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The 1b gene of raspberry bushy dwarf virus is a virulence component that facilitates systemic virus infection in plants

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ABSTRACT

A product translated from the 1b gene of raspberry bushy dwarf virus (RBDV) was specifically detected in RBDV-infected *Nicotiana benthamiana* plants by immunoblot analysis. To analyze the effects of the 1b gene on virus infection in host plants, an RBDV deletion mutant virus (RBΔ1bstop), which is unable to express the 1b gene, was constructed and inoculated to *N. benthamiana* plants. The results showed that accumulation of the virus genomic (g) RNAs 1 and 2 decreased in inoculated leaves, and that systemic virus spread was delayed compared with wild-type RBDV. In contrast, accumulation of the viral gRNAs 1 and 2 was elevated in RBΔ1bstop-infected leaf tissues during ectopic expression of the 1b gene. Furthermore, we found that the 1b has weak RNA silencing suppressor activity.

1. Introduction

Raspberry bushy dwarf virus (RBDV) is the only member of the genus *Idaeovirus* that has not been assigned to a family (MacFarlane, 2011). RBDV naturally infects *Rubus* species and grapevine, and experimentally infects fifty-five herbaceous plants including *Nicotiana benthamiana* (Barnett and Murant, 1970; Jones et al., 1982; Kokko et al., 1996; Strik and Martin, 2003; Mavrič et al., 2003). The RBDV genome consists of two positive-sense, single-stranded genomic (g) RNAs (RBDV gRNA1 and gRNA2; Fig. 1a). RBDV gRNA1 encodes an ~190 kDa replication protein (190 KP) with polymerase and helicase motifs, whereas gRNA2 encodes a 39 kDa movement protein (MP) and a 30 kDa coat protein (CP), which is expressed from a subgenomic RNA (sgRNA3) that is synthesized during replication and serves as the CP mRNA (Mayo et al., 1991; Natsuaki et al., 1991; Ziegler et al., 1992). These RNAs are encapsidated in three separate particles of slightly different densities that consist of gRNA1, gRNA2, and gRNA2 plus sgRNA3. Inoculation of RBDV gRNA1 and gRNA2 to *N. benthamiana* plants without sgRNA3 leads to a low level of infection because the CP translated from sgRNA3 activates replication (MacFarlane and McGavin, 2009). The RBDV 190 kDa protein contains methyltransferase, helicase and polymerase motifs that exhibit similarity to those of the two bromovirus replication proteins that are encoded separately by bromovirus gRNA1 and gRNA2. In the latter case, bromovirus gRNA1 encodes the 1a protein containing methyltransferase and helicase motifs, whereas gRNA 2 encodes the 2a polymerase protein (Jones, 2005; Ziegler et al., 1992). Moreover, RBDV shares some common characteristics with viruses belonging to the genus *Ilarvirus* in the family *Bromoviridae* (ilarviruses) in having easily deformable virus particles, and in being transmitted by pollen (Amari et al., 2009; Aparicio et al., 1999; Hamilton et al., 1984; Isogai et al., 2014, 2015; Murant et al., 1974; Murant, 1976). Therefore, it has been proposed that the genus *Idaeovirus* be included in the family *Bromoviridae* (Ziegler et al., 1993). Furthermore, phylogenetic analyses of the conserved polymerase, helicase and methyltransferase motifs have prompted Koonin and Dolja (1993) to postulate that RBDV bears a resemblance to the ancestor of viruses in the family *Bromoviridae*.

RBDV gRNA 1 potentially encodes a 1b gene in a different reading frame that partially overlaps the 190KP C-terminal coding region (Fig. 1a, gRNA1; Jones et al., 2005). However, the putative 1b translation product (1b protein) with a calculated MW of 12 kDa has not been detected in infected plants, and a BLASTP search shows no significant homology of the 1b protein with any proteins deposited in the GenBank/EMBL/DDBJ database. If RBDV bears a resemblance to the ancestor of viruses in the family *Bromoviridae* as suggested above, the 1b gene might be an orthologue of the 2b gene encoded by viruses belonging to the genera *Cucumovirus* and *Ilarvirus* subgroups 1 and 2 based on their gene positions on the viral gRNAs (Xin et al., 1998; Ding et al., 1994). The 2b protein translated from the cucumber mosaic virus (CMV) 2b gene has been shown to be an RNA silencing suppressor (RSS)

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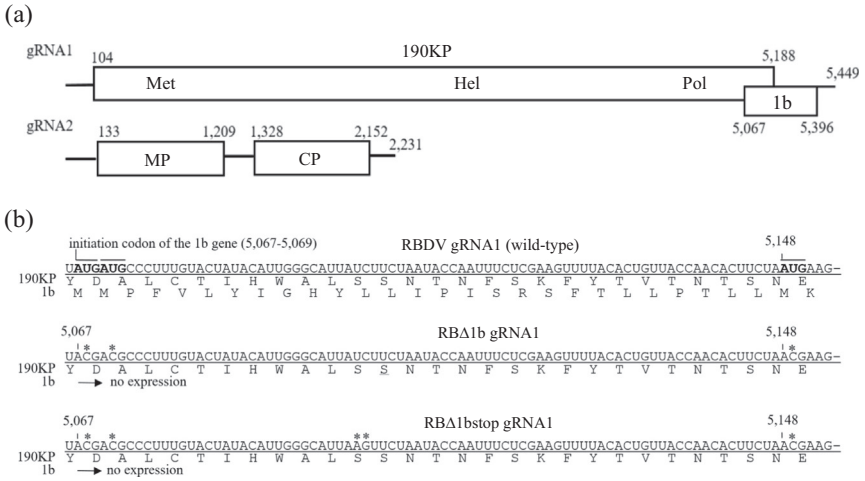


Fig. 1. Schematic representation of the genome organization of RBDV (a) and positions of the point mutations in the 1b gene (b). Boxes in genomic (g) RNA 1 represent open reading frames of the 190 kDa replication protein (190KP) showing the methyltransferase (Met), RNA helicase (Hel), and RNA-dependent RNA polymerase (Pol) domains and the 1b protein (1b). Boxes in gRNA 2 represent the movement protein (MP), and coat protein (CP). Bold nucleotides show the in-frame AUG codons of the 1b gene at nucleotide positions 5,067-5,069, 5,070-5,072, and 5,148-5,150 in the RBDV gRNA1 (wild-type). The RBΔ1b and RBΔ1bstop gRNA1s are not able to express the 1b protein by nucleotide substitutions (*). These substitutions did not change the 190 KP amino acid sequence.

that provides an antiviral defense mechanism to inhibit RNA degradation during infection (Burgyán and Havelda, 2011; Shimura et al., 2013). In general, RSS proteins encoded by different virus species lack significant amino acid sequence homology with those from other viruses (Csorba et al., 2015). Therefore, there is a possibility that the RBDV 1b protein and the CMV 2b protein have similar functions, even though no obvious amino acid sequence similarities have been detected.

In this paper, we have detected the 1b protein in RBDV-infected plants and have analyzed its effects on RBDV infection in plants by using mutant viruses that are unable to express the 1b gene. Moreover, RSS activity of the 1b protein was assessed by an *Agrobacterium*-mediated transient assay procedure and by a quantitative reverse transcription-polymerase chain reaction (RT-qPCR) test.

2. Results

2.1. The 1b protein is present in RBDV-infected plants

To detect the 1b protein in RBDV-infected plants, total protein extracts prepared from RBDV-infected *N. benthamiana* plants were separated in sodium dodecyl sulfate 20% polyacrylamide gels (20% SDS PAGE), blotted onto polyvinylidene difluoride (PVDF) membranes, and the membranes were then probed with an antiserum to the 1b protein. The results revealed a 14 kDa protein in infected *N. benthamiana* plants (Fig. 2a, lane 3 and 4), whereas the protein was not present in extracts from uninfected *N. benthamiana* plants (Fig. 2a, lane 1 and 2). In addition, total proteins from transgenic *N. benthamiana* plants expressing the 1b gene (1b-plants) were subjected to immunoblot analyses to identify the 1b protein. The antiserum to the 1b protein recognized a band at the same position as the 14 kDa protein obtained from RBDV-infected *N. benthamiana* plants (Fig. 2a, lane 5 and 6).

Subsequently, when the 1b gene was expressed in a cell-free transcription/translation wheat germ system, a 12 kDa protein was detected as a translation product (Fig. 2b, lane 3 and 4). In contrast, the 12 kDa protein was not detected in a control reaction lacking the 1b template (Fig. 2b, lane 2).

2.2. Deletion of the 1b gene decreases RBDV virulence

To analyze the effects of the 1b gene on RBDV infection in host plants, we constructed an RBDV 1b deficient mutant (RBΔ1b) by substituting the in-frame AUG codons of the 1b gene to ACGs (Fig. 1b, RBDV gRNA1 and RBΔ1b gRNA1). These substitutions resulted in alterations of the gRNA1 at nucleotide positions 5068, 5071, and 5149, but the substitutions did not change the 190 KP amino acid sequence. When RBΔ1b was inoculated to *N. benthamiana* plants by

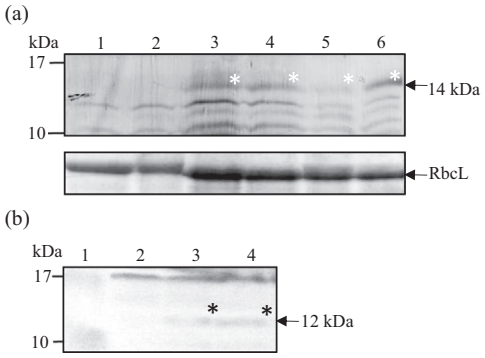


Fig. 2. Detection of the 1b protein in RBDV-infected and 1b gene-expressing transgenic *N. benthamiana* plants, and the cell-free translation product of the 1b gene. (a) Detection of the 1b protein in total protein extracts from RBDV-infected and 1b gene-expressing transgenic *N. benthamiana* plants by immunoblot analysis using an antiserum to the 1b protein; lanes 1 and 2, total proteins from uninfected *N. benthamiana* plants; lanes 3 and 4, total proteins from RBDV-infected *N. benthamiana* plants; lanes 5 and 6, total proteins from two transgenic *N. benthamiana* lines expressing the 1b gene (the 1b-plant line-2 and line-3, respectively). (b) Detection of the 1b protein in cell-free translation products by incorporation of biotinylated lysine; lane 1, marker proteins; lane 2, cell-free translation product with no template; lanes 3 and 4 cell-free translation products of the 1b gene. * indicates the 1b protein. RbcL (the large subunit of Rubisco) is shown as a loading control.

agroinfiltration, the mutant virus invaded the upper uninfiltrated leaves at eight days post infiltration (8 dpi), compared with ~ 6 dpi for the wild-type (wt) RBDV infections as assessed by RT-PCR assays.

To confirm that the 1b gene is dispensable for RBDV infection in *N. benthamiana* plants, we generated another RBDV 1b mutant (RBΔ1bstop) in which RBΔ1b gRNA1 at nucleotide positions 5099 (U) and 5100 (C) was changed to nucleotides A and G, respectively (Fig. 1b, RBΔ1bstop gRNA1). These substitutions also maintained the 190 KP amino acid sequence, but, the first and/or second in-frame AUG codons of the 1b gene were restored and the additional mutations resulted in UAA termination codons at positions 5,097-5,099 (Fig. 1b, RBDV gRNA1 and RBΔ1bstop gRNA1). We inoculated RBΔ1bstop to *N. benthamiana* plants by agroinfiltration, and the mutant virus was detected in the uninoculated upper leaves at 8 dpi by RT-PCR assays as was the case with the RBΔ1b mutant. In separate analyses, we cloned and sequenced a region comprising the substituted sequences by RT-PCR from the total RNAs extracted from the infected plants, and these sequences failed to reveal reversions in any of the twelve cDNA clones that were isolated. These results indicate that the 1b gene is a virulence component that assists in RBDV infection, but that 1b is not essential for

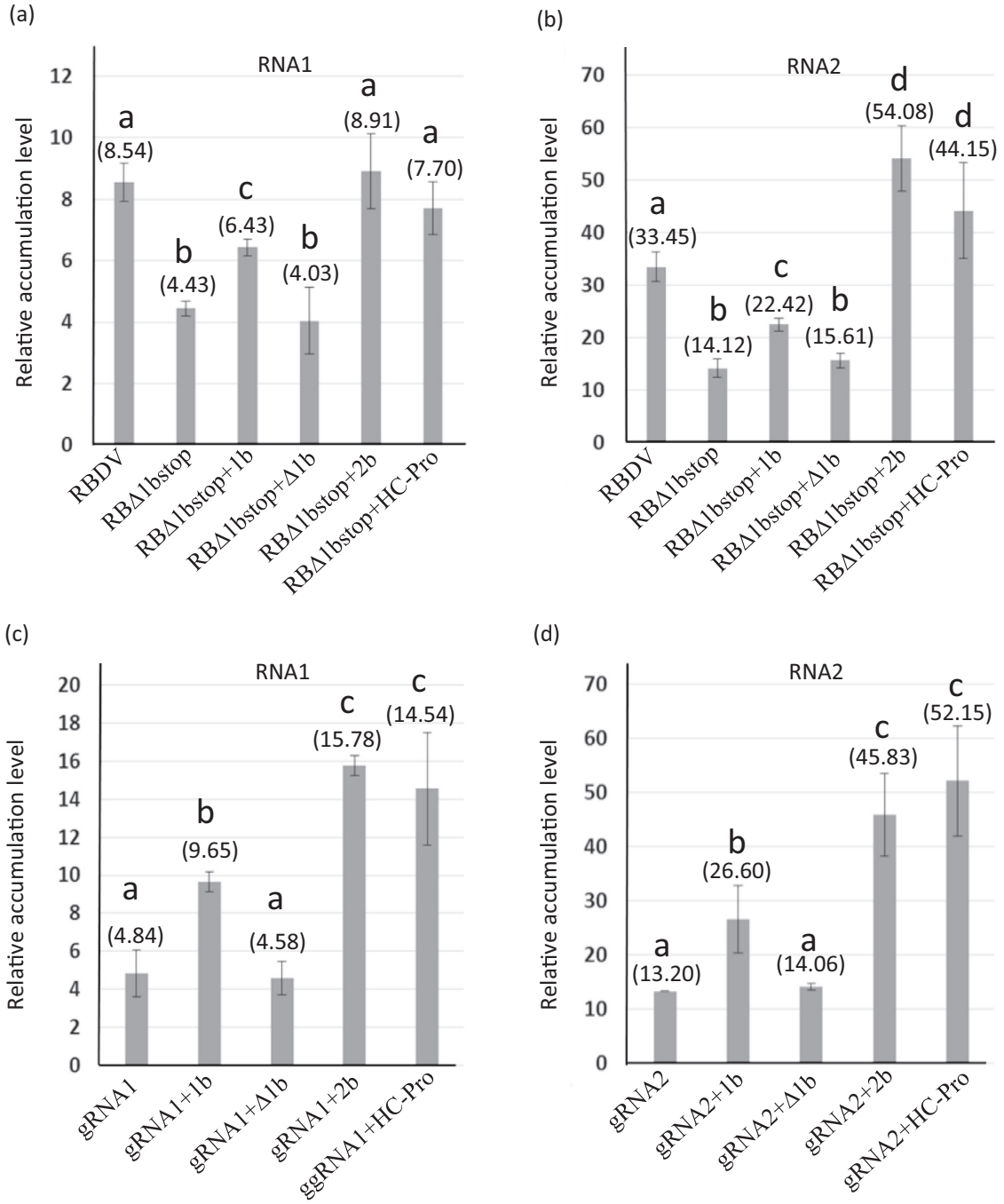


Fig. 3. Relative accumulation levels of RBDV gRNAs 1 and 2 in *N. benthamiana* leaves expressing the 1b gene. (a, b) Relative accumulation levels of gRNAs 1 (a) and 2 (b) at 3 days post infiltration (dpi) of *N. benthamiana* leaf tissues. wtRBDV and agrobacteria carrying the empty binary vector, pBE2113-P35T (RBDV) were inoculated by agroinfiltration of *N. benthamiana* leaf tissues. RBDV mutants were also inoculated by agroinfiltration with agrobacteria harboring pBE2113-P35T (RBΔ1bstop), 1b-agrobacteria (RBΔ1bstop + 1b), Δ1b-agrobacteria (RBΔ1bstop + Δ1b), 2b-agrobacteria (RBΔ1bstop + 2b), or HC-Pro-agrobacteria (RBΔ1bstop + HC-Pro). *Agrobacterium* suspensions for transcribing the RBDV gRNA1, gRNA2 and sgRNA3 were mixed with agrobacteria carrying pBE2113-P35T, 1b-agrobacteria, Δ1b-agrobacteria, 2b-agrobacteria, or HC-Pro-agrobacteria at a ratio of 1:1:1:1. (c, d) Relative accumulation levels of the RBDV gRNA1 (c) and gRNA2 (d) at 3 dpi. *N. benthamiana* leaf tissues expressing the RBΔ1bstop gRNA1 were inoculated by agroinfiltration with agrobacteria carrying pBE2113-P35T (gRNA1), 1b-agrobacteria (gRNA1 + 1b), Δ1b-agrobacteria (gRNA1 + Δ1b), 2b-agrobacteria (gRNA1 + 2b), and HC-Pro-agrobacteria (gRNA1 + HC-Pro). In *N. benthamiana* leaf tissues expressing RBDV gRNA2 was inoculated by agroinfiltration with agrobacteria carrying pBE2113-P35T (gRNA2), 1b-agrobacteria (gRNA2 + 1b), Δ1b-agrobacteria (gRNA2 + Δ1b), 2b-agrobacteria (gRNA2 + 2b), or HC-Pro-agrobacteria (gRNA2 + HC-Pro). *Agrobacterium* suspensions for transcribing the virus gRNA1 or gRNA2 was mixed with agrobacteria carrying pBE2113-P35T, 1b-agrobacteria, Δ1b-agrobacteria, 2b-agrobacteria, or HC-Pro-agrobacteria at a ratio of 1:1. Bars and error bars indicate mean values and standard deviations of three or more replicates. Different letters at the top of the bars indicate significant differences ($p < 0.05$) according to the Tukey-Kramer test.

systemic movement in *N. benthamiana* plants.

2.3. The 1b protein is required for optimal RBDV genomic RNA accumulation

As described above, infections of RBΔ1b and RBΔ1bstop in the upper uninfiltrated leaves were delayed by ~ 2 days, compared with wtRBDV. To determine whether the 1b gene affects the amounts of RBDV gRNAs in *N. benthamiana* leaf tissues, RBΔ1bstop was agroinfiltrated into *N. benthamiana* leaves, and the gRNA accumulation in the infiltrated tissue was quantified at 3 dpi by RT-qPCR. The results revealed that gRNAs 1 and 2 accumulation decreased by ~ 0.52 and ~ 0.42 times, respectively, in RBΔ1bstop infiltrations, compared with the wtRBDV agroinfiltrated leaf tissues (Fig. 3a and b, RBDV and RBΔ1bstop). Furthermore, to confirm that the presence of the 1b gene results in increased abundance of RBDV gRNAs in the infiltrated tissue, RBΔ1bstop was agroinfiltrated into *N. benthamiana* leaves in combination with agrobacteria harboring the pBE2113-1b gene (1b-agrobacteria), which was used for construction of the 1b-plants (Fig. 2, lane 5 and 6). RT-qPCR analyses of RBΔ1bstop/pBE2113-1b infiltrated tissue at 3 dpi revealed that RBDV gRNAs 1 and 2 increased by ~ 1.5 and ~ 1.6 times, respectively, when compared with tissue infiltrated with agrobacterial infiltrations harboring only RBΔ1bstop (Fig. 3a and b, RBΔ1bstop and RBΔ1bstop + 1b). In contrast, the amounts of gRNAs 1 and 2 were not significantly elevated in the leaf tissues in which RBΔ1bstop was agroinfiltrated with combinations harboring pBE2113-Δ1b (Δ1b-agrobacteria), carrying an untranslatable 1b gene (Fig. 3a and b, RBΔ1bstop and RBΔ1bstop + Δ1b). These results suggest that RBDV gRNA accumulation is increased by expression of the 1b protein *per se*, rather than by the 1b nucleotide sequence.

To compare the extent to which RBDV 1b, the CMV 2b, and clover yellow vein virus (CLYVV) HC-Pro proteins affect RBDV gRNA accumulation *in planta*, bacteria harboring RBΔ1bstop were agroinfiltrated into *N. benthamiana* along with 2b- or HC-Pro-agrobacteria, and the accumulation of RBDV gRNAs was quantified at 3 dpi by RT-qPCR. In comparison with tissues infiltrated with RBΔ1bstop, the amounts of gRNAs 1 and 2 increased by ~ 2.0 and ~ 3.8 times, respectively in the presence of 2b, while the gRNAs 1 and 2 increased by ~ 1.7 and ~ 3.1 times, respectively, in the presence of HC-Pro (Fig. 3a and b, RBΔ1bstop, RBΔ1b + 2b and RBΔ1b + HC-Pro). These results suggest that the 2b and HC-Pro proteins have high levels of RSS activity in inoculated tissues, whereas the 1b protein has more limited RSS activity.

2.4. The 1b gene elevates the abundance of RBDV gRNAs 1 and 2 separately

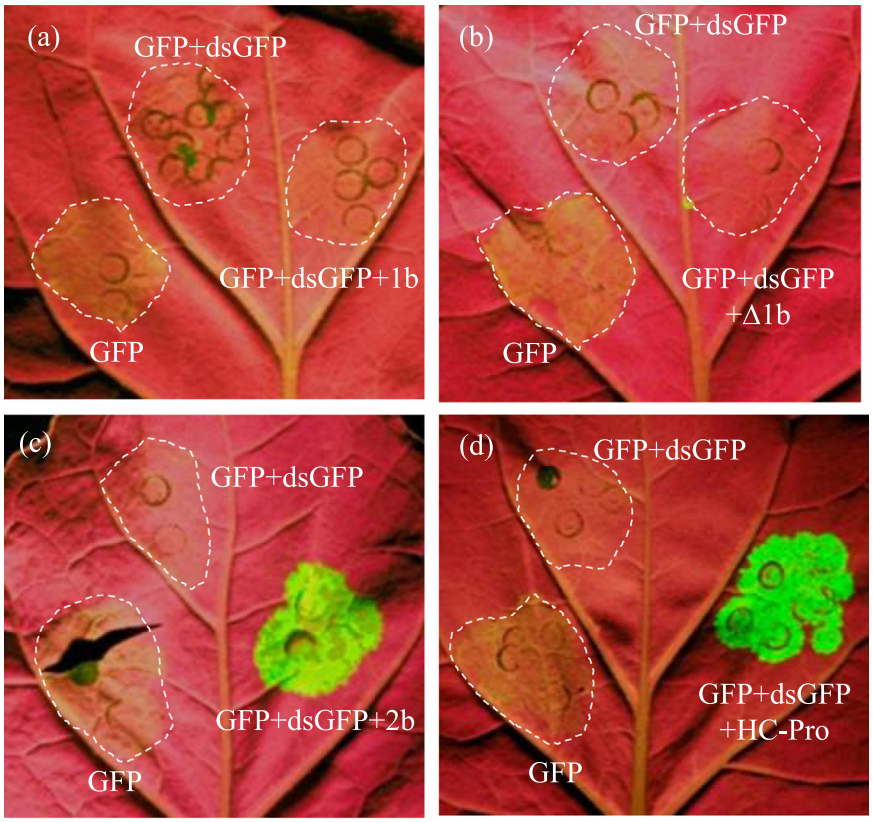
We sought to determine whether the 1b protein increases the amounts of RBDV gRNAs 1 and 2 separately. For this purpose, the RBΔ1bstop gRNAs 1 and 2 were agroinfiltrated individually into *N. benthamiana* leaves along with 1b-agrobacteria, and the accumulation of gRNAs in the infiltrated tissues were analyzed at 3 dpi by RT-qPCR. The results indicate that accumulation of gRNAs 1 and 2 increased by ~2 fold, when compared with those in *N. benthamiana* leaf tissues in which each gRNA was individually inoculated (Fig. 3c, gRNA1 and gRNA1 + 1b; Fig. 3d, gRNA2 and gRNA2 + 1b). On the other hand, when each RBΔ1bstop gRNA was agroinfiltrated into plants in combinations with Δ1b-agrobacteria, significant increases in the gRNAs were not detected by RT-qPCR (Fig. 3c, gRNA1 and gRNA1 + Δ1b; Fig. 3d, gRNA2 and gRNA2 + Δ1b). Taken together, the results show that the 1b protein elevates the abundance of RBDV gRNAs 1 and 2 separately, suggesting that the 1b protein is able to increase the amounts of the gRNAs in the absence of gRNA1 with the 190KP replication protein or gRNA2 with the cell-to-cell viral movement MP.

Subsequently, each RBΔ1bstop gRNA was inoculated to *N. benthamiana* leaf tissues by agroinfiltration in combination with 2b-agrobacteria or HC-Pro-agrobacteria, and the accumulation of gRNAs 1 and 2 in the infiltrated tissues was analyzed at 3 dpi by RT-qPCR. These results reveal that 2b increased the accumulation levels of gRNAs 1 and 2 by ~3.3 and ~ 3.5 times, respectively, and that HC-Pro increased the accumulation of gRNAs 1 and 2 by ~ 3.0 and ~ 4.0 fold, respectively, whereas 1b only increased the abundance of the gRNAs by ~ 2.0 fold (Fig. 3c, gRNA1, gRNA1 + 1b, gRNA1 + 2b, and gRNA1 + HC-Pro; Fig. 3d, gRNA2, gRNA2 + 1b, gRNA2 + 2b, and gRNA2 + HC-Pro). These results suggest that expression of the 1b protein results in limited elevation of the abundance of gRNAs 1 and 2 separately, and that the 2b and HC-Pro proteins have substantially increased effects over those of 1b on the accumulation of both RBDV gRNAs.

2.5. The 1b protein elicits weaker RSS activity than the 2b and HC-Proteins

We also analyzed possible RSS activity of the 1b protein by use of an agroinfiltration assay using *N. benthamiana* plants (Atsumi et al., 2015). In this assay, 1b-agrobacteria, harboring pBI-GFP (GFP-agrobacteria), which mediates expression of the GFP gene, and agrobacteria harboring pBI-dsGFP (dsGFP-agrobateria) for transcription of the GFP-inverted-repeat sequence were mixed, and infiltrated into *N. benthamiana* leaves. In these experiments, differences in GFP fluorescence at 3 dpi under long-wave UV light of leaf tissues infiltrated with GFP + dsGFP-agrobacteria and infiltrations with GFP + dsGFP + 1b-agrobacteria were difficult to assess (Fig. 4a, GFP + dsGFP and GFP + dsGFP + 1b), suggesting that only minimal 1b RSS activity is evident using this approach. Therefore, we performed RT-qPCR assays to quantify accumulation of GFP mRNA in the infiltrated tissues. The results revealed that accumulation of GFP mRNA in tissues infiltrated with GFP + dsGFP + 1b-agrobacteria at 3 dpi was ~ 1.8 times higher than in tissues infiltrated with GFP + dsGFP-agrobacteria (Fig. 4e, GFP + dsGFP and GFP + dsGFP + 1b). On the other hand, no significant differences in GFP fluorescence enhancement and GFP mRNA accumulation were observed between tissues infiltrated with GFP + dsGFP-agrobacteria and GFP + dsGFP + Δ1b-agrobacteria (Fig. 4b and e, GFP + dsGFP and GFP + dsGFP + Δ1b). In comparison assays, tissue infiltrated with GFP + dsGFP + 2b-agrobacteria and GFP + dsGFP + HC-Pro-agrobacteria showed strong GFP fluorescence (Fig. 4c and d), and the accumulation of GFP mRNAs were approximately 67 and 53 times higher than in leaf tissues infiltrated with GFP + dsGFP-agrobacteria (Fig. 4e, GFP + dsGFP, GFP + dsGFP + 2b and GFP + dsGFP + HC-Pro). In sum, our results provide evidence that the 1b protein exhibits RSS activity, albeit substantially lower than that of the more substantial activity of 2b or HC-Pro proteins.

Recently, it has been reported that the lettuce necrotic yellows virus (LNYV) P protein has weak RSS activity by co-expression with a GFP reporter without dsGFP, since dsGFP, which is a strong RNA silencing inducer, masks weak viral RSS activity (Mann et al., 2015; Mann and Dietzgen, 2017). Therefore, we compared GFP mRNA accumulation between *N. benthamiana* leaf tissues infiltrated with GFP-agrobacteria and GFP + 1b-agrobacteria, but no substantial differences in GFP fluorescence were observed under long-wave UV light in *N. benthamiana* tissues infiltrated with either combination (Fig. 5a and b). However, with the more sensitive RT-qPCR assays, the accumulation of GFP mRNA in tissue infiltrated with GFP + 1b-agrobacteria was ~1.7 times higher than in tissue singly infiltrated with GFP-agrobacteria (Fig. 5e; GFP and GFP + 1b). On the other hand, when GFP + Δ1b-agrobacteria were co-infiltrated into *N. benthamiana* leaves, enhancement of GFP fluorescence was not detected and increases in GFP mRNA accumulation were not observed at 3 dpi (Fig. 5b and e, GFP and GFP + Δ1b). In comparison assays, *N. benthamiana* leaf tissues infiltrated with GFP + 2b-agrobacteria and GFP + HC-Pro-agrobacteria showed strong GFP fluorescence (Fig. 5c and d), and the GFP mRNA accumulation was more than 200-fold higher than in leaf tissue singly infiltrated with GFP-agrobacteria at 3 dpi (Fig. 5e, GFP, GFP + 2b, and GFP + HC-Pro). Taken together, our results demonstrate that RBDV



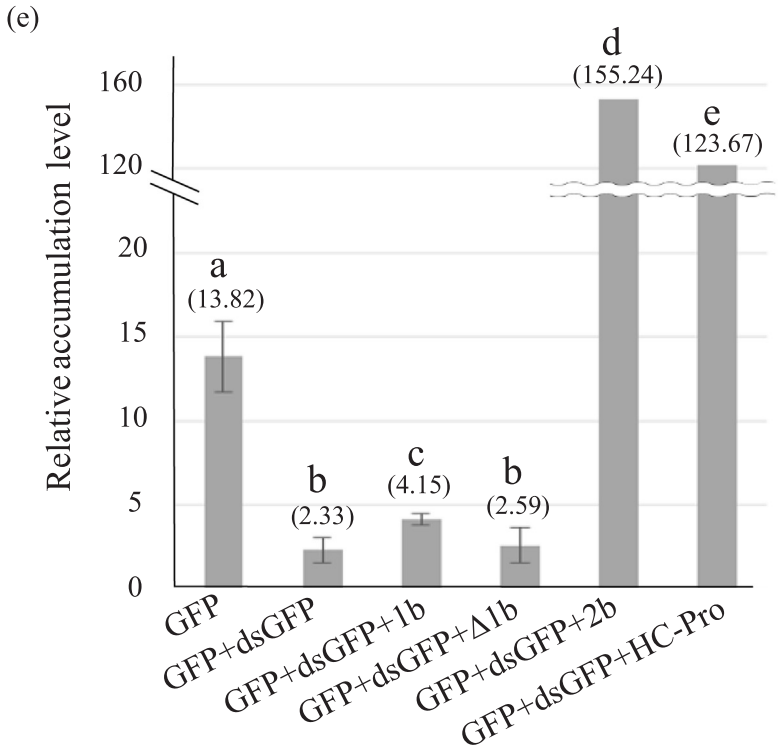
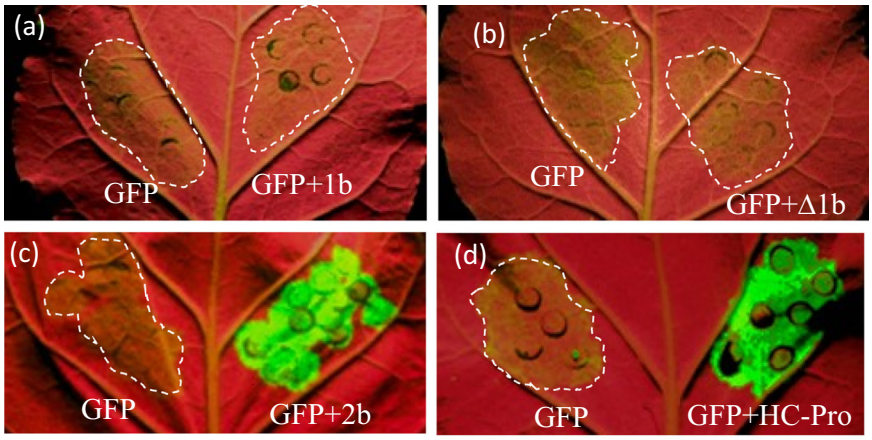


Fig. 4. Assessment of RNA silencing suppressor (RSS) activity of the 1b gene by an agroinfiltration assay of *N. benthamiana* plants. GFP fluorescence in *N. benthamiana* leaves was visualized at 3 dpi under a long-wave UV light (a-d) and by RT-qPCR analyses of relative accumulation levels of GFP mRNA in the infiltrated leaf tissues (e). GFP-agrobacteria and dsGFP-agrobacteria were mixed at a ratio of 10:1 (GFP + dsGFP-agrobacteria). Then, GFP + dsGFP-agrobacteria were added to agrobacteria carrying pBE2113-P35T (GFP + dsGFP), 1b-agrobacteria (GFP + dsGFP + 1b), Δ1b-agrobacteria (GFP + dsGFP + Δ1b), 2b-agrobacteria (GFP + dsGFP + 2b), or HC-Pro-agrobacteria (GFP + dsGFP + HC-Pro) at a ratio of 1:2, and the mixed agrobacteria were infiltrated to *N. benthamiana* leaf tissues. Bars and error bars indicate mean values and standard deviations of more than three replicates. Different letters at the top of the bars indicate significant differences ($p < 0.05$) according to the Tukey-Kramer test.

gRNA1 directs synthesis of a 14 kDa protein *in planta* and that this protein exhibits RSS activity that affects RBDV virulence, although its RSS activity against the GFP gene is much weaker than those of the 2b and HC-Pro RSS proteins.

3. Discussion

Immunoblot analysis using antiserum generated against the putative 1b protein, successfully identified a 14 kDa protein in RBDV-infected and in 1b gene-expressing transgenic plants (Fig. 2a). In contrast, the 14 kDa protein was not present in extracts from uninfected plants.



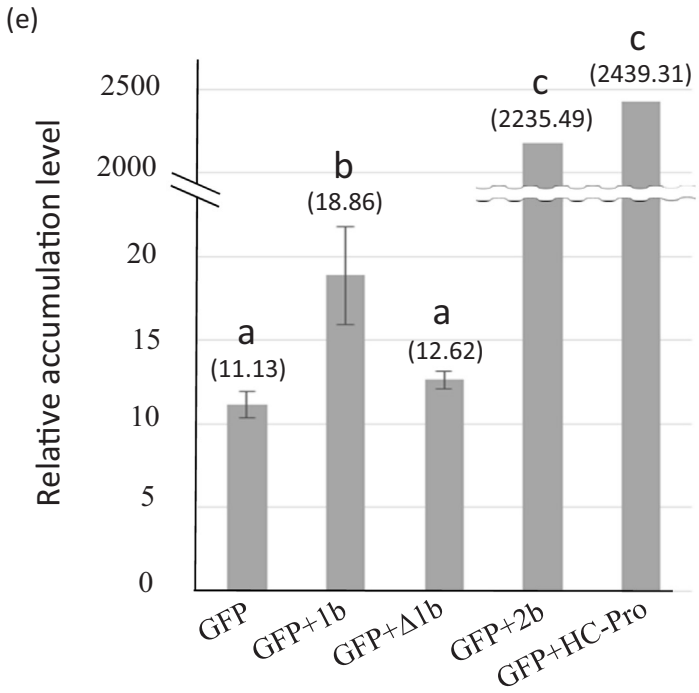


Fig. 5. RSS activity of the 1b gene against GFP in *N. benthamiana* plants during co-expression with the GFP gene. (a-d) GFP fluorescence visualized at 3 dpi under a long-wave UV light. (e) RT-qPCR analyses of relative accumulation levels of GFP mRNA in infiltrated leaf tissues at 3 dpi. GFP-agrobacteria were mixed with agrobacteria pBE2113-P35T (GFP), 1b-agrobacteria (GFP + 1b), Δ1bstop-agrobacteria (GFP + Δ1b), 2b-agrobacteria (GFP + 2b), or HC-Pro-agrobacteria (GFP + HC-Pro) at a ratio of 1:1, and then the mixtures were agroinfiltrated into *N. benthamiana* leaf tissues. Bars and error bars indicate mean values and standard deviations of more than three replicates. Different letters at the top of the bars indicate significant differences ($p < 0.05$) according to the Tukey-Kramer test.

Therefore, the 14 kDa protein appears to be the RBDV 1b protein expressed *in planta*, although its apparent size is larger than the 1b protein expressed in the wheat germ cell-free transcription/translation system (12 kDa protein; Fig. 2b). The wheat germ system for cell-free protein expression is low on protein modifying activities, where protein phosphorylation is commonly absent in the translation reactions and no glycosylation is observed (Harbers, 2014; Takai and Endo, 2010). Based on these results, we postulate that the 1b protein may be subjected to post-translational modification *in planta*, and this hypothesis, and its possible role in RSS activity will be evaluated in future experiments.

Deletions of the 1b gene resulted in decreases of RBDV gRNAs (Fig. 3a and b), but accumulation of the viral gRNAs was elevated in RBΔ1bstop-infected leaf tissues during ectopic expression of the 1b gene (Fig. 3a and b). Chiba et al. (2006) have reported that ectopic expression of RSS proteins can increase the accumulation of viral RNAs. Thus, it is reasonable to postulate that the 1b protein functions to provide RSS activities that increase the abundance of RBDV gRNAs during infection. In this study, we showed that expression of the 1b gene increases accumulation of RBDV gRNAs 1 and 2, respectively, in leaf tissues and that 1b activity facilitates systemic infection in plants. These RSS activities appear to be required for increased RBDV virulence by affecting both replicase and movement activities during infection. Thus, the results suggest that the 1b gene results in expression of a 14 kDa RSS protein that functions as an RSS that inhibits degradation of the RBDV gRNAs during infection. This conclusion is supported by our results that the 1b protein can increase accumulation of GFP mRNA in the RSS activity assays using a GFP reporter (Figs. 4e and 5e).

Recently, we reported that an RBDV-based vector producing a GFP gene fragment can induce virus-induced gene silencing (VIGS) of a GFP-transgene in *N. benthamiana* plants expressing the GFP gene (Shimura et al., 2016). Thus, we suggest that RNA silencing against the RBDV gRNAs is triggered by RBDV infection; hence raising the possibility that the putative 1b gene inhibits RNA silencing and has a role in inhibiting specific degradation of viral RNAs. Indeed, our RT-qPCR analyses revealed that gRNAs 1 and 2 accumulation are both elevated by expression of the 1b gene, although these levels are ~ 1.5 to ~ 2.0 times lower than RSS activities elicited by the 2b and HC-Pro proteins (Fig. 3c and d). However, the 1b protein appears to be more effective against RBDV derived RNAs than ectopically expressed GFP mRNA because elevation by the 1b gene was ~ 118 and ~ 129 times lower than with the 2b and HC-Pro genes, respectively (Fig. 5). Additional studies should allow us to elucidate these mechanisms whereby the 1b protein differentially

affects degradation of the virus gRNAs at levels similar to those of the 2b and HC-Pro proteins *versus* much weaker RSS activity against GFP gene expression.

It has been postulated that an ancestral bromovirus gRNA1, which has a resemblance to the RBDV gRNA1, evolved to transition into bromovirus gRNAs 1 and 2 by segmentation (Koonin and Dolja, 1993). This notion is supported by our results showing that the 1b and 2b proteins functions are similar and that both increase the accumulation of viral gRNAs and suppress RNA silencing against GFP mRNA (Figs. 3–5), and these results strengthen our hypothesis that the 1b and 2b genes may be orthologous. Recently, black currant leaf chlorosis associated virus and privet leaf blotch-associated virus have been reported to be putative members in the genus *Idaeovirus* (James and Phelan, 2017; Navarro et al., 2017; Thekke-Veetil et al., 2017). However, these putative members have not been reported to encode orthologues of the 1b gene. Our results reveal that the 1b gene is not absolutely essential for RBDV infection in *N. benthamiana* plants. Thus, there is a possibility that the dispensable RSS activities of the 1b gene could permit emergence of idaeoviruses lacking the 1b gene.

4. Materials and methods

4.1. Virus isolate and plant growth condition

RBDV-J1 was used as the wtRBDV in this experiment (Isogai et al., 2012). *N. benthamiana* plants were grown in plant pots in a plant growth room at 25 °C with a 16 h photoperiod.

4.2. Western blot analysis

Proteins were extracted from leaves of RBDV-infected *N. benthamiana* plants in 1 × SDS gel-loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The extracted proteins were separated by 20% SDS PAGE (Laemmli, 1970) and electroblotted onto a PVDF membrane (Immobilon-P^{SQ}; Merck Millipore, Carrigtwohill, Cork, IRL). Detection of the membrane bound 1b proteins was carried out as described previously (Blake et al., 1984).

4.3. Preparation of antiserum to the 1b protein

A DNA fragment of the 1b-encoding cDNA sequence was amplified by PCR using the 1b-F1/1b-R1 primer pair (Table 1). The amplified products were cloned into a T vector, pMD20, using the Mighty TA-cloning kit (Takara, Shiga, Japan). The resulting amplified sequences were restricted with *Nde* I and *Eco*R I, and the fragments were ligated into the equivalent *Nde* I and *Eco*R I regions of pCold ProS2 (Takara). A protein from the pCold ProS2 construct was expressed in *Escherichia coli* BL21, according the manufacturer's protocol. The resulting antibody, prepared by Eurofins Genomics (Tokyo, Japan), was raised by immunization of rabbits with the bacterially expressed protein.

4.4. In vitro protein synthesis of the 1b gene

Cell-free transcription/translation of the 1b gene was performed using the TNT coupled wheat germ extract systems (Promega, Madison, WI, USA). We constructed a plasmid, pIVT-1b, to use as a template DNA for the cell-free transcription/translation. The 1b-encoding cDNA sequence (Fig. 1a, nt 5,067-5,396 of the RBDV RNA1) was amplified by PCR using the 1b-F1/1b-R2 primer pair (Table 1). The amplified product was restricted with *Nde* I and *Bam*H I, and the resulting fragment was inserted between the *Nde* I and *Bam*H I sites of the DHFR plasmid DNA (New England Biolabs, Ipswich, MA, USA) to create pIVT-1b. For cell-free transcription/translation, 1 μg of pIVT-1b linearized by *Hind* III was added to the reaction mix (50 μl), which contained 25 μl of TNT Wheat Germ Extract, 2 μl of TNT Reaction Buffer, 1 μl of TNT T7 Wheat Germ Polymerase, 1 μl of 1 mM complete amino acid mixture, 1 μl (40 units) of Murine RNase Inhibitor (New England Biolabs), and 2 μl of biotilated lysine tRNA (Transcend tRNA; Promega). The reaction mixture was incubated at 30 °C for 1.5 h and the biotilated 1b protein was detected by Transcend non-radioactive translation detection system, according the manufacturer's protocol (Promega).

4.5. Infectious cDNA clones of RBDV

We constructed infectious cDNA clones of RBDV-J RNAs 1, 2, and 3, and used these derivatives (pBICRB1, pBICRB2, and pBICRB3) for agroinfiltration into plant leaves (Shimura et al., 2016). Point mutations of all in-frame AUG codons in the 1b gene of pBICRB1 were also conducted (Fig. 1b). For these derivatives, DNA fragments covering nt positions 3,334-5,449 of the RBDV gRNA1 was amplified from pBICRB1 as the template by PCR with a primer pair F398 (5′-CGTGTATTTCAG GTTAACTCG-3′) and R434 (5′-GCGGGCCCACGTGAGCGAAACCCT ATA-3′). The amplified product was cloned into a plasmid vector, pCR2.1-TOPO using TOPO TA cloning (Invitrogen, Carlsbad, CA, USA). The resulting TA clone was used to introduce targeted mutations of the 1b gene using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with two primers, mutation-5149 (5′-ACACTTCTAACGAAGTGCGAAGGAACAGAAG-3′) and mutation-5068&5071 (5′-AAGCTGCACCTAGTTTATACGACGCCCTTTG TAC-3′) as described in the manufacturer's protocol. The resulting clone (pTA-Δ1b) was restricted with *Sac* I and *Pml* I, and the resulting fragment was ligated into the equivalent region of the infectious pUBP35R-RB1 RBDV gRNA1 cDNA clone (Shimura et al., 2016) to create pUBP35R-RB1Δ1b. For plant inoculation by agroinfiltration, a region of the 35S promoter to 35S terminator of pUBP35R-RB1Δ1b was transferred to the binary agrobacterium vector (pBIC18; Takeda et al., 2005) using the *Sse*8387 I restriction site, generating pBICRB1Δ1b. In addition, we generated another infectious RBDV gRNA1 cDNA clone mutated at the 1b gene (pBICRBΔ1bstop). For this purpose, site-directed mutagenesis was used to modify pTA-Δ1b using the QuikChange Multi Site-Directed Mutagenesis Kit with a primer, mutation-5099&5100 (5′-GTACTATACATTGGGCATTAAGTTCTAATACCAATTTCTCGAAG-3′). Then, the resulting clone (pTA-Δ1bstop) was used to generate pBICRB1Δ1bstop by the same processes used to construct pBICRB1Δ1b.

The infectious RBDV RNA cDNA clones (pBICRB1, pBICRB1Δ1b, pBICRB1Δ1bstop, pBICRB2, and pBICRB3) were introduced into *A. tumefaciens* GV3101 by electroporation. *A. tumefaciens* derivatives harboring each construct were cultured on LB medium containing gentamicin (50 μg/ml), kanamycin (50 μg/ml), and rifampicin (100 μg/ml)

Table 1
List of primers used for PCR cloning of the RBDV gRNA1.

Primer name	Primer sequence (5'- 3')	Restriction site underlined	Nucleotide positions of the RBDV gRNA1
1b-F1	GGAATTCCATATGATGCCCTTTGTACTATAC	*Nde* I	5,067-5,087
1b-R1	GGAATTCTACAGAATTATAATAGTGTTAAGTAAT	*Eco*R I	5,180-5,204
1b-R2	CGGGATCCTAGTGAGCTAGGCTTAAGA	*Bam*H I	5,377-5,396
1b-F2	GCTCTAGAATGATGCCCTTTGTACTATACA	*Xba* I	5,067-5,087
1b-R3	TGCGAGCTCCTAGTGAGCTAGGCTTAAGAA	*Sac* I	5,376-5,396
Δ1b-F1	GCTCTAGAACGACGCCCTTTGTACTATACA	*Xba* I	5,067-5,088

at 28 °C for 48 h, and then harvested and resuspended in an infiltration buffer (10 mM MES, pH 5.7, 10 mM $MgCl_2$, 150 μM acetosyringone) at a final OD_{600} of 1.0. After 1 h of incubation, equal volumes of the *A. tumefaciens* carrying pBICRB1 (or pBICRB1Δ1b, or pBICRB1Δ1bstop), pBICRB2, and pBICRB3 were mixed, and the mixtures were infiltrated into of *N. benthamiana* leaves. The resulting viruses were designated wtRBDV (pBICRB1, pBICRB2, and pBICRB3), RBΔ1b (pBICRB1Δ1b, pBICRB2, and pBICRB3), and RBΔ1bstop (pBICRB1Δ1bstop, pBICRB2, and pBICRB3), respectively.

4.6. Binary plasmid constructs for transient expression and RNA silencing suppression assays

The binary plasmid constructs of pBE2113-P35T (empty binary vector), pBE2113-HCPro, pBE2113-2b, pBI-GFP and pBI-dsGFP have been previously described (Yaegashi et al., 2007). In this report, we made two new binary plasmid constructs, pBE2113-1b and pBE2113-Δ1b. To construct pBE2113-1b, the 1b gene of pBICRB1 was amplified by PCR using the 1b-F2/1b-R3 primer pair (Table 1). The amplified fragment was ligated into the pBE2113 binary vector (Mitsuhara et al., 1996) between the *Xba* I and *Sac* I sites. To generate pBE2113-Δ1b, the mutated 1b gene in pBICRB1Δ1bstop was amplified using the Δ1b-F1/1b-R3 primer pair (Table 1), and the fragment was ligated into pBE2113 between the *Xba* I and *Sac* I sites. The constructs were introduced into *A. tumefaciens* C58C1 by a freeze-thaw method and used for agroinfiltration as described previously (Yaegashi et al., 2007). GFP fluorescence was observed under a long-wave UV light (Black Ray model B 100A; UV products, Upland, CA, USA) and photographed with a digital camera with yellow filter.

4.7. Transgenic N. benthamiana plants expressing the 1b gene

pBE2113-1b described above was used to produce transgenic plants expressing the 1b gene. The plasmid was transferred to *A. tumefaciencs* LBA 4404 by electroporation (Yaegashi et al., 2008), and the transformed bacteria were inoculated to leaves of *N. benthamiana*, as described by Horsh et al. (1985). Transformants were selected for resistance to kanamycin (100 μg/ml), induced to regenerate shoots and roots, transferred to soil and placed in a growth chamber. Each transgenic plant was self-fertilized and five homozygous transgenic lines (1b-plant line-2, line-3, line-5, line-7 and line-9) were selected. These transgenic lines had no obvious phenotypic response to the insertion of the 1b gene.

4.8. RT-PCR assays

Total RNAs were extracted from *N. benthamiana* and *C. quinoa* leaves as described previously (Isogai et al., 2011) and reverse transcription (RT) was carried out using a random hexamer (1.25 μM). The cDNA was amplified by PCR as described by Isogai et al. (2014). The reaction mix was analyzed by 1% TAE agarose (w/v) gel electrophoresis with ethidium bromide staining.

4.9. RT-qPCR analysis

Total RNAs were extracted from agroinfiltrated *N. benthamiana* leaf tissues (Isogai et al., 2011). Then, contaminating host genomic DNA was removed from the total RNA samples by a DNase treatment using DNase I (Takara) according to the manufacturer's instructions, and the DNase was inactivated by phenol/chloroform extraction. First-strand cDNAs were synthesized by reverse-transcription using 1 μg of total RNA, a random hexamer (1.25 μM), and RevaTra Ace (100 units; Toyobo, Osaka, Japan) in a 20 μl reaction. Then, we assessed the amount of cDNAs reverse-transcribed from the RBDV gRNA1 and gRNA2 using KAPA SYBR FAST qPCR Kit (KAPA Biosystem, Wilmington, MA, USA) with the Eco Real-Time PCR system (illumine, San Diego, CA, USA). Primers used for qPCR of RBDV gRNA1, gRNA2, GFP and actin genes are listed Supplemental Table 1. Target regions of RBDV gRNAs 1 and 2 were amplified by qPCR were located in the virus genome positions where subgenomic RNAs are not generated (Isogai et al., 2018). Each 20 μl reaction contained 10 μl of 2×KAPA SYBR FAST qPCR Master Mix, 0.4 μl of 10 μM of each forward and reverse primer, 1 μl of 10-fold dilution of the first-strand cDNAs. The thermal profile was activated at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s of PCR reaction, and then 95 °C for 15 s, 55 °C for 15 s, and 95 °C for 15 s of melting curve. The relative quantification of the gRNAs and GFP RNA values were calculated by normalizing the quantity of actin with the comparative cycle threshold (Ct), and the standard curves produced with serial 10-fold dilution of full-length cDNA from the RBDV gRNA1 and gRNA2 (Isogai et al., 2018). The amounts of the GFP and RBDV RNAs were evaluated by at least three replications.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.10.025.

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