The Arginine-Rich Motif of *Bamboo mosaic virus* Satellite RNA-Encoded P20 Mediates Self-Interaction, Intracellular Targeting, and Cell-to-Cell Movement

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Satellite RNA of *Bamboo mosaic virus* (satBaMV) has a single open reading frame for a nonstructural, RNA-binding protein, P20, which facilitates the long-distance movement of satBaMV in *Nicotiana benthamiana*. Here, we elucidate various biological properties of P20 and the involvement of a single domain in its activities. P20 displayed a strong self-interaction in vitro and in vivo, and cross-linking assays demonstrated its oligomerization. Domain mapping, using the bacterial two-hybrid system, indicated that the self-interacting domain overlaps the RNA-binding domain in the N-terminal arginine-rich motif (ARM) of P20. The deletion of the ARM abolished the self-interaction of P20 in vitro and in vivo and impaired its intracellular targeting and efficient cell-to-cell movement in *N. benthamiana* leaves. Moreover, RNA and protein accumulation of the ARM deletion mutant of satBaMV was significantly reduced in leaves systemically coinfecting with *Bamboo mosaic potexvirus* and satBaMV. This is the first report of the involvement of ARM in various biological activities of a satellite RNA-encoded protein during infection of its host.

Additional keyword: protein-protein interaction.

Satellite RNA of *Bamboo mosaic virus* (satBaMV) is associated with *Bamboo mosaic potexvirus* (BaMV) infection (Lin and Hsu 1994) and is the sole example in the *Potexvirus* genus. SatBaMV depends on BaMV for replication and encapsidation (Lin and Hsu 1994). The helper virus BaMV has a single-stranded, positive-sense RNA of approximately 6.4 kb, with a 94-nt 5' untranslated region (UTR), a 142-nt 3' UTR with a poly (A) tail, and five open reading frames (ORF) (Lin et al. 1994). ORF1 encodes a 155-kDa polypeptide whose amino acid sequence reveals three functional domains: an N-terminal methyltransferase (Huang et al. 2004; Li et al. 2001a), a central putative RNA helicase (Li et al. 2001b), and a C-terminal RNA-dependent RNA polymerase (Cheng et al. 2001; Li et al. 1998). A triple gene block comprising ORF2 through ORF4 encodes the movement proteins (MPs) TGBp1, TGBp2, and TGBp3, respectively (Chang et al. 1997; Lin et al. 1994, 2004), and ORF 5 encompasses the capsid protein (CP) coding region (Lin et al. 1994).

The satBaMV genome is composed of an 836-nt (excluding the poly[A] tail) single-stranded RNA that contains a single ORF for a protein of 20 kDa (P20). The P20 ORF is flanked by a 5' UTR of 159 nt and a 3' UTR of 125 nt. SatBaMV RNA shares no significant homology with BaMV except at the 5' UTR (Lin and Hsu 1994). P20 is a nonstructural protein of satBaMV (Lin and Hsu 1994), and it shares 46% identity with the CP of *Satellite panicum mosaic virus* (SPMV) (Berger et al. 1994; Liu and Lin 1995). From protein modeling, P20 also structurally resembles the 17-kDa SPMV CP, which consists of eight-stranded β -sheets to form a “jelly roll” structure (Ban and McPherson 1995; Liu et al. 1997). P20 is not essential for satBaMV replication, and its coding region can be replaced with a foreign gene (Lin et al. 1996). P20 is a strong RNA-binding protein, which displays high preference for the UTR of BaMV and satBaMV (Tsai et al. 1999). The amino terminal arginine-rich motif (ARM) was identified as the RNA-binding domain of P20 (Tsai et al. 1999). Mutations within the P20 ORF delay or impair the satBaMV systemic movement in infected plants, which indicates the facilitating role of P20 in satBaMV movement (Lin et al. 1996). Similarly, recent results indicated that SPMV CP has properties that facilitate and enhance systemic spread (Omarov et al. 2005).

In this study, we provide insight into the biological properties of P20 and the role of a single domain in various activities of P20, i.e., self-interaction in vitro and in vivo and oligomerization in vitro. Use of the bacterial two-hybrid (BTH) system identified the self-interacting domain, which overlaps the ARM. The ARM also potentiated the intracellular targeting and efficient cell-to-cell movement of P20 in *Nicotiana benthamiana* leaves. An ARM deletion mutant showed a reduced accumulation of RNA and protein in leaves systemically coinfecting with BaMV and satBaMV.

RESULTS

P20 interacts with itself in vitro.

P20 is an RNA-binding protein that binds preferentially to UTR of BaMV and satBaMV in a highly cooperative mode (Tsai et al. 1999). Self-interaction and oligomerization may be prerequisites for the specific interaction of P20 with BaMV or satBaMV RNA. Therefore, we used pull-down assays to determine whether P20 can interact with itself in vitro. P20-His (recombinant P20 containing a C-terminal eight-histidine tag) and P20 were translated in vitro in rabbit reticulocyte lysate

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(RRL) and were used for the assay. P20 was labeled with [^{35}S]-methionine, and the synthesis was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A, lane a). Recombinant P20-His was unlabeled during translation and preadsorbed to cobalt-sepharose beads. ^{35}S -P20 was mixed with the preadsorbed beads and, after incubation, the interacting protein was eluted from the beads and was analyzed by SDS-PAGE and autoradiography. Radiolabeled P20 eluted from the sepharose beads preadsorbed with P20-His indicated the interaction between ^{35}S -P20 and P20-His (Fig. 1A, lane b). Radiolabeled P20 did not bind to beads by itself (Fig. 1A, lane c).

P20 forms oligomers.

To analyze whether self-interaction of P20 would lead to oligomerization, P20-His was overexpressed in *Escherichia coli*, was purified (Tsai et al. 1999), and was cross-linked with glutaraldehyde for various times. Cross-linked P20 species were resolved by SDS-PAGE and were detected by Western blotting. Recombinant P20-His formed dimers and tetramers, and the amount of monomer decreased with increasing time of cross-linking (Fig. 1B). Dimers predominated in all the reaction mixtures. Subsequently, we analyzed P20 expressed in the BaMV and satBaMV coinfecting leaves. Coinfecting leaf-protein extracts containing P20 were cross-linked with glutaraldehyde for various times, and P20 species were detected as before. The results showed that dimers also formed in plants (Fig. 1C), and the predominance of dimers at different times and tetramerization were similar to that observed for the recombinant P20-His (Fig. 1B and C).

Self-interaction of P20 in vivo.

To validate the results regarding the self-interaction of P20 observed in vitro, the interaction was analyzed in vivo using the BTH system. The BTH system we used is based on interaction-mediated reconstitution of the adenylate cyclase activity in *E. coli* (Karimova et al. 1998). It exploits the fact that the catalytic domain of adenylate cyclase from *Bordetella pertussis* consists of two complementary fragments, namely T18 and T25. When these two fragments are fused to interacting polypeptides, heterodimerization of the hybrid proteins results in functional complementation between T18 and T25 fragments and, therefore, cyclic AMP (cAMP) synthesis. cAMP binds to the catabolic activator protein, and this complex regulates several resident genes, including *lac* and *mal* operons and, therefore, the *E. coli* become able to use lactose or maltose as the unique carbon source. Complementation mediated by the interaction between fused proteins leads to cAMP synthesis, which confers a *Cya*⁺ phenotype (red) to the recipient *cya* bacteria (white), which can be distinguished on the indicator medium. Functional complementation is quantified by measuring the cAMP level and β -galactosidase activities in *E. coli*.

Cya-deficient *E. coli* cotransformants harboring P20 in both the complementing plasmids pKT25 and pUT18 turned dark red after 48 h on MacConkey agar medium (Fig. 2A), and the phenotype was similar to that of the positive control harboring pKT25-zip and pUT18-zip. However, in the latter case, zip-zip interaction was already visible at 24 h. Both β -galactosidase activity and cAMP quantity were substantially increased in the cotransformants carrying pKT25-P20 and pUT18-P20, which verified that the functional complementation is mediated by a strong physical interaction between the full-length P20 encoded by the two plasmids (Fig. 2B). Control cotransformants that carried pKT25-P20/pUT18 or pUT18-P20/pKT25 remained white after 48 h, and their β -galactosidase activity and cAMP levels were comparable to that of the negative control carrying pKT25 and pUT18.

ARM is the self-interacting domain of P20.

To delineate the region that mediates the self-interaction of P20, we used the BTH system. The ORF of full-length P20 was cloned into pKT25, whereas the N-terminal (N3 to N8)

Fig. 1. Self-interaction of P20 in vitro. **A**, Autoradiograph showing self-interaction of P20 as determined by pull-down assay. P20-His, P20 (lane a), P18-His, and P18 (lane d) were translated in vitro by use of the reticulocyte lysate system. P20 and P18 were translated in the presence and the recombinant His-tagged proteins in the absence of [^{35}S]-methionine. P20-His (lanes b and e) or P18-His (lane f) was adsorbed on cobalt-sepharose beads and was incubated with ^{35}S -P20 (lanes b and c) or ^{35}S -P18 (lanes e and f). After being washed, the beads were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and the supernatant was resolved in 15% SDS-PAGE; the interacting proteins were detected by autoradiography. **B** and **C**, Oligomerization of P20. Purified recombinant P20-His (**B**) or crude protein extracts from *Nicotiana benthamiana* leaves coinfecting with *Bamboo mosaic potyvirus* and satellite RNA of *Bamboo mosaic virus* (**C**) were cross-linked with 0.0001 or 0.025% glutaraldehyde at 25 and 30°C, respectively, for different times. The reaction was quenched with 0.1 M glycine, pH 8. After the addition of 2x sample buffer, the proteins were resolved by 10% SDS-PAGE and were electroblotted onto a polyvinylidene difluoride membrane. P20 was probed with rabbit anti-P20 serum and was detected by an enhanced chemiluminescence plus Western blotting system. The molecular size is shown on the left.

and C-terminal fragments (C2 to C9) of the P20 gene were generated by polymerase chain reaction (PCR) (Fig. 3A) and were cloned into pUT18. BTH101 cells were complemented with pKT25-P20 (expressing full-length P20) and pUT18 carrying either of the truncated P20 versions. Among the cotransformants harboring the full-length P20 and either of the N-terminal fragments of P20, the cells hosting P20 and the N3 fragment (encoding amino acids 1 to 15) showed abundant β -galactosidase activity and cAMP production (Fig. 3B). The addition of amino acids to the N3 fragment (N4, N5, N6, and N7 fragments) did not increase the strength of interaction with full-length P20. Fragment N8 displayed a marked interaction with P20, because its amino terminal region represents about 85% of the whole P20 polypeptide. Only cotransformants hosting P20 and N3 and P20 and N8 appeared red, whereas all others containing P20 and the N-terminal fragment appeared pink after 72 h of incubation.

The deletion of amino acids 1 to 15 (fragment C2) abolished the self-interaction of P20 (Fig. 3B). The C-terminal fragments C3, C4, C5, C6, C7, C8, and C9 did not mediate P20 interac-

tion. All the cotransformants expressing full-length P20 and either of the C-terminal fragments invariably remained white after 72 h. All the control cotransformants harboring pUT18-N/C terminal fragments of P20 and empty pKT25 appeared white, with no significant production of β -galactosidase and cAMP (data not shown). The deletion mapping results are, therefore, consistent with the interpretation that the self-interacting domain comprises amino acids 1 to 15, and this domain is designated as D1. D1 is also the RNA-binding domain, which encompasses the ARM (Tsai et al. 1999).

The deletion

of ARM abolished the self-interaction of P20 in vitro.

To verify the role of ARM in the self-interaction of P20 with an independent method, P18 (N-terminal truncated P20) was translated in vitro, and its interaction with P20-His and P18-His (recombinant P18 containing an eight-histidine tag at the C terminus) was analyzed by pull-down assays. Only P18 was labeled with [35 S]-methionine, and the synthesis was confirmed by SDS-PAGE and autoradiography (Fig. 1A, lane d). Recombinant P20-His and P18-His were unlabeled and preadsorbed to cobalt-sepharose beads individually. 35 S-P18 was mixed with the preadsorbed beads, and under similar conditions as those used to show P20-P20 interaction, 35 S-P18 did not interact with either P20-His or P18-His (Fig. 1A, lanes e and f).

ARM is critical

for intracellular targeting and cell-to-cell movement of P20 in *N. benthamiana* leaves.

Since the self-interacting domain D1 of P20 was identified to contain the ARM, we subsequently analyzed whether the ARM has any roles in biological activities such as intracellular localization and cell-to-cell movement of P20 in the satBaMV host. Therefore, enhanced green fluorescent protein (EGFP)-tagged wild-type and mutant P20 were transiently expressed in detached leaves. Plasmids pCass-P20-EGFP, pCass-D1.P20-EGFP, and pCass- Δ D1.P20-EGFP were used for biolistic bombardment to express EGFP-tagged P20 (full-length P20), D1.P20 (only D1 of P20), and Δ D1.P20 (ARM-truncated P20, also represented as P18), respectively. Free EGFP expressed from pCass-EGFP was used as a control. Intracellular localization was scored after 24 h of postbombardment by confocal microscopy (Fig. 4A through D). Free EGFP localized in the nucleus and throughout the cytoplasm (Fig. 4A). Full-length P20-EGFP targeted to the cell wall in what appeared to be punctate spots (Fig. 4B). D1.P20-EGFP did not form such spots and consistently accumulated into large aggregates with smaller satellite inclusions (Fig. 4C). The EGFP-tagged Δ D1.P20 (Δ D1.P20-EGFP) did not target within the cell and generally exhibited a phenotype identical to that of the free EGFP (Fig. 4D).

Cell-to-cell movement of EGFP-tagged wild-type and mutant P20 was assessed after 48 h of postbombardment (Table 1; Fig. 4E through H). In most of the observed sites, free EGFP fluorescence was detected in single cells (Fig. 4E); occasionally however, EGFP moved to two or three cells. In contrast, P20-EGFP generated clusters of two to 24 fluorescent cells in about 84% of the observed sites, which indicates extensive intercellular trafficking of P20-EGFP (Fig. 4F). Cell-to-cell movement of D1.P20-EGFP was highly restricted to two or three cells in 20% of the observed sites (Table 1; Fig. 4G), and Δ D1.P20-EGFP was incapable of movement in 80% of the sites (data not shown). The ARM deletion mutant Δ D1.P20-EGFP was restricted in movement and could not move beyond five or six cells in 27% of the observed sites (Table 1; Fig. 4H) and was completely abolished in 73% of the sites (data not shown). Prolonged incubation (72 h) of the leaves did not greatly enhance the intercellular movement of the mutants (data not

Fig. 2. Self-interaction of P20 in vivo. **A**, Colorimetric analysis of the cotransformants obtained in the bacterial two-hybrid (BTH) system. BTH101 cells of *Escherichia coli* cotransformed with the indicated plasmids were screened on MacConkey agar containing 0.5 mM IPTG, 100 μ g of ampicillin per milliliter, and 50 μ g of kanamycin per milliliter after 48 h of incubation at 30°C. Cotransformants harboring pKT25-zip/pUT18-zip and pKT25/pUT18 are the positive and negative controls, respectively. Plasmids pKT25 and pUT18 are denoted as T25 and T18, respectively. Cotransformants harboring pKT25-P20 and pUT18-P20 turned red, which indicated the P20-P20 interaction, while the control harboring pKT25-P20/pUT18 or pKT25/pUT18-P20 remained white. **B**, Functional complementation mediated by P20-P20 interaction as determined by β -galactosidase and cAMP assays. BTH101 cotransformants harboring the indicated plasmids were grown in Luria Bertani medium overnight under the conditions mentioned in A, and the liquid cultures were used for the assays. The data represent the mean and standard deviation of four experiments.

shown). The movement frequency and range of Δ D1.P20-EGFP indicate inefficient cell-to-cell movement of the mutants and the importance of ARM in efficient intercellular trafficking of P20 in the host.

Accumulation of wild-type and ARM-deletion mutant satBaMV in infected *N. benthamiana* leaves.

To determine the functional role of ARM in planta, *N. benthamiana* leaves were coinoculated with BaMV-S RNA and transcripts of satBaMV. Transcripts were prepared from satBaMV cDNA clones pBSF4 (encoding wild-type P20) or pBSFM2 (ARM-deletion mutant, encoding P18) (Fig. 5A). The wild type or the mutant did not vary in their symptom phenotypes in the coinoculated leaves, and their accumulation at the RNA level was analyzed by dot-blot assay. Inoculated leaves (at 10 days post-coinoculation [dpi]) and uninoculated upper leaves (10, 20, and 30 dpi) were harvested for assays. BSFM2 could replicate in the presence of BaMV-S RNA (Fig. 5B and D); however, the RNA level was lower than that of the wild type in the inoculated and upper leaves collected at all times, as quantitated by Image Quant. In inoculated and upper leaves at 10 dpi, the BSFM2 RNA level was reduced to about 20 and 30%, respectively. At 20 and 30 dpi, the reduction in upper leaves was 35 to 40%.

When the protein expression of the wild-type and mutant satBaMV in the infected leaves was assessed by immunoblot analysis (Fig. 5E and F), anti-P20 serum detected full-length

P20 and a protein with a molecular mass of 16 kDa (P16) in the BSF4 coinoculated and upper leaves at 10 dpi. At 20 and 30 dpi, only P16 accumulated at high levels and P20 could not be observed in upper leaves. In BSFM2 coinoculated and upper leaves, because of the deletion of ARM (1 to 15 amino acids), a truncated protein with a predicted molecular mass of 18 kDa (P18) was detected. P18 predominated in the inoculated and upper leaves up to 20 dpi, and no low-molecular mass protein species could be detected. The accumulation of P18 was always much less than wild-type P20, and P18 could not be detected at 30 dpi (Fig. 5E and F).

DISCUSSION

In this study, we have elucidated various properties of the satBaMV RNA-encoded P20 protein and the critical roles of the ARM. Physical interaction of P20 with itself was very strong in vitro, and deletion of the ARM abolished the self-interaction. Colorimetric analysis, β -galactosidase assay and cAMP assay with the BTH cotransformants showed that the self-interaction of P20 also occurred in vivo. The BTH system was adopted, since it is a novel approach to analyze protein interaction, which need not take place near the transcription machinery (Karimova et al. 1998) as in the case of the yeast two-hybrid system. Quick analysis and high transformation efficiency is achieved in *E. coli*. Like the recombinant P20-His, P20 expressed in BaMV- and satBaMV-coinoculated *N. benthamiana* leaves formed oligomers in vitro; the possibility of

Fig. 3. Mapping of the self-interacting domain of P20 by the bacterial two-hybrid (BTH) system. **A**, N- and C-terminal fragments of the P20 gene were generated from pBSF4 by polymerase chain reaction. A DNA fragment encoding full-length P20 or a truncated fragment (N3/N4/N5/N6/N7/N8/C2/C3/C4/C5/C6/C7/C8/C9) was cloned in-frame into pKT25 and pUT18, respectively. BTH101 cells were cotransformed with pKT25-P20 (encoding full-length P20) and pUT18-N/C-P20 (encoding truncated P20) and were analyzed for their phenotype on MacConkey agar containing 0.5 mM IPTG, 100 μ g of ampicillin per milliliter, and 50 μ g of kanamycin per milliliter after 48 h of incubation at 30°C. Dark red, red, pink, and white colony phenotypes indicate strong, moderately weak, weak, and no protein-protein interaction, respectively. **B**, Functional complementation mediated by protein-protein interaction as determined by β -galactosidase and cAMP assays. Cotransformants harboring the indicated plasmids were grown in liquid medium overnight under the conditions mentioned in A and were used for the assays. The data represent the mean and standard deviation of four experiments.

Fig. 4. Confocal micrographs of *Nicotiana benthamiana* epidermal cells showing intracellular localization and cell-to-cell movement of enhanced green fluorescent protein (EGFP)-tagged wild-type and mutant P20. One-month-old plant leaves were bombarded with either pCass-EGFP (encoding free EGFP), pCass-P20-EGFP (encoding P20 fused with EGFP), pCass-D1.P20-EGFP (encoding only D1 of P20 fused with EGFP), or pCass-ΔD1.P20-EGFP (encoding D1 truncated P20 fused with EGFP). **A** through **D**, Intracellular localization of EGFP-tagged wild-type and mutant P20 after 24 h postbombardment. **A**, Free EGFP was localized in the nucleus and throughout the cytoplasm. **B**, P20-EGFP targeted to the cell wall as punctate-like bodies, presumed to be plasmodesmata (indicated by arrow head). **C**, D1.P20-EGFP formed large aggregates and small satellite inclusions. **D**, ΔD1.P20-EGFP did not target to the cell wall as punctate bodies and localized in the nucleus and throughout the cytoplasm. **E** through **H**, Cell-to-cell movement of EGFP-tagged wild-type and mutant P20 after 48 h postbombardment. **E**, Free EGFP was confined to a single cell. **F**, P20-EGFP displayed extensive cell-to-cell movement. **G**, Movement of D1.P20-EGFP was restricted to three cells. **H**, ΔD1.P20-EGFP could not move beyond six cells. Scale bars = 50 μm.

Table 1. Cell-to-cell movement of wild-type and mutant P20 in *Nicotiana benthamiana* leaves

Expressed protein	Molecular mass (kDa)	No. of cell clusters observed ^a	% Cells with EGFP in clusters ^b	Range of cells in cluster
EGFP	26	0/292	0	0
P20-EGFP	46	294/350	84	2 to 24
D1.P20-EGFP	27	82/414	20	2 to 3
ΔD1.P20-EGFP	44	114/423	27	2 to 6

^a Two or more cells exhibiting enhanced green fluorescent protein (EGFP) fluorescence. Number of cell clusters exhibiting EGFP fluorescence/total number of fluorescent sites observed.

^b Percentage of fluorescent cell clusters observed at 48 h postbombardment in six leaves in three independent experiments. D1.P20-EGFP and ΔD1.P20-EGFP were confined to a single cell and were movement defective in 80 and 73% of the observed sites, respectively.

oligomerization as a result of self-interaction of P20 or interaction between P20 and helper virus or host protein, or both, cannot be excluded because P20 was in the crude plant protein extract. Although viral movement proteins tend to aggregate and may interact and anchor other proteins associated with the replication complex, such as replicase and CP of *Tobacco mosaic virus* (TMV) (Mas and Beachy 1999), our study is the first demonstration of oligomerization of a plant satellite RNA-encoded protein.

We further identified the fragment encompassing amino acids 1 to 15 as the major self-interacting domain D1 of P20 by the BTH system and pull-down assay. D1 contains the ARM,

which is also the domain mediating the P20-satBaMV RNA interaction (Tsai et al. 1999). RNA-binding motifs of MPs of several viruses are rich in basic residues, including arginine and lysine (Bleykasten et al. 1996; Fujita et al. 1998; Lin et al. 2004; Marcos et al. 1999; Ryabov et al. 2001; Schoumacher et al. 1994; Vaquero et al. 1997). ARMs of viral proteins other than MP, such as CP (Annamalai et al. 2005; Ban and McPherson 1995; Choi and Rao 2000; Satheshkumar et al. 2004; Schmitz and Rao 1998), replicase (Boonrod et al. 2005; Rajendran and Nagy 2003), and helicase (Fernandez and Garcia 1996) are also known to be involved in RNA-binding activity. Although the significance of the overlap between the self-interaction do-

Fig. 5. **A**, Schematic representation of pBSF4 (encoding wild-type P20) and pBSFM2 (arginine-rich motif deletion mutant) constructs. **B**, Dot-blot analysis of accumulated wild-type and mutant satellite RNA of *Bamboo mosaic virus* (satBaMV) RNA. Three leaves of 1-month-old *Nicotiana benthamiana* plants were coinoculated with 0.1 µg of *Bamboo mosaic potyvirus-S* RNA and 0.1 µg of transcripts of BSF4 or BSFM2 satBaMV. Inoculated (IL) and uninoculated upper leaves (SL) were harvested at different times. SL 5, SL 6, and SL 8 indicate the position of the uninoculated upper leaves. Total RNA was extracted from inoculated (10 days postinoculation [dpi]) and upper (10, 20, and 30 dpi) leaves, and equal amounts of RNA was dot-blotted onto nylon membrane. Blots were hybridized with a specific riboprobe to detect positive-strand satBaMV RNA. Hybridization signals were detected in a PhosphorImager and were quantified by use of ImageQuant, version 5.2. **C**, Ethidium bromide-agarose gel prior to blotting shows equal loading as revealed by abundance of ribosomal RNA in each lane. Lanes 1, 3, 5, and 7 and 2, 4, 6, and 8 represent RNA of 10 dpi IL, 10 dpi SL 5, 20 dpi SL 6, and 30 dpi SL 8 of F4 and FM2, respectively. **D**, Total proteins were extracted from inoculated (10 dpi) and upper (10, 20 and 30 dpi) leaves separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane. Full-length or truncated P20 was probed with rabbit anti-P20 serum, was detected by an enhanced chemiluminescence plus Western blotting system, and was quantified by MetaMorph Offline, version 3.2. **E** and **F**, Quantitative analyses of levels of wild-type and mutant satBaMV RNA and encoded protein. Data show the average of three independent experiments. Level of BSF4 was normalized to 100% for all times.

main of P20 and the arginine-rich RNA-binding domain is not certain, the overlap may serve to alter the conformation of the RNA-binding domain or, vice versa, to facilitate the protein-RNA interactions during infection (Ansel-Mckinney et al. 1996; Draper 1995; Matsumoto et al. 1996).

In the confocal microscopic studies, the punctate-like spots observed for wild-type P20-EGFP, which presumably represented aggregations at the plasmodesmata, resembled those observed for MPs of TMV, *Potato virus X*, *Beet necrotic yellow vein virus*, *Cucumber mosaic virus*, and *Alfalfa mosaic virus*

Table 2. Primers used for cloning

Primer ^a	Sequence ^b	Amino acid in full-length P20 ^c
To generate open reading frames of full-length and truncated P20 for the bacterial two-hybrid system		
T25P20-5'	CGCTTACTGCAGGATGGTTCGGAGGAGA	Full-length
T25P20-3'	AGCCGGTTGTGGGTACCCGTCAACTGGTTGGTGCG	
T18P20-5'	GCTTACCTGCAGGATGGTTCGGAGGAGA	Full-length
T18P20-3'	TTGTGCTCGGTACCCGACTGGTTGGTGCACG	
T18N3-3'	AGTGAGGCTACCCGGATGTCTGGTTCATTTG	1 to 19
T18N4-3'	AAAGGTCGAGGTACCCGATTGGCCAACCCAGG	1 to 42
T18N5-3'	GCTTGGGGTACCCGGGGGGAGGCAGACGA	1 to 62
T18N6-3'	GTGGTTGGTACCCGTTGAAGCCCGGTGGA	1 to 84
T18N7-3'	GGCAGAGGTACCCGATGGTGAGTGTGTGG	1 to 94
T18N8-3'	CGTGGTTGGTACCCGTTTCAGCATTAACAGT	1 to 155
T18C2-5'	GCGTGTCTGCAGGATGACCGACATCATG	16 to 183
T18C3-5'	AATGACCTGCAGGATGTATGGCTCACTA	20 to 183
T18C4-5'	TGGGTTCTGCAGGATGGGAGATCGACCT	43 to 183
T18C5-5'	TCTGCCTGCAGGATGCTTTACCAAGCC	63 to 183
T18C6-5'	CACCGGCTGCAGGATGTCTGGAACCACT	85 to 183
T18C7-5'	ACACACCTGCAGGATGAGAGCTCTGCCA	95 to 183
T18C8-5'	CAACTGGTAACTGCAGGATGCCTACAACCACG	156 to 183
T18C9-5'	ACCACCTGCAGGACCTCCCAGAGGGG	162 to 183
To construct clones for biolistic bombardment		
G5-5' ^d	TCGGTACCATGGTGAGCAAGGGCGAGG	
G3-3' ^d	TCGAGCTCAATTTACTTGTACAGCAGCAGCTCGTCCAT	
P20-5'	TCAGGCCTA44AATGGTTCGGAGGAGAAATCGT	Full-length
P20-3'	TCGGTACCAGTGGTTGGTGCACGGTCA	
ΔD1-5'	AGGCCTATCGCGAGGCCTA44AATGACCGACATCATG	Δ16 to 183
ΔD1-3'	GCTCGGTACCAGTGGTTGGTGCACG	
N84-5'	GAAGGCCTA44AATGGTTCGGAGGAGAAATCGTCGCCAGA	1 to 15
	GATCGCGTGTCTCCAAATGGTGAGCAAGGGCGAGGAGCTGTTTC	
To construct clones for plant inoculation		
M2-5'	GACGATGACCGACATCATGT	16 to 183
BS-43-3'	GTCGACTCTAGAT ₂₀	

^a T25/T18 indicates T25/T18 segment in pKT25/pUT18. N/C indicates N-terminal/C-terminal fragment of P20. T18-P20-5' and T18-P20-3' were used as common forward and reverse primers to generate N- and C terminal fragments of P20, respectively, from pBSF4. 5' and 3' denote forward and reverse primer, respectively.

^b Sequence of primer from 5' to 3' end; restriction sites are underlined; introduced sequence are in bold italics; the enhanced green fluorescent protein (EGFP) sequence is in bold letters.

^c Amplified from cDNA clone of satBaMV (EMBL/GenBank accession number NC003497).

^d Primers used to amplify the EGFP gene from pEGFP.

Fig. 6. Overlapping functional domain encompassing the arginine-rich motif (ARM) in the biological activities of P20. In the amino acid sequence of P20, ARM is in bold letters. ARM is involved in RNA binding activity (Tsai et al. 1999), self-interaction, intracellular localization, and efficient cell-to-cell movement of P20.

(Atkins et al. 1991; Blackman et al. 1998; Erhardt et al. 2000; Huang et al. 2001; Solovveyev et al. 2000). The cell-to-cell movement activity we showed for P20 in the host leaves together with its RNA-binding activity (Tsai et al. 1999) suggests that P20 may have a significant role in promoting cell-to-cell trafficking of a ribonucleoprotein complex involving P20-satBaMV RNA in the satBaMV host (Kawakami et al. 2004). Truncation of the ARM impaired the intracellular targeting and cell-to-cell movement properties of P20 in *N. benthamiana* leaves. The variation in extent of movement between the wild-type and the ARM deletion mutant P20 suggests that the ARM may be important for the interaction of P20 with host factors required for the efficient cell-to-cell movement of P20. In addition, deleting ARM may greatly affect the structural integrity of P20, which may be required for the RNA or protein interactions, or both, that influence the P20 movement. Regardless of the small size of D1.P20-EGFP (only D1 fused with EGFP), it failed to move efficiently, possibly because of the high degree of aggregation and loss of overall conformation of the protein. Collectively, the results suggest that, in addition to the conformation, ARM is important for the intracellular targeting and efficient cell-to-cell movement of P20.

Furthermore, ARM has a functional role in the accumulation of satBaMV RNA and especially of P20 protein in the infected cells. The RNA level of BSFM2 was significantly lower than that of the wild type (Fig. 5), possibly because the replication efficiency of BSFM2 was found to be 10 to 15% lower than that of the wild type in the coinfecting *N. benthamiana* protoplasts (data not shown). Also, unlike P20, P18 failed to bind to satBaMV RNA and did not participate in a stable ribonucleoprotein complex (Tsai et al. 1999).

Although the translation efficiency of both the wild-type and mutant satBaMV was almost equal in the RRL system in vitro (data not shown), the protein level of BSFM2 was much lower in planta. In BSFM2-coinfecting leaves, a protein of 18 kDa was detected because of the truncation of ARM. In the wild-type satBaMV-coinfecting leaves, besides the full-length P20, a protein of about 16 kDa (P16) reacted with anti-P20 serum. P16 could not be detected in the early stage of satBaMV infection (data not shown), but it predominated at the later stage. Moreover, P16 was detected only in coinfecting plants and not in RRL (Lin and Hsu 1994) and wheat germ extract in vitro (data not shown), which suggests that P16 may be a degradation product of P20. The expression of P16 is, therefore, less likely to occur by translational reinitiation at an in-frame start codon at nt 286 (aatAUGgg), representing the satBaMV amino acids 43 to 183 (predicted molecular mass, 15.5 kDa) as predicted by the ribosome leaky scanning model (Kozak 1995; Qiu and Scholthof 2000). However, the underlying generation and biological significance of P16 is under investigation.

P20 shares high amino acid sequence and structural homology with SPMV CP (Berger et al. 1994; Liu and Lin 1995), and these two proteins have a common ARM in the RNA-binding domain at the N terminus (Desvoyes and Scholthof 2000; Tsai et al. 1999). Deletion of the N-terminal segment of SPMV CP significantly decreased the accumulation of SPMV RNA in the upper noninoculated leaves of foxtail millet (Omarov et al. 2005), the decline perhaps due to lack of interaction between N terminal-truncated CP and RNA, between SPMV CP and PMV CP, or both (Omarov et al. 2005). This observation, along with the present data, suggest that, besides the reduced replication efficiency, the significant difference in RNA and protein accumulation between wild-type and mutant satBaMV in the upper leaves may result from a lack of interaction between P18 and either satBaMV RNA (Tsai et al. 1999), with itself, or both, and helper components or host proteins in infected plants.

Collectively, these data suggest that the ARM of P20 has important roles in various biological activities, including RNA-binding activity (Tsai et al. 1999), self-interaction (Figs. 1, 2, and 3), intracellular targeting, and efficient cell-to-cell movement of P20 (Fig. 4). Therefore, the overlapping functional domain (Fig. 6) may harbor multiple roles in P20 activities during BaMV and satBaMV coinfection.

MATERIALS AND METHODS

Plasmids and constructs.

Plasmid pBSF4 contains the full-length cDNA of satBaMV (EMBL/GenBank accession number NC003497) (Lin et al. 1996). To construct recombinant clones for the BTH system, the two plasmids pKT25 and pUT18 (Karimova et al. 1998) were used. Plasmid pKT25 (kan^r) encodes the T25 fragment of *Bordetella pertussis* *cya*, corresponding to the first 224 amino acids of CyaA, and plasmid pUT18 (Amp^r) encodes the T18 fragment encompassing amino acids 225 to 399 of CyaA. The ORF of full-length or truncated P20 (Fig. 3B) were amplified by PCR from pBSF4. The full-length P20 ORF was fused in-frame to the C and N termini of T25 and T18 in pKT25 and pUT18, and the resulting plasmids were designated pKT25-P20 and pUT18-P20, respectively. The ORF of truncated P20 (N3/N4/N5/N6/N7/N8/C2/C3/C4/C5/C6/C7/C8/C9) (Table 2) was fused to the N terminus of T18, and the resulting plasmids were designated pUT18-NP20 or pUT18-CP20. The P20 ORF was cloned into *Pst*I/*Kpn*I sites of pKT25 and *Kpn*I/*Pst*I of pUT18. Plasmids pKT25-*zip* and pUT18-*zip* containing the leucine zipper gene *GCN4* of yeast served as positive controls, whereas the empty vectors were used as the negative controls (Karimova et al. 1998).

For transient expression assays, EGFP-tagged wild-type or mutant P20 was expressed under the control of 35S promoter of *Cauliflower mosaic virus* and the NOS terminator from pCass (Ding et al. 1995). To construct pCass-EGFP, the EGFP ORF was amplified from pEGFP with G5-5' and G3-3' as forward and reverse primers (Table 2), respectively, and after *Kpn*I and *Sac*I digestion, the EGFP ORF was inserted into pCass predigested with the same set of enzymes. The ORF of full-length P20 and the ARM deletion mutant P20 were generated from pBSF4 by PCR with the respective primers (Table 2) and were fused in-frame to the N terminus of the EGFP gene in pCass-EGFP, using *Stu*I and *Kpn*I as cloning sites; the clones were designated pCass-P20-EGFP and pCass-ΔD1.P20-EGFP. To construct pCass-D1.P20-EGFP, insert D1.P20 (only D1 of P20)-EGFP was amplified by PCR with the N84-5' and G3-3' primer pair and pCass-EGFP as a template. N84-5' is the forward primer, which contains the nucleotides of the D1 fragment of P20 and the amino terminus of EGFP. The PCR product was digested with *Kpn*I and *Sac*I and was used to replace the EGFP gene in pCass-EGFP. DNA manipulations were carried out according to the standard protocols (Sambrook et al. 2000).

For plant inoculation assay, pBSFM2 was constructed. The N terminal deletion DNA fragment of P20 gene was first amplified from pBSF4 (Lin et al. 1996) by PCR with the M2-5' and BS43-3' primer pair, followed by *Eco*NI digestion. The fragment was then used to replace the corresponding region of pBSF4.

All the constructed clones were shown to have the predicted DNA sequence and restriction enzyme digestion profile. The nucleotide sequence of the primers used for cloning is listed in Table 2.

In vitro translation.

P20, P20-His, P18, and P18-His were translated in vitro from transcripts of pBSF4, pET-P20 (Tsai et al. 1999),

pBSFM2, and pET-P18 (Tsai et al. 1999), respectively, in a TNT T7-coupled transcription/translation RRL system (Promega, Madison, WI, U.S.A.) as described by the manufacturer. P20 and P18 were labeled with [³⁵S]-methionine (Amersham Life Science, Buckinghamshire, U.K.) and the recombinant P20-His and P18-His were unlabeled.

Pull-down assay.

In vitro-translated P20-His or P18-His was immobilized on cobalt-sepharose beads (ProFound pull-down poly-His protein-protein interaction kit; Pierce Biotech, Boston) in wash buffer (WB) (25 mM Tris-HCl, pH 7.2, 0.15 M NaCl, and 40 mM imidazole) for 1 h at 4°C. The beads were then washed in WB and were incubated with ³⁵S-P20 or ³⁵S-P18 at 4°C for 2 h in WB. Assays were carried out with equimolar levels of input material. After being washed, the beads were boiled in SDS-PAGE sample buffer, and the protein bound to P20-His or P18-His was resolved by 15% SDS-PAGE and was detected by autoradiography.

Plant inoculation.

BaMV-S RNA was prepared from the BaMV-infectious cDNA clone pCB (EMBL/GenBank accession number AF018156) as previously described (Lin et al. 2004). SatBaMV RNA transcripts from pBSF4 or pBSFM2 were prepared by linearizing the plasmids with *Xba*I and transcription in vitro in the presence of a 5' cap analog (m⁷GpppG) with T7 RNA polymerase (Lin et al. 1996). Six leaves of 1-month-old *N. benthamiana* plants were coinoculated with 0.1 µg BaMV-S RNA and 0.1 µg of wild-type or mutant satBaMV RNA transcripts. Inoculated and uninoculated upper leaves were harvested at different times and were used for the extraction of total RNA and protein.

Chemical cross-linking.

P20 was expressed in *E. coli* cells harboring pET-P20 and was purified (Tsai et al. 1999). BaMV- and satBaMV-coinfected *N. benthamiana* leaves were harvested 5 dpi, and inoculated leaves were ground to powder in phosphate-buffered saline buffer. Homogenate was centrifuged at 3,000 × *g* for 5 min, and the crude protein was filtered through a Miracloth. For cross-linking, purified P20 in CAPS [3-(cyclohexylamino)-1-propane sulfonic acid] buffer (20 mM CAPS, pH 11, 0.1 M NaCl, 1 mM dithiothreitol, 10% glycerol) or crude protein extract from *N. benthamiana* leaves were incubated with 0.0001 or 0.025% glutaraldehyde at 25 or 30°C, respectively, for different time periods. The reaction was quenched with a final concentration of 0.1 M glycine, pH 8, and the samples were resolved by 10% SDS-PAGE. After Western blotting, P20 was probed with rabbit anti-P20 serum and was detected by an enhanced chemiluminescence (ECL) plus Western blotting detection system (Amersham Life Science).

BTH analysis.

A *cya*-deficient *E. coli* strain, BTH101 (*cya*⁻), was cotransformed with a combination of complementing plasmids: pKT25-P20/pUT18-P20, pKT25-P20/pUT18-NP20, or pKT25-P20/pUT18-CP20. The P20 coding region in the complementing plasmids harbored in the cotransformants was confirmed by PCR with the primer pairs used in cloning. Functional complementation mediated by the protein-protein interaction between P20 proteins fused to T25 and T18 was scored by colorimetric assay on MacConkey agar (Difco, Sparks, MD, U.S.A.) medium supplemented with 1% maltose, 0.5 mM IPTG, ampicillin (100 µg/ml), and kanamycin (50 µg/ml). The phenotype was analyzed after incubation at 30°C for two or three days. Functional complementation was quantified by β-galactosidase activity and

cAMP level in liquid culture of the cotransformants by using the β-galactosidase assay kit (Stratagene, La Jolla, CA, U.S.A.) and cAMP enzyme immunoassay system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), respectively. The magnitude of the two-hybrid reporter activity is associated with the strength of interaction between the two proteins. All protein fusions were tested for intrinsic complementation by cotransformation of pUT18 fusion plasmid and empty pKT25. Experiments were repeated four times, and data were analyzed statistically.

Biolistic bombardment.

Leaves of similar age were detached from 1-month-old *N. benthamiana* plants and were bombarded with 1 µg of pCass-EGFP, pCass-P20-EGFP, pCass-D1.P20-EGFP, or pCass-ΔD1.P20-EGFP coated onto 1-µm gold particles at a pressure of 450 psi by using a helium biolistic device (PDS 1000/He particle delivery system; BioRad, Hercules, CA, U.S.A.). Bombarded leaves were incubated in a humid chamber at 25°C for observation.

Microscopy.

Bombarded leaves were wet-mounted at the time of analysis. A Zeiss confocal laser scanning microscope (LSM 410; Carl Zeiss, Oberkochen, Germany) equipped with a 50 mW krypton/argon laser was used for scanning and imaging intracellular localization and cell-to-cell movement of EGFP-tagged wild-type and mutant P20. EGFP was excited and detected at 488 and 507 nm, respectively. Micrographs were prepared using PhotoShop 7.0 software (Adobe Systems, San Jose, CA, U.S.A.).

RNA dot-blot and Western blot analyses.

BaMV and satBaMV (BSF4 or BSFM2)-inoculated and upper leaves of *N. benthamiana* were harvested at different times. Total RNA was extracted (Verwoerd et al. 1989) from 0.15 g of leaves, and 0.2 µg of RNA was dot-blotted onto nylon membrane (PerkinElmer, Norwalk, CT, U.S.A.). Blots were hybridized with a specific riboprobe to detect positive-strand satBaMV RNA. For this purpose, ³²P-labeled RNA probe was transcribed from *Eco*RI-linearized pBSHE with T7 RNA polymerase (Lin et al. 1996). Hybridization signals were detected in PhosphorImager and were quantified by use of ImageQuant, version 5.2 (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Total proteins were extracted from 3.75 mg of inoculated or systemic leaves, were separated in 12.5% SDS-PAGE and then transferred onto polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA, U.S.A.). P20 or its truncated forms were probed with rabbit anti-P20 serum and were detected by an ECL plus Western blotting detection system, and the signals were quantified by use of MetaMorph Offline, version 3.2 (Universal Imaging Corporation, West Chester, PA, U.S.A.).

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