The movement protein encoded by gene 3 of rice transitory yellowing virus is associated with virus particles

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Gene 3 in the genomes of several plant-infecting rhabdoviruses, including rice transitory yellowing virus (RTYV), has been postulated to encode a cell-to-cell movement protein (MP). Trans-complementation experiments using a movement-defective tomato mosaic virus and the P3 protein of RTYV, encoded by gene 3, facilitated intercellular transport of the mutant virus. In transient-expression experiments with the GFP-fused P3 protein in epidermal leaf cells of Nicotiana benthamiana, the P3 protein was associated with the nucleus and plasmodesmata. Immunogold-labelling studies of thin sections of RTYV-infected rice plants using an antiserum against Escherichia coli-expressed His6-tagged P3 protein indicated that the P3 protein was located in cell walls and on virus particles. In Western blots using antisera against E. coli-expressed P3 protein and purified RTYV, the P3 protein was detected in purified RTYV, whilst antiserum against purified RTYV reacted with the E. coli-expressed P3 protein. After immunogold labelling of crude sap from RTYV-infected rice leaves, the P3 protein, as well as the N protein, was detected on the ribonucleocapsid core that emerged from partially disrupted virus particles. These results provide evidence that the P3 protein of RTYV, which functions as a viral MP, is a viral structural protein and seems to be associated with the ribonucleocapsid core of virus particles.

INTRODUCTION

Transitory yellowing disease of rice (Oryza sativa L.) was first described in Taiwan (Chiu et al., 1965) and later in Japan and Thailand (Inoue et al., 1986). The main symptoms are yellowing of lower leaves and fewer tillers, resulting in a loss in grain yields. On the basis of inoculation experiments with insect vectors (Nephotettix nigriceps, N. cincticeps and N. virescens) and electron microscopy of the diseased rice cells, the agent of transitory yellowing disease was identified as a rhabdovirus and named rice transitory yellowing virus (RTYV) (Chiu et al., 1965, 1968, 1990; Shikata & Chen, 1969). Another rhabdovirus, rice yellow stunt virus (RYSV), causes yellow stunt disease of rice, a serious problem in central and southern China (Chen et al., 1979; Fan et al., 1965). The virus is transmitted by the same insect vectors as for RTYV. As judged from the intracellular distribution of RTYV and RYSV, both viruses were assigned to the genus Nucleorhabdovirus. The complete nucleotide sequences of RTYV and RYSV indicated that both are strains of the same virus, rather than distinct viruses; however, symptoms on rice induced by RTYV infection differ slightly from those caused by RYSV infection (Hiraguri et al., 2010).

Rhabdoviruses have a non-segmented, negative-sense ssRNA genome that contains a 3′-leader sequence (3′-le), five genes and a 5′-trailer sequence (tr-5′) in the order 3′-le–N–P–M–G–L–tr-5′ (Dietzgen et al., 2011). In general, rhabdovirus particles are composed of a lipid envelope derived from host membranes and a ribonucleocapsid core consisting of a viral RNA genome bound to complexes of the nucleocapsid protein (N), the phosphoprotein (P) and polymerase (L), which are encoded by genes N, P and L, respectively. The glycoprotein (G), encoded by gene G, protrudes from the exterior of the lipid envelope, and the matrix protein (M), encoded by gene M, connects the envelope to the ribonucleocapsid core (Dietzgen et al., 2011). The genome organization of
RTYV (synonym of RYSV) is unique in having two genes in addition to the basic gene order (Hiraguri et al., 2010; Huang et al., 2003). The small P6 protein of RYSV, encoded by gene 6 between genes G and L, was detected in purified virus preparations by immunoblot analysis using antiserum against the glutathione S-transferase (GST)–P6 fusion protein (Huang et al., 2003). Gene 3, located between genes P and M, has been identified in the genomes of all plant-infecting rhabdoviruses, but not in animal-infecting rhabdoviruses (Scholthof et al., 1994; Tanno et al., 2000; Tsai et al., 2005; Wetzel et al., 1994). The P3 protein, encoded by gene 3, has been postulated to be a movement protein (MP; Melcher, 2000), and its function as an MP has been demonstrated for RYSV (Huang et al., 2005). However, the involvement of the P3 protein in virus particles has not been adequately shown.

Primary infection of plants, from the entry of the viruses into a plant cell with a mechanically damaged cell wall and plasma membrane, is mostly confined to a single cell. MPs are then responsible for transporting viral genomes or particles to adjacent uninfectected cells through plasmodesmata (PD). Plant viruses encode one or more MPs. Although MPs are highly variable in their amino acid sequences and protein structures, they share the ability to localize at PD and increase the size-exclusion limit of PD (Benitez-Alfonso et al., 2010; Lucas, 2006; Melcher, 1990). The requirement of MPs for cell-to-cell movement was first demonstrated in studies on a non-structural, 30 kDa protein (30K) of tobacco mosaic virus (TMV) in the genus Tobamovirus (Atabekov & Dorokhov, 1984). By a reverse-genetics approach using in vitro-constructed mutants with a frame shift in the 30K protein gene, the 30K protein has been revealed to be involved in cell-to-cell movement (Meshi et al., 1987). Additionally, a potexvirus mutant defective in cell-to-cell movement can spread when infectious RNA of the mutant virus was co-bombarded with a separately cloned 30K protein gene of TMV (Morozov et al., 1997). Several viral movement proteins were characterized as belonging to the ‘30K’ superfamily of MPs, with primary and secondary structural similarity to the 30K protein of TMV (Melcher, 2000).

Analyses of primary and secondary protein structures of the P3 protein of RYSV suggested that the P3 protein is a member of the ‘30K’ superfamily and can trans-complement cell-to-cell movement for a movement-deficient potato virus X (PVX) mutant in Nicotiana benthamiana leaves (Huang et al., 2005). In the present study, to demonstrate the ability of the P3 protein of RTYV to act as an MP, we used a particle bombardment-mediated trans-complementation assay with a cell-to-cell movement-defective tomato mosaic virus (ToMV) mutant and transient expression experiments with the GFP-fused P3 protein (P3–GFP). In Western blot analyses of purified virus and immunogold electron microscopy of the crude sap from RTYV-infected rice leaves, we found that the P3 protein is associated with virus particles, in particular the ribonucleocapsid core of virus particles.

RESULTS

**Trans-complementation of a movement-defective ToMV mutant by the RTYV P3 protein**

To investigate trans-complementation for the P3 protein as a cell-to-cell movement protein of the ‘30K’ superfamily, we used a particle bombardment-mediated trans-complementation assay with a ToMV-based chimeric virus. Gene 3 of RTYV was inserted into a plant expression plasmid, pE7133-GW (Hiraguri et al., 2011), then the plasmid was introduced into mature leaves of *N. benthamiana* via particle bombardment, together with a plasmid pILMRd.erg3 (Fig. 1a) (Hiraguri et al., 2011). When pILMRd.erg3 that encoded an endoplasmic reticulum (ER)-targeting GFP (ergFP) as a reporter was used alone to bombard *N. benthamiana* leaves, GFP fluorescence was restricted to single, isolated epidermal cells at 96, 100 and 100% of transfected sites at 1, 2 and 4 days post-bombardment, respectively [Fig. 1b(i), c], indicating that the ToMV mutant was able to express GFP in initially infected cells, but was defective in cell-to-cell movement due to the lack of viral MP. In contrast, when pILMRd.erg3 was co-bombarded with pE7133-RTYV P3, clusters of green-fluorescing cells at 1 day post-bombardment [Fig. 1b(ii)] and the number of fluorescing clusters (Fig. 1c) increased over time. In 76% of infection foci, the movement-defective virus had spread from the initially infected cells across one or more cell boundaries at 4 days post-bombardment. These results suggested that the defect in cell-to-cell movement of the ToMV mutant from pILMRd.erg3 was complemented by co-expression of the RTYV P3 protein. Similar results were obtained in a positive control: after pILMRd.erg3 was co-bombarded with plasmid pE7133-ToMV MP, ToMV MP was then expressed [Fig. 1b(iii)]. However, we noted that the ToMV MP was more efficient than the P3 protein in mediating cell-to-cell movement of the movement-defective virus (Fig. 1c).

**Subcellular localizations of RTYV P3-GFP in *N. benthamiana* epidermal cells**

To clarify the subcellular sites of the RTYV P3 protein in *N. benthamiana* epidermal cells, we bombarded leaves with an expression plasmid encoding the P3 protein fused with GFP (pE7133-RTYV P3:GFP) (Fig. 2a). As a control for the expression of free GFP, pE7133-GFP was used. In *N. benthamiana* leaves bombarded with pE7133-RTYV P3:GFP, green fluorescence from the fusion protein was detected predominantly as punctate structures at the cell periphery, presumably indicating PD [Fig. 2b(i)]. These punctate structures were stationary and occurred at discrete regions of the epidermal cell walls, regardless of the age or position of leaves used for the bombardments. RTYV P3 protein fusions were also detected in the nucleus, but the intensity of the fluorescence was much lower than that for free GFP. In contrast, when plasmid pE7133-GFP was used to bombard *N. benthamiana* leaves, green
Fig. 1. Trans-complementation of a movement-defective ToMV mutant by the RTYV P3 protein. (a) Schematic diagrams of an infectious ToMV expression plasmid (left) and viral MP expression plasmids (right). Genomic organization of wild-type ToMV is at the top. Open boxes represent ORFs of encoded proteins; a box delineated with broken lines represents an untranslatable sequence derived from the ToMV MP gene. Pentagons indicate the 35S RNA promoter of cauliflower mosaic virus (CaMV) (35S) and the nopaline synthase terminator (NOS). (b) Confocal laser micrographs of GFP in leaves of *N. benthamiana* 2 days post-bombardment with piLMRd.erG3 alone (i), piLMRd.erG3 and pE7133-RTYV P3 (ii) and piLMRd.erG3 and pE7133-ToMV MP (iii). Bars, 50 nm. (c) Frequency distribution of fluorescing epidermal cells in leaves of *N. benthamiana* bombarded with piLMRd.erG3 and various viral MP expression plasmids at 1, 2 and 4 days post-bombardment (p.b.). Numbers of sites with fluorescence from a single cell or from two, three or four or more cells are shown in the table, with corresponding percentages plotted on the graph. The experiment was repeated three times; the values shown are from a single representative experiment.
fluorescence from free GFP was generally detected in the nucleus and the cytoplasm of epidermal cells, but cell-wall-associated punctate structures were not observed [Fig. 2(i)].

The subcellular localization of the RTYV P3 protein was also revealed by immunogold electron microscopy using antiserum against the *Escherichia coli*-expressed His<sub>6</sub>-tagged P3 protein (anti-P3 serum) (Fig. 2c). Thin sections from RTYV-infected rice leaves were prepared and treated with anti-P3 serum, then treated with gold-conjugated secondary antibody. Numerous gold particles localized in the cell walls of infected leaves. This gold labelling pattern is similar to those reported for other viral MPs (Li *et al.*, 2004; Xiong *et al.*, 2008). Interestingly, gold particles were also observed on the virus particles in the host cells, which we inferred to be the result of an association of RTYV P3 protein with the virus particles [Fig. 2c(i)]. Gold particles were not observed in the cell walls in thin sections of healthy rice leaves probed with the anti-P3 serum [Fig. 2c(ii)] or in the cell walls or on the virus particles in thin sections of RTYV-infected rice leaves probed with a pre-immune serum (data not shown).

### Detection of RTYV P3 and N proteins in purified virus by Western blot

RTYV particles have been reported to constitute five proteins encoded by the RTYV genome: N, P, M, G and L. However, the association of the P3 protein with the virus particles has not been adequately demonstrated. To clarify the association of the RTYV P3 protein with virus particles, we used Western blot analysis of purified RTYV and antisera against *E. coli*-expressed viral proteins or the purified virus. When anti-P3 serum was used, RTYV P3 protein (32.8 kDa) was detected in the purified virus and in RTYV-infected rice extracts (Fig. 3, lanes 2 and 4), as well as in *E. coli*-expressed P3 protein (Fig. 3, lane 1). Due to the addition of the His<sub>6</sub> tag, the migration of P3 protein

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**Fig. 2.** Subcellular localization of the RTYV P3 protein. (a) Schematic diagram of plasmids used for transient expression of the free GFP (pE7133-GFP) and the RTYV P3–GFP fusion protein (pE7133-RTYV P3 : GFP) in leaf epidermal cells of *N. benthamiana*. (b) Confocal laser micrograph of the RTYV P3–GFP fusion protein (i) and the free GFP (ii) in epidermal cells of *N. benthamiana* at 2 days post-bombardment. Bars, 50 nm. Open arrowheads mark fluorescing nuclei. (c) Electron micrographs showing immunogold labelling of the RTYV P3 protein in the cell walls of RTYV-infected (i) and healthy (ii) rice leaves treated with anti-P3 serum, followed by treatment with gold-conjugated secondary antiserum. Bar, 250 nm.

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**Fig. 3.** Western blot analysis of P3 and N proteins from purified RTYV. (a) Detection of RTYV P3 protein with anti-P3 serum (lanes 1–4) and with anti-RTYV serum (lanes 5–8). Total protein from *E. coli*-expressed P3 protein (lanes 1 and 5), purified virus (lanes 2 and 6), healthy rice leaves (lanes 3 and 7) and RTYV-infected rice leaves (lanes 4 and 8) was separated by SDS-PAGE (12% polyacrylamide) followed by immunoblot analysis. (b) Detection of RTYV N protein with anti-N serum (lanes 1–4) and with anti-RTYV serum (lanes 5–8). Total protein from *E. coli*-expressed N protein (lanes 1 and 5), purified virus (lanes 2 and 6), healthy rice leaves (lanes 3 and 7) and RTYV-infected rice leaves (lanes 4 and 8) was used. Positions of His<sub>6</sub>-tagged P3 protein (His-P3), RTYV P3 protein (P3), RTYV M protein (M), His<sub>6</sub>-tagged N protein (His-N) and RTYV N protein (N) are marked on the right; molecular size markers are on the left (in kDa).
extracted from *E. coli* was slower than those of the P3 proteins from purified virus and from RTYV-infected rice. Antiserum against purified virus (anti-RTYV serum) reacted with *E. coli*-expressed His<sub>6</sub>-tagged P3 protein (Fig. 3a, lane 5). Furthermore, a weak band (32.8 kDa) with a molecular size slightly larger than that of RTYV M protein (31.3 kDa) was recognized as the P3 protein from the purified virus (Fig. 3a, lane 6). Similar results were obtained from experiments to detect *E. coli*-expressed or intact N proteins with antiserum against the *E. coli*-expressed His<sub>6</sub>-tagged N protein (anti-N serum). RTYV N protein was detected in purified virus and in RTYV-infected rice extracts (Fig. 3b, lanes 2 and 4), as well as in the *E. coli*-expressed N protein (Fig. 3a, lane 1). Anti-RTYV serum reacted with *E. coli*-expressed N protein (Fig. 3b, lane 5) and RTYV N protein from purified virus and RTYV-infected rice extracts (Fig. 3b, lanes 6 and 8).

Considering that N protein is a virus structural protein and one of the components of a ribonucleocapsid core, these results suggested that the RTYV P3 protein, similarly to the RTYV N protein, is associated with virus particles and might be a viral structural protein.

**Detection of RTYV P3 and N proteins in disrupted virus particles**

Immunogold electron microscopy of crude saps from RTYV-infected rice leaves was conducted to determine the location of the P3 protein in RTYV particles. When crude saps were smeared onto membrane-covered grids and observed by electron microscopy, bullet-shaped virus particles with enveloped structures (Chiu *et al.*., 1990; Shikata & Chen, 1969) were observed (Fig. 4a). However, when the crude saps were treated with antiserum solutions, the virus particles were unstable and disintegrated easily (Fig. 4b). When the crude saps were treated with anti-P3 serum and then labelled with immunogold, the gold particles associated with the RTYV particles; in particular, gold particles were observed near the outer shells of partly disrupted RTYV particles (Fig. 4c). In contrast, no gold particles were observed on the disrupted viral shells. Similar results were obtained when the crude saps were treated with anti-N serum, a component of the ribonucleocapsid core, which is located in virus particles (Fig. 4d). These results apparently indicated that these gold labels were on the ribonucleocapsid that had spilled from the disrupted RTYV particles and that the RTYV P3 and N proteins were associated with virus particles, specifically with the ribonucleocapsid core inside virus particles.

**DISCUSSION**

Most plant viruses have evolved to encode one or more MPs, which facilitate viral cell-to-cell movement through the PD of susceptible hosts. Among viruses of the family *Rhabdoviridae*, several plant-infecting rhabdoviruses, including RTYV, have one or more additional genes between genes P and M. A possible MP role for these non-structural proteins encoded by the genes was first proposed for the sc4 protein of sonchus yellow net virus (SYNV) (Scholthof *et al.*., 1994). On the basis of secondary structural predictions, the sc4 protein and related proteins, such as the P3 proteins of RYSV and maize mosaic virus, P4 protein of maize fine streak virus and 4b protein of lettuce necrotic yellows virus, are possible members of the ‘30K’ superfamily (Huang *et al.*, 2005; Melcher, 2000). In transient-expression experiments, the SYNV sc4 protein was localized at the periphery of epidermal cells of *N. benthamiana* (Goodin *et al.*, 2002) and formed punctate structures at the periphery of SYNV-infected cells (Goodin *et al.*, 2007), suggesting that the protein may act as an MP at or near the PD. The first direct evidence for the function of a non-structural protein as an MP was provided by *trans*-complementation with the RYSV P3 protein of a movement-deficient PVX mutant that contained a frame shift in the p25 gene, which encodes one of the three MPs that comprise the triple-gene block of PVX (Huang *et al.*, 2005). Our data provide evidence here that the RTYV P3 protein can *trans*-complement cell-to-cell movement of a movement-deficient ToMV mutant. We have also shown that P3 protein fused with GFP can accumulate at the cell periphery and form punctate structures in the absence of other viral proteins, similar to the subcellular accumulation observed for fluorescent-protein-fused MPs of the ‘30K’ superfamily of TMV and ToMV (Crawford & Zambrayski, 2000; Sasaki *et al.*, 2009). Furthermore, we showed that the RTYV P3 protein was localized in the cell walls of virus-infected rice plants, which is a common feature of MPs. However, the RTYV P3 protein appeared to be less effective than the ToMV MP for *trans*-complementation of movement-defective ToMV. This difference might be due to RTYV primarily infecting rice, a monocotyledonous plant; the RTYV P3 protein may not interact efficiently with specific host component(s) in a dicotyledonous plant such as *N. benthamiana*.
During virus movement in infected cells, a virally encoded MP interacts with a virus genome to be transported from virus replication sites along the cytoskeleton and/or ER networks to the PD in the cell wall of infected cells. Then, the virus moves through the PD in the form of an MP-associated ribonucleocapsid or virus particle complex (Lucas, 2006; Scholthof, 2005). In the case of plant-infecting rhabdoviruses, virus particles are at least one order of magnitude larger than PD, with a diameter of approximately 5 nm. Therefore, transit of intact particles would require enormous PD alterations, that ought to be easily visible by electron microscopy. Although numerous ultrastructural studies have focused on different rhabdoviruses, such intact particles within enlarged PDs have not been observed. Similar to other negative-sense RNA viruses with a ribonucleocapsid complex as a minimal infectious unit (Jackson et al., 2005; Kormelink et al., 2011; Scholthof, 2005), formation of an MP-associated ribonucleocapsid may be involved in cell-to-cell movement for plant-infecting rhabdoviruses. A specific interaction between genes 3 and N of RYSV was revealed in a GST pull-down assay with E. coli-expressed recombinant proteins, and a region between aa 61 and 90 of the P3 protein was shown to be involved in binding to the N protein (Huang et al., 2003). However, an additional gene such as gene 6 has not been found in the SYNV genome. These results indicated that the particle structures of SYNV and RTYV, as well as RYSV, may differ, even though these viruses are related closely in the same genus of the family Rhabdoviridae.

Although the virus particle structures of SYNV and RTYV may differ, the MPs can be associated with virus particles and can also be a structural protein. The presence of MPs in virus particles may yield significant advantages for rapid virus spread from the initially infected cell, where the enveloped virus particles are uncoated. In the infection process, numerous virus particles are thought to be injected into the plant cells. Some of these virus particles may start translation or replication in the infected cells. Others, if they are ready to move to neighbouring cells, will form cell-to-cell movement complexes with the assistance of the MP in the virus particles and could be released rapidly into neighbouring cells, which would provide multiple possibilities for the onset of viral infection.

Methods

Particle bombardment-mediated trans-complementation assay.

Plasmid piLMRd.erG3, a derivative of piLerG3, was constructed previously for trans-complementation experiments with a movement-defective ToMV mutant (Hiraguri et al., 2011). The plasmid lacks the start codon (from ATG to ACG) and a large part (18–557 nt) of the MP gene and contains the ER-targeting GFP (erGFP) gene instead of the ToMV CP gene. The plasmid generates an erGFP fluorescence reporter that is competent but MP- and CP-defective ToMV (L strain) mutant under the control of the 35S RNA promoter of caulimovirus mosaic virus (CaMV) after transfection by particle bombardment (Fig. 1a). Infection and spread of the ToMV mutant derived from piLMRD.erG3 can be monitored by observing the distribution of erGFP fluorescence.
For preparing a 35S promoter-driven cDNA clone of RTYV gene 3 and the ToMV MP gene, RTYV gene 3 and the ToMV MP genes were amplified from a cDNA library of RTYV and plasmid pTLW3 by PCR as described previously (Hiraguri et al., 2010) with appropriate primer sets (Table S1, available in JGV Online). The PCR products were cloned into pDONR221 (Invitrogen) using Gateway technology according to the manufacturer’s instructions. Then, RTYV gene 3 and the ToMV MP gene were moved into pE7133-GW (Hiraguri et al., 2011) downstream of the 35S promoter to generate the plant expression vectors pE7133-RTYV P3 and pE7133-ToMV MP, respectively.

These plasmids were introduced into epidermal cells of N. benthamiana via particle bombardment using the PDS-1000/He system (Bio-Rad Laboratories) as described previously (Sasaki et al., 2009). Mature leaves (6–9 cm long) of N. benthamiana (4–7 weeks old) were cut and placed on an empty plate at a target distance of 6 cm. Three milligrams of 16 cm. Three milligrams of 1

Western blot analysis. To elucidate whether RTYV P3 protein is a viral structural protein, we used Western blot analysis of the purified virus as described previously (Sasaya et al., 2001). E. coli-expressed P3 protein, purified virus protein and total proteins from healthy and RTYV-infected rice leaves were separated by SDS-PAGE (12 % polyacrylamide). The gels were transferred to PVDF membranes (Millipore) at 50 mA for 50 min. After blocking overnight with Tris-buffered saline (TBS) (20 mM Tris/HCl pH 7.5, 500 mM NaCl) containing 4 % skinned milk powder, the membranes were probed for 1 h on a shaker at room temperature with anti-P3 serum, anti-N serum or anti-RTYV serum diluted 1:5000. Subsequently, the membranes were incubated with alkaline phosphatase-conjugated anti-rabbit IgG (H + L) diluted 1 : 10000 (Jackson ImmunoResearch) for 1 h at room temperature. The detection signal was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Immunogold electron microscopy of thin sections of RTYV-infected rice leaves. Small leaf samples were excised from healthy and RTYV-infected rice plants and fixed in 50 mM PBS (pH 7.2) containing 1 % glutaraldehyde and 2 % formaldehyde for 3 h at 4 °C. Fixed samples were dehydrated through a graded ethanol series at 96 °C to 100 % containing 1 % glutaraldehyde and 2 % formaldehyde for 3 h at 4 °C. Fixed samples were dehydrated through a graded ethanol series at 20 °C and embedded in Lowicryl K4M resin (Electron Microscopy Sciences). Polymerization was allowed to proceed for 72 h at −20 °C. Sections were sectioned on an ultramicrotome (LKB Nova) with a diamond knife and then mounted on 200-mesh nickel collodion grids. The sections were placed on drops of anti-P3 serum diluted 1:100 in TBS, for 30 min at room temperature. After washing with TBS, the grids were probed with immunogold-labelled goat antibodies against rabbit IgG with 20 nm gold particles (British BioCals International). The grids were stained in 2 % uranyl acetate and lead citrate solutions and then observed with an H-7000 electron microscope (Hitachi).

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