Use of *Spring beauty latent virus* to identify compatible interactions between bromovirus components required for virus infection

Koki Fujisaki, Masanori Kaido, Kazuyuki Mise and Tetsuro Okuno

**Correspondence**
Kazuyuki Mise
kmise@kais.kyoto-u.ac.jp

**Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan**

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_Spring beauty latent virus* (SBLV) is a member of the genus *Bromovirus*, and is closely related to *Brome mosaic virus* (BMV) and *Cowpea chlorotic mottle virus* (CCMV). Compatible interactions between viral components are required for successful infection of plants by BMV and CCMV. To further our understanding of interactions between bromovirus components, we used SBLV to produce reassortants among the three bromoviruses. We found that SBLV RNA 2 functioned with heterologous bromovirus RNA 1 in infections of whole plants and protoplasts of *Nicotiana benthamiana*, although SBLV RNA 1 did not function with heterologous bromovirus RNA 2. A DNA-based transient assay for 1a and 2a proteins, which are encoded by RNAs 1 and 2, respectively further suggested that SBLV 2a protein may function in combination with heterologous bromovirus 1a protein. Moreover, analysis of the ability of reassortants to spread locally revealed that an RNA 2-mediated interaction between viral components may be required for efficient cell-to-cell movement of bromoviruses.

**INTRODUCTION**

Systemic infection of plants by viruses is established via three steps. These are genome replication in the initially infected cells, cell-to-cell movement into neighbouring cells through intercellular channels (plasmodesmata), and long-distance movement through the vasculature of host plants. In all three steps, compatible interactions between viral components and between viral and host components are necessary.

Bromoviruses are a group of icosahedral plant viruses, the genomes of which are divided into three positive-sense tripartite RNAs, designated RNA 1, RNA 2 and RNA 3 (Lane, 1981). RNAs 1 and 2 encode the 1a and 2a proteins, respectively, which are both required for genomic RNA replication in protoplasts (Kroner et al., 1989, 1990). RNA 3, which is not necessary for viral RNA replication, encodes the 3a protein required for virus cell-to-cell movement (Mise et al., 1993; Schmitz & Rao, 1996) and the coat protein (CP) translated from a subgenomic RNA designated RNA 4 (Sacher & Ahlquist, 1989). Some interactions between bromovirus factors necessary for successful infection have been demonstrated. For instance, the 1a and 2a proteins of *Brome mosaic virus* (BMV) form a complex to achieve successful replication (Kao et al., 1992; O'Reilly et al., 1997). Moreover, BMV 1a protein must interact with the intercistronic region of BMV RNA 3 for efficient amplification of RNA 3 (French & Ahlquist, 1987; Sullivan & Ahlquist, 1999). Recent studies have identified sequences in the CP and viral RNAs that are required for bromovirus encapsidation (Choi et al., 2002; Damayanti et al., 2002). However, the interactions between bromovirus components required for movement have not been examined closely.

Reassortment is a powerful strategy with which to study the compatibility of interactions among viral components in some viruses with divided genomes, such as bromoviruses. For example, reassortants in which only RNAs 1 or 2 are exchanged between BMV and *Cowpea chlorotic mottle virus* (CCMV) do not allow virus genomes to accumulate to detectable levels in barley protoplasts, whereas reassortants in which RNA 3 is exchanged allow this accumulation (Allison et al., 1988). This observation reflects the importance of compatible combinations of RNAs 1 and 2, and therefore of the 1a and 2a proteins, for bromovirus replication.

_Spring beauty latent virus* (SBLV) is a member of the genus *Bromovirus*, together with BMV and CCMV (Valverde, 1985). SBLV is closely related to BMV and CCMV, and biologically active SBLV cDNA clones have been constructed (Fujisaki et al., 2003). To further analyse the interactions between bromovirus components required for systemic infection, we created reassortants of SBLV, BMV and CCMV and examined their infectivity in *Nicotiana benthamiana*, which is a common systemic host of the three bromoviruses. In this paper, we show that SBLV RNA 2 in combination with heterologous bromovirus RNA 1
directs systemic infection when inoculated together with bromovirus RNA 3, but that SBLV RNA 1 in combination with heterologous bromovirus RNA 2 does not. This is the first report to demonstrate that SBLV RNA 2 directs successful virus infection in combination with RNA 1 of other virus species within the family Bromoviridae. We also show that the infectivity of these reassortants in N. benthamiana plants is critically determined by the level of virus accumulation in single cells, and discuss the interactions between bromovirus components required for virus infection.

METHODS

Plasmid constructs. Full-length cDNA clones of BMV (pB1TP3, pB2TP5 and pB3TP8), CCMV (pCC1TP1, pCC2TP2 and pCC3TP4) and SBLV (pSB1TP6, pSB2TP7 and pSB3TP9) have been described previously (Janda et al., 1987; Allison et al., 1988; Fujisaki et al., 2003). The plasmids for expression of the 1a and 2a proteins of BMV (pB1PA17 and pB2PA17, respectively) and CCMV (pCC1SD3 and pCC2SD3, respectively) (Dinant et al., 1993) were obtained from P. Ahlquist (Univ. Wisconsin-Madison, USA). To construct SBLV 1a and 2a expression plasmids (pSB1KF1 and pSB2KF1, respectively), the 1a and 2a genes of SBLV were inserted downstream from the Cauliflower mosaic virus (CaMV) 35S promoter of pRT101 (Töpfer et al., 1987) as those of BMV or CCMV had been done (Dinant et al., 1993). For pSB1KF1, the fragment containing the N-terminal half of the SBLV 1a gene and a part of the 5′-non-coding region comprising the 34 bp from the initiation codon were amplified by PCR using the 5′ primer 5′-TCCCGGGTGGTTTCGAAATTTGTTCC-3′ (with a SalI site at its 5′ end) and the 3′ primer 5′-GGGTATCTGCT-ATCTC-3′. The product was digested with SalI and NdeI, and inserted into the SalI and NdeI sites of pRT101. The 0.6 kb EcoRI fragment from this construct was subcloned into the EcoRI site of pRT101 to create pSB1N. The 30-kb fragment from pSB1TP6 digested with XbaI was then ligated into the XbaI-digested 30-kb fragment of pSB1N, to create pSB1KF1. For pSB2KF1, the 2.7-kb Clal fragment from pSB2TP7 was treated with T4 DNA polymerase and inserted into the SalI site of pRT101.

Preparation of N. benthamiana plants and protoplasts, and virus inoculation. N. benthamiana plants were grown as described previously (Fujisaki et al., 2003). Plants were mechanically inoculated with transcripts (0.3 μg μl−1) from cDNA clones of BMV, CCMV or SBLV, as described previously (Fujisaki et al., 2003). Isolation of N. benthamiana protoplasts and inoculation with in vitro transcripts were performed essentially as described previously (Okuno & Furusawa, 1978; Kroner & Ahlquist, 1992). N. benthamiana protoplasts were prepared from plants at the eight-leaf stage or older. Protoplasts were prepared by incubation for 3-5 h at 25 °C in an isolation solution (0.6 M mannitol, 10 mM CaCl2, 1% cellulase (Onozuka R-10; Yakult, Tokyo, Japan) and 0.05% macerozyme (R-10; Yakult), pH 5.5). Typically, 4×106 protoplasts were inoculated with 3-0 μg transcripts (RNAs 1+2+3 in total) or a DNA inoculum consisting of 8×0 μg 1a and 2a expression plasmids, 2×0 μg RNA 3 transcripts and 2 μg salmon sperm DNA (Clontech) as carrier.

RNA analysis. A tissue printing assay of whole plants was performed as described previously (Mise et al., 1993). In protoplast experiments, total RNA was extracted as described (Kroner & Ahlquist, 1992) from infected protoplasts at 24 h post-inoculation (p.i.). Northern blot analysis of total RNA was performed as described previously (Damayanti et al., 1999). Positive-strand RNAs of BMV and CCMV were detected using digoxigenin (DIG)-labelled SP6 transcripts from HindIII-linearized pBSPL10 (Kaido et al., 1995) and T3 transcripts from EcoRI-linearized pCC3RA518 (Allison et al., 1990), respectively. To construct pSB1MC501, pSB2MC502 and pSB3MS503 for the detection of SBLV RNA 1, RNA 2 and RNA 3, respectively, the 0.7 kb Clal/PstI fragments of pSB1TP6 and pSB2TP7, and the 0.7 kb SalI/PstI fragment of pSB3TP9, respectively, were cloned into pBluescript II KS(−) (Stratagene). These cDNA fragments correspond to the 3′-terminal regions of the genomic RNAs. Positive-strand SBLV RNAs 1, 2 and 3 were detected using DIG-labelled T7 transcripts from Clal-linearized pSB1MC501 and pSB2MC502 and SalI-linearized pSB3MS503, respectively. Viral RNAs were detected with anti-DIG AP Fab fragment (Roche Molecular Biochemicals) and CDP-star substrate (New England Biolabs) as described (Sasaki et al., 2001). Membranes obtained were exposed to X-ray films (Fuji Photo Film) and the image of viral RNA accumulation was densitometrically analysed with the NIH Image program version 1.61 (National Institutes of Health, USA). In each quantification analysis, samples containing known amounts of transcripts from the three bromoviruses (BMV RNA 3, CCMV RNA 3 and SBLV RNAs 1, 2 and 3) were included, and the signal intensities of the samples were measured to compare the specific activity of each probe. Minor data corrections were made as appropriate, to allow proper comparisons of the various reassortant signals.

Protein analysis. Press-blot analysis of the distribution of bromovirus CPs in N. benthamiana was performed as described previously (Takahashi et al., 2001). Briefly, inoculated leaves were hammered between two pieces of MMM paper. Residual green colour was removed by rinsing in 2% Triton X-100 prior to blocking with skim milk and immunodetection. Infected protoplasts were disrupted in Laemmli sample buffer (Laemmli, 1970) and subjected to SDS-PAGE. Immunoblot analysis was carried out as described previously (Damayanti et al., 1999), using an Immobilon-P transfer membrane (Millipore). A mouse anti-CCMV 3a protein monoclonal antibody (Sasaki et al., 2003) was used to detect CCMV 3a protein. Moreover, because anti-BMV antiserum cross-reacts with CCMV and SBLV CPs (Fujisaki et al., 2003), CCMV and SBLV CPs as well as BMV CP were detected using a rabbit anti-BMV antiserum (ATCC PVAS-718). The accumulation of CCMV 3a protein and CP was densitometrically quantified using the NIH Image program.

RESULTS

Infectivity of reassortants from three bromoviruses in N. benthamiana

To analyse the interactions of viral components, we created reassortants from SBLV and the related bromoviruses, BMV and CCMV, for which tripartite genomic RNAs were used in the construction of non-infectious reassortants, a mixture of five probes was used (refer to the next section for details). Although BMV, CCMV and SBLV systemically and efficiently infected N. benthamiana, some of the reassortants (S1B2B3, S1B2S3, S1C2C3, S1C2S3, B1C2S3, B1B2C3 and S1C2B3) were not detected in either inoculated or uninoculated upper leaves (data not shown). All these non-infectious reassortants had RNAs 1 and 2 that were derived from different virus species.
species. The strict requirement for homologous combinations of RNAs 1 and 2 for successful bromovirus replication has been demonstrated in experiments in which all heterologous combinations of RNAs 1 and 2 between BMV and CCMV failed to support detectable viral RNA accumulation in barley protoplasts (Allison et al., 1988). Consistent with this, the inability of these eight reassortants to infect *N. benthamiana* may have been due to incompatibility between heterologous RNAs 1 and 2. Interestingly, some other reassortants (B1S2B3, B1S2S3, C1S2C3, C1S2S3, B1S2C3 and C1S2B3) were infectious to *N. benthamiana*, even though these also contained heterologous combinations of RNAs 1 and 2 (Fig. 1, data not shown). Four of these (B1S2B3, B1S2S3, C1S2S3 and C1S2B3) infected poorly (Fig. 1, data not shown), and the other two (C1S2C3 and B1S2C3) infected efficiently (Fig. 1). These infectious reassortants containing heterologous RNAs 1 and 2 all contained the S2 component, indicating that SBLV RNA 2 supports virus infection in combination with heterologous BMV or CCMV RNA 1.

In contrast to the reassortants containing heterologous RNAs 1 and 2, all reassortants with homologous combinations of RNAs 1 and 2 were infectious. However, although systemic infectivity of the reassortants containing S1S2 (S1S2B3 and S1S2C3) was similar to that of wild-type combinations (B1B2B3, C1C2C3 and S1S2S3), the infectivity of the two reassortants containing B1B2 or C1C2 (B1B2S3 and C1C2S3) was low (Fig. 1).

**Replication assay of reassortants in *N. benthamiana* protoplasts**

To elucidate the mechanism determining the infectivity of the reassortants, protoplasts of *N. benthamiana* were inoculated with the reassortants, and the accumulation of viral RNAs was examined by Northern blot analysis. To estimate viral RNA accumulation, probes each recognizing the conserved 3′-terminal sequences of BMV RNAs (Kaido et al., 1995) or those of CCMV RNAs (Allison et al., 1990) were used. Moreover, three individual probes recognizing the 3′-terminal sequences of SBLV RNAs 1, 2 and 3 were prepared to detect SBLV RNAs because the similarity of the 3′-terminal sequences of SBLV RNAs 1, 2 and 3 was too low (Fujisaki et al., 2003) to cross-hybridize with a single probe in a preliminary experiment (data not shown). Therefore, a mixture of four or five probes was used to estimate viral RNA accumulation in reassortants.

![Fig. 1. Representative tissue print assays for systemic infection of *N. benthamiana* plants after inoculation with the transcripts from cDNA clones of wt BMV, CCMV, SBLV, or the reassortants created from them. Inoculated leaves (I) and uninoculated upper leaves (U1 and U2) were harvested at 14 days p.i. Leaf positions are shown at the right. Two leaves each of I (i1 + i2), U1 (1 + 2) or U2 (3 + 4) were gathered, rolled and pressed onto a nylon membrane. Viral RNAs were detected using a mixture of five DIG-labelled probes that recognize the 3′-terminal sequences of BMV, CCMV or SBLV RNAs. Only reassortants whose infection was detected are shown. Combinations of BMV [B], CCMV [C] or SBLV [S] transcripts for inoculation are indicated above, and infectivity (no. of systemically infected plants/no. of plants infected in inoculated leaves/no. of plants tested) are indicated below.](http://vir.sgmjournals.org)
constructed from SBLV and other bromoviruses, and equimolar amounts of B3, C3, S1, S2 and S3 were used as controls for the specific activities of these five probes. Consistent results were obtained from at least five independent experiments and representative data are shown in Fig. 2.

In reassortments between BMV and SBLV, all combinations containing RNAs 1 and 2 derived from one virus species (i.e. B1B2B3, B1B2S3, S1S2S3 and S1S2B3) accumulated to detectable levels (Fig. 2a). Of the four reassortants with heterologous combinations of RNAs 1 and 2, the viral RNAs of the two reassortants containing S2 (B1S2B3 and B1S2S3) accumulated to detectable levels. However, after inoculation with the other two (S1B2B3 and S1B2S3), which were non-infectious to N. benthamiana plants, no viral RNA was detected, demonstrating that S1 in combination with B2 did not direct viral RNA accumulation even at the single-cell level. Similar results were obtained with reassortants between SBLV and CCMV (Fig. 2b) and those in which RNAs 1, 2 and 3 were heterologous for all three viruses (Fig. 2c). These data demonstrate that S2 characteristically functions with heterologous RNA 1 to facilitate the accumulation of viral RNAs to detectable levels in reassortants constructed from all three bromoviruses.

Although the S2 component directed viral RNA accumulation when inoculated with B1 or C1, the levels of total viral RNA accumulated in plants inoculated with B1S2B3 or B1S2S3, both of which inefficiently infected N. benthamiana plants (Fig. 1), were about 10-fold lower than levels detected during wild-type (wt) SBLV infection (Fig. 2a). Because equimolar amounts of transcripts loaded as controls showed similar signal intensities (Fig. 2), the specific activity of each probe seemed to be comparable. Therefore the band intensities of viral RNAs after infection with each reassortant directly reflected the level of viral RNA accumulation. Thus, these data indicate that the B1S2 combination functioned in viral RNA accumulation, but was inefficient compared with wild-type viruses. This suggests that the low infectivity of the reassortants containing the B1S2 combination (Fig. 1) was caused by the low-level accumulation of viral RNAs in single cells. Total viral RNA accumulation after inoculation with C1S2C3 or C1S2S3 was around half to two-thirds the level seen during wt

Fig. 2. Northern blot analysis of viral RNA accumulation of reassortant genomes in N. benthamiana protoplasts. Protoplasts were inoculated with reassortants constructed from BMV and SBLV (a), or from CCMV and SBLV (b), or with reassortants in which RNAs 1, 2 and 3 were heterologous for all three viruses. (c) Total RNA was extracted from infected protoplasts 24 h.p.i., separated by electrophoresis on a 1·5 % agarose gel, transferred to a nylon membrane, and hybridized with DIG-labelled probes. Combinations of BMV [B], CCMV [C] or SBLV [S] transcripts for inoculation are indicated above each lane, and viral RNA positions are indicated at the left. The blots were hybridized with a mixture of four probes that recognize the 3′-terminal sequences of BMV or SBLV RNAs in (a), with a mixture of four probes that recognize the 3′-terminal sequences of CCMV or SBLV RNAs in (b), and with a mixture of five probes that recognize the 3′-terminal sequences of BMV, CCMV or SBLV RNAs in (c). Equimolar amounts (0·1 pmol each) of transcripts of RNAs 1 and 2 of SBLV (S1 and S2) and RNA 3 of BMV, CCMV and SBLV (B3, C3 and S3) were loaded as controls, and are indicated at the right of each panel. Ethidium bromide-stained ribosomal RNA from the same volume of each sample is shown below each lane.
CCMV and SBLV infections (Fig. 2b), which far exceeds the accumulation observed with B1S2B3 and B1S2S3. This indicates that the C1S2 combination was more compatible than the B1S2 combination, and facilitated more efficient virus multiplication in N. benthamiana protoplasts.

In this assay, all reassortants with homologous combination of RNAs 1 and 2 (B1B2S3, S1S2B3, C1C2S3 and S1S2C3) accumulated to a detectable level. However, the accumulation in protoplasts of B1B2S3 and C1C2S3, which showed poor infectivity in N. benthamiana plants, was significantly lower (Fig. 2a, b), suggesting that the low infectivity of these two reassortants was due to their inability to multiply efficiently in single cells. It is noteworthy that, during C1C2S3 infection, S3 and its subgenomic RNA 4 (S4) accumulated to quite low levels, although S3 accumulated to some degree during B1B2S3 infection. Because the combination of RNAs 1 and 2 was identical to that in wt CCMV, the low-level accumulation of viral RNAs in C1C2S3 infection may have been due to the incompatibility of C1C2 and S3.

**Assay of transiently expressed 1a and 2a proteins to facilitate viral RNA accumulation in protoplasts**

Bromovirus RNAs 1 and 2 encode the 1a and 2a proteins, respectively (designated B1a and B2a in BMV; C1a and C2a in CCMV; S1a and S2a in SBLV). B1a and B2a are components of viral replicase and function by interacting with each other (Kao et al., 1992). In this study, the B1S2 and C1S2 combinations directed viral RNA accumulation to a detectable level, suggesting that S2a protein interacts compatibly with B1a and C1a. In contrast, S1a failed to produce detectable accumulation of viral RNA when combined with heterologous B2 or C2. Its failure in S1B2 and S1C2 may reflect that S1a cannot form a functional replicase in combination with B2a or C2a. Alternatively, the replicase containing S1aB2a or S1aC2a may be functional but specifically unable to interact with cis-acting elements of RNA 1 and/or RNA 2 with sufficient compatibility, as assumed by Dinant et al. (1993) in BMV–CCMV reassortments. This may cause a reduction in the accumulation of RNA 1 and/or RNA 2 or in the expression of 1a and/or 2a proteins, which are indispensable for viral RNA replication. Consequently, the accumulation of total viral RNAs may be undetectable. To examine these possibilities, the 1a and 2a proteins were transiently expressed under the CaMV 35S promoter from expression plasmids (designated pB1, pB2, pC1, pC2, pS1 and pS2). The expression system using these plasmids is independent of any amplification of RNAs 1 and 2, because mRNAs transcribed from these plasmids lack both the 5′ and 3′ non-coding cis-acting sequences (Fig. 3a).

Several combinations of 1a and 2a expression vectors were transfected into N. benthamiana protoplasts together with RNA 3 of BMV, CCMV or SBLV, and the accumulation of the RNA 3s was estimated. In this assay, pB1pS2 and pC1pS2 combinations directed the accumulation of bromovirus RNA 3s (Fig. 3b). pS2 alone did not result in the detectable accumulation of RNA 3 (Fig. 3b), indicating that S2a requires the function of the 1a protein to successfully replicate viral RNA, as do B2a and C2a (Dinant et al.,...
These results confirmed that S2a forms a functional replicase in combination with heterologous B1a or C1a. The accumulation of RNA 3 and especially RNA 4 supported by the pB1pS2 combination was much lower than the levels that accumulated after inoculation with wild-type combinations (pB1pB2 or pC1pC2), whereas the pC1pS2 combination supported viral RNA accumulation to a level similar to that produced by wild-type combinations. These data are consistent with the results from the RNA-based replication assay of the reassortants containing the B1S2 and C1S2 combinations (Fig. 2), indicating that low-level accumulation of reassortants containing B1S2 in protoplasts was caused by poor compatibility between B1a and S2a. On the other hand, no bromovirus RNA 3s accumulated to a detectable level when inoculated together with pS1pB2 or pS1pC2 (Fig. 3c). Because transiently expressed 1a and 2a proteins facilitated detectable accumulation of RNA 3 in homologous combinations, the failure of S1aB2a and S1aC2a to do so must be due to incompatible interactions between S1a and heterologous 2a proteins rather than to any inefficiency in their expression. In addition to bromovirus RNA 3, pS1pB2 and pS1pC2 did not direct the detectable accumulation of genomic RNAs 1 and 2 homologous to their combinations (i.e. S1 or B2 together with pS1pB2 and S1 or C2 together with pS1pC2) (data not shown). Therefore, we conclude that S1a cannot function with B2 or C2 to accumulate detectable levels of viral RNAs primarily because S1a cannot form a functional replicase with heterologous B2a or C2a.

**Effects of virus movement on infectivity of reassortants**

A replication assay of reassortants indicated that the infectivity of the reassortants in *N. benthamiana* plants is critically determined by their ability to multiply in single cells. However, the possibility remains that the ability of reassortants to move locally also affects their ability to accumulate in inoculated and uninoculated leaves. For example, the systemic infectivity of C1S2S3 was much lower than that of B1S2C3, although C1S2S3 accumulated more in protoplasts than did B1S2C3 (Figs 1 and 2).

We examined the efficiency of virus movement in infections with C1S2C3, S1S2C3 or wt CCMV, which all carry the CCMV 3a MP gene and cause the accumulation of high levels of viral RNA in protoplasts (Fig. 2b). Although all three combinations efficiently infected *N. benthamiana* plants at 14 days p.i. (Fig. 1), the systemic infectivity of C1S2C3 and S1S2C3 at 7 days p.i. was lower than that of wt CCMV (data not shown). Moreover, press-blot analysis demonstrated that the local spread of C1S2C3 and S1S2C3 in *N. benthamiana* was significantly delayed compared with that of wt CCMV and SBLV at 2 days p.i. (Fig. 4). CCMV and SBLV had spread throughout the entire inoculated leaves at 4 days p.i., whereas the reassortants had spread only locally even at 8 days p.i. (data not shown). On the other hand, Western blot analysis demonstrated that the accumulation of movement-associated proteins, 3a protein and CP, in *N. benthamiana* protoplasts infected with C1S2C3 and S1S2C3 was similar to that during infection with wt CCMV (Fig. 5a, b). These data suggest that C1S2C3 and S1S2C3 infect *N. benthamiana* plants inefficiently because their cell-to-cell movement is slower, rather than because the viruses multiply to lower levels in single cells, when compared with CCMV and SBLV infections.

**DISCUSSION**

Several viral factors function co-ordinately rather than independently during successful infection in plants. In this
study, to gain further insights into interactions between viral components in virus infections, we created reassortants from SBLV and other related bromoviruses, BMV and CCMV, and found some interactions involving SBLV components.

**SBLV RNA 2 functions with heterologous bromovirus RNA 1 in virus multiplication in single cells**

Replication assays of the reassortants demonstrated that all reassortants containing S2 components replicated and accumulated to a detectable level in *N. benthamiana* protoplasts, even when the combination of RNAs 1 and 2 was heterologous (Fig. 2). To our knowledge, there has been no report of reassortants with heterologous combinations of RNAs 1 and 2 from different virus species that can direct tripartite viral genome accumulation to detectable levels, except those produced between strains of a certain virus species (e.g. in bromoviruses; Shang & Bujarski, 1993; De Jong & Ahlquist, 1995) or between progeny derivatives containing mutations that allow them to interact compatibly (Masuta *et al.*, 1998). Therefore, SBLV is the first virus in the family Bromoviridae with an RNA 2 known to function in combination with RNA 1 of other virus species.

BMV 1a and 2a proteins, which are encoded by RNAs 1 and 2, respectively, must interact with each other for successful replication (Kao *et al.*, 1992; O’Reilly *et al.*, 1997). This interaction has also been reported for *Cucumber mosaic virus* (Kim *et al.*, 2002) and *Alfalfa mosaic virus* (Maurice *et al.*, 2001), the genomes of which consist of three divided RNAs corresponding to RNAs 1, 2 and 3 of the bromoviruses, indicating that the 1a–2a interaction is a common characteristic in the family Bromoviridae. Detectable accumulation of viral RNA was directed by B1S2 and C1S2 combinations. Moreover, S2a protein expressed from a DNA plasmid supported detectable accumulation of bromovirus RNA 3s in combination with heterologous B1a or C1a protein. These results indicate that the SBLV 2a protein interacts compatibly with heterologous 1a proteins. Immune coprecipitation assays using mutant polypeptides made in an *in vitro* translation system demonstrated that the N-terminal segment of BMV 2a protein interacts directly with BMV 1a protein (Kao & Ahlquist, 1992). Similar experiments may help identify interacting domains between S2a and B1a or C1a. In contrast, no reassortants with heterologous combinations of RNAs 1 and 2 containing S1 (S1B2 and S1C2) accumulated to detectable levels. Transiently expressed SBLV 1a protein in combination with BMV or CCMV 2a protein did not support detectable accumulation of viral RNAs (Fig. 3), suggesting that SBLV 1a protein cannot form a functional replicate with either of the heterologous 2a proteins.

**Compatible combinations of RNAs 1 and 2 support efficient amplification of SBLV RNA 3**

In contrast to the heterologous combinations of RNAs 1 and 2, all homologous combinations of RNAs 1 and 2 directed the detectable accumulation of viral RNAs in protoplasts, consistent with previous results (Allison *et al.*, 1988). However, two reassortants with homologous RNAs 1 and 2, B1B2S3 and C1C2S3, showed low-level accumulations of total viral RNAs (Fig. 2a, b). In particular, inoculation with C1C2S3 produced quite low levels of S3 and S4 accumulation. The low-level accumulation of total viral RNA during C1C2S3 infection may be caused by inefficient encapsidation with heterologous SBLV CP. However, this is unlikely to explain the S3-specific reduction in accumulation. Alternatively, the replicate formed from C1a plus C2a might not interact compatibly with cis-elements in SBLV RNA 3. On the other hand, the C1S2S3 combination accumulated to high levels (Fig. 2b), indicating that, in addition to the C1a–S2a interaction, the replicate–S3 interaction was also compatible. Taken together, these data suggest that S2a may function more efficiently than C2a in the amplification of S3.

**Compatible combinations of viral genomic RNAs required for efficient virus movement**

All reassortants non-infectious to *N. benthamiana* plants failed to accumulate to detectable levels in single cells, and most of the reassortants showing low infectivity accumulated to lower levels than wild-types. These results suggest that infectivity of reassortants is primarily determined by their ability to multiply at the single-cell level. Furthermore, significant delay in local spread of C1S2C3 and S1S2C3 when compared with the spread of wt CCMV suggests that
virus cell-to-cell movement is also a crucial step determining infectivity of reassortants, because viral RNAs of C1S2C3, S1S2C3 and wt CCMV accumulated to high levels in protoplasts (Fig. 2). C1S2C3, S1S2C3 and wt CCMV have different combinations of RNAs1 and 2 but all contain the C3 component and therefore express CCMV MP and CP, and the accumulation levels of these proteins were similar in each inoculum (Fig. 5). SBLV RNAs 1 and 2 should be functional because wt SBLV spread in inoculated leaves of *N. benthamiana* as efficiently as wt CCMV (Fig. 4). Therefore, slower spread of C1S2C3 and S1S2C3 may have been caused by poor compatibility of interactions between the viral components rather than the difference in functions of an individual component. Together, these data suggest that a compatible combination with RNAs 1 and/or 2 may be required for C3 to function in virus cell-to-cell movement. Supporting this suggestion, viral genes for replication, such as the bromovirus 1a and 2a genes, function not only in viral RNA replication but also in virus movement (Traynor et al., 1991; Gal-On et al., 1994; Hirashima & Watanabe, 2001). Because the local spread of C1S2C3 was slower than that of wt CCMV, an RNA 2-mediated interaction at least may be necessary.

So far, most reassortment tests have been performed to map virus functions. Therefore, reassortants were created between virus species or virus strains with distinct features (Allison et al., 1988; Shang & Bujarski, 1993; Takahashi et al., 1994; De Jong & Ahlquist, 1995). However, the reassortants in this study were created from different virus species with similar infectivity in a common host plant to elucidate the compatibility of interactions between viral factors. We confirmed that compatibility is required for viral RNA replication, as reported previously (1a–2a interaction), and demonstrated that S2 can function in replication heterologously with either B1 or C1. This characteristic of RNA2 made it possible for us to discover an RNA2-mediated interaction necessary for the step after viral RNA replication. Analysis of the RNA2-mediated interaction required for bromovirus movement is in progress.

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