A New Strain of Southern Bean Mosaic Virus Derived at Low Temperatures

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ABSTRACT


A new strain of southern bean mosaic virus (SBMV), designated NCP, was derived when certain cowpea lines were inoculated with the cowpea strain (CP) and maintained at 21 and 24 C. NCP was serologically different from CP and three other strains of SBMV. Strain CP caused necrotic local lesions on cowpea cultivar Clay and plant introduction (PI) 399419, while NCP caused no symptoms on Clay and local chlorosis and systemic mosaic on PI 399419. The two strains also differed in susceptibility of their RNAs to protease K and virion accumulation in cultivar Clay. NCP partially overcame the resistance in cultivar Clay, but had no appreciable effect on the resistance of three other cultivars. Four interacting factors were responsible for the derivation of NCP: host, temperature, viral replication, and viral movement. We speculate that the new strain induces some substance that aids or is required for systemic movement of the virus within the host.

An understanding of the occurrence of viral mutations and subsequent development of viral strains during the host-parasite interaction is important for two primary reasons. First, in the control of plant virus diseases, mutant viruses may arise that are capable of increasing disease severity or infecting previously resistant cultivars; alternatively, mutants may be useful in crop-protection programs. Second, in the study of molecular genetics, viral mutations are useful for analysis of gene composition, gene function, and genetic regulation mechanisms.

Various physical and chemical agents mutagenically affect plant viruses, and the resulting mutants have been used widely in research (13). Mutants also occur in nature and are usually recognized by symptoms that differ from those caused by the parent strain. Experimentally, viral mutants arising in vivo frequently are detected after passage of the parent strain through certain hosts that are either able to cause or to screen for mutations. The host passage effect has been observed and studied for many plant viruses (23).

Four strains of southern bean mosaic virus (SBMV) have been isolated from field-grown plants: the bean or type strain (B) (25), the severe bean strain (Sv) (24), the cowpea strain (CP) (18), and the Ghana strain (GH) (10). Until a recent report by Valverde and Fulton (20), strain derivation via host passage had not been described for SBMV. They found new strains in resistant cultivars of bean (Phaseolus vulgaris L.) that had been inoculated with strain B. In studies with strain CP, we detected a new strain (designated NCP) in cultivars of cowpea (Vigna unguiculata (L.) Walp. subsp. unguiculata) that had been maintained at relatively low temperatures (14).

The purpose of the studies reported here was to characterize the new strain and to compare it with other strains of SBMV. An evaluation of some factors related to the derivation of the new strain also is included.

MATERIALS AND METHODS

Virus source and plant manipulation. SBMV strain CP was first isolated from field-grown cowpea plants in 1963 (5). It has been maintained in desiccated tissue or in cowpea cultivar California Blackeye in the greenhouse. The new strain was maintained in cowpea plant introduction (PI) 399419. Two SBMV strains (strain B obtained from O. P. Sehgal and strain Sv from R. I. Hamilton) were cultured in Bountiful bean; strain GH, obtained from R. I. Hamilton, was maintained in California Blackeye cowpea.

Plant inoculations with virus were made onto primary leaves of 8- to 10-day-old cowpea plants with a cheesecloth pad moistened in inoculum, which consisted of either sap from infected plants (1 g/9 ml of 0.01 M potassium phosphate buffer, pH 7) or purified virus adjusted to the appropriate concentration in the same buffer.

Plants were grown in a methyl bromide-treated soil mix containing sand, and vermiculite (2:1:1, v/v) in 10-cm-diameter plastic pots. The soil pH was about 6.8, and the plants were fertilized weekly with a complete fertilizer (20-20-20, NPK). In greenhouse studies, the temperatures ranged from 21 to 35 C in the daytime and from 18 to 24 C at night. Growth chambers were used to control specific temperatures ranging from 21 to 36 C; the photoperiod was 16 hr/day and illumination with both incandescent and fluorescent lights was approximately 10,000 lux.

Single-lesion isolations. Cultivar: Clay cowpea host plants were used in attempts to select an isolate of strain CP free of variants. CP caused smaller local lesions (area < 0.8 mm²) on Clay than on PI 399419 (area < 2.5 mm²) and systemic necrosis on PI 399419 only. However, it must be noted that virus does move into uninoculated leaves of Clay (6) and replicates at low levels. Single lesions, ground in phosphate buffer, were used as inoculum to transfer CP to new 8- to 10-day-old Clay plants every 3-4 days.

Purification. Purification of strains CP (cultured in California Blackeye) and NCP (cultured in PI 399419) followed a previously described procedure (6) with two exceptions: 0.2% NaCl was added to the extraction buffer and three cycles of differential ultracentrifugation were followed by sucrose density gradient (10-40%) centrifugation to obtain highly purified preparations. Virus concentration was analyzed spectrophotometrically (E 590nm = 5.8).

Serology. Antiserum to strains CP and NCP were prepared by injection of highly purified virus into young 1.82- to 2.77-kg (4-5 lb) female rabbits. One milligram of virus was injected intravenously and 10 mg intramuscularly each week for 4 wk. After bleeding and serum processing, the antisera were stored at 5 C.

Serological comparisons of the various SBMV strains were done by Ouchterlony agar double-diffusion tests (16) employing 1% Noble agar gels buffered at pH 7.0 with 0.01 M potassium phosphate buffer containing 0.85% sodium chloride and 0.1% sodium azide. Antiserum and antigens were placed in the center and outer wells, respectively, which were spaced 6 mm apart.

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Strain identification. Strains CP and NCP were identified by their reaction on PI 399419 plants maintained in the greenhouse (local necrotic lesions for CP and local chlorosis and systemic mosaic for NCP) and by spur formation in immunodiffusion tests with heterologous antiserum.

Host range. Twenty-seven species and cultivars of plants selected from seven genera and two families were inoculated with strains CP and NCP. Selection of the hosts was made to include those known to be either susceptible or resistant to CP. Inoculations of plants in the greenhouse were made with 0.1 mg of virus per milliliter. Sap extracts from inoculated primary and uninoculated trifoliolate leaves were assayed for the presence of virus.

Biological properties. Accumulation of CP and NCP virions in cowpeas with different levels of resistance to CP was tested by inoculating plants with 0.1 mg of purified virus per milliliter and maintaining the plants in the greenhouse. Two weeks after inoculation, the inoculated primary leaves were harvested and virus was extracted and frozen overnight (6). The supernatants were then thawed, and 1–2 ml of sap was layered onto 10–40% sucrose density gradient columns. Following centrifugation for 3.5 hr at 104,000 g, the viral zones were analyzed with an ISCO model 640 fractionator and a UV absorbance monitor (254 nm) quantitated by planimetry of the resulting graphs.

Seed transmission of both CP and NCP was evaluated by planting seeds from infected plants in 10-cm-diameter pots. To avoid transmission (21) by soil movement from pot to pot, they were placed on inverted clay saucers. Seedlings were observed and tested for specific strains 28–35 days after planting.

Virus properties. RNA was isolated from purified CP and NCP by the LiCl method (21) and centrifuged on sucrose density gradient columns according to the procedure of Rutgers et al (17). To minimize ribonuclease degradation of RNA, all experiments were performed in glassware that had been washed with 10% hydrochloric acid, rinsed with glass-distilled water, and heated at 200°C overnight. In addition, RNA was stored in 95% ethanol at −20°C.

Coat protein was also isolated from intact, purified virions by the LiCl method (21). The dissociated coat protein, which remained in the supernatant following the 8,000-g centrifugation used to concentrate the RNA, was collected and dialyzed overnight at 5°C against several changes of 0.01 M tris-HCl buffer adjusted to pH 8.25, and then frozen at −20°C until use.

Electrophoresis of viral coat protein was conducted according to the discontinuous slab gel procedure described by Laemmli (9). Low-molecular-weight standards used for molecular weight comparisons were obtained from Bio-Rad Laboratories, Richmond, CA.

Protease K treatments of RNA isolated from CP and NCP were conducted according to the procedure of Veerisetti and Sehgal (21). Infection of protease K-treated CP and NCP RNA was assayed in 8- to 10-day-old PI 399419 plants; CP RNA was denoted by systemic local lesions, while infection by NCP RNA was determined by systemic mosaic.

Factors affecting derivation. To determine the effect of temperature on strain derivation, plants were grown in the greenhouse and, immediately after inoculation, they were transferred to growth chambers for the duration of the study. Studies of mixed infections involving simultaneous inoculation with CP and NCP were carried out by inoculating PI 399419 plants with 0.1 mg of each strain per milliliter. Plants were maintained at 24 or 27°C for approximately 1 mo. At that time, strain identification was determined by symptomology on PI 399419 and serology.

RESULTS

New strain of SBMV. When PI 399419 cowpeas were inoculated with the CP strain and maintained in the greenhouse or at 24°C, the plants developed necrotic local lesions (Fig. 1) 3–4 days after inoculation and either systemic necrosis at 8–20 days or no systemic symptoms. In one study at 24°C, local lesions developed on all 90 plants, 16 plants developed systemic necrosis, 72 had no systemic symptoms, and two had systemic mosaic. Sap from tissue with necrosis, either local or systemic, caused the expected CP reaction on PI 399419. Sap from mosaic tissue caused no local symptoms (Fig. 1, right), or sometimes local chlorosis, and systemic mosaic on PI 399419 plants (Fig. 2), a reaction similar to that caused by strain CP on nonnecrotic, susceptible cowpea cultivars such as California Blackeye. Thus, it was suspected that a new strain of SBMV had been derived by passage of strain CP through PI 399419 plants. The lack of production of either local or systemic necrosis on PI 399419 plants, or on four other cowpea lines reported (7) to respond with necrosis to strain CP, suggested the name nonnecrotic cowpea strain (NCP) for the new variant of SBMV.

Single-lesion isolation. After the sixth transfer of single lesions caused by strain CP, a lesion was used to inoculate California Blackeye; sap from these infected plants was used to inoculate PI 399419 plants which were maintained at 24°C. About 10% of the PI 399419 plants developed systemic mosaic, and an isolate serologically similar or identical to strain NCP was obtained from the tissue.

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Fig. 1. Reaction caused on the inoculated primary leaves of cowpea PI 399419 by southern bean mosaic virus strains CP (left) and NCP (right).

Fig. 2. Mosaic and leaf distortion caused on cowpea PI 399419 by southern bean mosaic virus strain NCP (about 1 mo after inoculation of an 8-day-old seeding).
When nine additional single-lesion transfers were made and the tests repeated, no systemic mosaic symptoms were noted on the PI 399419 test plants. However, when tissue from symptomless, un inoculated leaves from the test plants was used as inoculum, about one-third of the next group of PI 399419 test plants had NCP symptoms.

Serology. In Ouchterlony double diffusion tests, strain NCP was determined to be serologically related to, but distinct from, CP. When CP and NCP virions were in adjacent outer wells, distinct spurS developed with heterologous antisera (Fig. 3A and B). Also, NCP was serologically distinct from the other known strains of SBMV. With either CP or NCP antiserum, spurs developed between NCP and strains B, S, and GH (Fig. 3C and D). When CP antiserum was used to compare strain NCP with strains B and S, double spurs developed (Table 1). In the same comparison test, however, strain GH was responsible for the only spur that developed, suggesting a closer serological relationship between CP and GH than between CP and NCP.

Host range. All hosts from numerous genera and species within the Leguminosae and Solanaceae families reacted similarly to strains CP and NCP, except four cowpea lines (Brabham, Clay, Georgia, and S) and PI 399419 which developed local necrotic lesions to CP. NCP caused symptoms (described above) only on PI 399419.

Contrary to previous reports regarding CP (5,18), both CP and NCP could be isolated from inoculated, but not from un inoculated, leaves of several symptomless bean cultivars: Blue Lake, Kentucky Wonder, Spartan Half Runner, Tennessee Green Pod, and White Half Runner.

Biological properties. The stability of NCP was tested by inoculating a nonnecrotic, susceptible cultivar (California Blackeye) with highly purified NCP. Six weeks later, virus in both inoculated and un inoculated tissue was identified as NCP. Furthermore, when NCP was transferred serially from susceptible to susceptible cowpeas, there was no indication of reversion to CP during a 6-mo period.

Accumulation levels of CP and NCP were studied in six cowpea lines known to react differently to CP (4). There appeared to be more CP than NCP in the susceptible line, and little difference was observed between the strains in the resistant lines (Table 2). One exception, however, was cultivar Clay, which had 7 to 10 times as much NCP as CP.

Neither CP nor NCP was transmitted through seeds of PI 399419. However, both strains were seed-transmitted in California Blackeye, and the frequency appeared to be greater for CP (15 of 407) than NCP (4 of 393).

Virus properties. The sedimentation properties of purified CP and NCP were compared by centrifugation (150,000 g for 2 hr) in 10 to 40% sucrose density gradient columns. A single virus zone was consistently observed in tubes evaluated with an ISCO model UA 5 absorbance monitor, with CP and NCP alone and also with a mixture of equal quantities of CP and NCP, indicating no major differences in sedimentation properties between the two strains.

There were no significant differences in the profiles (ultraviolet absorbance) of RNAs of CP and NCP when equal quantities of each were centrifuged on 5 to 20% sucrose columns. The major peaks of both strains sedimented similar distances, and similar quantities of low-molecular-weight RNA species were observed. Electrophoresis of coat proteins in both 10 to 15% acrylamide gels yielded a single protein species for both strains CP and NCP. Tests in which equal amounts of CP and NCP coat proteins were electrophoresed in adjacent wells revealed that both proteins migrated similar distances; the molecular weight of the protein was approximately 29,000 daltons.

Inoculation of CP RNA and NCP RNA with 1.0 µg of protease K per milliliter resulted in the loss of infectivity of both RNAs (Table 3). However, treatment with 0.1 µg of protease K per milliliter destroyed NCP RNA infectivity, while limited infection occurred when CP RNA was inoculated with that concentration of the enzyme.

| TABLE 1. Comparison of serological reactions of southern bean mosaic virus strains CP and NCP with strains B, S, and GH in agar double diffusion tests |
|---------------------------------|---------------------------------|
| Antigens compared               | Antiserum                        | Antigen causing spur formation |
| NCP/CP                          | CP                              | CP                             |
| NCP/CP                          | NCP                             | NCP                            |
| NCP/B                           | CP                              | Both                           |
| NCP/Sv                          | NCP                             | NCP                            |
| NCP/Sv                          | CP                              | Both                           |
| NCP/GH                         | CP                              | NCP                            |
| NCP/GH                         | NCP                             | NCP                            |

*NCP = Cowpea nonnecrotic strain, CP = cowpea strain, B = bean strain, GH = Ghana strain, and S = severe bean strain.

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<tr>
<th>TABLE 2. Accumulation of southern bean mosaic virus strains CP and NCP in inoculated primary leaves of six cowpea lines</th>
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<td>Cultivar</td>
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<td>Early Pinkeye</td>
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<td>Clay</td>
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<td>PI 186465</td>
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*Seedlings 8 to 10 days old were inoculated with a suspension containing 0.1 mg of virus per milliliter; primary leaves were harvested 2 wk later.
*S = Susceptible (mosaic, stunting), M = moderately resistant (mild mosaic), R = Resistant (symptomless), HR = highly resistant (symptomless), LL = necrotic local lesions.
Plants died before harvest.
Primary leaves were harvested 1 wk after inoculation.

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Factors affecting derivation. Since strain NCP was initially observed in cowpeas maintained at 24°C and had not been noted in greenhouse-grown plants, the derivation of the new strain was studied at constant temperatures of 21, 24, 27, 30, 33, and 36°C. On primary leaves of PI 399419 inoculated with CP, necrotic lesions developed at all temperatures except the two highest, 33 and 36°C, in which they developed either local chlorosis or no symptoms (Table 4). No systemic symptoms developed on any of the plants at 21°C or on the majority of the plants at 24°C. At 27 and 30°C, a general systemic necrosis, which occurred within 5-8 days, was followed by death. Systemic mosaic developed at 33 and 36°C. Strain NCP was found in unoinoculated leaves of 10-20% of plants maintained at 21 and 24°C, while strain CP only was found in systemically infected leaves of all plants at 27, 30, 33, and 36°C (Table 4). When plants at 33 and 36°C that had developed systemic mosaic were transferred to 24°C or the greenhouse, leaf tissue began to collapse within 24 hr and all plants died within 5-10 days.

When PI 399419 plants were inoculated with an equal mixture of CP and NCP, local symptoms (necrotic lesions) were similar at 24 and 27°C (Table 5). Systemically, mosaic occurred on all plants at 24°C, while at 27°C, approximately half of the plants had mosaic and the other half developed necrosis. The single infection control treatments reacted as expected from previous results (Table 4).

**DISCUSSION**

A variant (NCP) of SBMV strain CP could be derived repeatedly by passage of CP through plants of a cowpea line maintained at temperatures of 21 or 24°C. Although physicochemical properties of CP and NCP were very similar, several biological properties clearly differentiated the two strains. Distinct spurs developed in serological reactions with heterologous antisera, and NCP caused no symptoms or local chlorosis on cowpea lines reacting to CP with local necrotic lesions. The NCP-RNA was more susceptible to the action of proteinase K than CP-RNA, and the virion concentration of NCP was significantly greater than CP in one resistant cultivar.

Several factors were responsible for the derivation of strain NCP: plant host, temperature, viral movement, and viral replication. The ability of a virus to overcome the hypersensitive type of resistance, frequently controlled by a single dominant gene (2), is a relatively common phenomenon (13). Two recent reports, one concerning a bean strain of SBMV (20) and the other cowpea mosaic virus (3), describe in detail the derivation of several virus strains. Such a reaction appeared to occur with host PI 399419 in this study: the parent virus CP caused local necrosis and new strain NCP caused systemic mosaic. However, in plants of cultivar Clay (a cowpea line that reacts with local necrosis to CP), strain NCP caused no symptoms, even though its replication was increased about tenfold over that of CP.

The effect of temperature on virus replication and subsequent selection of mutants is well documented (8,11). In 1974, Lamptey and Hamilton (10) speculated that a combination of host passage and high temperature could give rise to new strains of SBMV under natural conditions. Strain NCP developed at a relatively low temperature, and it is possible that the strain might not evolve in the tropical and subtropical regions where cowpeas are normally grown. However, temperature studies were important in developing some understanding of the mechanism of derivation.

While systemic movement of CP was restricted in PI 399419 at 24°C, it occurred readily for NCP. Furthermore, in mixed infections of CP and NCP in the greenhouse, and at 24°C, the local symptom reaction was similar to CP but the systemic one was dominated by NCP. Therefore, we speculate that the NCP genome induces some substance that aids or is required for systemic movement in PI 399419. The possibility of virus-induced components affecting movement has been noted previously. Wyatt and Kuhn (22) found that RNA 1 of cowpea chlorotic mottle virus was required for systemic movement in cowpeas. Also, Leonard et al (12) speculated that tomato mosaic virus induces a peptide which may affect cell-to-cell movement.

Serial transfer of single local lesions is generally accepted as a method by which biologically pure virus strains can be obtained frequently, but not always (13). Boxall and MacNeill (1) reported they could produce genetically homogeneous isolates of tobacco mosaic virus by one passage of the virus through single lesions. Subsequently, they used what was believed to be a pure isolate to

| Table 4. Effect of temperature on the derivation of southern bean mosaic virus strain NCP in PI 399419 cowpea plants inoculated with strain CP. |
|---|---|---|---|---|
| Temperature (°C) | Disease reaction | Plants with systemic symptoms | Strain in unoinoculated leaves |
| CP | Local | Systemic | 
| 21 | NL | None | 0 of 20 | NCP
| 24 | NL | None | 0 of 20 | NCP
| 27 | NL | None | 0 of 20 | NCP
| 30 | NL | None | 0 of 20 | NCP
| 33 | NL | None | 0 of 20 | NCP
| 36 | NL | None | 0 of 20 | NCP

*Following inoculation with strain CP, the 8- to 10-day-old seedlings were immediately transferred to growth chambers.

**Strain determined serologically and by subinoculation to PI 399419.

***Necrotic lesions.

****NCP was recovered from unoinoculated leaves of four plants.

*****Plants died.

******When these plants were transferred to 23°C or the greenhouse after incubation in the growth chambers for 10-20 days, all plants developed necrosis and died within 5-10 days.

| Table 5. Effect of single and mixed infections of southern bean mosaic virus strains CP and NCP on symptoms that developed on cowpea PI 399419 plants maintained at 24 and 27°C. |
|---|---|---|---|---|
| Virus inoculum | Incubation temperature (°C) | Local | Systemic |
| Test | | | Mosaic | Necrosis | None |
| CP | 24 | NL | 4 | 11 | 25 |
| NCP+ | 24 | Chl | 40 | 0 | 0 |
| CP | 27 | NL | 40 | 0 | 40 |
| NCP | 27 | Chl | 40 | 0 | 40 |
| CP + NCP | 27 | NL | 23 | 17 | 0 |

*Inoculated primary leaves of 8- to 10-day-old seedlings (40 per treatment) with 0.1 ng/mL of virus.

**Tests were repeated at each temperature with similar results.

***NL = necrotic lesions, Chl = chlorosis.

****Number of plants.

*****Lesion numbers were similar, about 400 per leaf.

******First two trifoliate leaves had both mosaic and necrosis; subsequent new growth had mosaic only.

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infect the susceptible portion of an intergrafted pair of susceptible and resistant tomato plants, and a new virus strain was detected in the resistant plant (15). In our study, numerous and rigorous transfers from single local lesions caused by parental strain CP could have eliminated contaminating strains from the original SBMV population, although unequivocal evidence was not demonstrated. Thereafter, we suggest that a spontaneous or host-induced viral mutation occurred in PI 399419 at 24 C. The new mutant (NCP) replicated rapidly, its movement was not restricted, and a new symptom type was produced on uninoculated trifoliate leaves. Although numerous new and variable mutants probably arose, the repetitive derivation of NCP most likely was caused by a highly specific selection pressure in the host at a specific temperature.

Increased virulence often is associated with the development of new viral strains, particularly in resistant hosts. In this study, the reaction of new strain NCP in different cowpea lines indicates that cowpea has at least two types of resistance to SBMV: local necrotization controlled by a single dominant gene (2) and highly restricted viral replication and movement in symptomless plants. When NCP overcame the local necrotization gene in PI 399419 and Clay, the two hosts responded very differently. The first was fully susceptible with high concentrations of virus and strong systemic symptoms; the second had no symptoms and remained resistant, although to a lesser degree than it was to CP on the basis of level of viral replication. Furthermore, cowpea lines Iron and PI 186465 were similarly resistant (symptomless and very low virus concentration) to both CP and NCP.

LITERATURE CITED