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 nigropictus, 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 uninoculated 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. 2b(ii)].

The subcellular location of the RTYV P3 protein was also revealed by immunogold electron microscopy using antiserum against the Escherichia coli-expressed His6-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 antiserum 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 His6 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 His6-tagged P3 protein (His-P3), RTYV P3 protein (P3), RTYV M protein (M), His6-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 \textit{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 \textit{E. coli}-expressed His\textsubscript{6}-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 \textit{E. coli}-expressed or intact N proteins with antiserum against the \textit{E. coli}-expressed His\textsubscript{6}-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 \textit{E. coli}-expressed N protein (Fig. 3a, lane 1). Anti-RTYV serum reacted with \textit{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 R TYV N protein, is associated with virus particles and might be a viral structural protein.

**Detection of R TYV 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 \textit{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 R TYV 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 \textit{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 \textit{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 \textit{et al.}, 2005; Melcher, 2000). In transient-expression experiments, the SYNV sc4 protein was localized at the periphery of epidermal cells of \textit{N. benthamiana} (Goodin \textit{et al.}, 2002) and formed punctate structures at the periphery of SYNV-infected cells (Goodin \textit{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 \textit{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 \textit{et al.}, 2005). Our data provide evidence here that the RTYV P3 protein can \textit{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 & Zambryski, 2000; Sasaki \textit{et al.}, 2009). Furthermore, we showed that the R TYV P3 protein was localized in the cell walls of virus-infected rice plants, which is a common feature of MPs. However, the R TYV P3 protein appeared to be less effective than the ToMV MP for \textit{trans}-complementation of movement-defective ToMV. This difference might be due to R TYV primarily infecting rice, a monocotyledonous plant; the R TYV P3 protein may not interact efficiently with specific host component(s) in a dicotyledonous plant such as \textit{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 ribonucleoprotein 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., 2005). Our immunogold electron microscopy results suggest that the RTYV P3 protein is associated with the ribonucleocapsid core of RTYV. In contrast, the SYNV sc4 protein interacted specifically with the G protein and not with the N protein in bimolecular fluorescence complementation experiments among all pairwise interactions of SYNV-encoded proteins (Min et al., 2010). SYNV sc4 protein was considered to interact with the G protein, together with the SYNV M protein and several cytoplasm-tethered transcription activators, and to form an ER- and microtubule-associated movement complex. Protein Y, a putative MP, of potato yellow dwarf virus (PYDV) in the genus Nucleorhabdovirus interacts with the M protein, but not the N protein, of PYDV (Bandyopadhyay et al., 2010). A comparison of protein–protein interaction data between virus-encoded proteins for these three nucleorhabdoviruses suggests that strategies for cell-to-cell movement may differ even among closely related viruses in the genus Nucleo- rabdovirus. To form the postulated ER- and microtubule-associated movement complex, the P3 protein of RTYV and RYSV may bind directly to the N protein. In contrast, the SYNV sc4 protein and PYDV Y protein may interact directly with either the G or M protein and may not bind directly to the N protein.

The virus particles of the plant-infecting rhabdoviruses consist of five virus-encoded proteins, and the MPs have been believed to be non-structural proteins (Dietzgen et al., 2011; Jackson et al., 2005; Kormelink et al., 2011). In the case of RTYV and RYSV, these virus particles have been reported to consist of five virus-encoded structural proteins, N, P, M, G, and L, according to SDS-PAGE analysis of purified viruses (Chiu et al., 1990; Fang et al., 1994; Hayashi & Minobe, 1987). However, when these SDS-PAGE profiles of purified RTYV and RYSV were re-verified, a weak band with a molecular mass that differed slightly from that of the M protein, probably corresponding to the P3 protein, was noted. Our Western blot analysis using anti-RTYV serum and anti-P3 serum revealed definitively that P3 protein was in purified virus. Furthermore, our immunogold electron microscope observation of the thin sections and the crude saps from the virus-infected rice plants provided evidence that the P3 protein of RTYV was associated with the virus particles and seemed to be localized in the viral inner ribonucleocapsid core, rather than in the outer surface of virus particles. The association of an MP with virus particles was also shown in an analysis of disrupted SYNV particles that were fractionated by sucrose-gradient centrifugation; the sc4 protein co-sedimented with envelope-associated viral structural proteins G and M. In contrast to RTYV, the sc4 protein of SYNV is considered to be incorporated into the virus envelope that is derived by budding through the inner nuclear membrane (Scholthof et al., 1994). In addition, the small P6 protein of RYSV, encoded by a gene between genes G and L, was detected in purified virus and thought to be incorporated into virus particles (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 pLMRD.erG3, a derivative of pLMerG3, 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 replication-competent but MP- and CP-defective ToMV (L strain) mutant under the control of the 35S RNA promoter of cauliflower mosaic virus (CaMV) after transfection by particle bombardment (Fig. 1a). Infection and spread of the ToMV mutant derived from pLMRD.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 1 μm gold particles were coated with 1 μg plasmid DNA and the resultant particles were divided into three equal parts, each of which was used to bombard leaves with a rupture disc of 900 p.s.i. (6.21 MPa). Bombarded leaves were incubated at 25 °C in the dark for 1–4 days. GFP signals were observed with an LSM510 confocal laser microscope (Carl Zeiss). An excitation light of 488 nm produced by the argon laser and an emission filter of 505–530 nm allowed the detection of GFP-specific fluorescence. For each of the plasmids used in this research, the bombardment was repeated at least three times.

**Localization of RTYV P3 protein in *N. benthamiana* cells.** To localize RTYV P3 protein in cells, we constructed pE7133-RTYV P3-GFP, an expression plasmid for RTYV P3 fused with GFP (P3–GFP), which was expressed through the activity of a CaMV 35S RNA promoter. The GFP gene and the RTYV gene 3 lacking the stop codon were amplified by PCR with pBl-OX-GFP (inPLANTA) and pE7133-RTYV P3 as templates, respectively, using appropriate primer sets (Table S1). To construct pE7133-RTYV P3-GFP, we cloned the RTYV gene 3 into pE7133-GW GFP, which was altered by modifying pE7133-GW to express GFP fused to the C-terminal of arbitrary proteins (Fig. 2a) (Hiraguri et al., 2011). As a control for the expression of free GFP, the GFP gene was cloned into pE7133-GW using the Gateway technology to create pE7133-GFP (Hiraguri et al., 2011). These plasmids were used individually to bombard *N. benthamiana* epidermal leaf cells as already described. The bombarded *N. benthamiana* leaves were incubated at 25 °C in the dark for 24 h. GFP signals were observed with a LSM510 confocal laser microscope (Carl Zeiss).

**Purification of recombinant RTYV P3 protein and preparation of antiserum.** For producing anti-P3 serum or anti-N protein, genes 3 and N of RTYV were respectively amplified from a DNA library of RTYV, described previously (Hiraguri et al., 2010), by PCR with a specific primer set (Table S1). Amplified PCR fragments were ligated into plasmid pDONR221 and were transferred into plasmid pDEST17 using an LR clonase reaction to yield pDEST17-P3, in which the P3 and N sequences are fused in frame to the His6 tag sequence. The His6-tagged N fusion protein and the His6-tagged P3 fusion protein were expressed in *E. coli* strain BL-21AI (Invitrogen) by induction of arabinose and were purified with an Ni-NTA Purification Module kit according to the instructions provided by the suppliers (Qiagen). Approximately 1 mg of the purified His6-tagged N fusion protein or the His6-tagged P3 fusion protein was emulsified in Freund’s complete adjuvant and injected subcutaneously into New Zealand white rabbits. Four injections were given at intervals of 2 weeks; antisera were collected from bleeds 15 days after the final injection.

**Purification of virus.** RTYV was purified from RTYV-infected rice plants as described previously (Hayashi & Minobe, 1987). Leaves harvested from infected rice plants were homogenized in an extraction buffer composed of 0.1 M sodium phosphate, pH 7.4, 0.5 % 2-mercaptoethanol and 10 mM EDTA. The homogenate was centrifuged at 7000 r.p.m. for 10 min in a Hitachi R10A2 rotor. The supernatant was centrifuged at 45000 r.p.m. for 90 min in a Hitachi P50A2 rotor. The virus pellet was suspended in the same buffer as above and the suspension was subjected to rate zonal centrifugation at 16000 r.p.m. for 90 min in 5–20 % Percoll gradients in a Hitachi SRP28SA rotor. Fractions containing virus particles were pooled, and the virus was recovered by fractionation on a Sepharose CL-4B column (GE Healthcare) (Chiu et al., 1965).

**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 % skimmed 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 −20 °C and embedded in Lowycril 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 colloidion 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).

**Immunogold electron microscopy of crude sap from RTYV-infected rice leaves.** Leaves harvested from infected rice plants were homogenized in TBS, and the homogenate was centrifuged at 7000 r.p.m. for 10 min in a Hitachi R10A2 rotor. The supernatant was dropped onto membrane-coated 200-mesh nickel colloidion grids. The grids were placed on drops of anti-P3 serum diluted 1:50 or anti-N serum diluted 1:500 in TBS for 1 h at 37 °C. After washing with TBS, the grids were placed on drops of the immunogold-labelled goat antibodies against rabbit IgG with 10 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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