Novel Naturally Occurring *Bean pod mottle virus* Reassortants with Mixed Heterologous RNA1 Genomes

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ABSTRACT

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The comovirus *Bean pod mottle virus* (BPMV) is widespread in the soybean-growing regions in the United States. It has a bipartite genome consisting of RNA1 and RNA2, which are encapsidated separately. We previously have reported the occurrence in nature of two distinct subgroups of BPMV strains (subgroups I and II), as well as reassortants between the two subgroups. Here, we report the isolation and molecular characterization of naturally occurring partial diploid reassortant strains, which are diploid for RNA1 and haploid for RNA2. Whereas the RNA1s of the partial diploids are derived from two distinct strain subgroups (I

and II), the RNA2 is derived from either subgroup I or II. The partial diploid strains induced strikingly severe symptoms on soybean, which could be explained based on the presence of two distinct RNA1s in the same plant. This conclusion was supported by the finding that pseudo-recombinants constructed with two diverse RNA1s induced very severe symptoms on soybean that mimicked those produced by the naturally occurring partial diploids. No enhancement of symptom severity was observed with pseudorecombinants constructed with closely related RNA1s. Likewise, no enhancement of symptom severity was noted with pseudo-recombinants that are diploid for RNA2 and haploid for RNA1. The potential role of genetic reassortment in the epidemiology and pathogenesis of BPMV is discussed.

Viruses with multipartite RNA genomes are able to reassort their genome segments in either their hosts or insect vectors as a consequence of mixed infections. Genetic reassortment has been reported for viruses belonging to several families of multipartite RNA viruses, including the families *Bunyaviridae*, *Bromoviridae*, *Arenaviridae*, *Potyviridae* (genus *Bymovirus*), *Reoviridae*, and *Orthomyxoviridae* (2,9,10,18,21,27,32). Genetic reassortment plays an important role in the evolution, pathogenesis, and epidemiology of many of these segmented viruses.

Partial diploid reassortment, which arises as a consequence of dual infection, refers to the genotype of the virus progeny that is diploid for one or more genome segments and haploid for the other genome segments. The term “diploid” refers to the genotype of the virus, not to the individual virions. This distinction is important for viruses that separately encapsidate their genome segments. The phenomenon of diploid reassortment has been observed with both plant and animal viruses in the laboratory under experimental conditions (9,21,30). In most cases, viruses with partial diploid genomes are transient and evolve into either the parental genotypes or genome reassortants (21,30). It is not clear whether genetic reassortment in natural populations also results in the emergence of partial diploid reassortants.

Bean pod mottle virus (BPMV), a member of the genus *Comovirus* in the family *Comoviridae*, has a bipartite positive-strand RNA genome consisting of RNA1 (≈6.0 kb) and RNA2 (≈3.6 kb), which are encapsidated separately in isometric particles 28 nm in diameter. Both genomic RNAs are polyadenylated and have a small basic protein, VPg, covalently linked to their 5' termini (24). The BPMV genome is expressed via the synthesis and sub-

sequent cleavage of large polyprotein precursors (11). Based on comparison with CPMV (8,25), BPMV RNA1 codes for five mature proteins required for replication (from 5' to 3'), a protease cofactor (Co-pro), a putative helicase (Hel), a viral genome-linked protein (VPg), a protease (Pro), and a putative RNA-dependent RNA polymerase (RdRp), whereas RNA2 codes for a replication co-factor (RC), putative cell-to-cell movement protein (MP), and the two coat proteins (L-CP and S-CP).

BPMV is widespread in the major soybean-growing areas in many of the southern and southeastern states. BPMV strain K-G7 was the first strain to be well characterized biologically, structurally, and at the molecular level (7,8,12,13,17,25). Therefore, it is regarded as the type strain of BPMV. To facilitate the classification of BPMV isolates based on nucleic acid hybridization analysis, strain K-G7 previously was designated as the prototype of hybridization subgroup I (15). Strain K-Ha1, which does not hybridize with the K-G7-cloned cDNA probes, was designated as the prototype of a second hybridization subgroup (subgroup II). The complete nucleotide sequences of the two genomic RNAs of BPMV strains K-G7 and K-Ha1 have been reported (8,17,25). The values for percentage of nucleotide sequence identity between strains K-G7 and K-Ha1 are 83.6 and 84.4% for RNA1 and RNA2, respectively. Thus, the BPMV strain subgroups I and II can be distinguished clearly based on nucleic acid hybridization and nucleotide sequencing analyses (15).

A recent severe outbreak in BPMV incidence in the north central and northern Great Plains states had serious impact on the soybean industry in this region (14). Concurrent with the increased incidence of BPMV has been an increase in disease symptom severity and the emergence of apparently new and unusual severe strains. Molecular characterization of such severe BPMV isolates revealed that they are reassortants between different strains belonging to subgroups I and II (15, this study). In the present study, novel, naturally occurring, partial diploid reassortant strains of BPMV, which induce markedly severe symptoms on soybean, were isolated and characterized. The possible mecha-

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nism or mechanisms underlying the generation of partial diploid reassortant strains and their potential role in the epidemiology and pathogenesis of BPMV is discussed.

MATERIALS AND METHODS

Virus isolates. BPMV strains K-G7 and K-Ha1 have been described previously and their complete nucleotide sequences have been reported (8,17,25). Strains K-G7 and K-Ha1 induce moderate and mild symptoms on soybean, respectively (15). The reassortant strain K-Ho1 was described in a previous study and the nucleotide sequences of its genomic RNAs were determined (17). The reassortant strain IL-Cb1 was collected from a soybean field in Carbondale, IL and recently was characterized at the molecular level (17,33; C. Zhang, H. Gu, and S. Ghabrial, *unpublished data*). Strains K-Ho1 and IL-Cb1 induce very severe symptoms on soybean. The BPMV strains, which initially were subjected to three serial single-lesion transfers on *Phaseolus vulgaris* 'Pinto' were propagated in the soybean cv. Essex and infected tissues were used for virion purification, as previously described (12).

RNA extraction and nucleic acid hybridization analysis. Procedures for total RNA extraction from infected plants, slot blot, and Northern hybridization analyses and for preparation of radiolabeled probes were described previously (15,17). The youngest fully expanded systemically infected trifoliolate leaf was harvested 10 days postinoculation (dpi) of the primary leaves and used for total RNA isolation. In all, 10 µg of total RNA, as estimated by spectrophotometry, was used per slot. Levels of total RNA loading also were assessed by agarose gel electrophoresis of aliquots from the total RNA samples (1.0 µl) and ethidium bromide staining of ribosomal RNA. Following hybridization, the slot or Northern blots were exposed to a phosphorimager screen and the images were visualized with a PhosphorImager 445 SI system and analyzed with the ImageQuant 4.1 program (Molecular Dynamics, Sunnyvale, CA).

cDNA cloning and sequencing. Reverse-transcription polymerase chain reaction (RT-PCR) was used for cloning subgroup II RNA1 cDNA from BPMV strain K-Ho1. BPMV RNA1-specific primer R1-Rev1 (5'-TTTATATTAAACACACTCATTGCACAT-AG-3') was used for first-strand cDNA synthesis with SuperScript II (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The BPMV subgroup II RNA1-specific primer pair, Han-R1-For5 (5'-ATTTCAAGTGCTCCATACC-3') and Han-R1-Rev4 (5'-TGTTCTGTGCCACCAAC-3'), was used for subgroup II RNA1 sequence amplification. Following PCR, the product was cloned into the pGEM-T easy vector (Promega Corp., Madison, WI). Sequencing was done using a Big Dye Terminator DNA Sequencing Kit (Applied Biosystems, Foster City, CA) and the ABI Prism 310 genetic analyzer. Sequence analysis was performed using the DNA strider (CEA, France) and Vector NTI programs (Informax Inc., Frederick, MD).

RT-PCR. RT-PCR was used to amplify strain-specific fragments from full-length cDNA clones of Ho1-RNA1 (subgroup I) and G7-RNA1 (subgroup I). Strain-specific primers for subgroup I RNA1 from strains K-Ho1 and K-G7 were designed based on examination of sequence alignments. The forward primers specific for strains K-G7 and K-Ho1, which corresponded to nucleotide positions 254 to 281, were designated G7-spec-F (5'-CTCTTACCTCTCTTTCTC-3') and Ho1-Spec-F (5'-CTCCTATCTTTCTTTCTC-3'), respectively. The reverse primer specific for G7-RNA1, which is complementary to nucleotide positions 3,518 to 3,547, was designated G7-spec-R (5'-ATTCTGGGATAAT-ATTGT-3'). The reverse primer specific for Ho1 RNA1, which is complementary to nucleotide positions 3,519 to 3,547, was designated Ho1-Spec-R (5'-CATTCTGGGTAACTGTC-3'). RT-PCR was performed using the SuperScript one-step RT-PCR system with platinum *Taq* DNA polymerase (Invitrogen). Samples of total

RNA from systemically infected soybean leaves 14 to 17 dpi were used as templates for RT-PCR.

Plant growth conditions and symptom documentation. Soybean plants (cv. Essex) were kept in a greenhouse with 16 h of light and 8 h of dark. Disease symptoms were photographed with a digital camera (Olympus C-2500L).

RESULTS

Identification and characterization of BPMV partial diploid strains. Previous nucleic acid hybridization results suggested that the wild-type strain K-Ho1 was a reassortant strain and that its genomic RNA1 and RNA2 were derived from subgroup I and II strains, respectively (15). This later was confirmed by cDNA cloning and sequencing of viral RNAs derived from strain K-Ho1 (17). For brevity, the designation of subgroup affiliation (I or II) of the viral RNAs will be indicated in parenthesis immediately next to the individual RNAs. Although strain K-Ho1 induced very severe symptoms on soybean, inoculation of soybean plants with transcripts derived from full-length cDNA clones of Ho1-RNA1(I) and Ho1-RNA2(II) induced severe symptoms; however, not as severe as those induced by the wild-type strain (17). Examination of the published slot blot of strain K-Ho1 virion RNA, which was probed with a subgroup II RNA1-cDNA probe, revealed a weak hybridization signal (15), suggesting that isolate K-Ho1 may contain two distinct species of RNA1. To confirm this observation, slot blot hybridization analysis of total RNA from soybean plants previously inoculated with either strain K-Ho1 or with a transcript set consisting of Ho1-RNA1(I) + Ho1-RNA2(II) was carried out and the blots were probed with cloned cDNA probes specific for either RNA1(I) or RNA1(II). The results revealed quite clearly that total RNA derived from plants infected with strain K-Ho1 hybridized strongly with both RNA1 probes, whereas total RNA derived from plants infected with the Ho1 transcript set, Ho1-RNA1(I) + Ho1-RNA2(II), reacted only with the RNA1(I)-specific probe (Fig. 1).

To further verify the presence of RNA1(II) in strain K-Ho1, RT-PCR was performed using RNA1(II)-specific primers to amplify a 1,972-bp cDNA fragment. The nucleotide sequence of the RNA1(II) fragment was determined and deposited in the GenBank under accession number DQ139274. The nucleotide sequence of Ho1-RNA1(II) cDNA fragment was 99.7% identical with the corresponding regions from RNA1 of strain K-Ha1, the prototype of subgroup II strains, and 97.8% identical with RNA1(II) from the partial diploid strain IL-Cb1 (Table 1). These identity scores are similar to those obtained between full-length RNAs from strains in the same strain subgroup (Table 1). On the other hand, the percent nucleotide sequence identity between the Ho1-RNA1(II) cDNA fragment and the corresponding regions from known RNA1(I)s was ≈86% (Table 1). This value, likewise, is similar to those obtained between full-length RNAs from strains in different subgroups (Table 1). Therefore, based on nucleic acid hybridization and sequencing analyses, strain K-Ho1 is a partial diploid reassortant, diploid for RNA1 and haploid for RNA2.

Occurrence of BPMV partial diploid reassortant strains in soybean fields. To determine whether partial diploid reassortants occurred in nature, we examined the genotypes of several field isolates collected from soybean fields in six states (Table 2). Out of 43 field isolates examined during the period 2002 to 2004, 10 were found to be partial diploid reassortants, all of which caused very severe symptoms on soybean plants under field and greenhouse conditions, as exemplified by isolate IL-Cb1, collected from a soybean field in Carbondale, IL (Fig. 2). All 10 partial diploid reassortants (Table 2) were diploid for RNA1 and haploid for RNA2. In eight reassortants, the RNA2 was derived from a subgroup II strain, whereas the remaining two reassortants had their RNA2 derived from a subgroup I strain (Table 2). The type

II strains, which induce mild symptoms on soybean, were predominant (≈63%) among the naturally occurring isolates examined in the present study.

Northern hybridization analysis of virion RNA from partial diploid strains. The presence of two distinct types of RNA1 in the partial diploid strains K-Ho1 and IL-Cb1 was confirmed by Northern hybridization analysis (Fig. 3). Consistent with the previous results of slot blot hybridization of virion RNA (15), the Northern blots showed uneven distribution of the two types of RNA1s in virions purified from plants infected with partial diploid reassortants. Whereas subgroup I RNA1 was the predominant species in the case of strain K-Ho1, subgroup II RNA1 was the major species in infections with strain IL-Cb1. Interest-

ingly, the predominant species in each case was the heterologously encapsidated species. Thus, an explanation of the difference in the ratios of RNA1 species based on preferential encapsidation of homologous RNA1 can be ruled out. Because experiments with total RNA from plants infected with these same partial diploid strains indicated that the two types of RNA1 occur with similar frequencies (Fig. 4A and B, right columns), a more likely explanation for the differences between the two types of experiments may be related to the age of the infected tissue sampled. In experiments with total RNA, the youngest fully expanded trifoliate leaves were used; whereas, in experiments with virion RNA, all systemically infected leaves were pooled for virion purification. RNA1 is known to undergo degradation in situ in older

Fig. 1. Slot blot hybridization analysis of *Bean pod mottle virus* RNA. **A and B,** Total RNA (10 µg) from soybean plants previously inoculated with either infectious transcripts derived from cloned cDNAs of genomic RNAs from strain K-Ho1 (left column; extracts from two plants, 1 and 2, with “i” indicating inoculated leaf and “s” indicating systemic leaf), or extracts from plants infected with strain K-Ho1 (top two slots of the right columns). Control RNAs (200 ng) from purified virions of strains K-G7 (subgroup I) and K-Ha1 (subgroup II) were blotted onto the lower slots of the right columns. The blots were probed with cloned cDNA probes specific for **A**, subgroup I or **B**, subgroup II.

TABLE 1. Percent nucleotide sequence identity between *Bean pod mottle virus* strains^a

Virus strain	K-G7 (I)	IL-CB1 (I)	IL-CB1 (II)	K-Ho1 (I)	K-Ho1 (II)	K-Ha1 (II)
K-G7 (I)	...	97.9	83.1	97.9	86.8	83.6
IL-Cb1 (I)	96.6	...	85.0	99.1	86.4	85.3
IL-Cb1 (II)	NA	NA	...	82.4	97.8	98.5
K-Ho1 (I)	NA	NA	NA	...	86.4	82.8
K-Ho1 (II)	85.7	84.7	NA	NA	...	99.7
K-Ha1 (II)	84.4	83.5	NA	NA	98.8	...

^a Percent nucleotide identity of RNA1 (above diagonal) and RNA2 (below diagonal). Except for comparisons involving K-Ho1 RNA1(II), which were based on the sequence of a 1,972-bp fragment, all identity scores were based on full-length RNA sequences. Identity scores were calculated by the GAP program of the GCG package. GenBank accession numbers are as follows: K-G7 (RNA1; U70866, RNA2; M62738), IL-CB1 (RNA1(I); AY744931, RNA1(II); AY744932, RNA2; AY744933), K-Ho1 (RNA1(I); AF394608, RNA1(II); DQ139274, RNA2; AF394609), and K-Ha1 (RNA1; AF394606, RNA2; AF394607). Subgroup affiliation (I or II) of the viral RNAs is indicated in parenthesis next to the strain names. NA = not applicable; the specified type of RNA is not present in one or the other of the indicated strains.

TABLE 2. Genotypes of *Bean pod mottle virus* field isolates collected from six states^a

Location	No. of isolates tested	Genotype ^b		Symptoms ^c	Reference
		RNA1	RNA2		
Arkansas	1	II	II	M	Gu et al. (15)
Illinois	8	II	II	M	This study
	4	I + II	II	vS	This study
	2	I + II	I	vS	This study
	4	II	II	M	This study
Indiana	1	I + II	II	vS	This study
	1	I	I	Mo	Gu et al. (15)
	7	II	II	M	Gu et al. (15)
	3	I + II	II	vS	This study
Kentucky	4	I	II	S, Mo	Gu et al. (15)
	2	II	II	M	Gu et al. (15)
	1	I	I	Mo	Gu et al. (15)
	5	II	II	M	Gu et al. (15)

^a Strain subgroup affiliation (I or II) was determined by slot blot hybridization analysis.

^b Genotype classification: haploid = I or II and diploid = I + II.

^c Abbreviations: M = mild (green mottling with little or no stunting or blistering), Mo = moderate (green or yellow mottling, some stunting, leaf distortion, and blistering), S = severe (yellow mottling, stunting, and extensive blistering plus necrosis on inoculated leaves), and vS = very severe (yellow mottling and severe stunting and blistering plus local and systemic necrosis).

infections, but not in the youngest trifoliolate leaves, those that are of similar age to the ones used in our study for total RNA analysis (20,31). The reason why the heterologously encapsidated RNA1 is relatively more stable than that packaged in homologous capsids remains unclear.

Symptom induced by co-infection of soybean plants with two distinct BPMV RNA1s. It previously was established that enhancement of symptom severity maps to subgroup I RNA1 and that it is strain specific (17). Thus, it was of interest to determine whether the strikingly severe symptoms induced by the partial diploid reassortants require the presence of specific RNA1s, or whether the mere presence of two distinct RNA1s is sufficient. Partial diploid pseudorecombinants comprising RNA1(I) and RNA1(II) plus RNA2(I) or RNA2(II) were constructed from infectious transcripts derived from strains K-Ho1, K-G7, and K-Ha1. We have demonstrated previously that infection with transcripts of Ho1-RNA1(I), G7-RNA1(I), or Ha1RNA1(II), regardless of the origin of RNA2, induced severe, moderate, and mild symptoms, respectively, on soybean (17). Five sets of transcripts, designated pseudorecombinants 1 to 5 (Fig. 4, Ps-1 to Ps-5), comprised different combinations of RNA1(I), RNA1(II), and RNA2 (I or II). Sap from transcript-infected plants was used to inoculate healthy soybean plants and total RNA was extracted from systemically infected leaves 10 dpi and subjected to slot blot hybridization analysis. The results showed that both RNA1s, RNA1(I) and RNA1(II), were able to replicate in all plants inoculated with progeny from the partial diploid pseudorecombinants (Fig. 4A and B). RNA-2 from one subgroup (either I or II) also was detected (Fig. 4C and D). The presence of two genetically distinct species of RNA1 in naturally infected soybean plants is associated with enhanced symptom severity (Table 2). This phenomenon also was observed with comparable diploid RNA1 pseudorecombinants constructed with the pertinent infectious transcripts (Fig. 5). As could be predicted from the results with the diploid RNA1 pseudorecombinants (Fig. 5), co-infection of soybean with BPMV strain K-G7 (a moderate strain with subgroup I RNA1) and strain K-Ha1 (a mild strain with subgroup II RNA1) induced very severe symptoms, similar to those produced by natural and laboratory diploid RNA1 reassortants (data not shown). Although RNA2 diploid pseudorecombinants that contain one type of RNA1 and two types of RNA2 were shown to replicate in the infected plants, there was no enhancement in symptom severity. This finding is consistent with our previous results that showed

that symptom severity maps to RNA1 (17). Furthermore, these RNA2 diploids were unstable because only a single RNA2 type was detected after three serial passages (data not shown). This probably is not surprising, considering that no naturally occurring RNA2 diploids were detected previously (Table 2).

Co-infection of soybean plants with closely related RNA1s.

To determine whether infection with two RNA1s derived from strains belonging to the same subgroup also may enhance symptom severity, transcripts from Ho1-RNA1(I) and G7-RNA1(I) cDNA clones were combined with the Ho1-RNA2(II) transcript. Five plants were inoculated with the G7-RNA1(I) + Ho1-RNA1(I) + Ho1-RNA2(II) transcript set. Samples of total RNA isolated from systemically infected leaves were used as templates for RT-PCR using K-G7 and K-Ho1 RNA1-specific primers. Likewise, samples of total RNA from plants infected with the wild-type K-Ho1 and K-G7 strains were used as controls. The results indicated that two out of five plants contained both Ho1-RNA1(I) and G7-RNA1(I) (Fig. 6, lanes 1 and 3). G7-RNA1(I) was the only RNA1 detected in two other plants (Fig. 6, lanes 4 and 5), and Ho1-RNA1(I) alone was present in the remaining plant (Fig. 6, lane 2). Comparable results were obtained in two other independent experiments. Interestingly, one or the other RNA1 was lost in the subsequent three to four passages of progeny virus from plants containing both G7-RNA1(I) and Ho1-RNA1(I) (data not shown). Plants containing two RNA1s from subgroup I did not show enhancement of symptom severity and induced symptoms similar to those produced by infection with the transcript set of Ho1-RNA1(I) + Ho1-RNA2(II) (Fig. 6B, compare panels 1, 2, and 6). Infections involving two RNA1(II)s were not tested because infectious transcripts are available from only one subgroup II strain, strain K-Ha1. Considering the fact that infections involving RNA1(II) induce only mild symptoms, and determinants of symptom sever-

Fig. 2. A, Symptoms exhibited by a soybean plant infected with the partial diploid strain IL-Cb1. The plant is severely stunted. B, Younger leaves show severe blistering and distortion and C, older leaves show yellow mottling and necrosis. The photographs were taken 3 weeks postinoculation.

Fig. 3. Northern blot hybridization analysis of genomic RNA isolated from purified virions of several *Bean pod mottle virus* (BPMV) strains. The BPMV strain designations are indicated at the top of the figure. Strains K-G7 (G7) and K-Ha1 (Ha1) belong to subgroup I and subgroup II, respectively. Strains IL-Cb1 (Cb1) and K-Ho1 (Ho1) are partial diploid reassortants between the two subgroups, and t-Ho are virions purified from soybean plants previously inoculated with a transcript set derived from Ho1-RNA1(I) and Ho1-RNA2(II) cDNA constructs. A, Levels of RNA loading were assessed by ethidium bromide staining; B, probe was prepared using full-length Ho1-RNA1(I) cDNA as a template; C, probe was prepared using full-length Ha1-RNA1(II) cDNA as a template; D, probe was prepared using full-length Cb1-RNA2(I) cDNA as a template; and E, probe was prepared using full-length Ha1-RNA2(II) cDNA as a template.

ity map to RNA1(I) (17), it is unlikely that symptom enhancement would occur in infections with two RNA1(II)s.

DISCUSSION

This study documents the presence of partial diploid reassortants among field isolates of BPMV. The BPMV partial diploid reassortants were stable under greenhouse conditions because strain K-Ho1 has maintained its diploid status in soybean since 1998 by successive passages at monthly intervals via sap inoculation. Both subgroup I and II RNA1s were present in the infected greenhouse plants after at least 60 passages (Figs. 3 and 4). The partial diploid reassortants also were stable after four passages by beetle transmission (A. Kritzman and S. A. Ghabrial, unpublished data). The fact that we continued to detect naturally occurring partial diploid reassortants during the past few years with genotypes similar to those of strain K-Ho1 supports the idea that these reassortants are potentially stable in natural populations. The finding that partial diploid reassortants which induced very severe symptoms on soybean occurred at a relatively high frequency (23%) among field isolates collected from six states (this study) suggests that diploid reassortment may play an important role in the evolution, pathogenesis, and epidemiology of BPMV.

Emergence of new virus variants as a consequence of genomic reassortment has been observed in natural populations of animal and plant multipartite viruses (18,19,22,26). Contribution of reassortment to virus evolution, however, may vary. Whereas high

frequencies of reassortment have been reported for members of the family *Bunyaviridae* (2,28,30), reassortment was considered insignificant to the evolution of *Cucumber mosaic virus* (CMV) (9). In a study on the frequency and characteristics of hantavirus reassortments, Rodriguez and coworkers (30) observed that 30% of 294 progeny plaques contained more than one S or M segments derived from both parental strains after dual infection. They suggested that the viruses with partial diploid genomes were in a transient state of genetic reassortment because most of the progenies of these partial diploid viruses evolved into either the parental or reassortant genotypes. No confirmed partial diploid reassortants were detected among 217 field isolates of CMV, even though 16% of the field isolates showed evidence of mixed infections by type 1 and type 2 isolates in subgroup I (9). However, following inoculation access by aphids, which were allowed acquisition feeding on plants co-infected with CMV types 1 and 2 isolates, 1 of 64 infected plants was infected with a partial diploid reassortant of CMV (RNA1 and RNA3 from type 1 and RNA2 from both type 1 and type 2). Diploid reassortment also was demonstrated in plants inoculated with a mixture of two different isolates of *Barley mild mosaic virus* (21). The stability of these reassortants, however, was not tested.

Both the virus host and the vector could be the site for the generation of diploid reassortment for bunyaviruses (2,30). As for BPMV, the detection of partial diploid reassortants was coincident with the recent BPMV outbreak, which apparently was linked to the warm winters of the past few years that allowed the bean leaf

Fig. 4. Slot blot hybridization analysis of total RNA from soybean plants previously inoculated with different partial diploid pseudorecombinants (Ps-1 to Ps-5). **A** to **D**, Left column: Ps-1, Ho1-RNA1(I) + Ha1-RNA1(II) + Ho1-RNA2(II); Ps-2, Ho1-RNA1(I) + Ha1-RNA1(II) + G7-RNA2(I); Ps-3, G7-RNA1(I) + Ha1-RNA1(II) + G7-RNA2(I); Ps-4, Ho1-RNA1(I) + Ha1-RNA1(II) + Ha1-RNA2(II); and Ps-5, G7-RNA1(I) + Ha1-RNA1(II) + Ha1-RNA2(II). The field isolates (K-Ha1, K-G7, K-Ho1, and IL-Cb1) used to inoculate the soybean plants are indicated to the right. The blots were probed with subgroup I-specific cloned cDNA probes for **A**, RNA1 and **B**, RNA2 or subgroup II-specific probes for **C**, RNA1 and **D**, RNA2.

beetle, vector of BPMV, to overwinter and emerge in the spring in unprecedented numbers (14). Diploid reassortment may originate from mixed infections with two distinct BPMV strains because these strains may accumulate in the vector prior to feeding on healthy plants (13). With large beetle populations and concomitant high incidence of BPMV, individual beetles may have been able to accumulate more than one strain of BPMV and introduce the mixture into healthy plants. Simultaneous infection with different BPMV strains is a prerequisite for reassortment because it may not be possible to introduce different strains sequentially into the same plant via viruliferous beetles. The reason for this is that, once a soybean plant is infected with the first BPMV strain, it is cross-protected against infection with other strains (16). The finding that one of the RNA2 segments was lost in subsequent passages after plants initially were inoculated manually with an

RNA2 diploid pseudorecombinant (diploid for RNA2 but haploid for RNA1) is of interest in view of the fact that all the field isolates of partial diploid reassortants contain only one type of RNA2 (Table 2).

The question of whether the partial diploid reassortants may serve as intermediate forms leading to the generation of new reassortant genotypes with different virulence characteristics needs to be addressed. A small number of the local lesion isolates derived from the severe partial diploid strain, IL-Cb1, lost the diploid status and induced mild symptoms (33; C. Zhang, H. Gu, and S. A. Ghabrial, *unpublished data*). Because BPMV titer in viruliferous beetle declines during inoculation feeding on healthy plants (13), it is conceivable that haploid genotypes may be recovered as a consequence of inoculation feeding by viruliferous beetles containing progressively lower titers of the partial diploid reassortant.

Fig. 5. Symptoms induced by naturally occurring *Bean pod mottle virus* (BPMV) strains and laboratory pseudorecombinants. **A to D**, Systemic symptoms exhibited by soybean plants (cv. Essex) previously inoculated with various naturally occurring BPMV strains. **E**, A trifoliolate leaf from a healthy control plant. **F**, Symptoms induced by infection with the haploid pseudorecombinant consisting of Ho1-RNA1(I) plus Ho1-RNA2(II). **G to J**, Symptoms induced by infection with the partial diploid pseudorecombinants Ps-1, Ps-2, Ps-3, and Ps-5, respectively. **F to J**, Genotypes of each of the pseudorecombinants are indicated to the right of panels. In all cases, the fourth trifoliolate leaves were photographed 21 days postinoculation.

Naturally occurring partial diploid reassortants as well as comparable laboratory pseudorecombinants (i.e., containing both RNA1(I) and RNA1(II)) induced very severe symptoms when compared with those induced by haploid pseudorecombinants containing either RNA1(I) or RNA1(II). This suggested that enhancement of symptom severity is due to the presence of two distinct RNA1s in the same cells. The isolation of RNA1 recombinants from plants infected with partial diploid reassortants strongly supports the conclusion that two distinct RNA1s may replicate in the same cells (33). The mechanism underlying the apparent synergism in plants infected with two diverse RNA1s is not clear. We recently showed that the Co-pro and Hel coding regions of RNA1 were major determinants of symptom severity for subgroup I RNA1, which correlated with viral RNA accumulation (17). Previous studies with CPMV, the type species of comoviruses, indicated that CPMV Co-pro (32K protein) is involved in targeting the replication complex to the endoplasmic reticulum (ER) and that Co-pro and 60K (Hel + VPg) induce massive proliferation and vesiculation of ER (4–6). The augmentation of symptoms severity in infections involving two

distinct RNA1s may be related to an enhancement in ER proliferation and vesicle formation resulting in an increase in the total surface area accessible for virus replication. This, in turn, may account for the increase in viral RNA accumulation detected in infections with partial diploid reassortants. Such an enhancement apparently does not occur if the two RNA1s are closely related (in the same subgroup), perhaps because their gene products (Co-pro and 60K) may compete for the same sites for ER membrane binding.

Symptom severity determinants have been reported to function as suppressors of RNA silencing (1,3,29). Attempts to demonstrate RNA silencing suppression by the Co-pro, Hel, or both were unsuccessful (17). Liu et al. (23) showed that the S-CP of CPMV is a weak suppressor of RNA silencing. Sequence analysis demonstrated that the deduced amino acid sequences of S-CP from BPMV strains K-Ho1 and K-Ha1 are identical. However, the partial diploid reassortant K-Ho1 induced very severe symptoms and K-Ha1 elicited mild symptoms in infected plants. Thus, suppression of RNA silencing does not appear to play a direct role in determining symptom severity induced by BPMV strains.

Fig. 6. Reverse-transcription polymerase chain reaction (RT-PCR) analysis and symptom development on soybean plants (cv. Essex) previously inoculated with an RNA1(I) diploid pseudorecombinant. **A**, RT-PCR analysis of total RNA extracted from systemically infected leaves 10 days postinoculation (dpi) using K-G7 RNA1(I)-specific primers (left panel) or K-Ho1 RNA1(I)-specific primers (right panel). Lanes 1 to 5, total RNA from five individual plants (designated plants 1 to 5) inoculated with the transcript set, G7-RNA1(I) + Ho1-RNA1(I) + Ho1-RNA2(II); lanes 6 and 7, total RNA from plants previously inoculated with strains K-Ho1 and K-G7, respectively. **B**, Panels 1 and 2, photographs showing symptoms induced on plants 1 and 2 previously inoculated with the transcript set G7-RNA1(I) + Ho1-RNA1(I) + Ho1-RNA2(II); panels 6 and 7, photographs of plants previously inoculated with strains K-Ho1 and K-G7, respectively. A healthy plant is shown in the lower panel to the right. Photographs were taken 10 dpi.

In summary, we presented evidence for the occurrence of BPMV partial diploid reassortants in nature. Beetle transmission may intrinsically enhance the frequency of diploid reassortment in fields with high incidence of BPMV because individual beetles may accumulate more than one strain and introduce the mixture into healthy plants. If this is true, one can predict that partial diploid reassortants also may be present in natural populations of other comoviruses. The partial diploid reassortants may represent an intermediate form that leads to the emergence of new variants of haploid genomic reassortants and, thus, play an important role in the evolution and epidemiology of BPMV.

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