Identification of a Movement Protein of Rice Yellow Stunt Rhabdovirus

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Rice yellow stunt rhabdovirus (RYSV) encodes seven genes in its negative-sense RNA genome in the order 3'-N-P-3-M-G-6-L-5'. The existence of gene 3 in the RYSV genome and an analogous gene(s) of other plant rhabdoviruses positioned between the P and M genes constitutes a unique feature for plant rhabdoviruses that is distinct from animal-infecting rhabdoviruses in which the P and M genes are directly linked. However, little is known about the function of these extra plant rhabdovirus genes. Here we provide evidence showing that the protein product encoded by gene 3 of RYSV, P3, possesses several properties related to a viral cell-to-cell movement protein (MP). Analyses of the primary and secondary protein structures suggested that RYSV P3 is a member of the "30K" superfamily of viral MPs. Biolistic bombardment transcomplementation experiments demonstrated that RYSV P3 can support the intercellular movement of a movement-deficient potexvirus mutant in *Nicotiana benthamiana* leaves. In addition, Northwestern blot analysis indicated that the RYSV P3 protein can bind single-stranded RNA in vitro, a common feature of viral MPs. Finally, glutathione S-transferase pull-down assays revealed a specific interaction between the RYSV P3 protein and the N protein which is a main component of the ribonucleocapsid, a subviral structure believed to be involved in the intercellular movement of plant rhabdoviruses. Together, these data suggest that RYSV P3 is likely a MP of RYSV, thus representing the first example of characterized MPs for plant rhabdoviruses.

Rice yellow stunt virus (RYSV) is a member of the plant-infecting *Nucleorhabdovirus* genus of the *Rhabdoviridae* family (35). RYSV has a nonsegmented, negative-sense single-stranded RNA genome which contains a 5' trailer, a 3' leader, and seven open reading frames (ORFs) in the order 3'-le-N-P-3-M-G-6-L-tr-5' (14). Five of the ORFs, i.e., N, P, M, G, and L, encode the nucleocapsid protein (12), the phosphoprotein (39), the matrix protein (22), the glycoprotein (23), and the RNA polymerase (14), respectively, based on their map positions or sequence similarities when compared to the counterparts of other characterized rhabdoviruses. However, the functions of P3 and P6, encoded by ORF3 and ORF6, are unknown. Gene 3 is located between the P and M genes (9) where additional ORFs have been identified in the genomes of all plant rhabdoviruses examined so far (*Sonchus yellow net virus* [SYNV] [30], *Lettuce necrotic yellows virus* [LNYV] [38], *Northern cereal mosaic virus* [32], *Maize mosaic virus* [MMV] [GenBank accession no. NC_005975] and *Maize fine streak virus* [MFSV] [GenBank accession no. NC_005974]). Such genes have not been found in animal-infecting rhabdoviruses (10). In addition, RYSV P3 and its analogues SYNV sc4 (30), LNYV 4b (GenBank accession no. AAG32647), MMV P3 (GenBank accession no. YP_052852) and MFSV P4 (GenBank accession no. YP_052846) all have similar molecular masses of about 30 kDa. These unique plant viral proteins must possess special functions intrinsic to the life cycles of plant viruses, e.g., systemic spread in the plant host or some other aspect such as insect-host interactions.

The movement of a plant virus from the initial site of infec-

tion into adjacent cells and the long-distance transport of the virion are essential for establishing successful systemic infections of the plant host. Specialized viral-encoded movement proteins (MPs) have evolved to facilitate transport of infectious viral derivatives through the plasmodesmata for nearly all plant viruses studied. At least five types of MP have been described: the products of a triple gene block, the tymovirus MPs, a series of small polypeptides (less than 10 kDa), the hsp70-like proteins, and a large number of members of the so-called "30K" superfamily, named after the 30-kDa *Tobacco mosaic virus* (TMV) MP (1, 25). Two general mechanisms of the 30K superfamily MPs, i.e., the TMV-like mechanism and the tubule-based mechanism, for cell-to-cell movement of plant viruses have been described. In the TMV-like mechanism, viruses spread from cell to cell as nucleoprotein complexes consisting of viral RNA and MP without the involvement of the coat protein (CP). The tubule-based mechanism generally requires MP(s) as well as CP for cell-to-cell movement of an encapsidated form (a subviral or virus particle) (5, 18, 19). The majority of acknowledged MPs are from positive-strand RNA viruses, and in the case of an ambisense RNA virus, i.e., *Tomato spotted wilt tospovirus*, the MP has been characterized (31). However, little is known about how the plant rhabdoviruses spread in host cells. The negative RNA genome of rhabdoviruses is associated with the N protein to form the ribonucleocapsid in vivo, and its transcription and replication are predicted to also need the virus-encoded P and L proteins (4, 34). Therefore, the movement of plant rhabdoviruses should involve the ribonucleoprotein complex containing the viral RNA and the N, P, and L proteins, which represents the minimal infectious component of rhabdoviruses.

The 30K superfamily of viral movement proteins has been demonstrated to have a variety of activities, including the ability to bind nucleic acids, to move to neighboring cells after

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microinjection or biolistic bombardment, to facilitate movement of RNA to neighboring cells, and to form tubular structures. Although the 30K MPs have similar functions, they exhibit very limited conservation of amino acid sequence (2, 25). Instead, it has been suggested that a common three-dimensional structure built of a series of similarly organized secondary structure elements forms the basis for recognition of the putative 30K superfamily members (25). By the alignment of predicted secondary structures, a consensus core structure that contains a series of β -elements flanked by an α -helix on each end was generated. By using this criteria to evaluate 30K superfamily members that were not previously acknowledged, the SYNIV sc4 protein (25) and the LNYV 4b protein (<http://opbs.okstate.edu/Virevol/Web/Capillo.html>) were predicted to be putative MPs. In fact, earlier work showing that SYNIV sc4 is a membrane-associated protein has led to a postulation that sc4 may have a role in potentiating cell-to-cell movement during systemic infection (30). However, direct experimental evidence has not been generated to support such predictions.

In this study, we indicate that the predicted secondary structures of the RYSV P3 have a significant similarity with the consensus core structure of the 30K superfamily members. Furthermore we demonstrate the ability of the RYSV P3 protein to complement the cell-to-cell movement of a movement-defective *Potato virus X* (PVX) derivative by using a biolistic bombardment-mediated transcomplementation assay. In addition, we show that P3 can bind RYSV RNA and N protein in vitro, indicative of its capacity for recognition and transport of the RYSV nucleocapsid core. These data suggest the P3 protein is the RYSV MP and provide the first direct evidence for a MP in a plant rhabdovirus.

MATERIALS AND METHODS

Biolistic bombardment-mediated transcomplementation assay. The full-length cDNA clone of PVX, pP2C2S (8), was kindly provided by D. C. Baulcombe (The Sainsbury Laboratory, John Innes Centre, Norwich, United Kingdom). pP2C2S was linearized by Bsp120I at position 4945 in the ORF of p25 in the PVX genome, blunt-ended with the Klenow fragment of DNA polymerase I (Promega), and religated, to provide pP2C2S(TrP25), a movement-deficient clone of PVX expressing the C-terminally truncated product of the PVX p25 protein (1, 26).

In order to generate a PVX reporter derivative that was deficient in movement, the β -glucuronidase (GUS) gene fragment in pBI221 (17; from Clontech) was excised by double-digestion with BamHI and EcoRI (a neoschizomer of SacI) and inserted into pBlueScript-II-SK⁻ (pSK) (Stratagene) between the BamHI and EcoRV sites to form pSK-GUS. Then, pSK-GUS was double-digested with EcoRI and SalI, and the GUS gene fragment was inserted between the EcoRV and SalI sites of pP2C2S(TrP25) to obtain pP2C2S(TrP25)-GUS.

To prepare a *Cauliflower mosaic virus* 35S promoter-driven clone expressing movement-deficient PVX-GUS, the 5' 550-nucleotide (nt) sequence of the PVX genome was first amplified by PCR with the forward primer corresponding to the 5' end of the PVX genome (5'-ggactagtGAAACTAAACCATACAC-3') with an additional SpeI site (underlined; the additional nucleotides are in lowercase) and a reverse primer (5'-TCGGATCCAAGAGTGCAG-3') corresponding to nucleotides from positions 550 to 533 of the PVX genome, which contains a BamHI site (underlined). The resulting PCR product was double-digested with SpeI and BamHI and inserted between the XbaI and BamHI sites of pBI121 (17; from Clontech) to produce the plasmid pPVX550. Then pP2C2S(TrP25)-GUS was double-digested with BamHI (partial) and SacI and the PVX-GUS sequence was inserted into the BamHI and SacI sites of pPVX550 to yield pPVX(TrP25)-GUS.

To prepare a 35S promoter-driven cDNA clone of gene 3 of RYSV, gene 3 was first amplified by PCR with a gene 3-specific forward primer (5'-aaaacccgggATGGGCGAGGGGAAGAACACC-3') containing a SmaI site (underlined)

fused to the 5' end of ORF3 and a reverse primer (5'-aaaagagctcGGCTGCTTGTGGAGTTTGC-3') complementary to the 3' nontranslated region of the gene 3 sequence that was fused to an additional SacI site (underlined). The PCR product was ligated into the pGEM-T vector (Promega) to generate the plasmid pGEM-P3, in which the gene 3 was located downstream of the T7 promoter. Then, the gene 3 sequence was moved into pBI121 downstream of the 35S promoter via ligation into the SmaI and SacI sites to generate the plant expression vector pBI-P3.

To prepare a 35S promoter-driven clone expressing the PVX p25 protein, the p25 gene was first amplified by PCR with a specific forward primer (5'-tctaGAGGCACTGTCTCACTTT-3') with an additional XbaI site (underlined) and a reverse primer (5'-gagctcGACCTAATACTATGTACAC-3') with an additional SacI site (underlined). The PCR product was double-digested with XbaI and SacI and inserted between the XbaI and SacI sites of pBI221 to obtain pBI-P25.

Biolistic bombardments of *Nicotiana benthamiana* and *Oryza sativa* leaves were performed using a PDS1000 instrument (Bio-Rad). Briefly, 10 μ l of plasmid DNA (at 1 μ g/ μ l) was precipitated onto 5-mg of 1.0- μ m gold particles. In the bombardment experiments, 5- μ l DNA (at 1 μ g/ μ l) of each plasmid was mixed and applied to gold particles. Then, the coated particles were washed and resuspended according to the supplier's instructions. One milligram of coated particles was used in each shot. *N. benthamiana* and *O. sativa* leaves were placed in the center of a petri dish and bombarded at a target distance of 9 cm with 1,350-lb/in² rupture disks. For each of the constructs used in this research, the bombardment was repeated at least three times. The bombarded leaves were kept in the dark for 2 to 3 days before GUS expression was monitored by histochemical staining (26).

P3 protein expression. To provide a P3 derivative that could be immunodetected, pGEM-P3 was double-digested with SmaI and SacI, and the P3 sequence was inserted between the SacI site and a filled-in BamHI site of the pQE32 vector (QIAGEN) to yield pQE32-P3 in which the P3 sequence is in-frame fused to the His₆ tag sequence. Following transformation of *Escherichia coli* M15 (prep4) (QIAGEN) with pQE32-P3 and induction by isopropyl- β -D-thiogalactopyranoside (IPTG), the His₆-tagged P3 (His-P3) fusion protein was expressed and evaluated by immunoblotting with a Penta-His monoclonal antibody (QIAGEN) as the primary antibody, an AP-conjugated anti-mouse immunoglobulin G (Promega) as the secondary antibody, and 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (Promega) for color development.

Northwestern blot analysis for P3-RNA interactions. DNA sequences corresponding to 191 nt of the 5' trailer region and 194 nt of the 3' leader region of the RYSV genomic RNA (36), respectively, were obtained by PCR amplifications. The primer set for the 5' trailer region was 5'-gttAACTCTCATCGACGACAAAAC-3' (the additional nucleotides in lowercase were added to produce a HpaI site) and 5'-AATTAACCCCTCACTAAAACACCACCATATCCAAAGCC-3' (the T3 promoter is underlined). The primer set for the 3' leader region was 5'-gtACACCACCAGATACATTCTG-3' (the additional nucleotides in lowercase were added to produce a RsaI site) and 5'-AATTAACCCCTCACTAAAAGGCCTATCTCTTATTGACATAT-3' (the T3 promoter is underlined). The PCR products were cloned into the pGEM-T vector to construct the pGEM-trailer and pGEM-leader plasmids, respectively.

To prepare the RNA transcripts, the pGEM-trailer and pGEM-leader plasmids were first linearized by HpaI or RsaI, respectively, and then transcribed in vitro by using a T3 Riboprobe in vitro transcription system (Promega) and [α -³²P]UTP. The DNA template was removed by digestion with RNase-free DNase and the labeled RNA probes were purified over a Push-Column (Merck).

To prepare control RNA probes derived from the polylinker regions of pBlueScript-II-KS⁻ (pKS) (Stratagene) and pSK vectors, the plasmids were linearized by EcoRI or KpnI, respectively. The linear pSK was further treated with the Klenow fragment of DNA polymerase I to remove the 3'-overhanging nucleotides resulting from KpnI digestion. In vitro transcription and purification of RNA transcripts were essentially as described above.

For Northwestern blot assays, protein samples were separated by sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) and then transferred to an NC nitrocellulose membrane (Schleicher & Schuell) using a Semi-Dry electrotransfer (Bio-Rad). Then, the membrane was washed three times for 30 min by gently shaking at 30°C in the binding buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 0.1% Triton X-100, 0.02% Ficoll 400, and 1 $\times$ Denhardt's reagent) for renaturation of the proteins (27). This was followed by a 3-h incubation in the binding buffer in the presence of a ³²P-labeled RNA probe and three washes with the binding buffer. The membrane was air-dried followed by autoradiography.

GST pull-down assays for protein-protein interactions. To prepare a glutathione S-transferase (GST)-N fusion protein, the entire N protein ORF was

FIG. 1. The secondary structure alignment of RYSV P3 and putative rhabdovirus MPs with the central region of the 30K superfamily. The symbols $\>$, $\<$, $\>$, and $-$ represent α -helical structure, β -element, loop structure, and no prediction, respectively (25). The secondary structure predictions of RYSV P3, SYNV sc4, LNYV 4b, MMV P3, and MFSV P4 were derived by using the PHD server (28). A, B, C, D, E, NA, and NB: α -helices. 1, 2, 3, 4, 5, 6, 7, N1, and N2: β -elements. The predicted consensus secondary structure of the 30K superfamily central region is adapted from that of Melcher (25). Filled blocks refer to the α -helical structures and empty blocks the β -elements. Gaps are introduced to facilitate alignment.

excised from the pN plasmid that harbors the RYSV N gene (12), and the ORF was inserted between the BamHI and EcoRI sites of the pGEX-3X vector (Amersham Pharmacia) to provide an in-frame fusion with the GST gene. Following transformation of *E. coli* JM109 with the recombinant clone pGEX-3X-N and induction by IPTG, the GST-N fusion protein was expressed and purified with a Bulk GST Purification Module kit (Amersham Pharmacia).

To prepare the [35 S]Met-labeled P3 protein, pGEM-P3 was used as a template for TNT Quick Coupled Transcription & Translation System (Promega) with [35 S]Met (NEN) as a label. The protein products were separated by SDS-PAGE and detected by phosphorimaging and autoradiography. The [35 S]Met-labeled luciferase was prepared by using a template provided by a TNT kit.

For GST pull-down assays, the glutathione Sepharose 4B matrix (Amersham Pharmacia) was loaded with either the purified GST-N fusion protein, purified GST protein, or no protein and incubated in STE buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) for 1 h at room temperature. After the beads were washed with phosphate-buffered saline buffer three times, the [35 S]Met-labeled P3 protein or [35 S]Met-labeled luciferase (used as a control) was added to each tube for a second 1-h incubation at room temperature. After extensive washing, the proteins bound to the beads were eluted and denatured by adding 2 $\times$ sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.2% bromophenol blue, 2% mercaptoethanol) and heating for 3 min at 90°C. Proteins were resolved on SDS-12% PAGE and detected by autoradiography.

Deletion mutants of the P3 protein were expressed from subclones of pGEM-P3 or PCR-amplified fragments by using specific primers (see Fig. 6A). For preparation of the N-terminal deletion mutant M1, the gene 3 fragment between the BglII and SacI sites of pGEM-P3 was inserted into compatible sites of pSK to generate pSK-3BS. Then, the gene 3 fragment between the SmaI and SacI sites of pSK-3BS was recovered and inserted between the same sites of pGEM-P3. For generation of the deletion mutants M2 and M3, pGEM-P3 was first digested by SmaI and StuI or by NcoI, respectively, followed by self-ligation. For preparation of the C-terminal deletions M4 and M8, pGEM-P3 was double-digested with EcoRV and BsrBRI or with BglII and BclI, respectively, followed by self-ligation. For generation of C-terminal deletions M5, M6, and M7, the pGEM-P3 fragment located between the SmaI and SacI sites, which contained the entire gene 3 ORF, was first subcloned into pUC19 to form pUC19-P3. The 3'-truncated gene 3 fragments were amplified by PCR by using the forward primer T7-3-1 (5'-TAATACGACTCACTATAGGGCGAATGGGCGAGGGG AAGAACCACC-3') corresponding to the 5' end of ORF3 plus a T7 promoter (underlined) and one of three reverse primers: 3-447 (5'-ttaGGCCGGGTCCC ACGAT-3') for M5; 3-359 (5'-tTAGTTCCCTCCAGTGA-3') for M6; or 3-270 (5'-ttaAGTGTGATCTTCTTCGTC-3') for M7 (the additional nucleotides in lowercase were added to produce stop codons). For generation of the M9 mutant, the gene 3 fragment between BglII and BclI of pGEM-P3 was first subcloned into pSK at the BamHI site downstream of the T7 promoter to generate pSK-BB3, and the gene 3 fragment of pSK-BB3 between SmaI and SacI was isolated and inserted between the same sites of pGEM-P3. All of the P3 deletion mutants were synthesized by TNT with the [35 S]Met label and evaluated in GST pull-down assays essentially as described above.

RESULTS

Secondary structure predictions and comparisons with 30K superfamily MPs. Amino acid sequence comparisons using the BLAST program revealed no significant similarity between the RYSV P3 protein and other proteins in the databases, including plant viral MPs. In light of the proposed common core secondary structures for the 30K superfamily members (25), we carried out a secondary structure prediction for RYSV P3 using the PHD protein secondary structure prediction protocol (28). The analyses indicated that the P3 protein has a central region containing six β -elements interposed by four α -helical segments to form three core structures (Fig. 1). The central region is flanked by a long N terminus and a very short C terminus. The long N-terminal region of the P3 protein is predicted to contain two β -elements (N1 and N2) and two α -helices (NA and NB) and form an additional core structure together with the α -helix A (Fig. 1). The organization pattern of the secondary structures of the P3 central region is very similar to that of the central region of the 30K superfamily consensus structures, which consist of seven β -elements and four α -helical segments that can be divided into three core structures. In comparison to the related proteins encoded by other sequenced plant rhabdoviruses, MMV P3 and MFSV P4 also each harbor a region predicted to form the 30K superfamily-like secondary structures, while SYNV sc4 and LNYV 4b have a lower degree of similarity to the consensus structure in that they both have a series of β -elements flanked by α -helical segments without internal α -helices (Fig. 1). Unlike RYSV P3, sc4, 4b, and MSFV P4 all have a long C-terminal region rich in β -elements and α -helices or loop structures (Fig. 1). Although MMV P3 has a long N-terminal region like RYSV P3, it contains β -elements but no α -helices. The N-terminal region of RYSV P3 also shows an obvious similarity to the N terminus of the caulimoviral MPs (data not shown). It is known that this N-terminal domain is essential for caulimovirus movement. Deletions in the N-terminal, but not in the C-terminal, region of the *Cauliflower mosaic virus* MP were reported to destroy its ability to form tubules in cultured insect

FIG. 2. Histochemical analysis of GUS activity in leaves of *N. benthamiana* bombarded with pPVX(TrP25)-GUS (A and B), cobombarded with pBI-P3 plus pPVX(TrP25)-GUS (C and D), and with pBI-P25 plus pPVX(TrP25)-GUS (E and F). Infection foci from B, D, and F were photographed under an Olympus SZX9 stereomicroscope. Bars in B, D, and F represent 50, 200, and 300 μ m, respectively.

cells (33). We also aligned the amino acid sequence of RYSV P3 with 18 families of the 30K superfamily (25), and this alignment revealed the existence of Ile-Gln-Asp at positions 162 to 164 and Gly at position 232. This amino acid arrangement forms an IXDX₇₁G motif homologous to the LXDX₅₀₋₇₀G motif found in many 30K superfamily members (24). Similar motifs were also found in SYN_V sc4 (IXDX₅₄G), LNYV 4b (IXDX₅₅G), MMV P3 (IXDX₄₇G), and MFSV P4 (IXDX₄₆G). All these structural analyses indicate that RYSV P3, as well as its analogues like the SYN_V sc4, LNYV 4b, MMV P3, and MFSV P4 proteins, may be candidate members of the 30K superfamily.

Complementation of movement-defective PVX by RYSV P3.

Since the RYSV P3 protein was predicted to be a 30K MP, we investigated its potential function by using a biolistic bombardment-mediated transcomplementation assay. For this purpose, the 25-kDa MP-defective PVX cDNA clone expressing a GUS reporter gene was placed under the control of the 35S promoter. The plasmid, pPVX(TrP25)-GUS, containing this derivative was generated essentially as described by Chapman et al. (7) and Morozov et al. (26). In pPVX(TrP25)-GUS, four nucleotides inserted into the ORF encoding the p25 movement protein caused a reading frameshift mutation and thus truncated p25 from the C terminus by 72 amino acid residues and inactivated movement of PVX.

When pPVX(TrP25)-GUS was used alone to bombard the leaves of *N. benthamiana* plants, histochemical staining for GUS resulted in no blue spots visible to the naked eye at 3 days postbombardment (Fig. 2A), although GUS activity restricted

to single cells could be observed by stereo microscopy (Fig. 2B and Table 1). These results demonstrated that the PVX mutant was able to express GUS locally but was defective in cell-to-cell movement.

In contrast, when pPVX(TrP25)-GUS was cobombarded with pBI-P3 in which RYSV P3 was expressed under the control of the 35S promoter, blue foci after GUS staining could be observed in leaves with the naked eye at 3 days postbombardment (Fig. 2C). Microscopic observation also revealed that the GUS activity could be detected in a large number of cells (Fig. 2D). The infection foci induced by cobombardment had a much larger average size than those induced by pPVX(TrP25)-GUS alone (Table 1). The results indicate that RYSV P3 expressed ectopically can complement movement of the defective PVX and thus has the MP function. However, RYSV P3 seems to be less efficient in mediating cell-to-cell movement of the PVX mutant than PVX p25, with which the blue infection foci developed on cobombarded *N. benthamiana* leaves were obviously larger (Fig. 2F and Table 1).

pPVX(TrP25)-GUS was also cobombarded with pBI-P3 into cells of *O. sativa* leaves, to determine whether the RYSV P3 protein could complement the cell-to-cell movement of the PVX mutant in the natural host of RYSV. No blue cells were observed in the rice leaves bombarded with either pPVX(TrP25)-GUS alone or together with pBI-P3 by histochemical staining (data not shown).

RNA-binding properties of RYSV P3. The RNA-binding capacity of the RYSV P3 protein was tested by Northwestern analysis using His₆-tagged P3 protein (His-P3) expressed in *E. coli*. In an immunoblot assay using the Penta-His monoclonal antibody (QIAGEN) as a primary antibody, the bacterial-synthesized His-P3 is of the expected size (32.5 kDa) (Fig. 3, lane 7) when compared to the same sample run in adjacent lanes of the gel stained with Coomassie blue (Fig. 3, lane 5).

When the membrane blotted with the bacterial proteins containing His-P3 was probed with an in vitro ³²P-labeled transcript corresponding to the 3' leader of the RYSV genomic RNA, a signal corresponding to the position of the His-P3 protein could be detected (Fig. 3, lane 3), but similar bands were not observed in lanes containing the total proteins from *E. coli* M15(prep4) harboring pQE32-P3 before IPTG induction (Fig. 3, lane 2) or from induced *E. coli* harboring the pQE32 vector (Fig. 3, lane 1). Additional P3-RNA blot assays were performed by using ³²P-labeled RNA probes identical to the entire 191-nt 5' trailer sequence of the RYSV genome and, as nonspecific controls, probes derived from polylinker sequences of pSK and pKS. In these analyses, the His-P3 protein bound to all three RNA probes (Fig. 4). These RNA-binding

TABLE 1. Size analysis of the GUS-expressing foci in *N. benthamiana* leaves subjected to bombardment

Plasmids	Mean size of foci (μ m) ^a	SD	SE
pPVX(TrP25)-GUS	35.1	4.68	1.1
pPVX(TrP25)-GUS + pBI-P3	191.2	53.7	8.2
pPVX(TrP25)-GUS + pBI-P25	245.6	46.9	9.0

^a Data were generated from foci on three leaves in each experiment.

FIG. 3. Northwestern assay for interaction of RYSV P3 with leader RNA. (A) Northwestern blotting using the ^{32}P -labeled leader RNA transcript as a probe. (B) Coomassie blue staining of protein extracts from noninduced or IPTG-induced cells. (C) Immunoblot detection of His-P3 with the Penta-His antibody as a primary antibody. Lane 1, induced *E. coli* M15(prep4) harboring the pQE32 vector; lanes 2–7, *E. coli* M15(prep4) harboring pQE32-P3 before (lanes 2, 4, and 6) or after (lanes 3, 5, and 7) IPTG induction. Arrowheads, the His-P3 fusion protein.

assays indicate that the RYSV P3 protein has a single-stranded RNA-binding capacity that lacks sequence specificity.

Interaction of P3 protein with RYSV N protein. To further elucidate the possible function of RYSV P3 in movement of the RYSV ribonucleocapsid, we investigated the interaction between P3 and the RYSV N protein by GST pull-down assays. To carry out these experiments, the ^{35}S -labeled P3 protein was synthesized from pGEM-P3 by using a T7 TNT kit with [^{35}S]Met as a label. The GST-N fusion protein was expressed in *E. coli* JM109 and purified by glutathione affinity chromatography. In the GST pull-down assay, glutathione Sepharose 4B beads were loaded with purified GST-N fusion protein and then incubated with ^{35}S -labeled P3 protein, and after extensive washing, the bound proteins were eluted and analyzed by SDS-PAGE followed by autoradiography. As negative controls, GST-loaded beads and unloaded beads were used, respectively. The ^{35}S -labeled P3 protein bound to GST-N fusion protein but not to the GST protein or unloaded beads (Fig. 5). In addition, the control ^{35}S -labeled luciferase could not bind to GST-N fusion protein-loaded beads (data not shown). Thus, it is obvious that the P3 protein can bind specifically to the

FIG. 5. GST pull-down assay to probe the interaction between RYSV P3 and the N protein. Glutathione Sepharose 4B beads were loaded with purified GST-N fusion protein and then incubated with ^{35}S -labeled P3 protein. The bound protein was eluted and resolved on SDS-12% PAGE and autoradiographed (lane 2). GST-loaded beads (lane 3) and unloaded beads (lane 1) were used as controls, respectively. Lane 4, in vitro transcription and translation products using pGEM-P3 as a template. Black arrowhead, ^{35}S -labeled P3 protein; hollow arrowhead, a ^{35}S -labeled in vitro translation product that probably resulted from an internal ATG of the ORF3 sequence.

RYSV N protein. The additional lower-molecular-weight bands in lanes 2 and 4 of Fig. 5 probably represent an in vitro translation product initiated at an internal ATG of ORF3.

In order to localize the N protein-binding domain of the P3 protein, a series of nine overlapping deletions in the ORF3 were constructed. All the P3 deletion mutants were synthesized by using an in vitro T7 TNT kit and their abilities to bind to the GST-N fusion protein were assessed by pull-down assays. The N-terminal deletion mutant M1 could bind to the GST-N fusion protein, while M2 and M3 could not (Fig. 6B). These results indicate that the N protein-binding activity resides in the amino acid residues from position 59 to 148. Analysis of the C-terminal deletion mutants M4 to M8 further narrowed down the N protein-binding domain to a region between amino acid 61 to 90. This region contains the first core structure of the P3 protein that is composed of the N-terminal

FIG. 4. Sequence-nonspecific RNA binding of RYSV P3. P3-RNA blot assays were performed, respectively, by using ^{32}P -labeled RNA probes corresponding to the entire 5' trailer sequence (191 nt) of the RYSV genome (trailer RNA), polylinker sequences of pSK (SK RNA), and pKS (KS RNA). –, *E. coli* M15(prep4) harboring pQE32-P3 before IPTG induction; +, *E. coli* M15(prep4) harboring pQE32-P3 after IPTG induction. Arrowheads, the position of the His-P3 fusion protein in SDS-PAGE.

FIG. 6. GST pull-down assays of P3 mutants with GST-N. (A) Numbers in the constructs refer to the amino acid residue positions in RYSV P3. M1 to M9 are deletion mutants of P3. Shaded blocks refer to the N protein-binding domain. (B) After incubating with GST-N beads, RYSV P3 and its deletion mutants were eluted and resolved on SDS-10% PAGE. Those P3 mutants that are able to bind the N protein were visualized by autoradiography (marked with “+” under the autoradiograph). Black arrowhead, ^{35}S -labeled P3 protein.

β -elements (N1 and N2) flanked with two α -helices (NB and A) (Fig. 1).

DISCUSSION

Cell-to-cell movement of infectious viral material through plasmodesmata and long-distance transport of viruses are essential for establishing successful systemic infections in plants. For cell-to-cell movement, plant viruses encode specialized MPs and have evolved different movement strategies (5, 18). MP functions have been identified in many plant virus families and genera but so far not in a plant rhabdovirus. By comparison with animal rhabdoviruses, all plant rhabdoviruses characterized so far encode additional genes between the P and M genes. Although little is known about the functions of the protein products of those additional genes, they have been suggested to play a role in the spreading of virus as MPs. A possible role of SYNIV sc4 as a movement protein was first proposed by Scholthof et al. (30) and extended by Goodin et al. by showing its localization to plant cell walls (13). Secondary structure predictions have placed SYNIV sc4 and other plant rhabdoviral proteins MMV P3, MFSV P4, and LNYV 4b (25; <http://opbs.okstate.edu/Virevol/Web/Capillo.html>; this study) in the 30K superfamily as possible members. Moreover, LNYV 4b has also been shown to have a high amino acid sequence similarity with the trichoviral MPs such as the *Apple chlorotic leaf spot virus* 50-kDa protein and the *Grapevine berry inner necrosis virus* 39-kDa protein which have tubule-inducing activity in host cells (15, 29). However, other properties essential for a MP have not been described for the above-mentioned plant rhabdoviral proteins.

In this study, we discovered that the secondary structures of RYSV P3 have core regions common to the 30K superfamily of MPs and that P3 shows higher similarity with the 30K superfamily consensus secondary structure than SYNIV sc4 and LNYV 4b. Furthermore, we have presented experimental data to show that P3 can bind RNA transcripts in vitro that correspond to the 5' trailer and 3' leader regions of the RYSV negative-sense genomic RNA and that P3 can also bind non-specifically to other RNAs. Such nonspecific RNA binding in vitro is a common feature of MPs of both positive- and negative-strand RNA viruses.

The RYSV P3-N protein interaction demonstrated by the GST pull-down assays provided further evidence that P3 is a possible MP of RYSV if a model that rhabdoviruses move from cell to cell in the form of nucleocapsids (16) is taken into account. The N protein is a main constituent of the nucleocapsid and thus the specific recognition of N by P3 may be essential for intercellular movement of the RYSV nucleocapsid. A recent study showed that the MP (NSm) of *Tomato spotted wilt tospovirus*, also a negative-strand RNA virus, bound to its N protein in vitro (31). The MP-CP interactions necessary for virus local movement have already been described for a number of positive-strand RNA viruses (18). Evidence for direct binding between CP and MP both in vitro (*Maize streak virus* and *Cowpea mosaic virus* [CPMV]) and in vivo (*Maize streak virus*) has been reported (6, 21). Moreover, it has been shown that CPMV virions are transported through tubules formed with CPMV MP via MP-CP interactions and deposited in the adjacent cell (37). These results indicated that direct

binding of MP to CP is essential for the cell-to-cell movement of some plant viruses.

More-direct evidence for RYSV P3 functioning as a MP comes from the bombardment-mediated transcomplementation assay in which P3 supported the cell-to-cell movement of the movement-defective, GUS-tagged PVX mutant in *N. benthamiana*. The exchange ability and complementation of movement functions have been documented for many plant viral MPs with viruses of the same family or different families (3) and even with plant and insect viruses (11). In this context, it would not be surprising for a rhabdoviral MP to be able to replace the MP of a positive-strand RNA virus. However, the RYSV P3 protein was less effective than the PVX MP in complementation of the cell-to-cell movement of the PVX mutant in *N. benthamiana*, a dicot plant, as judged by the size of the blue GUS foci (Table 1). This could be due to the fact that RYSV naturally only infects the monocot rice plant or that the cell-to-cell movement of RYSV requires functions of other RYSV-encoded protein(s) or rice cellular components. On the other hand, bombardment experiments on rice leaves revealed that the RYSV P3 protein could not support the movement-defective PVX to establish an infection in rice cells, similar to the results when the rice dwarf phyto-reovirus S6 protein was used as a cobombardment partner (20). Failure in these complementations may result from the inability of PVX to infect rice.

Together, the structure comparisons with known 30K MPs, interactions of P3 with RYSV RNA and the N protein (two major components of the viral nucleocapsid), and complementation of the movement-defective PVX provide persuasive evidence that RYSV P3 is an MP of RYSV, the first MP identified in plant rhabdoviruses. Our work also suggests that the related proteins encoded by genes in similar locations in other plant rhabdoviruses probably also function as MPs.

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