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Il-Ryong Choi
University of Nebraska - Lincoln

Drake C. Stenger
University of Nebraska - Lincoln

Thomas Jack Morris
University of Nebraska - Lincoln, jmorris1@unl.edu

Roy C. French
University of Nebraska - Lincoln, rfrench2@unl.edu

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TECHNICAL ADVANCE

A plant virus vector for systemic expression of foreign genes in cereals

Il-Ryong Choi[1], Drake C. Stenger[2], T. Jack Morris[1] and Roy French[2,*]
[1]*School of Biological Sciences and*
[2]*USDA-ARS, 344 Keim Hall, University of Nebraska, Lincoln, NE 68583, USA*

Received 15 February 2000; revised 24 May 2000; accepted 24 May 2000.
*For correspondence (fax +1 402 472 2853; e-mail rfrench@unlnotes.unl.edu).

Summary

Inserts bearing the coding sequences of NPTII and β-glucuronidase (GUS) were placed between the nuclear inclusion b (NIb) and coat protein (CP) domains of the wheat streak mosaic virus (WSMV) polyprotein ORF. The WSMV NIb-CP junction containing the nuclear inclusion a (NIa) protease cleavage site was duplicated, permitting excision of foreign protein domains from the viral polyprotein. Wheat, barley, oat and maize seedlings supported systemic infection of WSMV bearing NPTII. The NPTII insert was stable for at least 18–30 days post-inoculation and had little effect on WSMV CP accumulation. Histochemical assays indicated the presence of functional GUS protein in systemically infected wheat and barley plants inoculated with WSMV bearing GUS. The GUS constructs had greatly reduced virulence on both oat and maize. RT–PCR indicated that the GUS insert was subject to deletion, particularly when expressed as a GUS–NIb protein fusion. Both reporter genes were expressed in wheat roots at levels comparable to those observed in leaves. These results clearly demonstrate the utility of WSMV as a transient gene expression vector for grass species, including two important grain crops, wheat and maize. The results further indicate that both host species and the nature of inserted sequences affect the stability and expression of foreign genes delivered by engineered virus genomes.

Keywords: virus gene expression vector, cereals, monocotyledonous plants.

Introduction

Several viruses infecting dicotyledonous plants have been modified to express foreign genes. Uses of plant virus gene vectors include interferon production (De Zoeten *et al.*, 1989), antigen display (Fitchen *et al.*, 1995; Joelson *et al.*, 1997; Koo *et al.*, 1999; Porta *et al.*, 1994; Sugiyama *et al.*, 1995; Turpen *et al.*, 1995; Usha *et al.*, 1993; Wigdorovitz *et al.*, 1999; Yusibov *et al.*, 1997), antibody production (McCormick *et al.*, 1999; Verch *et al.*, 1998), virus movement assays with reporter genes (Angell and Baulcombe, 1995; Baulcombe *et al.*, 1995; Casper and Holt, 1996; Chapman *et al.*, 1992; Dolja *et al.*, 1992; Scholthof *et al.*, 1993), gene silencing (Angell and Baulcombe, 1999; Baulcombe, 1999; Ruiz *et al.*, 1998), alteration of biochemical pathways (Kumagi *et al.*, 1993; Kumagi *et al.*, 1995; Kumagi *et al.*, 1998), functional characterization of pathogen avirulence genes and host resistance genes (Hammond-Kosack *et al.*, 1995; Rommens *et al.*, 1995; Tobais *et al.*, 1999), and various gain-of-function assays (Baulcombe *et al.*, 1993; Karrer *et al.*, 1998; Ryabov *et al.*, 1998; Scholthof *et al.*, 1995; Whitham *et al.*, 1999). Insert instability and loss of systemic movement were recurrent problems requiring significant effort for their solution, as exemplified in studies with gene vectors based on tobacco mosaic virus (TMV) (Culver *et al.*, 1993; Dawson *et al.*, 1989; Donson *et al.*, 1991; Hamamoto *et al.*, 1993; Kearney *et al.*, 1993; Shivprasad *et al.*, 1999; Takamatsu *et al.*, 1990).

Despite these advances, there is currently no suitable virus vector for systemic expression of foreign genes in monocotyledonous plants. Two modified RNA plant viruses, brome mosaic virus (BMV) (French *et al.*, 1986) and barley stripe mosaic virus (BSMV) (Joshi *et al.*, 1990), efficiently express reporter genes in monocotyledonous

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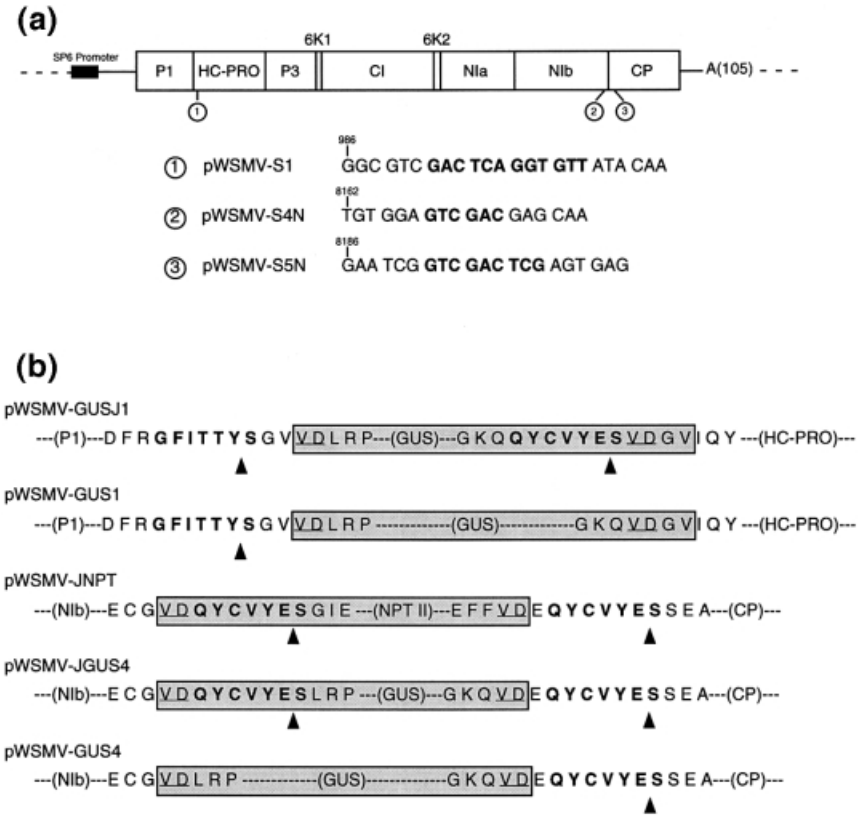

Figure 1. Construction of WSMV gene vectors.
(a) Positions (1,2,3) in the WSMV genome selected for insertion of 6–12 nucleotides (bold) containing engineered *Sal*I cleavage sites (GTCGAC) in constructs pWSMV-S1, pWSMV-S4N and pWSMV-S5N. (b) WSMV gene vector constructs bearing foreign sequences (shaded boxes) inserted at position 1 (pWSMV-GUSJ1 and pWSMV-GUS1) or position 2 (pWSMV-JNPT, pWSMV-JGUS4 and pWSMV-GUS4). Predicted viral protease cleavage sites are in bold with arrows denoting the location of the cleaved peptide bond. Underlined amino acids (VD) denote amino acid residues encoded by the inserted *Sal*I recognition sequence. Additional amino acid residues remaining at the N- or C-termini of reporter proteins after proteolytic processing and not present in native NPT II or GUS proteins are identified. Constructs pWSMV-JGUS1, pWSMV-JNPT and pWSMV-JGUS4 have viral protease cleavage sites flanking the reporter gene such that the foreign protein is completely excised from the viral polyprotein. Constructs with only a single protease cleavage site adjacent to the foreign protein result in fusion proteins where GUS remains as a C-terminal fusion with the NIb protein (pWSMV-GUS4) or as an N-terminal fusion with the HC-Pro protein (pWSMV-GUS1).

plant cells but do not move systemically in whole plants. Similarly, modified DNA genomes of the geminiviruses wheat dwarf virus and maize streak virus express foreign genes in monocotyledonous plant cells (Laufs *et al.*, 1990; Lazarowitz *et al.*, 1989; Matzeit *et al.*, 1991; Palmer *et al.*, 1999; Shen and Hohn, 1994; Shen and Hohn, 1995; Timmermans *et al.*, 1992; Ugaki *et al.*, 1991), but likewise fail to spread systemically.

The single ORF of bamboo mosaic virus satellite RNA (satBaMV) may be replaced to express a foreign gene when co-inoculated with its helper virus into *Chenopodium quinoa* (Lin *et al.*, 1996). Unfortunately, satBaMV and its helper virus have a narrow host range which includes only two monocotyledonous species (bamboo and barley). Although a foreign gene product delivered to barley by satBaMV was detected in systemically infected leaves, the level of expression was only 1/40th to 1/100th of that in inoculated leaves (Lin *et al.*, 1996).

Given the limitations of existing viral vectors to express foreign genes in monocotyledonous plants, we examined the potential of wheat streak mosaic virus (WSMV) as a gene vector. WSMV infects many grasses, including several key cereal crop species, and is readily mechanically transmissible. Recently, an infectious clone of WSMV has been constructed (Choi *et al.*, 1999), so that experiments to insert foreign sequences into the WSMV genome are now feasible. WSMV is the type member of the newly recognized genus *Tritimovirus*, with a genome organization typical of the family *Potyviridae* (Stenger *et al.*, 1998). We reasoned that the WSMV genome could be modified to accept foreign genes in a fashion similar to that demonstrated for the potyvirus, tobacco etch virus (TEV) (Dolja *et al.*, 1992; Dolja *et al.*, 1997; Dolja *et al.*, 1998; Whitham *et al.*, 1999). In this report, we describe the development of a WSMV-based gene vector capable of systemic expression of foreign genes in wheat and other cereals.

Results

Genomic location for insertion of foreign genes in WSMV

Three positions in the WSMV genome were targeted to accept foreign sequences by the insertion of a *Sal*I cleavage site (Figure 1a). Position 1 is immediately downstream of the predicted *cis* cleavage site of the P1 protein for excision from the viral polyprotein, and is the analogous position used in gene vectors based on TEV (Dolja *et al.*, 1992). Position 2 is located immediately upstream of the nuclear inclusion a (NIa) protease cleavage site at the nuclear inclusion b (NIb)–coat protein (CP) junction, whereas position 3 is located immediately downstream of this same proteinase cleavage site (Figure 1a). RNAs transcribed from pWSMV-S1, pWSMV-

S4N, and pWSMV-S5N (containing *Sal*I insertions at positions 1, 2 and 3, respectively) were inoculated onto wheat seedlings. RNA transcripts of pWSMV-S1 and pWSMV-S4N were infectious and produced systemic symptoms resembling those of wild-type RNA transcripts of pACYC-WSMV (Choi *et al.*, 1999), whereas RNA transcribed from pWSMV-S5N was not infectious (data not shown). Although the WSMV genome tolerated the small *Sal*I insert at position 1, attempts to insert β-glucuronidase (GUS) sequences (constructs pWSMV-GUSJ1 and pWSMV-GUS1) did not yield infectious transcripts (data not shown). Therefore, the WSMV gene vector constructs pWSMV-JNPT, pWSMV-JGUS4 and pWSMV-GUS4 (Figure 1b) described below used pWSMV-S4N as the cloning vector for insertion of foreign gene sequences.

NPT II expression in cereals

Wheat seedlings inoculated with RNA transcribed from pWSMV-JNPT expressed detectable NPT II protein (Figure 2a) as early as 6 days post-inoculation (dpi). Accumulation of NPT II protein in wheat peaked at 12 dpi (averaging 238 ng mg^{-1} soluble protein, or about 11 μg g^{-1} fresh leaf tissue), and by 18 dpi the amount of NPT II protein detected in infected wheat plants was reduced slightly to an average of 204 ng mg^{-1} soluble protein. The highest NPT II protein level observed in a single wheat plant extract was approximately 300 ng mg^{-1} soluble protein, or 14 μg g^{-1} leaf tissue. Barley, oat and maize seedlings inoculated with RNA transcribed from pWSMV-JNPT and assayed 3–7 days after the appearance of systemic symptoms also produced substantial amounts of NPT II protein (Figure 2b). Barley and oat plants contained levels of NPT II protein (averages of 176 and 201 ng mg^{-1} soluble protein, respectively) that were about the same as that observed in wheat, whereas in maize (averaging 78 ng mg^{-1} soluble protein) the level of NPT II protein accumulation was about 40% of that found in the other cereals.

NPT II protein, produced in wheat inoculated with RNA transcribed from pWSMV-JNPT, migrated in a polyacrylamide gel slightly more slowly than the commercially prepared and purified NPT II protein standard (Figure 3a). No NPT II protein was detected in uninoculated plants or in plants inoculated with wild-type WSMV (pACYC-WSMV) or WSMV bearing only the introduced *Sal*I cloning site (pWSMV-S4N). The N- and C-terminal residues of NPT II protein encoded by pWSMV-JNPT transcripts are different from those of the native protein. NPT II produced by pWSMV-JNPT is predicted to have serine replacing methionine at the N-terminus and nine additional C-terminal amino acid residues not present in native NPT II protein (Figure 1b). Thus, the slightly reduced electrophoretic mobility of NPT II protein produced in plants infected with pWSMV-JNPT transcripts was

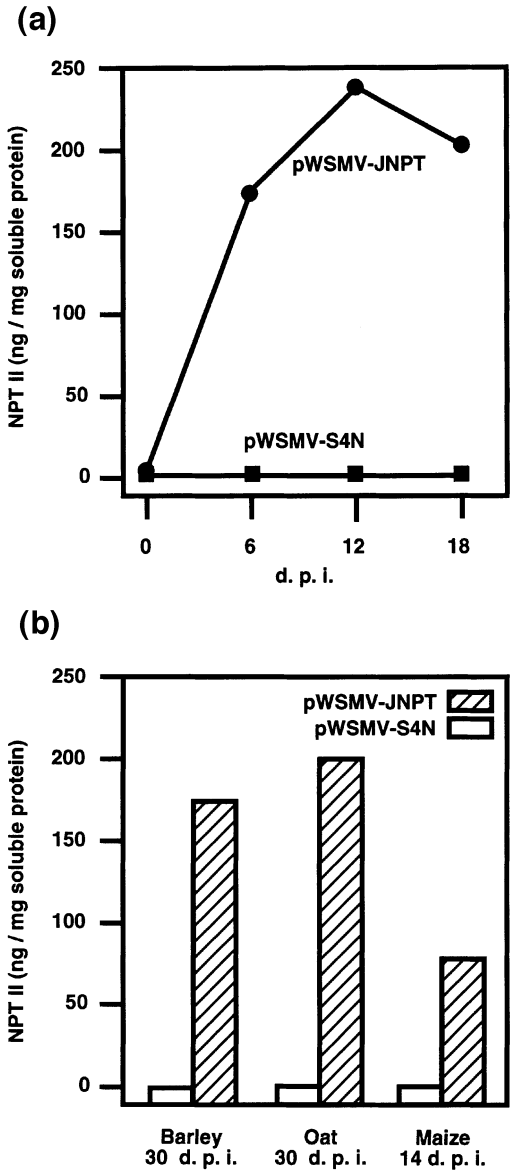

Figure 2. Accumulation of NPT II protein in plants systemically infected with WSMV bearing (pWSMV-JNPT) or lacking (pWSMV-S4N) the NPT II gene.
(a) Accumulation of NPT II protein in soluble protein samples extracted from wheat plants 0, 6, 12 and 18 days post inoculation (dpi). (b) Accumulation of NPT II protein in soluble protein samples extracted from barley, oats and maize at a single time point 3–7 days after the appearance of systemic symptoms. Concentration of NPT II protein present in soluble protein samples was determined by ELISA using commercially prepared and purified NPT II protein as a standard. NPT II concentrations presented are the mean of three plants sampled for each treatment

expected, and indicates that the WSMV NIa protease cleavage sites flanking NPT II sequences in pWSMV-JNPT were recognized and cleaved by NIa to release the foreign protein domain from the viral polyprotein.

Stability of the virus gene vector derived from pWSMV-JNPT was investigated by RT–PCR of total RNA samples

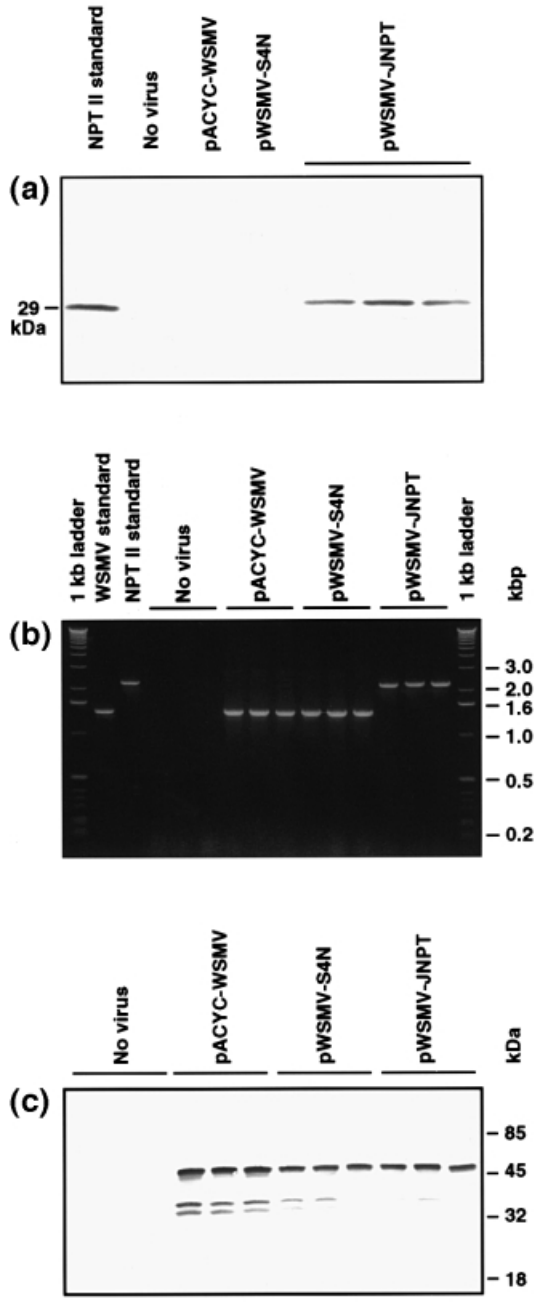

Figure 3. Analysis of wheat plants systemically infected with WSMV bearing or lacking the NPT II gene.
Wheat seedlings were inoculated with RNA transcribed from pACYC-WSMV (wild-type), pWSMV-S4N (WSMV modified to contain a *Sal*I site at the NIb–CP junction) or pWSMV-JNPT (WSMV bearing the NPT II coding sequence flanked by NIa protease cleavage sites and inserted into pWSMV-S4N). The 'no virus' treatment represents extracts from uninoculated wheat plants. (a) An immunoblot of total soluble protein samples extracted from wheat and probed with NPT II antibody. The NPT II standard (29 kDa) is commercially prepared and purified NPT II protein. (b) RT–PCR of RNA samples extracted from wheat plants 18 days post-inoculation. WSMV and NPT II standards represent PCR products derived from pACYC-WSMV and pWSMV-JNPT plasmid DNA templates, respectively. The sizes (kbp) of 1 kb ladder DNA standards are indicated on the right. (c) An immunoblot of total soluble protein samples extracted from wheat and probed with WSMV CP antibody. The sizes (kDa) and mobilities of protein standards are indicated on the right.

from wheat extracted 18 dpi (Figure 3b). RT–PCR of pWSMV-JNPT-infected wheat consistently yielded a product identical in size to a PCR product derived from pWSMV-JNPT plasmid DNA, indicating that the viral vector retained NPT II sequences upon systemic infection. RT–PCR analysis of samples extracted from wheat 6 and 12 dpi yielded the same products (data not shown) as at 18 dpi. Importantly, barley and oat plants infected with pWSMV-JNPT continued to produce NPT II protein at 30 dpi (Figure 2b).

The effect of inserted NPT II sequences on viral gene expression was assessed by immunoblotting of the WSMV CP (Figure 3c). Accumulation of WSMV CP in wheat plants 18 dpi with RNA transcribed from pWSMV-JNPT was only slightly less than for wild-type WSMV, and was essentially the same as for WSMV bearing the engineered *Sal*I site. Wheat plants extracted 6 and 12 dpi (data not shown) gave similar results.

GUS expression in cereals

A time-course study in wheat (Figure 4a) demonstrated the presence of functional GUS protein in plants inoculated with RNA transcripts of WSMV modified either to express GUS as a fusion protein with NIb (pWSMV-GUS4), or bearing GUS flanked by NIa cleavage sites for its complete excision from the viral polyprotein (pWSMV-JGUS4). Histochemical staining revealed that pWSMV-JGUS4 expressed GUS earlier and produced higher levels of functional GUS protein than did pWSMV-GUS4. This suggested that expression was more efficient when GUS was cleanly excised from the viral polyprotein, rather than remaining fused to the C-terminus of the NIb protein. Interestingly the spatial pattern of GUS expression mimicked the typical yellow–green mosaic symptoms of WSMV infection in wheat.

Expression of functional GUS protein in other cereal species was evaluated by histochemical staining (Figure 4b). Barley supported systemic expression of functional GUS protein 15 dpi with the pWSMV-JGUS4 gene vector. In contrast, GUS expression in oat was limited to clusters of cells at infection foci detected 3 dpi (Figure 4b). No GUS activity was detected in oat leaves assayed at 9 dpi (data not shown), indicating that GUS expression was both localized and transitory in this host. In maize, GUS activity was not observed at any time post-inoculation with pWSMV-JGUS4 (Figure 4b and data not shown).

Unlike the NPT II-bearing constructs, WSMV gene vectors bearing GUS tended to be unstable. RT–PCR analysis indicated that the accumulation of GUS sequences in wheat was both delayed and reduced for pWSMV-GUS4 relative to pWSMV-JGUS4 (Figure 5a). Furthermore, less than full-length RT–PCR products were observed for

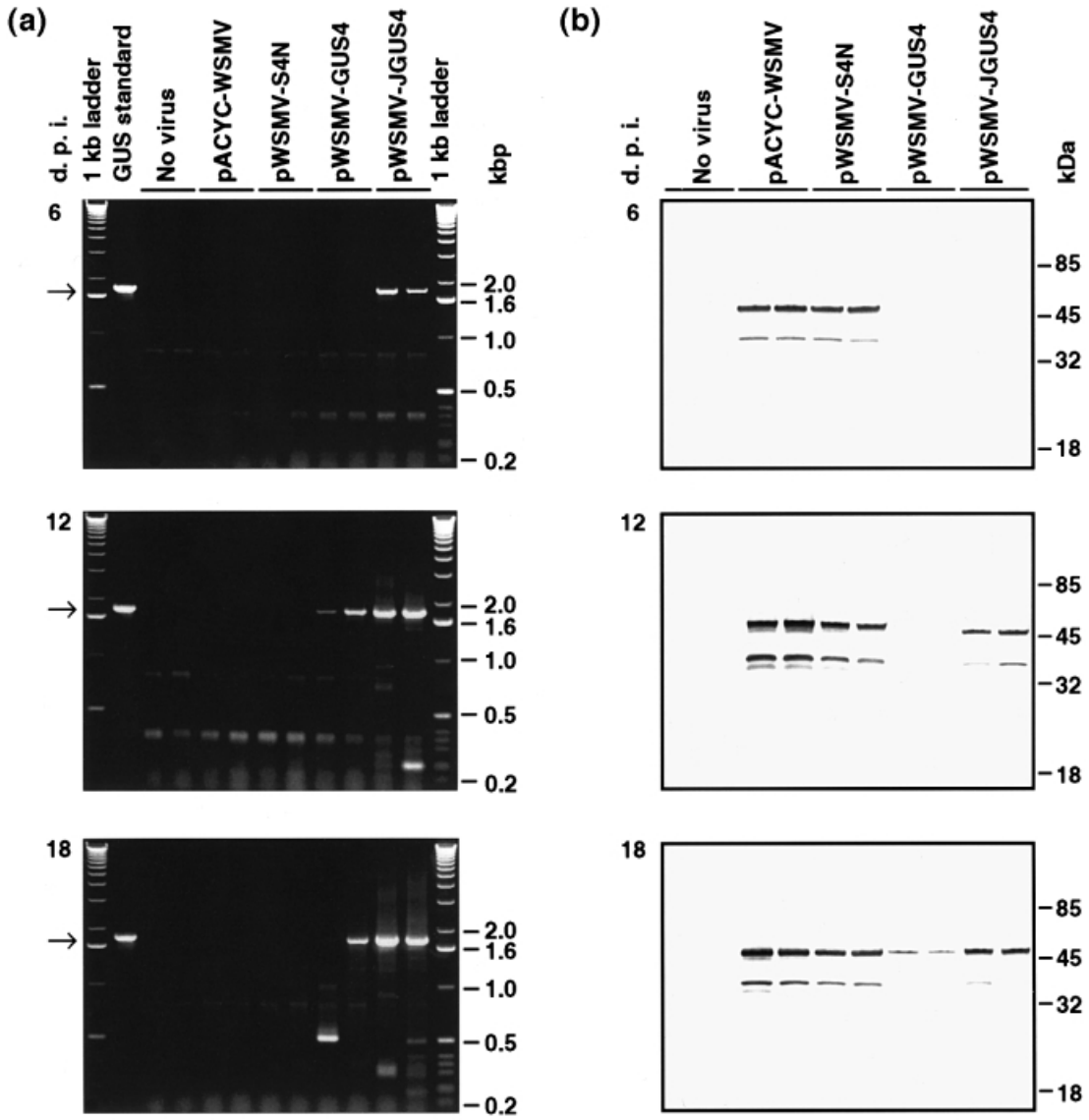

Figure 5. Analysis of wheat plants systemically infected with WSMV constructs bearing the GUS gene.
Wheat seedlings were inoculated with RNA transcribed from pWSMV-S4N (WSMV modified to contain a *Sal*I site at the NIb–CP junction), pWSMV-GUS4 (WSMV bearing the GUS coding sequence inserted into pWSMV-S4N to produce an NIb–GUS fusion protein), or pWSMV-JGUS4 (WSMV bearing the GUS coding sequence flanked by NIa protease cleavage sites and inserted into pWSMV-S4N). (a) RT–PCR of RNA samples extracted from wheat plants 6, 12 and 18 days post-inoculation (dpi). The GUS standard represents PCR product derived from pWSMV-JGUS4 plasmid DNA template, and the mobility of the expected full-length product is indicated with an arrow. The sizes (kbp) of 1 kb ladder DNA standards are indicated on the right. (b) Immunoblots of total soluble protein samples extracted from wheat plants at 6, 12 and 18 dpi and probed with WSMV CP antibody. The sizes (kDa) and mobilities of protein standards are indicated on the right.

the GUS-bearing constructs at both 12 and 18 dpi (Figure 5a), indicating spontaneous deletion of GUS sequences. The insertion of GUS sequences into the WSMV genome also affected CP expression (Figure 5b). Accumulation of CP in plants infected with either GUS construct was delayed and reduced, relative to wild-type virus or virus bearing only the *Sal*I cloning site. Attenuation of CP accumulation was most pronounced when GUS was expressed as a fusion with the NIb protein.

Expression of reporter genes in roots

Roots from systemically infected wheat plants inoculated with pWSMV-JGUS4 or pWSMV-JNPT were evaluated for GUS or NPT II expression 12 dpi. GUS activity was discontinuous and confined primarily to the vascular bundle region of the root stele (Figure 6). GUS activity was absent from root epidermis and root hairs, and was rarely observed in the root cortex. GUS activity was

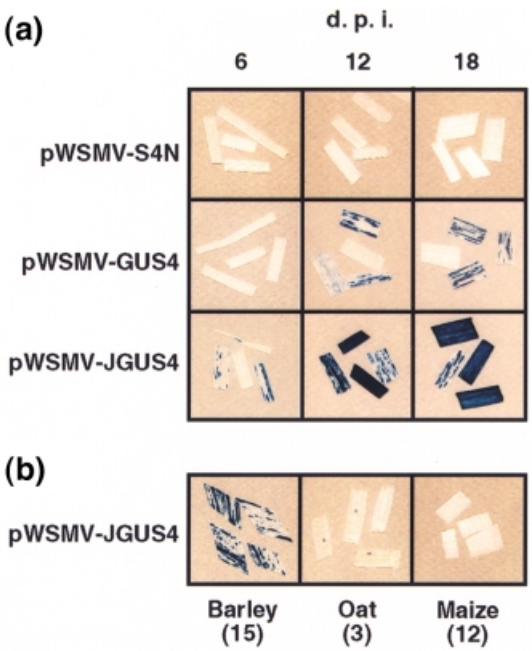

Figure 4. Histochemical detection of GUS protein activity in leaves of cereal species.
The blue colour indicates tissues containing GUS activity. (a) The results of a time-course experiment in wheat assayed for GUS activity at 6, 12 and 18 days post-inoculation. Wheat seedlings were inoculated with RNA transcribed from pACYC-WSMV (wild-type), pWSMV-S4N (WSMV modified to contain a *Sal*I site at the NIb–CP junction), pWSMV-GUS4 (WSMV bearing the GUS coding sequence inserted into pWSMV-S4N to produce an NIb–GUS fusion protein) or pWSMV-JGUS4 (WSMV bearing the GUS coding sequence flanked by NIa protease cleavage sites and inserted into pWSMV-S4N). (b) GUS activity observed in other cereal species inoculated with RNA transcribed from pWSMV-JGUS4. The number in parentheses indicates sampling time (days post-inoculation).

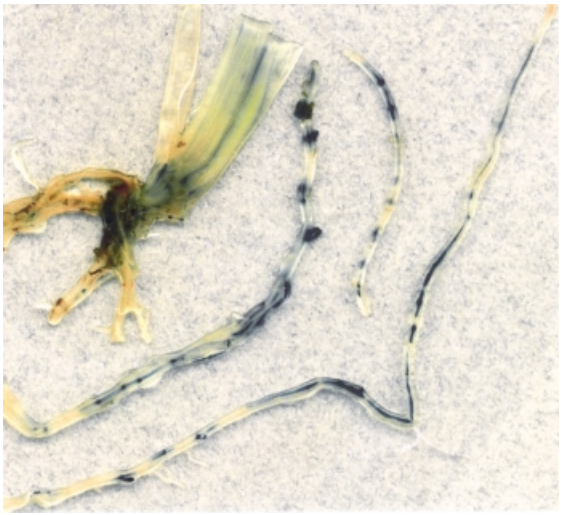

Figure 6. Histochemical detection of GUS protein activity in wheat roots and crown.
Wheat seedlings were inoculated with RNA transcribed from pWSMV-JGUS4. Tissues were assayed at 12 dpi.

Table 1. NPT II accumulation (ng mg^{-1} soluble protein) in wheat tissues 12 dpi with RNA transcribed from pWSMV-JNPT

Tissue	Non-inoculated plant	Inoculated plant 1	Inoculated plant 2	Inoculated plant 3
Leaf	7	208	233	452
Root	15	331	406	783

particularly prominent in regions in close proximity to lateral root primordia, as observed for TEV (Dolja *et al.*, 1992). Low levels of GUS activity were associated with tissues near the crown of the plant. NPT II was also expressed in roots. Although NPT II levels in roots (mean 507 ng mg^{-1} soluble protein) exceeded those observed in leaves (mean 298 ng mg^{-1} soluble protein) of the same plants (Table 1), soluble protein yields from roots were approximately 10% of those routinely recovered from leaves. Thus, on a fresh weight basis, more NPT II protein was present in leaves.

Discussion

Tolerance of foreign gene sequences by the WSMV genome

Our results indicate that choice of foreign sequence insertion site within the WSMV genome is an important consideration for expression vector development. Insertion of additional bases encoding a *Sal*I site was tolerated at positions 1 and 2, but not position 3 (Figure 1a). Upon insertion of gene-length sequences, only position 2 yielded viable transcripts. The WSMV polyprotein contains at least six other proteolytic cleavage sites which could serve as additional locations for insertion of foreign sequences. It is possible that one or more of these other locations might express foreign genes more efficiently, but this requires further experimentation. The sequential pattern of proteolytic processing of the WSMV polyprotein is not yet known, and predicted cleavage sites have yet to be experimentally confirmed. Nevertheless, cleaved forms of NPT II and CP were produced from pWSMV-JNPT, implying that the amino acid motif 'QYCVYES' does in fact encompass the NIb–CP cleavage site.

Considerable host effects on foreign gene expression were also noted. This could be due to differences in a wide range of virus–host interactions, such as translational efficiency, rate of virus replication, or requirements for systemic virus spread, among many other possibilities. Host differences in foreign gene expression were more pronounced with the larger GUS gene than for the NPT II

gene. Similarly, inserted NPT II sequences were more stable than GUS when expressed in wheat, the most permissive host found in this study.

Potential uses and limitations of WSMV-based gene vectors

We have demonstrated that the WSMV genome may be engineered to systemically express foreign genes in two major crop species, namely wheat and maize, as well as in two other cereals. As the host range of WSMV includes other monocotyledonous species, WSMV may prove useful as a gene vector in other grasses for which transformation protocols are lacking or poorly developed. However, given the differences in foreign gene expression and stability of different gene constructs in the same plant species, or the same gene construct in different species, the usefulness of WSMV as a gene vector will vary on a case by case basis. However, this is probably true for all RNA virus-based vector systems (Shivprasad *et al.*, 1999).

The restriction of GUS activity to specific root tissues suggests that WSMV movement functions are unable to efficiently move the virus beyond the endodermis surrounding the vascular system. This implies that wheat roots have either structural or physiological mechanisms to prevent movement of WSMV in root tissues external to the stele. We have not yet examined whether WSMV, or gene expression vectors based on WSMV, infect floral tissues. However, as WSMV is only infrequently seed-transmitted (Hill *et al.*, 1974), it is anticipated that WSMV gene vectors would not be suitable for ectopic expression of foreign genes in embryonic tissues.

The level of foreign gene expression obtained with the WSMV gene vector is similar to that reported for TEV, another potyviral gene expression vector (Dolja *et al.*, 1998). In systemically infected wheat leaves, WSMV normally accumulates to about 75 $\mu g\,g^{-1}$ leaf tissue (White and Brakke, 1983) which might be considered the upper limit for gene expression with this vector. The amount of NPT II protein accumulating in wheat was substantial, approaching one-fifth of this maximum level. Nevertheless, viral vectors based on higher-titre viruses such as potato virus X or TMV clearly remain the systems of choice in cases where production of high concentrations of foreign proteins, or epitope-tagged virions, is required. However, if the goal is to produce useful levels of foreign proteins in graminacious hosts, the WSMV gene expression vector described here provides an effective new research tool. The WSMV-based vectors should be particularly useful for applications to determine the biological properties of foreign proteins by gain-of-function assays, without the need for plant transformation. It should also find utility in applying functional genomics approaches to cereals.

Experimental procedures

WSMV gene vector construction

Six to 12 nucleotides containing the restriction cleavage site for *Sal*I were inserted in different locations of the WSMV cDNA in pACYC-WSMV (Choi *et al.*, 1999) by site-directed mutagenesis to create pWSMV-S1 (insertion of 5′-GACTCAGGTGTT-3′ at nt 892), pWSMV-S4N (insertion of 5′-GTCGAC-3′ at nt 8168) and pWSMV-S5N (insertion of 5′-GTCGACTCG-3′ at nt 8192), respectively (Figure 1a). DNA fragments of the gene for β-glucuronidase (GUS) with flanking nucleotide sequences encoding seven amino acid residues (QYCVYES) consisting of the predicted NIa proteolytic cleavage site between NIb and CP at the 5′ end (JGUS) or the 3′ end (GUSJ), and the gene for neomycin phosphotransferase (NPT II) with the nucleotide sequence for the NIa proteolytic cleavage site at the 5′ end (JNPT) were produced by PCR using Pfu polymerase (Stratagene). For GUS, the following primers were used: primer GUS-5, 5′-CATCGTCGACTTACGTCCTGTAGAAACCCC-3′ and primer GUS-3, 5′-CTCTGTCGACTTGTTTGCCTCCCTGCTGC-3′. For GUS plus 5′ proximal sequences encoding the proteinase cleavage site, primer JGUS-5, 5′-CATCGTCGACCAGTATTGTGTATACGAGTCATTACGTCCTGTAGAAACCCC-3′ and primer GUS-3 were used. For GUS plus 3′ proximal sequences encoding the proteinase cleavage site, primer GUS-5 and primer GUSJ-3, 5′-CCTCGTCGACTGACTCGTATACACAATATTGTTTGCCTCCCTGC TGC-3′ were used. For NPT II, primers JNPT-5 (5′-GAGAGTCGACCAATACTGCGTGTACGAATCGGGGATTGAA CAAGATGGA-3′) and NPT-3 (5′-GAGAGTCGACGGGATTGAACAAGATGGA-3′) were used. The DNA fragments produced by PCR were digested with *Sal*I and inserted into the engineered *Sal*I sites of pWSMV-S1 or pWSMV-S4N to obtain pWSMV-GUS1, pWSMV-GUSJ1, pWSMV-GUS4, pWSMV-JGUS4 and pWSMV-JNPT, respectively (Figure 1b).

Inoculation of plants with in vitro *transcripts*

Plasmid templates (12.5 μg per reaction) for *in vitro* transcription were linearized with *Not*I and transcribed in the presence or absence of 1 mM cap analogue [m7G(5′)ppp(5′)G] using the SP6 MAXIscript kit (Ambion) in a total volume of 250 μl as recommended by the manufacturer. An equal volume of 2% sodium pyrophosphate containing 2 $mg\,ml^{-1}$ of bentonite was added to the *in vitro* transcription products and the mixture was inoculated onto 15–20 plants with celite added as an abrasive.

RT–PCR

RT–PCR was performed to verify virus replication. Total nucleic acids were extracted from younger, non-inoculated leaf tissue (0.2 g) as described previously (Choi *et al.*, 1999). After reverse transcription using an oligo(dT) primer, 30 PCR cycles were performed with primers flanking the WSMV CP gene (CP1a, 5′-AGCTTATGAGCGCTATTATTGCAGCAT-3′ and CP2, 5′-CGATTTTTTTTTTTTTGCCGTCGCCCT-3′). PCR for specific amplification of the GUS gene employed primers GUS-5 and GUS-3. PCR products were analysed on 1% agarose gels.

Immunological detection of CP and NPT II

Proteins were extracted from leaf or root tissues by homogenization with 7 vol 0.25 M Tris–HCl (pH 7.8) containing 1 mM phenylmethylsulphonylfluoride. Homogenates were centrifuged at

7500 *g* for 30 min, and supernatants were analysed. Protein samples were mixed with 2 × loading buffer (0.2% bromophenol blue, 100 mM Tris–HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS and 20% glycerol) and denatured by boiling for 3 min before loading on 12% polyacrylamide gels. Approximately 20 μg of total soluble proteins were used for immunoblot analyses with antibodies for CP (Brakke *et al.*, 1990) or NPT II (Eppendorf-5 Prime, Boulder, Colorado, USA). Enzyme-linked immunosorbent assay (ELISA) of NPT II employed the NPT II ELISA kit (Eppendorf-5 Prime) as per the manufacturer's recommendations.

Histochemical detection of GUS

Leaf or root tissues were cut into small pieces and fixed for 30 min by vacuum infiltration in 0.7% formaldehyde. Fixed tissues were rinsed five times for 15 min with distilled water. The tissues were immersed in 50 mM phosphate buffer (pH 7.0) containing 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), and incubated at 37°C for up to 12 h. The tissues were then clarified with 70% ethanol and finally with 5% sodium hypochlorite.

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