The 25-kDa Movement Protein of PVX Elicits *Nb*-Mediated Hypersensitive Cell Death in Potato

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The potato gene *Nb* confers hypersensitive resistance to potato virus X (PVX). To characterize the viral elicitor of this resistance we introduced modifications into the genome of avirulent strains (ROTH1 and CP2) and virulent strains (UK3 and CP4) of PVX. From the analysis of the modified viral genomes, the *Nb* avirulence determinant was mapped in the PVX 25K gene coding for the 25-kDa movement protein. Furthermore, we showed that the isoleucine residue at position 6 of this protein was required for activation of the *Nb* response. Transient co-expression of the avirulent 25K gene with the β -glucuronidase (*GUS*) reporter gene introduced by particle bombardment in resistant and susceptible potato cells confirmed that the elicitor activity provided by the 25-kDa protein did not require other PVX-encoded proteins. To study cellular events associated with the *Nb* response, the 25-kDa proteins of PVX strains ROTH1 and UK3 were tagged with the green fluorescent protein (GFP) so that the dynamics of the subcellular distribution of the 25KGFP fusion proteins could be followed in living potato epidermal cells by laser scanning confocal microscopy. Using this method, we showed that the *Nb*-mediated response is associated with degradation of subcellular structures.

In plants, genetic control of resistance to pathogens is often determined by simple gene-for-gene interactions in which a resistance response is induced only if the pathogen encodes a strain-specific avirulence (*avr*) gene and the plant carries a corresponding resistance (*R*) gene (Flor 1971; Keen 1990). This type of interaction leading to resistance is termed incompatible. In contrast, when the plant *R* allele or the pathogen *avr* gene are absent or inactive, disease occurs and the interaction is defined as compatible.

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Resistance is typically manifested as a hypersensitive response (HR) that results in localized cell death at the site of pathogen entry (Morel and Dangl 1997). Associated with the HR, there is activation of general defense mechanisms throughout the plant by the production of signal molecules such as salicylic acid and reactive oxygen species (Dangl et al. 1996; Hammond-Kosack and Jones 1996). The HR may result from a programmed cell death (PCD) pathway that is induced by the specific interaction between pathogen *avr* gene products and host *R* gene products (Mittler et al. 1997; Morel and Dangl 1997). The molecular and cellular mechanisms involved in pathogen recognition and the consequent activation of the PCD pathway are poorly understood.

Potato virus X (PVX) and its interaction with potato *R* genes provides a well-characterized system in which to investigate the mechanisms of plant-pathogen recognition, induced resistance, and HR (Baulcombe et al. 1994). Two types of resistance to PVX have been identified in potato: HR resistance is controlled by the genes *Nb* and *Nx* and extreme resistance is provided by the *Rx1* and *Rx2* genes. *Rx1* and *Rx2* are located on chromosome XII and chromosome V, respectively (Ritter et al. 1991; Bendahmane et al. 1997) and were recently cloned (Bendahmane et al., *in press*; A. Bendahmane, K. Kanyuka, and D. C. Baulcombe, *unpublished data*). *Nb* has been mapped to the upper arm of chromosome V in the same region where other resistance genes are located (De Jong et al. 1997; Rouppe van der Voort et al. 1998). *Nx* was mapped on chromosome IX (Tommiska et al. 1998).

Previous studies have shown that two different avirulence determinants in the PVX coat protein gene are involved in *Rx*- and *Nx*-mediated resistance (Kavanagh et al. 1992; Santa Cruz and Baulcombe 1993). Analysis of frame-shift mutants and viral hybrids based on tobacco mosaic virus (TMV) in which the TMV coat protein was substituted with that of PVX showed that the coat protein of PVX avirulent strains can by itself elicit *Rx*-mediated resistance (Bendahmane et al. 1995).

The present report describes the identification of the PVX avirulence determinant of *Nb*-mediated resistance. PVX strains are classified on the basis of their interaction with potato *R* genes (Cockerham 1954). Strains belonging to group 1 and 2 are avirulent in potato cultivars carrying the *Nb* gene, whereas group 3 and 4 strains overcome *Nb* resistance (Cockerham 1954). Strain-specific avirulence determinants can therefore be identified by constructing recombinant hybrids in which genome segments of virulent strains (from

groups 3 and 4) are replaced by those derived from avirulent strains (from groups 1 and 2). Using this approach, we have mapped the avirulence determinant of *Nb* resistance in the N terminus of the 25-kDa movement protein of PVX strains ROTH1 and CP2. By transient expression of the PVX 25K gene together with two different reporter genes, we show that the 25-kDa protein alone elicits cell death in *Nb* potato leaves.

The 25-kDa protein retained *Nb* elicitor activity as a fusion with the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Chalfie et al. 1994). By monitoring the subcellular distribution of the 25KGFP fusion proteins in potato cells, we detected fragmentation of subcellular structures associated with the *Nb*-mediated response.

RESULTS

Mapping of the *Nb* avirulence determinant in the PVX genome.

To identify the PVX avirulence determinant that is involved in the activation of *Nb*-mediated HR in potato, four independent viral strains were selected (Table 1). ROTH1 (Malcuit 1998) and UK3 (Kavanagh et al. 1992) were isolated in Europe and CP2 was originally found in South America (Orman et al. 1990). CP4 was selected by serial passage of CP2 on susceptible and resistant plants (Jones 1985). Although nucleotide sequence analysis showed that ROTH1 and CP2 only shared 76% sequence homology at the nucleotide level (Table 1), both strains were able to induce *Nb*-mediated HR in inoculated potato leaves.

We took advantage of the fact that the avirulent strains ROTH1 and CP2 show a high percentage of homology with the resistance-breaking strains UK3 and CP4 (95 and 99%, respectively; see Table 1), so that only a few polymorphic sites needed to be examined for their involvement in the activation of *Nb*-mediated resistance. To map the *Nb* avirulence determinant in the PVX genome, a set of hybrid viruses was constructed by exchanging cDNA sequences between the avirulent strains ROTH1 or CP2 and the *Nb* resistance-breaking strains UK3 or CP4 (Figs. 1A and 2A). These chimeric constructs were designed to assess the PVX replicase and 25K genes because previous work had ruled out an involvement of the genes encoding the 12-kDa, 8-kDa, and coat proteins in *Nb* avirulence (Malcuit 1998).

The interaction between *Nb* and the ROTH1/UK3 viral hybrids was analyzed by particle bombardment of these chimeric genomes on potato plants (Fig. 1). Bombardment of detached resistant (*Nb*) potato leaves with viral cDNA hybrids carrying the 25K gene from ROTH1 (REP31 and FA313) or the wild-type, avirulent control pROTH1 led to the formation of necrotic lesions 3 days after bombardment, indicative of an in-

teraction between *Nb* and the corresponding PVX avirulence determinant (Fig. 1B). In contrast, there were no hypersensitive lesions with REP13 and FA131, containing the 25K gene from the virulent strain UK3, or with the wild-type virulent control pUK3 (Fig. 1B). Similarly, when *Nb* potato plants were graft-inoculated with REP31, FA313, and ROTH1, there was induction of systemic HR, whereas *Nb*-virulent constructs REP13, FA131, and UK3 induced systemic chlorotic symptoms in the graft-inoculated plants (data not shown). Parallel results were obtained with CP2/CP4 hybrids: chimeras containing the 25K gene from the avirulent strain CP2 triggered HR in graft-inoculated *Nb* potato plants whereas those carrying the 25K gene from the virulent strain CP4 infected the plants systemically (data not shown). All of these PVX chimeras induced the formation of symptoms in susceptible (*nb*) potato plants and accumulated to the same level as the wild-type strains, as measured by enzyme-linked immunosorbent

Fig. 1. Analysis of ROTH1/UK3 hybrids and UK3-Ile6 mutant by particle bombardment of detached *Nb* potato leaves. **A**, Schematic representation of the wild-type and modified potato virus X (PVX) genomes. REP31, REP13, FA313, and FA131 were generated by exchanging cDNA fragments between the PVX avirulent strain ROTH1 and the *Nb* resistance-breaking strain UK3. UK3-Ile6 is a viral mutant derived from UK3 in which the serine residue at position 6 in the 25-kDa protein is replaced by isoleucine. **B**, Responses induced on *Nb* leaves 3 days after bombardment with the hybrid and mutant cDNA constructs. Hypersensitive response is manifested by the appearance of necrotic lesions (black spots) at the site of impact.

Table 1. Description of potato virus X (PVX) strains

Strain	Group ^a	Interaction with <i>Nb</i>	Origin	Homology (%)
ROTH1	1	Incompatible	U.K.	95
UK3	3	Compatible	U.K.	
CP2	2	Incompatible	South America	99
CP4	4	Compatible	South America	

^a According to the strain classification of Cockerham (1954).

assay (ELISA) detection of the viral coat protein. Therefore, induction of the *Nb*-mediated resistance in potato is dependent upon either the PVX 25-kDa movement protein or the RNA sequence encoding it.

Fig. 2. Analysis of CP2/CP4 hybrids by graft inoculation of *Nb* potato plants. **A**, Schematic representation of the potato virus X (PVX) hybrid genomes generated by exchanging cDNA fragments between the avirulent PVX strain CP2 and the *Nb* resistance-breaking strain CP4. **B**, Responses induced on upper leaves of *Nb* potato plants graft-inoculated with the viral chimeras. Hypersensitive response is manifested by the appearance of necrotic lesions (black spots) on expanded leaves.

A single amino acid change in the PVX 25-kDa protein affects the outcome of *Nb*-mediated response.

Comparative sequence analysis of the 25-kDa proteins of ROTH1, UK3, CP2, and CP4 identified 32 amino acid substitutions in total (Malcuit 1998). However, there is only one amino acid difference when the 25-kDa protein sequences of ROTH1 and CP2 were compared with those of UK3 and CP4, respectively. The isoleucine at position 6 (Ile⁶) in the 25-kDa protein of ROTH1 and CP2 is replaced by a serine (Ser⁶) in UK3 and a threonine (Thr⁶) in CP4. This conservation of Ile⁶ in the two avirulent strains indicates that it is probably essential for activation of the *Nb* response. To test this hypothesis, three transcription clones were generated, each resulting in a single substitution in the 25-kDa protein sequence at position 6 (Ser⁶→Ile: UK3-Ile6 mutant; Thr⁶→Ile: BB424 hybrid of CP2 and CP4; Ile⁶→Thr: BB242 hybrid of CP2 and CP4) (Figs. 1A and 2A). When these constructs were tested on plants, UK3-Ile6 or BB424 induced *Nb*-mediated necrosis (Figs. 1B and 2B), whereas BB242 produced systemic chlorotic symptoms in graft-inoculated *Nb* plants (Fig. 2B). These data indicate that presence of Ile⁶ in the 25-kDa protein is required for activation of the *Nb* response.

Transient expression of the PVX 25-kDa protein elicits *Nb*-mediated cell death.

To study the effect of the expression of the PVX 25-kDa protein by itself on the activation of HR in *Nb* potato cells, we used a biolistic transient expression system (Mindrinos et al. 1994) in which the 25-kDa protein and the β -glucuronidase (GUS) reporter were produced from different cauliflower mo-

Fig. 3. Effects on β -glucuronidase (GUS) activity of transient expression of potato virus X (PVX) 25K gene in *Nb* and *nb* potato leaves. **A**, Diagrams representing plasmid constructs used in the bombardment experiments. 25K1, 25K2, and 25K3 represent the PVX genes coding for the 25-kDa proteins of strains ROTH1, CP2, and UK3, respectively. The cauliflower mosaic virus (CaMV) 35S promoter and terminator sequences are indicated by hatched and gray boxes, respectively. **B**, *Nb* and *nb* potato leaves were stained histochemically for GUS activity 3 days after bombardment with the p25KGUS constructs. **C**, Time course of GUS expression was monitored for 3 days. Each horizontal bar represents the mean relative number of blue spots observed on 10 *Nb* leaves with, as the 100% standard, the mean number of blue spots observed on 10 *nb* leaves the same day.

saic virus (CaMV) 35S expression cassettes carried by the same plasmid. If the 25-kDa protein alone was able to elicit *Nb*-mediated HR and subsequent cell death, GUS activity would be low or absent in bombarded *Nb* potato cells. However, if activation of *Nb*-mediated HR required the 25-kDa protein and other viral components, then the expression of the 25-kDa protein alone would not trigger HR. Consequently, the living *Nb* cells would contain high levels of GUS, similar to those observed in *nb* cells.

The results of these experiments (Fig. 3B) showed that, at 3 days post-bombardment, GUS activity was greatly reduced in *Nb* plants expressing either of the avirulent constructs p25K1GUS (derived from ROTH1) or p25K2GUS (derived from CP2). Control experiments showed that, when introduced in *nb* potato leaves, these constructs gave GUS activity similar to that observed when the 25-kDa protein from the virulent strain UK3 (p25K3GUS) was expressed in either *Nb* or *nb* potatoes (Fig. 3B). These findings indicated that the 25-kDa protein elicited *Nb*-mediated response in the absence of other virus-encoded proteins.

To confirm that the elicitation of *Nb*-mediated cell death was provided by the 25-kDa protein rather than the corresponding RNA, an untranslatable version of the 25K gene was generated by deleting the first ATG in the coding sequence (pΔATG, Fig. 3A). As expected, GUS activity from this construct was the same in *Nb* and *nb* leaves (Fig. 3B): cell death could not be triggered in *Nb* plants because the 25-kDa protein elicitor was not produced.

To investigate the timing of cell death induced by the 25-kDa elicitor in *Nb* cells, we followed the time course of GUS expression at 1, 2, and 3 days after bombardment (Fig. 3C). GUS expression in *Nb* leaves was determined as a percentage of the GUS expression observed in *nb* leaves (number of blue spots) measured on the same day. In plants bombarded with p25K1GUS and p25K2GUS, the level of GUS expression was the same in resistant and susceptible plants on day 1; GUS activity was reduced to 40 and 54%, respectively, on day 2 and it was considerably reduced on day 3 (5 and 10%, respectively) (Fig. 3C). GUS activity remained the same in *Nb* and *nb* plants bombarded with p25K3GUS or pΔATG throughout the time course experiment (Fig. 3C). These data suggest that the bombarded DNA was expressed within 1 day and that the elicitation of the *Nb*-mediated HR was activated shortly afterwards. Cell death occurred within 2 to 3 days.

Dynamics of the subcellular distribution of 25KGFP fusion proteins in relation to the *Nb*-mediated response.

GFP has proven to be a very useful tool for monitoring cellular trafficking and subcellular localization of proteins in living cells (Leffell et al. 1997). To investigate the cellular dynamics associated with pathogen-induced HR, GFP was used as a marker to follow the localization of the 25-kDa protein in potato cells and monitor cellular changes specifically associated with the activation of *Nb*-mediated HR. We used *mgfp4*, a mutant form of the *gfp* gene modified for optimum expression of GFP in plant cells (Haseloff et al. 1997). This gene was fused to the 3' end of the 25K gene of ROTH1 and UK3 (p25K1GFP and p25K3GFP; Fig. 4). The fusion constructs were delivered into potato cells by particle bombardment and subcellular distribution of the fusion proteins in epidermal cells was monitored by laser scanning confocal microscopy over a 3-day period.

Fig. 4. Real time imaging of the subcellular distribution of 25KGFP fusion proteins in *Nb* and *nb* potato cells. Green fluorescent protein (GFP) fluorescence was detected by laser scanning confocal microscopy. Each picture shows the distribution of GFP fluorescence in different epidermal cells at different times after bombardment of detached *Nb* and *nb* potato leaves with (A) p25K1GFP, (B) p25K3GFP, and (C) pGFP. The cauliflower mosaic virus (CaMV) 35S promoter and terminator sequences are indicated by hatched and gray boxes, respectively. h: hours post-bombardment. Position of the nucleus (n) is indicated by an arrow.

GFP fluorescence was detected 6 h after bombardment in epidermal cells for all the constructs tested (Fig. 4). Free GFP expressed from the CaMV 35S promoter (pGFP construct) accumulated in the nucleus and was found in the cytoplasm, as described previously (Haseloff et al. 1997; Fig. 4C). In contrast, GFP fluorescence in potato cells expressing the 25K1GFP and 25K3GFP fusion proteins was initially localized in inclusions in the cytoplasm of *Nb* and *nb* cells and also seemed to be present in or associated with the nucleus (Fig. 4A and B). After 28 h, most of the 25KGFP fluorescence was localized in one large inclusion adjacent to the nucleus (Fig. 4A). Eventually, starting between 28 and 31 h after bombardment, these inclusion bodies fragmented into progressively smaller pieces in *Nb* potato cells bombarded with the avirulent construct p25K1GFP (Fig. 4A). After 48 h, there was no remaining GFP fluorescence in *Nb* cells bombarded with p25K1GFP, indicating that the cell death process was complete (data not shown). In control experiments involving bombardment of *nb* plants with p25K1GFP and *Nb* plants with p25K3GFP, the green fluorescence was still visible after 48 h and the large inclusions remained intact (Fig. 4A and B). These data suggest that the changes in GFP fluorescence reflect degradation of subcellular structures specifically associated with *Nb*-mediated HR.

DISCUSSION

The results reported here show that the PVX 25-kDa protein is the elicitor of *Nb*-mediated HR. In compatible interactions the role of this protein is to facilitate cell-to-cell movement of the virus, probably by interacting with plasmodesmata (Angell et al. 1996; Morozov et al. 1997). At this stage we do not know whether these two different functions are interrelated. It could be, for example, that *Nb* prevents the 25-kDa protein from interacting with a cellular component involved in cell-to-cell movement. However, it is also possible that *Nb*, like other *R* genes, is part of a surveillance system for detection of foreign molecules in plant cells (de Wit 1997). If that is the case, the resistance mechanism would be independent of the movement role of the 25-kDa protein. Although there is no firm evidence ruling out either alternative as a basis of *Nb*-mediated resistance, the second possibility seems more likely than *Nb*-mediated suppression of movement protein function. Many of the known *Nb* responses, including callose deposition, accumulation of phenolic compounds, and cell death (I. Malcuit and M. R. Marano, *unpublished data*), are common to other *R* gene responses (Morel and Dangel 1997).

Interestingly, the 30-kDa movement proteins of other viruses such as TMV and tomato mosaic virus (ToMV) function as avirulence determinants in tomato plants carrying the *Tm-2* or *Tm2²* *R* genes, respectively (Meshi et al. 1988). Analysis of resistance-breaking strains and movement protein deletion mutants of TMV has shown that *Tm2* avirulence determinants are found in the N-terminal portion of the 30-kDa movement protein (Meshi et al. 1988), whereas two amino acids in the carboxy-terminal 30 amino acid domain of the ToMV 30-kDa protein are critical *Tm2²* avirulence determinants (Weber and Pfitzner 1998). These carboxy-terminal residues are dispensable for movement indicating that, as proposed for *Nb*, the *Tm2²* resistance most probably involves a surveillance mecha-

nism rather than alteration of the movement protein function (Weber and Pfitzner 1998).

Analysis of both resistance-inducing (ROTH1 and CP2) and resistance-breaking (UK3 and CP4) strains showed that the elicitor function involves the isoleucine at position 6 (Ile⁶) in the 25-kDa protein. This amino acid is conserved in the two avirulent strains ROTH1 and CP2 and is substituted by a serine or a threonine in the resistance-breaking strains UK3 and CP4, respectively. Surprisingly, however, Ile⁶ is also present in the 25-kDa protein encoded by the PVX strain HB, a strain that is known to overcome *Nb*-mediated resistance (Querici et al. 1993). This suggests that Ile⁶ may act as a determinant of the three-dimensional structure of an avirulence domain that is specifically recognized by the *Nb* gene product. Presumably, in PVX strain HB, amino acid substitutions elsewhere in the 25-kDa protein alter the recognition domain such that it no longer elicits *Nb*-mediated HR.

We used 25KGFP fusions to study subcellular distribution of the PVX 25-kDa protein in living potato cells, and to monitor its fate and other cellular events associated with *Nb*-mediated cell death. The HR-eliciting property of the 25-kDa protein was not affected by the fusion with GFP (I. Malcuit and M. R. Marano, *unpublished data*) and it allowed the visualization by laser scanning confocal microscopy of a cellular process specifically associated with the *Nb*-mediated response. The large, GFP-labeled, perinuclear bodies in the bombarded potato cells expressing the 25KGFP fusion may represent the lamellar inclusions detected previously by immunocytochemistry and in ultrastructural studies (Davies et al. 1993). These inclusion bodies are intimately associated with the endoplasmic reticulum and with actin microfilaments (Karl Oparka and Simon Santa Cruz, *personal communication*). As the *Nb*-elicited cells died these perinuclear structures fragmented and disappeared. In animal cells, PCD was associated with cleavage of microfilament proteins by caspases (Brancolini et al. 1995). Previous studies in plants have implicated caspase-like enzymes in disease resistance (Del Pozo and Lam 1998). Therefore, it is possible that the fragmentation of the 25KGFP inclusion bodies during the *Nb*-mediated response is due to the action of a caspase-like protease.

The use of the 25KGFP fusions may also help establish how subcellular location relates to the role of the PVX 25-kDa protein in cell-to-cell movement. Cell-to-cell trafficking of plant virus movement protein fused to GFP was recently reported for the cucumber mosaic virus 3a protein (Itaya et al. 1998). It was previously suggested that the PVX 25-kDa protein was itself able to move between cells as the virus moves (Angell et al. 1996; Morozov et al. 1997). In contrast, in none of the experiments associated with the data described in Figure 4 did we observe movement of the 25KGFP fusion protein out of the bombarded cell. By bombardment of the 25KGFP fusion to transgenic plants expressing the other PVX movement proteins, we will be able to determine whether these proteins interact with and modify the compartmentalization of the 25-kDa protein.

Spectral variants of GFP fused to the appropriate signal sequences can be used to mark specific organelles and other cellular compartments (Tsien 1998). By expression of these GFP fusions and the 25-kDa elicitor protein in *Nb* potato cells it will be possible to monitor various aspects of cellular structure and organelle integrity, in real time, during the

course of *Nb*-mediated HR. The use of GFP in these ways allows non-invasive monitoring of cells undergoing PCD.

MATERIALS AND METHODS

PVX strains and potato cultivars.

A description of the viral strains used in this study is given in Table 1. PVX-ROTH1 was obtained from AFRC Rothamsted (U.K.). PVX strains UK3, CP2, and CP4 were described previously (Jones 1985; Orman et al. 1990; Kavanagh et al. 1992). Percentages of homology between viral genomic sequences (Table 1) were calculated with the program CLUSTAL W (Thompson et al. 1994). Resistant (*Nb*) and susceptible (*nb*) plants were obtained from a selfed progeny of the tetraploid potato cultivar Pentland Ivory that carries the *R* gene *Nb* in the simplex condition (De Jong et al. 1997). Plants were considered resistant if they displayed HR (necrotic lesions) on leaves inoculated with ROTH1 or CP2 avirulent strains and if no virus was detected on non-inoculated leaves. Plants were considered susceptible if after challenging with ROTH1 or CP2, the virus infected the plants systemically and could be detected in non-inoculated leaves.

Viral chimeric constructs and mutant.

Routine DNA manipulations were performed according to Sambrook et al. (1989). Purification of PVX viral RNA from virions was as described previously (Kavanagh et al. 1992). PVX cDNA synthesis was carried out with purified viral RNA with a cDNA synthesis kit (Boehringer Mannheim, Lewes, U.K.). A full-length cDNA copy of the ROTH1 genome was cloned under the control of the CaMV 35S promoter (Odell et al. 1984; pROTH1). The plasmid pUK3 is a derivative of pTXS (Kavanagh et al. 1992) in which the T7 promoter driving the genomic cDNA of PVX strain UK3 was replaced by the CaMV 35S promoter. Plasmids pCP2 (Santa Cruz 1993) and pCP4 (Goulden et al. 1993) contain the entire genomic cDNA of PVX strains CP2 and CP4, respectively, under the control of a phage T7 promoter. In vitro transcription experiments (T7 constructs) and direct inoculation of plants with plasmid DNA (35S constructs) were performed as described previously (Kavanagh et al. 1992; Malcuit 1998). These clones were modified by polymerase chain reaction (PCR)-based procedures, as described below. The sequences of the primers indicated below can be obtained on request.

ROTH1/UK3 hybrid viral genomes were constructed by exchanging sequences between pROTH1 and pUK3. This was accomplished by a PCR strategy that enables the creation of seamless junctions between DNA molecules in a restriction site-independent manner. The primers FK1 and FK2 carry a 5'-terminal *FokI* recognition site and complementary PVX sequences. Plasmids pUK3 and pROTH1 were used as templates in two PCRs with *Pfu* DNA polymerase (Stratagene, Cambridge, U.K.): (i) primers REP7 and FK2 were used to produce a 523-bp fragment (positions 3909 to 4477 in the PVX genome); and (ii) primers FK1 and MOV1 were used to produce a 583-bp fragment (positions 4456 to 5034). The REP7-FK2 fragments were subsequently digested with *AvrII* and partially digested with *FokI*. The FK1/MOV1 fragments were digested with *ApaI* (position 4949) and *FokI* (position 4565) and ligated in pROTH1 and pUK3 transcription clones to produce FA313 and FA131 hybrids, respectively (Fig. 1A).

REP31 and REP13 hybrids were generated by exchanging the *ApaI*-*XhoI* fragments (positions 4909 to 6302) between FA313 and FA131 (Fig. 1A). The hybrids BB424 and BB242 were produced by exchanging a *BspEI*-*BsaBI* restriction fragment (positions 4408 to 4783) between pCP2 and pCP4 (Fig. 2A).

UK3-Ile6 mutant was generated by PCR with the overlap extension method (Ho et al. 1989). The mutation results in an amino acid change at position 6 in the 25-kDa protein (Ser⁶ → Ile). pUK3 was used as template in the PCRs with *Pfu* DNA polymerase (Stratagene). In the first PCR, two fragments of 641 bp (positions 3874 to 4515) and 1,348 bp (positions 4491 to 5870) were amplified with two pairs of primers, REP3/25K3MB and 25K3MA/ROTH3, respectively. The fragments generated in the first PCR step were gel purified, denatured, annealed to each other, and used in the second PCR step ("overlap extension"). The extension products were used in the final PCR step with primers REP3 and ROTH3. The REP3-ROTH3 fragments were digested with *AvrII* (position 3939) and *ApaI* (position 4949) and cloned into pUK3 to produce UK3-Ile6 (Fig. 1A). The presence of the mutation (G → T substitution at position 4502 in PVX-UK3 genome) was confirmed by sequencing.

25K constructs for transient gene expression assays.

The plasmid pJIT60 (kindly provided by F. Guerineau, John Innes Institute) is a derivative of pUC19 (Yanisch-Perron et al. 1985) carrying a duplicated CaMV 35S minimal promoter and CaMV polyadenylation signal separated by a multiple cloning sequence. The sequences of the primers indicated below can be obtained on request. The 25K genes of PVX strains ROTH1, CP2, and UK3 were amplified by PCR with primers 25K1 and 25K2 and cloned in pJIT60 to produce p25K1, p25K2, and p25K3 constructs, respectively. The gene coding for GUS (Jefferson et al. 1987) was excised as an expression cassette from the plasmid pSLJ4K1 (Jones et al. 1992) with *EcoRI* and *HindIII* and cloned into the p25K constructs to produce the corresponding p25KGUS constructs (Fig. 3A). An untranslatable version of the 25K gene (lacking the first ATG) was PCR amplified from pROTH1 with 25KHA and 25K2 primers and digested with *HindIII*. p25K1GUS was digested with *HindIII* and *SmaI*, the fragment containing the vector was gel purified and ligated with the digested 25KHA/25K2 PCR product to produce the pΔATG construct (Fig. 3A).

The gene coding for a modified version of GFP (mGFP4) was PCR amplified from the plasmid pBIN-35S-GFP4 (Haseloff et al. 1997) with primers GFPB and GFPS and digested with *BamHI* and *SalI*. The 25K genes of ROTH1 and UK3 were amplified from pROTH1 and pUK3 with primers 25KH1/25KB and 25KH3/25KB, respectively, and digested with *HindIII* and *BamHI*. The 25K and GFP fragments were cloned into the pJIT60 vector in a three-way ligation to produce p25K1GFP and p25K3GFP constructs (Fig. 4A and B). The gene coding for mGFP4 was excised as an expression cassette from the plasmid pBIN-35S-GFP4 (Haseloff et al. 1997) with *HindIII* and *EcoRI* and cloned in pUC19 to generate pGFP (Fig. 4C).

Bombardment of detached potato leaves and graft inoculation experiments.

The biolistic methods employed for transient expression were as described previously (McCabe and Christou 1993;

Marano and Baulcombe 1998). Graft inoculation experiments were made as described (Kavanagh et al. 1992) with tomatoes infected with PVX as rootstocks onto which resistant and susceptible potato scions were grafted. To monitor virus accumulation in infected plants, ELISA reactions were carried out with polyclonal antibodies and monoclonal antibodies MAC58 or MAC72 (Torrance et al. 1986) prepared against the PVX coat protein.

Confocal microscopy.

Imaging of GFP fluorescence was performed with a TCS-NT confocal laser scanning microscope (Leica, Heidelberg, Germany) equipped with a 20X or 40X dry objective. Laser illuminations at 488 and 568 nm (argon ion laser) were recorded through a 530 nm or 600 to 630 nm bandpass filter normally used for the detection of fluorescein and Texas red dyes, respectively. Scanning was recorded with a Diamond 21TX computer (Mitsubishi Electric, Birmingham, U.K.) equipped with TCS-NT version 1.5.451 software (Leica, Heidelberg, Germany). Images were composed with Photoshop (Adobe Systems, Mountain View, CA).

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