Bromovirus Movement Protein Genes Play a Crucial Role in Host Specificity

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Monocot-adapted brome mosaic virus (BMV) and dicot-adapted cowpea chlorotic mottle virus (CCMV) are closely related bromoviruses with tripartite RNA genomes. Although RNAs 1 and 2 together are sufficient for RNA replication in protoplasts, systemic infection also requires RNA3, which encodes the coat protein and the nonstructural 3a movement protein. We have previously shown with bromoviral reassortants that host specificity determinants in both viruses are encoded by RNA3 as well as by RNA1 and/or RNA2. Here, to test their possible role in host specificity, the 3a movement protein genes were precisely exchanged between BMV and CCMV. The hybrid viruses, but not 3a deletion mutants, systemically infected Nicotiana benthamiana, a permissive host for both parental viruses. The hybrids thus retain basic competence for replication, packaging, cell-to-cell spread, and long-distance (vascular) spread. However, the hybrids failed to systemically infect either barley or cowpea, selective hosts for parental viruses. Thus, the 3a gene and/or its encoded 3a protein contributes to host specificity of both monocot- and dicot-adapted bromoviruses. Tests of inoculated cowpea leaves showed that the spread of the CCMV hybrid containing the BMV 3a gene was blocked at a very early stage of infection. Moreover, the BMV hybrid containing the CCMV 3a gene appeared to spread farther than wt BMV in inoculated cowpea leaves. Several pseudorevertants directing systemic infection in cowpea leaves were obtained from plants inoculated with the CCMV(BMV 3a) hybrid, suggesting that the number of mutations required to adapt the hybrid to dicots is small.

Systemic infection can be achieved when viruses have the ability to replicate, to move from cell to cell, and to move long distances through the vasculature of a host plant (12). In many cases, the inability of a virus to infect a particular plant is thought to result from a lack of cell-to-cell movement of the viruses (32, 42). Such cell-to-cell movement is frequently mediated by virus-encoded nonstructural “movement proteins,” which therefore might be important candidates for host specificity factors. Recently, there has been a rapid increase in knowledge concerning such movement proteins and their possible functions (9, 16, 23, 24).

Brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) are closely related bromoviruses and have different host ranges. Although both BMV and CCMV systemically infect Nicotiana benthamiana (29, 30), the major systemic hosts of BMV and CCMV are monocotyledonous grasses (e.g., barley) and dicotyledonous legumes (e.g., cowpea), respectively (13, 29).

To study the host specificity and other properties of bromoviruses, we have previously constructed biologically active cDNA clones from which infectious transcripts of the three genomic RNAs of BMV (2, 3, 25) and CCMV (5) can be synthesized in vitro. These genomes are divided among three separate messenger-sense RNAs (1, 4, 6, 10, 18). Genomic RNAs 1 and 2 together are sufficient for RNA replication in protoplasts (20, 26) and encode nonstructural proteins 1a and 2a, respectively, which are trans-acting factors required for viral RNA replication (27, 28, 43, 44). Systemic infection also requires dicistronic RNA3 (8), which encodes the nonstructural 3a protein (4), and the coat protein, which is translated from subgenomic RNA4 (11).

Deletion analyses of CCMV RNA3 show that both the 3a and coat genes are required for systemic infection of cowpea (8). Mutations in the 3a gene block viral RNA accumulation even in inoculated leaves (8, 14). Moreover, fluorescent antibody and other localization studies show that the CCMV 3a gene is necessary even for initial cell-to-cell movement of infection from primary infected cells (35).

Reassortants in which RNA3 is exchanged between BMV and CCMV show that host specificity determinants exist on RNA1 and/or RNA2 and also on RNA3 (5). Thus, two or more host specificity determinants must exist, presumably reflecting both the interaction of more than one virus gene product with the host and the wide genetic distance between monocot and dicot plants. Since the exchange of RNA3 between the two viruses simultaneously transfers the 3a and coat genes and the noncoding sequences, it is not clear which factor(s) in RNA3 contributes to host specificity.

To test the role of the 3a movement protein gene in bromovirus host specificity, we have analyzed the systemic infection abilities of hybrid viral RNAs constructed by precisely exchanging the 3a genes between BMV and CCMV. The resulting hybrids were able to direct systemic infection of N. benthamiana, indicating that they retain basic competence for infection spread. However, their failure to systemically infect barley or cowpea demonstrated that, for successful bromovirus infection of these selective hosts, the 3a gene must be specifically adapted to the host.

MATERIALS AND METHODS

Wild-type BMV and CCMV and hybrid cDNA clones. Viral cDNA clones and their transcripts and progeny RNAs will be referred to by the brief descriptive names listed below, with laboratory plasmid designations following in parenthe-
FIG. 1. (A) Schematic diagram of BMV RNA3 (B3), CCMV RNA3 (C3), and their derivatives and hybrids. The 3a genes of BMV (B3a) and CCMV (C3a) and the coat protein genes of BMV (Bcp; open boxes) and CCMV (Ccp; shaded boxes) are indicated. The surrounding noncoding regions of BMV and CCMV are indicated by thin and thick lines, respectively. B3 and C3 derivatives B3(BmBg) and C3(Bm) contain the indicated BamHI and BglII sites immediately flanking the ends of the 3a gene. These sites were used to create hybrids B3(C3a) and C3(B3a), in which the 3a gene segments were exchanged between BMV and CCMV RNA3. Brackets above B3 and below C3 indicate the sequence deleted in B3(Δ3a) and C3(Δ3a), respectively. (B) Nucleotide sequence showing the BamHI and BglII sites (over- and underlined) created just 5' and 3', respectively, to the 3a gene. A BglII site following the 3a gene occurs naturally in the wt C3 sequence. Asterisks mark bases altered by oligonucleotide-directed mutagenesis, with the preexisting wt bases shown outside the asterisks.

The RNA3 clones are illustrated in Fig. 1. B1 (pB1TP3), B2 (pB2TP5), and B3 (pB3TP8) are wild-type (wt) cDNA clones of BMV RNA1, RNA2, and RNA3, respectively (25). C1 (pCC1TP1), C2 (pCC2TP2), and C3 (pCC3TP4) are wt cDNA clones of CCMV RNA1, RNA2, and RNA3, respectively (5). B3(Δ3a) (pB3BC1), C3(Δ3a) (pCC3RA3), B3 (BmBg) (pB3TP10), and pCC3TP8 have been described previously (19, 37, 39). B3(BmBg) (37) was made by site-directed mutagenesis of B3, followed by using the mutagenized DNA fragment containing the T7 promoter region and the region from the 5' end of BMV RNA3 to the SalI site (nucleotide [nt] 1 to 1253) to replace the corresponding fragment in B3. To confirm the desired mutations and to ensure that no additional mutations had been introduced, nt 1 to 1253 was then sequenced. C3(Bm) (pCC3WD21; kindly provided by W. De Jong) was made by site-directed mutagenesis of pCC3TP8 with the oligonucleotide d(GGGATCC AAAACAGCAA), which anneals to bases 221 to 237 to change base 229 from A to T, eliminating an extra ATG codon just upstream of the start codon of the 3a gene. The mutagenized DNA fragment containing the T7 promoter region and the 5' noncoding region of CCMV RNA3 (nt 1 to 231) was then used to replace the corresponding fragment in pCC3TP8, creating C3(Bm), and nt 1 to 231 was sequenced. In B3(BmBg), C3(Bm), and pCC3TP8, the 3a gene is flanked by a BamHI site at the 5' end and by a BglII site at the 3' end (Fig. 1).

The BMV and CCMV 3a genes (hereafter designated B3a and C3a, respectively) were exchanged as follows: B3(C3a) (pBC3AJ5) and C3(B3a) (pBC3KM11) were constructed by replacing the BamHI-BglII 3a open reading frame cassettes of B3(BmBg) and C3(Bm) with the corresponding cassettes of pCC3TP8 and B3(BmBg), respectively, and confirmed by restriction digest mapping. Since B3(BmBg) and C3(Bm) have additional BglII sites immediately 3' to the oligo(A) tract (4) and inside the C3a gene (6), respectively, these plasmids were partially digested with BglII and the required partial digest fragments were isolated from agarose gels before ligation.

In vitro transcription and plant inoculation. Before transcription, B3, B3(BmBg), B3(Δ3a), and C3(C3a) were linearized with EcoRI (3), while C3, C3(Bm), C3(Δ3a), and C3(B3a) were linearized with XbaI (5). In vitro transcription was carried out with T7 RNA polymerase as described previously (5, 19, 27).

All plants used for protoplast isolation or systemic infection assay were grown under previously described conditions (38) unless otherwise stated. In all solutions to which protoplasts were exposed, 0.6 M mannitol was included as an osmotic stabilizer. Barley protoplasts were prepared from 6-day-old plants (Hordeum vulgare L. cv. Morex) essentially as described previously (36), except that 1% Cellulase Onozuka R-10 (Kinki Yakult Co., Nishinomiya, Japan) was replaced with 2% Cellulysin (Calbiochem) in the protoplast isolation medium. Cowpea protoplasts were prepared from 15- to 20-day-old plants [Vigna sinensis (Torner) Savi. cv. Blackeye No. 5] essentially as described previously (21), except that 1% Cellulase Onozuka R-10 was replaced with 1% Cellulysin for protoplast isolation. For both barley and cowpea plants, the abaxial epidermis of primary leaves was
peeled off before enzyme treatment. RNA inoculation and incubation of protoplasts were carried out as described previously (27).

To initiate whole plant infections, the two largest leaves of 2-month-old N. benthamiana plants, the primary leaves of 7-day-old barley plants, or both primary leaves of 9-day-old cowpea plants were inoculated with in vitro transcriptions as described previously (2, 5).

**Tissue printing and press-blotting techniques.** Nylon hybridization membranes (Hybond N; Amersham) were used, and tissue prints (31, 34) were prepared as follows. Two or 3 weeks after inoculation, leaves were removed and subjected to tissue printing in descending order from the top of the plant. N. benthamiana leaves were first rolled into a tight cylinder, with the midrib parallel to the long axis of the cylinder, and then cross-sections were cut perpendicular to the midrib axis. Barley leaves were folded into sections 2 to 3 cm in length before being cut across the long axis of the leaves. For cowpea leaves, three trifoliate leaflets or two primary leaves were first piled and then rolled into a tight cylinder and cut in the same way as for N. benthamiana. Using a new razor blade for each plant, cross-sections were manually cut at positions one-fourth, one-half, and three-fourths along the length of the long axis of each rolled or folded leaf or set of leaflets. Each cut surface was pressed onto a membrane side by side to obtain three separate prints for each leaf sample. The three prints from each leaf sample gave consistent results in all cases, and representative results are shown in Fig. 3 and 5.

Press blots (33) were prepared as follows. Two pieces (9.5 by 8 cm) of Whatman 3MM filter paper were placed below a piece (9.5 by 4 cm) of dry nylon membrane. To improve the resolution of the viral RNA hybridization signal, the abaxial epidermis was carefully peeled from the cowpea primary leaf samples, which had previously been inoculated with transcripts on their adaxial surfaces. The leaves were then cut along the midrib and a half leaf was placed onto the membrane with the peeled abaxial surface in direct contact with the membrane. This sandwich was wrapped in Saran Wrap, and the air between the sample and the wrap was expelled through a pore made with a needle. The sandwich was placed between two flat aluminum blocks (13 by 10 by 2.5 cm), and the blocks were pressed for 1 min with the 4-cm-diameter piston of a hydraulic press operated at 500 kg/cm². The sandwich was disassembled, and the membrane was allowed to air dry.

After membranes had been prepared by tissue printing or press blotting as described above, the viral RNA was fixed by baking it at 80°C for 2 to 3 h. Membranes were stored at room temperature and then subjected to hybridization as previously described (27), except that prehybridization was performed for 20 to 24 h.

**RNA isolation and Northern (RNA) blot analysis.** Total nucleic acids were extracted from transfected protoplasts as described previously (27). Virion RNA was isolated from uninoculated leaves of N. benthamiana or cowpea plants as described previously (45). Total and viral RNAs were separated by electrophoresis through 1.0% agarose gels after glyoxalation and Northern blots were prepared and analyzed by using 32P-labeled RNA probes as previously described (27). Probes to detect positive-strand BMV or CCMV RNAs have been described previously (8, 37), pB3BY3, kindly provided by B. M. Young, was constructed by cloning the BamHI-BglII B3a gene cassette of pB3TP10 into the BamHI site of the vector Bluescribe M13 (+) (Stratagene), and a T3 RNA polymerase transcript from this plasmid was used to detect positive-strand B3a sequences. A T3 RNA polymerase transcript from pCC3RA516, constructed by cloning a BglII fragment (bases 430 to 1150) of pCC3TP4 into the BamHI site of pT7T3-18U (Pharmacia), was used to detect positive-strand C3a sequences. RNA3 and RNA4 levels were determined by analyzing Northern blots with a Betagen digital radioactive imaging system. To provide an internal standard for comparison between lanes, the RNA3 and RNA4 levels were normalized to the combined counts present in the RNA1 and RNA2 bands of the same lane.

**RESULTS**

To examine the possible contribution of the 3a movement protein genes to bromovirus host adaptation, we exchanged the 3a genes between BMV and CCMV by manipulating biologically active cDNA clones (5, 25). Subsequent inoculation of plants and protoplasts was then performed with infectious in vitro transcripts synthesized from the desired wt or modified plasmids.

To facilitate 3a gene exchange, we first used site-directed mutagenesis to introduce BamHI and BglII restriction sites into equivalent positions immediately flanking both ends of the 3a gene in BMV RNA3 (B3) and CCMV RNA3 (C3) (37; Fig. 1). The resulting B3 and C3 derivatives, B3(BmBg) and C3(Bm), each bear two or three nucleotide substitutions at the site of the introduced restriction sites (Fig. 1). To determine whether these noncoding substitutions had any significant effect on virus replication, B3(BmBg) and C3(Bm) were tested in protoplasts and plants with the corresponding, homologous RNAs 1 and 2. When transcripts of B3(BmBg) plus BMV RNAs 1 and 2 (B1 + B2) were inoculated into protoplasts of the natural BMV host, barley, and C3(Bm) plus CCMV RNAs 1 and 2 (C1 + C2) were inoculated into protoplasts of the natural CCMV host, cowpea, each substitution mutant was replicated and produced subgenomic RNA4 (Fig. 2). Analysis of the resulting Northern blots with a digital radioactive imaging system and comparison of three
experiments showed that the level of B3(BmBg) and C3(Bm)
relative to RNA1+2 was consistently 70 to 80% that of
the corresponding wt RNA3.

When whole cowpea plants were inoculated with either
C1+C2+C3 or C1+C2+C3(Bm), 100% became systemically
infected (data not shown). In initial experiments with barley
plants grown at a density of 30 plants per 11-cm-diameter
pot, 90 to 100% of the barley plants inoculated with either
B1+B2+B3 or B1+B2+B3(BmBg) developed systemic
infection. In later experiments with six barley plants per pot,
systemic infection still occurred in 90 to 100% of plants
inoculated with B1+B2+B3 but in only 50% of plants
inoculated with B1+B2+B3(BmBg). Both before and after
inoculation, barley plants grew faster at 6 rather than 30
plants per pot. This growth difference might account for
the different infection rates, since systemic infection might
be determined in part by a race between infection spread
and plant development (see Discussion). To ensure that the
results of subsequent experiments in barley would not be
altered by this planting density effect, multiple tests under
both planting densities were used to evaluate the B3(C3a)
hybrid (see below), which retained the BamHI-BglII substi-
tution, 75% of B3(BmBg). As described below, B3(C3a) consis-
tently failed to produce systemic infections whether under
the low-density growth conditions in which B3(BmBg) sup-
pported 50% systemic infection and under the high-density
conditions in which B3(BmBg) gave nearly 100% systemic
infection.

B3(BmBg) and C3(Bm) were then used to exchange 3a
genes between BMV and CCMV, creating the hybrids
B3(C3a) and C3(B3a) (Fig. 1). To determine their replication
characteristics, B3(C3a) was assayed with B1+B2 in barley
protoplasts and C3(B3a) was assayed with C1+C2 in cowpea
protoplasts (Fig. 2). B3(C3a) and C3(B3a) were replicated
and accumulated to 30 and 25%, respectively, of the level of
the corresponding wt RNA3, while their respective subge-
nomic coat gene mRNAs accumulated to 75 and 50% of the
level of the corresponding wt RNA4 (on the basis of repro-
ducible measurements from Fig. 2 and two other experi-
ments).

As described in the sections below, the infectivity of each
hybrid RNA3, in combination with B1+B2 or C1+C2, was
then examined in common and selective systemic hosts of
the parental viruses. Each inoculum-plant combination was
tested in three or more independent experiments, using at
least four plants per inoculum in each experiment. For each
plant so inoculated, viral RNA accumulation in both the
inoculated and uninoculated leaves was tested by tissue
printing (31, 34). Three tissue prints were obtained from
independent sections of each leaf sample as described in
Materials and Methods. In all cases, the results of different
prints from the same leaf sample were consistent and represen-
tative tissue printing results are presented as examples in
Fig. 3 and 5.

Systemic infection of N. benthamiana by the hybrid viruses.
N. benthamiana is a permissive host for BMV and CCMV
(29, 30). The tissue print results shown in Fig. 3 confirmed
this and revealed differences in the distribution of BMV and
CCMV in the leaves of infected N. benthamiana. In BMV
infections, the one or two lowest uninoculated leaves con-
sistently escaped virus infection (Fig. 3 and data not shown).
By contrast, in CCMV infections, the first two to three
uninoculated leaves consistently accumulated high levels of
virus, whereas little viral RNA was detected in the fourth
and higher uninoculated leaves (Fig. 3).

To ensure that the relative permissiveness of N. benthami-
amina for BMV, CCMV, and many other plant viruses (12)
does not reflect the lack of a requirement for the 3a gene, N.
benthamiana was inoculated with B1+B2+B3(A3a) or
C1+C2+C3(Δ3a). B3(Δ3a) and C3(Δ3a) each lacks approxi-
mately the C-terminal half of the 3a gene (Fig. 1) and is
replication competent in protoplasts (19, 39). As shown in
Fig. 3, no viral RNA was detected in either inoculation,
showing that BMV and CCMV need a functional 3a cell-to-
cell movement gene to spread from primary infection sites in
inoculated N. benthamiana leaves and to systemically infect
this plant.

To investigate the effect of exchanging the 3a genes
between BMV and CCMV, N. benthamiana was inoculated
with B1+B2+B3(C3a) or C1+C2+C3(B3a). Each hybrid
accumulated in the inoculated leaves and in certain uninoc-
ulated leaves, showing that the hybrids retained basic com-
petence for systemic infection (Fig. 3). However, the distri-
bution of viral RNA in uninoculated leaves differed between
the two hybrids. In plants inoculated with B1+B2+B3(C3a),
the distribution of virus was similar to that of wt BMV, i.e.,
the first one or two uninoculated leaves consistently escaped
infection, but virus accumulated in all higher leaves (Fig. 3
and data not shown). Conversely, in plants inoculated with
C1+C2+C3(B3a), virus distribution was more similar to that
of CCMV than BMV, in that the first uninoculated leaf
invariably became infected, and virus accumulation was
greater in the first two to three uninoculated leaves than in
higher uninoculated leaves (Fig. 3). However, the level of
C1+C2+C3(B3a) in the first to third uninoculated leaves was
often lower than that seen with wt CCMV.

To examine the nature of the progeny viral RNA in the
infected N. benthamiana plants, virion RNA was extracted
from uninoculated leaves 3 weeks after inoculation and
subjected to Northern blot analysis (Fig. 4). Parallel hybrid-
ization reactions were performed with probes specific for the

FIG. 3. Representative tissue print assays for systemic infection of N. benthamiana by the 3a gene-exchange hybrid viruses and the 3a deletion mutants. The two largest leaves (labeled i1 and i2 in the schematic) of 2-month-old N. benthamiana plants were inoculated with in vitro transcripts from cDNA clones of the indicated RNA3 (Fig. 1), together with transcripts from wt RNA1 and RNA2 cDNA clones of BMV (B1+B2) or CCMV (C1+C2) (Materials and Meth-
ods). Three weeks after inoculation, leaves were removed from the
plant and each was rolled into a tight cylinder before being cross-
sectioned in with a razor blade. Each cut surface was pressed onto
a nylon membrane, which was then hybridized with a mixture of
probes specific for BMV and CCMV positive-strand viral RNAs. The
autoradiograms show representative Northern blot analysis of
viral RNAs in inoculated leaves (i1 and i2), and uninoculated upper
leaves (l to 7). Mock, RNA from leaves inoculated with buffer only.
3' ends of all BMV and CCMV positive-strand RNAs and for the positive strands of the BMV and CCMV 3a genes. The results confirmed that all four RNAs of the hybrids are packaged into virions and that the 3a gene sequence is maintained in the progeny RNA (Fig. 4). As in protoplasts (Fig. 2), progeny from each hybrid virus infection contained a lower level of RNA3 relative to RNA1+2 than did that from wt virus infection.

**Failure of hybrid viruses to systemically infect barley and cowpea.** Barley and cowpea are selective hosts for systemic infection by BMV and CCMV, respectively (13, 29). To investigate whether the 3a gene contributes to host specificity, barley and cowpea were inoculated with the 3a-exchange hybrids and were tested for systemic infection (Fig. 5).

Of 32 barley plants inoculated with B1+B2+B3(C3a) in five separate experiments, none displayed systemic infection, i.e., no viral RNA signal was detected in uninoculated leaves even after prolonged exposure (Fig. 5A). An extremely weak signal was detected in barley leaves inoculated with B1+B2+B3(C3), but the level of the signal was consistently at least 10^4 times weaker than that for wt BMV infection (Fig. 5A; note the 500-fold reduced exposure for the wt B3 samples). Similarly, inoculation with B1+B2+B3(A3a) or with C1+C2 in combination with C3, C3(A3a), or C3(B3a) failed to produce systemic infection and, in inoculated leaves, yielded only weak hybridization signals similar to those of B1+B2+B3(C3a) (Fig. 5A). The magnitude of the difference in signal between leaves inoculated with B1+B2+B3 and those inoculated with B1+B2+B3(C3a) and the lack of a significant difference in the signals in leaves inoculated with B1+B2+B3(C3a) or B1+B2+B3(A3a) suggest that B3(C3a) supported little or no cell-to-cell movement in inoculated barley leaves.

Of 42 cowpea plants inoculated with C1+C2+C3(B3a), four developed systemic infections due to the appearance of pseudorevertant mutants, as shown in the next section. In the remaining 38 plants, no viral RNA was detected in uninoculated leaves (Fig. 5B). Clear hybridization signals, weaker and more localized than that for wt CCMV infection, were detected in the tissue prints from both C1+C2+C3 (B3a)- and B1+B2+B3(C3a)-inoculated leaves (Fig. 5B). No significant or reproducible signal was detected in tissue prints of cowpea primary leaves inoculated with the 3a deletion mutants or with B1+B2+B3 (Fig. 5B).

To examine the level and distribution of wt CCMV, wt BMV, and the hybrids C1+C2+C3(B3a) and B1+B2+B3(C3a) in inoculated cowpea primary leaves, we applied the
FIG. 6. Time course and distribution of viral RNA accumulation in cowpea leaves inoculated with wt or hybrid viruses. Leaves were inoculated with in vitro transcripts from cDNA clones of the indicated RNA3 derivatives (Fig. 1), together with transcripts from cDNA clones of RNA1 and RNA2 of CCMV (C1+C2) or BMV (B1+B2), and were harvested at 2, 4, 6, and 10 dpi, as indicated at the left. Viral RNA in inoculated primary half leaves was visualized by a press-blot hybridization procedure (33; Materials and Methods). Press blotting of each half leaf was done at the time of harvesting and, after being baked to fix viral RNA, the resulting nylon membranes were stored for subsequent hybridization with a mixture of probes specific for BMV and CCMV positive-strand viral RNAs.

press-blotting technique (33). This technique involves the use of high pressure to transfer viral RNA from intact leaves to a membrane, which is then analyzed by hybridization. Inoculated leaves were tested at different intervals after inoculation for the presence of viral RNA. When cowpea leaves were inoculated with wt CCMV, viral RNA signals were first detected as a set of discrete spots that were still highly localized at 2 days postinoculation (dpi) (Fig. 6). By 4 dpi, viral RNA was detectable over 50% or more of the leaf area. At this stage, viral RNA signals were seen extending along the full length of many vascular channels, often flanked by as yet uninfected interveinal tissue (Fig. 6). By 6 and 10 dpi, viral RNA was detectable throughout virtually the entire leaf (Fig. 6). In contrast, inoculation with wt BMV gave little or no detectable signal in the inoculated leaves, whereas inoculation with C1+C2+C3(B3a) or B1+B2+B3 (C3a) gave localized signals in the inoculated leaves from 4 to 10 dpi (Fig. 6).

In addition to the above results, B1+B2+C3(B3a) and C1+C2+B3(C3a) were each inoculated onto both barley and cowpea plants. When analyzed by tissue printing tests equivalent to those described in the legend to Fig. 5, none of these combinations showed systemic infection (data not shown) and the signals in inoculated barley or cowpea leaves were equivalent to those of inoculations with B1+B2+B3 (Δ3a) or C1+C2+C3(Δ3a) (Fig. 5).

Recovery of pseudorevertants after C1+C2+C3(B3a) inoculation. As noted above, 4 of 42 cowpea plants inoculated with C1+C2+C3(B3a) showed systemic infection, using the tissue printing assay as described in the legend to Fig. 5. In inoculated leaves, no differences were observed among the hybridization signals of viral RNA from these 4 plants and those from the other 38 plants (data not shown). However, in uninoculated upper leaves, the level of viral RNA signals from these four plants was similar to those from wt CCMV infection, except that the first uninoculated trifoliate leaf escaped virus infection in the plant from which the C3 (B3a)-7 isolate (Fig. 7) was obtained (data not shown). To investigate whether these systemic infections might have been caused by secondary mutation(s) of the hybrid virus, a Northern blot analysis of rare pseudorevertant virion RNAs (see main text) recovered at 2 weeks after inoculation from uninoculated trifoliate leaves of certain cowpea plants inoculated with in vitro transcripts from cDNA clones of wt CCMV RNAs 1 and 2 and hybrid C3(B3a). The four pseudorevertant virion samples shown arose from independent plants and are designated C3(B3a)-7, -9, -25, or -36. For comparison, an equivalent virion RNA sample from a cowpea plant inoculated with transcripts of wt CCMV RNAs 1 and 2 and wt CCMV RNA3 (C3) is shown at the left of each blot. Samples from equivalent amounts of tissue (3.0 mg) were glyoxylated before electrophoresis, and the final membranes were hybridized with 3' probe to detect all four positive-strand RNAs of CCMV (CCMV-3') or with probe to detect the 3a gene sequence of CCMV (C3a) or BMV (B3a). The positions of virion RNAs are indicated at the left.
virus was isolated from un inoculated upper leaves for reinoculation tests. As a control, virus was isolated from the inoculated leaves of a cowpea plant that had been inoculated with C1+C2+C3(B3a) and had not shown systemic infection. Each of the four systemic virion isolates and the control virion sample were diluted to a concentration of 5, 25, or 50 μg/ml, and three cowpea plants were inoculated with each dilution. The resulting 45 cowpea plants were tested by tissue printing of inoculated and uninoculated leaves 3 weeks after inoculation. All 36 plants inoculated with the four possible pseudorevertant isolates became systemically infected, confirming their pseudorevertant nature (data not shown). By contrast, only the third trifoliate leaf of one plant inoculated with 50 μg of the control C1+C2+C3(B3a) virion preparation per ml became infected, presumably by another independent pseudorevertant.

To examine the nature of the progeny virion RNA in the four original pseudorevertant-yielding plants, viral RNA was extracted from each virion preparation and subjected to Northern blot analysis (Fig. 7). Hybridization with a probe complementary to the conserved 3′ end of all CCMV positive-strand RNAs showed that, for all four pseudorevertants, the RNA3 level relative to RNA1+2 was much lower than that for wt RNA3 (Fig. 7, left panel). Hybridization with probes specific for the positive-strand of the BMV or CCMV 3a gene ruled out possible wt CCMV contamination (Fig. 7, center panel) and confirmed that the B3a gene sequence is maintained in the progeny viral RNA (Fig. 7, right panel). When the levels of RNA3 or RNA4 were compared, all four pseudorevertants were distinguishable from each other and from wt CCMV (Fig. 7, left and right panels), suggesting that the isolates are not identical. In addition, careful comparisons showed that RNA3 of isolate C3(B3a)-25 migrated slightly slower than the other RNA3 species in electrophoresis (Fig. 7, left and right panels and other blots). Similar results were obtained when total RNA extracted from the uninoculated upper leaves of the original pseudorevertant-yielding plants was analyzed (data not shown). Two relatively strong lower-molecular-weight bands were seen with the B3a probe (Fig. 7, right panel). These may be degradation products related to the low accumulation level of the hybrid RNA3.

DISCUSSION

3a movement protein genes contribute to bromovirus host specificity. To examine the possible contribution of the bromovirus 3a cell-to-cell movement gene to host specificity, we have precisely exchanged the 3a genes between the closely related BMV and CCMV (6). These hybrids, but not 3a deletion mutants, systemically infected N. benthamiana, a common host for both parental viruses. This result and the analysis of virion RNAs from uninoculated leaves revealed that both BMV and CCMV require the 3a gene to infect this species and show that the hybrids retain at least the basic functions required for replication, packaging, cell-to-cell spread, and long-range (vascular) infection spread.

The four possible combinations of the two hybrids with B1+B2 or C1+C2 were tested in both barley and cowpea, the selective hosts for the parental viruses. No combination showed systemic infection of barley or cowpea except for C1+C2+C3(B3a) in cowpea, for which pseudorevertants were obtained from approximately 10% of the inoculated plants. In contrast, although sunn-hemp mosaic tobamovirus (SHMV) and CCMV differ in basic morphology and basic genetic organization and their movement proteins have no significant sequence similarity (6), a hybrid constructed by transferring the movement protein gene from SHMV to CCMV showed systemic infection in cowpea, a common host for both parental viruses (14). Thus, cell-to-cell movement genes are functionally exchangeable between a bromovirus and a highly divergent tobamovirus adapted to the same host but not between two bromoviruses adapted to different hosts. These results indicate that movement protein genes make a significant contribution to bromovirus host specificity and systemic infection.

Press-blotting results (Fig. 6) show that the initial spread of C1+C2+C3(B3a) infections was seriously impaired even in directly inoculated cowpea leaves, indicating that host-specific adaptation of viral movement protein is needed to achieve even effective local spread in inoculated tissues. Consistent with this finding, the level of B1+B2+B3(C3a) accumulation in inoculated cowpea leaves was higher than that of wt BMV (Fig. 6), suggesting that the cowpea-adapted C3a gene supports better local movement of BMV in cowpea than does the barley-adapted B3a gene. To determine the precise stage at which C1+C2+C3(B3a) or B1+B2+B3(C3a) infection spread is arrested in inoculated tissues, further analysis is now being conducted with higher-resolution localization techniques to detect infection at the single cell level.

To test the possibility that the failure of systemic infection by the 3a-exchange hybrids might result from incompatibility between 3a genes and RNAs 1+2, C1+C2+B3(C3a) and B1+B2+C3(B3a) were inoculated onto barley and cowpea leaves, respectively. However, neither produced systemic infection, showing that exchanging RNAs 1+2 does not overcome the misadaptation of C3a and B3a to barley and cowpea, respectively. These results are consistent with the systemic competence of the above-mentioned CCMV-BMV movement gene hybrid, which implies that the movement protein functions independently of sequence-specific interaction with other viral components or sequences (14).

Taken together, the results show that the 3a gene contributes a host-specific function to the spread of bromovirus infection in inoculated leaves, but this contribution is not sufficient to achieve full adaptation for systemic infection of barley or cowpea. This is consistent with previously reported RNA reassortment experiments, which show that host specificity determinants are also carried by RNA1 and/or RNA2, which encode the viral RNA replication genes (5). Host-specific effects in these genes might be explained by a model in which full systemic infection of a plant involved a race between the rates of viral replication and movement and the rate of plant growth (12).

Similarly, the patterns of BMV and CCMV systemic spread in N. benthamiana differed from each other and were not affected by the exchange of the 3a movement protein genes, suggesting that a factor(s) other than the 3a gene influences bromovirus distribution in this plant. These other factors might be encoded by RNAs 1 and 2, as noted above, or by other portions of RNA3, as noted below.

Possible role of RNA3 noncoding sequences in host specificity. A side result of these studies was the observation that B1+B2+C3(B3a) and C1+C2+B3(C3a) failed to systemically infect barley and cowpea, respectively. Other recent experiments in our laboratory show that the coat protein genes of RNA3 (Fig. 1) can be exchanged between BMV and CCMV without altering the ability of these viruses to systemically infect barley and cowpea, respectively (7). Together, these two sets of results suggest that the BMV and CCMV RNA3 noncoding sequences, as well as the 3a genes,
contain some host-specific features required for systemic infection of barley and cowpea. The possible contribution of RNA3 noncoding sequences to host specificity may result from interactions between the noncoding sequences and viral or host factors involved in replication or translation.

Substantially reduced RNA3 levels can support systemic infection. In some cases, the failure of bromovirus derivatives to spread systemically in plants has been accompanied by decreased accumulation of RNA3 (9, 17). However, recent results show that the level of RNA3 accumulation can be substantially reduced without preventing systemic infection. First, a CCMV RNA3 derivative in which the 3a gene was replaced with the SHMV movement gene accumulated at least 10- to 20-fold less than wt C3, but was still able to spread systemically (14). Second, the results in N. benthamiana show that the two hybrid viruses in which the 3a genes were exchanged between BMV and CCMV do spread systemically even with low amounts of RNA3 in progeny virus (Fig. 4). Finally, the reduced level of C3(B3a) accumulation (Fig. 2B) remained unchanged in three of four pseudorevertants derived from C1+C2+C3(B3a) and was even reduced further in the remaining pseudorevertant C3(B3a)-25 (Fig. 7). For these reasons, the failure of the 3a-exchange hybrids to spread in barley and cowpea cannot be explained solely because the accumulation of the hybrid RNA3s in protoplasts was three- to fourfold lower than that of wt RNA3 (Fig. 2).

Pseudorevertants. Serial transmission experiments showed that the four progeny viruses recovered from cowpea leaves inoculated with C1+C2+C3(B3a) are pseudorevertants. RNA3 of isolate C3(B3a)-25 showed slower electrophoretic migration than the RNA3 from the other three isolates, suggesting that recombination may have been involved in its generation. In addition, differences in RNA3 and RNA4 levels show that the progeny RNA3s in all four pseudorevertant isolates are all genetically distinct and must differ at least by one or more base substitutions. The high average substitution rates (10^{-3} to 10^{-2} per base copied) estimated for replication by most RNA viruses (22, 40, 41), the large virus population of each CCMV-infected cell (more than 10^9 to 10^{10}), and the multiple primary infection sites in inoculated leaves (estimated at 10^9 to 10^7 per leaf from Fig. 6) all favor the appearance of mutations. Even so, the selection of pseudorevertants in as many as 10% of cowpea plants inoculated with C1+C2+C3(B3a) suggests that the number of changes necessary to achieve systemic infection is probably not large. Accordingly, although the BMV and CCMV 3a proteins differ at 48% of their amino acids (6), the residues differentiating their host specificity may be relatively few or confined to specific domains. Consistent with this, several nucleotide changes inducing amino acid changes in the B3a gene have been shown to direct BMV to systemically infect cowpea line TVu-612 (13, 15).

In recent additional tests, 3% of barley plants inoculated with B1+B2+B3(C3a) have become systemically infected, apparently by pseudorevertants resembling those derived from C1+C2+C3(B3a) in cowpea. Further characterization of both types of pseudorevertants will be presented elsewhere.

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