Rice black streaked dwarf virus P9-1, an α-helical protein, self-interacts and forms viroplasms in vivo

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Replication and assembly of viruses from the family Reoviridae are thought to take place in discrete cytoplasmic inclusion bodies, commonly called viral factories or viroplasms. Rice black streaked dwarf virus (RBSDV) P9-1, a non-structural protein, has been confirmed to accumulate in these intracellular viroplasms in infected plants and insects. However, little is known about its exact function. In this study, P9-1 of RBSDV-Baoding was expressed in Escherichia coli as a His-tagged fusion protein and analysed using biochemical and biophysical techniques. Mass spectrometry and circular dichroism spectroscopy studies showed that P9-1 was a thermostable, α-helical protein with a molecular mass of 41.804 kDa. A combination of gel-filtration chromatography, chemical cross-linking and a yeast two-hybrid assay was used to demonstrate that P9-1 had the intrinsic ability to self-interact and form homodimers in vitro and in vivo. Furthermore, when transiently expressed in Arabidopsis protoplasts, P9-1 formed large, discrete viroplasm-like structures in the absence of infection or other RBSDV proteins. Taken together, these results suggest that P9-1 is the minimal viral component required for viroplasm formation and that it plays an important role in the early stages of the virus life cycle by forming intracellular viroplasms that serve as the sites of virus replication and assembly.

INTRODUCTION

The formation of viral inclusion bodies, or viroplasms, is a common feature of double-stranded (ds)RNA viruses from the family Reoviridae with genomes composed of 10–12 segments (Brookes et al., 1993; Fabbretti et al., 1999; Fukushi et al., 1962; Petrie et al., 1984; Rhim et al., 1962; Shikata & Kitagawa, 1977; Touris-Otero et al., 2004). These inclusion bodies are mainly composed of viral dsRNA, viral proteins and partially and fully assembled viral particles (Dales et al., 1965; Fabbretti et al., 1999; Isogai et al., 1998; Rhim et al., 1962; Silverstein & Schur, 1970; Touris-Otero et al., 2004; Wei et al., 2006). In addition, the inclusion bodies have been shown to contain microtubules and thinner ‘kinky’ filaments suggested to be intermediate filaments (Dales, 1963; Dales et al., 1965; Spendlove et al., 1964). Although the formation mechanism of these inclusions is largely unknown, they are thought to play an important role in viral infections because they are probable sites of viral genome replication, protein synthesis and virus assembly (Fabbretti et al., 1999; Isogai et al., 1998; Petrie et al., 1984; Rhim et al., 1962; Wei et al., 2006).

Rice black streaked dwarf virus (RBSDV), a member of the genus Fijivirus within the family Reoviridae, is the causal agent of rice black streaked dwarf and maize rough dwarf diseases, both of which are responsible for severe yield losses in some countries (Bai et al., 2002; Fang et al., 2001; Shikata & Kitagawa, 1977). RBSDV is an icosahedral, double-layered particle with a diameter of about 75–80 nm and contains 10 segments (S1–S10) of linear dsRNA ranging in size from approximately 1.8 to 4.5 kb (Wang et al., 2003; Zhang et al., 2001). Sequence analysis suggests that S1 encodes a putative RNA-dependent RNA poly-

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merase (168.8 kDa) with similarities to that encoded by S1 of *Nilaparvata lugens* reovirus (NLRV), whilst S2 and S4 are both monocistronic and probably encode the core protein and outer-shell B-spike protein, respectively (Zhang et al., 2001). S3-encoded protein has been assigned as a possible guanylyltransferase, with similarities to VP3 of mycoreovirus 1 (Supyani et al., 2007), and S6-encoded protein functions as a viral RNA-silencing suppressor (Zhang et al., 2005). Western blot analysis of the different components of viral particles suggests that S8 and S10 encode a core capsid and a major outer-capsid protein, respectively, whereas S7 and S9 encode non-structural proteins (Isogai et al., 1998).

The gene encoding RBSDV S9 is approximately 1900 nt long and contains two large non-overlapping open reading frames (ORFs; S9-1 and S9-2), which encode a 39.9 kDa (P9-1) and a 24.2 kDa (P9-2) polypeptide, respectively. No sequence similarity has been found between P9-1 and P9-2. Both ORF1 and ORF2 are conserved in RBSDV S9, maize rough dwarf virus S8 and NLRV S9, indicating that proteins encoded by RBSDV S9 may play a role in the virus life cycle. Studies to date have shown that P9-2 is not detected in RBSDV-infected plants and insects, whilst P9-1 has been confirmed to accumulate in intracellular viroplasms, suggesting that, as a component of viroplasms, P9-1 may play an important role in viroplasm formation and viral morphogenesis (Isogai et al., 1998). However, little is known about the biochemical and biophysical properties and the exact function of P9-1. In this study, using a series of biochemical and biophysical analyses, we demonstrated that P9-1 is a thermostable, α-helical protein and has an intrinsic ability to self-interact and form homodimers *in vitro* and *in vivo*. Furthermore, to address the hypothesis that P9-1 plays a role in viroplasm formation, we transiently expressed P9-1 fused with green fluorescent protein (GFP) at its C terminus in *Arabidopsis* protoplasts. The results suggest that P9-1 forms large, discrete viroplasms in the absence of infection or other RBSDV proteins *in vivo*.

**METHODS**

**RNA extraction, RT-PCR and plasmid constructs.** Maize plants infected with RBSDV were gathered from Baoding, China. Viral genomic RNAs were isolated according to previously published methods (Uyeda et al., 1998) and were reverse transcribed in the presence of a 9 nt random primer with Superscript reverse transcriptase (TaKaRa) according to the manufacturer’s instructions. S9-1 of RBSDV-Baoding was then amplified using the specific primers T-S9-F (5′-GGGGATCCATGGCAGACCAAGAGCGGAG-3′; added BamHI site underlined) and T-S9-R (5′-GGGGTGCGACCAAGGCAAGCGGAG-3′; added Xhol site underlined) based on the published sequence of a Japanese isolate of RBSDV (GenBank accession no. AB011403) and cloned into the pMD18-T vector (TaKaRa) to generate pMD-S9-1. The sequence of S9-1 was submitted to GenBank after sequencing.

For bacterial expression of P9-1, the full-length S9-1 gene from pMD-S9-1 was digested with BamHI and Xhol and then inserted into the vector pET21a (+) (Novagen) cut with the same enzymes, resulting in the prokaryotic expression plasmid pET-S9-1.

The yeast two-hybrid constructs were generated by fusing the S9-1 gene in frame to the GALA activation domain (AD) in pGADT7 (Clontech) and to the GALA DNA-binding domain (BD) in pGBKTK7 (Clontech). The detailed strategy was as follows: S9-1 was amplified from plasmid pMD-S9-1 with primers S9-AB-F (5′-GGGGATCCATGGCAGACCAAGAGCGGAG-3′; added BamHI site added next to the +2 nucleotide underlined) and S9-AB-R (5′-GGGGTGCGACCAAGGCAAGCGGAG-3′; added Xhol site underlined), and then cloned into the BamHI/Xhol sites of pGADTK7 to generate pGAD-S9-1 and the BamHI/Secl sites of pGBKTK7 to generate pGBK-S9-1.

For expression of GFP or GFP fusion proteins in *Arabidopsis* protoplasts, S9-1 was amplified from plasmid pMD-S9-1 with primers 221-S9-F (5′-GGGGATCCATGGCAGACCAAGAGCGGAG-3′; added BamHI site underlined) and 221-S9-R (5′-GGGGTGCGACCAAGGCAAGCGGAG-3′; added Xhol site underlined) based on the published sequence of a Japanese isolate of RBSDV (GenBank accession no. AB011403) and cloned into the pGBKT7 vector (BD Biosciences Clontech), resulting in pBI221-S9-GFP.

All constructs were confirmed by DNA sequencing.

**Protein expression and purification.** A freshly transformed overnight culture of *Escherichia coli* BL21(DE3) (Novagen) containing the plasmid pET-S9-1 was transferred to 1 l Luria broth with 30 μg kanamycin ml⁻¹ at 37 °C until the OD₆₀₀ reached 0.6–1.0 and then induced with isopropyl-β-D-galactopyranoside (IPTG) at a final concentration of 0.5 mM. After 3 h at 22 °C, the cells were harvested, resuspended in Tris-buffered saline [TBS: 25 mM Tris/HCl (pH 8.0), 125 mM NaCl] and lysed by sonication. The lysate was then incubated with 1% Triton X-100 for 30 min at 0 °C and centrifuged at 12,000 g for 30 min at 4 °C. The clarified supernatants were loaded onto a Ni²⁺-chelated Sepharose Fast Flow column (Amersham Pharmacia Biotech) pre-equilibrated with TBS. The column was subsequently washed with 10 column volumes of TBS and the His-tagged proteins were eluted with a linear gradient of 10–500 mM imidazole in TBS. Eluate fractions collected from the column were analysed by SDS-PAGE and Western blotting, and the fractions containing target proteins were pooled, concentrated to a suitable concentration by ultrafiltration (10 kDa cut-off) and stored at −70 °C until further analysis. The protein concentration was determined using a BCA protein assay kit (Pierce Biochemicals).

**Western blot analysis.** Proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes using a semi-dry blotting apparatus (Bio-Rad) according to the manufacturer’s instructions. The membranes were blocked with 2.5% sodium caseinate in PBS containing 0.02% Tween 20 (PBST) and incubated with rabbit anti-His-tag polyclonal antibody (Sigma). The secondary antibody was horseradish peroxidase-labelled goat anti-rabbit IgG (Sigma). After several washes with PBST, the membrane was developed using 3,3′-diaminobenzidine as the substrate.

**Gel-filtration analysis.** The purified proteins were loaded onto a Hilo load Superdex 200 HR 10/30 column (Amersham-Pharmacia) of an AKTA Explorer FPLC system (Amersham-Pharmacia). The fractions under the peak were collected and analysed by 12% SDS-PAGE and Western blotting, and the fractions containing target proteins were pooled, concentrated to a suitable concentration by ultrafiltration (10 kDa cut-off) and stored at −70 °C until further analysis. The protein concentration was determined using a BCA protein assay kit (Pierce Biochemicals).

**Mass spectrometry.** For matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis, proteins purified by gel-filtration chromatography were dialysed against 20 mM Tris/HCl (pH 8.0) and then analysed with a Voyager-DE-STR.
MALDI/TOF mass spectrometer (PerSeptive BioSystems) as described previously (Barr et al., 1996).

**Chemical cross-linking.** The purified proteins described above were cross-linked with various concentrations (0.5, 1, 2, 3 and 4 mM) of ethylene glycol bis(succinimidyl succinate) (EGS) for 2 h on ice. The reaction was then terminated with 50 mM Tris/HCl (pH 8.0) and further incubated for 5 min at room temperature. Aliquots of the reaction mixture were analysed by 12% SDS-PAGE.

**Circular dichroism (CD) spectroscopy.** CD spectroscopy was performed on a Jasco J-715 spectrophotometer with dialysed proteins in PBS buffer (pH 7.3) and wavelength spectra were recorded at 25 °C in a cuvette of 0.1 cm path length. Thermodynamic stability was measured at 222 nm by monitoring the CD signals in the temperature range 25–80 °C with a scan rate of 5 °C min⁻¹.

**Yeast two-hybrid assay and β-galactosidase assays.** Yeast two-hybrid assays were performed using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech) according to the manufacturer’s protocols. *Saccharomyces cerevisiae* strain AH109 was transformed with the yeast two-hybrid constructs described above using the small-scale lithium acetate method (Clontech) and plated on synthetic defined (SD) minimal medium lacking adenine, histidine, leucine and tryptophan (SD/−Ad/−His/−Leu/−Trp). A β-galactosidase colony-lift filter assay using 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) as a substrate was then performed to assess the interaction. Yeast co-transformed with pGADT7-T (encoding a fusion of the DNA-activation domain with the SV40 large T antigen) and pGBKTK7-p53 (encoding a fusion of the DNA-binding domain with murine p53 protein) was used as a positive control, and yeast co-transformed with pGADT7-T and pGBKTK7-Lam (encoding a fusion of the DNA-binding domain with human lamin C) was used as a negative control.

**Arabidopsis protoplast transient expression assays.** *Arabidopsis* protoplasts were isolated and transfected using a modified polyethylene glycol method as described previously (Abel & Theologis, 1994). Typically, 1 × 10⁶–2 × 10⁷ protoplasts were transfected with 30–50 μg pBI221-GFP or pBI221-S9-GFP and then incubated at 22 °C for 24 h in the dark. Expression of GFP or P9-1–GFP fusion protein in *Arabidopsis* protoplasts was observed using a Zeiss LSM510 confocal laser-scanning fluorescence microscope with a GFP filter. Images were captured using Zeiss LSM510 software, converted to TIFF for export and processed using Adobe Photoshop software (Adobe Systems).

## RESULTS AND DISCUSSION

**Cloning and sequence analysis**

The full-length S9-1 gene was successfully amplified using specific primers, cloned into pMD18-T vector and sequenced. Sequence analysis showed that S9-1 of RBSDV-Baoding consisted of 1041 nt encoding a protein of 347 aa with a calculated molecular mass of 39.9 kDa and shared 89.1–99.0% nucleotide identity and 92.8–99.1% amino acid identity with other published S9-1 sequences of RBSDV isolates. However, considerable variations occurred, mainly between aa 102 and 164 (data not shown).

**Expression and purification of P9-1**

To obtain sufficient quantities of P9-1 for biochemical and biophysical analysis, the expression vector pET-S9-1 containing the full-length S9-1 gene was designed and used to transform *E. coli* strain BL21(DE3). After induction with IPTG, the expression of recombinant protein was confirmed by SDS-PAGE.

Initially, the His-tagged fusion protein (apparent molecular mass 40 kDa) existed mainly in the form of inclusion bodies when the cultures were induced with 1 mM IPTG for 3 h at 28 °C (Fig. 1a, lanes 2 and 3). Further optimization showed that large amounts of soluble

**Fig. 1.** Expression and purification of His-tagged P9-1. (a) SDS-PAGE of P9-1 expressed under different conditions. Lanes: 1, molecular mass markers; 2 and 3, supernatant and pellet of BL21(DE3) with pET-S9-1 induced with 1 mM IPTG at 28 °C; 4 and 5, supernatant and pellet of BL21(DE3) without pET-S9-1 induced with 1 mM IPTG at 28 °C; 6 and 7, supernatant and pellet of BL21(DE3) with pETS9-1 induced with 0.3 mM IPTG at 22 °C. (b) SDS-PAGE of P9-1 purified by Ni²⁺-affinity chromatography. Lanes: 1, molecular mass markers; 2–7, proteins eluted with 10, 20, 50, 100, 200 and 500 mM imidazole, respectively. (c) Western blot analysis of His-tagged P9-1. Lanes: 1, supernatant of BL21(DE3) without pET-S9-1, induced with 1 mM IPTG at 28 °C; 2, supernatant of BL21(DE3) with pETS9-1 induced with 0.3 mM IPTG at 22 °C; 3, purified P9-1 eluted with 500 mM imidazole. The arrow shows the 40 kDa band corresponding to P9-1.
proteins to form hetero-oligomers through homo-oligomers or might interact with other viral or host components of the viroplasms, P9-1 might self-interact to form homodimers and the purified P9-1 was concentrated to about 20 mg ml\(^{-1}\) in TBS for further analysis.

As reported for other proteins, the presence of the His tag does not affect the secondary structure during CD measurement. Moreover, it causes low interference during the crystallization process, suggesting that His-tagged fusion proteins can be used for direct structural analysis (Bucher et al., 2002; Mohanty & Wiener, 2004; Reyes et al., 2006). Thus, the His-tagged P9-1 protein obtained in this study was suitable for the following structural analysis.

**P9-1 is a thermostable, z-helical protein**

Determining the secondary structure content of P9-1 protein by CD spectroscopy was the first step towards a detailed characterization of its three-dimensional structure, which may supply us with a structural basis for P9-1 function. The CD spectra showed that P9-1 was a typical z-helical protein, with double minima at 208 and 222 nm (Fig. 2a). The z-helix content decreased gradually with increasing temperature and a significant loss occurred only when the temperature was raised to 75 °C (Fig. 2b), indicating that P9-1 was very stable in PBS.

The z-helical coiled coil, despite its simplicity, is a highly versatile folding motif, and coiled-coil-containing proteins exhibit a broad range of different functions, especially molecular recognition and protein refolding (Burkhard et al., 2001). Also, the z-helical coiled coil is one of the principal oligomerization motifs in proteins: coiled-coil-containing proteins have the potential to form homo-oligomers or to associate with coiled-coil regions of other proteins to form hetero-oligomers (Burkhard et al., 2001; Lupas, 1996). Previous studies have shown that viroplasms in cells infected by viruses from the family Reoviridae contain viral dsRNA, viral proteins and partially and fully assembled viral particles, and that there are many possible interactions, including complex protein–protein and protein–RNA interactions, involved in the formation of the viroplasms (Dales et al., 1965; Fabbretti et al., 1999; Isogai et al., 1998; Rhim et al., 1962; Silverstein & Schur, 1970; Touris-Otero et al., 2004; Wei et al., 2006). Therefore, we hypothesized that, as a component of the viroplasms, P9-1 might self-interact to form homo-oligomers or might interact with other viral or host proteins to form hetero-oligomers through z-helical coiled-coil interactions.

**P9-1 self-interacts and forms homodimers in vitro and in vivo**

To determine whether P9-1 had the intrinsic ability to self-interact in vitro, a combination of MALDI-TOF MS, gel filtration chromatography and chemical cross-linking was performed as described in Methods.

To determine the molecular mass of P9-1, the purified protein was analysed by MALDI-TOF MS. The result revealed a major peak at 41.804 kDa (data not shown), which was close to the predicted molecular mass estimated by SDS-PAGE and Western blot analysis (Fig. 1).

When analysed by gel filtration chromatography, P9-1 eluted between the corresponding positions of 150 kDa (alcohol dehydrogenase) and 66 kDa (BSA) (Fig. 3a), suggesting that P9-1 might exist as oligomers in vitro. Peak fractions were collected and analysed by 12 % SDS-PAGE. Bands at 40 and 80 kDa (twice the apparent molecular mass of P9-1) were detected when the samples were not heated prior to SDS-PAGE (Fig. 3b). However, heating (100 °C, 5 min) resulted in loss of the 80 kDa band (Fig. 3c), indicating that P9-1 self-interacts to form homodimers in vitro. Chemical cross-linking was performed to determine further the homodimerization of P9-1. As shown in Fig. 3(d), although the cross-linking was not very efficient (most P9-1 remained as monomers because of the denaturation during SDS-PAGE), EGS treatment generated the 80 kDa (dimer) band again and the intensity of the
A yeast two-hybrid assay was performed to evaluate further the self-interaction of P9-1 \textit{in vivo}, followed by a $\beta$-galactosidase assay. First, the expression of the fusion proteins AD–P9-1 and DBD–P9-1 containing the entire P9-1 sequence was verified by Western blot analysis (data not shown). Also, it was verified that expression of AD–P9-1 and DBD–P9-1 was not toxic for AH109 yeast cells and that these fusion proteins did not bind or transactivate the \textit{lacZ} reporter gene non-specifically when expressed separately (data not shown). As shown in Fig. 4, yeast transformants containing pGAD-S9-1 and pGBK-S9-1, as well as those containing pGADT7-T and pGBK7-p53, grew well on SD/–Ade/–His/–Leu/–Trp medium and turned blue when assayed for $\beta$-galactosidase activity resulting from activation of the \textit{lacZ} reporter gene, whereas no growth was observed for the negative control (Fig. 4). These results indicated clearly that P9-1 can specifically self-interact \textit{in vivo} as well as \textit{in vitro}.

Previous studies have shown that self-interaction of viral proteins is important for many of their functions in the virus life cycle (Haas \textit{et al.}, 2005; Khu \textit{et al.}, 2001; Nakai \textit{et al.}, 2006; Takemoto & Hibi, 2005; Tanaka \textit{et al.}, 2002), and that self-interaction is likely to be critical for their ability to interact with several cellular proteins simultaneously (Harada \textit{et al.}, 2001; Tanaka \textit{et al.}, 2002). Thus, we suggest that P9-1 has the intrinsic ability to self-interact and that this self-interaction may be a prerequisite for its biological functions.

**P9-1 forms viroplasms \textit{in vivo}**

Replication and assembly of viruses from the family Reoviridae are thought to take place in cytoplasmic inclusion bodies, and several non-structural viral proteins have been reported to be involved in the formation of these structures (Brookes \textit{et al.}, 1993; Dales, 1963; Fabbretti \textit{et al.}, 2003). Fig. 3. Dimerization of P9-1 \textit{in vitro}. (a) Gel filtration chromatography of purified P9-1. P9-1 was eluted from the column between the corresponding positions of 150 kDa (alcohol dehydrogenase) and 66 kDa (BSA), indicating that P9-1 can form oligomers. (b, c) SDS-PAGE of P9-1 in the peak fractions collected from gel filtration chromatography. The dimer band was detected when the proteins were not heated before SDS-PAGE (b), whereas heating resulted in the loss of the band (c), indicating that P9-1 forms homodimers \textit{in vitro}. (d) Chemical cross-linking studies showing the dimerization of P9-1. After treatment with various concentrations of EGS as indicated, the dimer band was detected again and the intensity of the band increased with the increase in the cross-linker concentration. Bands corresponding to monomers and dimers are indicated.

Fig. 4. Self-interaction of P9-1 \textit{in vivo}. Yeast cells containing pGAD-S9-1 and pGBK-S9-1, as well as those containing pGADT7-T and pGBK7-p53, grew well on SD/–Ade/–His/ –Leu/–Trp medium (a) and turned blue in the $\beta$-galactosidase assays (b), indicating that P9-1 can self-interact \textit{in vivo}. Yeast co-transformed with pGADT7-T and pGBK7-Lam was used as a negative control.
as shown in Fig. 5, after incubation at 22 °C for 16–24 h, large discrete inclusion bodies were observed within the cytoplasm of Arabidopsis protoplasts transfected with pBI221-S9-GFP, and these inclusion bodies were formed by numerous smaller punctate aggregates (Fig. 5a, b). In contrast, transfection with pBI221-GFP resulted in the diffuse distribution of GFP throughout the cytoplasm and no formation of inclusion bodies in the protoplasts (Fig. 5c, d), indicating that GFP fused to the C terminus of P9-1 had no significant effect on the ability of GFP-fused P9-1 to self-assemble and form inclusion bodies in Arabidopsis protoplasts. These results are consistent with those of rice dwarf virus Pns12 protein (Wei et al., 2006), indicating that expression of P9-1, even in the absence of other RBSDV proteins, is sufficient for the formation of viroplasms in vivo.

In addition, approximately 30% of Arabidopsis protoplasts transfected with pBI221-S9-GFP contained aggregates of variable size but less than 5 μm in diameter (Fig. 5e, f), which could be ascribed to the early stages of viroplasm formation.

Taken together, our findings suggest that RBSDV P9-1 is the minimal viral component required for viroplasm formation. Like some non-structural proteins of other viruses from the family Reoviridae, RBSDV P9-1 may play an important role in the early stages of the virus life cycle by forming intracellular viroplasms that serve as the site of virus replication and assembly. We believe that these results will facilitate further studies of the exact mechanisms of viroplasm formation in RBSDV infection.

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