Non-structural protein P6 encoded by rice black-streaked dwarf virus is recruited to viral inclusion bodies by binding to the viroplasm matrix protein P9-1

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Like other members of the family Reoviridae, rice black-streaked dwarf virus (RBSDV, genus Fijivirus) is thought to replicate and assemble within cytoplasmic viral inclusion bodies, commonly called viroplasms. RBSDV P9-1 is the key protein for the formation of viroplasms, but little is known about the other proteins of the viroplasm or the molecular interactions amongst its components. RBSDV non-structural proteins were screened for their association with P9-1 using a co-immunoprecipitation assay. Only P6 was found to directly interact with P9-1, an interaction that was confirmed by bimolecular fluorescence complementation assay in Spodoptera frugiperda (Sf9) cells. Immunoelectron microscopy showed that P6 and P9-1 co-localized in electron-dense inclusion bodies, indicating that P6 is a constituent of the viroplasm. In addition, non-structural protein P5 also localized to viroplasms and interacted with P6. In Sf9 cells, P6 was diffusely distributed throughout the cytoplasm when expressed alone, but localized to inclusions when co-expressed with P9-1, suggesting that P6 is recruited to viral inclusion bodies by binding to P9-1. P5 localized to the inclusions formed by P9-1 when co-expressed with P6 but did not when P6 was absent, suggesting that P5 is recruited to viroplasms by binding to P6. This study provides a model by which viral non-structural proteins are recruited to RBSDV viroplasms.

INTRODUCTION

Many RNA viruses induce the formation of cellular compartments, known as viral inclusion bodies or viroplasms, in which viral replication occurs and which possibly protect the viral genome from cytoplasmic nucleases or host immune responses (Netherton et al., 2007). Viroplasm formation is consistently associated with reovirus infections. Reoviruses have between 9 and 12 dsRNAs in their genome and a multilayered virus particle (Attoui, 2011). It is believed that the formation of a viral inclusion body is essential for the reovirus life cycle because viral RNA synthesis and particle assembly take place in the inclusion body (Patton et al., 2006; Roy & Noad, 2006). Viroplasms of reoviruses usually contain multiple viral proteins, viral RNAs, partially and fully assembled virus particles, and some cellular components (Arnold et al., 2008; Becker et al., 2001; Broering et al., 2004; Wei et al., 2006). This localized concentration of viral RNAs and proteins is thought to occur by protein–protein or protein–RNA interactions, but the detailed processes of viroplasm formation remain largely unknown (Patton et al., 2006). Studies on mammalian, avian and plant-infecting reoviruses have all shown that each virus encodes a viroplasm matrix protein that is a key component of the viral inclusion body and which also recruits other viral proteins into the viroplasm (Becker et al., 2003; Contin et al., 2010; Touris-Otero et al., 2004a; Wei et al., 2006).

Rice black-streaked dwarf virus (RBSDV), a member of the genus Fijivirus, infects maize and rice plants and causes significant yield losses in Asia (Bai et al., 2002). RBSDV is transmitted in nature by the small brown planthopper (Laodelphax striatellus) and replicates in cells of both host plants and insect vectors. It has a genome of ten dsRNAs (S1 – S10) encased in icosahedral double-shelled particles and encodes at least ten primary translation products, which are named from their respective genome segment (Zhang et al., 2001). Six of these proteins (P1, P2, P3, P4, P8 and P10) are structural components of the viral particle.

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Two supplementary figures are available with the online version of this paper.
P9-1 self-interacts and forms cytoplasmic viroplasm-like inclusion bodies in Spodoptera frugiperda (Sf9) cells

Previously, RBSDV P9-1 was shown to be able to self-interact in vivo and in vitro and to form inclusion bodies when expressed alone in Arabidopsis protoplasts (Akita et al., 2012; Zhang et al., 2008). To determine whether P9-1 also possesses similar characteristics when expressed in Sf9 cells using the baculovirus expression system, we utilized a BiFC assay (Hu & Kerppola, 2003), because this method allows simultaneous examination of protein interactions and subcellular distribution. P9-1 was fused to the N- and C-terminal portions of eYFP [eYFP(n) and eYFP(c)], cloned into pBacDual plasmids and then the recombinant plasmids were co-transfected into Sf9 cells. The reconstruction of eYFP fluorescence, indicating a protein–protein interaction, was visualized by using a confocal laser scanning microscope (CLSM) 48 h post-transfection (p.t.). As shown in Fig. 1(a), bright eYFP fluorescence was observed in the cells expressing eYFP(n)–P9-1 and eYFP(c)–P9-1, similar to that seen in cells expressing wild-type unfused eYFP. In contrast, no fluorescence was observed when the cells were co-transfected with unfused N- or C-terminal portions of eYFP plus eYFP(n)–P9-1 or eYFP(c)–P9-1. Close examination of the cells expressing eYFP(n)–P9-1 and eYFP(c)–P9-1 showed that, at 48 h p.t., eYFP fluorescence localized to numerous punctate inclusions throughout the cytoplasm. As time progressed (72 h p.t.), these structures became much fewer but markedly increased in size (Fig. 1b). The inclusion structures observed in this experiment closely resemble those formed by P9-1 in Arabidopsis protoplasts (Zhang et al., 2008) or formed by P9-1 of SRBSDV or MCRV in Sf9 cells (Jia et al., 2012; Maroniche et al., 2010). This result demonstrates that RBSDV P9-1 is able to self-interact and to form viroplasm-like inclusion bodies in the absence of other viral factors in cultured Sf9 cells.

P9-1 binds to non-structural protein P6

The total proteins of leaves of RBSDV-infected maize plants were extracted under non-denaturing conditions (Fang et al., 2001; Isogai et al., 1998a; Liu et al., 2007a, b; Shikata & Kitagawa, 1977; Zhang et al., 2001). P1, P2 and P8 are core particle proteins and P10 is an outer capsid protein (Isogai et al., 1998a; Liu et al., 2007a, b). Bioinformatics analyses indicate that P1, P3 and P4, have conserved domains for RNA-dependent RNA polymerase (RdRp), guanylyltransferase and N-myristoylation, respectively, suggesting that P1 has RNA polymerase activity and P3 has capping enzyme activity (Isogai et al., 1998b; Supyani et al., 2007; Wang et al., 2003). P4 may be the outer-shell B spike protein (Zhang et al., 2001). There are six non-structural proteins, P5, P6, P7-1, P7-2, P9-1 and P9-2. The function of P5 is unknown, while P6 has RNA silencing suppression activity (Zhang et al., 2005). S7 and S9 both encode two ORFs and so potentially each express two proteins, namely P7-1, P7-2, P9-1 and P9-2 (Isogai et al., 1998a). P7-1 forms the cytoplasmic tubular-like structures that serve as a conduit for virion movement between cells in the insect vector (Liu et al., 2011). P7-2 and P9-2 are probably expressed in low amounts and their functions are still unknown. P9-1 is the major component of viroplasms (Akita et al., 2012; Isogai et al., 1998a) and is a thermostable, α-helical protein that is able to self-interact and to form inclusion-like structures when expressed alone in Arabidopsis protoplasts (Zhang et al., 2008). Crystallographic analysis shows that P9-1 forms cylindrical octamers via lateral hydrophobic interactions (Akita et al., 2012). Accordingly, expression of P9-1 encoded by southern rice black-streaked dwarf virus (SRBSDV) or mal de Río Cuarto virus (MRCV), two closely related viruses belonging to the genus Fijivirus, induces formation of viroplasm-like structures in non-host insect cells (Jia et al., 2012; Maroniche et al., 2010). Thus, P9-1 is a major viroplasm matrix protein and the minimal viral component required for viroplasm formation.

To date, the viral protein components that are specifically recruited by RBSDV P9-1 to the viroplasm have not been identified. In this study, several RBSDV-encoded proteins were investigated for their association with P9-1 using co-immunoprecipitation (Co-IP) and bimolecular fluorescence complementation (BiFC) assays. Our results demonstrate that P6 directly interacts with P9-1 and is recruited into RBSDV viroplasms and that P5 interacts with P6 and is also recruited into RBSDV viroplasms.

RESULTS

P9-1 self-interacts and forms cytoplasmic viroplasm-like inclusion bodies in Spodoptera frugiperda (Sf9) cells

Fig. 1. P9-1 self-interacts and forms inclusions in Sf9 cells. (a) BiFC assay to examine the P9-1 self-interaction. Sf9 cells were co-transfected with recombinant baculovirus vectors containing the constructs indicated above the images. YFP fluorescence was observed using CLSM at 48 h p.t. Bars, 250 μm. (b) Subcellular distribution of YFP in Sf9 cells co-transfected with eYFP (n/c)-P9-1. Bars, 5 μm.
and subjected to co-immunoprecipitation assay using a P9-1 specific antibody. Protein complexes were detected by Western blotting using P5, P6, P7-1, P7-2, P8, P9-2 and P10 specific antibodies. First, to test the capability of the P9-1 antibody for immunobinding, we immunoprecipitated human influenza haemagglutinin (HA)-tagged P9-1 (HA–P9-1) expressed by the baculovirus expression system in Sf9 cells and detected the immunocomplex with HA antiserum. The immunoblotting result shows that HA–P9-1 was efficiently immunoprecipitated by the P9-1 antibody (Fig. 2a, upper panel). When the immunocomplexes derived from RBSDV-infected maize cells were probed using the antibodies specific for the different RBSDV-encoded proteins, only P6 was found to co-precipitate with P9-1 (Fig. 2a and data not shown). This result suggests that P6 directly binds to P9-1 in planta. To further confirm the interaction between P6 and P9-1, we performed a BiFC assay in Sf9 cells. The Sf9 cells expressing eYFP(c)–P9-1 plus eYFP(n)–P6 or eYFP(n)–P9-1 plus eYFP(c)–P6 showed bright yellow fluorescence, indicating protein–protein interactions (Fig. 2b). Notably, many cells showed the localization of yellow fluorescence to large inclusions or aggregated structures in the cytoplasm (Fig. 2b), suggesting that the interaction occurs in inclusion structures. In control experiments, no interaction was observed between P9-1 and P5 or P10, or P6 and P10 (Fig. 2b). Together, these results suggest that P6 directly binds to the viroplasm matrix protein P9-1. Using the yeast two hybrid (YTH) assay, we have recently identified an interaction between the P6 and P5 proteins of SRBSDV (Li et al., 2013). In the BiFC assay, interaction between P6 and P5 of RBSDV was also observed (Fig. 2b).

**P6 and P5 co-localize with P9-1 in electron-dense inclusion structures**

Because P9-1 and P6 interact, we carried out immunogold electron microscopy to analyse the presence of P6 in viral inclusion bodies. Ultrathin sections were prepared from the leaves of RBSDV-infected plants. The viroplasm structures were readily recognized as amorphous, granular, electron-dense cytoplasmic bodies with sizes ranging from 0.2 to 57 μm (Fig. 3). The RBSDV viroplasms had two regions. One was a dark osmiophilic area that surrounded the second, lighter areas. Within and around the dark osmiophilic portion, there were numerous mature and core particles of RBSDV (Fig. 3). This may suggest the association of the dark osmiophilic areas with particle

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**Fig. 2.** Interaction of P9-1 with P6. (a) Co-immunoprecipitation assay to investigate the association of P9-1 with other viral proteins. The total proteins extracted from virus free (VF) or RBSDV-infected maize leaves were immunoprecipitated with P9-1 antibody and then analysed by Western blot using P6, P5 and P10 antibodies. In parallel, total crude proteins extracted from Sf9 cells expressing HA-tagged P9-1 (HA–P9-1) and non-transfected (NT) Sf9 cells were immunoprecipitated with P9-1 antibody and were then analysed by Western blot using HA antibody. (b) BiFC assay to examine the interaction of P9-1 with P6, P5 or P10 and P6 with P5 or P10. Sf9 cells were co-transfected with recombinant baculovirus vectors containing the constructs indicated above the images. YFP fluorescence was observed using CLSM at 48 h p.t. The bright-field images were merged with fluorescent images. Bars, 50 μm.
Fig. 3. Co-localization of P9-1, P6 and P5 in electron-dense inclusion bodies. Electron micrographs showing immunogold labelling of RBSDV viroplasms using P9-1 (a), P6 (b), P5 (c) and P10 (d) antibodies or pre-immune serum (e). Ultrathin sections were prepared from leaves of RBSDV-infected maize plants. Gold particle (10 nm)-conjugated goat antibody against rabbit IgG was used as the secondary antibody. Immunogold particles are marked with arrows and virus particles with black arrowheads. Bars identify the degree of magnification in each image.
maturation. The P9-1 antibody labelled both dark and light regions of the viroplasms (Fig. 3a), while the P6 antibody almost exclusively labelled the dark portion (Fig. 3b, labelled with arrowheads). When the antibodies specific for the remaining non-structural proteins (P5, P7-1, P7-2 and P9-2) were used for immunoelectron microscopy, only the P5 antibody labelled viroplasms (Fig. 3c). P9-1, P6 and P5 antibodies consistently labelled RBSDV viroplasms when immunolabelling was carried out on larger numbers of ultrathin sections prepared from the leaves of infected maize or rice plants (data not shown). In contrast, antibodies to the P10 structural proteins labelled virus particles but not the electron-dense inclusion (Fig. 3d). No gold-particle labelling was observed in inclusion bodies when pre-immune serum was used (Fig. 3e). These results indicate that P6 and P5 are constituents of RBSDV viroplasms.

P9-1 recruits P6 to viroplasm-like structures in Sf9 cells

Because P6 interacts with P9-1 and is a constituent of the viroplasm, we wanted to examine the subcellular distribution of P6 when expressed alone or together with P9-1. P6 was fused to the C terminus of red fluorescent protein (RFP–P6) and expressed in Sf9 cells. When the fluorescence was observed by CLSM, RFP–P6 was diffusely distributed throughout the cytoplasm (Fig. 4a), which indicates that P6 alone was not able to form inclusion structures. However, when RFP–P6 was co-expressed with P9-1 fused to the C terminus of enhanced green fluorescent protein (eGFP–P9-1) in Sf9 cells, RFP–P6 co-localized with eGFP–P9-1 in inclusion structures in co-transfected cells (Fig. 4a), indicating that P9-1 recruits P5 to inclusions. The red fluorescence did not fully overlap the green fluorescence as RFP–P6 mostly formed smaller inclusions than eGFP–P9-1. This was more pronounced at 72 h p.t., when inclusion sizes were much bigger (Fig. 4a). Thus, it seems that P9-1 encloses P6 or that P6 accumulates in the interior region of the inclusion structures formed by P9-1. To further examine whether the co-localization with P9-1 also occurred when P6 was expressed as an unfused protein, Sf9 cells expressing P6 and HA–P9-1 were analysed by immunofluorescence staining using P6 and HA antibodies. At 48 h p.t., the immunostaining indicated that HA–P9-1 and P6 co-localized in inclusion structures in the cytoplasm (Fig. S1, available in JGV Online).

When P5 fused to the C terminus of RFP (RFP–P5) was expressed alone in Sf9 cells, RFP–P5 localized to numerous irregular structures of various sizes in the cytoplasm (Fig. 4b). These structures appeared quite similar to the inclusion structures formed by P9-1 (Figs 1b and 4a). When co-expressed with eGFP–P9-1, the structures formed by RFP–P5 did not co-localize with P9-1-derived inclusions (Fig. 4b), indicating that P5 is not recruited by P9-1 to viroplasm-like structures. This observation therefore concurs with the result that P5 does not interact with P9-1 (Fig. 2b). In a control experiment, P10 fused to the C terminus of RFP (P10-RFP) was diffusely distributed throughout the cytoplasm whether expressed alone or together with eGFP–P9-1 (Fig. 4c).

Cell lysis with a Triton X-100-containing buffer has been used previously to discriminate between free and inclusion-associated protein (Tourís-Otero et al., 2004b). To further confirm the association of P6 with inclusions, we performed a similar experiment, in which Sf9 cells expressing P6, P9-1 or P6 plus P9-1 were incubated with Triton X-100-containing buffer, followed by cellular fractionation to obtain soluble and insoluble fractions. Western blot analysis revealed that P9-1 was predominantly associated with the insoluble fraction (Fig. 5). When expressed alone, P6 was exclusively detected in the soluble fraction but became associated with the insoluble fraction when co-expressed with P9-1 (Fig. 5). The reliability of the fractionation was verified by the association of tubulin with the insoluble fraction (Fig. 5). This result further indicates that P9-1 recruits P6 into the viroplasm.

P5 is recruited to viroplasm-like structures by binding to P6

Because P6 and P5 interact (Fig. 2b), we then examined the subcellular distribution of P6 and P5 when they were expressed together in Sf9 cells. P6 fused to the C terminus of enhanced green fluorescent protein (eGFP–P6) co-localized with RFP–P5 into aggregated structures whereas eGFP–P6 was diffusely distributed throughout the cytoplasm when expressed alone (Fig. 6a). Thus, it seems that the subcellular distribution of P6 is substantially altered by P5 when these two proteins are expressed together. Because P5 is a constituent of RBSDV viroplasms (Fig. 3c) but does not directly interact with P9-1 (Fig. 2), we further questioned whether P5 is able to localize to viroplasms through its association with P6. To assess this possibility, we simultaneously expressed RFP–P5, eGFP–P6, eYFP(n)–P9-1 and eYFP(c)–P9-1 in Sf9 cells. As expected, both RFP–P5 and eGFP–P6 co-localized with P9-1-derived inclusion structures that were visualized through eYFP fluorescence reconstructed by P9-1 self-interaction (Fig. 6b). When eGFP–P6 or RFP–P5 was expressed alone with eYFP(n)–P9-1 and eYFP(c)–P9-1, eGFP–P6 but not RFP–P5 overlapped the eYFP fluorescence reconstructed by P9-1 self-interaction (Fig. S2). These observations suggest that P6 recruits P5 into the viroplasm.

DISCUSSION

The results of our analyses of RBSDV P9-1 using the baculovirus expression system in Sf9 cells were consistent with the previously reported characteristics of P9-1 (Akita et al., 2012; Zhang et al., 2008). The ability of P9-1 to self-interact in vivo and to form inclusions in the absence of other viral protein was confirmed by BiFC assay or when P9-1 was expressed as a fluorescent fusion protein (Figs 1
Thus, P9-1 shows striking similarities to the key proteins of viroplasm formation in other reoviruses, such as μNS of avian and mammalian reoviruses, NS2 of rotaviruses, NSP5 of bluetongue virus (BTV, genus Orbivirus) and Pns12 of rice dwarf virus (RDV, genus Phytoreovirus) (Becker et al., 2003; Contin et al., 2010; Thomas et al., 1990; Touris-Otero et al., 2004a; Wei et al., 2006). Interestingly, observations at 48 and 72 h p.t. revealed that the first-formed inclusions fused to form bigger structures. This resembles the progression in viroplasm size observed in cells infected with avian reovirus or RDV (Touris-Otero et al., 2004b; Wei et al., 2006). Co-immunoprecipitation assays indicated that of the five non-structural proteins only P6 directly interacted with P9-1 (Fig. 2a). This confirms results of a previous study showing that P9-1 and P6 interacted in the yeast two-hybrid system, and which also showed that the central region of P6 was necessary for the interaction with P9-1 (Wang et al., 2011). Thus, this direct interaction is likely responsible for the recruitment of P6 into the viroplasm (Figs 3b, 4b and 5). A recent study of the subcellular localization of proteins encoded by MRCV in Sf9 cells similarly showed that P6 alone was distributed in the cytoplasm but when co-expressed with P9-1 was partially localized to inclusion structures (Maroniche et al., 2012).

Together, these observations may suggest that the interaction between P9-1 and P6 is conserved among viruses belonging to the genus Fijivirus. The recruitment of a viral non-structural protein by a viroplasm matrix protein by direct interaction has also been documented for other reoviruses. For example, μNS of avian or mammalian reoviruses interacts with σNS and recruits it into viroplasms (Becker et al., 2003; Touris-Otero et al., 2004b). A similar relation was also observed between NSP5 and NSP2 of rotaviruses (Afrikanova et al., 1998; Fabbretti et al., 1999). In the case of RDV, Pns12 is the
minimal factor required for formation of viroplasms but two other non-structural proteins, Pns6 and Pns11, also appear to be constituents of viroplasms (Wei et al., 2006) although no direct interactions with Pns12 have been demonstrated. RBSDV P5 is also a constituent of viroplasms and interacted with P6 but not with P9-1 (Figs 2b and 3c). Interestingly, P5 localized to P9-1-derived inclusions when co-expressed with P6 but did not when P6 was absent (Figs 4b and 6b). In summary, our results suggest a model in which P9-1 directly interacts with P6 and recruits it into viroplasms while it indirectly recruits P5 into the viroplasms through the association of P6 and P5.

It is important to note that many of the viroplasm-associated proteins such as σNS, NSP2, NS2 and Pns11 share the ability to bind single-stranded RNAs (ssRNAs) (Gillian et al., 2000; Kattoura et al., 1992; Xu et al., 1998; Zhao et al., 1994), which suggests that these proteins may play an important role in viral RNA replication or packaging in the viroplasm. The ability of P6 or P5 to bind ssRNA is not known, but RBSDV P9-1 and the closely related MRCV P9-1 can bind ssRNAs (Akita et al., 2012; Maroniche et al., 2010), suggesting additional roles for P9-1 in viral RNA replication or packaging. Previously, P6 was shown to have an RNA silencing suppression activity in plants (Zhang et al., 2005). Given that viroplasms contain high concentrations of viral RNAs, the presence of an RNA silencing suppressor in the viroplasm seems to provide an effective strategy to protect viral RNAs from the host antiviral defence. In fact, our deep sequencing analysis of small RNAs revealed that abundant RBSDV-derived small interfering RNAs are generated in rice plants and insect vectors infected with RBSDV (unpublished results), demonstrating that RBSDV replication indeed triggers RNA silencing-mediated antiviral defence in the cells. The precise nature of the aggregate structures formed by P5 fused to RFP in S9 cells is still unclear (Fig. 4b). When fused with the C terminus of GFP and expressed in S9 cells, MRCV P5-1, which is the counterpart of RBSDV P5, accumulates in vesicle-like structures that do not co-localize with a peroxisomal marker (Maroniche et al., 2012). MRCV P5-1 contains two predicted transmembrane domains and a C-terminal putative type I PDZ domain-binding motif that was essential for its distribution to vesicle-like structures (Maroniche et al., 2012). Analysis of RBSDV P5 using several online transmembrane segment prediction programs showed that it contains transmembrane domains, but no known PDZ domain-binding motif was found in its C terminus. Further work is needed to investigate whether P5 expression induces endomembrane rearrangement as commonly observed for membrane-associated proteins.

**METHODS**

**Virus and S9 cell line.** RBSDV-infected maize plants were collected from a field in Jiaxing city, Zhejiang Province, China. Primary cultures of S9 cells were purchased from Invitrogen and maintained in SFH-900 medium containing 10% FBS (Gibco) at 28 °C.

**RNA extraction, reverse transcription PCR (RT-PCR) and plasmid construction.** Total RNA was extracted from leaf tissue using Trizol (Invitrogen) according to the manufacturer’s protocol. The RBSDV coding regions were amplified by RT-PCR using total RNA extracted from the leaves of RBSDV-infected maize plants. First strand cDNAs were synthesized using ReverTra Ace reverse transcriptase (Toyobo) and amplified by PCR using Blend Tag DNA polymerase (Toyobo) according to the manufacturer’s protocols. PCR primers were designed to introduce additional restriction sites at the 5’- and 3’-terminal ends of the clones. PCR fragments were cloned into pGEM-T Easy (Promega) and sequenced. Details of primers used for all PCR amplifications are available upon request.

To express glutathione S-transferase (GST)-tagged recombinant proteins in *Escherichia coli*, DNA fragments were inserted between *Bam*HI and *Not*I restriction sites in pGEX-6p-1 (Invitrogen). For baculovirus expression in S9 cells, DNA fragments were inserted between the *Bam*HI and *Not*I sites of the pBacDual vector (Invitrogen). To construct the plasmid vector for the BicF assay in S9 cells, the N- (aa 1–175) and C-terminal (aa 175–245) portions of eYFP were amplified, respectively, from plasmids pSAt6-eYFP and pSAt6-eYFP (Clontech) and cloned into BglII–*Bam*HI-digested pBacDual plasmid to produce pBD-eYFP(n) and pBD-eYFP(c). Subsequently, DNA fragments were inserted between the *Bam*HI and *Not*I restriction sites located downstream of the N-terminal or C-terminal portions of the YFP gene. To construct plasmid vectors for expression of fluorescent protein fusions in S9 cells, eGFP and RFP genes were amplified from the plasmids pGFPN1 and pRFPN1 (Invitrogen), respectively. The PCR products were cloned into the *Bgl*II–*Bam*HI-digested pBacDual vector to generate pBD-eGFP and pBD-RFP. Subsequently, DNA fragments were inserted between the *Bam*HI and *Not*I restriction sites located downstream of the eGFP or RFP gene. To construct HA–tagged P9-1, the P9-1 gene was amplified by PCR using primers in which the coding sequence of the HA peptide had been introduced into the forward primer. The PCR product was inserted between *Bam*HI and *Not*I sites of the pBacDual plasmid.

**Baculovirus expression in S9 cells.** Recombinant baculoviruses were prepared using the Bac-to-Bac Baculovirus Expression System (Invitrogen) and transfected into S9 cells using lipofectamine (Invitrogen). Culturing of S9 cells and preparation of cell lysates were performed as described previously (Sun & Suzuki, 2008).

**Antibody preparation.** GST-fused viral proteins (RBSDV P5, P6, P7-1, P7-2, P8, P9-1 and P9-2) were expressed in *E. coli* strain BL21 (Novagen) and purified using glutathione-agarose (Sigma) according to the manufacturer’s protocol. Antibodies were raised in rabbits as described previously (Sun et al., 2013). A Sepharose affinity column (NHS-activated Sepharose 4 Fast Flow; Amersham Pharmacia Biotech) was used to purify antibodies specific for each protein described above. RBSDV P10 mAb was provided by Jianxiang Wu (Zhejiang University, China).

**Co-immunoprecipitation assay and Western blot analysis.** Crude total protein of S9 cells or leaves was extracted with a binding buffer [20 mM Tris/HCl (pH 7.0), 0.2 M NaCl, 1 mM EDTA, 0.5% NP-40 and 1 x protease inhibitor cocktail (Roche)]. Cell lysate or leaf extracts were immunoprecipitated by addition of polyclonal antibody specific to P9-1 and protein A–Sepharose beads (New England Biolabs) followed by incubation with rotation overnight at 4 °C. The beads were then washed three times with binding buffer, resuspended in SDS-PAGE sample buffer, boiled for 8 min, separated by SDS-PAGE and analysed by Western blot as described previously (Sun & Suzuki, 2008). For detection of P5, P6, P7-1, P7-2, P8 and P9-2, primary polyclonal antibodies (1:1000) and secondary polyclonal alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (1:5000; Invitrogen) and polyclonal antibody specific to P9-1 were added to the Western blot.
Sigma) were used. P10 and HA–P9-1 were detected using primary anti-P10 and anti-HA monoclonal serum (1:1000; Sigma) and secondary AP-conjugated goat anti-mouse IgG (1:5000; Sigma).

**Immunogold electron microscopy.** Preparation of ultrathin sections of plant leaf tissues and immunogold labelling generally followed the procedures described by Xiong et al. (2008). The grids were first incubated with antibodies specific for RBSDV non-structural proteins or P10 (1:50) and then with gold (10 nm)-conjugated goat IgG anti rabbit (1:100) (Sigma). The grids were examined by transmission electron microscopy (H-7650; Hitachi) and photographed by a Gatan 830 CCD camera.

**Immunofluorescence staining.** For immunofluorescent microscopy, S9 cells were treated as described by Wei et al. (2006) and were incubated with the following antibodies: mouse anti-HA monoclonal antibody (1:500), rabbit anti-P6 polyclonal antiserum (1:200), secondary antibody goat IgG anti-rabbit conjugated to FITC (1:200) and goat IgG anti-mouse conjugated to Alexa 594 (1:200; Sigma-Aldrich).

**Fluorescent protein imaging.** Fluorescent protein expressions in S9 cells were observed using a Leica TCS SP5 confocal laser scanning microscopy. The fluorescent signals were visualized using laser excitation/emission filter 488/500–508 nm for eGFP, 514/580–600 nm for eYFP, 543/610–655 nm for RFP, 543/570–635 nm for Alexa 594 and 488/505–550 nm for FITC.

**Cell fractionation.** S9 cells (approx. 1 x 10^6) were harvested and washed with 0.1 mM PBS (pH 7.3) three times prior to lysis in a buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % (v/v) NP-40, protease inhibitor cocktail (Sigma) and 1 % (v/v) Triton X-100 at 4 °C for 2 h. The soluble (supernatant) and insoluble (pellet) fractions were separated by centrifugation at 1500 g for 15 min. The pellets were washed once in lysis buffer. The soluble and insoluble fractions were run on SDS-PAGE and subjected to Western blotting. Tubulin was detected using primary (1:1000) anti-tubulin monoclonal serum (Sigma) and secondary (1:5000) AP-conjugated goat anti-mouse IgG (Sigma).

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