected wheat, causing increased disease severity and yield losses (Byamukama et al. 2012, 2014; Tatineni et al. 2010). WSMV and TriMV virions contain single 9.4- and 10.3-kilobase (kb) genomic RNA, respectively, encoding a single open reading frame (ORF) of about 350-kDa polyproteins. The polyproteins of both WSMV and TriMV are likely processed into mature proteins by three virus-encoded proteinases, P1, HC-Pro, and NIa-Pro. The P1 proteins of WSMV and TriMV have been identified as suppressors of RNA silencing and enhancers of pathogenicity (Tatineni et al. 2012; Young et al. 2012). HC-Pro of WSMV is dispensable for systemic infection of wheat but is required for mite transmission (Stenger et al. 2005a and b, 2006). Independent of its function for virion production, WSMV coat protein (CP) is identified as a multifunctional protein required for cell-to-cell movement and is a long-distance transport determinant for extension of virus host range (Tatineni and French 2014; Tatineni et al. 2011b, 2014). Additionally, P3N-PIPO is also implicated in cell-to-cell movement of WSMV (Choi et al. 2005).

In wheat, genetic resistance has been used for management of viral diseases by deployment of nonallelic *Wsm1* or *Wsm2* genes (Graybosch et al. 2009; Lu et al. 2011). *Wsm1*, a single dominant *R* gene present in wheat cultivar Mace, was originally transferred from intermediate wheatgrass [*Thinopyrum intermedium* (Host) Barkworth & D. R. Dewey] (Graybosch et al. 2009). *Wsm2*, a single dominant *R* gene of unknown origin, was originally identified in wheat germplasm line CO960293-2 (Haley et al. 2002) and has been incorporated into wheat cvs. Snowmass (Haley et al. 2011) and RonL (Seifers et al. 2007). Wheat cv. Mace is genetically near homogeneous for virus resistance, while 'Snowmass' is heterogeneous in its reaction to WSMV, as approximately 20% of the plants remain susceptible to WSMV at 18°C (Haley et al. 2011). Both *Wsm1* and *Wsm2* are temperature-sensitive genes conferring high-levels of resistance to WSMV and TriMV (*Wsm1*) and WSMV (*Wsm2*), at or below 18°C but are ineffective at higher temperatures (Graybosch et al. 2009; Haley et a. 2011; Seifers et al. 1995, 2007; Tatineni et al. 2010). Although the molecular basis for dominant genetic resistance is understood for a few virus-host systems, none include cereals (de Ronde et al. 2014; Kang et al. 2005a). The mechanisms of temperature-dependent virus resistance

of the *Wsm1* and *Wsm2* genes in wheat cvs. Mace and Snowmass, respectively, are not known.

Additionally, resistance to WSMV is also reported in maize inbred line Pa405 (McMullen et al. 1994). WSMV resistance in maize is controlled by *Wsm1*, *Wsm2*, and *Wsm3* dominant genes located on chromosome 6, 3, and 10, respectively (Jones et al. 2011; McMullen et al. 1994). WSMV-resistant genes in wheat and maize were named as per the convention of naming host genes based on phenotype. However, there is no evidence to suggest that WSMV-resistant genes in wheat and maize are related.

Previously, fluorescent protein–tagged WSMV and TriMV were used to monitor virus movement in wheat and to identify viral determinants involved in cell-to-cell and long-distance movement (Tatineni et al. 2011a and b, 2014, 2015; Tatineni and French 2014). In this study, red fluorescent protein (RFP)-tagged WSMV and green fluorescent protein (GFP)-tagged TriMV were used to explore the resistance mechanisms of *Wsm1* and *Wsm2* genes in wheat cvs. Mace and Snowmass, respectively. At 18°C, both WSMV and TriMV efficiently replicated and moved cell-to-cell in inoculated leaves of resistant wheat cultivars but the long-distance transport was debilitated. Additionally, the failure of long-distance movement of WSMV and TriMV in resistant wheat cultivars was found to be due to inability of these viruses to enter the vasculature. Taken together, these data suggest that *Wsm1* and *Wsm2* gene–based resistance in wheat cultivars is due to temperature-dependent impairment of viral long-distance movement with no significant effect on virus replication and cell-to-cell movement.

RESULTS

Development of RFP-tagged WSMV.

Availability of fluorescent protein–tagged viruses facilitates examination of viral resistance mechanisms of wheat cvs. Mace and Snowmass. Recently, a GFP-tagged TriMV was developed with stable and efficient expression of cycle 3 GFP (Fukuda et al. 2000) in local and systemically infected wheat leaves (Fig. 1) (Tatineni et al. 2015). Previously, the cycle 3 GFP ORF fused to a heptapeptide NIa-Pro cleavage site located between the 6K1/CI cistrons in WSMV was efficiently expressed as aggregate-like fluorescent structures, which facilitated efficient tracking of the virus in wheat (Tatineni and French 2014; Tatineni et al. 2011a, 2014). In the present study, an RFP-tagged WSMV was developed by fusing an RFP ORF (Campbell et al. 2002) to a heptapeptide cleavage site located between the 6K1/CI cistrons to obtain WSMV-RFP-6K1/CI(7aa) (Fig. 1A). The RFP is released by a cleavage of P1 in *cis* at the C-terminus of P1 between tyrosine and glycine residues and a cleavage at the C-terminus of RFP in the 6K1/CI heptapeptide cleavage site by NIa-Pro in *trans* between glutamine and serine residues (Fig. 1A).

In vitro transcripts of WSMV-RFP-6K1/CI(7aa) (here after named as WSMV-RFP) and TriMV-GFP-NIb/CP(9aa) (renamed as TriMV-GFP) elicited infection foci on inoculated leaves of susceptible wheat cv. Tomahawk, followed by efficient systemic infection in upper noninoculated leaves at 9 to 14 days post-inoculation (dpi) (Fig. 1B). These results demonstrate that fluorescent protein–tagged WSMV and TriMV can be used to track the viruses in resistant wheat cvs. Mace and Snowmass and ascertain mechanisms of virus resistance in these cultivars.

Wheat cvs. Mace and Snowmass screening for resistance against WSMV and TriMV.

The reaction of 'Mace' and 'Snowmass' to wild-type WSMV or TriMV at 18°C (in a growth chamber) and 24°C (in a greenhouse, at 20°C minimum and 26°C maximum, with a mean temperature of 24°C) was examined by inoculating wheat seedlings at the two-leaf stage, with a 1:20 dilution of crude sap

extracted from WSMV- or TriMV-infected wheat leaves. Susceptible wheat cv. Tomahawk was included as a positive control. The upper noninoculated leaves were examined for symptom development at 14 and 21 dpi. Wheat cvs. Tomahawk and Snowmass inoculated with WSMV or TriMV elicited symptoms by 14 dpi in 100% of plants at 24°C (Table 1). Both WSMV and TriMV caused stunting of plants and elicited severe mosaic and mottling symptoms on 'Tomahawk' and 'Snowmass' plants at 24°C by 21 dpi (Fig. 2A). Additionally, WSMV induced large chlorotic stripes and yellowing of lower noninoculated leaves but TriMV did not. At 24°C, WSMV and TriMV elicited systemic infection in 92 to 95% and 100% of wheat cv. Mace by 14 and 21 dpi, respectively (Table 1). Both WSMV and TriMV induced slight stunting of plants with chlorotic streaks and mosaic and mottling symptoms on wheat cv. Mace by 21 dpi. Also, WSMV but not TriMV induced large

chlorotic stripes and yellowing of a few lower leaves by 21 dpi (Fig. 2A). These results confirmed that wheat cvs. Mace and Snowmass are not resistant to WSMV and TriMV at the 24°C mean temperature present in the greenhouse.

At 18°C, wheat cv. Mace inoculated with WSMV or TriMV elicited systemic symptoms only in 0 to 2% of plants by 14 dpi and in 2 to 3% of plants by 21 dpi (Table 1; Fig. 2A), confirming that 'Mace' is resistant to both WSMV and TriMV at 18°C. TriMV efficiently infected wheat cv. Snowmass in 100% of inoculated plants at 14 dpi, with moderate mosaic and mottling symptoms in upper noninoculated leaves and stunting of plants by 21 dpi (Table 1; Fig. 2A). In contrast, at 18°C, WSMV infected only 34 and 37% of inoculated 'Snowmass' plants by 14 and 21 dpi, respectively (Table 1; Fig. 2A). Though Haley et al. (2011) reported 20% of 'Snowmass' plants were susceptible to WSMV at 18°C, we found a slightly higher percentage (34 to
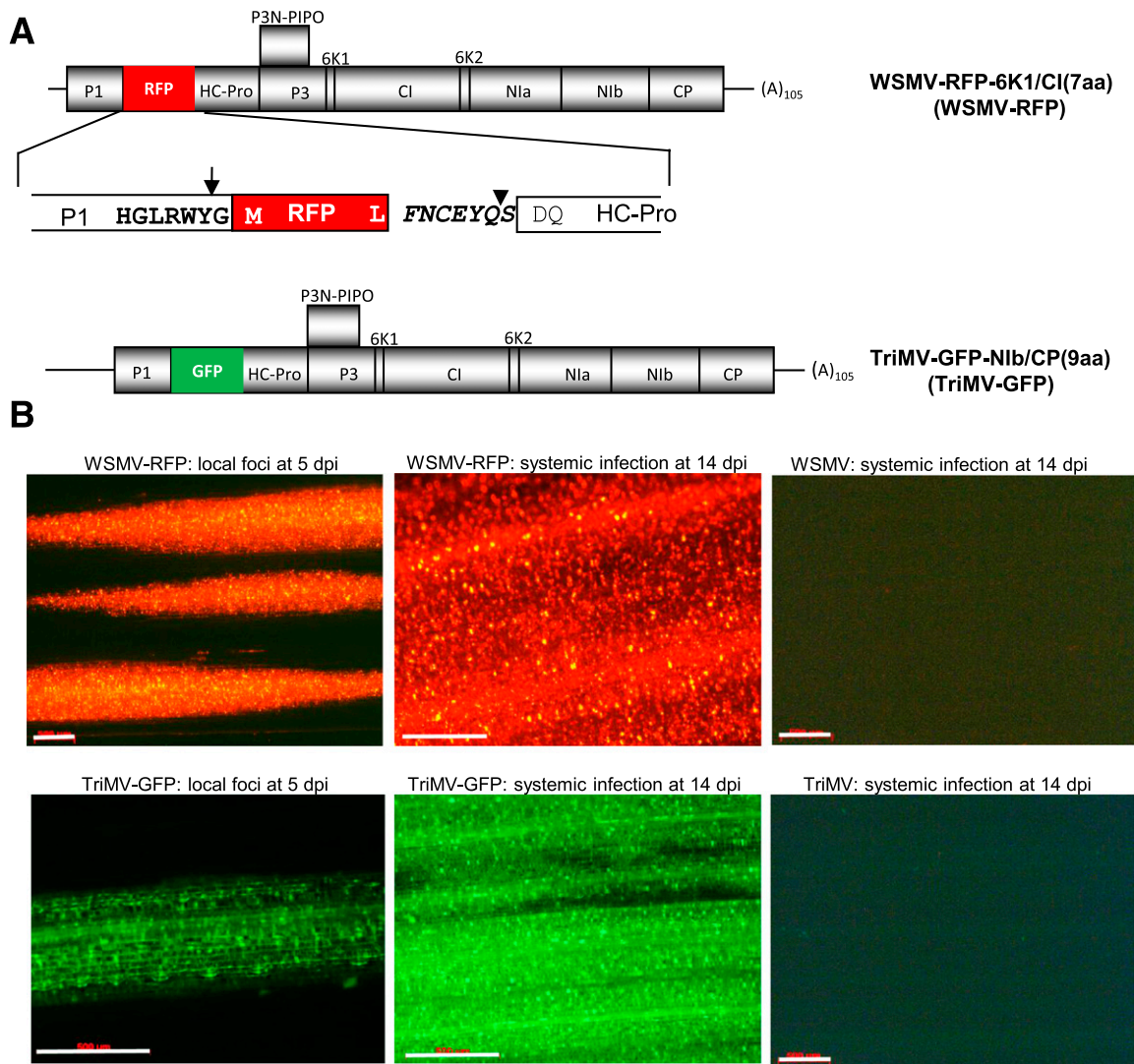

Fig. 1. Visualization of infection elicited by fluorescent protein–tagged *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV). **A,** Schematic diagrams of genome organization of red fluorescent protein–tagged (RFP, indicated by a red rectangle) WSMV (WSMV-RFP-6K1/CI[7aa]) and green fluorescent protein–tagged(GFP, indicated by a green rectangle) *Triticum mosaic virus* (TriMV) (TriMV-GFP-NIb/CP[9aa]). The expanded view below the genome organization map of WSMV-RFP-6K1/CI(7aa) shows the C-terminus of the P1 cistron, followed by the RFP open reading frame fused to the NIa-Pro heptapeptide cleavage site located between 6K1/CI cistrons and the N-terminus of HC-Pro. The first and last amino acids of RFP are indicated. The locations of P1 cleavage in *cis* and NIa-Pro cleavage in *trans* at the engineered NIb/CP heptapeptide cleavage sites are indicated with an arrow and arrowhead, respectively. Construction and characteristic features of TriMV-GFP-NIb/CP(9aa) were described previously by Tatineni et al. (2015). For simplicity, WSMV-RFP-6K1/CI (7aa) and TriMV-GFP-NIb/CP(9aa) are renamed as WSMV-RFP and TriMV-GFP, respectively. **B,** Visualization of local infection foci on inoculated leaves at 5 days postinoculation (dpi) and systemic infection in upper noninoculated leaves at 14 dpi elicited by in vitro transcripts of WSMV-RFP and TriMV-GFP on wheat cv. Tomahawk. Inoculated leaves were observed under a Stereo Discovery V12 fluorescence microscope using a RFP or GFP filter. The brightness of wild-type WSMV- and TriMV-infected leaf images were adjusted to see the leaf background under RFP and GFP filters, respectively. Bars represent 500 µM.

37%) of 'Snowmass' was susceptible to WSMV (Table 1). It is unknown if susceptible plants lack the *Wsm2* gene or if this variable response is due to differential gene expression among plants of this cultivar.

To exclude the possibility of either symptomless infection, low levels of accumulation of viruses in wheat cultivars, or both, total RNA extracted from upper noninoculated leaves of wheat plants at 21 dpi was used as the template for reverse transcription polymerase chain reaction (RT-PCR) amplification with CP-specific primers of WSMV or TriMV. Both WSMV and TriMV infected all wheat cultivars at 24°C, as RT-PCR products were readily obtained (Fig. 2, lanes 4 to 6). No RT-PCR product was amplified from 18°C incubated 'Mace' plants inoculated with WSMV or TriMV (Fig. 2, lane 1). At 18°C, RT-PCR amplification was obtained from 'Snowmass' plants inoculated with TriMV but not from WSMV-inoculated asymptomatic plants (Fig. 2, lane 2). In 'Tomahawk', RT-PCR products were obtained from WSMV or TriMV-inoculated plants at 18°C (Fig. 2, lane 3). These data revealed that, at 18°C, the *Wsm1* gene in 'Mace' confers resistance to WSMV and TriMV and *Wsm2* of 'Snowmass' provides resistance to WSMV but not to TriMV.

Wheat cvs. Mace and Snowmass exhibit similar levels of resistance to fluorescent protein–tagged WSMV and TriMV.

The fluorescent protein–tagged viruses facilitate examination of the involvement of viral replication, cell-to-cell movement, long-distance transport, or a combination thereof in wheat cultivar resistance to WSMV and TriMV. It is important to know whether wheat cultivars elicit similar levels of resistance to fluorescent protein–tagged WSMV and TriMV compared with wild-type viruses. Wheat cvs. Mace, Snowmass, and Tomahawk were inoculated at the two-leaf stage with crude sap of WSMV-RFP, TriMV-GFP, or both at a 1:20 dilution. The upper non-inoculated leaves of wheat cultivars were examined for the presence of fluorescent foci in wheat plants inoculated with WSMV-RFP, TriMV-GFP, or both. A wheat plant was considered positive for virus infection if a single fluorescent focus was observed in upper noninoculated leaves.

At 24°C, WSMV-RFP and TriMV-GFP efficiently infected 'Mace' and 'Snowmass' at 81 to 100% by 14 dpi and 100% by 21 dpi, similar to infections on 'Tomahawk', a susceptible wheat cultivar (Table 2). At 18°C, WSMV-RFP and TriMV-GFP infected Mace at 0 to 6% by 14 dpi and 6 to 13% by 21 dpi compared with 100% infection of 'Tomahawk' (Table 2). As expected, TriMV-GFP infected 100% of 'Snowmass' plants by 14 dpi and WSMV-RFP infected 22 and 28% of plants by 14 and 21 dpi, respectively (Table 2). These data suggested that 'Mace' and 'Snowmass' are similarly resistant to wild-type

(Table 1) and fluorescent protein–tagged (Table 2) viruses. Slightly increased infection rate of 'Mace' by WSMV-RFP or TriMV-GFP could be due to more stringent assay with fluorescent protein–tagged viruses, as a single infection focus in upper noninoculated leaves was considered positive for virus infection.

Coinoculation of wheat cvs. Mace and Snowmass with WSMV and TriMV failed to overcome temperature-dependent resistance.

WSMV and TriMV interact synergistically in coinfected wheat, with increased disease severity and virus concentration (Tatineni et al. 2010). Coinoculation of 'Mace' and 'Snowmass' with WSMV-RFP and TriMV-GFP was evaluated to determine whether coinfection can overcome temperature-dependent resistance of these wheat cultivars. At 18°C, in coinoculated 'Mace', WSMV-RFP and TriMV-GFP systemically infected 0 and 4.5% of the plants by 14 dpi and 9.0% of plants, each, by 21 dpi, respectively, similar to infection by the individual viruses (Table 2). Similarly, no significant increase in 'Snowmass' infection by WSMV-RFP was observed in plants coinoculated with TriMV-GFP (Table 2). These data revealed that coinoculation of 'Mace' and 'Snowmass' with WSMV and TriMV did not alter wheat cultivar resistance.

Wheat cultivar resistance to WSMV and TriMV is not at virus cell-to-cell movement.

The above data demonstrates that expression of fluorescent proteins by WSMV or TriMV had no discernible effect on ability to infect wheat cvs. Tomahawk, Mace, or Snowmass. Hence, fluorescent protein–tagged viruses were used to explore whether resistance of 'Mace' and 'Snowmass' affects cell-to-cell or long-distance movement. Cell-to-cell movement of WSMV and TriMV in wheat cvs. Mace, Snowmass, and Tomahawk was examined, using fluorescent protein–tagged viruses by measuring the extent (size of fluorescent focus) and number of foci on virus-inoculated leaves. Cell-to-cell movement of WSMV and TriMV in 'Tomahawk', a susceptible cultivar, by 5 dpi at the nonpermissible temperature (18°C) was similar to that 3 dpi at permissible (24°C) temperatures (Figs. 3, 4, and 5). This result is probably because replication of WSMV and TriMV are reduced at 18°C. Nevertheless, WSMV-RFP and TriMV-GFP similarly elicited foci on inoculated leaves of 'Mace', 'Snowmass', and 'Tomahawk' by 5 dpi at 18°C and by 3 dpi at 24°C (Fig. 3A to F). Additionally, coinoculation of wheat cultivars with WSMV-RFP and TriMV-GFP elicited foci similar to infection by individual viruses, except that several foci were doubly-infected with WSMV and TriMV (Fig. 3E and F). Cell-to-cell movement of WSMV and TriMV in wheat cultivars was compared within a cultivar between 18 and 24°C

Table 1. Number of wheat plants systemically infected with wild-type *Wheat streak mosaic virus* (WSMV) or *Triticum mosaic virus* (TriMV) in a growth chamber and greenhouse

Wheat cultivar[y]	Virus	Growth chamber (18°C)[z]		Greenhouse (24°C)[z]	
		14 dpi	21 dpi	14 dpi	21 dpi
'Mace'	WSMV	0/38 (0.0%)	1/38 (2.6%)	36/39 (92%)	39/39 (100%)
	TriMV	1/43 (2.3%)	1/43 (2.3%)	36/38 (95%)	38/38 (100%)
	Buffer	0/17 (0.0%)	0/17 (0.0%)	0/16 (0.0%)	0/16 (0.0%)
'Snowmass'	WSMV	12/35 (34%)	13/35 (37%)	40/40 (100%)	40/40 (100%)
	TriMV	36/36 (100%)	36/36 (100%)	38/38 (100%)	38/38 (100%)
	Buffer	0/19 (0.0%)	0/19 (0.0%)	0/18 (0.0%)	0/18 (0.0%)
'Tomahawk'	WSMV	34/34 (100%)	34/34 (100%)	34/34 (100%)	34/34 (100%)
	TriMV	35/35 (100%)	35/35 (100%)	17/17 (100%)	17/17 (100%)
	Buffer	0/17 (0.0%)	0/17 (0.0%)	0/16 (0.0%)	0/16 (0.0%)

[y] Wheat seedlings at the two-leaf stage were mechanically inoculated with WSMV or TriMV at 1:20 dilution in 20 mM sodium phosphate buffer, pH 7.0. The top noninoculated leaves were visually observed for symptom development at 14 and 21 days postinoculation (dpi).

[z] Number of plants systemically infected/number of plants mechanically inoculated, followed by % infection in parentheses.

but not between cultivars, as the wheat cultivars evaluated are differentially susceptible to these viruses.

Number of fluorescent foci on inoculated leaves. The number of foci elicited by WSMV-RFP and TriMV-GFP on inoculated leaves of wheat cultivars was counted in 15 to 20 plants per virus per cultivar. In 'Tomahawk', WSMV and TriMV produced, respectively, 7.9 and 5.9 foci per square centimeter at 18°C, compared with 8.3 and 4.3 foci per square centimeter at 24°C (Fig. 4). In coinoculated 'Tomahawk' plants, WSMV and TriMV elicited foci similarly to single-virus inoculations with 7.4 and 5.5 foci per square centimeter at 18°C, compared with 7.5 and 4.8 foci per square centimeter at 24°C (Fig. 4). In 'Mace', WSMV-RFP and TriMV-GFP elicited 3.9 and 2.6 foci per square centimeter leaf at 18°C, compared with 2.9 and 1.4 foci per square centimeter leaf at 24°C (Fig. 4). In coinoculated 'Mace', WSMV and TriMV elicited 5.9 and 3.4 foci per square centimeter at 18°C, compared with 3.5 and 1.9 foci per square centimeter at 24°C (Fig. 4). These data suggest that WSMV and TriMV elicited similar or slightly more foci on inoculated leaves of 'Mace' at 18°C, compared with those at 24°C. In 'Snowmass', WSMV and TriMV elicited 6.4 and 5.9 foci per square centimeter leaf at 18°C and 6.6 and 5.2 foci per square centimeter at 24°C, respectively
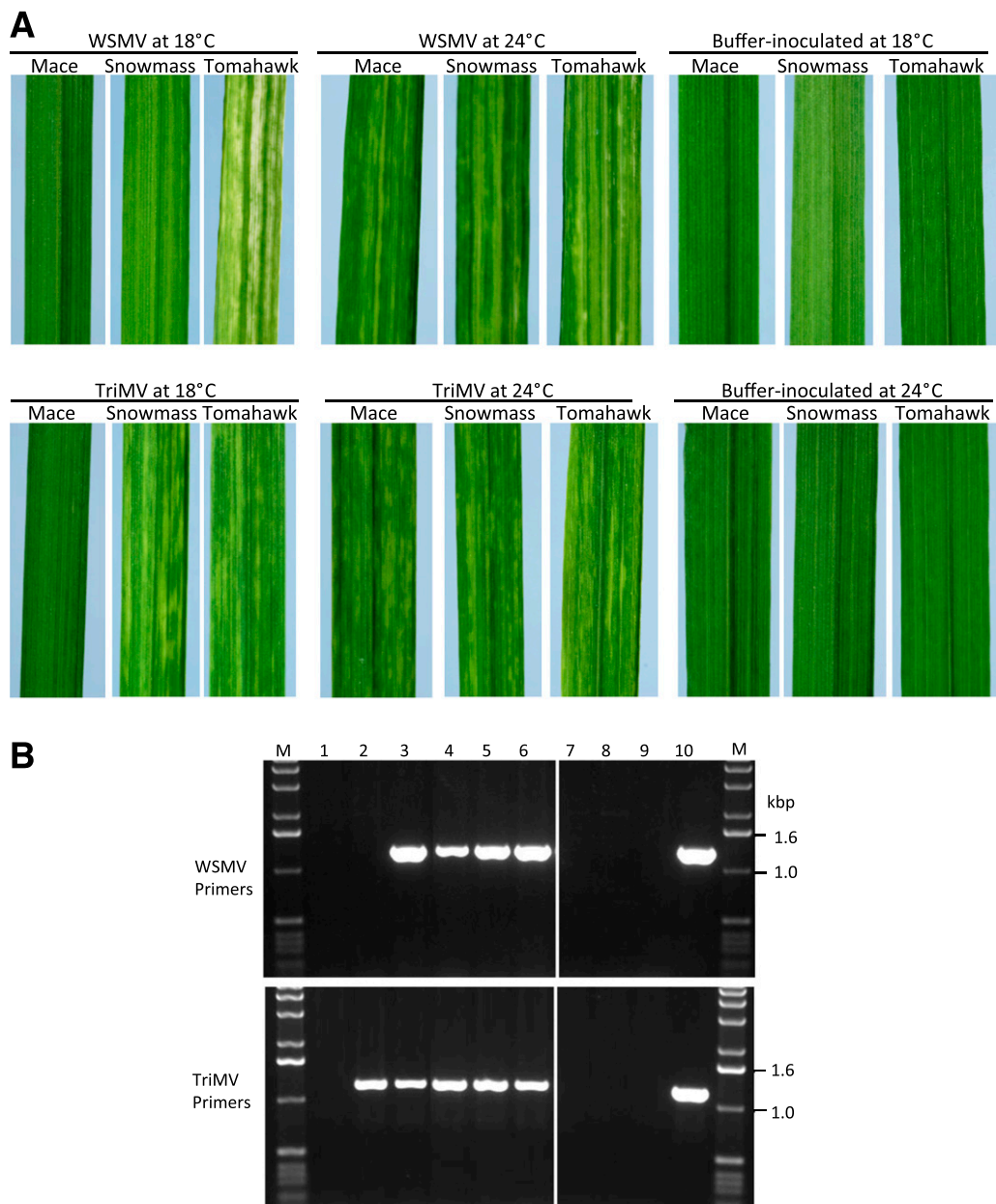

Fig. 2. Wheat cultivar screening for resistance against *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV) at 18 and 24°C. **A,** Systemic infection of wheat cvs. Mace, Snowmass, and Tomahawk by WSMV or TriMV at 21 days postinoculation (dpi) at 18 and 24°C. **B,** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of total RNA isolated from 'Mace', 'Snowmass', and 'Tomahawk' inoculated with WSMV and TriMV at 21 dpi at 18 and 24°C. Agarose gel electrophoresis of WSMV- and TriMV-specific RT-PCR products from wheat cultivars mechanically inoculated with wild-type WSMV or TriMV. Virus-inoculated wheat plants were maintained in a growth chamber at 18°C (lanes 1 to 3) and in the greenhouse at 24°C (lanes 4 to 6). RT-PCR products from 'Mace' (lanes 1 and 4), 'Snowmass' (lanes 2 and 5), and 'Tomahawk' (lanes 3 and 6) were mechanically inoculated at the two-leaf stage with WSMV (top panel, lanes 1 to 6) or TriMV (bottom panel, lanes 1 to 6). Total RNA isolated from healthy 'Mace' (lane 7), 'Snowmass' (lane 8), and 'Tomahawk' (lane 9) was included as negative control. pSP6-WSMV and pTriMV-R were included as positive controls for PCR assays in lane 10 of top and bottom panels, respectively. Lane M: 1.0-kbp DNA ladder.

(Fig. 4), suggesting that both virulent (WSMV) and avirulent (TriMV) viruses elicited foci similarly on inoculated leaves. In coinoculated 'Snowmass' plants, WSMV and TriMV elicited, respectively, 8.9 and 6.9 foci per square centimeter at 18°C, compared with 6.7 and 4.8 foci per square centimeter at 24°C (Fig. 4A and B). Collectively, these data suggested that WSMV and TriMV elicited foci on inoculated leaves of resistant 'Mace' and 'Snowmass' similarly at 18 and 24°C. The differences in the number of foci elicited on 'Mace', 'Snowmass', and 'Tomahawk' at 18 and 24°C by WSMV ('Mace', $P = 0.292$; 'Snowmass', $P = 0.855$; 'Tomahawk', $P = 0.684$) and TriMV ('Mace', $P = 0.191$; 'Snowmass', $P = 0.721$; 'Tomahawk', $P = 0.227$) were not statistically significant.

Size of fluorescent foci on inoculated leaves. Though WSMV and TriMV elicited foci on inoculated leaves of resistant wheat cultivars similarly at permissible and nonpermissible temperatures, it is possible that resistance to virus infection might have affected foci size. The size of foci elicited by WSMV-RFP and TriMV-GFP on inoculated leaves of wheat cultivars was measured from 20 foci per virus per cultivar. In 'Tomahawk', WSMV and TriMV elicited 0.94- and 0.60-mm^2 foci at 18°C, compared with 0.86- and 0.75-mm^2 foci at 24°C (Fig. 5). In coinoculated 'Tomahawk', WSMV and TriMV produced 0.91- and 0.56-mm^2 foci at 18°C, compared with 0.80- and 0.66-mm^2 foci at 24°C, suggesting that, by 5 dpi, foci sizes at nonpermissible temperature are comparable to those at permissible temperatures by 3 dpi. In 'Mace', WSMV and TriMV elicited 0.89- and 0.57-mm^2 foci at 18°C, compared with 0.73- and 0.58-mm^2 foci at 24°C (Fig. 5). In coinoculated 'Mace', WSMV and TriMV produced, respectively, 0.74- and 0.53-mm^2 foci at 18°C, compared with 0.73- and 0.56-mm^2 foci at 24°C (Fig. 5). In 'Snowmass', WSMV and TriMV elicited 1.35- and 0.86-mm^2 foci at 18°C, compared with 0.90- and 0.75-mm^2 foci at 24°C (Fig. 5). In coinoculated 'Snowmass', WSMV and TriMV produced foci similar to those in single-virus inoculations. These data indicated that, in resistant wheat cultivars, WSMV and TriMV formed foci with approximately similar sizes at 18 and 24°C. The differences in foci sizes at 18 and 24°C elicited by WSMV ('Mace', $P = 0.518$; 'Snowmass', $P = 0.613$; 'Tomahawk', $P = 0.527$) and TriMV ('Mace', $P = 0.705$; 'Snowmass', $P = 0.494$; 'Tomahawk', $P = 0.380$) in resistant and susceptible wheat cultivars were not statistically significant.

Taken together, the number and size of foci elicited by WSMV and TriMV on inoculated leaves of wheat cvs. Mace, Snowmass, and Tomahawk at 18°C are comparable to those at 24°C, suggesting that 'Mace' and 'Snowmass' resistance at 18°C does not affect local virus cell-to-cell movement.

WSMV and TriMV efficiently replicated in inoculated leaves of resistant wheat cultivars.

We next examined whether cell-to-cell movement of WSMV and TriMV in resistant cultivars resulted from efficient virus replication. Total RNA, extracted at 5 and 3 dpi from virus-inoculated leaves of wheat plants incubated at 18 and 24°C, respectively, was used for absolute quantification of WSMV and TriMV genomic RNAs as described (Tatineni et al. 2010). The number of genomic RNA copies of WSMV and TriMV in single- or double-virus inoculated leaves of 'Mace', 'Snowmass', and 'Tomahawk' at 18 and 24°C is presented in Table 3. In all wheat cultivars inoculated with individual viruses at 18°C, genomic RNA of TriMV accumulated at enhanced levels while WSMV accumulated at reduced levels, compared with those at 24°C (Table 3). The lower accumulation of WSMV genomic RNA in resistant as well as susceptible wheat cultivars at 18°C, suggests that WSMV might replicate at reduced levels at 18°C. In coinoculated (WSMV+TriMV) wheat leaves, both WSMV and TriMV genomic RNAs accumulated at higher levels at 18°C, compared with those at 24°C in all cultivars (Table 3). The increased levels of WSMV accumulation in coinoculated plants compared with single-virus inoculations could be due to synergistic interaction between WSMV and TriMV (Tatineni et al. 2010). Collectively, these data suggest that WSMV and TriMV replicated efficiently in inoculated leaves of resistant and susceptible wheat. Differences in accumulation of genomic RNAs of WSMV ('Mace', $P = 0.066$; 'Tomahawk', $P = 0.952$) and TriMV ('Snowmass', $P = 0.090$; 'Tomahawk', $P = 0.150$) in resistant and susceptible wheat cultivars at 18 and 24°C were not statistically significant but were significantly different in 'Snowmass' for WSMV (0.0007) and in 'Mace' for TriMV ($P = 0.006$).

WSMV and TriMV exhibit cultivar-specific and temperature-dependent long-distance movement.

Wheat cvs. Mace, Snowmass, and Tomahawk were inoculated with crude sap of WSMV-RFP or TriMV-GFP at the

Table 2. Number of wheat plants systemically infected with red fluorescent protein–tagged *Wheat streak mosaic virus* (WSMV-RFP) and green fluorescent protein–tagged *Triticum mosaic virus* (TriMV-GFP) at 18 and 24°C

Cultivar[y]	Virus	Growth chamber (18°C)[z]		Greenhouse (24°C)[z]	
		14 dpi	21 dpi	14 dpi	21 dpi
'Mace'	WSMV-RFP	0/17 (0.0%)	1/17 (5.9%)	17/21 (81%)	21/21 (100%)
	TriMV-GFP	1/16 (6.0%)	2/16 (13%)	22/22 (100%)	22/22 (100%)
	DI: WSMV-RFP	0/22 (0.0%)	2/22 (9.0%)	17/18 (94%)	18/18 (100%)
	DI: TriMV-GFP	1/22 (4.5%)	2/22 (9.0%)	16/17 (94%)	17/17 (100%)
	Buffer	0/14 (0.0%)	0/14 (0.0%)	0/16 (0.0%)	0/16 (0.0%)
'Snowmass'	WSMV-RFP	4/18 (22%)	5/18 (28%)	17/17 (100%)	17/17 (100%)
	TriMV-GFP	17/17 (100%)	17/17 (100%)	17/17 (100%)	17/17 (100%)
	DI: WSMV-RFP	4/19 (21%)	6/19 (32%)	18/18 (100%)	18/18 (100%)
	DI: TriMV-GFP	16/17 (94%)	16/17 (94%)	18/18 (100%)	18/18 (100%)
	Buffer	0/17 (0.0%)	0/17 (0.0%)	0/16 (0.0%)	0/16 (0.0%)
'Tomahawk'	WSMV-RFP	16/16 (100%)	16/16 (100%)	17/17 (100%)	17/17 (100%)
	TriMV-GFP	20/20 (100%)	20/20 (100%)	19/19 (100%)	19/19 (100%)
	DI: WSMV-RFP	19/19 (100%)	19/19 (100%)	14/14 (100%)	14/14 (100%)
	DI: TriMV-GFP	19/19 (100%)	19/19 (100%)	14/14 (100%)	14/14 (100%)
	Buffer	0/15 (0.0%)	0/15 (0.0%)	0/17 (0.0%)	0/17 (0.0%)

[y] Wheat seedlings at the two-leaf stage were mechanically inoculated with WSMV-RFP, TriMV-GFP, or WSMV-RFP+TriMV-GFP at 1:20 dilution in 10 mM sodium phosphate buffer, pH 7.0. Two upper noninoculated leaves per plant were observed for fluorescent protein under a Stereo Discovery V12 fluorescence microscope. The presence of at least a single fluorescent focus was considered as positive for infection.

[z] Number of plants systemically infected/number of plants mechanically inoculated, followed by % infection in parentheses. DI: double inoculation with WSMV-RFP+TriMV-GFP; dpi: days postinoculation.

two-leaf stage to track viral long-distance movement by observing fluorescent protein in upper noninoculated leaves. By 21 dpi, both WSMV and TriMV moved long distance at 24°C in all wheat cultivars, as fluorescent proteins were readily observed in 100% of inoculated plants (Fig. 6C and D). However, fluorescent proteins were not observed in upper noninoculated leaves of WSMV-RFP- or TriMV-GFP-inoculated 'Mace' at 18°C (Fig. 6A and B) but developed local (fluorescent) foci on 100% of inoculated leaves (Fig. 3A and B), suggesting that WSMV and TriMV failed to move long distance in 'Mace' at 18°C. TriMV-GFP moved long distance in 'Snowmass' at 18°C, as fluorescent protein was observed in upper noninoculated leaves while RFP was observed in only 28% of upper noninoculated leaves of plants inoculated with WSMV-RFP, which is in agreement with approximately 20% susceptibility of 'Snowmass' to WSMV (Fig. 6A and B; Table 2) (Haley et al. 2011). Coinoculation (WSMV-RFP+TriMV-GFP) of 'Tomahawk', 'Snowmass', and 'Mace' plants resulted in efficient systemic coinfection by both viruses at 24°C and, in 'Tomahawk', at 18°C by 21 dpi (Fig. 6E and F). As expected, neither virus infected wheat cv. Mace

systemically in coinoculated plants at 18°C (Fig. 6F). However, coinoculation of 'Snowmass' at 18°C resulted in systemic infection by TriMV-GFP, but only approximately 30% of plants were doubly-infected with WSMV-GFP and TriMV-GFP (Fig. 6F; Table 2).

Development of local foci by WSMV-RFP in all inoculated 'Snowmass' leaves at 18°C together with failure to infect approximately 70% of plants systemically suggest that WSMV failed to move long distance in most 'Snowmass' plants. These data revealed that both viruses moved long distance in all wheat cultivars at 24°C and only cell-to-cell but not long distance at 18°C in resistant cultivars, suggesting that WSMV and TriMV exhibit cultivar-specific and temperature-dependent long-distance movement.

WSMV and TriMV failed to enter the vasculature of resistant wheat cultivars at 18°C.

Plant viruses fail to move long distance, possibly due to inability to one or both enter or exit the vasculature at a distal place for the subsequent spread by cell-to-cell movement.
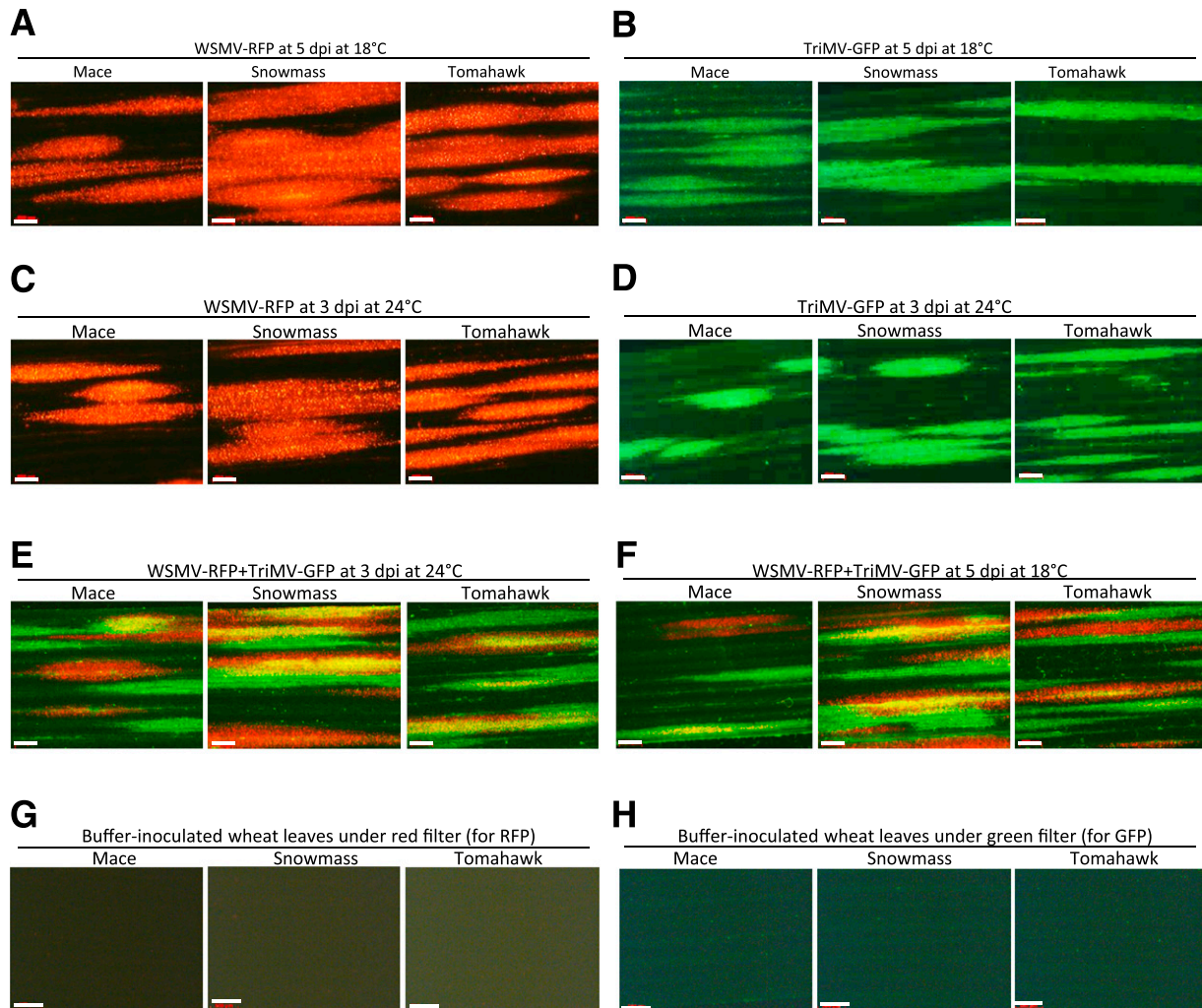

Fig. 3. *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV) facilitated efficient cell-to-cell movement in resistant wheat cultivars at 18°C. **A** and **C,** Local foci elicited by WSMV-RFP (red fluorescent protein), **B** and **D,** TriMV-GFP (green fluorescent protein), and **E** and **F,** WSMV-RFP+TriMV-GFP at 18°C (A, B, and F) and 24°C (C, D, and E) in inoculated wheat leaves. Note that WSMV-RFP and TriMV-GFP facilitated efficient cell-to-cell movement in inoculated leaves of 'Mace' and 'Snowmass' at 18°C (A and B). As a control, susceptible wheat cv. Tomahawk' was included as a positive control for cell-to-cell movement of WSMV-RFP and TriMV-GFP. **G,** Buffer-inoculated wheat leaves were observed under RFP or **H,** GFP filters as negative controls. Inoculated wheat leaves were observed under a Stereo Discovery V12 fluorescence microscope using RFP or GFP filters. Merged images of RFP and GFP from mixed inoculation were presented in E and F. Note that colocalization of RFP and GFP foci resulted in a yellow color in mixed inoculations. The brightness of buffer-inoculated images in G and H were adjusted to see the background of objects under RFP and GFP narrow-band filters, respectively. Bars represent 500 μM.

Reasons for lack of long-distance movement of WSMV and TriMV in resistant wheat cultivars at 18°C were examined by inoculating resistant cultivars Mace and Snowmass and susceptible cultivar Tomahawk with WSMV-RFP or TriMV-GFP. The inoculated leaves and leaf sheaths and crowns from plants incubated at 18°C were observed at 21 dpi, for the presence of fluorescent proteins (Fig. 7A). Local foci spread from initial infection sites throughout most of the inoculated leaves, further confirming efficient cell-to-cell movement of WSMV and TriMV in resistant and susceptible wheat cultivars alike (Fig. 7B). In 'Tomahawk', fluorescent proteins were readily detected in WSMV-RFP- or TriMV-GFP-inoculated leaf sheaths (Fig. 7C). In contrast, no fluorescent proteins were detected in leaf sheaths of WSMV-RFP- or TriMV-GFP-inoculated leaves of 'Mace' (Fig. 7C). Since approximately 20% of 'Snowmass' plants were susceptible to WSMV systemic infection (Haley et al. 2011), leaf sheaths of 'Snowmass' plants that WSMV-RFP failed to systemically infect (but had local foci) were examined for the presence of RFP in leaf sheaths. No RFP was detected in leaf sheaths of WSMV-RFP–inoculated leaves of 'Snowmass' that contained local foci (Fig. 7C). In contrast, fluorescent protein was readily detected in leaf sheaths of TriMV-GFP–inoculated 'Snowmass' plants (Fig. 7C). These data revealed that WSMV and TriMV efficiently moved cell to cell from initial infection foci in resistant wheat cultivars at 18°C but failed to enter the vasculature of leaf sheaths to establish a successful systemic infection. At 24°C, fluorescent proteins were readily detected at 21 dpi in the leaf sheaths of 'Mace', 'Snowmass', and 'Tomahawk' plants inoculated with WSMV-RFP or TriMV-GFP (data not shown).

The crowns of wheat plants inoculated with WSMV-RFP or TriMV-GFP were examined for the presence of fluorescent proteins as both WSMV and TriMV must infect the crown before moving systemically. The crowns of 'Mace' inoculated with WSMV-RFP or TriMV-GFP were free from fluorescent proteins (Fig. 7D), suggesting that WSMV and TriMV failed to move into crowns at detectable levels. Similarly, the crowns of 'Snowmass' that WSMV-RFP failed to infect systemically but elicited local foci were also free from detectable levels of RFP (Fig. 7D). In contrast, fluorescent proteins were readily detected in the crowns of 'Tomahawk' inoculated with WSMV-RFP or TriMV-GFP, which is consistent with 'Tomahawk's susceptibility to WSMV and TriMV. These data suggest that WSMV and TriMV are unable to reach the crowns of resistant wheat cultivars at 18°C.

To exclude the possibility that WSMV or TriMV might be present in the crowns at levels undetectable by fluorescence assays, total RNA extracted from three crowns per sample was
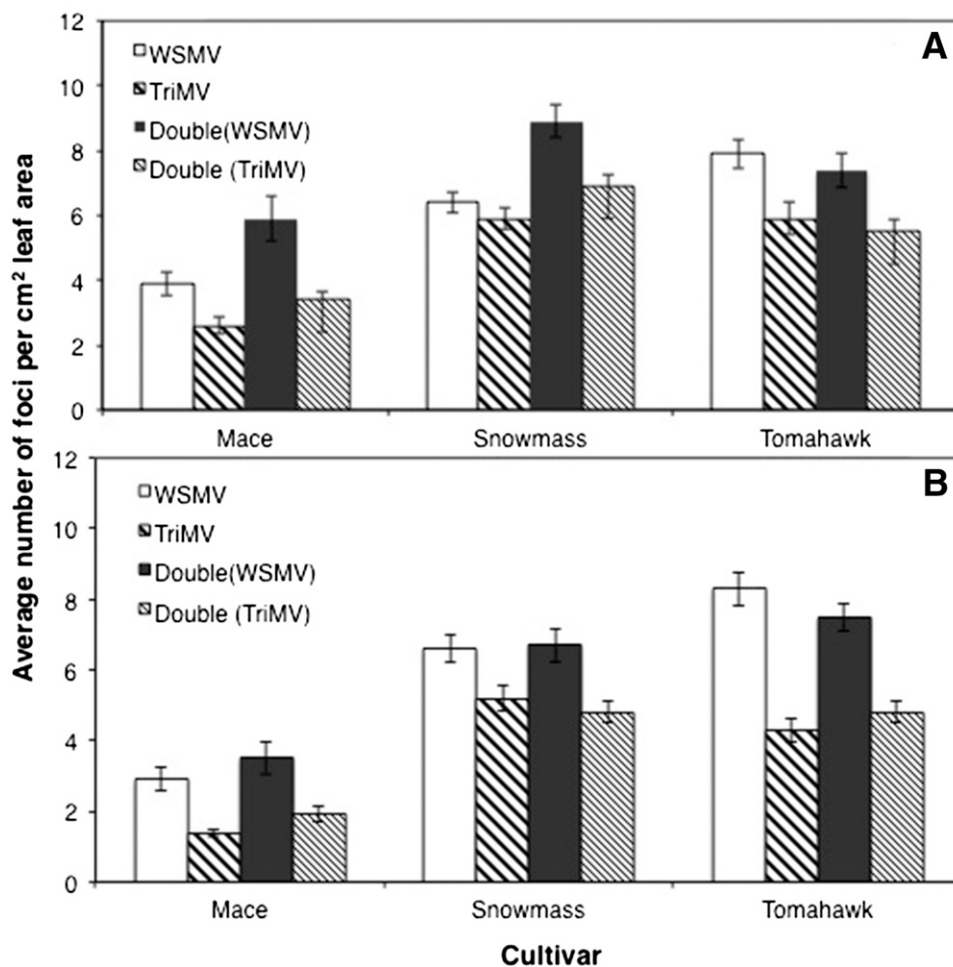

Fig. 4. A, The average number of virus infection foci per square centimeter of leaf area (mean ± standard error) in inoculated leaves of wheat cvs. Mace, Snowmass, and Tomahawk at 18°C at 5 days postinoculation (dpi) and **B,** at 24°C at 3 dpi. Vertical bars represent standard error. Wheat seedlings were inoculated with crude sap of either red fluorescent protein–tagged *Wheat streak mosaic virus* (WSMV-RFP), green fluorescent protein–tagged *Triticum mosaic virus* (TriMV-GFP), or both, at a 1:20 dilution in 20 mM sodium phosphate buffer, pH 7.0, and plants were maintained at 18 or 24°C. Double: coinoculation with WSMV-RFP and TriMV-GFP. The differences in the number of foci elicited on 'Mace', 'Snowmass', and 'Tomahawk' at 18 and 24°C by WSMV (P = 0.292, P = 0.855, and P = 0.684, respectively) and TriMV (P = 0.191, P = 0.721, and P = 0.227, respectively) were not statistically significant.

used as a template for RT-PCR using CP-specific primers of WSMV (Fig. 7F, top panel) and TriMV (Fig. 7F, bottom panel). No RT-PCR product was obtained from 'Mace' crowns inoculated with one or both WSMV or TriMV (Fig. 7F, lanes 1 to 3). In 'Snowmass', as expected, RT-PCR amplification was obtained from TriMV-GFP-inoculated plants but not from WSMV-RFP-inoculated plants (Fig. 7F, lanes 4 to 6). In 'Tomahawk', RT-PCR product was obtained from plants inoculated with one or both WSMV or TriMV (Fig. 7F, lanes 7 to 9). Taken together, these data demonstrate that WSMV and TriMV failed to move long
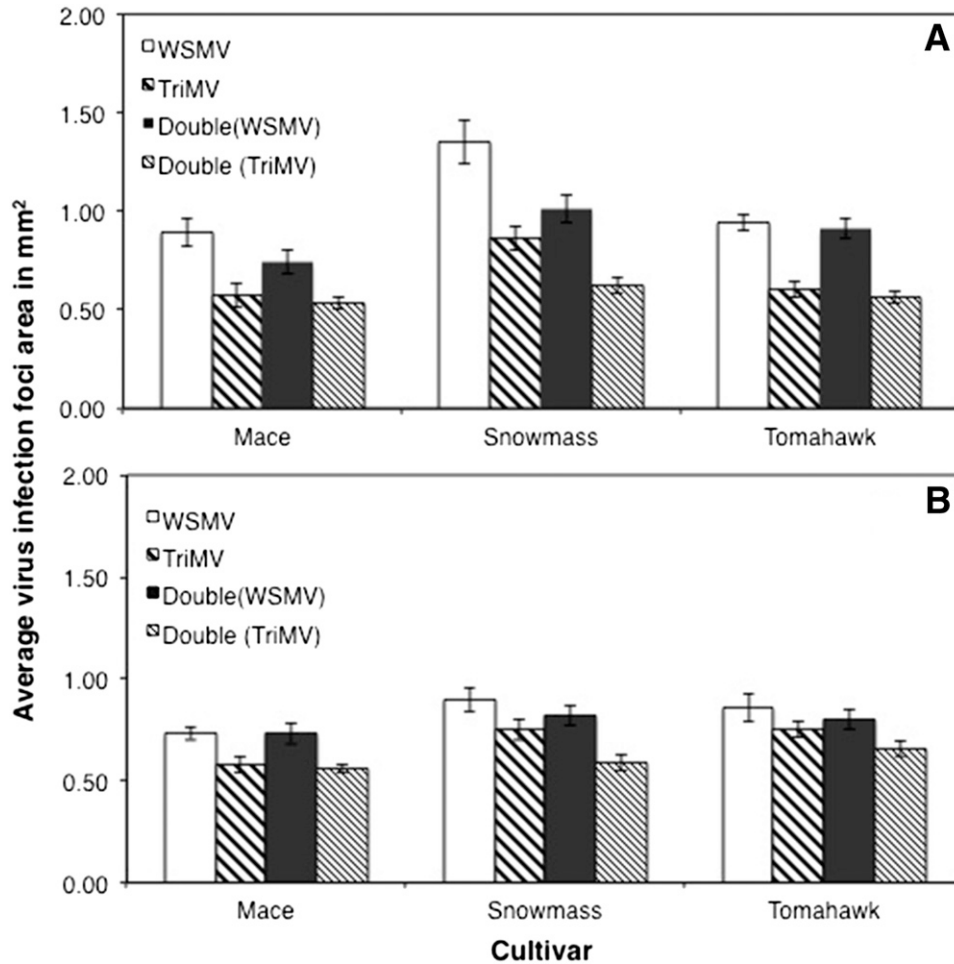

Fig. 5. A, The average size of virus infection foci in square millimeters (mean ± standard error) in inoculated leaves of 'Mace', 'Snowmass', and 'Tomahawk' at 18°C at 5 days postinoculation (dpi) and **B,** at 24°C at 3 dpi. Vertical bars represent standard error. Wheat seedlings were inoculated with crude sap of either red fluorescent protein–tagged *Wheat streak mosaic virus* (WSMV-RFP), green fluorescent protein–tagged *Triticum mosaic virus* (TriMV-GFP), or both, at 1:20 dilution in sodium phosphate buffer, pH 7.0, and were incubated at 18 or 24°C. Double: coinoculation with WSMV-RFP and TriMV-GFP. The differences in foci sizes at 18 and 24°C elicited elicited on 'Mace', 'Snowmass', and 'Tomahawk' by WSMV ($P = 0.518$, $P = 0.613$, and $P = 0.527$, respectively) and TriMV ($P = 0.705$, $P = 0.494$, and $P = 0.380$, respectively) in resistant and susceptible wheat cultivars were not statistically significant.

Table 3. Absolute quantification of *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV) genomic RNAs in inoculated leaves of three wheat cultivars held at 18 and 24°C

Virus[y]	Degrees	Number of genomic RNA copies in wheat cultivars[z]		
		'Mace'	'Snowmass'	'Tomahawk'
WSMV-RFP	18°C	$1.43 \times 10^4 \pm 4.91 \times 10^3$ a	$3.64 \times 10^4 \pm 5.21 \times 10^2$ b	$3.25 \times 10^4 \pm 4.90 \times 10^3$ a
	24°C	$4.35 \times 10^4 \pm 1.19 \times 10^4$ a	$1.50 \times 10^5 \pm 2.30 \times 10^4$ a	$5.50 \times 10^4 \pm 1.17 \times 10^4$ a
TriMV-GFP	18°C	$1.53 \times 10^4 \pm 2.36 \times 10^3$ a	$5.35 \times 10^4 \pm 7.97 \times 10^3$ a	$6.38 \times 10^4 \pm 3.74 \times 10^3$ a
	24°C	$3.40 \times 10^3 \pm 9.10 \times 10^2$ b	$2.48 \times 10^4 \pm 9.67 \times 10^3$ a	$1.60 \times 10^4 \pm 7.97 \times 10^3$ a
DI: WSMV-RFP	18°C	$5.20 \times 10^4 \pm 1.75 \times 10^3$ a	$1.35 \times 10^5 \pm 2.14 \times 10^4$ a	$7.74 \times 10^4 \pm 1.85 \times 10^4$ a
	24°C	$2.58 \times 10^4 \pm 8.50 \times 10^3$ a	$9.10 \times 10^4 \pm 3.13 \times 10^4$ a	$4.68 \times 10^4 \pm 9.96 \times 10^3$ a
DI: TriMV-GFP	18°C	$1.39 \times 10^4 \pm 5.16 \times 10^3$ a	$4.38 \times 10^4 \pm 5.34 \times 10^3$ a	$2.08 \times 10^4 \pm 2.78 \times 10^3$ a
	24°C	$8.54 \times 10^3 \pm 2.69 \times 10^3$ a	$2.59 \times 10^4 \pm 6.91 \times 10^3$ a	$1.26 \times 10^4 \pm 2.13 \times 10^3$ a

[y] Wheat seedlings at the two-leaf stage were mechanically inoculated with crude sap of wheat leaves systemically infected with WSMV-RFP, TriMV-GFP, and WSMV-RFP+TriMV-GFP (DI) at 1:20 dilution in 20 mM sodium phosphate buffer, pH 7.0. DI: double virus inoculation.

[z] Number of genomic RNA copies (mean ± standard error) was detected in inoculated leaves at 5 and 3 days postinoculation from plants incubated at 18°C (growth chamber) and 24°C (greenhouse), respectively, by the real-time reverse transcription polymerase chain reaction as described by Tatineni et al. (2010). Mean genomic RNA copies with the same letter within columns for each virus are not significantly different (Tukey-Kramer test $P = 0.05$). WSMV or TriMV genomic RNA copies were not detected from buffer-inoculated wheat cultivars at 18 and 24°C.

distance in resistant wheat cultivars, as these viruses failed to enter the vasculature of resistant wheat at 18°C.

DISCUSSION

In this study, we found that temperature-dependent impairment of viral long-distance transport explains resistance in wheat cv. Mace against WSMV and TriMV and cv. Snowmass against WSMV at 18°C. WSMV and TriMV replicated and moved cell to cell efficiently in inoculated leaves but failed to establish systemic infection, as both viruses did not enter the vasculature of resistant wheat cultivars at 18°C. Thus, wheat cvs. Mace and Snowmass provide resistance by blocking viruses from entering the vasculature, thereby precluding long-distance movement without significant effects on virus replication and local cell-to-cell movement.

The molecular basis for genetic resistance to viruses in cereal hosts is poorly understood (Kang et al. 2005a). The fluorescent protein–tagged WSMV and TriMV facilitated examination of the mechanisms of *Wsm1-* and *Wsm2-*conferred resistance in wheat cvs. Mace and Snowmass, respectively. Both WSMV and TriMV replicated and moved cell to cell in inoculated leaves of resistant and susceptible wheat cultivars similarly at permissive

and nonpermissible temperatures. The difference in replication and cell-to-cell movement of WSMV and TriMV between 5 dpi at 18°C and 3 dpi at 24°C was similar in both susceptible and resistant wheat cultivars. These data suggest that WSMV and TriMV replicate and facilitate cell-to-cell movement similarly in resistant and susceptible cultivars at 18°C. However, at 18°C, both WSMV and TriMV failed to move long distance in wheat cv. Mace; also TriMV moved long distance in 'Snowmass' but WSMV did not. The debilitated long-distance movement of WSMV and TriMV in resistant wheat cultivars at 18°C is due to blocked virus entry into the vasculature. These data revealed that resistance in 'Mace' and 'Snowmass' is not at viral replication and local cell-to-cell movement steps of the virus life cycle but is due to temperature-dependent impairment of viral long-distance transport.

Many host *R* genes against members of family *Potyviridae* are recessive alleles of genes encoding translation initiation factors such as eIF4E and its isoforms (Kang et al. 2005a; Mazier et al. 2011; Ruffel et al. 2002; Wang and Krishnaswamy 2012; Yeam et al. 2007). Recessive resistance, in most cases, results in an undetectable level of virus multiplication in inoculated leaves, as shown for *pvr2* in pepper against TEV and *Pepper vein mottle virus* (Kang et al. 2005b; Ruffel et al. 2006), *mo1* in lettuce against *Lettuce mosaic virus* (Nicaise et al. 2003), *pot-1* in tomato
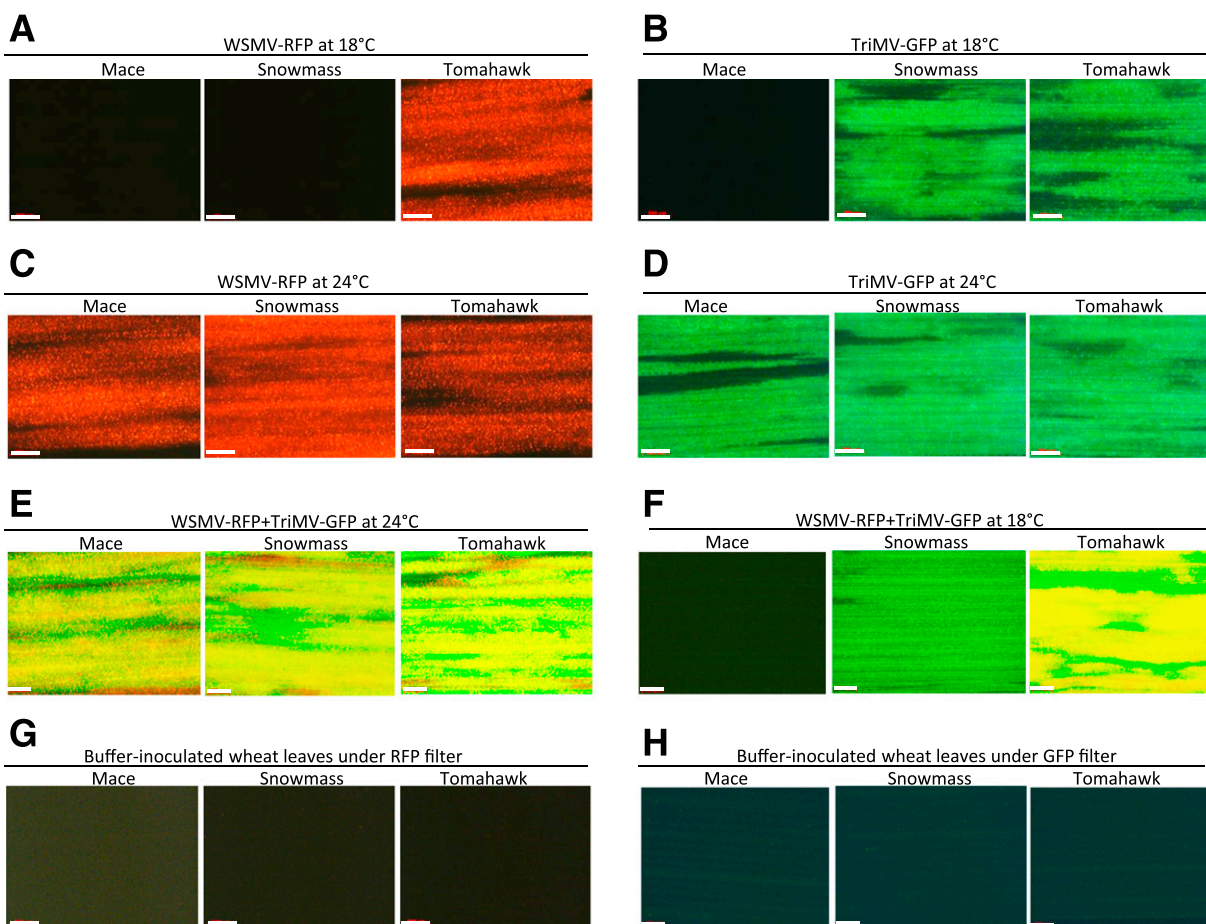

Fig. 6. *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV) facilitated cultivar-specific temperature-dependent long-distance transport in resistant wheat cultivars. **A** and **C,** Systemic infection of wheat cultivars Mace, Snowmass, and Tomahawk by red fluorescent protein tagged WSMV (WSMV-RFP), **B** and **D,** green fluorescent protein–tagged *Triticum mosaic virus* (TriMV-GFP), and **E** and **F,** WSMV-RFP+TriMV-GFP (mixed inoculation), at 21 days postinoculation (dpi) at 18°C (A, B, and F) and 24°C (C, D, and E). **G,** Buffer-inoculated leaves of 'Mace', 'Snowmass', and 'Tomahawk' were observed under RFP or **H,** GFP filters as negative controls. The upper noninoculated wheat leaves were observed under a Stereo Discovery V12 fluorescence microscope using RFP or GFP narrow-band filters. Merged images of RFP and GFP from mixed inoculation are presented in E and F. Note that colocalization of RFP and GFP resulted in a yellow color. The brightness of buffer-inoculated images in G and H were adjusted to see the background of objects under RFP and GFP filters, respectively. Note that WSMV-RFP does not infect 'Mace' and 'Snowmass' systemically (A) and TriMV-GFP does not infect 'Mace' but efficiently infected 'Snowmass' at 18°C (B).

against *Potato virus Y* and TEV (Ruffel et al. 2005), *rym4* and *rym5* in barley against *Barley yellow mosaic virus* (Kanyuka et al. 2005; Stein et al. 2005), *sbm1*, *wlv*, and *cyv* in pea against *Pea seed-borne mosaic virus*, *Bean yellow mosaic virus*, and *Clover yellow vein virus*, respectively (Bruun-Rasmussen et al. 2007; Gao et al. 2004), and *bc-3* in beans against *Bean common mosaic virus* (Naderpour et al. 2010). Recently, Yang and associates (2014) demonstrated that loss-of-function HvPDIL5-1 alleles at the recessive *rym11* resistance locus conferred broad-spectrum resistance to several strains of bymoviruses. However, recessive

virus *R* genes are unlikely to be found in hexaploid wheat. Dominant virus *R* genes are characterized by induction of a hypersensitive or extreme response when plants are inoculated with viruses containing the corresponding avirulence factor (Fraser 1990; Moffett 2009). However, *Wsm1* and *Wsm2* do not induce a hypersensitive or extreme response on inoculated wheat leaves at 18°C, suggesting that these genes are nonconventional *R* genes.

The other nonconventional *R* genes also provide viral resistance by debilitating one or more steps that led to systemic infection of plants. For example, the *RTM* genes of *Arabidopsis*
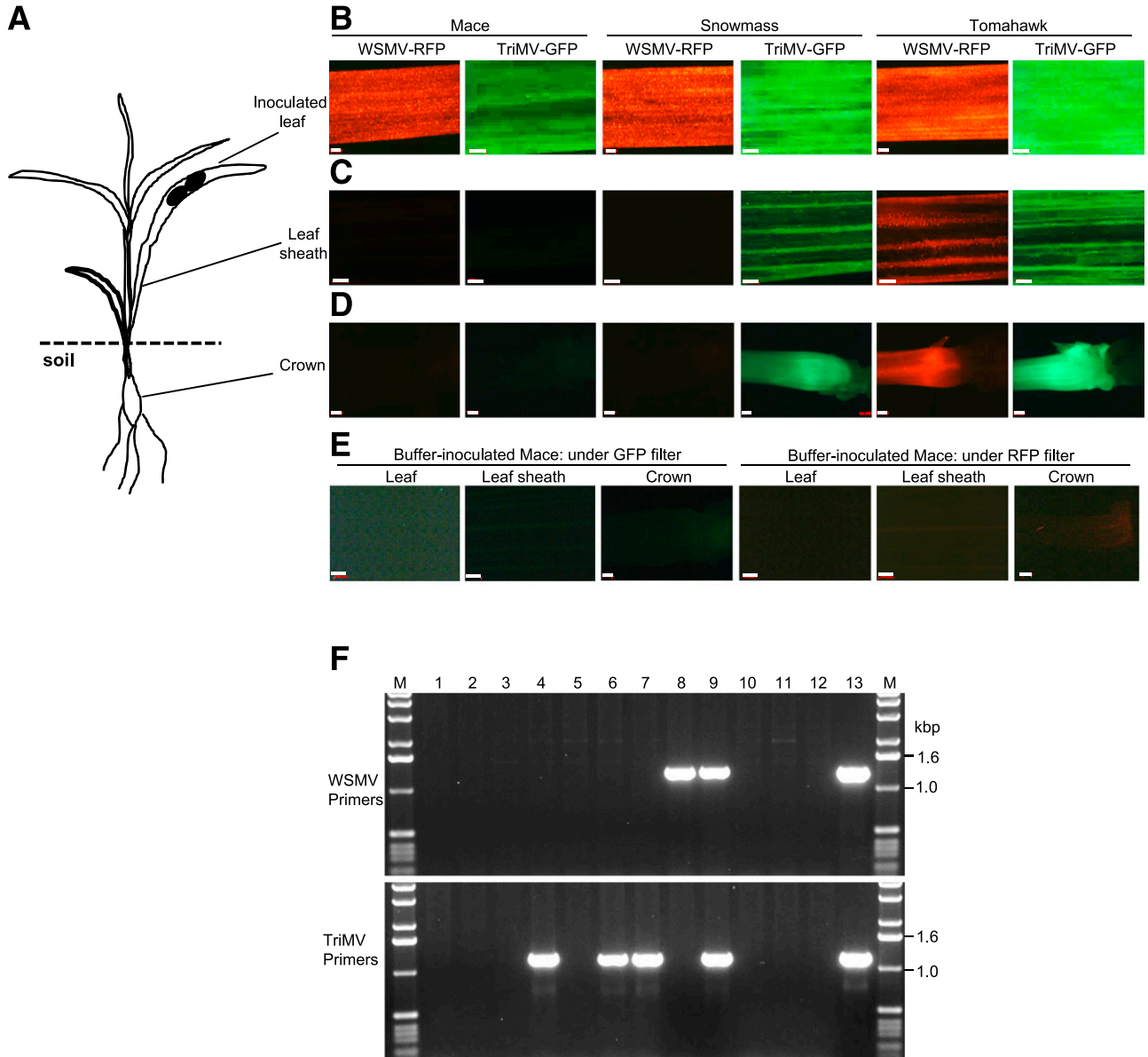

Fig. 7. *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV) failed to enter the vasculature of resistant wheat cultivars at 18°C. **A,** Schematic diagram of a wheat plant showing the point of inoculation (dark circles), leaf sheath, and crown. **B,** Local foci elicited by red fluorescent protein–tagged WSMV (WSMV-RFP) or green fluorescent protein–tagged TriMV (TriMV-GFP) in inoculated leaves of resistant ('Mace' and 'Snowmass') and susceptible ('Tomahawk') wheat at 21 days postinoculation (dpi). **C** and **D,** Detection of fluorescent protein–tagged viruses in the leaf sheaths of inoculated leaves (C) and in crowns (D). Note that fluorescent protein was not observed at detectable levels in the leaf sheaths and crowns of resistant wheat cultivars but abundant levels were observed in susceptible wheat cultivars. **E,** Buffer-inoculated healthy leaf, leaf sheath, and crown observed under GFP or RFP narrow-band filters are shown as negative controls. The brightness of buffer-inoculated images were adjusted to see the background of objects under RFP and GFP filters. Bars represent 500 μM. **F,** Reverse transcription-polymerase chain reaction (RT-PCR) assay of total RNA isolated from wheat crowns at 21 dpi. Agarose gel electrophoresis image showing RT-PCR products of WSMV and TriMV from the crown samples of 'Mace' (lanes 1 to 3), 'Snowmass' (lanes 4 to 6), and 'Tomahawk' (lanes 7 to 9) inoculated with TriMV-GFP (lanes 1, 4, and 7), WSMV-RFP (lanes 2, 5, and 8), and coinoculated with TriMV-GFP and WSMV-RFP (lanes 3, 6, and 9). Note that WSMV and TriMV did not accumulate at detectable levels in the crowns of 'Mace' and 'Snowmass', or 'Mace', respectively. Total RNA from healthy crowns of 'Mace' (lane 10), 'Snowmass' (lane 11), and 'Tomahawk' (lane 12) was used as negative control. Total RNA from 'Tomahawk' leaves infected with WSMV (top panel, lane 13) and TriMV (bottom panel, lane 13) was used as a positive control for RT-PCR. Lane M: 1.0-kbp DNA ladder.

prevent systemic infection but not cell-to-cell movement of TEV (Chisholm et al. 2001; Decrocq et al. 2009; Mahajan et al. 1998; Whitham et al. 2000). The *Rsv4* gene of soybean restricts SMV movement and accumulation in inoculated leaves and also prevents systemic infection (Khatabi et al. 2012). The *Tm-1* gene of tomato prevents the formation of viral replication complex on membrane surfaces, thus inhibiting ToMV replication (Ishibashi and Ishikawa 2013; Ishibashi et al. 2007). The above-mentioned nonconventional *R* genes, including *Wsm1* and *Wsm2*, do not elicit HR-mediated resistance but provide resistance by affecting, singly or in combination, viral replication, cell-to-cell, or long-distance movement. However, it remains to be seen whether these nonconventional dominant *R* genes can be considered as a new class of *R* genes.

Differential long-distance movement of viruses in a host at two temperatures with no appreciable effects on virus replication and cell-to-cell movement is unusual. It is possible that interaction of *Wsm1* and *Wsm2* gene products with viral proteins is temperature-dependent, and such interactions might be required for long-distance transport in resistant wheat cultivars. Potyviral P1, P3, 6K2, NIa-VPg, NIa-Pro, and CP have been reported to be involved in differential infection of certain hosts (Carbonell et al. 2013; Chen et al. 2008; Decrocq et al. 2009; Rajamäki and Valkonen 1999; Salvador et al. 2008; Schaad et al. 1997; Tatineni and French 2014; Tatineni et al. 2011b). In the above-mentioned examples, viral gene products are required for infection of certain specific hosts. However, the situation with WSMV and TriMV is different, as wild-type viruses exhibited temperature-dependent long-distance movement in resistant wheat cultivars. The members of family *Potyviridae* do not encode dedicated long-distance movement proteins (Revers and García 2015). Instead, several potyviral proteins are involved in long-distance movement in addition to other unrelated functions. Recently, the WSMV CP was determined to be a long-distance transport factor for systemic infection of maize by specifically allowing the virus to enter the vascular system (Tatineni and French 2014; Tatineni et al. 2011b). These data suggest that the N- and C-terminal regions of CP may be involved in interactions with the host proteins for a successful invasion of hosts by WSMV. Viral determinants for long-distance movement of TriMV are not known. However, availability of TriMV infectious cDNA clone (Tatineni et al. 2015) will facilitate determination of viral determinants for long-distance movement.

Viruses establish successful systemic infection in a host plant by performing repeated cycles of cell-to-cell movement through plasmodesmata and long-distance transport by entry and exit through the vasculature. Since viruses encode only a limited number of proteins, viruses must interact with several host factors to survive in plant hosts. Incompatible interactions between viral and host factors at any of these steps could prevent viruses from infecting plants systemically. In this study, both WSMV and TriMV efficiently replicated and moved cell to cell in resistant wheat cultivars but failed to move long distance at 18°C, indicating that incompatible interactions between viral and host factors for long-distance transport as the most likely explanation for resistance. It is interesting that nonallelic *Wsm1* and *Wsm2* genes elicit resistance in wheat cultivars with a similar mechanism of specifically blocking viral long-distance transport at 18°C but not at higher temperatures.

Though several host factors restricting viral long-distance movement process were genetically identified (Mandadi and Scholthof 2013; Revers and Nicaise 2014), only the *RTM* genes from *Arabidopsis* were cloned and characterized (Cosson et al. 2010; Mahajan et al. 1998; Whitham et al. 2000). Although both *Arabidopsis RTM* genes and wheat *Wsm1* and *Wsm2* genes provide resistance by blocking virus long-distance movement,

the *Wsm*-based resistance differs from that of *RTM* as *Wsm*-based resistance is strictly temperature sensitive. The *Wsm*-based resistance blocking viral long-distance transport at 18°C might be due to the following reasons: i) sequestration of one or both virus particles or ribonucleoprotein complex while loading into the vasculature, ii) the *Wsm*-gene products limit accessibility of host factors for virus long-distance transport, or iii) expression of host proteins required for viral long-distance transport were silenced or expressed at suboptimal levels at 18°C. The fact that both WSMV and TriMV efficiently infected resistant wheat cultivars at higher temperatures suggests that incompatible interactions between viral and host factors at 18°C, temperature-dependent expression of *Wsm1* and *Wsm2* genes in wheat, or the silencing or low-level expression of *Wsm* genes encoded host factors for viral long-distance transport result in *Wsm*-based resistance in wheat cultivars.

MATERIALS AND METHODS

Wheat cultivars.

Wheat cvs. Mace (PI 651043) (Graybosch et al. 2009) and Snowmass (PI 658597) (Haley et al. 2011), containing the *Wsm1* and *Wsm2* genes, respectively, and cv. Tomahawk (PI 552814), a susceptible cultivar, were used in this investigation. PI numbers indicate accession designations of these cultivars in the United States Department of Agriculture (USDA)-Agricultural Research Service National Small Grains Collection, Aberdeen, ID, U.S.A.

Construction of RFP-tagged WSMV.

The RFP ORF was fused to the heptapeptide cleavage site located between the 6K1/CI cistrons by overlap extension PCR (Ho et al. 1989). The RFP ORF with a cleavage peptide sequence at its 3′ end was precisely inserted between the P1 and HC-Pro cistrons by overlap extension PCR, using three individual PCR fragments with 18- to 21-bp overlapping sequences as described (Tatineni et al. 2011a). Briefly, PCR fragment 1 contained the SP6 RNA polymerase promoter sequence, followed by WSMV sequence through the end of P1 cistron plus the first six codons of RFP. PCR fragment 2 contained the last six codons of P1, followed by RFP ORF with a cleavage site plus the first seven codons of HC-Pro. PCR fragment 3 comprised the last seven codons of RFP, followed by a cleavage site plus the HC-Pro cistron and part of the P3 cistron sequence until nucleotide 3,960. Overlap extension PCR was performed with gel-eluted PCR fragments 1, 2, and 3 as templates. Amplification of PCR fragments 1 to 3 and overlap extension PCR were performed with Herculase II Fusion DNA polymerase (Agilent Technologies, Santa Clara, CA, U.S.A.). The overlap extension PCR fragments were digested with NgoMIV (engineered upstream of an SP6 RNA polymerase promoter sequence in the plus-sense primer) and *Afl*II (nucleotide 3,905) and were ligated into similarly digested pSP6-WSMV (Choi et al. 1999).

Viruses.

Wild-type WSMV isolate Sidney 81 and TriMV isolate Nebraska were obtained from in vitro transcripts of pSP6-WSMV (Choi et al. 1999) and pTriMV-R (Tatineni et al. 2015), respectively. Cycle 3 GFP-tagged TriMV (pTriMV-GFP-NIb/CP [9aa]) was described previously (Tatineni et al. 2015). In vitro transcripts of pSP6-WSMV-RFP-6K1/CI(7aa) and pTriMV-GFP-NIb/CP(9aa) were inoculated to wheat seedlings to obtain WSMV-RFP and TriMV-GFP, respectively.

In vitro transcription reaction of each construct was carried out in a 40-μl reaction volume consisting of 1.0 μg of linearized plasmid DNA, 40 mM Tris-HCl, pH 7.9, 20 mM dithiothreitol,

8.5 mM MgCl$_2$, 2 mM spermidine, 1.2-mM concentrations each of ATP, CTP, UTP, and Cap analog (m7G[5′]ppp[5′]G; Cellscript, Madison, WI, U.S.A.), 0.048 mM GTP, 20 U of rRNasin ribonuclease inhibitor (Promega) and 50 U of SP6 RNA polymerase (Cellscript™). The reaction was incubated for 15 min at 37°C. The concentration of GTP was increased to 0.5 mM with additional 2-h incubation at 37°C. The integrity and quality of transcripts was examined by analyzing 1 μl of in vitro transcripts on 1.0% native agarose gel in 1× Tris-acetate-EDTA (TAE) buffer. Freshly prepared in vitro transcripts were inoculated to wheat seedlings at the single leaf stage as described (Tatineni et al. 2011b). Infected wheat leaves collected at 14 dpi were stored at −20°C for inoculation of wheat seedlings.

Screening wheat cultivars for virus resistance.

Frozen wheat leaves infected with WSMV or TriMV at 14 dpi were ground in 20 mM sodium phosphate buffer, pH 7.0, at 1:20 dilution (1 g per 19 ml) with a mortar and pestle. Wheat plants were raised in a pasteurized soil mix that consisted of 33% each of clay loam soil and peat moss and 16.5% each of sand and vermiculite. Wheat cvs. Mace, Snowmass, and Tomahawk at the two-leaf stage were mechanically inoculated with WSMV or TriMV (approximately 20 seedlings per 15-cm earthen pot). Two inoculated pots per cultivar per virus were incubated in the greenhouse at a mean temperature of 24°C (20°C minimum and 26°C maximum temperatures) with 14 h of daylight or supplemental light. Another set of inoculated wheat seedlings (two pots per cultivar per virus) was incubated in a growth chamber at 18°C with 14 h of light. Wheat plants were observed for symptom development at 14 and 21 dpi.

Examination of virus movement.

Two sets of wheat seedlings were mechanically inoculated at the two-leaf stage with crude sap of WSMV-RFP, TriMV-GFP, or both at a 1:20 dilution in 20 mM sodium phosphate buffer, pH 7.0. One set of inoculated plants were incubated at 18°C in a growth chamber and another set in a greenhouse at a mean temperature of 24°C with 14 h of light. Cell-to-cell movement of WSMV-RFP and TriMV-GFP was monitored by examining the formation of fluorescent foci on inoculated wheat leaves under a Zeiss Stereo Discovery V12 fluorescence microscope, using narrow band filter sets of RFP or GFP, as described (Tatineni et al. 2011a). The fluorescent images were taken with an AxioCam MRc5 camera attached to the V12 fluorescence microscope using an RFP filter set 43 (533 to 558 nm excitation and 571 to 641 nm emission) or GFP filter set 38 (400 to 450 nm excitation and 450 to 490 nm emission) (Carl Zeiss MicroImaging, Inc., New York), as described (Tatineni et al. 2011a, 2015). The fluorescent foci sizes were measured using a program provided with the AxioCam MRc5 camera. Long-distance movement of WSMV-RFP and TriMV-GFP in wheat cultivars was examined in upper noninoculated leaves by observing for the presence of RFP or GFP fluorescence.

Quantification of genomic RNAs of WSMV and TriMV.

Total RNA was extracted from inoculated wheat leaves using Tripure reagent, as described (Tatineni et al. 2010). One microgram of total RNA was used to synthesize the first-strand cDNA, using random primers. One microliter of 1:10-diluted first-strand cDNA reaction was used for real-time PCR, using primers and probe as described by Tatineni et al. (2010).

RT-PCR assay.

Total RNA was extracted from upper fully expanded wheat leaves and crowns, as described (McNeil et al. 1996). The first-strand cDNA was synthesized in a 10-μl reaction volume with random primers, as described previously (Tatineni et al. 2010).

One microliter of first-strand cDNA was used for PCR, in a 25-μl reaction volume with plus- and minus-sense CP-specific primers of WSMV (Tatineni et al. 2014) or TriMV (Bartels et al. 2016), with the following PCR program: 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 90 s and one cycle at 72°C for 10 min. The RT-PCR products were analyzed on 1.0% agarose gels in TAE buffer.

Data analyses.

Data analyses were performed using SAS software version 9.4 (SAS Institute Inc. Cary, NC, U.S.A.). Number of foci and foci size was tested for differences using PROC GLIMMIX with Poisson and normal distribution, respectively. Genomic RNA molecule data were log transformed and were tested for differences by using PROC GLIMMIX, but nontransformed data were reported. The LSMEANS statement was used to obtain least squares means and the Tukey-Kramer test at $P = 0.05$ was used for pairwise comparison of treatment means. Means and standard errors for numbers of foci, foci size, and genomic RNA molecules were obtained using the PROC MEANS.

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