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Participation of the *Cowpea mosaic virus* protease in eliciting extreme resistance

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ABSTRACT

Extreme resistance of Arlington line cowpea (*Vigna unguiculata*) to *Cowpea mosaic virus* (CPMV) is under control of a dominant locus designated *Cpa*. We transiently expressed, using *Tomato bushy stunt virus* (TBSV) vectors and *Agrobacterium tumefaciens*, in nearly isogenic *Cpa*/*Cpa* and *cpa*/*cpa* cowpea lines, sequences from RNA1, the larger of two CPMV genomic RNAs. Activation of a *Cpa*-specific response mapped to the CPMV 24K protease (24KPro). Mutational analysis of the 24KPro gene implicated protease activity, rather than 24KPro structure, in *Cpa*-mediated recognition of CPMV invasion. A 24KPro with alanine replacing the active site cysteine [24KPro(C-A)], but not wildtype 24KPro, accumulated after agroinfiltration of the corresponding binary vector constructions into *Cpa*/*Cpa* cowpea. In *cpa*/*cpa* cowpea, both protease versions accumulated, with 24KPro(C-A) in greater abundance. Thus, enzymically active 24KPro was recognized by both cowpea genotypes, but in *Cpa*/*Cpa* cowpea the suppression of 24KPro accumulation was very strong, consistent with extreme resistance to CPMV.

Introduction

More than one thousand cowpea (*Vigna unguiculata*) lines were tested for resistance to *Cowpea mosaic virus* (CPMV) (Beier et al., 1977). About 6% of the lines, among them line Arlington, showed extreme resistance as seedlings. Protoplasts, recovered from the primary leaves of seedlings corresponding to these resistant lines and inoculated with CPMV, gave yields of CPMV capsid antigen comparable to those achieved for protoplasts from susceptible cowpea cultivar Blackeye 5. The exception was line Arlington, for which the yield was only 1–10% of the Blackeye 5 protoplast yield (Beier et al., 1979). Line Arlington cowpea protoplasts also showed reduced accumulation of CPMV virion RNA and RNA complementary to virion RNA (Beier et al., 1979; Bruening and Kiefer, 1981; Eastwell et al., 1983). Hence, line Arlington was selected for further study. Cowpea protoplasts from all lines tested, whether resistant or susceptible to CPMV as seedlings, were similar in their ability to support an increase in another comovirus, *Cowpea severe mosaic virus* (CPSMV) (Beier et al., 1979, 1977; Bruening and Kiefer, 1981; Eastwell et al., 1983).

CPMV is the type member of genus *Comovirus*. Comoviruses have a divided genome, with RNA1 and RNA2 separately encapsidated in icosahedral capsids to form the bottom (B) and middle (M) virions, respectively, named according to their positions in density gradients. Each genomic RNA has a 5′-linked protein, designated VPg, and a 3′-polyadenylate. The capsid is composed of 60 copies each of two coat proteins which, with a movement protein, are encoded in RNA2. RNA1 (Fig. 1A) encodes proteins that mediate replication. Polyproteins translated from the two genomic RNAs, and cleavage of these polyproteins by the only CPMV protease, the RNA1-encoded 24K protease (24KPro), yields 15 complete and partial cleavage products, including free 24KPro itself (Goldbach and Wellink, 1996; van Kammen et al., 2001).

The resistance to CPMV shown by Arlington cowpea is extreme and robust. Blackeye 5 seedlings are uniformly infected after inoculation of CPMV at 1 μg/mL and develop a systemic mosaic. Arlington seedlings inoculated with up to the greatest CPMV concentration tested, 10,000 μg/mL, did not develop symptoms or accumulate detected CPMV virions, and rub inoculation of extracts from the inoculated Arlington leaves to Blackeye 5 seedlings did not result in infection (Beier et al., 1979, 1977; Bruening and Kiefer, 1981; Eastwell et al., 1983). When Arlington seedlings were approach-grafted to CPMV-infected Blackeye 5 seedings, the Arlington portion of the chimera did not become infected (Beier et al., 1979).

Resistance of seedlings was inherited as a simple, dominant locus in Blackeye 5 × Arlington crosses (Kiefer et al., 1984; Saayer-Riep and de Jager, 1988). The locus is designated here *Cpa*, for resistance to CPMV derived from Arlington. Inheritance as a simple, dominant locus also is characteristic of extreme resistance to CPMV shown by cowpea lines Black (Beier et al., 1977) (Bruening, unpublished result) and TVu470 (Sterk and de Jager, 1987). Although protoplasts from lines Black and TVu470 do not show resistance to CPMV (Beier et al., 1979; Sterk and de Jager, 1987), nothing excludes the possibility that the extreme resistance loci of lines Black and TVu470 correspond to *Cpa*.

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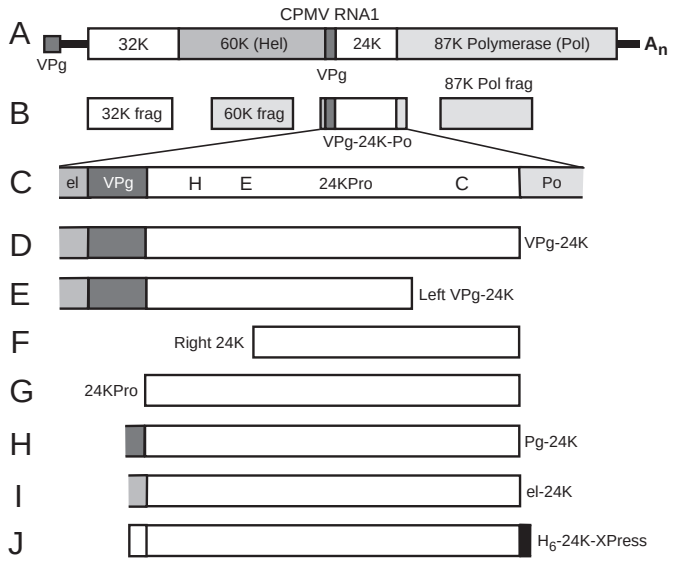

Fig. 1. Genetic map of *Cowpea mosaic virus* (CPMV) RNA1 and representation of RNA1 fragmentary sequences tested by expression from a *Tomato bushy stunt virus* (TBSV) vector and *Agrobacterium* binary vectors. A. CPMV RNA1 has a 5′-linked VPg, 3′-polyadenylate, a single open reading frame (ORF) encompassing five protein products (represented by large rectangles), and terminal untranslated regions. B. Four large fragments of the RNA1 ORF were tested by expression using a TBSV vector. Codon coordinates of the fragments in the 1866-codon CPMV RNA1 ORF are: 32K fragment, 85–269; 60K fragment, 424–651; VPg-24K-Po fragment, which includes the 208 amino acid residue 24KPro sequence, 913–1162; 87K Pol fragment, 1314–1590. C. Expanded diagram of the VPg-24K-Po fragment showing the approximate locations of the 24KPro catalytic triad: H, histidine 40; E, glutamic acid 76; C, cysteine 166 (Dessens and Lomonossoff, 1991). D–J. Each insert corresponds to a contiguous RNA1 sequence except for construction el-24K (I), in which the VPg sequence has been deleted to join the 5′ end of the *24KPro* s equence to the 3′ end of the 60K helicase gene, and construction H_6-24K-Xpress (J), which has the non-CPMV sequences MGH_6AG (small white rectangle) and DLYDDDDK (small black rectangle), respectively, flanking the 5′- and 3′-ends of the 24KPro sequence. In el-24K, the sequence GAFSAEPQ/MSL replaces the cleavage site provided by the VPg-24K junction, RRVWADAQ/MSL. For comparison, the helicase-VPg junction sequence is GAFSAEPQ/SRK. Insert left VPg-24K (E) includes codons 1–126 of the 24KPro sequence, whereas right 24K (F) corresponds to codons 46–208. "el" and "Pg" represent 10 and 12 codons, respectively, of the carboxyl-end regions of the 60K helicase and VPg. Each of the represented fragmentary sequences has an in-frame stop codon inserted at the 3′ end. The construction 24K ORF (G) begins with the methionine codon of the native 24KPro sequence.

Co-inoculation of CPMV (serving as the "protecting virus") and CPSMV (the "challenging virus") to primary leaves of Arlington cowpea seedlings reduced the numbers of CPSMV-induced lesions (Bruening et al., 1979). Similar results were obtained for CPMV and CPSMV co-inoculation (Eastwell and Kalmar, 1997; Sterk and de Jager, 1987) using CPMV-resistant lines Black and TVu470, respectively. The CPMV protection phenomenon is not limited to comoviruses. Cowpea lines showing extreme resistance to CPMV have also developed fewer lesions when CPMV was co-inoculated with *Cherry leafroll virus* (nepovirus), *Southern cowpea mosaic virus* (sobemovirus), *Cowpea chlorotic mottle virus* (bromovirus) and *Blackeye cowpea mosaic virus* (potyvirus) (Bruening et al., 2000; Sterk and de Jager, 1987). CPMV can be replaced as the protecting virus by CPMV RNA or preparations of the RNA1-containing virion B. However, CPMV ribonucleoprotein component M, CPMV empty capsids (top component, T) (Bruening, 1969; Sterk and de Jager, 1987) or ultraviolet-inactivated CPMV or CPMV RNA preparations were not protective (Bruening et al., 2000; Eastwell and Kalmar, 1997; Sterk and de Jager, 1987). Sequential inoculation of CPMV and CPSMV greatly reduced the observed protection against CPSMV, compared to the results from co-inoculation experiments (Bruening et al., 2000, 1979; Bruening and Kiefer, 1981; Eastwell and Kalmar, 1997).

The term concurrent protection was proposed (Ponz and Bruening, 1986) to describe a reduction in challenging virus infection (observed number of infection centers or measured titer) due to co-inoculation (but not sequential inoculation) with a protecting virus that does not accumulate or induce symptoms in the host plant. When CPMV-susceptible cowpea seedlings, rather than seedlings from the lines showing extreme resistance to CPMV, were inoculated with CPMV and a challenging virus, the results based on symptom development were obscured by CPMV-induced symptoms. However, by selection of challenging viruses that produced symptoms very distinct from those produced by CPMV, by assessing capsid antigen accumulation rather than symptoms, or by using purified CPMV component B in place of CPMV for the protecting virus, it was possible to show that challenging virus accumulation and symptom induction were not significantly impaired in CPMV-susceptible cowpea lines by co-inoculation with CPMV (Bruening et al., 2000; Eastwell and Kalmar, 1997). Concurrent protection also was not observed for co-inoculation of CPSMV and CPMV to Arlington cowpea protoplasts (Ponz and Bruening, 1986). Plant virus systems that appear to exhibit concurrent protection have been reviewed (Bruening et al., 2000).

The occurrence of the phenomenon of concurrent protection in association with *Cpa*-mediated extreme resistance to CPMV and the reliance of that phenomenon on intact protecting CPMV RNA1 allow insight into *Cpa*-mediated extreme resistance. Although cowpea lines bearing the *Cpa* locus apparently do not accumulate new CPMV virions or show infection symptoms, presumably the activation of concurrent protection requires the expression of one or more CPMV genes, most likely a single gene under the gene-for-gene framework (Chisholm et al., 2006). Since the RNA1-containing CPMV component B is sufficient to initiate concurrent protection against a variety of cowpea-infecting viruses (Bruening et al., 2000) and CPMV RNA1 infections are constrained to the inoculated cell (Rezelman et al., 1982), the gene in question must be encoded by RNA1 and presumably acts within the inoculated cell for both extreme resistance and concurrent protection (Bruening et al., 2000). Here we examine expression of CPMV RNA1 genes in cowpea genotypes *Cpa*/*Cpa* (homozygous resistant) and *cpa*/*cpa* (susceptible) and attribute elicitor action to enzymically active CPMV 24KPro. Results presented here are derived in major part from the PhD dissertation of Qiuling Fan (Fan, 2008).

Results

Elicitor capability for Cpa-conferred resistance resides in the CPMV 24KPro gene

Sequences derived from CPMV RNA1 cDNA were expressed in cowpea leaves under the control of *Cauliflower mosaic virus* (CaMV) 35S promoters using a virus vector or one of two *Agrobacterium* binary vectors. *Tomato bushy stunt virus* (TBSV; gene map presented in Supplement 1, Fig. 1A) coat protein replacement vectors (Scholthof, 1999) were pHST12 and pXHST34, both of which induced chlorotic lesions on both *Cpa*/*Cpa* and *cpa*/*cpa* genotype cowpea seedlings. pHST12 has the first two ATGs of the TBSV coat protein gene mutated to AGG and therefore relies on the inserted sequence to provide a start codon. pHST12 also has an inactivated TBSV P19 suppressor of silencing (Supplement 1, Fig. 1B). pXHST34 was derived from pHST34 (Scholthof, 1999) and has an ATG and Xpress (Invitrogen) epitope tag preceding a multiple cloning site (SnaBI–XhoI–NotI) which is followed by a hexahistidine sequence (Supplement 1, Fig. 1C).

Sequences from the five genes encoded in CPMV RNA1 (Fig. 1A) were incorporated into four inserts of TBSV constructions, with complete sequences for both VPg and 24KPro being incorporated into the VPg-24K-Po fragment of RNA1 (Fig. 1B). Each of these constructions was inoculated to primary leaves of *Cpa*/*Cpa* and *cpa*/*cpa* genotype cowpea seedlings. Wildtype TBSV local lesion induction was little affected by co-inoculation with CPMV (Bruening et al., 2000), i.e., the concurrent protection effect was very weak. However, unlike co-

inoculation in a concurrent expression experiment, incorporation of potential elicitor-encoding sequences directly into the TBSV genome should lock TBSV replication directly to elicitor expression, temporally and spatially, just as CPMV replication and elicitor expression are linked in CPMV replication. Therefore, our expectation was that the extreme resistance to CPMV shown by *Cpa*/*Cpa* cowpea would be reflected by a similar restriction on increase of TBSV for whichever TBSV construction is bearing the RNA1-derived elicitor of the *Cpa* gene. For three of the constructions (Figs. 2A–C), no consistent difference in symptom development on the two cowpea genotypes was observed. However, VPg-24K-Po, expressed from the pHST12 vector, induced predominantly necrotic local lesions on *Cpa*/*Cpa* cowpea and predominantly chlorotic lesions on *cpa*/*cpa* cowpea (Fig. 2D). This differential, though not corresponding to extreme resistance, was taken as evidence for localization of a *Cpa* elicitor-encoding sequence within the VPg-24K-Po (Fig. 1B) region of RNA1.

In preliminary experiments, cell suspensions of *Agrobacterium tumefaciens* strain GV2260 bearing binary plasmid pCB4NN-GFP were pressure infiltrated into cowpea primary and trifoliate leaves. Compared to trifoliate leaves, primary leaves took up larger volumes at each infiltration site and larger areas could be infiltrated. However, far more cells became fluorescent at infiltration sites of trifoliate leaves. Fluorescence was detected under a fluorescence microscope with 488 nm illumination. The ability of cowpea lines to recognize and react to polypeptides encoded by the VPg-24K region of RNA1 was tested using *Agrobacterium* bearing the binary vector pCB4NN (Supplement 1, Fig. 2). As shown by the right halves of the leaflet images in Figs. 3A–G, constructions that included the 24KPro sequence and all or part of the VPg sequence induced a hypersensitive reaction (HR) in *Cpa*/*Cpa* but not *cpa*/*cpa* genotype cowpea, consistent with results in Fig. 2 using TBSV vectors.

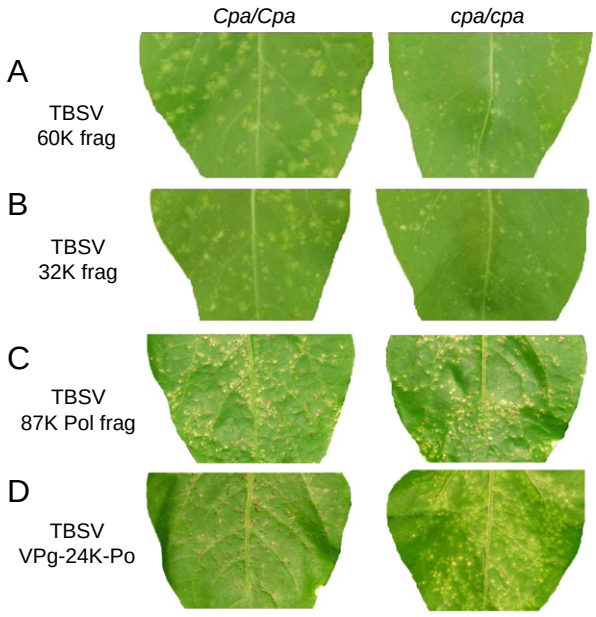

Fig. 2. Comparison of symptoms resulting from the inoculation of *Tomato bushy stunt virus* (TBSV) vector with inserts designed to express fragments (Fig. 1B) of the CPMV RNA1 polyprotein. The 60K helicase (A) and 32K (B) fragment sequences were inserted in TBSV vector pXHST34. A polymerase-derived fragment (C) and the full 24KPro sequence, flanked by the 3′ end sequence of the 60K helicase and complete VPg sequence and, on its 3′ side, by the 5′ end sequence of the polymerase gene (D), were installed in the pHST12 vector. Primary leaves of resistant (*Cpa*/*Cpa*) and susceptible (*cpa*/*cpa*) genotype cowpea were inoculated. Symptoms were photographed at 5–7 days after inoculation. Although leaf and lesion appearance varied among experiments, necrotic local lesions predominated only for the VPg-24K-Po and *Cpa*/*Cpa* combination, and only 24KPro-Encoding inserts showed a differential when inoculated to *Cpa*/*Cpa* and *cpa*/*cpa* genotype cowpea lines.

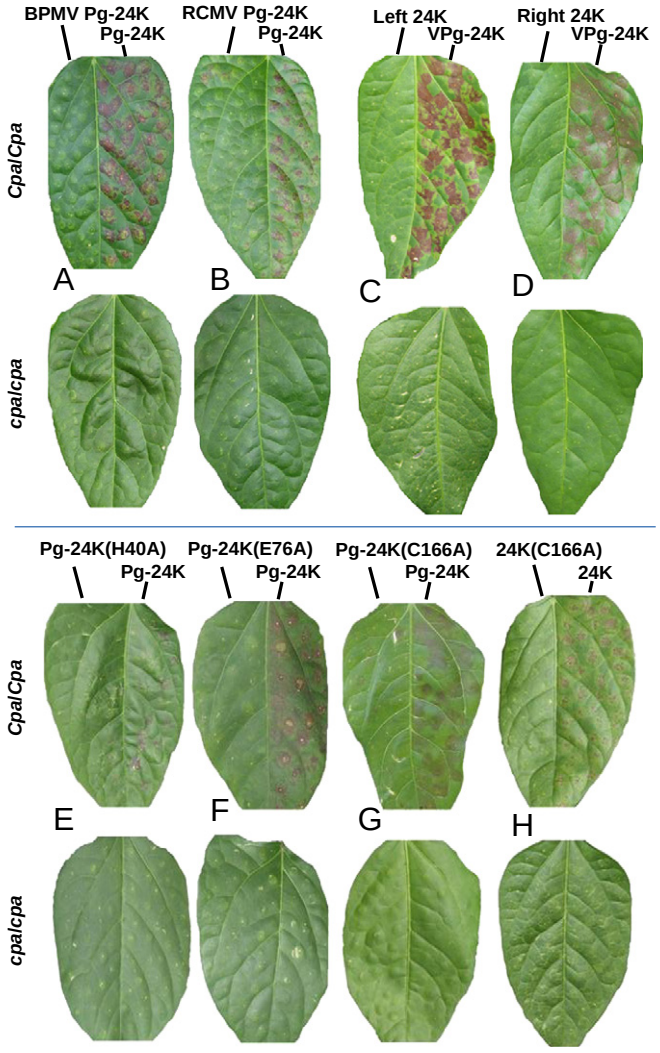

Fig. 3. Cowpea genotype-specific induction of a hypersensitive reaction by agroinfiltration for expression of intact CPMV 24KPro. Cowpea trifoliate leaflet halves were pressure infiltrated at multiple sites with *Agrobacterium tumefaciens* strain GV2260 bearing binary vector pCB4NN with inserts for expression of VPg-24K (Fig. 1D), Pg-24K (Fig. 1H), or 24KPro (Fig. 1G, corresponding to binary vector pCB4NN-24K). What is shown as the right half of each leaflet received constructions with an intact CPMV 24KPro sequence, as indicated by labeling above the upper leaflet for panels A–H, each panel consisting of an image of a *Cpa*/*Cpa* leaflet above an image of a *cpa*/*cpa* leaflet. The left panels of leaflets received (A and B) full 24KPro inserts from *Bean pod mottle virus* (BPMV) and *Red clover mottle virus* (RCMV), each with the 3′ 12 codons from the corresponding VPg gene; (C and D) two inserts, corresponding to the incomplete 24KPro sequences Left VPg-24K (Fig. 1E) and Right 24K (Fig. 1F), infiltrated into the left half of the leaflet. (E, F and G) Three alanine-substitution variants of the 24KPro sequence, at sites H, E and C of the Fig. 1C diagram and in the Pg-24KPro form (Fig. 1H), were agroinfiltrated into the left half of the leaflet. For (H), the right panel was agroinfiltrated with a 24KPro construction, with no flanking sequence, and the left panel received a construction of the same form but with an alanine substitution at C166. A HR was observed only for constructions expected to produce unmutated CPMV 24KPro (right leaflet half agroinfiltrated) and only for the *Cpa*/*Cpa* genotype.

The two comoviruses that appear to be most closely related to CPMV are *Bean pod mottle virus* (BPMV) and *Red clover mottle virus* (RCMV). All three comoviruses have a similar cysteine 24KPro (Chen and Bruening, 1992; Di et al., 1999; Shanks and Lomonossoff, 1990). Constructions of the Pg-24K type (Fig. 1H), but derived from RNA1 of BPMV RNA1 and RCMV RNA1 rather than CPMV RNA1, were prepared in pCB4NN. Neither of these comovirus Pg-24K constructions induced an HR in either *Cpa*/*Cpa* or *cpa*/*cpa* cowpea (Figs. 3A and B), suggesting that recognition of the Pg-24K polypeptide by *Cpa*/*Cpa* cowpea is specific to CPMV. Expression of CPSMV 24KPro also did not result in

any apparent difference in symptoms for *Cpa*/*Cpa* and *cpa*/*cpa* cowpea (unpublished observations).

Cowpea genotype-specific recognition of CPMV 24KPro requires that the protease is active

Results shown in Figs. 3C and D suggest that cowpea genotype-specific recognition of VPg-24K polypeptide does not depend on recognition of a motif or motifs in the full length sequence. Approximately two-thirds length amino-end and carboxyl-end polypeptides derived from the VPg-24K sequence, although they together represent the entire VPg-24K polypeptide, failed to induce an HR in trifoliate leaves of *Cpa*/*Cpa* cowpea when agroinfiltrated separately (Figs. 3C and D) or together (not shown). To test whether recognition relies on CPMV 24KPro enzymic activity, each of the three amino acid residues that form the catalytic triad, H40, E76 and C166 (Fig. 1C) (Bazan and Fletterick, 1988; Dessens and Lomonossoff, 1991; Gorbalenya et al., 1989), was individually replaced with an alanine residue in otherwise identical TBSV constructions of the el-24K-Po form. The necrotic lesions that are characteristic of the interaction between *Cpa*/*Cpa* genotype cowpea and TBSV constructions bearing an intact 24KPro sequence (Fig. 4A, left image; Fig. 2D) were not observed for the catalytic triad mutants (Figs. 4B–D). Consistent with results for other CPMV 24KPro constructions, *cpa*/*cpa* cowpea developed predominantly chlorotic TBSV lesions for all four constructions (Fig. 4).

The phenotypes of the 24KPro catalytic triad mutants also were examined by agroinfiltration, using constructions of the Pg-24K form (Figs. 3E–G). As expected, HR developed for infiltration sites receiving the wild type sequence but not for sites receiving any of the three catalytic triad mutant versions of the Pg-24K sequence. To eliminate any possibility of VPg sequences participating in the induction of the observed HR, a construction consisting of the 24KPro sequence alone (Fig. 1G), in both the wildtype and C166A mutant forms, was tested by agroinfiltration [binary plasmids designated pCB4NN-24K and pCB4NN-24K(C-A), respectively]. A HR was observed only at infiltration sites on *Cpa*/*Cpa* cowpea receiving the wildtype construction (Fig. 3H). These results connect the cowpea genotype-specific HR to expression and, specifically, function of 24KPro.

Cowpea non-genotype-specific recognition of the 24KPro

Extract was prepared from leaf lamella tissue of both *Cpa*/*Cpa* and *cpa*/*cpa* trifoliate leaves (like those of Fig. 3H) that had been agroinfiltrated with pCB4NN-24K. No signal was detected when the extract was analyzed by electrophoresis through sodium dodecyl sulfate-permeated polyacrylamide gel (SDS-PAGE) and immunoblotted using anti-24KPro antibody. The pEAQ-HT binary vector (Sainsbury et al., 2008; Sainsbury and Lomonossoff, 2008) is derived from CPMV RNA2 untranslated sequences. pEAQ-HT-GFP has been demonstrated to produce very high levels of GFP expression in *Nicotiana benthamiana*. Agroinfiltration of pEAQ-HT-GFP binary plasmid into *N. benthamiana* leaves resulted in a fluorescence signal very easily observed under a hand-held 360 nm UV light. A strong band with a migration distance expected for GFP protein was detected when extracts of the infiltrated leaf lamella were analyzed by SDS-PAGE and stained with coomassie brilliant blue dye. In contrast, when pCB4NN-GFP was infiltrated into *N. benthamiana* leaves, a microscope was required to detect a 488 nm-activated fluorescence signal, and no band was observed after leaf extract was analyzed by SDS-PAGE and staining (not shown).

Proteolytically active CPMV 24KPro has been generated in *N. benthamiana* after agroinfiltration of pEAQ-HT-24K (Saunders et al., 2009). We constructed binary plasmid pEAQ-HT-24K(C-A) with a C166A substitution for expression of proteolytically inactive CPMV 24KPro. Agroinfiltration of pEAQ-HT-24K and pCB4NN-24K gave very similar, *Cpa*/*Cpa*-specific reactions after agroinfiltration into cowpea leaves (Supplement 3 result compared to the Fig. 3H result). pEAQ-HT-24K was agroinfiltrated into *cpa*/*cpa* and into *Cpa*/*Cpa* trifoliate leaves. 24KPro accumulation was observed for *cpa*/*cpa* leaves (Fig. 5, lane 10)

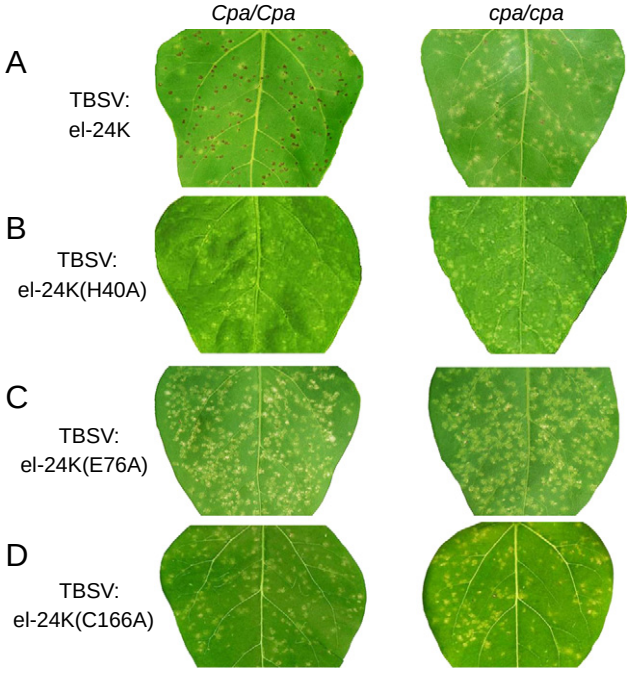

Fig. 4. TBSV vector expression of 24KPro sequences with alanine substitutions of catalytic triad amino acid residues. Cowpea primary leaves were inoculated with plasmid pXHST34 DNA bearing the el-24K insert (Fig. 1I diagram) as the wildtype sequence and as three alanine-substitution variants at the sites H, E and C shown in the Fig. 1C diagram. Necrotic lesions predominated only for *Cpa*/*Cpa* leaves inoculated with the construction designed to express a full length, unmutated CPMV el-24K sequence.

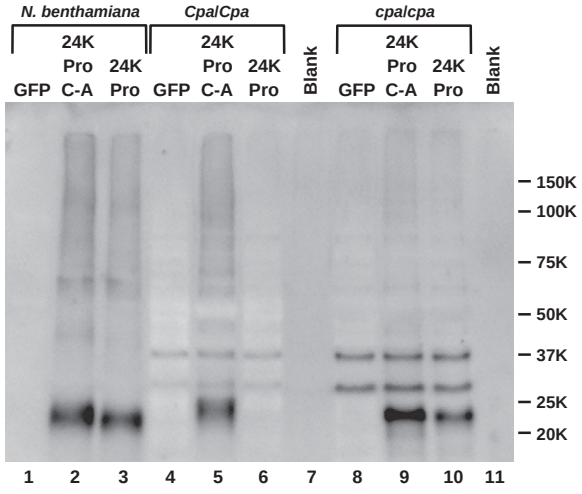

Fig. 5. Transient expression of CPMV 24KPro and its catalytic triad C166A mutant in cowpea and *Nicotiana benthamiana*. Agroinfiltration was performed as for Fig. 3 but with binary plasmid pEAQ-HT with inserts for expression of the two versions of 24KPro or GFP. Extract introduced into the gel well corresponded to 1 mg of leaf tissue for *Cpa*/*Cpa* (lanes 4–6) and *cpa*/*cpa* (lanes 8–10) cowpea and to 50 μg for *N. benthamiana* (lanes 1–3) leaf tissue, collected at 5 days after infiltration. After electrophoresis through 8–16% gradient polyacrylamide gel with SDS in the upper reservoir buffer, immunoblots were prepared and exposed to primary antibody against CPMV 24KPro. As is indicated above the chemiluminescence image, extracts were from leaves infiltrated with the GFP construction (lanes 1, 4 and 8), the 24KPro mutant C166A construction (lanes 2, 5 and 9), and the wildtype 24KPro construction (lanes 3, 6 and 10). The 24KPro antibody consistently reacted with two additional bands of unknown origin, with migration distances corresponding to 28K and 37K, in extracts from cowpea of either genotype.

but not for *Cpa*/*Cpa* leaves (Fig. 5, lane 6). Agroinfiltration of pEAQ-HT-24K(C-A) generated a band, detected by immunoblotting, at the 24KPro migration position for both genotypes (Fig. 5, lanes 5 and 9). The apparent ability of constructions, designed to express 24KPro, to in fact suppress 24KPro accumulation is paralleled by results from co-agroinfiltration of pCB4NN-24K and pCB4NN-GFP. Co-agroinfiltration results in suppression of GFP accumulation compared to GFP accumulation after agroinfiltration of pCB4NNGFP alone (Supplement 4).

Both 24KPro and 24KPro(C-A) accumulated in agroinfiltrated leaf lamella of *N. benthamiana* plants and in amounts more than 20-fold greater, per unit mass of leaf extracted, than was observed in cowpea (Fig. 5, lanes 2 and 3 compared to lanes 5, 9 and 10). Neither 24KPro or 24KPro(C-A) protein was detected in extracts of the *N. benthamiana* leaf extract by coomassie stain of these gels. Agroinfiltration of the pEAQ-HT-GFP construction gave no detected 24KPro signal on immunoblots with anti-24KPro primary antibody, as expected (Fig. 5, lanes 1, 4 and 8).

The relative amounts of 24KPro protein, in the bands that migrated to the 24K position, were estimated from summed pixel densities in rectangles outlining the bands, after background subtraction of pixel densities from corresponding areas of lanes receiving leaf extract after GFP agroinfiltration. The results were expressed as ratios. The average and standard deviation for the ratio of 24KPro accumulation in *Cpa*/*Cpa* cowpea to 24KPro accumulation in *cpa*/*cpa* cowpea was 0.0 ± 0.04 for 9 measurements from 3 agroinfiltration experiments, confirming the apparent lack of 24KPro accumulation in *Cpa*/*Cpa* cowpea (Fig. 5, lane 6). The average and standard deviation for the ratio of 24KPro accumulation over 24KPro(C-A) accumulation in *N. benthamiana* were 0.85 ± 0.09 (6 measurements from 2 agroinfiltrations), whereas the values for the same ratio for *cpa*/*cpa* cowpea were 0.51 ± 0.05 (5 measurements from 2 agroinfiltrations). The unpaired *t* test found the difference between the results for *N. benthamiana* and for *cpa*/*cpa* cowpea to be significant ($P < 0.0001$).

Discussion

CPMV-mediated concurrent protection has been strictly associated with *Cpa*-conferred resistance in progeny of *cpa*/*cpa* x *Cpa*/*Cpa* crosses, and inoculation of the RNA1-containing virion B was sufficient to provide concurrent protection in *Cpa* cowpea against the same virus species that were subject to concurrent protection by mixtures of M and B virions (Bruening et al., 2000). Therefore, we focused on the products of RNA1-encoded genes as candidates for the elicitor of *Cpa*-controlled resistance.

Of the virus vectors tested in preliminary experiments, derived from *Potato virus X*, TBSV and *Tobacco mosaic virus*, only the TBSV vector consistently induced easily scored lesions. The TBSV vector also tolerated CPMV-derived cDNA inserts well, with only a few fold dimunition in lesion numbers when vectors with and without insert were compared (G. Bruening and J.M. Buzayan, unpublished observations). Fifty percent of the RNA1 sequence was tested as four fragments (Figs. 1B and 2). The four fragments contained part or all of the five RNA1 genes and were of similar size, with the intent of placing a similar burden on the TBSV expression vector. In our application of the two distinct expression systems, based on TBSV vectors and *Agrobacterium* binary plasmids, the results were consistent in those instances where the same or very closely related inserts were tested.

Results comparing the phenotypes of the constructions shown in Figs. 1B–F, presented in Figs. 2 and 3C and D, mapped a *Cpa* genotype-specific reaction to the RNA1-encoded intact 24KPro or to short flanking sequences thereof. To exclude the possibility of a contribution to elicitation from the sequences flanking 24KPro, the precise 24KPro construct, without any flanking sequences (Fig. 1G), was tested by agroinfiltration of binary plasmid pCB4NN-24K. This construction elicited a HR in *Cpa*/*Cpa* cowpea (Fig. 3H). These observations suggest that the intact 24KPro protein, the only protease encoded in the CPMV genome, rather than any epitope within it or the RNA encoding it, is responsible for activation of *Cpa*-mediated extreme resistance to CPMV.

Although expression of the full-length 24KPro sequence appears to be sufficient for elicitation of a *Cpa*-genotype-specific HR, other results are consistent with 24KPro proteolytic function being a necessity. Previous work demonstrated that replacement of cysteine166 by a serine residue reduced, but did not abolish, 24KPro proteolytic activity in the processing of the RNA1 polyprotein as it was synthesized in an in vitro protein synthesis system (Dessens and Lomonossoff, 1991). The substitution of alanine for cysteine166 would be expected to actually or very nearly eliminate 24KPro activity by leaving no nucleophile at the 166 position. Similarly, alanine substitutions for the 24KPro His40 or Glu76 would be expected to result in no or at most very marginal proteolytic activity (Dessens and Lomonossoff, 1991). All three catalytic triad 24KPro mutants gave no significant induction of HR and hence no *Cpa* versus *cpa* genotypic differential (Figs. 4 and 3E–H). 24KPro accumulated after agroinfiltration of pEAQ-HT-24K into *cpa*/*cpa* but not *Cpa*/*Cpa* cowpea trifoliate leaves (Fig. 5 and pixel density measurements). All of these results strongly support the notion that enzymically active CPMV 24KPro is required for activation of extreme resistance against CPMV and presumably for concurrent protection against other viruses co-inoculated to *Cpa* cowpea.

We contend that induction of HR and induction of extreme resistance to CPMV, both of which are *Cpa*-genotype-specific, are reflective of elicitation by the enzymic action of CPMV 24KPro. As anticipated by this contention and the specificity of virus proteases, two other 24KPro enzymes, those of the comoviruses BPMV and RCMV, did not induce a *Cpa*-specific response (Figs. 3A and B). Like CPMV 24KPro, the BPMV and RCMV 24KPro enzymes are cysteine-active site, chymotrypsin-like proteases (Di et al., 1999; Shanks and Lomonossoff, 1990), and they show 50% and 54%, respectively, identity to the CPMV 24KPro. However, amino acid sequences surrounding the sites cleaved in the polyprotein substrates of BPMV and RCMV 24KPro are different from those for the CPMV polyprotein substrates (Chen and Bruening, 1992; Di et al., 1999; Shanks and Lomonossoff, 1990). BPMV infects both *Cpa* and *cpa* cowpea poorly (our unpublished result). The infectivity of RCMV in cowpea was not tested due to importation limitations on this virus.

The CPMV-*Cpa* system resembles the system of *Potato virus Y* (PVY), a potyvirus, and *Ry* gene-mediated potato resistance against this virus. *Ry* confers extreme resistance to PVY. The PVY gene *NIa* encodes the nuclear inclusion protein a. NIa has two domains. The amino portion of NIa comprises the VPg of PVY, and the carboxyl portion is a protease analogous to CPMV 24KPro, designated NIaPro. A proportion of NIa molecules are cleaved by the action of NIaPro to release VPg and NIaPro (Adams et al., 2005). Transient expression of PVY NIaPro by agro-infiltration elicited a HR in *Ry*/*Ry* but not in *ry*/*ry* potato. A similar genotypic differential was observed after agro-infiltration for expression of the NIaPro of the potyvirus *Pepper mottle virus*, which matches the cleavage specificity of PVY NIaPro, whereas the corresponding proteases of two other potyviruses, with different cleavage specificities, did not induce HR (Mestre et al., 2000). Various PVY NIaPro alanine-substitution mutants were tested by an *in planta* assay for protease activity and by agro-infiltration into *Ry*/*Ry* potato and assessment of HR development (Mestre et al., 2003). No mutant form of NIaPro that lacked protease activity also showed elicitor activity. However, there were three mutants that retained full protease activity in the *in planta* assay but failed to induce, or induced only limited, HR. It remains to be determined whether the phenotype of mutants of this last type reflects a requirement for an activity other than host protein cleavage or is the result of an alteration in substrate specificity in one or more host protein cleavage reactions. Non-catalytic-triad substitutions into CPMV 24KPro were not tested here.

For both of the transient-expression systems applied here, the cowpea genotype-specific and functional CPMV 24KPro-specific reaction was HR development rather than complete suppression of

TBSV or *Agrobacterium* accumulation. As described in the previous paragraph, this substitution of HR for extreme resistance during the interaction of a transiently expressed virus elicitor in a host showing extreme resistance was also demonstrated for potato expressing the *Ry* gene (Mestre et al., 2003). Similar results also were obtained for the potato *Rx* gene conferring extreme resistance to PVX, a system that exhibits concurrent protection (Köhm et al., 1993). Transient expression of the *Rx* gene in tobacco transformed to generate PVX coat protein, which is the *Rx* elicitor, resulted in a HR (Bendahmane et al., 1999, 2000). It has been suggested that extreme resistance may be a form of highly localized HR in which virus infection is terminated in the inoculated cell, consistent with our observation of no detected accumulation of 24KPro protein in agroinfiltrated *Cpa*/*Cpa* leaf tissue. Others (Ghazala and Varrelmann, 2007; Goulden et al., 1993; Köhm et al., 1993) provide other speculations about the connection between HR development following expression of a virus elicitor and extreme resistance to infection by the corresponding virus.

Accumulation of 24KPro(C-A) in pEAQ-HT-24K(C-A)-agroinfiltrated cowpea trifoliate leaves was much less than its accumulation in similarly infiltrated *N. benthamiana* leaves (Fig. 5). This difference is consistent with the much greater accumulation of GFP in pEAQ-HT-GFP-agroinfiltrated *N. benthamiana* than in agroinfiltrated cowpea. Nevertheless, degradation of CPMV 24KPro in *cpa*/*cpa* cowpea trifoliate leaves may also be contributing to the low recovery of this protease. In general, in analyses of extracts of CPMV-infected cowpea tissue or cells, anti-24KPro antibody reveals a trace of, or no, free 24KPro. Most of the signal is associated with more slowly migrating proteins, presumably incompletely processed CPMV RNA1 polyprotein products, rather than free 24KPro protein itself [results for cowpea leaf protoplasts in lane 1 of Fig. 4 of (Garcia et al., 1987) and results for infected cowpea trifoliate leaves from our unpublished immunoblots]. The animal picornaviruses and comoviruses have similar patterns of gene expression based on proteolytic processing of polyproteins; the picornavirus protease homologous to the comovirus 24KPro is 3C protease. The 3C proteases of picornaviruses hepatitis A and encephalomyocarditis virus 3C are efficiently degraded by an ubiquitin pathway (Gladding et al., 1997). Versions of CPMV 24KPro that have flanking amino acid sequences, and are likely to have retained those sequences when expressed in cowpea (Figs. 1I and J), also retained genotype-specific HR-inducing activity (Fig. 4A). That 24KPro has proteolytic activity in its polyprotein forms is indicated by cleavage at the 32K-Hel junction (Fig. 1A) while 24KPro still is embedded in the 1866 amino acid RNA1 polyprotein and the general pattern of RNA1 polyprotein processing (Goldbach and Wellink, 1996; Lomonossoff and Shanks, 1983). These results and deductions suggest that the principal inducer of Cpa-mediated extreme resistance to CPMV may be proteolitically active 24KPro still embedded in the partial cleavage products of the RNA1 polyprotein, rather than free 24KPro.

Our observations on 24KPro and 24KPro(C-A) accumulation in *cpa*/*cpa* and *Cpa*/*Cpa* cowpea after agroinfiltration of the cognate binary constructs suggest that plants of both genotypes recognize enzymically active 24KPro and suppress its accumulation, but *Cpa*/*Cpa* cowpea is far more effective in this regard. Results related to *Cpa*-mediated cowpea resistance to CPMV appear to be consistent with the guard hypothesis (Rafiqi et al., 2009; van der Biezen and Jones, 1998; van der Hoorn and Kamoun, 2008). Under this hypothesis, CPMV 24KPro recognizes and cleaves one or more cowpea proteins, generating a factor which ignites a plant defense response with the outcomes of genotype-specific HR or extreme resistance to CPMV and CPMV-mediated concurrent protection. Considering various picornaviruses, the host protein substrates of the various 3C proteases form a diverse set (Armer et al., 2008; Papon et al., 2009; Perera et al., 2007; Weng et al., 2009; Zaragoza et al., 2006). Therefore, it is reasonable to expect that CPMV 24KPro may have multiple protein substrates in the cowpea cell, and 24KPro may act as a virulence factor of CPMV.

Consistent with the ability of active 24KPro to suppress its own accumulation (Fig. 5), as a result of its action on a host cell protein, is the phenomenon of concurrent protection and our observation that co-agroinfiltration for expression of 24KPro and GFP results in decreased accumulation of GFP specifically in *Cpa*/*Cpa* cowpea (Supplement 4); all of these observations suggest inhibition of protein synthesis in cells receiving active 24KPro.

The concept of the microbe-associated molecular patterns (MAMP) was developed primarily on the basis of analyses of bacterium–plant interactions, including interactions in which protease action plays a key role (Jones and Dangl, 2006; Rafiqi et al., 2009). The greater accumulation of 24KPro(C-A) compared to 24KPro, in pEAQ-HT-agroinfiltrated cpa/cpa cowpea (Fig. 5 and pixel density calculations), suggests that superimposed on the *Cpa*-mediated extreme resistance is a general, not cowpea genotype-specific, recognition of functional 24KPro protease. Proteolytically active CPMV 24KPro, and the proteases of other plant viruses, may act not only to mediate virus gene expression but also as effectors of virulence and as MAMPs.

Materials and methods

Materials

As described by Bruening et al. (2000), nearly isogenic cowpea lines 9405CII and 9405Gii, genotypes *Cpa*/*Cpa* and *cpa*/*cpa*, respectively, were derived from a backcross series initiated with a cross of Blackeye 5 × Arlington (Beier et al., 1977). The sources of comovirus RNA1 cDNA clones were, for CPMV, plasmid pCP1 from George Lomonossoff, John Innes Centre, Norwich, UK, GenBank X00206.1 (Dessens and Lomonossoff, 1993); for BPMV, plasmid pGG7R1 from Said Ghabrial, University of Kentucky, GenBank U70866.1 (Gu and Ghabrial, 2005) and for RCMV, plasmid pBS22, George Lomonossoff, GenBank X64886.1 (Shanks et al., 1996). Herman Scholthof , Texas A&M University, provided TBSV coat protein replacement vectors pHST12 and pHST34 (Scholthof, 1999). Vectors pUNCB5 (Harvey et al., 2008) and pCB4NN and *A. tumefaciens* strain GV2260 were provided by James Lincoln, University of California at Davis. pEAQ-HT, pEAQ-HT-GFP and pEAQ-HT-24K were generously supplied by George Lomonossoff, John Innes Centre, Norwich, UK. The nucleotide sequences of oligodeoxyribonucleotide primers and adaptors (d-oligos), purchased from Operon, are presented in Supplement 2, Table 1. Restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs. Cloned Pfu Taq DNA polymerase was from Stratagene. Qiagen kits were applied for purification of PCR products, extraction of gel electrophoresis bands, and plasmid minipreps.

TBSV plasmid constructions

Maps for pHST12 and pXHST34 are presented in Supplement 1, Figs. 1B and C. Standard molecular techniques were as described by Sambrook and Russell (2001). Constructions were verified by sequencing at the University of California, Davis, Sequencing Facility or at Davis Sequencing, Davis, CA. Plasmid pXHST34 was created from TBSV vector plasmid pHST34 (Scholthof, 1999) by inserting an Xpress epitope tag (DLYDDDDK, Invitrogen) and a hexahistidine tag flanking the 5′ and 3′ ends, respectively, of a multiple cloning site (XhoI, SnaBI and NotI) (Supplement 1, Fig. 1C). Two d-oligos, TBSV-Xpress-lower and TBSV-6xHis-TAG-upper, were 5′-phosphorylated using T4 polynucleotide kinase and were annealed to TBSV-ATG-Xpress-upper and TBSV-6xHis-lower, respectively (Supplement 2, Table 1). The two products of the annealing reactions were ligated with T4 DNA ligase. After electrophoresis through 8% polyacrylamide gel in Tris–borate–EDTA (TBE) buffer and ethidium bromide staining, the double-stranded DNA product of interest was recovered from the gel. After 5′phosphorylation of the DNA, it was ligated into NotI- and SnaBI-cut pHST34. Sequencing reactions were primed with d-oligo TBSV p92 (Supplement 2, Table 1).

Inserts for TBSV vectors pHST12 and pXHST34 were amplified by PCR using plasmid pCP1 as template and primer pairs noted in Supplement 2, Tables 2 and 3. A typical thermocycling program was 94 °C 3 min; 25 cycles (94 °C 30 s, 52 °C 30 s, 72 °C 1 min); 72 °C 7 min. The PCR products were digested with XhoI and SnaBI and ligated into XhoI- and SnaBI-cut pHST12 or pXHST34. Transformants were confirmed by sequencing using primer TBSV p92.

Construction of pCB4NN binary plasmids bearing comovirus 24KPro sequences

Comovirus 24K-derived segments (Fig. 1) were amplified by PCR using primer pairs designated in Supplement 2, Table 4. A typical thermocycling program was: 94 °C 3 min; 25 cycles (94 °C 30 s, 55 °C 30 s, 72 °C 1 min); 72 °C 7 min. The PCR products were digested with EcoRI and XhoI and ligated into intermediate plasmid pUNCB5 (Supplement 1, Fig. 2) that had been cut with the same enzymes. Clones in pUNCB5 were digested with NotI to release the 24K sequence flanked by a *Cauliflower mosaic virus* 35S promoter and an octopine synthase terminator (OCS) and were ligated into NotI-cut pCB4NN. Transformants were selected on LB plates containing 50 μg/mL kanamycin, 50 μg/mL X-gal and 50 mM IPTG (0.05 M) (Harvey et al., 2008). Plasmid sequencing primers were CB4F and CB4R (Supplement 2, Table 1).

Replacement of the 24K protease catalytic triad codons with an alanine codon

Alanine substitutions were performed by overlapping PCR (Higuchi et al., 1988) using two primer pairs, one pair corresponding to the pair used in cloning the 24KPro-encoding sequence and a second pair with complementary 5′ regions encoding the nucleotide substitutions, as indicated in Supplement 2, Tables 1, 2 and 4. For construction of binary plasmid pEAQ-HT-24K(C-A), the 24KPro(C-A)-encoding sequence of plasmid pCB4NN-24K(C-A) was transferred to pEAQ-HT by PCR amplification and oriented cloning at the NruI and XhoI sites (Supplement 2, Tables 1 and 5).

TBSV inoculation

The TBSV plasmid constructions were inoculated at a DNA concentration of 50 μg/mL in 30 mM K_2HPO_4, 20 mM KH_2PO_4, 1 mM Na_2EDTA by rub inoculation to cowpea primary leaves that had been dusted with 600 grit carborundum. The inoculated cowpea plants were maintained in a glass house for 5–7 days for symptom development.

Preparation of A. tumefaciens electro-competent cells and leaf infiltration

Preparation of electro-competent cells of *A. tumefaciens* strain GV2260 and inoculum were as described (Harvey et al., 2008). The pCB4NN constructs were transformed into *A. tumefaciens* strain GV2260 by electroporation (Bio-Rad *E. coli* Pulser) (Shen and Forde, 1989). Cowpea leaves were infiltrated with or without nicking the lower epidermis of the leaf lamella with a 26 G needle and then immediately placing a needle-less syringe over the puncture, supporting the opposite surface of the upper epidermis, and slowly and steadily pressure infiltrating the *Agrobacterium* cell culture. Photographs were taken 4–5 days after infiltration.

Analysis of extracts from infiltrated tissue

Tissue samples (100 mg) of infiltrated and uninfiltrated of *N. benthamiana* or cowpea leaf lamella regions were homogenized with a Mini-Beadbeater-16 cell disrupter (BioSpec Products, Bartlesville, OK) in 100 μL of QB extraction buffer (100 mM potassium phosphate buffer, pH 7.8, 1 mM sodium EDTA, 1 mM dithiothreitol (DTT), 1 mM 1% Triton-X-100, and 10% glycerol), and the resulting lysate was clarified by centrifugation at 16,000×*g* at 4 °C for 15 min (Ni et al., 1996). The supernatant was diluted with an equal volume of 2× high pH disruption solution (10 M urea, 136 mM dithiothreitol, 192 mM Tris, 45 mg/mL SDS, 50 μg/mL brom phenol blue tracking dye) and heated in a boiling water bath for 4 min. The solution was cooled to room temperature on ice, and 0.045 volumes of 2 M HCl were added before applying the sample to 8–16% SDS-polyacrylamide gradient gels (precast gel, Thermo Scientific). Proteins were electroblotted to polyvinylidene fluoride (PVDF) membrane (LeGendre and Matsudaira, 1988). Membranes were exposed to blocking solution [PBST (10 mM Na_2PO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, autoclaved; add Tween 20 to 1 μg/mL and non-fat dry milk to 50 mg/mL] for an hour to overnight and then were incubated with a 1:10,000 dilution of anti-CPMV 24K polyclonal antiserum (gift from Joan Wellink, Agricultural University, Wageningen, The Netherlands) in immunoblotting buffer for an hour at room temperature with constant shaking.

After rinsing the PDVF membrane twice for 15 min each with PBST solution, the membrane was incubated with 1:10,000 dilution of goat anti-rabbit immunoglobulin, conjugated to horseradish peroxidase (Thermo Pierce) in immunoblotting buffer (PBST, 10 mg/mL non-fat dry milk) for 1 h with shaking and at room temperature followed by two rinses with PBST. The membrane was incubated for 5 min with super signal west dura extended duration substrate (Thermo Pierce). Chemiluminescence was visualized using a Kodak Image Station 2000R with no excitation illumination or filter. Exposures were for 3–100 min. Analyses of pixel density were performed with the ImageJ program (Ferreira and Rasband, 2010).

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.04.022.

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