Cell-to-Cell Movement of the 25K Protein of *Potato virus X* Is Regulated by Three Other Viral Proteins

Yang Yang,¹ Biao Ding,² David C. Baulcombe,³ and Jeanmarie Verchot¹

¹Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater 74078, U.S.A.;

²Botany Department, Oklahoma State University, Stillwater 74075, U.S.A.; ³Sainsbury Laboratory, Norwich Research Park, Colney NR4 7UH, U.K.

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The 25K, 12K, and 8K proteins and coat protein (CP) of *Potato virus X* (PVX) are required for virus cell-to-cell movement. In this study, experiments were conducted to determine whether the PVX 25K protein moves cell to cell and to explore potential interactions between the PVX 25K, 12K, and 8K proteins and CP. The PVX 25K gene was fused to the green fluorescent protein (GFP) gene and inserted into plasmids adjacent to the cauliflower mosaic virus 35S promoter. These plasmids were introduced by biolistic bombardment to transgenic tobacco expressing the PVX 12K, 8K, and CP genes. The GFP:25K fused proteins moved cell to cell on nontransgenic tobacco and tobacco expressing either the 12K or 8K proteins. However, the GFP:25K proteins did not move on transgenic tobacco expressing the combined 12K/8K genes or the CP gene. Thus, movement of the PVX 25K protein through plasmodesmata may be regulated by interactions with other PVX proteins.

Additional keywords: potexvirus, triple gene block proteins, viral movement protein.

Potexviruses contain a block of three partially overlapping reading frames termed the “triple gene block” (TGB) that encode proteins required for virus cell-to-cell movement (Huisman et al. 1988; Koonin and Dolja 1993). In the case of *Potato virus X* (PVX), these three proteins are named, in accordance with their molecular mass, 25K, 12K, and 8K proteins (Huisman et al. 1988). It is likely that these three proteins interact with each other to facilitate viral intercellular transport, but the nature of these interactions remains poorly understood. Studies of the 25K and 26K proteins of the potexviruses PVX, *White clover mosaic virus* (WCIMV), and *Fox-tail mosaic virus* have revealed that these proteins can bind RNA, increase the size exclusion limit of plasmodesmata, and move from cell to cell (Rouleau et al. 1994; Angell et al. 1996; Lough et al. 1998; Kalinina et al. 1998). Specific functions of the PVX 12K and 8K proteins are still obscure although they are suggested to have membrane-binding activities (Skryabin et al. 1988). The PVX coat protein (CP) is also

required for viral cell-to-cell movement (Chapman et al. 1992; Forster et al. 1992; Baulcombe et al. 1995). The CP accumulates in plasmodesmata in infected cells but does not gate plasmodesmata (Oparka et al. 1996). It has been postulated for PVX and WCIMV that either virions or a complex containing the 25K (26K of WCIMV) protein, CP, and viral RNA are trafficking through plasmodesmata (Oparka et al. 1996; Santa Cruz et al. 1998; Lough et al. 1998), although such a complex has not been identified *in vivo*. Based on these limited studies, it is assumed that the PVX 25K protein and CP are central to the viral movement process by providing most of the activities required for intercellular RNA trafficking. The 12K and 8K proteins would therefore provide activities accessory to the 25K proteins. We suggest that there are two alternative roles for the 12K and 8K proteins. These proteins, functioning as membrane anchors, may play a more direct role by regulating viral RNA trafficking potentiated by 25K proteins, or they may promote formation of a ribonucleoprotein trafficking complex early in the movement process. Alternatively, they may restrict 25K protein plasmodesmatal gating activities late in infection as a means to restore normal intercellular communication.

In two previous studies, DNA constructs containing the green fluorescent protein (GFP) gene fused to the 3' end of the PVX 25K gene under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV) were bombarded onto tobacco or potato leaves (Malcuit et al. 1999; Morozov et al. 1999). The resulting 25K:GFP fusion products were expressed in single cells and did not move cell to cell. In one study (Morozov et al. 1999) the 25K:GFP fusion proteins could complement cell-to-cell movement of 25K-deficient PVX viruses. These data suggested that cell-to-cell movement of the 25K protein may not be essential for viral cell-to-cell movement. To investigate the cell-to-cell movement of the PVX 25K protein we used biolistic bombardment to introduce similar constructs, containing the GFP gene fused to the 5' or 3' end of the 25K gene, into tobacco leaves. The GFP:25K proteins did move cell to cell and complemented viral cell-to-cell movement defects. In addition, these constructs were introduced by bombardment into transgenic tobacco expressing the 12K, 8K, combined 12K/8K, or CP genes. These same transgenic tobacco lines were used in previous studies and the transgenic proteins were able to complement movement-defective PVX viruses (Verchot et al. 1998; Spillane et al.

Corresponding author: Jeanmarie Verchot; Telephone: 1-405-744-7895; Fax: 1-405-744-6039; E-mail: verchot@okstate.edu

1997). On transgenic tobacco expressing the 12K/8K genes together or the CP gene, GFP:25K proteins did not move cell to cell.

RESULTS

Fusion of GFP to the N terminus of the PVX 25K protein retained function of the 25K protein in viral cell-to-cell movement.

The GFP:25K fused genes were expressed directly from the genome of 25K-deficient PVX. The plasmids pPVX204 and pPVX204.Δ25K contain cDNA copies of the PVX genome (Baulcombe et al. 1995) fused to the CaMV 35S promoter. Both plasmids contain the GFP gene fused to a duplicated CP subgenomic (sg) RNA promoter inserted into the viral genome. pPVX204.Δ25K contains a mutation deleting 357 nucleotides of the 25K gene that inhibits viral cell-to-cell movement (Verchot et al. 1998). In pPVX204.GFP25K, the GFP gene in pPVX204.Δ25K was replaced with the GFP:25K fused genes (Fig. 1A). To determine if the GFP:25K fused genes could restore infectivity to the 25K-deficient PVX virus, these three plasmids were delivered by biolistic bombardment to detached tobacco leaves. Viral cell-to-cell movement was monitored for 5 days by epifluorescence microscopy to detect GFP activity. Expanding infection foci were observed on leaves bombarded with the pPVX204 and

pPVX204.GFP25K plasmids but not on leaves bombarded with pPVX204.Δ25K plasmids (Fig. 1B, C). These data indicate that the GFP:25K fused genes were able to restore viral cell-to-cell movement to 25K-deficient PVX viruses.

Cell-to-cell movement of GFP:25K fusion proteins was inhibited in transgenic tobacco expressing both 12K and 8K genes or the CP gene.

To directly determine whether the 25K protein moves cell to cell, three different DNA constructs were prepared. Plasmids p25K:GFP and pGFP:25K contain the GFP gene fused to the 3' and 5' ends of the PVX 25K gene, respectively. Plasmid pGFP contains only the GFP gene (Fig. 2). Expression of all genes is driven by the CaMV 35S promoter. These plasmids were delivered to single epidermal cells of tobacco leaves by biolistic bombardment, and epifluorescence microscopy was used to study cell-to-cell movement of GFP, 25K:GFP, and GFP:25K proteins. These plasmids were also bombarded onto transgenic tobacco leaves expressing the PVX 12K, 8K, both 12K and 8K, or CP genes to determine if these three viral proteins affect movement of the 25K protein. In addition, the pattern of protein accumulation was monitored to determine if there are differences in how the 25K:GFP and GFP:25K proteins accumulate.

Transgenic tobacco line TGB100 expresses the PVX 25K gene, line TGB200 expresses the 12K gene, line TGB300 ex-

Fig 1. A, Schematic representation of plasmids containing cDNA copies of the *Potato virus X* (PVX) genome (open boxes) fused to the 35S promoter of *Cauliflower mosaic virus* (CaMV). Gray boxes indicate green fluorescent protein (GFP) genes inserted into the PVX genome next to a duplicated coat protein (CP) subgenomic (sg) RNA promoter. Deletion of 357 nucleotides in the 25K gene is indicated in the plasmid pPVX204.Δ25K and pPVX204.GFP25K by a black box. **B**, Nontransgenic and TGB600 transgenic tobacco expressing the PVX 12K and 8K genes were inoculated with transcripts or DNAs and GFP fluorescence was used to monitor viral cell-to-cell movement. Expansion of infection foci was monitored for 5 to 10 days after inoculations. Numbers of leaves in which expanding infection foci were observed per total number of inoculated leaves are indicated. ND = not determined. **C**, Examples of infection sites observed at 5 days post bombardment on nontransgenic tobacco leaves with pPVX204 and pPVX204.GFP25K.

presses the 8K gene, line TGB600 expresses the 12K and 8K genes, and line CP expresses the CP gene (Fig. 3). The TGB600 line contains coding sequence for a bicistronic mRNA that resembles the bicistronic PVX sgRNA2. Previous studies demonstrated that viral proteins produced in these transgenic tobacco lines provide in *trans* complementation of defects in cell-to-cell movement of mutant PVX viruses

(Verchot et al. 1998; Spillane et al. 1997). Thus, we reasoned that in planta interactions between the viral proteins produced from the transgenic plants and the 25K protein produced from plasmid bombardment would resemble functional interactions occurring during virus infection.

Plasmids were bombarded to sink and source tobacco leaves of the nontransgenic and transgenic tobacco leaves.

Fig. 2. Images of tobacco cells bombarded with pGFP, p25K:GFP, and pGFP:25K plasmids observed by fluorescence microscopy. Transgenic and non-transgenic cells are identified below images. **A** and **B**, Nontransgenic tobacco cells at 1 day post bombardment (dpb) with pGFP and p25KGFP plasmids. Images were taken with a $\times 40$ and a $\times 60$ objective lens, respectively. **C**, Images of nontransgenic ($\times 20$ objective lens) and TGB200 ($\times 40$), TGB300 ($\times 40$), TGB600 ($\times 60$), and coat protein (CP; $\times 60$) transgenic tobacco cells 1 dpb with pGFP:25K. Arrows indicate nucleus in each cell.

The physiological status of these leaves was determined by carboxyfluorescein (CF) translocation studies (Oparka et al. 1994; Roberts et al. 1997). Briefly, a young leaf that imported and unloaded CF was determined to be a sink and a fully expanded leaf that was unable to import CF was a source (data not shown). Between four and 14 nontransgenic or transgenic leaves were bombarded with each plasmid. Approximately 50% of the leaves bombarded with each plasmid were identified as sink leaves. There was little difference in the pattern of

Fig. 3. Schematic representation of transgenes present in transgenic plants. The 35S promoter of *Cauliflower mosaic virus* (hatched boxes) is fused to the open reading frames of *Potato virus X* (open boxes).

Table 1. Cell-to-cell movement of green fluorescent protein (GFP) and 25K:GFP, GFP:25K and 3a:GFP proteins

Plants	Proportion of sites containing GFP activity in multiple cells ^a			
	GFP ^b	25K:GFP ^b	GFP:25K ^b	3a:GFP
Nontransgenic				
1 dpb	0 (0/142)	0 (0/120)	34 (10/29)	20 (21/104)
3 dpb	0 (0/28)	0 (0/40)	37 (4/11)	ND
TGB100				
1 dpb	3 (3/104)	ND ^c	ND	ND
3 dpb	4 (1/23)	ND	ND	ND
TGB200				
1 dpb	0 (0/117)	0 (0/79)	50 (16/32)	ND
3 dpb	0 (0/23)	0 (0/10)	53 (8/15)	ND
TGB300				
1 dpb	0 (0/100)	4 (2/81)	50 (21/42)	ND
3 dpb	0 (0/27)	0 (0/10)	61 (13/21)	ND
TGB600				
1 dpb	0% (0/138)	5% (9/174)	6% (3/52) ^d	17 (22/131)
3 dpb	0 (0/33)	0 (0/30)	4 (1/25)	ND
CP				
1 dpb	0 (0/60)	0 (0/26)	5 (2/41) ^d	18 (20/112)
3 dpb	0 (0/18)	0 (0/10)	5 (1/19)	ND

^a Percentage of fluorescent cell clusters is indicated. Total number of cell clusters relative to total number of sites containing GFP (single cells + cell clusters) is indicated in parentheses. Approximately four to 14 leaves of each nontransgenic and transgenic tobacco line were bombarded with each plasmid to obtain total numbers indicated.

^b Some leaves bombarded with pGFP, p25K:GFP, and pGFP:25K plasmids were maintained for 3 to 5 days post bombardment (dpb). Thus, there are less sites indicated at 3 dpb than at 1 dpb although the proportion of sites remains constant.

^c Not determined.

^d All TGB600 and CP leaves bombarded with pGFP:25K plasmids were maintained for 3 to 5 dpb. There is a difference between total number of sites observed between 1 and 3 dpb because a proportion of the sites faded and could not be detected. In these cases no sites were clearly observed at 5 dpb.

protein accumulation and trafficking on source and sink leaves bombarded with each plasmid. At 1 day post bombardment (dpb), GFP and 25K:GFP proteins were detected in single epidermal cells in nontransgenic tobacco and in TGB100, TGB200, TGB300, TGB600, and CP transgenic tobacco leaves (Table 1; Fig. 2A,B). GFP expression was observed in two adjacent cells in 3 to 5% of the sites on nontransgenic and transgenic tobacco leaves bombarded with pGFP and p25K:GFP plasmids. Some leaves were maintained for 3 to 5 dpb and the GFP or 25K:GFP proteins still remained in single cells (Table 1). On rare occasions, neighboring cells may have expressed the bombarded genes simultaneously. Similar background levels were reported in related studies in which viral movement protein genes were fused to the GFP gene and delivered to tobacco cells by biolistic bombardment (Itaya et al. 1997; Morozov et al. 1997). GFPs were also reported to move after plasmids containing only the GFP gene were delivered into young leaves (Oparka et al. 1999). It was reported that proteins as large as 50 kDa in size (the GFP is approximately 27 kDa) can move through plasmodesmata located between immature epidermal cells but not between mature epidermal cells (Oparka et al. 1999). In this study, there were occasions when GFP expression was observed in two adjacent cells but the protein was not observed to move from the initial one or two cells during extended incubations (data not shown).

In contrast, at 1 dpb, GFP:25K proteins accumulated in clusters of neighboring cells in nontransgenic tobacco leaves. The GFP:25K proteins were detected in clusters of at least two to three cells in 34% of the sites analyzed (Fig. 2C; Table 1). The number of multiple cell clusters observed on the TGB200 and TGB300 transgenic tobacco bombarded with the pGFP:25K plasmids was slightly higher. Approximately 50% of the sites observed consisted of multiple cell clusters (Fig. 2C). Protein movement was monitored by measuring the number of fluorescent cells across the diameter of each cell cluster over a period of 5 days. As indicated in Table 2, the fluorescent cell clusters expanded from approximately two to three cells to four to five cells over a 5-day period. To our surprise, GFP:25K fusion protein was restricted to single cells in leaves of TGB600 and CP transgenic tobacco lines (Fig. 2C; Table 1). Only 5 to 6% of the sites observed at 1 dpb on the TGB600 and CP leaves contained GFP:25K proteins in two adjacent cells. This level is similar to the background level observed on leaves bombarded with pGFP and p25K:GFP plasmids. After 3 dpb, GFP:25K still remained in single cells in most cases in TGB600 and CP transgenic tobacco leaves (Table 1). These data indicate that intercellular trafficking of

Table 2. Average number of GFP:25K-containing cells across the diameter of each cell cluster observed during a 5-day period

Plants	Average cell cluster diameter ^a		
	1 dpb	3 dpb	5 dpb
Nontransgenic	2.14 ± 1.65	2.06 ± 1.57	3.71 ± 0.39
TGB200	1.97 ± 1.42	2.11 ± 1.51	3.60 ± 0.73
TGB300	2.12 ± 1.26	2.79 ± 1.55	4.80 ± 1.28
TGB600	1.06 ± 0.24	1.08 ± 0.57	ND
CP	1.11 ± 0.43	1.11 ± 0.47	ND

^a Number of cells across the diameter of each cell cluster was counted; averages and standard deviations are indicated. Total numbers of cell clusters in this table are identical to the numbers listed at 3 days post bombardment (dpb) in Table 1. ND = not determined.

GFP:25K fusion proteins was inhibited in the presence of PVX CP or a combination of 12K/8K proteins.

Inhibition of PVX 25K intercellular movement by PVX CP or 12K/8K proteins was specific.

Inhibition of PVX CP and 12K/8K to PVX 25K intercellular trafficking could specifically affect interactions between these PVX proteins or between these PVX and certain host proteins. Alternatively, there could be a general inhibitory mechanism provided by transgenic PVX CP and 12K/8K proteins that may also block intercellular trafficking of heterologous viral proteins. To distinguish between specific and general inhibition, we bombarded CP and TGB600 transgenic as well as nontransgenic tobacco plants with pRTL2-MP3a:GFP (Itaya et al. 1997). This plasmid carries a DNA construct that expresses GFP fused to the 3a movement protein of *Cucumber mosaic virus*. 3a:GFP trafficks from cell to cell in tobacco epidermis upon biolistic DNA bombardment (Itaya et al. 1997, 1998). As shown in Table 1, 3a:GFP trafficked from cell to cell in nontransgenic, TGB600, and CP transgenic tobacco leaves. Thus, we concluded that inhibition of PVX 25K intercellular trafficking by PVX CP or the combined 12K and 8K proteins in the transgenic plants is a specific process.

The 25K:GFP and GFP:25K fusion proteins were localized to different subcellular compartments.

During our analysis of the trafficking functions of 25K:GFP and GFP:25K fusion proteins, we also noticed they had different subcellular localization patterns. In both cases, the fusion proteins were not observed to be associated with the cell wall or plasmodesmata. The 25K:GFP proteins accumulated primarily in cytoplasmic inclusions (Fig. 2B) (Davies et al. 1993). The GFP:25K fusion protein, on the other hand, was dispersed throughout the cytoplasm (Fig. 2C). Since both 25K:GFP and GFP:25K proteins can promote virus cell-to-cell movement (Morozov et al. 1999; this study) we cannot determine whether differences in the subcellular targeting of these two fusion proteins, as viewed by fluorescence microscopy, are significant for protein function. In the transgenic tobacco leaves, the 25K:GFP proteins also accumulated in cytoplasmic inclusions, suggesting that the transgenically expressed proteins had no effect on its subcellular targeting. In transgenic leaves expressing the 12K, 8K, CP, or the combined 12K and 8K genes, the GFP:25K proteins appeared soluble throughout the cytoplasm. The fusion proteins also accumulated in nuclei of cells that initially produced the fusion proteins and in cells that imported the proteins (Fig. 2C).

DISCUSSION

In this paper, we have characterized two properties of the 25K protein of PVX. Similar to the 26K protein of WCIMV (Lough et al. 1998), the PVX 25K protein can move from cell to cell and this intercellular movement does not require the presence of other PVX-encoded proteins. The second finding reported here is that the potential of the 25K protein to move between cells is reduced in the presence of the combined PVX 12K and 8K proteins or in the presence of the PVX CP.

Lough et al. (1998) proposed a general model describing potexvirus cell-to-cell movement. According to this model, the PVX 25K protein forms a complex with the 12K and 8K

proteins that is anchored to the cytoskeleton. Within this complex, the 25K protein binds viral RNAs and trafficks to the cell wall. There the 25K protein interacts with the viral CP to transport viral RNA through the plasmodesma. We demonstrated in this study that the PVX 25K protein interacts with the CP and the 12K/8K proteins in plant cells in a manner that inhibits 25K protein cell-to-cell trafficking. When purified WCIMV 26K protein was injected into transgenic tobacco expressing the 13K and 7K proteins (homologues of the 12K and 8K proteins), similar interactions were not observed. Movement of the 26K protein was not blocked in those transgenics (Lough et al. 1998). Differences in these observations may be due to differences between the viruses or may be due to the different manners in which the protein was produced.

Based on the WCIMV model, the PVX 12K/8K proteins are predicted to function as membrane anchors for the viral movement complex. The 25K protein and CP would form a complex with the 12K and 8K anchors and then bind viral RNA to transport it through the plasmodesmata. The data presented in this paper provide evidence of these protein-protein interactions and also suggest that movement of the 25K protein through the plasmodesmata is regulated by these protein-protein interactions. In addition to functioning as membrane anchors, the 12K/8K proteins may also act in combination to temporarily restrict movement of the 25K protein into the plasmodesmata. This may promote assembly of the viral RNA within the movement complex, especially with the 25K protein. Perhaps binding of viral RNA to the 25K protein, or formation of the ribonucleoprotein complex, triggers release of the 25K protein from the anchorage site to engage in plasmodesmata trafficking.

PVX movement requires additional participation of the CP. PVX CP accumulates in, but does not gate, plasmodesmata (Oparka et al. 1996). Perhaps the CP blocks sites within the plasmodesmata preventing attachment, and therefore intercellular trafficking, of the 25K protein. Alternatively, there may be direct interactions between the cytoplasmic CP and 25K protein that restrict 25K protein trafficking. At the plasmodesmata, the 25K protein may act to push the viral RNA inside while the CP acts as a conduit for transport of the viral RNA into adjacent cells. Immunolabeling of PVX-infected leaves revealed that the 25K protein accumulates primarily in the cytoplasm and has never been seen associated with the interior of the plasmodesmata (Davies et al. 1993). While there is evidence that plasmodesmatal gating and ATPase activity are important functions of the 25K protein (Angell et al. 1996; Morozov et al. 1999), there is no evidence yet indicating that cell-to-cell movement of the 25K protein itself is required for virus cell-to-cell transport.

The data obtained with these transgenic tobacco may reveal some protein-protein interactions that occur during the progression of the viral infection cycle. It is also possible that autonomous movement of the 25K protein may represent an early stage in the infection cycle before there is substantial accumulation of the 12K and 8K proteins. Expression of the TGB proteins in PVX and related viruses involves two subgenomic RNA species and readthrough translation (Verchot et al. 1998). In principle this could represent a strategy for regulation of the different phases in the infection cycle. The reduced movement capability of the 25K protein in the presence of the transgenically expressed, PVX-encoded proteins could

represent a late stage in the infection cycle when the 12K and 8K proteins and free CP accumulate to higher levels. In an earlier study, it was shown that PVX does not replicate in every cell it enters and may continue to move through several cells before replication is initiated (Angell and Baulcombe 1995). Thus, there may be a population of 25K proteins that accumulate early in infection that move at the infection front as independent molecules or associated with virions or viral RNA to ensure plasmodesmatal gating for rapid cell-to-cell movement. This could ensure synchronous infection of cells at the infection front as well as ensuring that virus movement is always toward noninfected cells. This may also serve to regulate 25K protein-induced plasmodesmatal gating and prevent the spread of defense signaling molecules that may follow the virus through the gated plasmodesmata. Third, the 25K protein may be sequestered away from the plasmodesmata when its plasmodesmatal gating activity is no longer required by the virus. This may be necessary to restore normal intercellular communication in cells that are behind the infection front.

The TGB of PVX is a genetic module that is conserved among the hordei-, carla-, furo-, and other potexviruses (Skryabin et al. 1988; Morozov et al. 1989). The first encoded TGB proteins in all cases are similar to the 25K protein of PVX in that they share sequence motifs, ATPase activity, and RNA binding activity (Gorbalenya and Koonin 1989; Bleykasten et al. 1996; Donald et al. 1997; Lough et al. 1998; Kalinina et al. 1998; Rouleau et al. 1994). It is likely that these proteins, like the 25K protein of PVX, undergo regulated trafficking between cells and that these interactions are required for movement of the virus. A future challenge will be to characterize how these proteins interact with each other, with the viral genome, and with host factors, and to determine the importance of such interactions during virus infection.

MATERIALS AND METHODS

Bacterial strains and plasmids.

All plasmids were constructed and used to transform *Escherichia coli* DH10B (Sambrook et al. 1989). All plasmids were delivered to tobacco leaves by the biolistic bombardment method (see below). The pPVX204 plasmid and its derivatives (Fig. 1A) contain cDNA copies of the PVX genome. The GFP genes were inserted into the PVX genomes under the control of a duplicated sgRNA promoter as described previously (Baulcombe et al. 1995). The pPVX204 plasmid contains the CaMV 35S promoter and nos terminator. The plasmid pPVX204.Δ25K has 357 nucleotides deleted from the central region of the 25K gene and was prepared previously (Verchot et al. 1998). The pPVX204.GFP25K plasmid was derived from pPVX204.Δ25K. By polymerase chain reaction (PCR), the GFP gene was fused to a wild-type 25K gene (see below) and inserted adjacent to the duplicated sgRNA promoter. The GFP:25K fused genes were PCR amplified from pGFP:25K plasmids (see below) with oligonucleotides that introduced a *SalI* restriction site at the 3' end of the 25K gene. The GFP:25K PCR products and pCX.GFPΔ25K were digested with *PmII* (a unique restriction site in the GFP gene) and *SalI* and then were ligated together.

Two plasmids containing the PVX 25K gene fused to the 5' end of GFP, and the PVX 25K gene fused to the 3' end of GFP were used for biolistic bombardment of tobacco leaves (Fig.

2A,B, and C). These two plasmids were pUC19 derivatives containing the CaMV 35S promoter and polyadenylation sequences. The p25K:GFP and pGFP:25K translational fusion constructs were prepared with "overlapping" PCRs. The plasmid p25K:GFP contains the GFP gene fused to the 3' end of the 25K gene and was prepared previously (Malcuit et al. 1999). The pGFP:25K plasmid contains the GFP gene fused to the 5' end of the 25K gene and was prepared by a two-step PCR procedure. First, two oligonucleotides were used to PCR amplify the GFP gene. The 5' oligonucleotides (YY1) contain an additional *BamHI* site and the 3' oligonucleotides (YY2) contain 12 additional nucleotides that overlap sequences in the 5' end of the 25K gene. The 25K gene was also PCR amplified with 5' oligonucleotides (YY3) containing an additional 12 nucleotides that overlap sequences in the 3' end of the GFP gene and 3' oligonucleotides (YY4) containing a *SstI* site. In the second step, the overlapping PCR products were denatured and annealed to each other. The oligonucleotides YY1 and YY4 were used to amplify the GFP:25K fusion. The final PCR products were digested with *BamHI* and *SstI* and gel purified. The p25K:GFP plasmid was also digested with *BamHI* and *SstI*, gel purified, and used in ligation reactions with the gel-purified PCR products. The plasmid pGFP was prepared previously (Voinnet and Baulcombe 1997). The plasmid pRTL2-MP3a:GFP was described previously (Itaya et al. 1998).

Plant material.

Transgenic and nontransgenic *Nicotiana tabacum* (cv. Petit Havana) plants were used in all experiments. The transgenic tobacco lines TGB100, TGB200, TGB300, TGB600, and CP contain transgenes encoding the PVX 25K, 12K, 8K, 12K/8K, or CP genes of PVX, respectively (Fig. 3). These transgenic tobacco plants are able to complement cell-to-cell movement of defective PVX viruses and were described previously (Spillane et al. 1997; Verchot et al. 1998).

Particle bombardment of detached tobacco leaves.

To identify source and sink tobacco leaves, a mature leaf was excised above the petiole. CF dye (Sigma, St. Louis, MO) was applied to the petiole and its direction of movement was monitored by fluorescence microscopy. The CF dye moves through the phloem from source to sink leaves (Oparka et al. 1994; Roberts et al. 1997). Therefore, accumulation of dye in the upper leaves identified them as sink tissue. Source tissues did not accumulate dye. Microprojectile bombardment of tobacco leaves was conducted with the Biolistic PDS 1000/He System (BioRad, La Jolla, CA) at a pressure of 7,500 kPa with 26 Hg vacuum in the chamber. Ten micrograms of plasmid DNA was combined with 1 mg of 1-μm gold particles (BioRad) that was sonicated in 40 μl of double-distilled (dd) H₂O. Then, 50 μl of 2.5 M CaCl₂ and 20 μl of 0.1 M spermidine were added and mixed for 15 min at 4°C. The DNA-coated gold particles were washed by 70% ethanol and suspended in 60 μl of absolute ethanol. Ten microliters of this mixture was loaded on carrier disk and used for bombardment of detached tobacco leaves (Itaya et al. 1997).

Microscopy.

GFP was detected with a Nikon Optiphot-2 epifluorescence microscope with a blue excitation filter (420 to 490 nm). Fluor-

rescent images were captured with a Hamamatsu C2400 CCD camera and processed with the Hamamatsu Argus 20 image-processing system (Hamamatsu Photonics KK, Hamamatsu, Japan).

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