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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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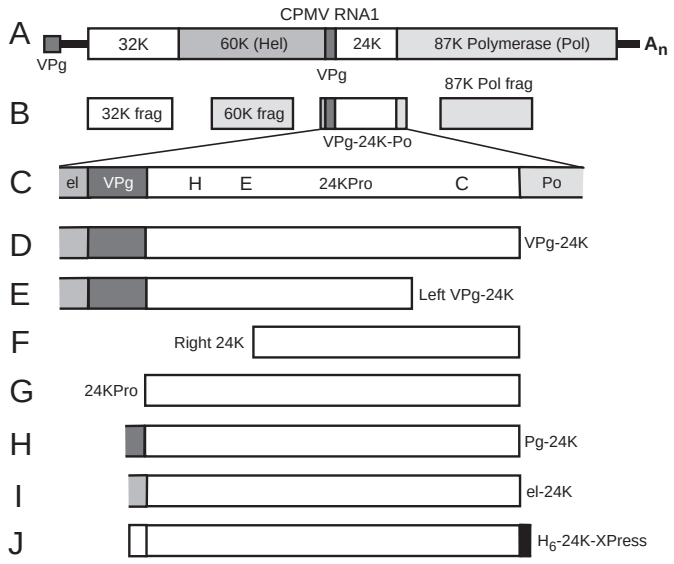

Fig. 1. Genetic map of *Cowpea mosaic virus* (CPMV) RNA1 and representation of RNA1 fragmentary sequences tested by expression from a *Tomato bushy stunt virus* (TBSV) vector and *Agrobacterium* binary vectors. A. CPMV RNA1 has a 5′-linked VPg, 3′-polyadenylate, a single open reading frame (ORF) encompassing 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-