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Characterizing the Interference between Two Comoviruses in Cowpea

Kenneth C. Eastwell
Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, B.C., Canada, V0H 1Z0

Gabriel B. Kalmar
Kinetek Pharmaceuticals, Inc., Suite 150-520 West 6th Avenue, Vancouver, B.C., Canada V5Z 1A1

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Abstract. **In certain cultivars of cowpea [*Vigna unguiculata* (L.) Walp.] that are operationally immune to cowpea mosaic virus strain SB (CPMV), coinoculation of CPMV with cowpea severe mosaic virus strain DG (CPSMV) reduces severity and delays expression of symptoms normally induced by CPSMV alone. In cultivars susceptible to both viruses, coinoculation delays development of symptoms in response to CPSMV. Using monoclonal antibodies for serological assays and virus-specific RNA probes for hybridization, it is demonstrated that the presence of CPMV in the inoculum yields a concomitant delay in the synthesis of CPSMV coat protein and replication of CPSMV RNA and restricts the transport of CPSMV out of infection centres. Only bottom component of CPMV containing RNA1 is required to offer protection against CPSMV. Destroying the integrity of CPMV RNA eliminates its protective capability. In cowpea cultivars that are operationally immune to CPMV, the presence of CPSMV in the inoculum is unable to compensate for events of CPMV replication that are inhibited. The lack of complementation suggests a high degree of specificity in the replication of these two comoviruses.**

Cowpea mosaic virus (CPMV) and cowpea severe mosaic virus (CPSMV) are comoviruses. Once considered strains of a single virus, CPMV and CPSMV share many attributes including serological epitopes (Kalmar and Eastwell, 1989; Swaans and van Kammen, 1973) and genome organization (de Souza, 1987). Beier et al. (1977) have demonstrated that a number of cowpea (*Vigna unguiculata*) cultivars are operationally immune to CPMV. Seedlings of 'Arlington' are immune to infection by CPMV, while protoplasts isolated from 'Arlington' support CPMV replication, but at a greatly reduced level relative to that in protoplasts of highly susceptible cultivars (Beier et al., 1979). This is unique among all other cultivars tested in that protoplasts prepared from leaf tissue of other cultivars are highly susceptible to infection by CPMV regardless of the susceptibility of intact seedlings (Beier et al., 1979). The immunity to CPMV infection exhibited by 'Arlington' cowpeas has been examined at the biochemical and genetic levels. A dominant allele at a single locus or tightly linked loci are responsible for conferring the immune phenotype (Kiefer et al., 1984; Ponz et al., 1988). The presence of the immunity factor in backcross generations to susceptible cultivars correlates with a proteinaceous component that inhibits the proteolytic cleavage of CPMV RNA2 translation products; functional cleavage products are essential for the transport of progeny CPMV to adjacent tissues (Wellink and van Kammen, 1989; van Lent et al., 1990). Immunity or resistance to CPSMV was not observed in 1000 cowpea cultivars screened for immunity (Bruening et al., 1979), although some examples of resistance to CPSMV have been described (e.g., Rios and das Neves, 1982). It has been shown that CPMV and CPSMV interact when inoculated onto some lines of cowpeas. The presence of CPMV in the inoculum limits expression of symptoms in response to CPSMV (Bruening et al., 1979). This phenomenon has been referred to as interference rather than cross-protection since, in the former, the effect is greatest when both agents are introduced simultaneously. However, because of similarity of the viruses at the molecular level, investigations of this interaction have been confined to cowpea cultivars that are immune to the inducing virus so that its replication would not confound analysis of the challenge virus.

Using selective assay techniques, the constituent coat proteins and RNAs of each virus can be estimated independently; the analysis of virus replication is not limited to the formation of infectious particles. Moreover, the interference between two viruses in hosts that are highly susceptible to both viruses is possible without sacrificing accuracy of diagnosis.

The present study seeks to confirm that CPMV in the inoculum mitigates the symptoms elicited by CPSMV. This relationship has been reported in 'Arlington', but we now extend the examination of this phenomenon to include two other cowpea cultivars, California Blackeye 5 and Black, each exhibiting different responses to inoculation by CPMV alone. Furthermore, data are presented to address whether symptom expression alone is affected, or whether the replication of CPSMV or its individual components are also affected by the presence of CPMV in the inoculum.

Materials and Methods

Virus and plant stocks. CPMV-strain SB was obtained from the American Type Culture Collection (#PV272) and CPSMV-strain DG was obtained from the virus collection of G. Bruening (Univ. of California, Davis, Calif.). Both viruses were maintained in cowpea 'California Blackeye 5' (Vermont Bean Seed Co., Fair Haven, Vt.) and isolated according to Bruening (1969). Seeds of 'Arlington' and 'Black' cowpea were provided by G. Bruening.

CPMV was fractionated into top, middle, and bottom components by density gradient centrifugation (Bruening, 1969).

Plant inoculation. Cowpea seeds were planted in trays of vermiculite and grown at 23 °C under fluorescent light with a 16-h photoperiod providing ≈115 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR. When second-

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ary leaves were visible but <0.5 cm in length, plants were placed under reduced light (≈1 μmol·m^{-2}·s^{-1} PAR) for ≈1 h and dusted with carborundum (325 grit), and leaves were inoculated with virus or viral RNA diluted in inoculation buffer (0.05 M potassium phosphate, pH 7.0). Inoculated leaves were rinsed with distilled water. For sequential inoculations, the leaves were blotted dry with filter paper before they were inoculated again. After inoculation, plants were returned to reduced light for 30 min before resumption of the normal light regimen.

Detection of virus capsid protein. Preparation and characterization of murine monoclonal antibodies specific for CPMV and CPSMV and their use in triple antibody sandwich enzyme-linked immunosorbent assays (TAS–ELISA) have been previously described (Kalmar and Eastwell, 1989). Antibody SB2 reacts with the large capsid subunit (VP37) of CPMV in both native virions and fully denatured protein preparations, while antibody DG11 reacts with native virions of CPSMV. These monoclonal antibodies are specific for their respective target virus with no cross-reactivity with the other virus used in this study.

Recombinant plasmids and nucleic acid probes. Oligo-d(T) primed cDNAs were synthesized using viral RNA1 and RNA2 as templates (D'Alessio and Gerard, 1988). Methylmercury hydroxide was added to remove secondary structure (Jelkmann et al., 1989). Double-stranded cDNA was cloned into plasmid vectors pGEM2 or pGEM4Z (Promega) (Melton et al., 1984). Plasmid pG2SB1 contained the 310-base Sau3AI fragment from CPMV-SB RNA1 (bases 3547 to 3856) in the BamHI site of the vector, and pG2SB2 contained the 342-base TaqI fragment from CPMV-SB RNA2 (bases 1622 to 1963) in the AccI site. Plasmid pG2DG1 contained the 740-base TaqI fragment from CPSMV RNA1 cloned into the AccI site of pGEM2 and pG4ZDG2 contained the 845-base BglII restriction fragment from CPSMV RNA2 cloned into the BamHI site of plasmid pGEM4Z. DNA inserts were sequenced by the dideoxy-ribonucleotide method (Sanger et al., 1977). Clones pG2SB1, pG2SB2, pG2DG1, and pG4ZDG2 were transcribed with DNA-dependent RNA polymerases T7, SP6, T7, and T7, respectively, to generate [^{32}P]-labelled RNA probes complementary to positive-sense viral RNAs. Each probe, selected from plasmid libraries, was shown not to hybridize to the remaining three viral RNAs encountered in these studies, thus ensuring its utility for monitoring the replication of viral RNAs with no interference from other virus sequences.

A recombinant plasmid (provided by D. Bhattacharya, Simon Fraser Univ., Burnaby, B.C.) containing the gene for the 18S ribosomal RNA cloned from *Costaria costata* (Bhattacharya and Druehl, 1988) was digested with restriction enzyme EcoRI. The 1.91-kilobase pair insert was subcloned into pGEM4Z (Promega) and radiolabelled transcripts for hybridization were produced by T7 DNA-dependent RNA polymerase.

RNA analysis in plant extracts. A leaf tissue disk (≈100 mg) was excised with a #10 cork borer and stored at –20 °C or used fresh. Each disk was homogenized in 425 μL grinding buffer (0.2 M glycine; 0.1 M NaH_2PO_4; 0.6 M NaCl; 1% sodium dodecyl sulfate (SDS); 1% 2-mercaptoethanol, pH 9.6) and extracted once with an equal volume of phenol, once with 200 μL each of phenol and chloroform, and twice with 400 μL chloroform. The aqueous phase was diluted with 1.5 volumes 10× SSC (0.15 M trisodium citrate; 1.5 M NaCl, pH 7.2). One-fifth of the sample, ≈4.4 μg total RNA as indicated by spectrophotometry, was applied in the first column of a slot-blot onto a nylon membrane (GeneScreen; Du Pont, Boston), with 1:10 serial dilutions in adjacent columns.

All prehybridization and hybridization procedures were modifications of the protocol outlined by the manufacturer of the nylon membrane (Du Pont). Prehybridization and hybridization of filters were performed at 60 °C. Hybridized filters were subjected to two washes in 2× SSC (5 min each at room temperature), three washes in 0.1× SSC and 0.1% SDS (20 min each at 65 °C), three washes in 0.1× SSC and 0.1% SDS (20 min each at room temperature), incubation in 2× SSC and 1 μg·mL^{-1} RNase A (Sigma) (10 min at room temperature), and a final wash in 0.1× SSC and 0.1% SDS (40 min at 50 °C). XK-1 film (Kodak) was exposed to membranes at –70 °C; video images of resulting autoradiographs were analyzed with VISTA Image Processing Program (NORAN; Middleton, Wis.)

Extraction of RNA from virus. RNA was extracted from virus particles by established methods (Daubert et al., 1978). The RNA was further purified on a 1-mL spin-column of Sepharose CL-2B-300 (Pharmacia) (Maniatis et al., 1982).

Ultraviolet irradiation of viral RNA. Purified viral RNA (0.625 mg·mL^{-1} in water) was placed on a wax membrane (Parafilm) and irradiated at 1200 μW·cm^{-2} UV light (254 nm) for 5 min.

Results

Biological characteristics of interference. Inoculating primary leaves of 'California Blackeye 5', 'Black', and 'Arlington' seedlings with 1.0 μg·mL^{-1} CPSMV, the lowest concentration tested, resulted in symptom development in all inoculated plants. Large areas of necrosis were visible on inoculated primary leaves

Table 1. Comparison between the coinoculation of 'Black' with CPSMV and CPMV virions, and coinoculation with ssRNA purified from the two viruses.

Inoculum[z] (μg·mL^{-1})	Plants inoculated (no.)	Surviving plants[y] (%)
No CPMV:		
7.5 CPSMV	6	0
1.0 CPSMV	7	0
1.5 CPSMV RNA	8	0
0.2 CPSMV RNA	7	0
[CPMV] : [CPSMV] = 10:1		
75 CPMV + 7.5 CPSMV	83	20
15 CPMV RNA + 1.5 CPSMV RNA	52	75
[CPMV] : [CPSMV] = 75:1		
75 CPMV + 1.0 CPSMV	38	21
15 CPMV RNA + 0.2 CPSMV RNA	49	90

[z]On a molar basis, the concentration of virus and RNA are the same within each experiment based on the assumption that RNA represents 20% (w/w) of the virus.
[y]Evaluated at 14 d post inoculation.

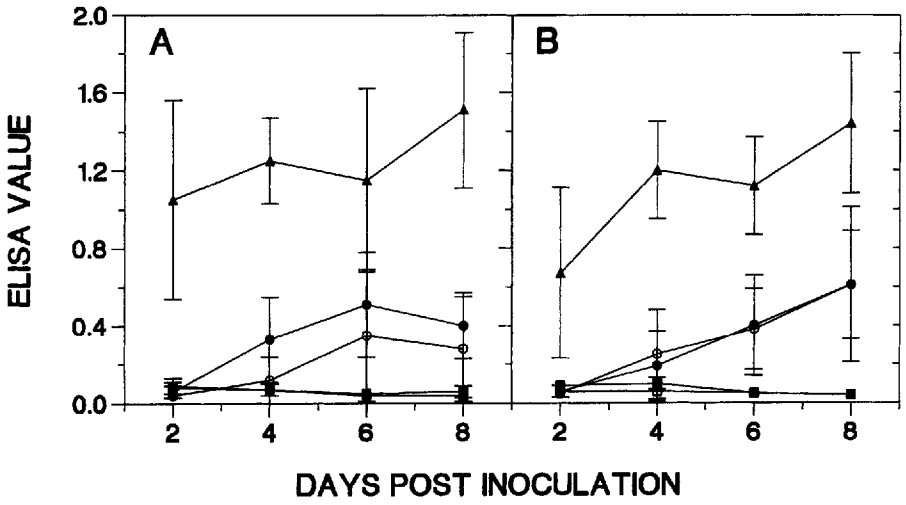

Fig. 1. Comparison of virus capsid protein accumulation in primary leaves of 'Black' (**A**) and 'Arlington' (**B**) seedlings that exhibit symptomless or symptomatic secondary leaves. Plants were coinoculated with 75 μg·mL^{-1} CPMV and 7.5 μg·mL^{-1} CPSMV. Viral coat proteins were estimated by TAS–ELISA: CPMV in plants with symptomless (□) and symptomatic (■) secondary leaves; CPSMV in plants with symptomless (○) and symptomatic (●) secondary leaves. 'Black' and 'Arlington' seedlings were inoculated with 7.5 μg·mL^{-1} CPSMV alone for comparison (▲). Each point corresponds to the mean of five test plants in each treatment, assayed twice; bars correspond to SD. ELISA value = $A_{405} - A_{620}$; background from uninoculated control plants <0.100.

3 to 4 d post inoculation (DPI), followed, in most cases, by vascular necrosis and seedling collapse 6 to 8 DPI. Vascular necrosis was observed in all inoculated 'Arlington' seedlings and all the plants died. At the lowest inoculum concentration, 50% of 'California Blackeye 5' survived and developed secondary leaves with chlorotic lesions.

Inoculation of 'Black' and 'Arlington' seedlings with CPMV alone did not result in visible symptom expression, while 'California Blackeye 5' seedlings developed a yellow mosaic characteristic of CPMV infection.

When either 'Black' or 'Arlington' seedlings were inoculated with 7.5 μg·mL^{-1} CPSMV, no plants survived. However, when the inoculum contained 7.5 μg·mL^{-1} CPSMV plus 75 μg·mL^{-1} CPMV, 20% (17/83) of 'Black' seedlings (Table 1) and 30% (17/57) of 'Arlington' seedlings survived. In 'Black' and 'Arlington', surviving plants exhibited characteristic discrete, large, irregular necrotic lesions on primary leaves surrounded by apparently healthy tissue compared to the overall dull-green desiccated appearance of plants that did not survive. Thus, the presence of CPMV in the inoculum provided protection from CPSMV to a substantial number of seedlings of 'Black' and 'Arlington'.

Differences between interference elicited in 'Black' seedlings by purified RNA and by intact virions were also examined (Table 1). At a constant 10 CPMV : 1 CPSMV ratio, the fraction of surviving plants increased from 20%, when 'Black' seedlings were inoculated with virions, to 75% when inoculated with the same molar concentrations of RNA. This relationship was also observed at an elevated [CPMV] : [CPSMV] ratio of 75:1. Similar results were observed for 'Arlington' seedlings (data not shown). From these experiments, it was evident that coinoculation with purified CPMV RNA and CPSMV RNA, relative to coinoculation with purified virions, was more effective in eliciting the interference response.

The time interval between introduction of the two viruses is critical for protecting 'Black' and 'Arlington'. Plants were inoculated with 75 μg·mL^{-1} CPMV followed by 7.5 μg·mL^{-1} CPSMV at intervals of 0 to 144 h. When both viruses were coincidentally introduced into 'Black' and 'Arlington' seedlings, 20% and 30%, respectively, of the plants survived and developed secondary leaves. However, a delay of 5 min between inoculation with CPMV and CPSMV reduced the survival rate to 10% in both cultivars. Delays of 24 h or longer between sequential inoculations reduced the survival rate to 6% and 4% for 'Black' and 'Arlington', respectively.

'Black' and 'Arlington' seedlings were inoculated with CPSMV plus either top, middle, or bottom component of CPMV. Each inoculum was applied to 40 'Arlington' and 60 'Black' seedlings. Only unfractionated virus or bottom component (containing RNA1) of CPMV interfered with the replication of CPSMV. Neither middle (containing RNA2) nor top (empty capsid) components of CPMV were able to attenuate the development of symptoms in 'Black' or 'Arlington' cowpeas. When 'Arlington' cowpeas were inoculated with 7.5 μg·mL^{-1} CPSMV, the addition to the inoculum of 37.5 μg·mL^{-1} CPMV enriched for bottom component reduced the average number of necrotic local lesions formed on primary leaves from 18 per leaf in plants inoculated with CPSMV alone to <1 per leaf. An equimolar amount of unfractionated CPMV reduced the lesion number to 4 per leaf. Similarly, the percentage of 'Black' and 'Arlington' seedlings to collapse before 21 DPI decreased substantially from 100% to 36% in either cultivar when the CPSMV inoculum was augmented with a CPMV fraction enriched for bottom component.

Accumulation of virus capsid polypeptides in coinoculated cowpeas. Seedlings were inoculated with 75 μg·mL^{-1} CPMV and 7.5 μg·mL^{-1} CPSMV. Plants were individually tagged at the time of inoculation, and the same plants were sampled at various times.

Fig. 2. Slot-blot analyses of the accumulation of CPMV and CPSMV plus-sense RNA1 in extracts of primary leaves of 'Black' inoculated with 75 μg·mL^{-1} CPMV and 7.5 μg·mL^{-1} CPSMV. Blots were hybridized with [^{32}P]-labelled virus-specific probes to detect CPMV in plants with symptomless (□) and symptomatic secondary leaves (○); CPSMV in plants with symptomless (■) and symptomatic secondary leaves (●). RNA extracts from seedlings inoculated with CPSMV alone were assayed for comparison (▲). Each point represents the average of two samples that had been diluted 1:10.

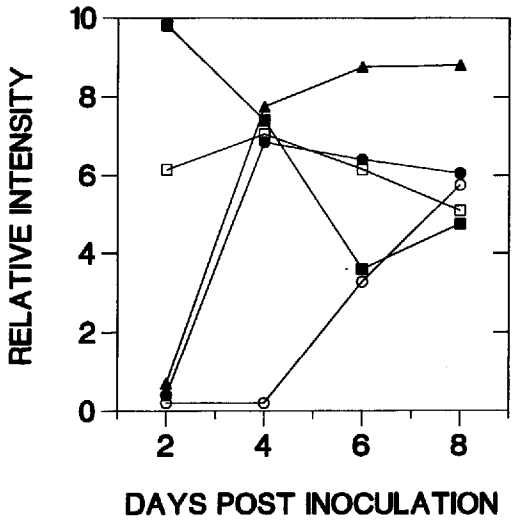

Table 2. Testing acquired systemic resistance against CPSMV in 'Black' seedlings previously inoculated with CPMV.

Inoculum[z] (μg·mL^{-1})		Plants	Plants in each disease class (%)			
Primary leaf	Secondary leaf	inoculated	Symptom rating[y]			
CPMV	CPSMV	(no.)	0	1	2	3
0	1.0	32	6	3	6	84
75	1.0	18	0	33	17	50
250	1.0	18	11	39	17	33
15 RNA	1.0	29	62	7	10	21
0	7.5	32	0	9	6	84
75	7.5	20	10	20	35	35
250	7.5	19	16	21	21	50
15 RNA	7.5	20	10	40	15	35

[z]Primary leaves were inoculated with CPMV virions or RNA and, when the first set of secondary leaves were 2 to 3 cm long, they were inoculated with CPSMV.

[y]Symptoms on challenged leaves were classified as 0 = none visible, 1 = less than five necrotic lesions per leaf, 2 = more than five necrotic lesions per leaf, and 3 = total vascular collapse.

At 14 DPI, plants were classified as either having symptomless secondary leaves or symptomatic secondary leaves (i.e., vascular necrosis). Primary leaves of coinoculated 'Black' and 'Arlington' seedlings were assayed for CPSMV and CPMV coat protein accumulation by TAS–ELISA (Fig. 1 A and B, respectively). CPMV capsid protein was not detected in either 'Black' or 'Arlington' seedlings. There was no difference in the accumulation of CPSMV protein in primary leaves of 'Black' or 'Arlington' when comparison was made between seedlings that developed symptomatic versus symptomless secondary leaves. The accumulation of CPSMV protein in 'Black' and 'Arlington' seedlings inoculated with both viruses, relative to seedlings inoculated with CPSMV alone, was significantly reduced and the onset of this accumulation was delayed by 2 d.

Accumulation of virus RNA within individual coinoculated cowpeas. Replication of viral RNA was analyzed by nucleic acid hybridization using probes complementary to positive-sense viral RNAs. Hybridization assays to detect the much less abundant minus-sense viral RNA did not provide adequate sensitivity under these experimental conditions. Blots of plant extracts were also probed with an RNA transcript complementary to 18S RNA cloned from *C. costata* to confirm that each extract contained approximately equivalent amounts of total RNA; analysis of 16 samples yielded 15% variability in blot intensity (data not shown).

The dynamics of CPSMV RNA replication in 'Black' parallels the synthesis of capsid proteins. In seedlings inoculated with CPSMV alone, the yield of RNA increased to significant levels by 4 DPI with no further increase to 8 DPI (Fig. 2), possibly because of the advanced state of senescence associated with the infected symptomatic tissue. In 'Black' seedlings coinoculated with CPMV and CPSMV, increased CPSMV RNA was detected in inoculated primary leaves 4 or 6 DPI depending on whether the plant developed symptomatic or symptomless secondary leaves, respectively. The onset of CPSMV RNA replication in plants with symptomless secondary leaves relative to plants with infected secondary leaves was delayed 2 d. Thus, not only has symptom expression been delayed and diminished, but synthesis of CPSMV progeny RNA has been delayed for a similar period of time. Residual CPMV RNA from the high inoculum concentrations was evident in the primary leaves but declined with time.

Samples from symptomless and symptomatic secondary leaves were assayed for the presence of CPMV and CPSMV RNA by hybridization with probes complementary to positive-sense viral RNA. CPMV RNAs were not detected in samples from either symptomatic or symptomless secondary leaves indicating that CPMV was not transported out of the primary inoculation site, as is the case in 'Arlington' and 'Black' cowpeas inoculated with CPMV alone (data not shown). CPSMV RNAs were detected in extracts from all symptomatic secondary leaves, but not in extracts from symptomless secondary leaves. Therefore, in plants with symptomless secondary leaves, CPSMV RNA was localized in primary inoculated leaves, with no apparent movement of infection to the secondary leaves.

Acquired systemic resistance induced by CPMV. Primary leaves of 'Black' cowpeas were inoculated with 75 or 250 μg·mL^{-1} CPMV, or with 15 μg·mL^{-1} CPMV RNA, and the secondary leaves were challenged with either 1 or 7.5 μg·mL^{-1} CPSMV (Table 2). For comparison, CPSMV was inoculated on secondary leaves of previously uninoculated plants. In all examples of sequential inoculation, symptoms induced by CPSMV on secondary leaves of plants previously inoculated with CPMV were reduced compared to CPSMV-inoculated secondary leaves of uninoculated seedlings. The concentrations of CPMV or CPSMV used to inoculate seedlings had little or no effect on the degree of symptom development in response to CPSMV. The use of CPMV RNA and intact virions yielded similar results. The degree of protection offered by spatially and chronologically separated inoculations is much less than by coinoculation.

'Black' seedlings were coinoculated with 75 μg·mL^{-1} CPMV and 7.5 μg·mL^{-1} CPSMV. At 21 DPI, samples from plants with symptomless secondary leaves were analyzed by TAS–ELISA for CPSMV. Plants testing negative were repotted and retested 4 weeks later for CPSMV. Of 127 coinoculated plants, 44 (35%) plants had symptomless secondary leaves and 40 plants were maintained and grown to seed. Sixty-eight progeny from selfed 'Black' plants that had not been systemically infected by CPSMV were coinoculated with CPMV and CPSMV. Sixteen (24%) plants had symptomless secondary leaves 21 DPI compared to 20% in the parental generation. Therefore, a predisposition to interference was not transmitted to surviving generations.

Requirement of infectious CPMV RNA for interference. Purified CPMV RNA was rendered noninfectious by UV irradiation. The irradiated RNA was examined by denaturing agarose gel electrophoresis and found to be degraded to lengths ranging predominantly from 1000 to 2000 bases. Residual biological activity was assayed by inoculation of 'California Blackeye 5'

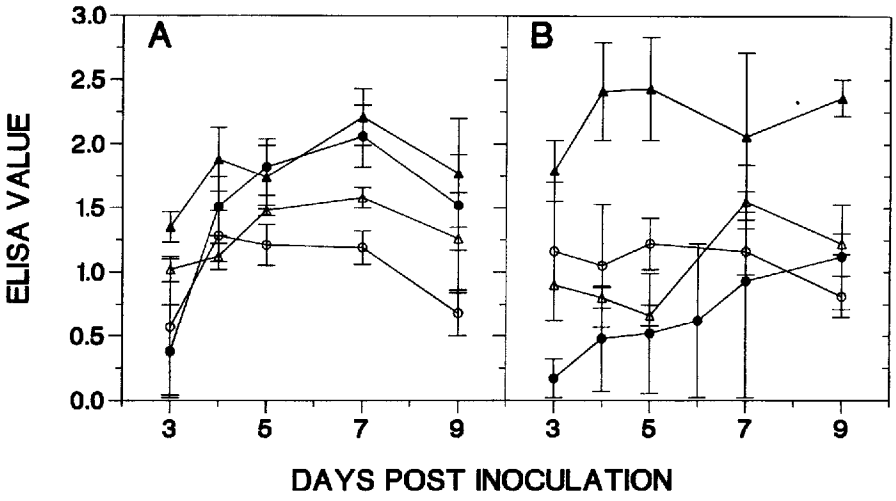

Fig. 3. Comparison of virus protein accumulation as determined by TAS–ELISA in 'California Blackeye 5' inoculate singularly or in combination, with CPMV and CPSMV virus or RNA. (**A**) Seedlings are inoculated with 200 μg·mL^{-1} CPMV virus (Δ), 5.8 μg·mL^{-1} CPSMV virus (▲), or 200 μg·mL^{-1} CPMV virus plus 5.8 μg·mL^{-1} CPSMV virus (○ = CPMV; ● = CPSMV). (**B**) Seedlings are inoculated with 40 μg·mL^{-1} CPMV ssRNA (Δ), 1.2 μg·mL^{-1} CPSMV ssRNA (▲), or with 40 μg·mL^{-1} CPMV ssRNA plus 1.2 μg·mL^{-1} CPSMV ssRNA (○ = CPMV; ● = CPSMV). Each point corresponds to the mean of six test plants, each of which was assayed twice; bars correspond to SD. ELISA value = $A_{405} - A_{620}$; background from uninoculated control plants <0.100.

with the irradiated RNA sample; no infection was observed. Interference was not observed when irradiated CPMV RNA was coinoculated with intact CPSMV RNA on 'Black' seedlings. Thus, the genome of the inducing virus must be biologically active to elicit the interference response.

Interference in permissive hosts. 'California Blackeye 5' is highly susceptible to infection by CPMV and CPSMV (Beier et al., 1977). When seedlings were coinoculated with a mixture of intact virions consisting of 200 μg·mL^{-1} CPMV and 5.8 μg·mL^{-1} CPSMV, both viruses accumulated in primary inoculated leaves (Fig. 3A). The initiation of CPSMV capsid protein synthesis was delayed by 2 d (Fig. 3A), but quickly attained levels similar to those detected in plants inoculated with CPSMV alone. There was also a small but significant reduction in the accumulation of CPMV protein in coinoculated plants relative to those inoculated with CPMV alone. When plants were coinoculated with 40 μg·mL^{-1} CPMV RNA and 1.2 μg·mL^{-1} CPSMV RNA, the accumulation of CPSMV protein in primary inoculated leaves was delayed and dramatically suppressed relative to inoculation with CPSMV RNA alone (Fig. 3B).

Discussion

All published results to date on the interference of CPMV and CPSMV in cowpea seedlings immune to CPMV have revealed suppression of symptoms but no information regarding the effects of interference on the replication of the two viruses. An earlier report on mixed infections of 'Arlington' protoplasts indicated that recovery of infectious CPSMV from coinoculated preparations is reduced relative to that from protoplasts inoculated with CPSMV alone (Bruening et al., 1979). The synthesis of virus proteins and progeny viral RNA can only be inferred from that data. In the present study, nucleic acid probes and monoclonal antibodies were used to analyze the accumulation of virus specific RNA and coat proteins, respectively, of the individual viruses. It is evident that, in addition to symptom amelioration, the presence of CPMV restricts CPSMV replication and transport. Although complete containment of CPSMV is not always achieved, the spread of the challenge virus has been curtailed, allowing the development of virus-free tissue.

In coinoculated plants, CPSMV replicates at or near the site of initial infection, but accumulation of CPSMV coat protein and RNA1 is delayed. The primary response of interference in cowpea is the restricted of movement of the challenging virus. Whether this is achieved by direct virus–virus interactions or mediated through a plant response cannot be determined with certainty from these data. However, it is evident from studies with a host permissive for both viruses, i.e., 'California Blackeye 5', replication of CPSMV is delayed in response to the presence of CPMV in the inoculum. Thus, interference occurs independent of the cultivars' susceptibility to CPMV.

In seedlings of either 'Black' or 'Arlington' coinoculated with CPMV and CPSMV, it is evident that the presence of CPSMV is unable to complement the functions of CPMV replication that are inhibited in these cowpea lines. Consequently, the CPMV remains entrapped at the initial site of infection, with no long-distance transport function being observed. The CPMV replication cycle is interrupted in 'Arlington' at the proteolytic cleavage of the RNA2 translation product (Sanderson et al., 1985), and a host-encoded inhibitor of viral protease is implicated in providing immunity (Ponz et al., 1988). The presence of CPSMV in the inoculum does not enhance the replication of CPMV, suggesting that the cleavage function is not provided by CPSMV. Indeed, the proteases associated with the cleavage of comovirus polyproteins are very specific for polyproteins of the parental virus strain (Goldbach and Krijt, 1982). In addition to long distance transport to secondary leaves, it is likely that the cell-to-cell transport of CPMV is also impaired. Functional 58 kDa/48 kDa and capsid proteins are required for movement of CPMV (Wellink and van Kammen, 1989). Processing the polyprotein that releases these proteins occurs at the cleavage site targeted by the putative protease inhibitor in 'Arlington' (Sanderson et al., 1985). It is also evident that the transport function(s) of CPSMV are unable to accommodate CPMV.

Experiments were conducted to identify the constituent of the inoculum that restricts migration of the challenging virus. As was the case in 'Arlington' protoplasts (Bruening et al., 1979), the presence of coat protein from the protecting virus is not essential for interference. This has been demonstrated by the observation that the top component of CPMV, consisting of empty capsid particles only, is unable to induce interference, while isolated viral RNAs were more effective in promoting the interference phenomenon than were intact virions. The uncoating process of the challenge virus was not a factor in this system of interference because CPSMV RNA was more susceptible to interference than intact CPSMV virions. Furthermore, the CPMV bottom component containing RNA1 was more effective in promoting protection that was the CPMV middle component containing RNA2; RNA2 encodes the transport and coat proteins. These results are consistent with observations reported for other cowpea cultivars (Sterk and de Jager, 1987). Therefore, CPMV coat proteins derived either from virions or from in vivo translation products of RNA2 are not required for interference. These results are contrary to the conclusions drawn from experiments with transgenic plants in which coat

protein expression is capable of limiting virus replication (Powell et al., 1990; Register and Beachy, 1988; van Dun et al., 1987, 1988). Furthermore, in the present study, interference of CPSMV replication is not circumvented by replacing the inoculum with CPSMV RNA, while the ability to overcome coat protein-mediated protection in transgenic plants with isolated viral RNA is a common (Loesch-Fries et al., 1987; Nelson et al., 1987) but not universal (Hemenway et al., 1988) phenomenon.

Interference is associated with either CPMV RNA1 or a product of its replication. Biologically active RNA1 is essential as UV-irradiated RNA does not elicit interference. This is a common requirement observed in similar systems (Bruening et al., 1979; Saayer-Riep and de Jager, 1988; Sterk and de Jager, 1987). Morch et al. (1987) demonstrated that replication of the tymovirus genome could be blocked by preincubation in vitro with transcripts of the 3' termini. This is evidently not the situation in mixed infections of comoviruses in vivo.

Interference in 'Black' and 'Arlington' is similar to that in 'TVu470' (Sterk and de Jager, 1987). All three cowpea cultivars are immune to the strain of CPMV used in the respective studies (Beier et al., 1979; Sterk and de Jager, 1987), but only 'Arlington' maintains immunity as protoplasts. Although data from protoplasts suggests two distinct forms of immunity may be involved, all three cowpea cultivars share similar characteristics with respect to interference.

There are clearly two levels of interaction between the infection of the host plant with the inducing virus and the replication and/or transport of the challenge virus. Interference dramatically reduces symptom development and this is associated with the reduced capacity of the challenge virus to spread throughout the plant. A more subtle form of acquired systemic resistance is also evident when secondary leaves are challenged by CPSMV. Although a systemic infection is established, the symptoms are ameliorated.

The stringent requirement for simultaneous introduction of the inducing virus and the challenging virus to obtain optimal protection precludes the application of this strategy for field management of these viruses. However, these data support an increasing number of studies that demonstrate that the replicase gene can offer protection in transgenic plants (Scholthof et al., 1993), including the replicase gene derived from CPMV (Sijen et al., 1995). In the latter case, the RNA rather than the protein product was required for the protection and protection was offered only against other strains of CPMV, but not CPSMV. The current study indicates that intact RNA1 of CPMV does provide protection against CPSMV, suggesting incorporation of additional sequence information may result in an extended range of viruses to which transgenic plants would be immune or resistant.

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