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An Inhibitor of Polyprotein Processing with the Characteristics of a Natural Virus Resistance Factor

Fernando Ponz, Christopher B. Glascock, and George Bruening

Department of Plant Pathology, College of Agricultural and Environmental Sciences, University of California, Davis, California 95616, U.S.A.
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Seedlings of cowpea (*Vigna unguiculata* cv. Arlington) are operationally immune to cowpea mosaic virus (CPMV), giving no detected increase of inoculated virus, but they are susceptible to another comovirus, cowpea severe mosaic virus (CPSMV). Arlington-derived immunity against CPMV is controlled by a single dominant locus in crosses to susceptible line Blackeye 5. Previous results revealed an inhibitor of the proteolytic processing of a CPMV polyprotein in extracts of Arlington cowpea protoplasts, and the inhibitor was postulated to effect the observed immunity. We found this inhibitory activity in partially fractionated extracts of Arlington cowpea leaves. Two other activities also were candidates for immunity factor(s) by virtue of their greater potency in Arlington than in Blackeye 5 cowpea leaf extracts: inhibitor(s) of the translation of CPMV RNAs and proteinase(s) that degrade CPMV-encoded proteins. The proteinases degraded CPSMV and CPMV proteins equally well, and the activities were not coinherited with immunity against CPMV in progeny of cowpea crosses. The inhibitor of CPMV polyprotein processing possessed both the virus specificity and the coinheritance that are expected for an agent conveying immunity to CPMV. The inheritance of the translation inhibitor activities was complex, and our results show that one or more of these activities may contribute to the immunity against CPMV.

Additional key words: cowpea mosaic virus, cowpea severe mosaic virus, inhibitor of translation, inhibitor of virus proteinase, proteinases, proteinase inhibitor

The comovirus cowpea mosaic virus (CPMV) failed to increase to a detected level in seedlings of the cultivar Arlington cowpea (*Vigna unguiculata*); and among the more than 1,000 lines tested, only Arlington cowpeas gave leaf protoplasts that resisted infection by CPMV (Beier *et al.* 1977, 1979). Another comovirus, cowpea severe mosaic virus (CPSMV), infects Arlington and Blackeye 5 cowpeas and increases to a high titer in both seedlings and protoplasts. The virus-specific, operational immunity of Arlington cowpea seedlings to CPMV was inherited, in crosses with the susceptible Blackeye 5 cowpea, as if it is controlled by a simple dominant locus. Kiefer *et al.* (1984) correlated, among the progeny of such crosses, the resistance of protoplasts to CPMV with the operational immunity of the corresponding seedlings. They showed that CPMV-inoculated Arlington cowpea protoplasts accumulated less capsid antigen and less of the RNA that is complementary to the genomic CPMV RNAs, per unit of genomic RNAs, than did CPMV-inoculated Blackeye 5 cowpea protoplasts. Based on these results, Kiefer *et al.* (1984) speculated that Arlington cowpea protoplasts and seedlings specifically restrict the replication of CPMV, but not CPSMV, by reducing or preventing the production of CPMV proteins, such as capsid proteins and RNA-dependent RNA polymerase.

CPMV proteins have, as their precursors, polyproteins that are translated from the CPMV genomic RNAs 1 and 2 (e.g., Wellink *et al.* 1986). RNA 2 encodes the M_r 95,000 polyprotein (95K) from which is released two proteins, 48K, from the amino terminal portion, and 60K, the precursor of the two capsid proteins. Initiation of *in vitro* translation closer to the 5′ end of RNA 2 generates the polyprotein 105K, which upon cleavage gives 58K and 60K. The larger RNA 1 is translated into the 200K precursor of several nonvirion proteins and the 5′-linked protein of the genomic RNAs, VPg. Among the activities of proteins encoded by RNA 1 is one that results in the cleavage of 95K to produce 48K and 60K (Franssen *et al.* 1982; Wellink *et al.* 1986). Sanderson *et al.* (1985) compared extracts of Arlington and Blackeye 5 cowpea protoplasts for their ability to interfere with *in vitro* processing of 95K and found an Arlington-specific inhibitor activity. This result suggests that a specific inhibitor of 95K polyprotein processing is a, or the, CPMV-resistance factor of Arlington cowpeas, through its action in specifically preventing the production of CPMV proteins. The result also is consistent with the virus specificity of Arlington cowpea resistance against CPSMV, since the polyprotein processing activity of CPMV was not active against the 95K polyprotein of CPSMV (Goldbach and Krijt 1982).

The objectives of the research reported here were to determine whether extracts of Arlington cowpea leaves, like the previously examined extracts of Arlington cowpea protoplasts, have an activity that interferes with the proteolytic processing of the CPMV 95K polyprotein and whether that activity has characteristics expected for a mediator of immunity to CPMV. Our *in vitro* assays of partially fractionated leaf extracts revealed not only inhibitor(s) of CPMV polyprotein processing but two other activities as well, proteinase(s) that degrade CPMV proteins and inhibitor(s) of the translation of CPMV RNAs. All three activities were more potent in leaf extracts of Arlington cowpeas than of Blackeye 5 cowpeas and thus were candidate mediators of immunity to CPMV. All three were tested for two other characteristics deemed to be essential for CPMV-restricting factors of Arlington

cowpeas: specificity for CPMV over CPSMV and coinheritance, in progeny of cowpea crosses, with immunity against CPMV.

MATERIALS AND METHODS

Cowpeas, viruses, and virus RNA. The origins of the SB isolate of CPMV and of CPSMV isolate DG have been described (Beier *et al.* 1979). Virions were purified as previously described (Bruening 1969). Cowpea cultivar Blackeye 5 and line Arlington (U.S. Department of Agriculture Plant Introductions 293457 and 293453, respectively) were from the collection in this laboratory. Cowpeas were grown in vermiculite wetted with a modified Hoagland's nutrient solution in a growth chamber maintained at 28° C during the 16-hr light cycle and 23° C during the dark portion of the cycle. Inoculations were according to Kiefer *et al.* (1984).

Preparations enriched in the RNA 1-containing bottom or the RNA 2-containing middle components of CPMV or CPSMV were obtained by repeated centrifugation in CsCl gradients (Bruening 1969) or/and by rate zonal centrifugation in linear-log sucrose gradients (Brakke and van Pelt 1970). RNA was isolated from virions by the procedure of Mandeles and Bruening (1968), and its integrity was analyzed by electrophoresis through 1.5% agarose-urea gels (Locker 1979) in the buffer of Loening (1967). RNA was aliquoted in sterile deionized water and stored at −80° C.

Cowpea genetic crosses. The female parent of each cowpea cross was Blackeye 5 or another homozygous susceptible (ii) cowpea (Beier *et al.* 1979; Kiefer *et al.* 1984). Pollen was from Arlington or other homozygous immune (II) cowpea or from a heterozygous (Ii) cowpea, all of which were derivatives of Blackeye 5 × Arlington. We determined operational immunity or susceptibility of the progeny by inoculating with CPMV and assessing symptoms 7–10 days later (Beier *et al.* 1977). The genotypes of F_2 progeny were determined by selfing them and scoring the F_3 progeny for immunity and susceptibility. The F_3 seedlings from any F_2 cowpea scored as all immune or all susceptible or had a 3:1 ratio of immune to susceptible progeny, in the expected ratios and as observed previously (Kiefer *et al.* 1984). On this basis the F_2 progeny were assigned the genotypes II, ii, or Ii, respectively.

An F_3 II line was obtained from a sib cross of II and ii F_2 progeny from Blackeye 5 × Arlington. The F_3 II line was crossed to Blackeye 5 to produce the progeny of what we designate as the first backcross. Progeny of each succeeding backcross to Blackeye 5 cowpea were selected only for operational immunity (Beier *et al.* 1977) to CPMV, through seven serial crosses. F_3 progeny of the last cross were scored to identify one cowpea line each of the genotypes ii and II. These susceptible and immune lines are expected to be nearly isogenic with each other and with Blackeye 5 cowpeas.

Preparation and fractionation of extracts. Uninoculated cowpeas were grown for 3 wk in a controlled environment chamber before harvest of leaf tissue. Tissue was used immediately or was stored at −20° C for up to 2 mo before extraction. Fifty-gram samples of leaf tissue were homogenized in 200 ml of ice-cold extraction buffer (EB = 80 mM $Na_2B_4O_7$, 480 mM H_3BO_3, 290 mM NaCl, 1 mg/ml sodium ascorbate; modified from Quayle *et al.* [1980]) in which phenylmethylsulfonyl fluoride was freshly dissolved to 1 mM. The slurry was filtered through four layers of cheesecloth and centrifuged in a Sorvall GSA rotor for 10 min at 10,000 rpm. The supernatant was loaded immediately onto a 46 cm × 5.4 cm column of cross-linked dextran beads (Sephadex G-25 coarse, calibrated for void volume with blue-dyed dextran) equilibrated with EB. The green colored void volume fractions were collected and combined with 1.2 volumes of ammonium sulfate solution that had been saturated at 4° C and been brought to pH 7 with aqueous ammonia. Precipitation overnight and subsequent steps were at 4° C.

The precipitate was collected by centrifugation in a Sorvall GSA rotor for 15 min at 10,000 rpm. The precipitate was dissolved at room temperature in 20 ml of TDTT buffer (20 mM Tris-HCl, pH 6.8, 1 mM dithiothreitol). The solution was then clarified by centrifugation in a Sorvall SS-34 rotor for 5 min at 10,000 rpm. Five milliliters of the supernatant was loaded onto a 65 cm × 1.5 cm column of cross-linked 6% agarose beads (Sepharose Cl-6B) that was equilibrated and eluted with TDTT. Fractions of 1.8 ml were collected at a flow rate of 10 ml/hr. Fractions were pooled according to the A_{280} profile and were stored at −20° C until assayed. Protein concentrations were determined using the BCA microassay (Pierce Chemical Co., Rockford, Illinois) with bovine serum albumin as the protein standard.

***In vitro* translation and polyprotein processing.** Micrococcal nuclease-treated rabbit reticulocyte lysate with 140 mM potassium acetate, 1 mM magnesium acetate, and unspecified concentrations of spermidine, dithiothreitol, hemin, creatine phosphate, creatine phosphokinase, and calf liver tRNA was from Promega Biotec (catalog no. L4157). Most reactions mixtures were of 10 μl final volume, of which 6 μl contained components needed for translation: 4.1 μl of lysate, 16 μCi of [^{35}S]methionine (Amersham, >800 Ci/mmol), 20 μM each amino acid other than methionine, 4.3 units of ribonuclease inhibitor RNasin (Promega-Biotec), and 1.0–1.2 μg of CPMV RNA or CPSMV RNA. The amount of RNA for each batch of lysate was set to give maximum incorporation after 1 hr at 30° C into materials precipitated by trichloroacetic acid (Promega Biotec Technical Bulletin 003). Four microliters of fractions of cowpea leaf extract or of TDTT buffer was added either before any incubation or after an initial incubation for translation without added extract fraction. Preliminary experiments showed that the dilution from 6 μl to 10 μl had little effect on the extent of incorporation or the pattern of polypeptides synthesized; supplementing the reaction mixtures with potassium acetate or magnesium acetate did not increase the extent of incorporation. Reaction mixtures were analyzed directly or stored at −20° C.

Electrophoresis and fluorography. PAGE (Laemmli 1970) in 12.5%, $NaDodSO_4$-permeated gels, usually of dimensions 10 cm × 12 cm × 0.75 mm (BioRad Mini-Protean II cell), employed a modified sample solution containing 4.4 M urea in place of glycerol. Electrophoresis generally was at 200 volts for 40 min. One microliter of mercaptoethanol was added to 9 μl of sample buffer immediately before use for each sample. The equivalent of 1–2 μl of each assay reaction was loaded per well. The gel was permeated with fluor using En^{3}Hance as recommended by New England Nuclear, Inc. The Kodak XAR-5 film was preexposed before fluorography (Laskey and Mills 1975).

RESULTS AND DISCUSSION

Fractionation of extracts of cowpea leaves. The early

steps in the fractionation procedure were designed to minimize accumulation of pigments associated with the action of polyphenol oxidases. A borate buffer, modified from Quayle *et al.* (1980), should esterify phenolic compounds, and exclusion chromatography on cross-linked dextran separated the bulk of the phenols from the macromolecular constituents of the extract. Extracts so treated remained green on storage.

We found that extracts of Arlington cowpea leaves have strong proteinase activities and inhibitor(s) of *in vitro* translation. These activities prevented assay for the inhibitor(s) (Sanderson *et al.* 1985) of the processing of CPMV polyproteins in the extract. Therefore, we further fractionated the extract. Exclusion chromatography of cowpea extracts on cross-linked 6% agarose beads, of material eluted from the cross-linked dextran column, resolved four broad "peaks" (A_{280} profile, Fig. 1). Peak I was green and contained membrane fragments. All four zones emerged before the total volume of the column had been eluted, indicating their macromolecular composition.

Proteinase activity that degraded virus polyproteins. Polyproteins translated from CPMV and CPSMV genomic RNAs, especially RNA 2, were substrates in assays for cowpea proteinase activity. As expected (e.g., Beier *et al.* 1981), each RNA 2 preparation directed the synthesis of the 95K and 105K polyproteins (Fig. 2, lanes 1, 6). Assays by PAGE showed that the products of cleavages of polyproteins by the cowpea proteinases were unlike the products of the same polyproteins cleaved by the *in vitro* proteolytic processing activities of the corresponding virus. The products of extensive digestions by cowpea proteinases had mobilities greater than those of the bulk of the *in vitro* processing products, so sequences within the mature CPMV and CPSMV proteins must be substrates of the cowpea proteinases. The cowpea proteinase activities were strongest in peak II fractions (Fig. 1) from Arlington cowpea extracts (Fig. 2, compare lane 3 and buffer control lane 1) but were detected weakly in peak III fractions and very weakly in peak IV fractions, as well (not shown).

The peak II proteinase activities in extracts of Blackeye 5 cowpeas, although readily detected, were much weaker than those of Arlington cowpeas (Fig. 2, lanes 2, 3), a result that might be expected if such proteinases contribute to the operational immunity that is characteristic of Arlington cowpeas. However, by two criteria the Arlington cowpea proteinase activities appear not to contribute to immunity: the proteinase activities lacked the expected specificity for proteins of CPMV, and they were not inherited in parallel with immunity to CPMV in cowpea genetic crosses.

At equal concentrations of protein in the peak II fractions, the Arlington cowpea proteinase activities were equally effective in degrading CPMV and CPSMV polyproteins (Fig. 2, compare lanes 1 with 3 and 4 with 6). The peak II fractions from extracts from several F_1 and F_3 progeny of Blackeye 5 × Arlington were examined. F_3 progeny cowpeas that scored as homozygous susceptible (ii) to CPMV produced an extract that had much stronger proteinase activities than were observed for extracts from immune F_1 (Ii) progeny cowpeas (Fig. 2, lanes 7, 8). These

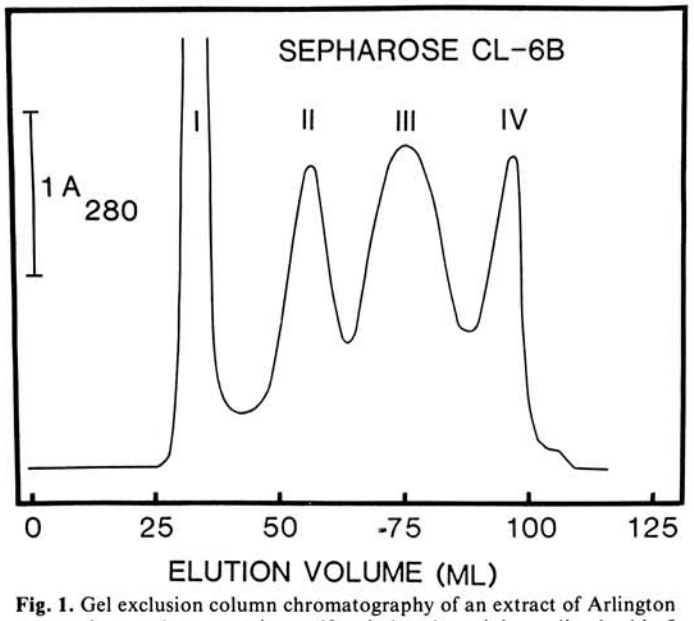

Fig. 1. Gel exclusion column chromatography of an extract of Arlington cowpea leaves. An ammonium sulfate-induced precipitate, dissolved in 5 ml of 20 mM Tris-HCl, 1 mM dithiothreitol, pH 6.8, was applied to a 115-ml bed volume column of cross-linked, 6% agarose beads, equilibrated, and eluted with the same buffer. The elution rate was 10 ml/hr, and the effluent was monitored continuously at 280 nm. Four broad zones of eluted material are numbered I–IV.

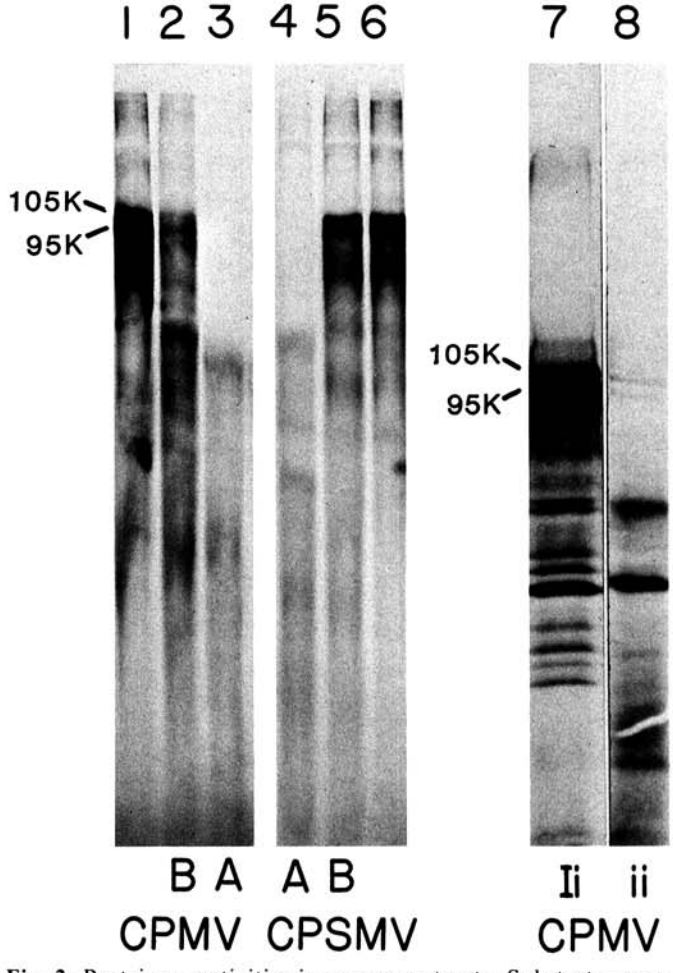

Fig. 2. Proteinase activities in cowpea extracts. Substrates were [^{35}S]methionine-labeled products of 1-hr translations of cowpea mosaic virus (CPMV) RNA 2 (lanes 1–3, 7, 8) or cowpea severe mosaic virus (CPSMV) RNA 2 (lanes 4–6) in rabbit reticulocyte lysate. The incubated translation reaction mixtures (6 μl) were combined with 4 μl of buffer (lanes 1, 6) or of fraction II (see Fig. 1) from exclusion chromatography of cowpea extracts from cowpea leaves: Arlington (A, 78 μg/ml, lanes 3, 4); Blackeye 5 (B, 78 μg/ml, lanes 2, 5); derivative from Blackeye 5 × Arlington, F_1, heterozygous immune to CPMV (Ii, 450 μg/ml, lane 7) and F_3, homozygous susceptible to CPMV (ii, 500 μg/ml, lane 8). Solutions were incubated at 30°C for 1 hr before analysis by PAGE and fluorography. Locations of zones for 95K and 105K CPMV polyproteins are indicated.

results are inconsistent with a role for the Arlington cowpea proteinases, as assayed *in vitro*, in the immunity of Arlington seedlings against CPMV.

An inhibitor of the *in vitro* translation of CPMV RNAs. The inhibitor(s) of translation (Fig. 3) found in extracts of Arlington cowpea leaves were almost entirely confined to peak IV (Fig. 1) after exclusion chromatography of the extracts on 6% agarose beads. The inhibitors of translation apparently do not prevent translation by acting as nucleases because the CPMV RNA supplied to the translation system is no more degraded when incubated with reticulocyte lysate and peak IV extract than when incubated with lysate alone. They were effective not only against the translation of comovirus RNAs but also genomic RNAs of brome mosaic virus, tobacco mosaic virus, and tobacco ringspot virus and globin mRNA (results not shown).

The inhibitors reduced the accumulation of products directed by mixed CPMV RNAs, in a nuclease-treated reticulocyte lysate system, to below what was observed without added mRNA (Fig. 3, compare lanes 1, 2, 4). The peak IV fractions from an extract of Blackeye 5 cowpeas were much less effective inhibitors of translation than the fractions from Arlington cowpeas (Fig. 3, lanes 2–4). This is a result that might be expected if the operational immunity of Arlington cowpeas is effected at least in part by the cowpea inhibitors of *in vitro* translation.

Although the inhibitors of translation from Arlington cowpea were effective against CPSMV RNA as well as CPMV RNA (Fig. 3, lanes 5- 8), up to a 10-fold greater concentration of the peak IV proteins was required to inhibit the translation of CPSMV RNAs. In preliminary trials we determined the greatest concentration of the peak IV proteins that would allow nearly uninhibited levels of translation of CPMV RNAs and of CPSMV RNAs. Peak IV proteins in the reaction mixtures analyzed in Figure 4, lanes 4 and 11, were at these concentrations of 23 and 230 μg/ml, respectively.

The extracts from genotype II cowpea, derived from a seven step backcross series, were more potent inhibitors of *in vitro* translation of CPMV RNAs than were the corresponding extracts from the genotype ii cowpea (Fig. 3, lanes 9–12). Thus at least one inhibitor of translation seems to have been inherited in parallel with immunity to CPMV in crosses of Blackeye 5 and Arlington. However, among the several cowpea lines we have derived from Blackeye 5 and Arlington, one ii derivative from a series, which was distinct from the seven step backcross, gave a peak IV extract that totally inhibited the translation of CPMV RNAs at a final

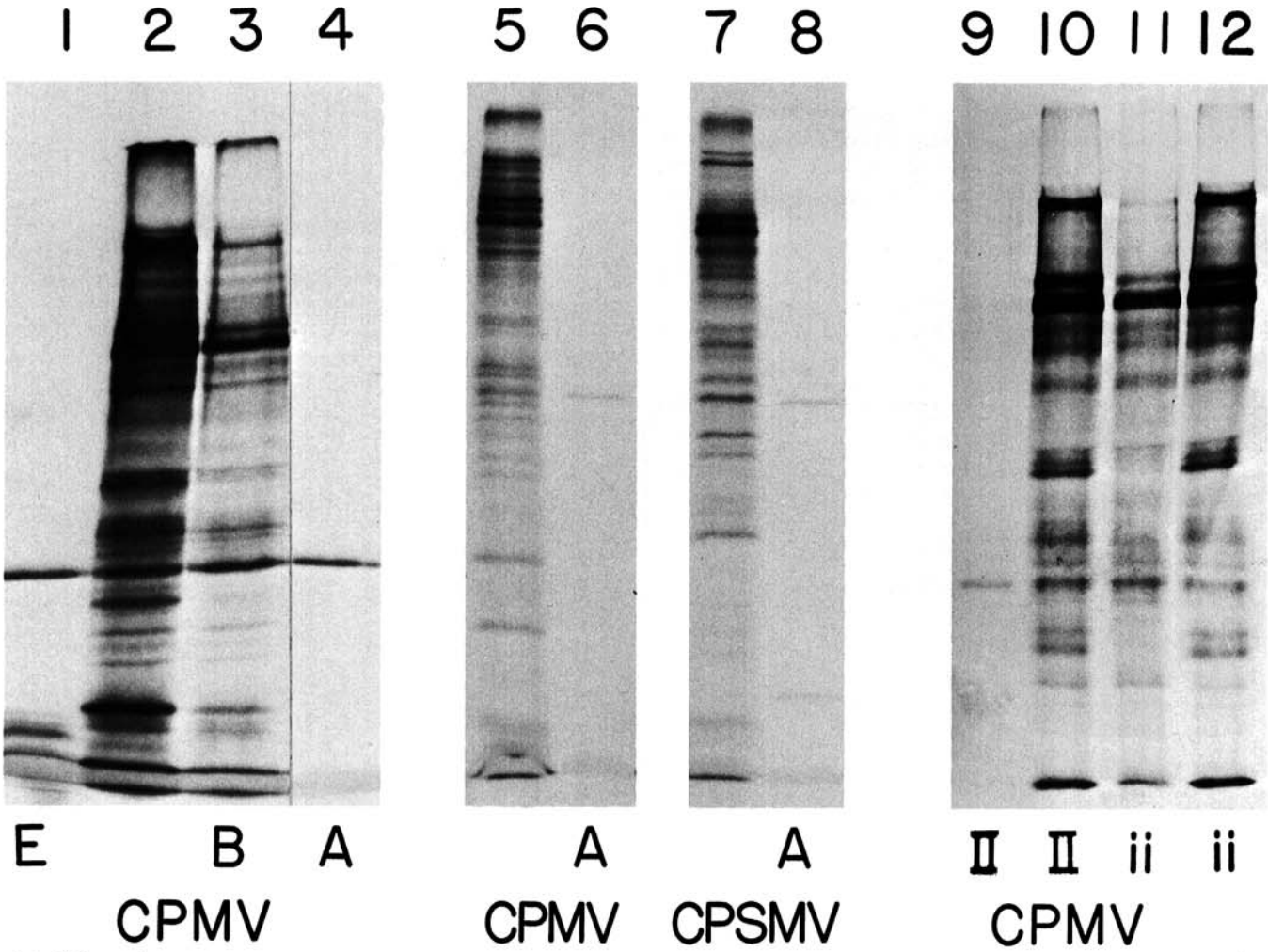

Fig. 3. Inhibitors of *in vitro* translation in extracts of cowpea leaves. The RNAs that programmed the 10 μl-translation reaction mixtures, and the corresponding lanes on the polyacrylamide gel, were unfractionated genomic RNAs of CPMV (lanes 2–6, 9–12) or of CPSMV (lanes 7, 8) or none (endogenous, E, lane 1). Reaction mixtures included 4 μl of buffer (lanes 1, 2, 5, 7) or 4 μl of diluted or undiluted peak IV fractions of extracts chromatographed on 6% agarose beads (as in Fig. 1). The sources of cowpea extracts and the final concentrations of protein contributed to the reaction mixtures are, for Blackeye 5, 16 μg/ml (B, lane 3) and, for Arlington, 12 μg/ml (A, lanes 4, 6, 8). Extracts from the two F_3 derivatives from a seven step backcross series also were tested, one homozygous immune (II, lanes 9, 10), the other homozygous susceptible (ii, lines 11, 12). The protein concentrations were 360 (lanes 9, 11) and 18 μg/ml (lanes 10, 12). Analysis was by PAGE and fluorography.

protein concentration of 20 μg/ml. The *in vitro*-assayed inhibitors of translation appear to be controlled by several genetic loci.

Our results on the quantitative differences in virus specificity and on the inheritance of inhibitors of translation do not allow us to eliminate the possibility that one or more of these activities may have some role in the immunity of Arlington cowpeas against CPMV. Several general inhibitors of translation have been identified in plant extracts (Jiménez and Vázquez 1985). One of particular interest is the pokeweed antiviral protein. This protein inhibits the mechanical transmission of plant viruses and, in pokeweed tissue sections, appears to be confined to matrix of the cell wall (Ready *et al.* 1986). If one or more of the inhibitors of translation from Arlington seedlings is localized in the cell wall, this might explain the greater resistance of Arlington cowpea seedlings, than of protoplasts (Beier *et al.* 1979), to CPMV.

An inhibitor of proteolytic processing of CPMV polyproteins. Pelham (1979) showed that the translation of CPMV RNA 1 in a cell-free system, containing extract from rabbit reticulocytes, generated sufficient proteolytic processing activity to convert the polyproteins derived from CPMV RNA 2, 95K (and 105K), to 60K and 48K (and 58K). Our assays for inhibitor of CPMV polyprotein processing rely on the formation of the 60K and 48K processing products on long incubation of the *in vitro* translation reaction mixture programmed by genomic CPMV RNAs (Fig. 4, lanes 1, 2). In preliminary experiments, an activity that inhibits the processing of the polyproteins translated from CPMV RNA 2 was detected (Fig. 4, compare lanes 2 and 4) in peak IV fractions from Arlington. This activity was not detected in peak I and peak III fractions and could not be assayed in peak II fractions because of the proteinases.

Two variations of the assays for inhibitors of polyprotein processing were designed to avoid the effects of the inhibitors of translation. In one the peak IV fractions were sufficiently diluted to have ineffective concentrations of the inhibitors of translation. In the other, the peak IV fractions were added 30 min after initiating *in vitro* translation, a time

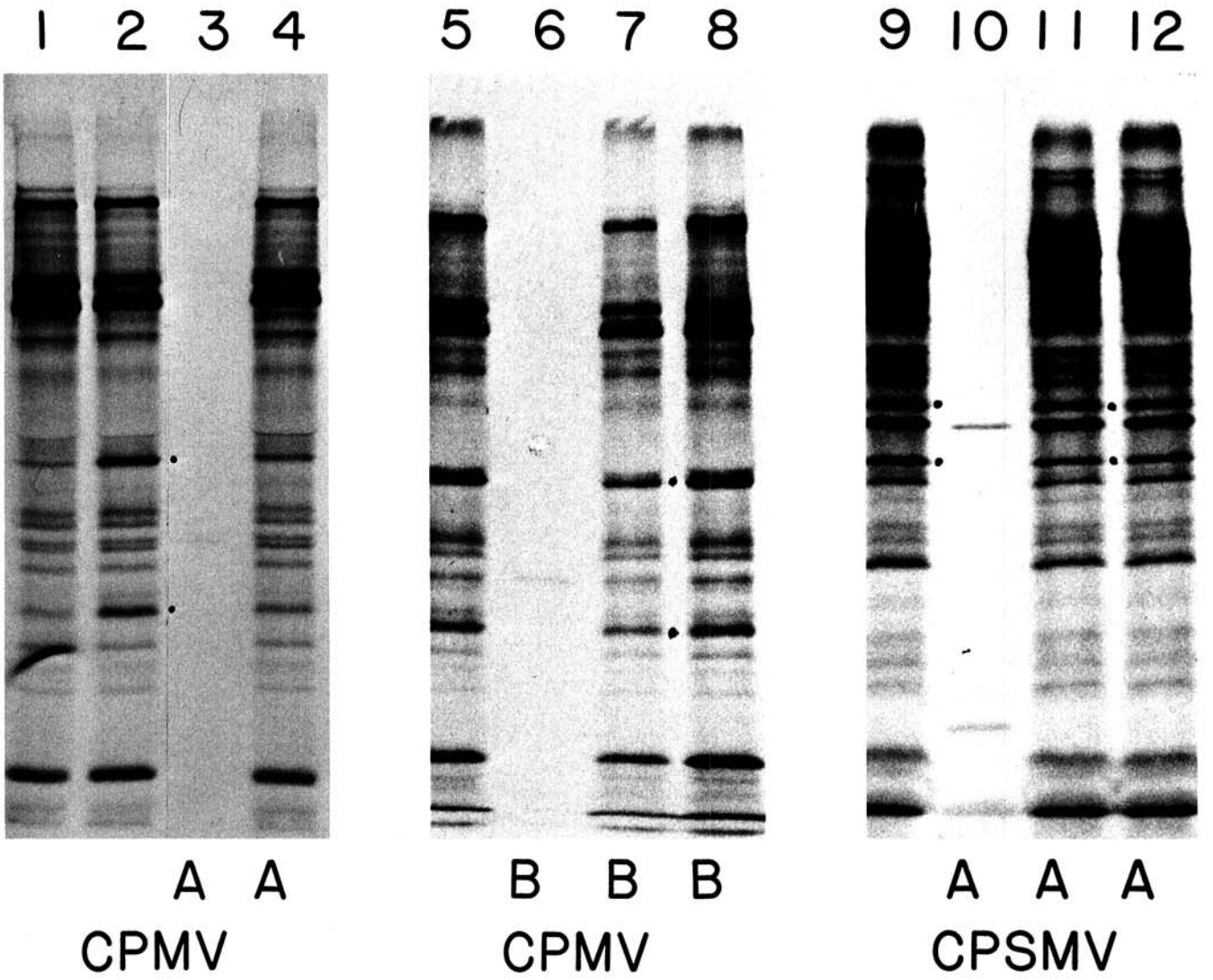

Fig. 4. Inhibitors of proteolytic processing of a CPMV polyprotein. RNAs programming the 10 μl translation reaction mixtures were unfractionated genomic RNAs of CPMV (lanes 1–8) and of CPSMV (lanes 9–12). Incubations were for 90 min, except for 30 min for lane 1. Reaction mixtures that contained 4 μl of buffer and no cowpea extract were applied to lanes 1, 2, 5 and 9; each of the other reaction mixtures included 4 μl of peak IV chromatography fractions (defined in Fig. 1). The preparation from the same Arlington cowpea extract was supplied at four final protein concentrations, 1.1 mg/ml (lanes 3, 10) and 23, 230, and 164 μg/ml (lanes 4, 11, 12, respectively). Similarly, final protein concentrations from the same Blackeye 5 cowpea extracts were 370, 37, and 12 μg/ml (lanes 6, 7, 8, respectively). Analysis was by PAGE and fluorography. Dots locate products of polyprotein processing reactions, as indicated by their late appearance in the translation reaction mixtures (early time of incubation not shown for CPSMV RNA).

after which little translation but most of the proteolytic processing occurred (Fig. 4, lanes 1, 2). In timed assays (not shown) we identified products of polyprotein processing reactions of CPSMV, and zones for these are marked in Figure 4, lanes 9, 11, and 12 (compare Beier *et al.* 1981). However, translation and polyprotein processing were not clearly temporally separated in the CPSMV system, and we assessed the potential of dilute fractions of cowpea extracts to interfere with CPSMV polyprotein processing by adding them when the *in vitro* translation was initiated.

A 1:50 dilution of pooled peak IV fractions from an extract of Arlington cowpea leaves caused almost no reduction in the accumulated translation products of CPMV RNAs but reduced the production of the 60K and 48K processing products (Fig. 4, lanes 2, 4) by 80%, as determined by densitometric tracings of fluorograms. As noted, the peak IV fractions from extracts of Blackeye 5 cowpea leaves were less potent inhibitors of translation than those from Arlington cowpeas. With additions of increasingly more dilute Blackeye 5 peak IV fractions (Fig. 4, lanes 5–8), the extent of translation and the extent of polyprotein processing increased in parallel.

The translation of CPSMV RNAs showed a sharp increase at a critical dilution of added Arlington peak IV fractions (Fig. 4, lanes 9–11). No dilution of Arlington peak IV fractions, which allowed translation of CPSMV RNAs, caused a specific diminution of the zones previously identified as products of the proteolytic processing of CPSMV polyproteins. Thus these assays revealed no evidence for an inhibitor of polyprotein processing in the extracts of Blackeye 5 cowpea leaves and no action of the inhibitor from Arlington cowpea leaf extracts against the processing of CPSMV polyproteins. The decrease in the products of polyprotein processing that were observed in reactions containing the Blackeye 5 peak IV fractions can be attributed to decreased translation rather than decreased processing *per se*.

Extracts (peak IV) of F_3 cowpeas from the seven step backcross series showed a differential effect when added at 30 min to incubated translation reaction mixtures programmed by CPMV RNAs. At a similar concentration of cowpea proteins, the fraction from the genotype II cowpea effectively interfered with polyprotein processing (Fig. 5, lane 1), whereas the fraction from a homozygous susceptible plant did not (lane 3). Dilution of the former fraction allowed only limited processing (lane 2). In these assays, as has been observed by others (Franssen *et al.* 1982), the 48K zone appeared as a doublet.

The observed characteristics of the inhibitor of polyprotein processing of Arlington cowpea extracts are entirely consistent with its postulated role as a, or the, factor that mediates the immunity of Arlington cowpeas against CPMV. No dilution of peak IV fractions from Blackeye 5 cowpeas that allowed extensive translation of CPMV RNAs showed any evidence of an inhibitor of CPMV polyprotein processing, nor was the activity apparent in peak I or III fractions from Blackeye 5 (data not shown). Hence the inhibitor of polyprotein processing was Arlington cowpea-specific. It showed the expected virus specificity and the expected coinheritance with immunity in a backcross series selected only for the character of operational immunity to CPMV. Thus the gene or genes that specify the inhibitor of polyprotein processing must, at the very least, be closely linked to genes that control operational immunity to CPMV.

We speculate that the Arlington cowpea-derived characters of immunity of seedlings against CPMV and inhibition of proteolytic processing of the 95K polyprotein are controlled principally by a single gene, a virus-specific resistance gene. Possibly a second, closely linked gene for an inhibitor of translation also has a role in virus resistance. Under our hypothesis, the product of the first gene is a protein that is a specific proteinase inhibitor. The activity of this postulated protein now has been detected by *in vitro* assays of fractionated extracts of both leaves and leaf protoplasts (Sanderson *et al.* 1985) of the Arlington cowpea.

That protein inhibitors of polyprotein processing may be of general utility in restricting infections by certain viruses is indicated by the results of Korant *et al.* (1985). Cultured human cells were exposed to cystatin, a 116 amino acid residue protein from chicken egg white that is an inhibitor of cysteine proteinases. No alteration in the pattern of newly synthesized proteins was detected in uninfected cells, but the yield of poliovirus after subsequent inoculation, and the accumulation of correctly processed poliovirus proteins,

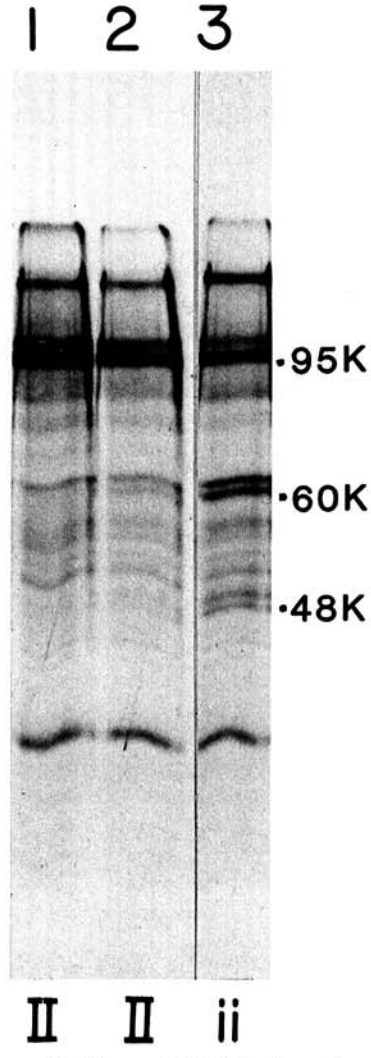

Fig. 5. Coinheritance of inhibitors of CPMV polyprotein processing and operational immunity to CPMV. Assay conditions were as given in Figure 4 except that 6 μl reaction mixtures were incubated at 30° C for 30 min and only then were brought up to the full 10 μl-volume by addition of a peak IV chromatography fraction of cowpea leaf extract. Incubations were continued for 60 min. Extracts were from homozygous immune (II, lanes 1, 2) and homozygous susceptible (ii, lane 3) cowpeas derived from the immunity-selected seventh backcross of Blackeye 5 to Arlington. Final protein concentrations contributed by the peak IV fractions are 120 μg/ml (lanes 1, 3) and 36 μg/ml (lane 2). Zones for the 95K polyprotein and its 60K and 48K (doublet) proteolytic processing products are marked.

both were reduced. Proteinaceous inhibitors of proteinases that effect resistance of plants against insects have been well characterized (Cleveland *et al.* 1987).

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