Interstrain Pseudorecombinants of Cowpea Chlorotic Mottle Virus: Effects on Systemic Spread and Symptom Formation in Soybean and Cowpea

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Received 1 June 1993. Accepted 30 August 1993.

Full-length complementary DNA (cDNA) copies of genomic RNA1, RNA2, and RNA3 segments of cowpea chlorotic mottle virus (CCMV) strains D, N, and S were synthesized using polymerase chain reaction and were cloned downstream of a T7 RNA polymerase promoter. Mixtures of the homologous *in vitro*-transcribed RNAs produced typical CCMV symptoms when inoculated on soybean (cv. Bragg) and cowpea (cv. California Blackeye) plants. Using either gel-purified or *in vitro*-transcribed CCMV RNA components, the pseudorecombinants SSD, SSN, DDS, and NNS were constructed. The properties of these pseudorecombinants were tested by comparing the induced symptoms, virus concentration, and systemic spread. These studies revealed a direct involvement of RNA3 genetic information in necrotic lesion formation and in breakage of CCMV resistance in soybean. Exchanges between RNA1 or RNA2 components further affected the biological features of reassorted CCMV variants. The true nature of the pseudorecombinants was confirmed by reconstitution of the parental strains from the corresponding pseudorecombinant RNA components.

Cowpea chlorotic mottle virus (CCMV) is a member of the bromoviruses (Lane 1979, 1981), a group of plus-stranded tripartite RNA viruses of plants (Kuhn 1964). The genome of CCMV is divided among three separately encapsidated RNAs designated RNA1, RNA2, and RNA3. The type (T) strain (Kuhn 1964) has been cloned (Allison *et al.* 1988) and sequenced (Allison *et al.* 1989; Dzianott and Bujarski 1991), and infectious *in vitro* transcripts have been synthesized (Allison *et al.* 1988). CCMV RNA1 (3.2 kb) and RNA2 (2.8 kb) encode two replicase proteins (Kroner *et al.* 1989; Traynor *et al.* 1991), whereas the RNA3 segment (2.2 kb) encodes the movement protein and coat protein. These latter two polypeptides are dispensable for viral RNA replication (Allison *et al.* 1990; Pacha *et al.* 1990).

Virally encoded, host-related genetic information has been studied for CCMV and for brome mosaic virus (BMV), a related bromovirus (reviewed by De Jong and Ahlquist 1991). Pseudorecombinant exchanges between two BMV strains revealed that the information that determines their host range was located within the RNA3 component (De Jong and Ahlquist 1991). Also, the use of nitrous acid mutants of two CCMV strains indicated that systemic symptoms on cowpea were genetically directed by RNA3 (Kuhn and Wyatt 1979). However, reassortants between BMV and CCMV (strain T) demonstrated that host specificity determinants are encoded in all three genomic RNAs (Allison *et al.* 1988; Bancroft and Lane 1973). To map RNA3 sequences that specify the reactions of bromoviruses with their hosts, hybrid BMV/CCMV RNAs were constructed by precise exchanges of the BMV and CCMV 3a genes (Mise *et al.* 1993). This revealed that bromovirus movement proteins must be specifically adapted for successful bromovirus infection. We have recently observed that mutations in the BMV coat protein gene affect long-distance movement in *Chenopodium hybridum* (S. Flasinski, A. Dzianott, S. Pratt, and J. J. Bujarski, unpublished results). In alfalfa mosaic virus (AlMV), construction of pseudorecombinants between two strains revealed that symptom differences observed on tobacco plants mapped to RNA3. The use of chimeric RNA3 molecules further mapped some of the essential AlMV sequences to the coat protein open reading frame (Neeleman *et al.* 1991).

In addition to T strain, other CCMV strains have been described in the literature (Kuhn 1964; 1968; Fulton *et al.* 1975). Strain S was isolated from soybean (Kuhn 1968). Both the T and S strains induced necrotic lesions on inoculated leaves of resistant soybean cultivars Bragg and Williams, but, in contrast to strain T, the S strain produced delayed symptoms in cowpea and caused more virus to be produced in this host (Kuhn 1968). Strains D and N were derived by passage of strain BYS through soybean cv. Bragg (Paguio *et al.* 1988). Unlike strains T and S, D and N have the ability to overcome the hypersensitive resistance in the soybean cultivars Bragg and Williams.

To contribute to our knowledge concerning host-related genetic information in bromoviruses, we have generated viable CCMV pseudorecombinants by exchanging individual genomic RNA components among S, D, and N strains. This demonstrated that the genetic information encoded by RNA 3 is involved in the induction of anti-CCMV resistance and symptom formation in soybean. RNA1 and RNA2 components also contributed to the interactions of CCMV with its hosts.

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MPMI Vol.I. 6, No. 6, 1993, pp. 755-763

RESULTS

Characterization of parental virus strains.

The infectivity of CCMV D, N, and S strains, was evaluated 10 days postinoculation on soybean cultivar Bragg and cowpea cultivar California Blackeye. The symptoms observed were consistent with those described in the literature (Kuhn 1968; Paguio *et al.* 1988). Specifically, on soybean cv. Bragg, strain S caused local necrotic lesions on inoculated leaves and no systemic symptoms, whereas strains D and N caused chlorosis on inoculated leaves and systemic mosaic symptoms. Strain D but not strain N caused distortion of systemic leaves and plant stunting. On cowpea, strain S caused mottling symptoms that developed more slowly and were less intense than those of strains T (Kuhn 1968), D, or N. Electrophoretic analysis revealed that the concentration of RNA1 in D and N strains was significantly lower than that of RNA2 (Fig. 1A). RNA1 and RNA2 of the S strain migrated more slowly and faster, respectively, than the corresponding RNA segments of the D and N strains, indicating possible differences in their lengths.

Generation of cDNA clones and the infectivity of *in vitro* transcripts.

The polymerase chain reaction (PCR) approach was used to obtain full-length clones of all nine genomic RNAs for three CCMV strains (S, D, and N) using corresponding 5′ and 3′ primers (Fig. 2). An electrophoretic analysis of the PCR-amplified cDNA products of the D strain showed single bands of 3.1, 2.8, and 2.1 kb, which correspond to the predicted length of RNA1, RNA2, and RNA3, respectively (Fig. 1B). Since the same pair of primers was used for the cDNA amplification of RNA1 and RNA2, a small amount of RNA1 cDNA was detected in the RNA2 reaction.

As shown in Figure 1C, the RNAs obtained by *in vitro* transcription from the linearized CCMV cDNA clones comigrated with the corresponding virion RNA components in a 1% agarose gel. To examine the infectivity, mixtures of capped RNA1, RNA2, and RNA3 transcripts were inoculated on soybean and cowpea plants. After a 3- to 5-day delay (as compared with virion RNA infections), the inoculated plants developed characteristic symptoms of the S, D, and N strains. Electrophoretic analysis of progeny virion RNAs demonstrated comigration of each RNA component with the corresponding natural virion RNA segments (data not shown). This confirmed that the use of PCR generated infectious cDNA clones and that any possible PCR-generated mutations did not affect the biological properties of the corresponding synthetic CCMV RNAs.

Characterization of pseudorecombinants on soybean.

The RNA3 segments in CCMV strains D or N were replaced with the corresponding S RNA3, to determine the contribution of RNA3 in controlling the ability of CCMV to overcome resistance in soybean cv. Bragg. Pseudorecombinant DDS and NNS RNA mixtures were prepared by combining equimolar amounts of the corresponding *in vitro*-transcribed CCMV RNAs. Symptoms generated by DDS (Fig. 3B, panel DDS) and NNS (not shown) RNA mixtures on inoculated soybean leaves were similar to those induced by infection with the S strain (panel SSS in Fig. 3B): the two mixtures induced local necrotic lesions. Neither DDS (Fig. 3A) nor NNS (Table 1) induced symptoms on systemic leaves of soybean cv. Bragg. A similar experiment was performed using gel-purified virion RNA fractions. Except for a several-day delay, the *in vitro*-transcribed RNA mixtures and the corresponding gel-purified virion RNA fractions produced indistinguishable symptoms. These data suggest that the genetic information of the S RNA3 component elicits the necrotic reaction on resistant soybean plants.

To confirm these results, the *in vitro*-transcribed RNA3 component of the S strain was replaced by that of either the D or the N strain. The SSD pseudorecombinant induced a systemic leaf distortion on soybean (Fig. 3A, panel SSD), indicating that the RNA3 sequences are responsible for this characteristic feature of strain D (Fig. 3A, panel DDD). Also, both the N strain and the SSN pseudorecombinant inocula produced systemic mosaic symptoms on soybean (Table 1),

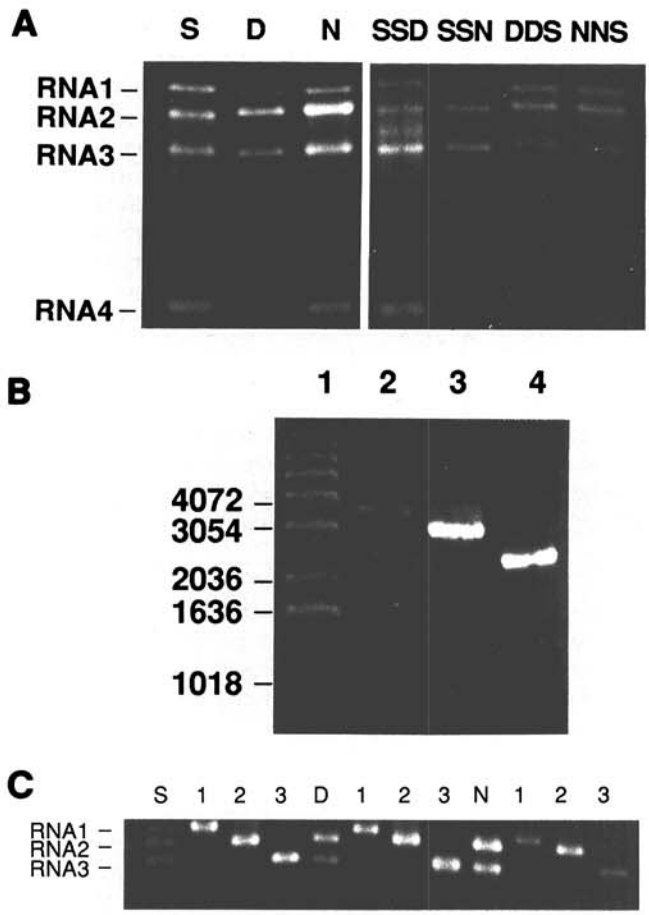

Fig. 1. Electrophoretic analysis (1% agarose gel stained with ethidium bromide) of cowpea chlorotic mottle virus (CCMV) virion RNAs, their full-length cDNA, and *in vitro* transcripts. **A,** Formaldehyde-denatured RNAs of S, D, and N strains and of pseudorecombinants SSD, SSN, DDS, and NNS. **B,** Polymerase chain reaction (PCR)-amplified cDNA products of D strain RNAs. Lane 1, marker DNA (BRL); lane 2, full-length cDNA of RNA1; lane 3, mixture of full-length cDNA of RNA1 and RNA2; lane 4, full-length cDNA of RNA3. The sizes of marker bands (bp) are shown on the left. **C,** Synthetic RNAs obtained by transcription *in vitro* from linearized cDNA clones of individual S, D, and N RNA comonents. From left to right: S virion RNA; *in vitro* transcripts of S strain cDNA1, -2, and -3, respectively; D virion RNA; *in vitro* transcripts of D strain cDNA1, -2, and -3, respectively; N virion RNA; *in vitro* transcripts of N strain cDNA1, -2, and -3, respectively.

indicating that the RNA3-encoded genetic information contributed to symptom formation by CCMV in infected soybean.

The concentration of viral RNA was estimated by dot blot hybridization to quantify the systemic spread of pseudorecombinants and to determine whether the RNA3 component controlled the level of CCMV RNA accumulation. As shown in Figure 4A and B, the S strain derived from *in vitro*-transcribed RNAs accumulated poorly in the inoculated leaves of soybean and did not spread systemically in this host. In contrast, the D and S strains accumulated to a high level in the inoculated leaves and readily spread to the systemic leaves. The DDS and NNS pseudorecombinants produced an amount of virus RNA comparable to that of the S strain in the inoculated leaves, whereas reciprocal exchanges of RNA3 component caused systemic spread of the SSD and SSN variants and virion accumulation similar to that of strains D and N. These results confirmed that RNA3-encoded information was responsible for overcoming CCMV resistance in soybean cv. Bragg, and participated in virus RNA accumulation in both inoculated and systemic soybean leaves. Similar data were obtained using pseudorecombinants generated with gel-purified virion RNA fractions (not shown).

Virion RNAs were extracted from pseudorecombinants SSD, SSN, DDS, and NNS after propagating on soybean cv. Bragg. Electrophoretical comparison with the RNAs of parental strains (Fig. 1A) revealed the characteristic patterns of individual RNA components, including lower accumulation of RNA1 than of RNA2 in D and N strains or differences in migration of RNA1 and RNA2 components between S, D, and N strains. This confirmed the generation of desired CCMV pseudorecombinants. The extra band between RNA2 and RNA3 in sample SSD may come from either the degradation of RNA1 or RNA2 components or may represent a defective interfering RNA derived from RNAs 1 or 2, as observed in broad bean mottle virus (Romero *et al.* 1993).

The low viral RNA concentration of SSD and SSN pseudorecombinants in systemic soybean leaves (Fig. 4A) suggested that RNA1 and/or RNA2 components of the S strain are involved in virus accumulation. To analyze further the effects of RNA1 and RNA2 sequences on CCMV-host reactions, these components were exchanged using *in vitro*-transcribed RNAs. The resulting pseudorecombinants were tested on soybean cv. Bragg (Table 1). Increased distortion and stunting compared to that from D and N strains were observed on

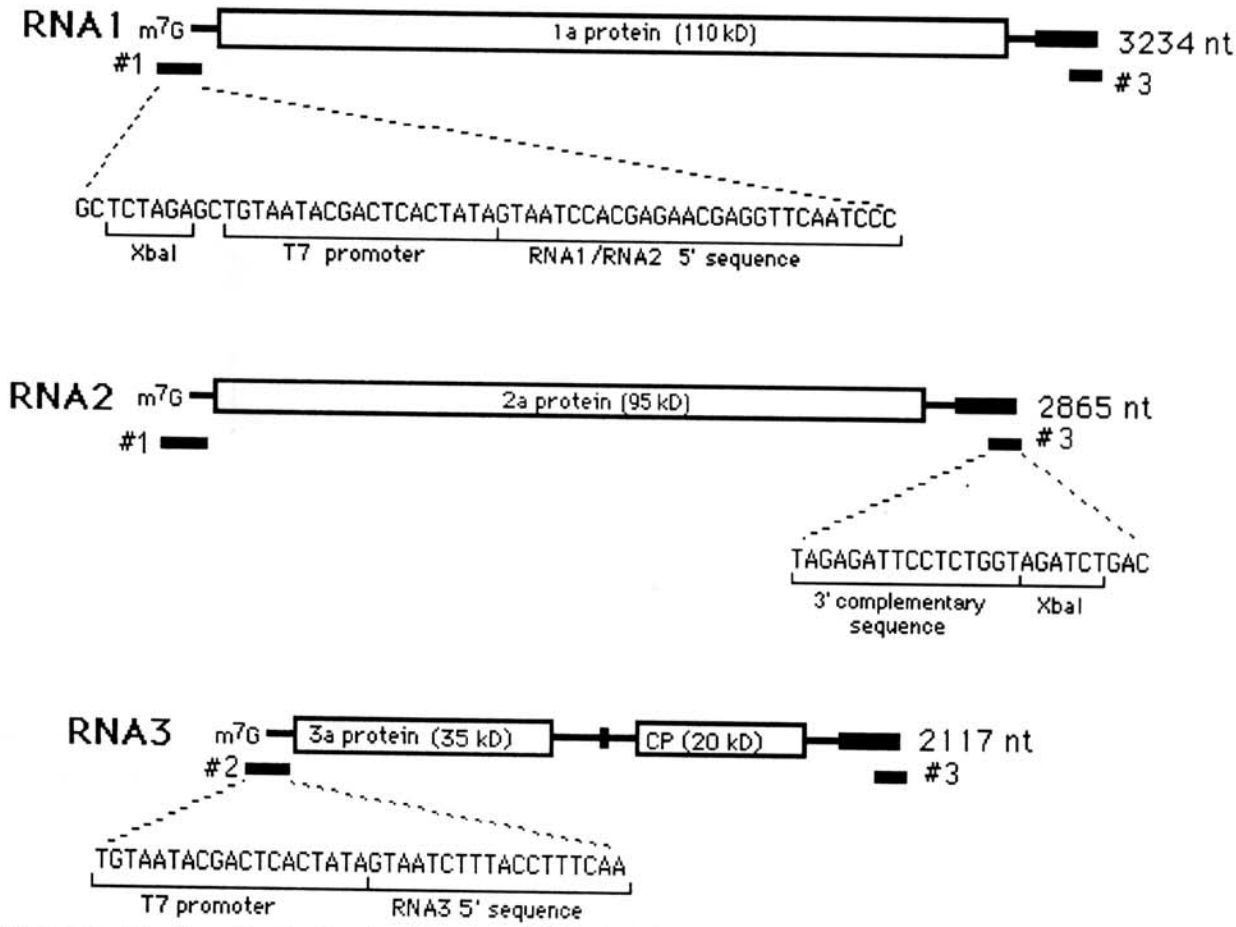

Fig. 2. Sequences of the oligonucleotide primers used to generate full-length cDNA clones of S, D, and N cowpea chlorotic mottle virus (CCMV) RNAs. Open boxes symbolize open reading frames for four CCMV proteins. Short black lines at both ends of three CCMV RNAs as well as in the middle of RNA3 depict noncoding sequences. The largest black boxes indicate 3′ noncoding sequences common between three CCMV RNAs; smaller black boxes represent the locations of the oligonucleotide primers; while the very small box within the RNA3 intercistronic region depicts the initiation site of subgenomic RNA 4. Primer 1 contains an *Xba*I restriction site, T7 RNA polymerase promoter, and 28 bases of CCMV RNA1 or RNA2 common 5′ complementary sequences. Primer 2 contains an *Xba*I restriction site, T7 RNA polymerase promoter, and 18 5′-end RNA3 nucleotides. Primer 3 contains 16 nucleotides of common 3′-end complementary sequences and the *Xba*I restriction site.

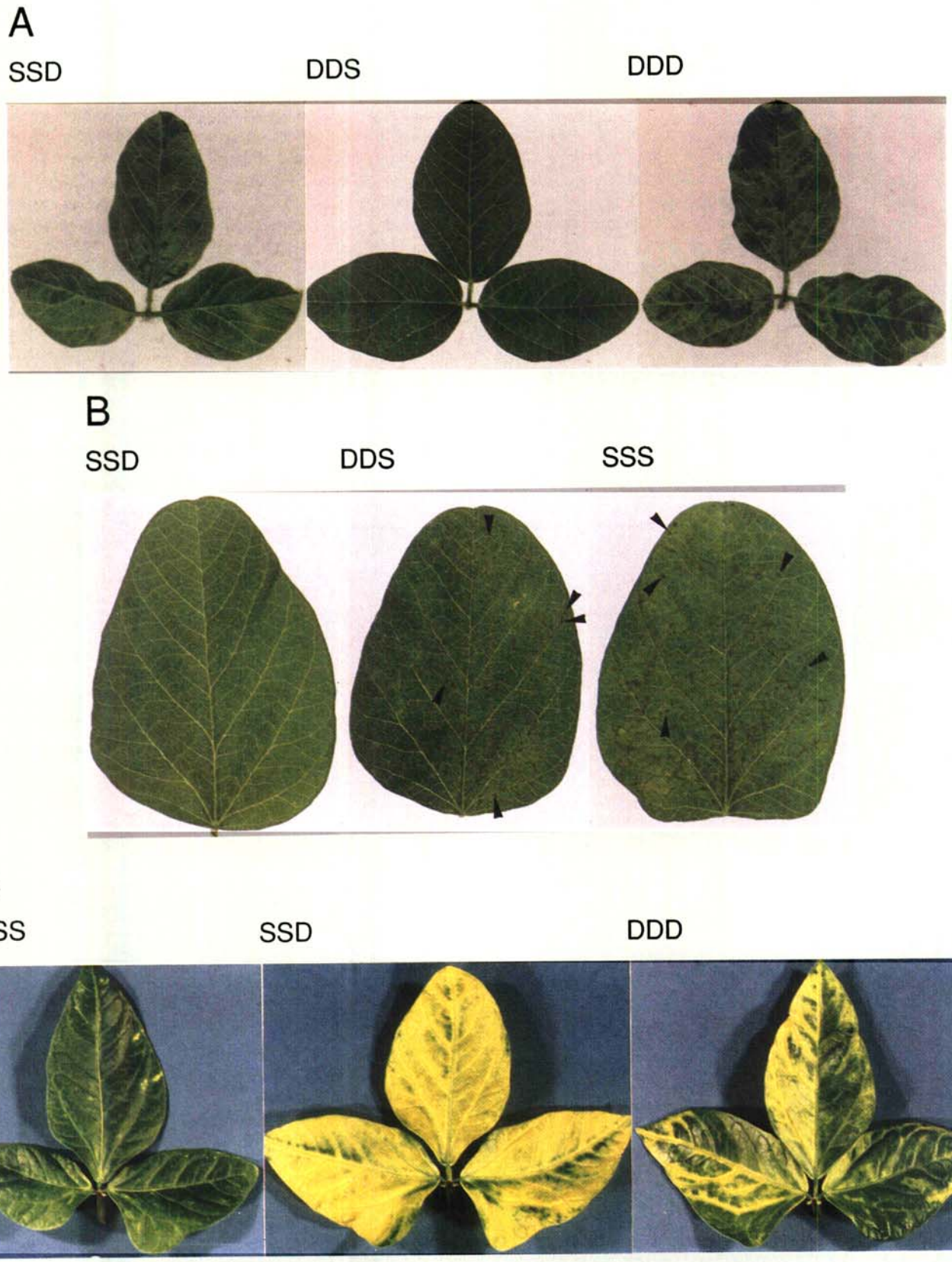

Fig. 3. Symptoms induced by cowpea chlorotic mottle virus (CCMV) S and D strains or by pseudorecombinant RNA mixtures SSD and DDS on leaves of soybean (cv. Bragg) and cowpea (cv. California Blackeye) using *in vitro*-transcribed CCMV RNA components. **A,** Soybean, systemically infected trifoliate leaves; **B,** soybean, inoculated primary leaves; **C,** cowpea, systemically infected trifoliate leaves.

systemically infected soybean leaves after inoculation with the pseudorecombinants DND, NND, and NDD. NSD and SND showed milder symptoms, similar to those of the D strain. This indicated possible interactions of RNA1 and RNA2 with RNA3 components (either at the RNA or protein levels) during symptom formation on soybean.

Comparison between the virus concentration presented in Figure 4A and the symptoms shown in Figure 3A demonstrates that there is no direct correlation between virus concentration and symptom severity on soybean. For instance, strain D and pseudorecombinant SSD caused similar mottling symptoms and leaf distortion on systemic leaves of soybean, although the latter accumulated to a much lower level. A similar phenomenon was observed for strain N and pseudorecombinant SSN (Fig. 4A and Table 1).

Characterization of pseudorecombinants on cowpea.

The original D, N, and S strains as well as DDS, NNS, SSD, and SSN pseudorecombinants were able to spread systemically in cowpea plants. Both DDS and NNS caused mild systemic mottling (Table 1), similar to those of the S strain (Fig. 3C, panel SSS), suggesting that the genetic information of the S RNA3 component determined mild symptoms in susceptible cowpea plants. However, SSD and SSN pseudorecombinants produced more severe symptoms in this host (Fig. 3C; Table 1; Shang and Bujarski 1992) than parental D or N strains did. This indicated that RNA1 and/or RNA2 components also contributed to symptom formation in cowpea. Dot blot hybridization (Fig. 4C) revealed that the parental D, N, and S strains as well as the SSD, SSN, DDS, and NNS pseudorecombinants accumulated to high levels in second systemic leaves of soybean. The S strain and DDS and NNS variants consistently produced less virus, suggesting that RNA3 sequences contributed to virus accumulation in this host. As in soybean, there was no direct correlation between virus concentration (Fig. 4C) and the intensity of symptoms (Fig. 3C and Table 1) in systemic cowpea leaves.

Restoration of parental strains from pseudorecombinants.

To confirm the true nature of the pseudorecombinants, the parental D, N, and S strains were regenerated by mixing the RNA components of virion preparations isolated from cowpea plants. The S components from SSD were mixed with those from DDS, D component from DDS with those of SSD, and N components from NNS with those of SSN. The electrophoretic patterns of the restored virion RNAs as well as the induced symptoms were identical with those of the original CCMV strains (data not shown).

DISCUSSION

The pseudorecombinant CCMV RNA exchanges described here provide evidence that the RNA3-encoded genetic information participates in several host-related interactions on soybean and cowpea plants, including systemic symptom formation, virus concentration, necrotic lesion formation, and, most importantly, the ability to overcome a necrotic-type resistance. It

Table 1. Symptoms induced by D, N, and S strains of cowpea chlorotic mottle virus (CCMV) and by interstrain CCMV pseudorecombinants on soybean cv. Bragg and on cowpea cv. California Blackeye

		Soybean cv. Bragg			Cowpea cv. California Blackeye
		Systemic			
Pseudorecombinant	Local lesion[a]	Distortion	Mosaic	Severity[b]	Chlorotic mottle severity[b]
SSS	+	−	−	+	+
SSD	−	+	+	+++	++++
SSN	−	−	+	++	++++
SDS	+	−	−	+	...
SNS	+	−	−	+	...
SDD	−	+	+	+++	...
SNN	−	−	+	++	...
SDN	−	−	+	++	...
SND	−	+	+	+++	...
DDD	−	+	+	+++	+++
DDS	+	−	−	+	+
DDN	−	−	+	+++	...
DSD	−	+	+	+++	...
DND	−	+	+	++++	...
DSS	+	−	−	+	...
DNN	−	−	+	++	...
DNS	+	−	−	+	...
DSN	−	−	+	++	...
NNN	−	−	+	++	+++
NNS	+	−	−	+	+
NND	−	+	+	++++	...
NSN	−	−	+	++	...
NDN	−	−	+	++	...
NSS	+	−	−	+	...
NDD	−	+	+	++++	...
NDS	+	−	−	+	...
NSD	−	+	+	+++	...

[a] + = symptoms present, − = symptoms not present.
[b] + = mild, ++ = moderate, +++ = severe, ++++ = very severe, ... = not tested.

has been shown that a hypersensitive-type resistance gene, *rcv*, controls localization of the T and S strains of CCMV in soybean cv. Bragg (Bijaisoradat and Kuhn 1985). The fact that the host resistance response in Bragg is elicited by a factor that maps to CCMV RNA3 suggests that the 3a protein and/or coat protein (CP) may directly or indirectly interact with this resistance gene. In BMV, exchanges of the 3a open reading frames (ORFs) between two natural mutants affected the systemic infection in cowpea plants (De Jong and Ahlquist 1991).

Two hypotheses have been suggested to explain localization of a virus within necrotic lesions. One proposes that movement of the virus is prevented due to death of the local-lesion necrotic tissue (Kimmins and Wuddah 1977). An alternative suggestion is that, although virus replication is retarded in necrotic tissue, virus movement is prevented by other mechanism(s). Movement of CCMV may be altered by modified interaction(s) with host factors essential for either cell-to-cell or long-distance virus transport. It is generally accepted that movement proteins are involved in cell-to-cell virus spread (Hull 1991). Therefore, the fact that the S strain is able to induce local lesions indicates that it spreads at least between the cells on the inoculated leaf. It further suggests that 3a protein-mediated cell-to-cell virus transport might not be the only factor limiting the spread of this CCMV strain in cv. Bragg.

The involvement of CP ORF sequences in the induction of necrotic symptoms has been documented for some plant RNA viruses, including AlMV (Neeleman *et al.* 1991), cucumber mosaic virus in tobacco (Shintaku *et al.* 1992), and tobacco mosaic virus (TMV) in *N. sylvestris* (Saito *et al.* 1987). However, we have recently removed the entire CP ORF from BMV RNA3, and this did not prevent induction of necrotic lesions by BMV in *C. hybridum* (S. Flasinski, A. Dzianott, and J. J. Bujarski, unpublished results). If the same is true for the CCMV-soybean system, the 3a protein or noncoding RNA3 sequences might elicit the necrotic reactions of the T and S strains in soybean. CP ORFs were not required for local lesion formation by turnip crinkle virus on *C. amaranticolor* (Hacker *et al.* 1992) or by TMV on tobacco Xanthi-nc (Dawson *et al.* 1988).

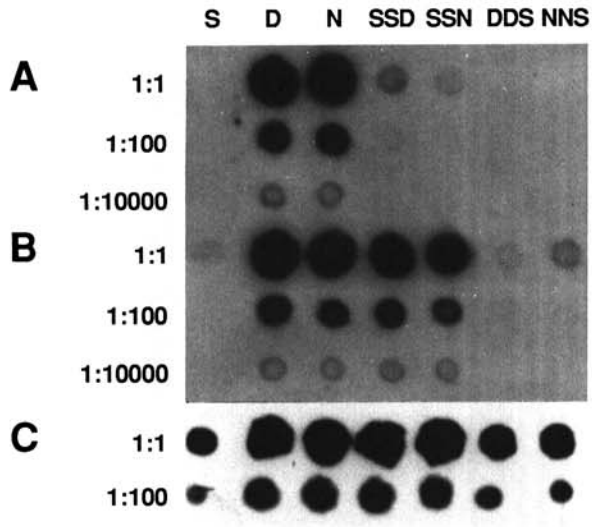

Fig 4. Determination of virus concentration by dot-blot hybridization analysis in systemic (**A**) and inoculated (**B**) soybean leaves as well as in systemically infected cowpea (**C**) leaves. Plants were infected with cowpea chlorotic mottle virus (CCMV) strains S, D, or N or with pseudorecombinants SSD, SSN, DDS, and NNS. All viral infections were initiated using *in vitro*-transcribed viral RNAs. Total leaf RNA preparations were extracted, diluted, attached to the membrane, and probed as described in the text.

We have found that mutations in the BMV CP gene impaired long-distance virus movement in *C. hybridum* (S. Flasinski, A. Dzianott, and J. J. Bujarski, unpublished results). Also, the CCMV CP gene is required for systemic infection of the T strain in cowpea (Allison *et al.* 1990). Changes in CP genes might then participate in breaking soybean resistance with strains D or N. The use of chimeric interstrain CCMV RNA3 molecules should help us to map further the genetic information involved. A comparison of RNA3 nucleotide sequences among the D, N, and S strains will identify sequence differences potentially responsible for the observed strain-specific phenotypes.

In addition to hypersensitive anti-CCMV resistance in soybean Bragg, at least three other types of cultivar-specific nonnecrotic resistance were found against the S strain (Paguio *et al.* 1988). One of them, observed on soybean PI 346304, was related to blockage of long-distance movement (Goodrick *et al.* 1991). It would be interesting to determine whether the viral sequences responsible map to the same CCMV RNA regions as those responsible for necrotic resistance. Studies demonstrate that distinct resistance mechanisms against CCMV can operate in soybean and in cowpea plants. For instance, CCMV R and PSM strains can overcome resistance in cowpea PI 186465 (Paguio et al. 1988), but neither of these strains could overcome any type of anti-CCMV resistance in soybean (Kuhn *et al.* 1981).

Consistent with previous observations (Bancroft and Lane 1973; Wyatt and Kuhn 1980; Kuhn *et al.* 1981; Allison *et al.* 1988), our data demonstrate that, in addition to that from the RNA3 component, RNA1- and/or RNA2-encoded genetic information contributes to symptom formation and virus accumulation. For instance, both SSD and SSN pseudorecombinants induced more severe symptoms on cowpea California Blackeye than did the D or N strains. Also, these pseudorecombinants accumulated less virus on systemically infected leaves of soybean Bragg. Pseudorecombinants DND, NND, and NDD induced more severe distortion on soybean Bragg. The mechanism of these effects is not well understood. Data of other plant viruses reveal the involvement of replicase genes in host reactions. For instance, in cucumber mosaic virus, a nonrandom distribution of sequence differences between RNA1 and RNA2 components has been found among several strains that differed in their reactions with selected hosts (Rizzo and Palukaitis 1988, 1989; Shintaku *et al.* 1992). Also, in TMV, the products encoded by the putative replicase genes were found to interact with the products of the tomato *Tm-1* resistance gene (Meshi *et al.* 1988). Further studies are required to dissect the corresponding molecular mechanism for the observed effects in CCMV.

MATERIALS AND METHODS

Virus strains and hosts.

The D, N, and S strains were recovered initially from dried stocks by mechanical inoculation on the primary leaves of 10-

day-old resistant soybean cultivar (cv. Bragg) or susceptible cowpea (cv. California Blackeye) seedlings in 10 mM sodium phosphate, pH 7.0. Plants were grown in 10-cm-wide square plastic pots (three soybean or two cowpea plants per pot) containing Pro-Mix BX (Premier Brands Inc., Stamford, CT) amended with a complete fertilizer. Plants were maintained in a greenhouse with summer temperatures of 25–35° C and winter temperatures of 21–28° C, with approximately 5,000 lux units illumination for 12 hr a day. To retard abscission of the inoculated primary leaves, the new trifoliate leaves were routinely removed from all soybean plants. The D and N isolates were propagated in California Blackeye and sap diluted 1:10 with phosphate buffer. Dot blot hybridization analysis done at several occasions revealed that this adjusted the virus concentration to 30–60 mg/ml.

Virus purification and separation of genomic RNAs.

Virus was harvested 8–12 days postinoculation. Data of Kuhn (1968) established that viral concentration had reached a maximum after this period. Virus preparations were purified from the infected leaves as described (Lane 1981). To extract the RNAs, virus preparations were treated with 2% sodium dodecyl sulfate followed by phenol-chloroform extraction and ethanol precipitation. To separate individual RNA components, RNA preparations were denatured with 6% formaldehyde followed by heating at 65° C for 10 min (Hsu and Chiu 1988). RNAs were then fractionated by two cycles of electrophoresis in 1% low-melting-point agarose (SeaPlaque GTG agarose, FMC Corp.) gels. To prevent possible cross-contamination, the RNA3 molecules in the RNA1+2 fractions were removed by cleavage with RNaseH in the presence of oligo $d(T)_{10}$, as described by Smirnyagina and Atabekov (1989).

Construction of infectious cDNA clones, *in vitro* transcription, and infectivity tests.

The first-strand cDNA primer (primer no. 3 in Fig. 2) was 5′-d(CAGTCTA-GAGTGGTCTCCTTAGAGAT)-3′ (extra sequences containing *Xba*I restriction site are underlined) for all three CCMV RNAs of the D, N, and S strains. Second-strand cDNA primers were 5′-d(GCTCTAGAGCTGTAATACGACTCACTATAGTAATCCACGAGAACGAGGTTCAATCCC)-3′ for RNA1 and RNA2 (primer no. 1) and 5′-d(TGTAATACGACTCACTATAGTAATCTTTACCTTTCAA)-3′ for RNA3 (primer no. 2) (extra sequences with *Xba*I or *Hind*III restriction sites and T7 RNA polymerase promoter are underlined). The first-strand cDNA primer was annealed to unfractionated virion RNA preparations and extended with reverse transcriptase (GIBCO/BRL), as described by Allison *et al.* (1988). After purification in 1% low-melting-point agarose gels, the cDNAs were mixed with first- and second-strand synthesis primers and amplified using PCRs. PCR reaction mixtures (100 µl) contained 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100, 100 µg/ml BSA, 2 mM dNTPs (dATP, dCTP, dGTP, and dTTP), 1 µg of each primer and 2 units of a thermostable VENT DNA polymerase (New England BioLabs). After three initial thermocycles (92° C for 1 min, 55° C for 2 min, 72° C for 3 min) and 25 amplification cycles (92, 60, and 72° C for 1, 2, and 3 min, respectively), the PCR products were extracted with phenol-chloroform, precipitated with ethanol, and purified by electrophoresis in a low-melting-temperature agarose gel. The full-length cDNA products were then ligated into *Sma*I-cleaved pUC19 cloning vector. The clones were confirmed by restriction mapping and by sequencing of the 5′ and 3′ termini (Shang and Bujarski 1991).

Transcription reaction mixtures (100 µl) contained 5 µg of linearized plasmid DNA (cut with *Sph*I, *Pst*I, or *Eco*RI restriction enzymes for RNA1, RNA2, or RNA3 components, respectively), 10 U T7 RNA polymerase, and other reagents, as described by French and Ahlquist (1987). The DNA template was removed by digestion with RQ1 DNase (Promega); the RNAs were mixed with an inoculation buffer (30 mM K_2HPO_4, pH 9.2, 50 mM glycine, 0.2% bentonite, and 0.2% Cellite) and applied to primary leaves of 10-day-old soybean or cowpea seedlings. Symptoms were observed 10 days after inoculation.

Construction of pseudorecombinants using fractionated virion RNA.

Using gel-purified fractions, virion RNA(1+2) from the D or N strains was mixed with RNA3 of the S strain, and RNA3 from the D or N strains was mixed with RNA(1+2) of the S strain, each RNA at a concentration of 100 µg/ml, with approximately a 1:1:1 molar ratio. After adding RNA inoculation buffer (above), these mixtures were inoculated onto a local-lesion host (*Chenopodium hybridum*). The progeny virus of DDS (RNA1 and RNA2 of strain D, RNA3 of strain S), NNS, SSD, and SSN reassortants was isolated 7 days later from a single lesion by homogenization in 10 mM sodium phosphate buffer, pH 7.0, and then was mechanically inoculated onto the primary leaves of soybean or cowpea seedlings. Symptoms were examined 10 days after inoculation, and pseudorecombinant virion RNA preparations were obtained by the same method used for purifying the RNA from parental isolates. The RNA composition of each pseudorecombinant was confirmed by direct sequencing of the 5′ ends with specific primers and AMV reverse transcriptase. To regenerate the parental virus strains, the RNA(1+2) and RNA3 fractions were separated by the procedures described for construction of pseudorecombinants, and the regenerated parental virus was used to inoculate hosts for symptom comparisons.

Construction of pseudorecombinants using *in vitro* transcripts.

The pseudorecombinants SSD, SSN, DDS, and NNS (see above) as well as other pseudorecombinants of CCMV (Table 1) were constructed by mixing corresponding RNA *in vitro* transcripts. The RNA mixtures were inoculated on CCMV hosts, as described above. Symptoms were observed 2 wk after inoculation.

Dot blot hybridizations.

Virus concentrations were determined by dot blot hybridization, using a protocol described by Kafatos *et al.* (1979) and Thomas (1980). Briefly, virus samples were extracted from 1 g of infected leaf tissue, and 10 µl of 1-, 100-, or 10,000-fold dilutions were loaded on a nylon membrane (Hybond-N+, Amersham) saturated with 20× SSC buffer (3 M sodium chloride, 0.3 M sodium citrate). The membranes were dried at room temperature, and RNAs were bound to the membrane for 2 hr at 80° C under vacuum. The membrane was prehy-

bridized for 1 hr at 60° C with a mixture of 2 mg/ml sheared denatured salmon sperm DNA, 4× Denhardt's solution, 4× SSC buffer, and 1% sodium dodecyl sulfate. Afterwards, the membrane was hybridized with a ^{32}P-labeled cDNA probe that represented a 220-bp 3′ terminal fragment isolated from a full-length CCMV cDNA3 clone of S strain after digestion with *Eco*RI and *Ava*I restriction enzymes. The probe was made radioactive according to a random priming protocol (Sambrook *et al.* 1989) that involved denaturation of cDNA at 100° C for 5 min, quenching on ice, and mixing with a final concentration of 67 mM HEPES, 17 mM Tris (pH 6.8), 1.7 mM $MgCl_2$, 0.02 mM of dCTP, dGTP, and dTTP, 0.05 mM of α-^{32}P-dATP, 3.3 mM β-mercaptoethanol, 0.133 mg/ml BSA, 1.8 OD_{260} U/ml random primers, and 3 U of Klenow DNA polymerase. After incubation at room temperature for 4 hr, the reaction was terminated by adding 20 mM ethylenediamine tetraacetic acid, pH 7.5 (final concentration), and the probe was precipitated with ethanol.

ACKNOWLEDGMENTS

We thank C. Kuhn for the D, N, and S strains of CCMV, H. R. Boerma for seeds of soybean Bragg, and D. Stenger for a critical review of the manuscript.

This research was supported by the U.S. Department of Agriculture under grant 90 CRCR-37262-5418 and by the Plant Molecular Biology Center at Northern Illinois University.

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