Nucleic acid-binding properties and subcellular localization of the 3a protein of brome mosaic bromovirus

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Brome mosaic bromovirus (BMV) 3a protein is required for cell-to-cell movement of the virus in host plants. The BMV 3a protein (B3a) was produced in Escherichia coli using an expression vector. Gel retardation analysis and UV cross-linking experiments demonstrated that B3a bound single-stranded RNA cooperatively without sequence specificity. Binding competition analysis showed that B3a bound to single-stranded nucleic acids more strongly than to double-stranded nucleic acids. Deletion mutagenesis located a nucleic acid-binding domain to amino acids 189–242. Western blot analysis of fractionated proteins of BMV-infected barley using monoclonal antibodies against B3a indicated that B3a may interact with membrane materials and form complexes in the cytoplasm. Immunogold labelling of thin sections of infected barley tissues revealed that B3a was associated with plasmodesmata and cytoplasmic inclusions.

Introduction

The cell-to-cell movement of plant viruses is assumed to occur through plasmodesmata, which are intercellular connections between plant cells that permit movement of macromolecules (reviewed by Lucas, 1995; Carrington et al., 1996). Virus-encoded movement protein (MP) has been shown to be involved in this process. Plant viruses can be categorized by their mechanism of cell-to-cell movement. Several groups of viruses, including tobacco mosaic virus (TMV), do not require coat protein (CP); its encoded MP induces an increase in the size exclusion limit of the plasmodesmata permeable space (Ding et al., 1995; Lucas, 1995; Wolf et al., 1989) and possesses nonspecific single-stranded (ss) RNA-binding activity to shape viral nucleic acids into a transferable form (Citovsky et al., 1990, 1992). Another group, including cowpea mosaic virus (CPMV) and cauliflower mosaic caulimovirus (CaMV), does require CP and its encoded MP participates in the formation of tubular structures which extend from plasmodesmata and in which virus-like particles are detected (van Lent et al., 1991). The fact that CaMV MP also has nucleic acid-binding properties suggests that two mechanisms of movement may exist concomitantly in a single virus infection (Citovsky et al., 1991). Besides these viruses, tobacco etch potyvirus (Dolja et al., 1994) and potato virus X (Oparka et al., 1996) require CP but tubule-mediated virion transport has not been reported.

Brome mosaic virus (BMV), a member of the Bromoviridae, is a tripartite RNA plant virus. It has three separately encapsidated positive-strand genomic RNAs. RNA1 and RNA2 encode nonstructural proteins 1a and 2a, respectively, both of which are required for viral RNA replication (Kroner et al., 1989, 1990). RNA3 encodes the nonstructural 3a protein (B3a) and CP, which are dispensable for RNA replication but required for the spread of infection in plants. Fluorescent in situ hybridization analysis reveals that the 3a gene is involved in virus cell-to-cell movement from initially infected cells (Schmitz & Rao, 1996). B3a dictates host specificity (Mise et al., 1993) and modulates symptom expression in susceptible hosts (Fujita et al., 1996; Rao & Grantham, 1995). Recently, BMV has been shown to require CP along with B3a for virus cell-to-cell movement (Schmitz & Rao, 1996) and virus-like particles have been observed in tubules extending from protoplasts transfected with BMV (Kasteel et al., 1997).

Speculation concerning the function of B3a has been based on sequence similarity with other viral proteins described above, but has not been studied directly in vitro. To gain information on the MP of BMV, we have examined the nucleic acid-binding properties of B3a produced in Escherichia coli and identified the RNA-binding domain using deletion mutants. Furthermore, we have observed the localization of B3a in infected tissues immunologically by fractionation and electron microscopy, and discuss here the mechanism of BMV cell-to-cell movement.
Methods

Expression and purification of B3a and its deletion mutants. Plasmid pBC3KM11 (Mise et al., 1993) was digested with BamHI and BglII, and the resulting 0.9 kb fragment, which contains the entire region of the 3a gene, was cloned into the BamHI site of the T7 polymerase vector pET3b (Novagen) to generate pEB1. An NdeI site was introduced at the translation initiation codon of the 3a gene in pBC3KM11 by PCR using the sense primer BC3NdeI (5′ GGATC- CATATGTCTAAGA 3′, where the NdeI site is underlined) and the antisense primer KM11-4 (5′ GCCGGTTCTGATTCTTC 3′). The PCR-amplified fragment was digested with NdeI and ClaI and then replaced with the corresponding fragment of pEB1 to generate pB3a. The NdeI restriction site of pET3b induces the translation initiation signal so that plasmid pB3a expresses the 3a protein without alterations to the coding sequence.

To obtain a set of deletions of the 3a ORF (Fig. 1), we used a series of PstI restriction sites introduced by mutagenesis (Δ) are represented in the upper diagram (WT) with amino acid numbering given above. The deleted region and the remaining portions of the wild-type sequence are represented by horizontal lines and shadowed rectangles, respectively.

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**Fig. 1.** Schematic illustration of the deletion mutants constructed within B3a and their ability to bind ss RNA. The position of PstI restriction sites introduced by mutagenesis (Δ) are represented in the upper diagram (WT) with amino acid numbering given above. The deleted region and the remaining portions of the wild-type sequence are represented by horizontal lines and shadowed rectangles, respectively.

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**Fig. 2.** Analysis of the expression and purification of wild-type and deletion mutants of B3a. Proteins were analysed on a 15% polyacrylamide–SDS gel and visualized by Coomassie blue staining. The positions of the molecular mass markers (kDa) are indicated.

**Fig. 3.** Electrophoretic gel retardation assay for RNA binding by B3a. The indicated amount of B3a was incubated in 10 µl of buffer B with 1 ng of 32p-labelled transcripts derived from BMV RNA3 cDNA clone (A) and bacterial expression vector (B), and the mixture was electrophoresed in a 4% nondenaturing polyacrylamide gel. (C) Competition analysis with B3a. B3a (400 ng in 10 µl) was incubated with 1 ng of labelled ss RNA probe either in the absence (lane 2) or presence (lanes 3–10) of nonlabelled or labelled competitors: ss RNA (BMV RNA, lanes 3 and 4), ds RNA (RDV RNA, lanes 5 and 6), ss DNA (heat-denatured plasmid DNA fragment, lanes 7 and 8), ds DNA (plasmid DNA fragment, lanes 9 and 10). Two levels of competitor were used: 250 ng (lanes 3, 5, 7 and 9) or 1 µg (lanes 4, 6, 8 and 10). Lane 1, probe only.
of plasmids designated pBC3OMn (n = 1–7) (kindly provided by N. Okitsu and Y. Fujita, Kyoto University, Kyoto, Japan) which each have a unique PstI site at various locations in the 3a ORF of pBC3KM11. The PstI (CTGCAG) sites were introduced into pBC3KM11 by inserting four nucleotides (TGCA) between two adjacent codons. The third nucleotide of the upstream codon was substituted with a guanosine. The internal deletions were created by reassembling the small NdeI–PstI fragment and the large NdeI–PstI fragment from two mutants carrying the PstI site at successive locations. Four extra nucleotides in the PstI site were eliminated by PstI digestion, treatment with T4 DNA polymerase and subsequent religation to generate pBC3OM. To express 3a proteins, the pB3a and pB∆1–3), respectively, by PCR as above. The PCR fragment containing deletions in the 3a ORF was digested with Ndel and BglII and cloned into the Ndel–BglII fragment of pBC3a. To create mutants pB∆2, pB∆3 and pB∆4, an Ndel site was introduced into pBC3OMn, respectively, by PCR as above. The PCR fragment containing deletions in the 3a ORF was digested with Ndel and BglII and cloned into the Ndel–BglII sites of pET3b. Mutant pB∆5 was created by ligation of the large Scal fragment of pBC3OMn with the small Scal fragment of pB3a while mutants pB∆6 and pB∆7 were generated by ligation of large Clal fragments of pBC3OMn and pBC3OMn, respectively, with the small Clal fragment of pB3a. To create mutant pB∆8, pBC3OMn linearized with PstI digestion and blunt-ended with T4 DNA polymerase was first ligated to a phosphorylated fragment containing deletions in the 3a ORF was digested with BglII and cloned into the pET3b. The small Clal fragment of the resulting plasmid was used to replace the corresponding Clal fragment of pB3a.

To express 3a proteins, the pB3a and pB∆n (n = 1–8) plasmids were transfected into E. coli strain BL21(DE3) (Studier & Moffatt, 1986) and overexpressed as indicated in the manufacturer’s instructions (Novagen). The cells were harvested and lysed by sonication for 15 min at 4 °C (sonic source Kubota 201M, 150 W) in buffer B (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 100 mM NaCl). The insoluble fraction was pelleted by centrifugation at 3000 g for 5 min at 4 °C. The pellets were washed twice with buffer B containing 0.5% Triton X-100 and 10 mM EDTA, and then washed twice with buffer B. Proteins were solubilized with buffer B containing 8 M urea and renatured by dialysis at 10 °C against buffer B. The insoluble debris resulting from the dialysis was pelleted and the concentration of the soluble fractions was determined by the Bradford assay (Bradford, 1976) using BSA (fraction V, Sigma) as a standard.

Gel retardation analysis and UV cross-linking assay. pB3TP8 (Janda et al., 1987), containing a full-length cDNA copy of RNA3 of BMV, was digested with Clal, and the first 0.6 kb of the BMV RNA3 was uniformly labelled with [5-32P]UTP in an in vitro transcription reaction using T7 RNA polymerase. Similarly, the bacterial vector plasmid pBluescript II KS+ (Stratagene) was digested with Ddel and 0.6 kb labelled transcript was synthesized using T7 RNA polymerase. The resulting RNA transcripts were purified over a Sephadex G-50 (Phar-
Fig. 6. For legend see facing page.
macia) column. Typically, 1 ng of transcripts and 0.05–2 µg of proteins in a total volume of 10 µl were used in a binding reaction. Reaction mixtures were incubated in buffer B for 30 min on ice, and the mixture was either subjected to PAGE in nondenaturing 4% polyacrylamide gels in TBE buffer (Sambrook et al., 1989) or irradiated with UV light for 10 min using a UV light source (CSL-4LC; Cosmo Bio, 4 W at 254 nm), treated with RNase A and analysed by SDS–PAGE (Laemmli, 1970). The gels were dried and autoradiographed or analysed by a digital radioactive imaging analyser (Fuji BAS2000; Fuji). For competition analysis, BMV RNA (ss RNA), rice dwarf virus (RDV) RNA [double-stranded (ds) RNA], Sambrook et al. (1989) or irradiated with UV light for 10 min using a UV light source (CSL-4LC; Cosmo Bio, 4 W at 254 nm), treated with RNase A and analysed by SDS–PAGE (Laemmli, 1970). The gels were dried and autoradiographed or analysed by a digital radioactive imaging analyser (Fuji BAS2000; Fuji). For competition analysis, BMV RNA (ss RNA), rice dwarf virus (RDV) RNA [double-stranded (ds) RNA], Smal-digested and heat-denatured vector plasmid pUC118 (Takara Shuzo) (ss DNA) and Smal-digested pUC118 (ds DNA) were used as competitors.

**Production of monoclonal antibodies.** Mouse monoclonal antibody specific to B3a was produced using E. coli-expressed B3a as immunogen. B3a was purified as above and injected into BALB/c mice intraperitoneally. Spleen cells of the immunized mice and sp2/O-Ag14 myeloma cells were fused by polyethylene glycol as previously described (Galfre et al., 1977). Two clones of anti-B3a monoclonal antibodies, designated B3-1 and B3-16, were used for subsequent experiments.

**Subcellular fractionation of barley and immunodetection.** The systemic host barley (Hordeum vulgare cv. Hinode-hadaka) was grown in a plant growth room as previously described (Fujita et al., 1996), infected with BMV-M1 (Ahlquist et al., 1984) and plant tissue extract was fractionated as previously described (Rouleau et al., 1994). Briefly, 1 g of leaf tissue from BMV-infected barley secondary leaves (4 days post-inoculation) was homogenized with a pestle and mortar in 4 ml homogenization buffer. The homogenate was filtered through Miracloth (Calbiochem) to obtain the cell wall (CW) fraction. The filtrate was centrifugated at 10000 g for 10 min to recover the pellet fraction (P1). The supernatant solution was centrifuged at 30000 g for 30 min to generate a supernatant fraction (S30) and pellet fraction (P30). S30 was further centrifuged at 100000 g for 60 min to generate a supernatant fraction (S100) and pellet fraction (P100). The CW fraction was washed with homogenization buffer containing 2% Triton X-100. Proteins were extracted from each fraction as previously described (Laemmli, 1970), and separated by SDS–PAGE. Immunoblot analysis was carried out as previously described (Towbin, 1979) using an Immobilon-P transfer membrane (Millipore). B3a was detected using mouse monoclonal antibody B3-1 and an alkaline phosphatase-conjugated goat anti-mouse secondary antibody.

**Immunocytochemical methods.** Barley secondary leaves infected with BMV-KU2 (Nagano et al., 1997) or BMV-M1 were cut and fixed with 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde. After dehydration, the specimens were soaked in propylene oxide and 0.5–2% paraformaldehyde and 0.1% glutaraldehyde. Embedding was carried out as previously described (Towbin, 1979) using an Immobilon-P transfer membrane (Millipore). B3a was detected using mouse monoclonal antibody B3-1 and an alkaline phosphatase-conjugated goat anti-mouse secondary antibody.

**Results**

**Expression and purification of the 3a proteins**

B3a was expressed in E. coli cells carrying plasmid pB3a, which contains the coding region of B3a linked to a strong bacteriophage T7 promoter. Induction of expression of the T7 RNA polymerase resulted in the synthesis of a protein with the expected size (molecular mass 32 kDa). B3a formed insoluble aggregates which could be separated from the soluble bacterial proteins by centrifugation. However, B3a could be solubilized in 8 M urea and remained soluble after removal of urea. The purified B3a gave a single band on SDS–PAGE followed by Coomassie blue staining (Fig. 2, lane 1). The deletion mutants of B3a using plasmids pΔn (n = 1–8) were expressed and purified as above (Fig. 2, lanes 2–9, respectively).

**RNA-binding properties**

Binding of B3a to ss RNA was assayed by electrophoretic gel retardation in polyacrylamide gels. Fig. 3(A) and (B) illustrate the binding of B3a to two kinds of 32P-labelled 0.6 kb RNA, transcribed from a BMV RNA3 cDNA clone or bacterial vector plasmid (pBluescript), respectively. At low protein concentrations, the RNA3 fragment probe migrated to the same distance as the protein-free RNA, but when 0.4 µg or more of B3a was added, the probe barely entered the gel matrix (Fig. 3 A), indicating that the RNA formed a complex with B3a. Moreover, B3a also bound to pBluescript-derived transcript in the same manner (Fig. 3 B), suggesting that B3a has no sequence specificity in binding to RNAs. When B3a was heat-treated prior to assay, RNA-binding activity of B3a was abolished (data not shown). The relative stability of B3a–RNA complexes at different salt concentrations was analysed by a UV cross-linking assay. B3a bound RNA maximally at 50 mM NaCl, at 200 mM NaCl, the B3a–RNA complexes were fully dissociated (data not shown).

We also investigated whether B3a binds preferentially to particular types of nucleic acid. Competition binding assays were performed by incubating 32P-labelled RNA probe and unlabelled competitor nucleic acids together with the B3a, followed by gel retardation analysis. The ss nucleic acids were able to compete efficiently with the labelled transcript for
binding to B3a at low mass ratio (Fig. 3 C, lanes 3, 4, 7 and 8), while ds nucleic acids were able to compete only at higher mass ratio (Fig. 3 C, lanes 5, 6, 9 and 10), suggesting that B3a binds to ss nucleic acids more strongly than to ds nucleic acids.

RNA binding by B3a deletion mutants

To map the B3a domains involved in RNA binding, we expressed and purified a series of deletion mutants of B3a (Fig. 2, lanes 2–9). The RNA-binding activity was assessed by a UV cross-linking assay as described in Methods. A comparison of the RNA-binding activity of B3a deletion mutants 1–8 showed that mutant 6 (Δ189–242) failed to bind RNA, while the other mutants all bound RNA at readily detectable levels (Fig. 4 A).

Subcellular localization of B3a in barley tissues

The monoclonal antibodies raised against the bacterially produced B3a allowed investigation of its subcellular localization in BMV-infected barley. On Western blots, both of the two monoclonal antibodies, B3-1 and B3-16, reacted with B3a synthesized in E. coli and in barley plants infected with BMV-M1 or KU2 strain, while no proteins were immunoreactive in total protein from mock-inoculated plant homogenates (data not shown). We conclude that the monoclonal antibodies specifically react with B3a. To determine the intracellular localization of B3a, BMV-infected barley leaves were fractionated by differential centrifugation to produce CW, 1000 g pellet (P1), 30000 g pellet (P30) and 30000 g supernatant (S30) fractions and were then analysed by Western blotting for their B3a content. Immunodetection revealed that B3a was found predominantly in the P30 and S30 fractions (Fig. 5, lanes 3 and 4), although trace amounts of B3a were also detected in the CW fraction (Fig. 5, lane 1). When the S30 fraction was further fractionated by 100 000 g centrifugation, part of the B3a was pelleted into the P100 fraction (Fig. 5, lane 5).

Intracellular localization

To investigate further the location of B3a in planta, ultrathin sections of the non-inoculated secondary leaves of mock-inoculated and BMV-infected barley were immunogold-labelled using the anti-B3a antibodies. In mock-infected barley tissues, no specific labelling with anti-B3a antibodies was observed (Fig. 6 A). B3a was found to be associated with electron-dense inclusions in the cytoplasm (Fig. 6 B). These inclusions were BMV infection-specific and contained membranous materials and some vesicle-like regions. B3a was also observed in the longitudinal (Fig. 6 C, D) and cross sections (Fig. 6 E, F) of plasmodesmata. No specific labelling with anti-B3a antibodies was present inside cellular organelles such as the chloroplasts, mitochondria and microbodies, and the central vacuoles were devoid of labelling. There was no difference between results from inoculations with BMV-KU2 and BMV-M1 strains (data not shown).

Discussion

In this study, we have investigated the functional properties of B3a which are required for BMV cell-to-cell movement. We expressed B3a in E. coli and showed that B3a possesses RNA-binding activity in vitro. Immunological studies using monoclonal antibodies against B3a revealed that B3a localized in plasmodesmata and cytoplasmic inclusions in BMV-infected barley tissues.

RNA-binding properties

B3a cooperatively bound RNA in a sequence-nonspecific manner similar to the other viral MPs studied previously. The RNA–protein complexes formed by B3a were more sensitive to NaCl than are those formed by the TMV 30 kDa MP (Citovsky et al., 1990) and were almost as sensitive as those formed by the MPs of CaMV (Citovsky et al., 1991), alfalfa mosaic virus (AlMV) (Schoumacher et al., 1992) and cucumber mosaic virus (CMV) (Li & Palukaitis, 1996). B3a also bound ss DNA, ds RNA and ds DNA and, like AlMV MP (Schoumacher et al., 1992), showed higher affinity to ss than to ds nucleic acids. Furthermore, we showed that there is an RNA-binding domain located between amino acids 189 and 242. This is a predominantly hydrophilic region with relatively high surface probability similar to the RNA-binding domain of TMV (Citovsky et al., 1992), AlMV (Schoumacher et al., 1994), red clover necrotic mosaic virus (Osman et al., 1993), CaMV (Thomas & Maule, 1995) and CMV (Vaquero et al., 1997). This region includes a large number of positively-charged residues which could form ionic bonds with negatively-charged phosphate groups. BMV and CMV are both members of the family Bromoviridae and an amino acid homology search showed that the RNA-binding domain of B3a is related to that of CMV MP (Melcher, 1990; Vaquero et al., 1997) (Fig. 4 B). Additionally, the RNA-binding activity of B3a was affected by heat treatment, suggesting that the activity is structure-dependent, similar to CMV (Li & Palukaitis, 1996).

Localization

Subcellular fractionation analysis showed that B3a was present in the CW fraction. Consistent with this, immunogold labelling of ultrathin sections of BMV-infected barley revealed that B3a was localized in plasmodesmata within the cell wall. These data suggest the involvement of B3a in virus movement through plasmodesmata, including modification of plasmodesmata. However, we have not observed the distinct tubules with virus-like particles in plasmodesmata which are typical of CPMV-like plant viruses (van Lent et al., 1991), and we could not affirm how BMV moves through plasmodesmata.

We have shown that B3a was also associated with electron-dense inclusions containing membranous materials. This was consistent with the results of subcellular fractionation analysis.
showing that considerable amounts of B3a were present in the P30 fraction which should be rich in cytoplasmic membrane material (Deom et al., 1990). It has previously been shown by immunocytochemical analysis using polyclonal antibody against B3a (Hosokawa et al., 1992) that B3a localizes in cytoplasmic inclusions characteristic of BMV infections. We have confirmed this using monoclonal antibodies against B3a.

In subcellular fractionation studies, at least part of the B3a in the 30000 g supernatant fraction was pelleted by 100000 g centrifugation, suggesting that B3a may form complexes, rather than exist as free protein. These complexes could be associations between B3a and fragmented subcellular structures such as the endoplasmic reticulum. Alternatively, these structures might be nucleoproteins involved in BMV cell-to-cell movement as speculated for TMV (Citovsky et al., 1990).

Cell-to-cell movement of BMV

The results presented here suggest that B3a may form complexes with BMV RNA which function as intermediates in virus cell-to-cell movement. On the other hand, recent studies have provided evidence that BMV requires CP for successful cell-to-cell movement (Schmitz & Rao, 1996) and possesses tubule-forming properties (Kasteel et al., 1997), suggesting that BMV moves through tubules assembled in plasmodesmata as virus particles. Similarly, CaMV and AIMV MP also induce tubular structures (Linstead et al., 1988; Kasteel et al., 1996, 1997) and possess RNA-binding activity (Citovsky et al., 1991; Schoumacher et al., 1992). This suggests that at least two mechanisms of movement may co-exist in a single virus infection (Citovsky et al., 1991), and that BMV infection may be an example.

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References


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