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Satyanarayana Tatineni  
USDA-ARS, Satyanarayana.Tatineni@ars.usda.gov

Anthony J. McMechan  
University of Nebraska-Lincoln, justin.mcmechan@gmail.com

Gary L. Hein  
University of Nebraska-Lincoln, ghein1@unl.edu

Roy French  
University of Nebraska-Lincoln, Roy.French@ars.usda.gov

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Efficient and stable expression of GFP through *Wheat streak mosaic virus*-based vectors in cereal hosts using a range of cleavage sites: Formation of dense fluorescent aggregates for sensitive virus tracking

Satyanarayana Tatineni a,⁎, Anthony J. McMechan b, Gary L. Hein b, Roy French a

a United States Department of Agriculture–Agricultural Research Service, and Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, NE 68583, USA
b Department of Entomology, University of Nebraska-Lincoln, Lincoln, NE 68583, USA

Introduction

The use of viral vectors as gene-expression vehicles to transiently express specialty products in plants for pharmaceutical and biotechnological purposes and to examine the functions of plant genes by virus-induced gene silencing (VIGS) has been growing rapidly (for example, Arazi et al., 2002; Atkinson et al., 1998; Burch-Smith et al., 2004; Golovkin et al., 2007; Hammond-Kosack et al., 1995; Lim et al., 2010; Liu et al., 2002; Sablowski et al., 1995; Scofield and Nelson, 2009; Scofield et al., 2005; Yusibov et al., 1999; Zhang et al., 2010). Transient expression of foreign genes in plants through viral-vectors is considered to be an alternative or supplemental approach to stable transformation of plants because of the difficulty to transform some plant species. Several viruses infecting dicotyledonous plants were developed as transient expression vectors to express green fluorescent protein (GFP) or β-glucuronidase (GUS) as marker proteins (for example, Baulcombe et al., 1995; Dolja et al., 1992; Donson et al., 1991; Golimov et al., 2007; Copina et al., 2000; Roberts et al., 1997; Santa Cruz et al., 1996; Shivprasad et al., 1999; Zhang and Ghabrial, 2006). In contrast, only a few viruses have been modified to express foreign genes systemically in monocotyledonous plants (Choi et al., 2000; Haupt et al., 2001; Holzberg et al., 2002). Plant viral vectors with GFP or GUS as marker proteins were successfully used to monitor virus trafficking (Dolja et al., 1992; Golimov et al., 2007; Oparka et al., 1999; Roberts et al., 1997; Sudarshana et al., 1998) and to define the function(s) of viral genes (Dolja et al., 1994; Ju et al., 2005; Prokhnevsky et al., 2002). However, the intrinsic problem with viral-based vectors is the amount of foreign protein being produced and the stability of foreign genes in viral vectors in host plants (Gleba et al., 2007; Pogue et al., 2002; Scholthof et al., 1996). Thus, viral vectors with low levels of foreign gene expression and/or insert instability in plants are not useful for biotechnological and functional genomics purposes.

*Foot-and-mouth disease virus* (FMDV) of the family *Pota* is a single-stranded positive-sense RNA genome of 9384 nucleotides (nt) excluding the poly A tail at the 3' end (Stenger et al., 1998). The genomic organization of FMDV is similar to that of the members of the *Potyvirus* and *Rymovirus* genera with a single large open reading frame, or polypeptide. The polypeptide is processed into at least 10 mature proteins by three virus-encoded proteinases, P1, HC-Pro and Nla-Pro (Stenger et al., 1998). FMDV is semipersistently transmitted by *wheat curl mites* (*Accretosichella Keifer*) (Slykhuis, 1955). For **i**t is dispensable for systemic infection of wheat, but it has been identified as a viral determinant for mite transmission (Stenger et al., 2005a,b). Recently, P1 was identified as the suppressor of RNA silencing instead of HC-Pro, which is the silencing suppressor of members of the *Potyvirus* genus (Stenger et al., 2007; French, unpublished).
Previously, WSMV vectors were developed by introducing the NPTII or GUS cistrons between the Nil and CP cistrons, and the GUS cistron between the P1 and HC-Pro cistrons. Duplicated Nla-Pro heptapeptide cleavage sites were introduced to release NPTII or GUS proteins from the WSMV polyprotein (Choi et al., 2000, 2002). Although NPTII and GUS were expressed from WSMV in wheat, these vectors were unstable as less than full-length RT-PCR products were observed by 12 dpi (Choi et al., 2000). Moreover, WSMV with the GUS cistron failed to infect Zea mays inbred line SDp2; thus, GUS insertion affected the host range of WSMV (Choi et al., 2000, 2002). This suggests that the large size of the GUS cistron possibly affected replication efficiency and/or invisiveness of the virus in certain hosts, which prevents the study of virus–host interactions and virus movement in SDp2. Additionally, movement and trafficking of WSMV in wheat and other cereal hosts has not been examined thoroughly. There is no information on the role(s) of WSMV proteins in cell-to-cell or long-distance movement of the virus, except that mutations in the putative PIPO (Chung et al., 2008) affected movement of the virus with minimal affect on virus replication (Choi et al., 2005). Availability of a stable and efficient WSMV-GFP vector(s) will greatly enhance our abilities to examine virus–vector interactions, virus movement, trafficking, and virus–host interactions in monocotyledons. In this study, we engineered the cycle 3 GFP (GFP) cistron (Chaffie et al., 1994; Crameri et al., 1996) between the P1 and HC-Pro cistrons of WSMV with Foot-and-mouth disease virus (FMDV) catalytic peptides or with a range of Nla-Pro cleavage peptides of WSMV, and analyzed their efficiencies to release GFP from HC-Pro. We found that failure to cleave GFP from HC-Pro resulted in expression of GFP as a weak diffuse fluorescence exhibited with efficient cleavage of GFP from HC-Pro. We further examined the stability of WSMV-GFP vectors in wheat, wheat curl mite transmissibility of WSMV-GFP vectors, and the distribution of WSMV in wheat using a GFP-tagged virus.

Results

Vector construction

Previously, Choi et al. (2000, 2002) examined the expression of GUS at three different positions in the WSMV genome, and found that the GUS cistron inserted between the P1 and HC-Pro cistrons resulted in a relatively efficient expression vector. Thus, we chose to insert the GFP cistron between the P1 and HC-Pro cistrons in the genome of WSMV by fusing cleavage/catalytic peptides to the C-terminus of GFP. GFP would then be released from WSMV polyprotein by a cleavage at the N-terminus of GFP by WSMV P1 proteinase in cis and cleavage at the C-terminus of GFP by Nla-Pro in trans at an engineered homologous cleavage site, or by cotranslational cleavage using peptides derived from either FMDV 2A or 1D/2A. In this study, we fused WSMV Nla-Pro recognition cleavage peptide sequences representing those located between P3/6K1, 6K1/CI, Nla/Nlb, or Nlb/CP cistrons to the C-terminus of GFP. For convenience, we named the Nla-Pro recognition cleavage peptides based on their location in the WSMV genome: P3/6K1, 6K1/CI, Nla/Nlb, or Nlb/CP cleavage peptides/sites.

WSMV-GFP vector with FMDV 1D/2A or 2A cleaved GFP from HC-Pro and produced weak fluorescence in wheat

Initially, we inserted a GFP cistron with FMDV 1D/2A or 2A catalytic peptide sequence between the P1 and HC-Pro cistrons of the WSMV genome to avoid the use of homologous cleavage peptides that might affect vector stability (Gopinath et al., 2000). FMDV 2A is a short peptide of 18 aa that cleaves the FMDV polyprotein at the 2A/2B junction cotranslationally (Ryan et al., 1991). Cleavage takes place between a glycine residue at the C-terminus of 2A and a proline residue at the beginning of the FMDV 2B protein. Fusion of the 2A peptide plus a proline residue to GFP allowed processing of the polyprotein and release of 50 to 60% GFP in free form from plant viral vectors (Gopinath et al., 2000; Santa Cruz et al., 1996). However, the cleavage efficiency of the 2A peptide was greater than 99% when 2A was preceded by 14 aa residues from the C-terminus of FMDV 1D (1D/2A; Donnelly et al., 2001).

We fused sequences encoding FMDV 2A (TLNFDLLKLAGDVESNPGP) or 1D/2A (EARHKQIVAPVKQTLNFDLLKLAGDVESNPGP) peptides to the C-terminus of GFP and inserted these between the P1 and HC-Pro cistrons of WSMV to obtain WSMV-GFP-2A and WSMV-GFP-1D/2A, respectively (Fig. 1A). Because cleavage by 2A and 1D/2A peptides occurs between G/P residues of the 2A peptide, GFP proteins expressed from WSMV-GFP-2A and WSMV-GFP-1D/2A possess either 18 or 32 non-GFP amino acid residues at their C-termini, respectively (Fig. 1A). As a result, proline becomes the N-terminal amino acid of HC-Pro (Fig. 1A). Cleavage at the N-terminus of GFP will be mediated in cis by WSMV P1 proteinase and a glycine residue resulting from P1 protease cleavage will become the N-terminal amino acid of GFP (Fig. 1A).

In vitro transcripts from pSP6-WSMV-GFP-2A and pSP6-WSMV-GFP-1D/2A were inoculated to wheat seedlings at the single-leaf stage, inducing systemic chlorotic streaks at 5–9 dpi, followed by mosaic and mottling symptoms similar to those of wild-type virus (data not shown). These GFP constructs infected more than 90% of wheat seedlings, suggesting that insertion of GFP cistron with FMDV catalytic peptide sequences did not affect infection efficiency and symptom phenotype of WSMV. Expression of GFP was examined from infected wheat leaves at 9 and 14 dpi, and it was found that WSMV-GFP-1D/2A expressed GFP mostly as free protein with a few aggregate-like structures in wheat leaves based on the distribution of fluorescence (Fig. 1B, left panel), which was only slightly stronger than the chlorophyll fluorescence emitted from comparable wheat leaves infected with wild-type virus (Fig. 1B, top panel, left). In contrast, WSMV-GFP-2A expressed slightly brighter fluorescence, which appeared as free form as well as small aggregate-like fluorescent structures (Fig. 1B, middle panel).

Western blot analysis of total proteins from WSMV-GFP-1D/2A-infected wheat leaves using a GFP-specific monoclonal antibody (Clontech, Mountain View, CA) detected a 30 kDa protein, which was the expected size for GFP plus 32 aa resulting from the fused 1D/2A catalytic peptide (Fig. 1C, top panel, lane 1). Two minor proteins of 28.5 and 27 kDa were also observed (Fig. 1C, top panel, lane 1). The origin of these two minor proteins is not clear; however, these proteins might have been generated by proteolysis at the end of GFP and in the middle of the 1D/2A catalytic peptide. The ~70 kDa GFP:HC-Pro fusion protein was detected from longer exposures of X-ray films on immunoblots (data not shown). Based on the ratio of cleaved and uncleaved GFP from HC-Pro, the cleavage efficiency of 1D/2A catalytic peptide was estimated to be ~90%. Immunoblot analysis of total proteins from WSMV-GFP-2A-infected wheat detected ~70 kDa and 28.5 kDa GFP-specific proteins (Fig. 1C, top panel, lane 2). The 28.5 kDa protein was the expected size for GFP plus 18 aa of the 2A peptide, whereas the ~70 kDa protein is most likely the GFP:HC-Pro fusion protein resulting from incomplete cleavage of GFP from HC-Pro by the FMDV 2A catalytic peptide. We also found a minor protein of 27 kDa (Fig. 1C, top panel, lane 2), which might have been generated by proteolysis near the C-terminus of GFP. The efficiency of GFP cleavage from HC-Pro by 2A was estimated to be ~40 to 60% based on the ratio of ~70 kDa and 28.5 kDa proteins. Even though plants infected by WSMV-GFP-1D/2A and WSMV-GFP-2A accumulated similar amounts of P1 (Fig. 1C, middle panel), GFP accumulated differentially with 5 and 15 µg GFP per g fresh leaf tissue, respectively (Fig. 1C, top panel).

WSMV-GFP vectors with homologous Nla-Pro heptapeptide cleavage sequences failed to release GFP from HC-Pro and formed dense fluorescent aggregates

We next examined whether a GFP cistron fused with a range of Nla-Pro recognition cleavage peptides from WSMV would result in

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efficient expression of GFP in wheat. The heptapeptide cleavage sites of P3/6K1, 6K1/Cl, Nla/Nlb, or Nlb/CP were fused to the C-terminus of GFP in WSMV-GFP-P3/6K1(7aa), WSMV-GFP-6K1/Cl(7aa), WSMV-GFP-Nla/Nlb(7aa) and WSMV-GFP-Nlb/CP(7aa), respectively (Fig. 2A, a–d). Nla-Pro of WSMV should recognize predicted heptapeptide cleavage sequences in trans and cleavage was expected to occur between the 6th (P1 position) and 7th (P1′ position) amino acid of the heptapeptide sequence (Adams et al., 2005), leaving six amino acid residues at the C-terminus of GFP and the 7th amino acid at the N-terminus of HC-Pro (Fig. 2A, a–d). Possible homologous recombination due to duplicated cleavage peptide sequences in the WSMV genome, which could affect the stability of the GFP cistron, was
Fig. 2. WSMV-GFP vectors with homologous Nla-Pro heptapeptide cleavage sites failed to release GFP from HC-Pro and produced dense aggregate-like structures in wheat. A. Genomic organization of WSMV with GFP cistron between the P1 and HC-Pro cistrons. The expanded view of GFP cistron with the C-terminal and N-terminal junctions of P1 and HC-Pro, respectively, is indicated. The heptapeptide cleavage sequences of P1-6K1 (a), 6K1/Cl (b), Nla/Nlb (c), Nlb/CP (d), or reverse orientation of Nlb/CP (e) from WSMV were fused to the C-terminus of GFP, or GFP was fused to the N-terminus of HC-Pro (f). The cleavage sequences of P1 proteinase and Nla-Pro are indicated with bold and italic bold, respectively. The positions of P1 and NIa-Pro cleavages are indicated with arrows and arrowheads, respectively. All WSMV-GFP vectors in (A) are similarly expressed GFP, but representative pictures of GFP expression in wheat leaves infected with WSMV-GFP-6K1/CI(7aa) (B-1) and WSMV-GFP-Nlb/CP(R) (B-2 and B-3) at 14 dpi are shown. Wheat leaves viewed under stereo-fluorescence microscope (B-1, B-2 and B-4) and confocal laser scanning microscope (B-3). Expression of GFP in Nicotiana benthamiana leaves infiltrated with Agrobacterium cells harboring pCAM-WSMV-GFP-6K1/CI(7aa) at 7 dpi is shown in B-4. Bars represent 200 μm (B-1, B-2 and B-4) and 50 μm (B-3). C. Western blot hybridization of total proteins extracted from wheat leaves at 18 dpi with GFP (α-GFP) and WSMV P1 (α-WSMV P1) antibodies. Healthy (lane 1), WSMV (lane 2), 2.5 ng of GFP control (lane 3), WSMV-GFP-P3/6K1(7aa) (lane 4), WSMV-GFP-6K1/Cl(7aa) (lane 5), WSMV-GFP-Nla/Nlb(7aa) (lane 6), WSMV-GFP-Nlb/CP(7aa) (lane 7), WSMV-GFP-Nlb/CP(R) (lane 8), and WSMV-GFP/HC-Pro (lane 9). A Coomassie Brilliant Blue stained SDS-PAGE gel showing the large subunit of Rubisco protein of wheat was a control for the amount of protein loaded per well. M, protein standards. Note the free form of GFP produced similarly from WSMV-GFP vectors with or without a functional cleavage peptide.
minimized by changing at least one nt of each amino acid codon, wherever possible, in accordance with WSMV codon usage.

In vitro transcripts of WSMV-GFP-P3/6K1(7aa), WSMV-GFP-6K1/C(7aa), WSMV-GFP-Nla/Nlb(7aa) and WSMV-GFP-Nlb/CP(7aa) infected 90 to 100% wheat seedlings and produced chlorotic streaks/spots, mosaic and mottling symptoms similar to wild-type virus, indicating that insertion of GFP with heptapeptide cleavage sequences did not affect efficiency of infection or symptom phenotype in wheat. These four vectors accumulated GFP at abundant levels in wheat, which was found mostly as bright fluorescent aggregate-like structures with minimal amount of free form of GFP (Figs. 2, B-1). We next examined the expression profile of GFP from total proteins of infected wheat leaves by immunoblot analysis and found that all four vectors accumulated high levels of a ~70 kDa putative GFP:HC-Pro fusion protein and a minor 27 kDa free form of GFP at a ratio of 10 to 15:1 (Fig. 2C, top panel, lanes 4–7). Plants infected by these WSMV-GFP vectors produced 20 to 25 μg GFP per g fresh leaf tissue, which was more than GFP yields from plants infected by WSMV-GFP vectors with 1D/2A or 2A catalytic peptides.

To exclude the possibility that expression of GFP as aggregate-like structures in wheat might be due to a host-specific effect, we transferred WSMV-GFP-6K1/C(7aa) into pCAMBIA 1380 to obtain pCAM-WSMV-GFP-6K1/C(7aa). Agrobacterium cells harboring pCAM-WSMV-GFP-6K1/C(7aa) and S35-TuMV-HC-Pro (a suppressor of RNA silencing) were co-infiltrated into Nicotiana benthamiana leaves, and it was found that WSMV-GFP-6K1/C(7aa) expressed GFP as aggregate-like structures at 7 dpi similar to those in wheat leaves (Fig. 2B-4). These data suggested that expression of GFP as aggregate-like structures in wheat by WSMV-GFP vectors with heptapeptide cleavage sites is not host-specific, but is most likely due to accumulation of abundant levels of GFP:HC-Pro fusion protein as the result of inefficient cleavage by Nla-Pro.

We next examined the GFP expression profiles of WSMV-GFP with Nlb/CP heptapeptide cleavage site in reverse orientation [WSMV-GFP-Nlb/CP(R)] and with GFP fused directly to the N-terminus of HC-Pro ([WSMV-GFP-HC-Pro]) (Fig. 2A, e, f). These mutants infected wheat and expressed GFP mostly as aggregate-like structures similar to WSMV-GFP vectors with homologous heptapeptide cleavage sites (Fig. 2, B-2 and B-3; compare B-2 with B-1). Immunoblot analysis of total proteins from infected wheat showed that WSMV-GFP-Nlb/CP(R) and WSMV-GFP-HC-Pro accumulated ~70 kDa and 27 kDa proteins corresponding to a GFP:HC-Pro fusion protein and free GFP, similar to those with homologous cleavage sites (Fig. 2C, top panel, compare lanes 8 and 9 with 4–7). The low level accumulation of 27 kDa free form of GFP from these constructs might be due to proteolytic activity at the junction of GFP:HC-Pro as reported for Tobacco etch virus (TEV) (Dolja et al., 1998). Moreover, WSMV-GFP vectors with heptapeptide cleavage sites, WSMV-GFP-Nlb/CP(K), and WSMV-GFP-HC-Pro accumulated 40 kDa protein specific to WSMV P1 antiserum (Fig. 2C, middle panel), suggesting that cleavage occurred between the P1 and GFP as expected, and the ~70 kDa protein is the result of GFP:HC-Pro fusion.

Insertion of spacer amino acids at either side of heptapeptide cleavage site or a deletion in HC-Pro facilitated efficient cleavage of GFP from HC-Pro by Nla-Pro

Expression of GFP mostly as GFP:HC-Pro fusion with a range of heptapeptide cleavage sites indicated that WSMV Nla-Pro was poorly able to process these sites, perhaps because the surrounding GFP and/or HC-Pro sequences provide a suboptimal context and/or make heptapeptide cleavage sites inaccessible. To examine this possibility, we added one amino acid on either side of heptapeptide cleavage sites of 6K1/C and Nlb/CP in WSMV-GFP-6K1/C(9aa) and WSMV-GFP-Nlb/CP(9aa), respectively (Fig. 3A, a, b). We further added two amino acids on either side of Nlb/CP heptapeptide sequence in WSMV-GFP-Nlb/CP(11aa) (Fig. 3A, c). These three vectors infected wheat in a similar manner to vectors with heptapeptide cleavage sites and wild-type virus. WSMV-GFP vectors with one or two spacer amino acids on either side of the Nlb/CP cleavage peptide produced mostly free GFP with a few small aggregate-like structures in wheat leaves (Fig. 3B). In contrast, WSMV-GFP-6K1/C(9aa) with one spacer amino acid on either side of the 6K1/C cleavage site produced mostly large fluorescent aggregates (Fig. 3B).

Immunoblot analysis of total proteins from infected wheat leaves revealed that WSMV-GFP-6K1/C(9aa) accumulated GFP:HC-Pro fusion protein and free GFP at a ratio of 4:1, compared to 12:1 from WSMV-GFP-6K1/C(7aa), with no significant increase in accumulation of 27 kDa free GFP (Fig. 3C, top panel, compare lanes 1 and 2). A different GFP expression profile was observed from WSMV-GFP-Nlb/CP(9aa)– or WSMV-GFP-Nlb/CP(11aa)-infected wheat, in which the GFP:HC-Pro fusion protein and free GFP accumulated at a ratio of 1:9 (Fig. 3C, top panel, lanes 4 and 5). Taken together, these data suggested that the 6K1/C and Nlb/CP cleavage peptides with one spacer amino acid on either side of heptapeptide cleavage sites are differentially processed by Nla-Pro; thus, the number of spacer amino acids for optimal context of heptapeptide cleavage sites located between the 6K1/C and Nlb/CP are different. WSMV-GFP vectors with one or two spacer amino acids on either side of the Nlb/CP heptapeptide cleavage site accumulated 20 to 22 μg GFP per g fresh tissue, which was comparable to WSMV-GFP vectors without spacer amino acids.

The previously mentioned experiments suggested that spacer amino acids are required for optimal context of heptapeptide cleavage site for efficient cleavage by Nla-Pro. We next examined whether deletion in HC-Pro would increase the optimal context or accessibility of heptapeptide cleavage site for Nla-Pro processing. HC-Pro is not necessary for systemic infection of wheat (Stenger et al., 2005a), thus aa 17 to 148 were deleted in HC-Pro citron in WSMV-GFP-Nlb/CP (7aa) to obtain WSMV-GFP-Nlb/CP(7aa)–ΔNHC-Pro. Immunoblot analysis of total proteins from wheat leaves infected with WSMV-GFP-Nlb/CP(7aa)–ΔNHC-Pro suggested that Nla-Pro efficiently cleaved the GFP from HC-Pro and accumulated only free 27 kDa GFP (Fig. 3C, top panel, lane 9). The GFP:HC-Pro fusion protein (~70 kDa) failed to accumulate at detectable levels even with longer exposures of X-ray films on immunoblots. These data suggested that deletion in the N-terminus of HC-Pro facilitated efficient cleavage by Nla-Pro at the inserted Nlb/CP heptapeptide site at the C-terminus of GFP.

GFP:HC-Pro fusion protein formed insoluble aggregate-like fluorescent structures in wheat

We examined the nature of aggregate-like fluorescent structures formed in wheat by WSMV-GFP vectors by comparing the soluble and insoluble forms of GFP produced by WSMV-GFP-6K1/C(7aa) and WSMV-GFP-Nlb/CP(11aa), which expressed GFP mostly as aggregate-like structures and mostly as free-form of GFP, respectively (Fig. 4A). WSMV-GFP-6K1/C(7aa) accumulated GFP mostly as a GFP:HC-Pro fusion protein (~70 kDa) in 3K and 30K pellets, which contained soluble and membrane bound proteins, respectively (Fig. 4B, lanes 2 and 4). A small amount of GFP:HC-Pro and free GFP, which were detected in the soluble protein fraction (~30K supernatant) at a ratio of 1:1.5 (Fig. 4B, lane 3). The ratio between soluble and insoluble forms of GFP accumulated in all three fractions by WSMV-GFP-6K1/C(7aa) was 1:12 (Fig. 4B, compare lane 3 with lanes 2 and 4). In contrast, WSMV-GFP-Nlb/CP(11aa) accumulated more free GFP in the 30K supernatant (Fig. 4B, lane 3) and lesser amounts of GFP in the 3K and 30K pellets compared to corresponding fractions of WSMV-GFP-6K1/C(7aa) (Fig. 4, compare lanes 2 and 4 in B and B). The soluble and insoluble forms of GFP from all three fractions of WSMV-GFP-Nlb/CP(11aa)-infected wheat leaves accumulated at a ratio of 2:1 (Fig. 4B, compare lane 3 with lanes 2 and 4). These data suggested that aggregate-like
Fig. 3. Effect of spacer amino acids on either side of the heptapeptide cleavage site, or a deletion in HC-Pro on the release of GFP from HC-Pro by NIA-Pro. A. Schematic representation of WSMV genome with GFP cistron. An expanded view of GFP cistron in the WSMV genome with portions of P1 and HC-Pro cistrons is indicated below the genomic organization. Cleavage peptides with one spacer amino acid on either side of the heptapeptide cleavage sites of 6K1/CI (a) and Nib/CP (b), and two spacer amino acids on either side of the Nib/CP heptapeptide cleavage site (c) fused to the C-terminus of GFP. The cleavage sequence of P1 and NIA-Pro are indicated in bold and italic bold, respectively. The spacer amino acids are indicated with lower case letters. The positions of P1 and NIA-Pro cleavages are indicated with arrows and arrowheads, respectively. B. Expression of GFP in wheat leaves at 14 dpi as indicated. Bars represent 200 μm. C. Western immunoblot analysis of total proteins with a GFP-specific monoclonal antibody from wheat leaves at 18 dpi. WSMV-GFP-6K1/CI(7aa) (lane 1), WSMV-GFP-6K1/CI(9aa) (lane 2), WSMV-GFP-Nib/CP(7aa) (lane 3), WSMV-GFP-Nib/CP(9aa) (lane 4), WSMV-GFP-Nib/CP(11aa) (lane 5), 2.5 ng of GFP control (lane 6), WSMV (lane 7), healthy (lane 8), and WSMV-GFP-Nib/CP(7aa)ΔN/HC-Pro (lane 9). A Coomassie Brilliant Blue stained SDS-PAGE gel showing the large subunit of Rubisco protein of wheat was a control for the amount of protein loaded per well. Lane M, protein standards.
fluorescent structures formed by WSMV-GFP vectors are due to excessive accumulation of insoluble GFP:HC-Pro fusion protein.

Stability of WSMV-GFP vectors in wheat

The stability of introduced GFP cistron in a series of WSMV-GFP vectors [WSMV-GFP-1D/2A, WSMV-GFP-2A, WSMV-GFP-P3/6K1 (7aa), WSMV-GFP-6K1/CI(7aa), WSMV-GFP-Nla/Nlb(7aa), WSMV-GFP-Nlb/CP(7aa), WSMV-GFP-Nlb/CP(9aa), WSMV-GFP-Nlb/CP (11aa) and WSMV-GFP-Nlb/CP(R)] in wheat plants was examined by two methods: 1. The progeny viruses derived from wheat leaves infected with in vitro transcripts were passaged serially in wheat at 14 dpi intervals for seven passages. At each passage, total RNA was isolated and examined for the integrity of GFP sequences by RT-PCR. 2. Wheat plants inoculated with in vitro transcripts at the single-leaf stage were incubated in a greenhouse, and total RNA was extracted at 30-day intervals until 120 dpi. The presence of intact GFP cistron in the WSMV genome was examined by RT-PCR.

We performed reverse transcription with random primers (Promega Corporation, Madison, WI), followed by PCR with oligonucleotides W-211 (corresponding to nts 1021 to 1048) and W-212 (complementary to nts 1249 to 1223). These primers flank the GFP cistron in the 3′ end of the P1 cistron and in the 5′ end of the HC-Pro cistron (Fig. 5A). RT-PCR amplification would result in a ~950 bp product with intact GFP in the WSMV genome and a 229 bp product from the wild-type virus (Fig. 5A). RT-PCR analysis of total RNA from passages 1 through 7 from wheat plants infected with WSMV-GFP vectors obtained a product of ~950 bp, which was the expected size for the presence of an intact GFP cistron in the WSMV genome (Fig. 5B). No other minor PCR products were detected from these samples. RT-PCR analyses of total RNA from wheat plants infected with WSMV-GFP vectors at 30, 60, 90, and 120 dpi yielded a PCR product of ~950 bp, but minor amounts of less than full-length RT-PCR products were only observed from WSMV-GFP-2A and WSMV-GFP-Nlb/CP(7aa)-infected plants at 120 dpi (Figs. 5C and D, lanes 2 and 6). All WSMV-GFP vectors expressed GFP fluorescence continuously in wheat at 120 dpi (data not shown). Thus, WSMV-GFP vectors
are remarkably stable for a long period of time as revealed by two independent assays. We did not keep WSMV-GFP vector-infected wheat plants beyond 120 dpi because the average life span of wheat is around 120 days.

Stability of WSMV-GFP vectors in wheat is not due to their ability to compete effectively with wild-type-like recombinants. This possibility was analyzed by examining the ability of WSMV-GFP vectors to compete with wild-type virus. Wheat seedlings were co-inoculated with wild-type virus plus one of the following WSMV-GFP vectors: WSMV-GFP-6K1/CI(7aa), WSMV-GFP-NIb/CP(7aa), or WSMV-GFP-NIb/CP(11aa). Expression of GFP and the presence of GFP insert in singly and doubly infected wheat leaves were examined at 9 dpi. We found abundant levels of GFP expression from singly infected wheat (Fig. 6B), but not from co-infected plants (Fig. 6C). RT-PCR analysis revealed that, as expected, a ~950 bp product was obtained from singly inoculated plants, whereas only RT-PCR products corresponding to the wild-type virus (229 bp) were obtained from co-infected plants (Fig. 6D). Thus, WSMV-GFP vectors were apparently unable to compete with wild-type virus in co-infection experiments. These data suggested that the stability of WSMV-GFP vectors in wheat plants is not due to a competitive advantage over wild-type-like recombinants.

Host range of WSMV-GFP vectors

The stability of WSMV-GFP vectors could be due to their ability to compete effectively with wild-type-like recombinant viruses with deletions in GFP cistron. This possibility was analyzed by examining the ability of WSMV-GFP vectors to compete with wild-type virus. Wheat seedlings were co-inoculated wild-type virus plus one of the following WSMV-GFP vectors: WSMV-GFP-6K1/CI(7aa), WSMV-GFP-Nl/CP(7aa), or WSMV-GFP-Nl/CP(11aa). Expression of GFP and the presence of GFP insert in singly and doubly infected wheat leaves were examined at 9 dpi. We found abundant levels of GFP expression from singly infected wheat (Fig. 6B, lane 1), but not from co-infected plants (Fig. 6C). RT-PCR analysis revealed that, as expected, a ~950 bp product was obtained from singly inoculated plants, whereas only RT-PCR products corresponding to the wild-type virus (229 bp) were obtained from co-infected plants (Fig. 6D). Thus, WSMV-GFP vectors were apparently unable to compete with wild-type virus in co-infection experiments. These data suggested that the stability of WSMV-GFP vectors in wheat plants is not due to a competitive advantage over wild-type-like recombinants.
Fig. 6. WSMV-GFP vectors failed to compete with wild-type virus in wheat. Progeny virus derived from WSMV-GFP-6K1/Cl(7aa), WSMV-GFP-Nlb/CP(7aa) or WSMV-GFP-Nlb/CP(11aa)-infected wheat leaves was inoculated either singly or co-inoculated with wild-type virus. Representative pictures of expression of GFP from WSMV-GFP-6K1/Cl(7aa) infection (B) and co-infection with wild-type virus (C) at 9 dpi are given, and WSMV-infected wheat at 9 dpi was used a negative control (A). Note the lack of GFP expression in co-infected wheat leaves. Bars represent 200 μm. (D) RT-PCR analysis of total RNA extracted from wheat leaves infected either singly (lanes 1, 3, and 5) or co-infected with wild-type virus (lanes 2, 4, and 6) at 9 dpi. Lanes 1 and 2: WSMV-GFP-6K1/Cl(7aa) and co-infection with WSMV; lanes 3 and 4: WSMV-GFP-Nib/CP(7aa) and co-infection with WSMV; lanes 5 and 6: WSMV-GFP-Nlb/CP(11aa) and co-infection with WSMV; lane 7: WSMV; lane 8: healthy; and lane 9: water control. RT-PCR products were separated through 1.0% agarose gel in 1× TAE buffer. M: DNA size markers.

Fig. 7. Host range of WSMV-GFP vectors. The test plants were mechanically inoculated with progeny virus derived from wheat leaves infected with in vitro transcripts of WSMV-GFP-6K1/Cl(7aa), WSMV-GFP-Nib/CP(7aa), or WSMV-GFP-Nlb/CP(11aa) at a 1:10 dilution. Systemically infected leaves from host plants expressing GFP due to the infection of WSMV-GFP-6K1/Cl(7aa) are given as representative of WSMV-GFP vectors. Bars represent 200 μm.
wheat plants infected with WSMV-GFP-6K1/CI(7aa). Large, bright plants. Fluorescent structures were observed by confocal microscopy in different parts of wheat: stem, roots, awns, lemma, and florets (Figs. 8B–G). Large amounts of GFP fluorescence were found on the surface of the stem at 14 dpi (Figs. 8B and B†). Examination of cross-sections of the stem from an 18 dpi plant consisting of leaf sheaths and young leaves revealed that the center youngest leaf was mostly free of GFP fluorescence and the outer leaf sheaths contained large amounts of GFP (Figs. 8C and C†). GFP fluorescence was also detected in roots with brighter fluorescence in rootlets and their tips compared to the main secondary roots (Figs. 8D and D†). Examination of heads of spring wheat at 45 to 50 dpi revealed the presence of GFP fluorescence in the awns and lemma of florets (Figs. 8E, F, and F†). GFP fluorescence was found in large amounts in the basal portions of florets and fluorescence was also found in anther filaments, style and ovary (Figs. 8G and G†). Wheat seeds from 80 to 90-day old spring wheat contained a large amount of fluorescence in seed coats, but not in the endosperm (data not shown). Collectively, WSMV was shown to be distributed in most wheat tissues examined with the exception of the seed endosperm.

Discussion

Previously, Choi et al. (2000) developed WSMV-based vectors to transiently express GUS and NPTII in wheat. In the present study, we modified the WSMV genome to express GFP using FMDV catalytic peptides and homologous P3/6K1, 6K1/Cl, Nla/Nib and Nib/CP cleavage peptides. WSMV-GFP-vector with the FMDV 1D/2A catalytic peptide expressed mostly free GFP while the 2A peptide mediated only partial release of GFP from HC-Pro. Even though WSMV-GFP-1D/2A and WSMV-GFP-1A accumulated similar amounts of P1 (Fig. 1C, middle panel) and CP (data not shown) in wheat, accumulation of GFP was much lower by the former virus. Because WSMV utilizes a polypeptide as a genome expression strategy, the final individual proteins from WSMV polypeptide should yield equimolar amounts. Thus, it is probable that free form of GFP expressed from WSMV-GFP-1D/2A is subject to increased proteolytic turnover due to the presence of 1D peptide sequence at the C-terminus. Similarly, expression of Beet yellows virus CP in a TEV vector also resulted in less than equimolar protein accumulation, which was attributed to protein misfolding and destabilization (Dolja et al., 1998).

WSMV-GFP vectors with homologous Nla-Pro heptapeptide cleavage sites unexpectedly expressed GFP as aggregate-like fluorescent structures, with abundant levels of a ~70 kDa protein and a 27 kDa minor protein. Detection of P1 protein of the predicted size (40 kDa) from these constructs (Fig. 2C, middle panel), suggested that the ~70 kDa GFP-specific protein detected in immunoblots was a GFP:HC-Pro fusion protein. Moreover, WSMV-GFP vectors with Nib/CP heptapeptide cleavage sequence in reverse orientation or with GFP/HC-Pro fusion expressed free GFP and GFP:HC-Pro fusion proteins similar to those with homologous heptapeptide cleavage sites. This implies that WSMV-GFP vectors with heptapeptide cleavage sites were poorly processed to release GFP from HC-Pro.

In previous studies, it has been shown that the Nib/CP heptapeptide is sufficient for cleavage of Gus from WSMV HC-Pro (Choi et al., 2002) and release of free NPTII from WSMV CP (Choi et al., 2000). The minimal size of Nla-Pro cleavage sites of other potyviruses also have been identified through mutational analyses and sequence alignments as heptapeptides (Adams et al., 2005; Carrington and Dougherty, 1988; Carrington et al., 1988), and heptapeptide Nla-Pro cleavage sites were used to express foreign genes from potyvirus-based viral vectors (Dolja et al., 1998; Seo et al., 2009). On the other hand, sequence outside a heptapeptide of Plum pox virus has been shown to affect cleavage by Nla-Pro (Garcia et al., 1992). Thus, it is possible that the introduced WSMV heptapeptide cleavage sites at the C-terminus of GFP might be either inaccessible and/or in suboptimal contexts for

Table 1
Wheat curl mite transmission assays of Wheat streak mosaic virus (WSMV) isolate Sidney 81 bearing the GFP cistron between the P1 and HC-Pro cistrons.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Transmission to test plants*</th>
<th>Total # of plants infected/total # of plants inoculated</th>
<th>% wheat curl mite transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source plant 1a</td>
<td>Source plant 2</td>
<td>Source plant 3</td>
<td></td>
</tr>
<tr>
<td>WSMV</td>
<td>10/10</td>
<td>9/10</td>
<td>6/10</td>
</tr>
<tr>
<td>WSMV-GFP-Nib/CP(R)†</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>WSMV-GFP-Nib/CP(7aa)</td>
<td>2/10</td>
<td>0/10</td>
<td>1/1</td>
</tr>
<tr>
<td>WSMV-GFP-Nib/CP(9aa)</td>
<td>7/10</td>
<td>4/10</td>
<td>2/10</td>
</tr>
<tr>
<td>WSMV-GFP-Nib/CP(11aa)</td>
<td>2/10</td>
<td>6/10</td>
<td>4/10</td>
</tr>
<tr>
<td>No virus</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Number of test plants infected/number of test plants inoculated with a single mite transferred from each source plant. The source and test plants were assayed for WSMV-GFP vector infection and for the presence of GFP insert by RT-PCR and GFP fluorescence.
† The source plants were mechanically inoculated with respective progeny virus derived from transcript-inoculated wheat plants.
‡ Reverse orientation of Nla-Pro cleavage site located between Nib/CP cistrons.

Expression of GFP in different parts of wheat plants

Even though WSMV-GFP-6K1/Cl(7aa) (producing mostly aggregate-like punctate fluorescent structures) and WSMV-GFP-Nib/CP(11aa) (producing mostly free GFP) accumulated similar amounts of GFP in wheat at 20 to 25 μg per g fresh tissue, the GFP expressed from WSMV-GFP-6K1/Cl(7aa) was found to be a sensitive marker protein for virus trafficking in plants. Moreover, GFP expression from WSMV-GFP-6K1/Cl(7aa) can be readily detected and easily distinguished from background fluorescence of WSMV-infected or healthy wheat plants.

GFP expression in different parts of wheat was examined using wheat plants infected with WSMV-GFP-6K1/Cl(7aa). Large, bright fluorescent structures were observed by confocal microscopy in mesophyll cells of infected wheat leaves at 14 dpi (Figs. 8A and A†). WSMV-GFP fluorescence was further observed with a stereo epifluorescence microscope in different parts of wheat: stem, roots, awns, lemma, and florets (Figs. 8B–G). Large amounts of GFP fluorescence were found on the surface of the stem at 14 dpi (Figs. 8B and B†). Examination of cross-sections of the stem from an 18 dpi plant consisting of leaf sheaths and young leaves revealed that the center youngest leaf was mostly free of GFP fluorescence and the outer leaf sheaths contained large amounts of GFP (Figs. 8C and C†). GFP fluorescence was also detected in roots with brighter fluorescence in rootlets and their tips compared to the main secondary roots (Figs. 8D and D†). Examination of heads of spring wheat at 45 to 50 dpi revealed the presence of GFP fluorescence in the awns and lemma of florets (Figs. 8E, F, and F†). GFP fluorescence was found in large amounts in the basal portions of florets and fluorescence was also found in anther filaments, style and ovary (Figs. 8G and G†). Wheat seeds from 80 to 90-day old spring wheat contained a large amount of fluorescence in seed coats, but not in the endosperm (data not shown). Collectively, WSMV was shown to be distributed in most wheat tissues examined with the exception of the seed endosperm.
processing by Nla-Pro. Consistent with this idea, WSMV-GFP vectors with one or two spacer amino acids on either side of the NIb/CP heptapeptide cleavage site efficiently released GFP from polyprotein, which resulted in ~70 kDa GFP:HC-Pro fusion protein and 27 kDa free GFP at a ratio of 1:9. However, one spacer amino acid on either side of the 6K1/CI heptapeptide did not significantly increase the cleavage efficiency, suggesting that the minimum number of spacer amino acids differ among NIA-Pro recognition sites. Alternatively, the heptapeptide cleavage site of 6K1/CI, a poorly characterized cleavage site, might not be efficiently processed by NIA-Pro; thus, addition of spacer amino acids did not improve the cleavage efficiency of GFP cleavage from HC-Pro by NIA-Pro. Changing the context of the NIb/CP cleavage site by deleting a portion of HC-Pro also resulted in efficient release of free GFP. Taken together, these data suggest that GFP and/or HC-Pro sequences in the polyprotein might have blocked the accessibility of heptapeptide cleavage sequence inserted between the GFP and HC-Pro cistrons, and thus failed to be recognized by NIA-Pro.

Subcellular fractionation of proteins from wheat leaves infected with WSMV-GFP vectors producing aggregates of GFP:HC-Pro revealed that proteins reacting with GFP antibody were mostly in an insoluble form or associated with membranes, whereas nearly all free GFP was in the soluble protein fraction. As a consequence, plants infected with WSMV-GFP-Nlb/CP(11aa) produced mainly free GFP and had a diffuse fluorescence under UV light, while plants infected with WSMV-GFP-6K1/CI(7aa) produced GFP:HC-Pro fluorescent aggregates. This bright punctate fluorescence was readily observed with an epi-fluorescence dissecting microscope.

WSMV-based vectors with intact GFP remained as the major component of the population after seven serial passages and at 120 dpi, which was significant considering the roughly 120 day life span of wheat. Citrus tristeza virus (CTV)-based vectors expressing GFP were stable for more than 5 years after infection of citrus trees by being able to efficiently compete with recombinants arising in infected citrus plants (Folimonov et al., 2007). In contrast, the stability of WSMV-based vectors reported in this study was likely not due to their ability to compete with the recombinants because WSMV-GFP vectors did not accumulate in plants co-infected with wild-type virus. These data suggest that the mechanism of stability of WSMV-based vectors in wheat is different from CTV in citrus trees. It is possible that the smaller proportion of recombinants observed at 120 dpi might overtake WSMV-based vector eventually, if wheat plants survive beyond that time frame. In contrast, a WSMV-vector with an insertion

Fig. 8. Detection of GFP fluorescence in different parts of the wheat plant. Wheat seedlings were inoculated with progeny virus derived from wheat leaves infected with in vitro transcripts of WSMV-GFP-6K1/CI(7aa) (A–G) or WSMV (A′–D′) or healthy (E–G′). A and A′: Confocal laser scanning microscope pictures showing the expression of GFP as large aggregate-like fluorescent structures in wheat leaves at 14 dpi. Bars represent 50 μm. B and B′: Wheat stem surface view with top leaf sheath removed at 14 dpi showing abundance of fluorescence. C and C′: Stem cross section with GFP fluorescence in outer leaf sheaths at 14 dpi. Note that the young leaf located in the center of the stem is mostly free from GFP fluorescence. D and D′: GFP fluorescence in roots at 14 dpi. Note the bright fluorescence at root tips (arrow). GFP fluorescence in awns (E and E′), lemma (F and F′), and floret (G and G′) of spring wheat cv. ID 0580 at 45–50 dpi. Note the abundant GFP fluorescence in basal part of floret, and the GFP fluorescence in the anther filament and stigma. Bars represent 200 μm.
of the GUS cistron between Nb/CP was unstable in wheat as less than full-length RT-PCR products were detected by 12 dpi (Choi et al., 2000). Thus, either the location (between P1 and HC-Pro vs between Nb and CP) or the size of the foreign gene (GUS, 1800 nts vs GFP, 720 nts) might play important roles in the stability of WSMV-based vectors.

Insertion of foreign genes in viruses can have considerable effects on virus biology, replication and movement in different host plants. For example, *Tobacco mosaic virus* with a GFP insertion failed to move systematically in tobacco, a propagation host, and affected symptom severity in *N. benthamiana* plants (Shivprasad et al., 1999). WSMV with a GUS insertion failed to infect *Z. mays* inbred line Sdp2 (Choi et al., 2000). In contrast, WSMV-GFP vectors infected all of the cereal hosts examined with a 1–5 day delay and elicited symptoms similar to those of the wild-type virus. These data suggest that GFP insertion in WSMV did not significantly affect the ability of virus to move systematically in different cereal hosts.

Additionally, WSMV-GFP vectors retained wheat curl mite transmissibility. The efficiency of mite transmission increased with increasing amounts of free GFP produced by WSMV-GFP vectors, which would also affect the amount of free HC-Pro being produced. Mite transmission efficiency increased from 0% with WSMV-GFP vectors containing the Nb/CP cleavage peptide in reverse orientation to 10% with the heptapeptide cleavage site, to 40% with 1 or 2 spacer amino acids on either side of the Nb/CP heptapeptide cleavage site. When compared to 83% mite transmission of wild-type virus, the reduced mite transmission rates of WSMV-GFP vectors with 1 or 2 spacer amino acids on either side of the Nb/CP heptapeptide cleavage site might be due to a substantial amount of GFP:HC-Pro fusion products being produced by these constructs, which proportionately limits the amount of free HC-Pro produced. These data suggest that efficiency of wheat curl mite transmission is correlated with the amount of free HC-Pro being produced by WSMV-GFP vectors, which further supports the role of HC-Pro in mite transmission (Stenger et al., 2005b, 2006). The availability of differentially mite transmissible WSMV-GFP vectors would allow a wide range of experiments such as the use of non-transmissible WSMV-GFP vectors to express specialty products in fields, and examining virus-mite interactions under greenhouse conditions using mite transmissible WSMV-GFP vectors.

The availability of GFP-tagged WSMV provides a powerful tool to examine viral replication, movement and trafficking, as well as virus-host and virus-vector interactions. Recently, by using WSMV-GFP-6K1/CI(7aa), we identified the N-terminal region of CP involved in strain- and host-specific long-distance movement (Tatineni et al., 2011). Moreover, the availability of a series of WSMV-based vectors with a range of catalytic/cleavage peptides producing different levels of free and GFP:HC-Pro fusion proteins would provide useful tools for examining the biological properties of foreign proteins in cereal hosts. The WSMV-based vectors can also be used as a VIGS vector to examine viral gene functions because WSMV infects a wide range of cereal hosts.

**Materials and methods**

**Construction of GFP vectors**

pSP6-WSMV, an infectious cDNA clone of WSMV isolate Sidney 81 (Choi et al., 1999), was the basis of all constructs used in this study. The cycle 3 GFP cistron (Cramer et al., 1996) with a catalytic or cleavage peptide sequence at its 3′ end was precisely inserted into the WSMV genome between the P1 and HC-Pro cistrons by overlap extension PCR (Ho et al., 1989) of three individual PCR fragments with 18 to 21 bp overlapping sequences. PCR 1 contains the SP6 RNA polymerase sequence, followed by the 5′ end of WSMV sequence through the end of the P1 cistron, plus the first six codons of GFP. PCR 2 contains the last six codons of P1, followed by GFP cistron with a catalytic or cleavage sequence, plus the first seven codons of HC-Pro. PCR 3 contains the last six or seven codons of GFP, followed by a catalytic or cleavage site, plus the HC-Pro cistron through nt 3961. The three PCR fragments were amplified with Pfu Ultra II Fusion HS DNA polymerase (Agilent Technologies Inc., Santa Clara, CA). The PCR 1 fragment was common for all WSMV-GFP constructs, whereas the PCR 2 and 3 will differ from each other in the catalytic or cleavage site located at the 3′ and 5′ ends of the PCR 2 and PCR 3 fragments, respectively. The overlap extension PCR was performed using gel-isolated PCR 1, 2, and 3 products as templates with a pair of oligonucleotides, W-124 (with NgoMIV restriction site followed by SP6 RNA polymerase sequence and nts 1 to 32 of WSMV) and W-1 (complementary to nts 3961 to 3927). The overlap extension PCR was carried out with Herculase II Fusion DNA polymerase (Agilent Technologies Inc.). The overlap extension PCR products were digested with NgoMIV and AvfI restriction enzymes, and ligated into similarly digested pSP6-WSMV (NgoMIV was introduced upstream of SP6 RNA polymerase sequence and AvfI sites was located at nt 3905 in WSMV genome). The positive clones for the GFP cistron were screened for the presence of an additional NcoI site located in the GFP cistron.

**pSP6-WSMV-GFP-1D/2A and pSP6-WSMV-GFP-2A**

The plus- and minus-sense primers encoding the last seven codons of cycle 3 GFP, followed by 32 aa codons of FMDV 1D/2A or 18 aa codons of FMDV 2A plus a proline residue, and the first seven amino acids of HC-Pro were designed to amplify the PCR 2 and PCR 3 fragments. The overlap extension PCR of PCR 2 and PCR 3 products was performed using Herculase II Fusion DNA polymerase (Agilent Technologies Inc., Santa Clare, CA). The PCR 1 fragment was common for all WSMV-GFP constructs, whereas the PCR 2 and 3 products were digested with NgoMIV and AvfI restriction enzymes, and ligated into similarly digested pSP6-WSMV between NgoMIV and AvfI restriction sites to obtain pSP6-WSMV-GFP-1D/2A and pSP6-WSMV-GFP-2A, respectively.

**pSP6-WSMV-GFP-P3/6K1(7aa), pSP6-WSMV-GFP-6K1/CI(7aa), pSP6-WSMV-GFP-Nla/Nib(7aa), and pSP6-WSMV-GFP-Nib/CP(7aa)**

Seven amino acid codons of P3/6K1, 6K1/CI, Nla/Nib, or Nib/CP cleavage peptides were fused to the 3′ end of GFP cistron by overlap extension PCR as described earlier. The plus and minus-sense primers encoding the last seven amino acid codons of GFP, followed by seven amino acid codons of cleavage peptides located between the P3/6K1, 6K1/CI, Nla/Nib, or Nib/CP cistrons and the first seven amino acid codons of HC-Pro were used to amplify PCR 2 and PCR 3 fragments. The overlap extension PCR products containing the GFP with heptapeptide cleavage sites were ligated into pSP6-WSMV between NgoMIV and AvfI restriction sites to obtain pSP6-WSMV-GFP vectors with respective heptapeptide cleavage sites.

**pSP6-WSMV-GFP-Nib/CP(R) and pSP6-WSMV-GFP/HC-Pro**

The reverse orientation of Nib/CP heptapeptide was fused to the 3′ end of GFP cistron by overlap extension PCR in pSP6-WSMV-GFP-Nib/CP(R). In another construct oligonucleotides were designed such that the GFP cistron was fused to the 5′ end of HC-Pro in pSP6-WSMV-GFP/HC-Pro. The overlap extension PCR products were ligated into pSP6-WSMV between NgoMIV and AvfI restriction sites as described earlier.

**pSP6-WSMV-GFP-6K1/CI(9aa)**

The nt sequence encoding for KFNCEYQSG amino acids was fused to the 3′ end of GFP cistron by overlap extension PCR in pSP6-WSMV-GFP-Nib/CP(R). The plus and minus sense primers with nt sequence encoding the last six amino acids of GFP, followed by nt sequence encoding EQYCVYESS or GEQYCVYESSE, and the first seven amino acids of HC-Pro were used to amplify PCR 2 and PCR 3 fragments. The overlap extension PCR was performed using PCR 1, PCR 2 and PCR 3 as templates, and ligated into pSP6-WSMV between the NgoMIV and AvfI

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sites to obtain pSP6-WSMV-GFP-N1byCP(9aa) and pSP6-WSMV-GFP-N1byCP(11aa).

The 5′ and 3′ junctions of GFP in the WSMV genome were verified by sequencing plasmid DNAs at the University of Florida ICBR Core DNA Sequencing Facility using an Applied Biosystems 3730 model sequencer.

Plasmid DNA isolation, in vitro transcription and inoculation of wheat seedlings

Plasmid DNAs were prepared from an overnight-grown Escherichia coli culture at 30 °C using the Bio-Rad Plasmid Midi prep kit (Bio-Rad, Hercules, CA) as per the manufacturers' instructions. Transcription reaction was carried out in a 40 μl reaction volume consisting of 1.0 μg of NotI-linearized plasmid DNA, 40 mM Tris–HCl, pH 7.9, 20 mM DTT, 8.5 mM MgCl₂, 2 mM spermidine, 1.2 mM concentration of each of ATP, CTP, UTP, and Cap analog (m7G[5′]ppp[5′]G; Epicentre Biotechnologies, Madison, WI), 0.048 mM GTP, 20 U of rRNasin ribonuclease inhibitor (Promega) and 50 U of SP6 RNA polymerase (Epicentre Biotechnologies), and 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 transcribed on 1.0% native agarose gel in 1× TAE buffer.

Wheat seedlings (12 to 18 per transcription reaction) at the single-leaf stage were inoculated with freshly prepared in vitro transcripts that were mixed with an equal volume of 2% sodium pyrophosphate, pH 9.0 containing 1% baked celite. Inoculated wheat seedlings were incubated in a greenhouse with 24 to 30 °C max and 18 to 20 °C min temperatures with a 14–16 h photoperiod and observed for symptom development from 5 dpi onwards. Three independent clones per each WSMV-GFP vector were inoculated to wheat, and the results of one representative clone per construct were presented.

Detection of GFP fluorescence in wheat

Fully expanded upper leaves from plants infected with WSMV-GFP vectors at 9 and 14 dpi were used to examine the expression of GFP. Expression of GFP in different parts of wheat was examined with a Zeiss Stereo Discovery V12 Fluorescence Microscope (Carl Zeiss MicroImaging, Inc., New York, NY) using GFP narrow band filters for stem, leaves, stems, roots and heads or with FITC long pass filters for stem cross sections and flowers. The GFP fluorescence pictures were taken using AxioCam MRc5 camera attached to a V12 Fluorescence Microscope. Wheat leaves infected with WSMV-GFP vectors were also observed under an Olympus FluView 500 confocal laser scanning microscope (Olympus America Inc., Center Valley, PA).

Protein extraction and Western immunoblot analysis

Total proteins were isolated from 18 dpi wheat leaves by freezing 0.5 g tissue in liquid nitrogen and macerating into a fine powder, and adding 3 ml of TPE buffer (50 mM Tris-acetate pH 7.4, 10 mM potassium acetate, 1 mM EDTA, 5 mM DTT) containing 1 Complete Mini Protease Inhibitor Cocktail tablet (Roche, Indianapolis, IN) per 10 ml of TPE buffer. Thoroughly ground macerate was mixed with an equal volume of 2× sample buffer (100 mM Tris–Cl pH 6.8, 4% SDS, 20% glycerol, 5% β-mercaptoethanol, and 0.2% bromophenol blue), boiled for 3 min, and centrifuged at 16,000 g for 3 min. The supernatant containing the total proteins was stored at −20 °C until used for Western blot analysis.

The TPE macerate was further fractionated by centrifuging 1.0 ml at 3000 g for 15 min at 4 °C. The resulting supernatant was centrifuged at 30,000 g for 30 min to separate soluble proteins in the supernatant from membrane bound proteins in the pellet. The supernatant was diluted with an equal volume of 2× sample buffer, and the pellet was suspended in 0.5 ml of 1× sample buffer. The 3000 g pellet, containing cellular debris and insoluble material, was washed with 1.0 ml of TPE buffer and re-centrifuged at 3000 g. This final pellet was suspended in 1.0 ml of 2× sample buffer. All protein samples were boiled for 3 min and stored at −20 °C.

Proteins were separated on 4–20% gradient Tris-Glycine-SDS polyacrylamide gels (Invitrogen, Carlsbad, CA), followed by Coomassie Brilliant Blue R 250 staining or transferred onto PVDF membranes using iBlot dry blotting system (Invitrogen) followed by immunoblotting. The membranes were blocked with 5% (w/v) nonfat dry milk powder and probed either with GFP-specific monoclonal antibody (Clontech) at 1:10,000 dilution or with WSMV P1 antiserum raised against a P1-specific peptide at 1: 5000 (unpublished). Either anti-mouse (for GFP antibody) or anti-rabbit (for P1 antiserum) HRP conjugate was used at 1:50,000 dilution as secondary antibodies. Immobilon Western Chemiluminescent HRP substrate was used as per instructions (Millipore, Billerica, MA). The SDS–PAGE showing the large subunit of Rubisco protein of wheat was used as a control for the amount of protein loaded per well for immunoblot analyses. The amount of GFP accumulated in wheat leaves infected by WSMV-GFP vectors was quantified by including known amounts of GFP (BD, Franklin Lakes, NJ) in immunoblots, and GFP-specific bands on X-ray films (BioMax Light Film, Kodak, Rochester, NY) were quantified with the Molecular Imager ChemiDoc XRS system (Bio-Rad).

Mite transmission assays

The transmissibility of selective WSMV-GFP vectors by wheat curl mites was performed as described (Stenger et al., 2005b), except the use of a single mite per test plant. Wheat seedlings inoculated at the two-leaf stage with progeny virus prepared from in vitro transcript-inoculated wheat leaves were used as the source plants at 8 dpi for mite transmission. The source plants were kept in individual cages and infested with ~10 mites per source plant from an avirulent mite colony. The plants were allowed a 3-week period for further propagation of mites and inoculum acquisition. Three source plants were used per virus, and 10 two-week old wheat seedlings per source plant were used to transfer single mites, and a total of 30 test seedlings were used per virus. The test plants were kept in individual cages and incubated in a growth chamber for 3–4 weeks. The number of mites found on test plants was rated under the microscope, and the presence of GFP was observed under Discovery V12 Fluorescence microscope as described earlier. The wild-type WSMV and water-inoculated wheat seedlings were used as positive and negative controls, respectively. The test plants were also examined by RT-PCR for the presence of GFP cistron.

RT-PCR analysis

Total RNA was extracted from wheat leaves essentially as described by McNeil et al. (1996). One μg of total RNA was used to perform reverse transcription with random primers in a 20 μl reaction with AMV reverse transcriptase (Roche) (0.5 U per μl reaction volume). One μl of cDNA was used to perform PCR with oligonucleotides W-211 and W-212 in a 25 μl reaction with Herculase II Fusion DNA polymerase (Agilent Technologies Inc.). Ten μl of PCR reaction was analyzed through 1.0% agarose gel electrophoresis in 1× TAE buffer.

Acknowledgments

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References


