Transgenic rice plants that overexpress transcription factors RF2a and RF2b are tolerant to rice tungro virus replication and disease

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Rice tungro disease (RTD) is a significant yield constraint in rice-growing areas of South and Southeast Asia. Disease symptoms are caused largely by infection by the rice tungro bacilliform virus (RTBV). Two host transcription factors, RF2a and RF2b, regulate expression of the RTBV promoter and are important for plant development. Expression of a dominant negative mutant of these factors in transgenic rice resulted in phenotypes that mimic the symptoms of RTD, whereas overexpression of RF2a and RF2b had essentially no impact on plant development. Conversely, lines with elevated expression of RF2a or RF2b showed weak or no symptoms of infection after *Agrobacterium* inoculation of RTBV, whereas control plants showed severe stunting and leaf discoloration. Furthermore, transgenic plants exhibited reduced accumulation of RTBV RNA and viral DNA compared with nontransgenic plants. Similar results were obtained in studies after virus inoculation by green leafhoppers. Gaining disease resistance by elevating the expression of host regulators provides another strategy against RTD and may have implications for other pararetrovirus infections.

basic leucine zipper | host factors | resistance

Rice tungro disease (RTD) accounts for ≈ $1.5 billion annual loss in rice production worldwide (1, 2), and epidemics of tungro disease in the last century caused famines and great loss of human life (1–5). RTD results from coinfection by rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV). Typical disease symptoms include stunting and discoloration of infected plants, reduced tillering, and small and/or sterile panicles. When plants are infected in the early seedling stage, yield losses can be as much as 100% (4, 6, 7). The disease is transmitted by green leafhoppers (GLH) (*Nephotettix virescens*) in a semipersistent manner. RTBV is the causative reagent of RTD symptoms, whereas RTSV is required for disease transmission (8). Disease-tolerant cultivars have thus far been selected by plant breeders; many such lines rely on resistance to insect transmission (5). Pathogen-derived resistance against RTD was reported as being only partially effective, although recent reports involving an RNAi construct are encouraging (5, 9). Nevertheless, because of the limitations of current breeding programs and disease management, RTD remains a serious threat to rice production in regions of South and Southeast Asia.

RTBV is a plant pararetrovirus with a circular 8-kb dsDNA genome (10). Transcription of the RTBV DNA genome is regulated by a promoter located in the intergenic region between ORF IV and ORF I. RTBV accumulates in vascular tissues and activity of the RTBV promoter is largely restricted to vascular tissues. Several *cis*-acting regulatory elements were identified as contributing to the regulation of expression of this promoter (11, 12), including a unique box II element located immediately upstream of the TATA box (12, 13). Two basic leucine zipper (bZIP)-type rice proteins, RF2a and RF2b, were shown to interact with BoxII and activate transcription from the RTBV promoter in vitro and in vivo (13–15). RF2a and RF2b are also important for rice development, and transgenic rice lines in which their levels were reduced by (−)sense RNA exhibited phenotypes that, in part, resembled the symptoms of RTD (14, 15). In addition, constitutive expression of a dominant negative mutant of RF2a in transgenic tobacco plants caused severe stunting (16). These observations led us to hypothesize that RTBV causes redistribution of important host transcription factors, including RF2a and RF2b, to favor transcription of the RTBV viral promoter over host genes. We propose that favoring the RTBV promoter may perturb the expression of genes that are important for plant growth and development and/or disease defense resulting in development of disease symptoms. In this study we show that overexpression of RF2a and RF2b in transgenic rice plants reduces virus accumulation and gene expression and leads to tolerance to RTBV.

Results

Expression of a Dominant Negative Mutant of RF2a Produces RTD-Like Phenotypes. To test the hypothesis that RF2a and RF2b are involved in symptoms of RTD (13, 15), we introduced into rice a gene encoding the bZIP domain of RF2a (referred as RF2a-3Δ) (see Fig. S1 for sequence information) to act as a dominant negative factor to down-regulate the expression of genes that are controlled by RF2a and RF2b. As shown in Fig. 1*B*, expression of RF2a-3Δ from the maize ubiquitin gene promoter (Ubi:RF2a-3Δ) (14) caused an abnormal phenotype that mimics the primary symptoms of RTD. When the RF2a-3Δ sequence was controlled by promoters expressed primarily in vascular tissues, including the promoters from RTBV (15, 17, 18), the maize Shrunken1 (Sh1; ref. 17) and bean phenylalanine ammonia lyase2 (PalII; ref. 18) genes, transgenic plants were stunted (Fig. 1; E:RF2a-3Δ, Sh1:RF2a-3Δ, and PalII:RF2a-3Δ). However, expression of RF2a-3Δ under the control of the green tissue-specific promoter from the gene encoding chlorophyll a/b binding protein (Cab3) did not cause noticeable changes in plant growth or development (Cab3:RF2a-3Δ; Fig. 1*D* and Fig. S2). These results indicated that the negative effects of RF2a-3Δ were likely caused by effects on genes that are expressed in vascular tissues, including genes controlled by RF2a and RF2b.

Overexpression of RF2a and RF2b Reduces Symptoms of RTBV Infection. The results of the dominant negative mutant studies (Fig. 1) support the hypothesis that RF2a and RF2b are involved in RTD

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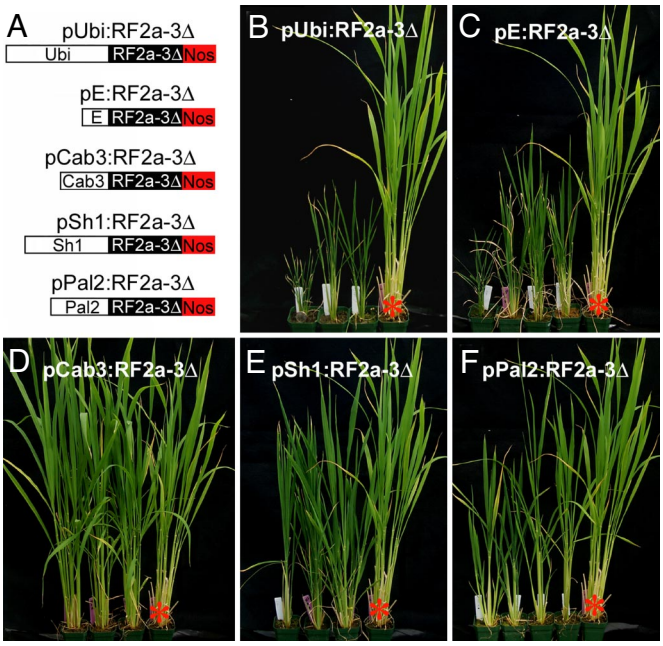

Fig. 1. Overexpressing the bZIP domain of RF2a reduces rice growth and development. (*A*) Diagram of gene constructs that carry the coding sequence of the bZIP domain of RF2a (referred as RF2a-3Δ) under the regulation of promoters with different tissue specificities. PalII, promoter from bean phenylalanine ammonia-lyase gene; Cab3, promoter from *A. thaliana* chlorophyll a/b binding protein gene; E, promoter from RTBV; Ubi, promoter from maize ubiquitin gene; Sh1, promoter from maize shunken 1 gene; Nos, terminator sequence of *A. tumefaciens* nopaline synthase gene. (*B–F*) Transgenic plants carrying different gene constructs as labeled. Multiple T_0 transgenic plants regenerated from independent transgenic events were included and compared with the transgenic plant that carried the selection marker gene (p35S:hpt) alone (labeled ∗). All images were taken 34 days after transplanting.

symptom development. It logically follows that transgenic lines in which levels of RF2a or RF2b are elevated may affect symptom development caused by RTD and/or RTBV accumulation. Such transgenic lines may gain tolerance to RTD based on sufficient levels of RF2a or RF2b to support plant development and virus replication. Alternatively, transgenic lines may develop more severe symptoms and/or lead to higher levels of virus accumulation.

We developed transgenic lines that overexpress either RF2a or RF2b by using strong constitutive promoters. Overexpression of RF2a was accomplished by using the maize ubiquitin promoter (Ubi:RF2a), and overexpression of RF2b was accomplished by using the promoter isolated from cassava vein mosaic virus (ref. 19; CsVMV:RF2b). All transgenic lines used in these studies contain a single gene at a single genetic locus, and transgenic lines had significantly higher levels of RF2a or RF2b compared with nontrangenic plants (Fig. S3). All transgenic lines were similar to nontransgenic plants in appearance, growth, and development under greenhouse conditions except that they were slightly delayed in flowering time (Fig. S3). Two independent transgenic lines (homozygous) carrying the Ubi:RF2a gene, RF2a modified TP309 lines RaMT1 and RaMT3, were selected for virus challenge assays. Similarly, 2 RF2b modified TP309 lines, RbMT6 and RbMT9, with the CsVMV::RF2b gene were tested.

Inoculation with RTBV was conducted by injecting the "stem" of young seedlings with *Agrobacterium tumefaciens* EHA105 cells that carried the RTBV infectious clone [pRTRB1162 (8)]. As shown in Fig. 2 *A* and *B*, the growth of nontransgenic TP309 plants was significantly reduced ($P < 0.05$) at 21 days after

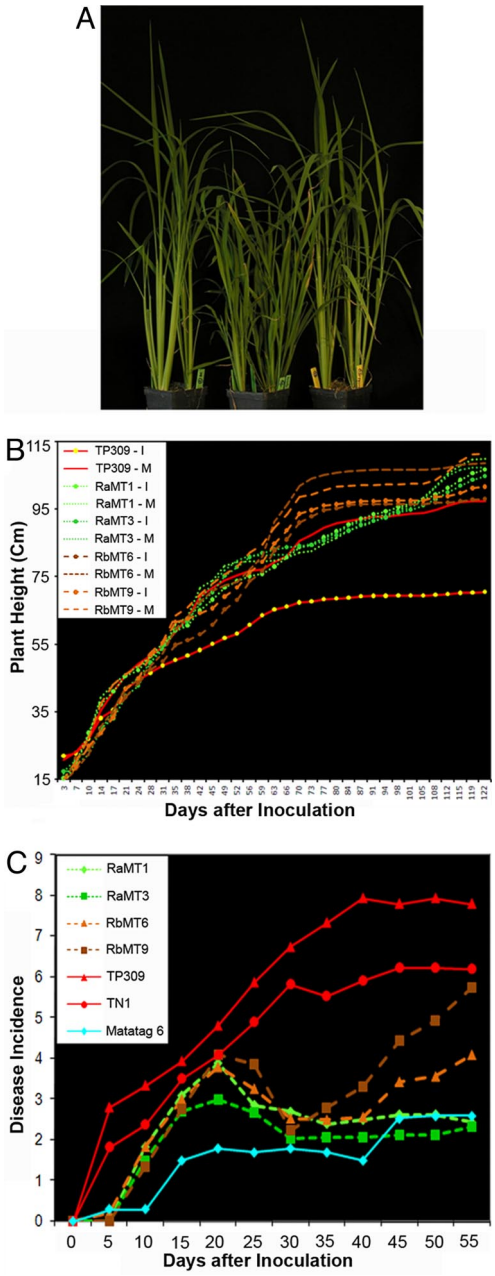

Fig. 2. Transgenic rice lines with elevated expression of RF2a or RF2b are resistant to tungro disease. (*A*) Transgenic lines with elevated expression of RF2a gained resistance to RTBV infection. Eleven-day old RaMT1 plants (*Left*) and TP309 wild-type plants (*Center*) were inoculated with an *Agrobacterium* strain carrying the RTBV infectious clone pRTRB1162. Similarly, TP309 plants (*Right*) were inoculated with control plasmid pRTBV:GUS, through *Agrobacterium* infiltration. The image was taken 45 days after inoculation. (*B*) Infection of RTBV did not cause severe stunting in transgenic rice lines with elevated RF2a or RF2b. For each treatment, 30 plants of each line were planted. All plants were inoculated at 11 days after planting and maintained in growth chambers; plants were transferred to a greenhouse at 60 DAI. The growth rate of each line is the average height of 30 plants. Plants were agro-inoculated with RTBV pRTRB1162 (−I) or control plasmid pRTBV:GUS (−M). TP309, nontransgenic plants; RaMT1 and RaMT3, T_5 generation homozygous lines that contain Ubi:RF2a; RbMT6 and RbMT9, T_4 generation homozygous lines that contain CsVMV:RF2b. (*C*) Transgenic rice lines with elevated levels of RF2a or RF2b had greatly reduced tungro disease incidence after GLH insect-mediated inoculation with RTBV and RTSV. Disease incidence was recorded by following a standard method established by the International Rice Research Institute. Each line represents the average disease incidence index of 30 inoculated plants. TN1, a tungro disease-susceptible rice variety; Matatag 6, a tungro-tolerant rice variety bred by the Philippine Rice Research Institute.

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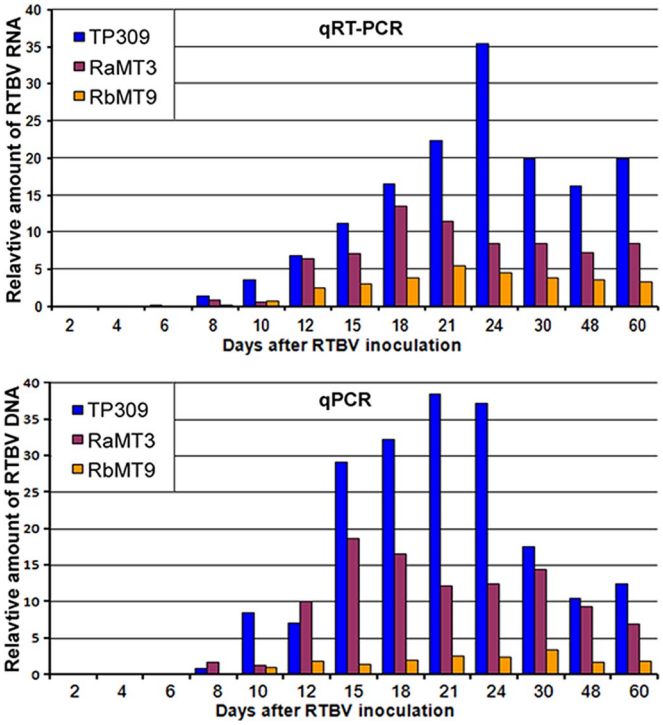

Fig. 3. Reduced accumulation of RTBV transcripts and genomic DNA in transgenic rice plants with elevated levels of RF2a or RF2b after *Agrobacterium* inoculation with RTBV. (*Upper*) Accumulation level of RTBV transcripts in nontransgenic and transgenic plants in a time-course study. For each time point, RNA sample was prepared from 30 pRTRB1162 inoculated plants and subjected to qRT-PCR analysis. Each bar represents the main value of relative amount of RNA of 3 qRT-PCRs after normalization against the amount of actin3 mRNA. (*Lower*) Accumulation of RTBV genomic DNA in the study described above. For each time point, DNA was prepared from 30 inoculated plants and subjected to qPCR analysis. Each bar represents the main value of relative amount of DNA produced by 3 qPCRs after normalizationd against the amount of the rice actin3 gene PCR product. TP309, wild-type plants with which transgenic plants were developed; RaMT3, RF2a overexpression line; RbMT9, RF2b overexpression line.

inoculation (DAI) and plant growth ceased by 63 DAI. In contrast, the growth of plant lines RaMT1 and RaMT3 was not affected by RTBV infection. In comparison, the growth of RbMT6 and RbMT9 plants was retarded by 32 DAI ($P < 0.05$) for several weeks, after which growth resumed and no other symptoms were observed. During the heading period, internode elongation of RbMT6 and RbMT9 plants was repressed by virus infection. However, panicle development of RbMT6 and RbMT9 plants was not affected by infection, unlike nontransgenic plants where panicle development was severely reduced by infection.

Transgenic Lines with Elevated Levels of RF2a and RF2b Are Resistant to RTBV Infection. To determine whether the transgenic lines were tolerant or resistant to RTBV infection, a time course study was undertaken to monitor the accumulation of virus transcript (vRNA) and vDNA in transgenic lines RaMT3 and RbMT9 after *Agrobacterium*-mediated inoculation. In this study, 30 plants were collected at each time point and samples were combined for analyses. Results from these studies revealed that accumulation of vRNA and vDNA was greatly reduced in transgenic lines compared with nontransgenic plants (Fig. 3). Accumulation of vRNA and vDNA in nontransgenic plants continued to increase through 21–24 DAI, after which the amount of each was reduced and remained stable for the remainder of the study. Transgenic

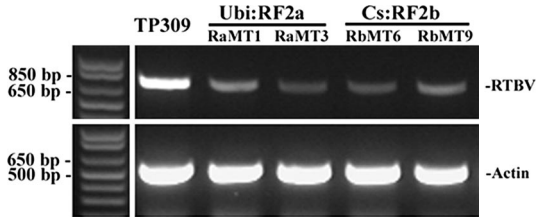

Fig. 4. Rice callus cultures that overexpress RF2a or RF2b are resistant to RTBV. Rice calli induced from mature seeds of TP309 or transgenic rice lines RaMT1, RaMT3, RbMT6, and RbMT9 were inoculated with pRTRB1162 by coculturing. RNA samples were prepared from RTBV-inoculated calli and analyzed by RT-PCR. (*Upper*) Products of RT-PCR resolved in a 1% agarose gel. (*Lower*) The same amount of RNA sample used in *Upper* was used for amplification of rice actin 3 gene transcript using RT-PCR. RT-PCR products were resolved in 1% agarose gel.

line RaMT3 accumulated lower amounts of vRNA and vDNA than control plants throughout the experiment, although the amount of vDNA in control and RaMT3 plants was approximately the same near the end of the study. In contrast, the amount of vRNA and vDNA was much less in RbMT9 than in either control plants or in line RaMT9 throughout the study.

To confirm the impact of elevated expression of RF2a or RF2b on accumulation of vRNA in another system, callus cultures made up of nondifferentiated cells were derived from mature seeds of transgenic lines RaMT1, RaMT3, RbMT6, and RbMT9. Established cultures were cocultured with the *A. tumefaciens* strain EHA105 that carries pRTRB1162. Calli were sampled at 3 days after coculture, and the amount of RTBV vRNA was determined by RT-PCR analyses. In comparison with callus from nontransgenic TP309 seeds, the amount of RTBV vRNA in transgenic calli was significantly lower (Fig. 4). The data gathered from these studies in nondifferentiated cells suggested that RF2a and RF2b may be involved in regulating defense-related genes that reduce virus replication; such genes are apparently activated in nondifferentiated cells and differentiated plant tissues.

To confirm that increased resistance to RTBV was a consequence of expression of the transgenes rather than induced expression of endogenous RF2a and RF2b genes, the effect of infection on the accumulation of endogenous RF2a and RF2b mRNAs was examined in nontransgenic plants at different times after *Agrobacterium*-mediated inoculation with RTBV. These studies revealed that virus infection did not change the amounts of RF2a and RF2b in wild-type plants (Fig. 5). It was therefore concluded that resistance to RTD in the transgenic lines is a

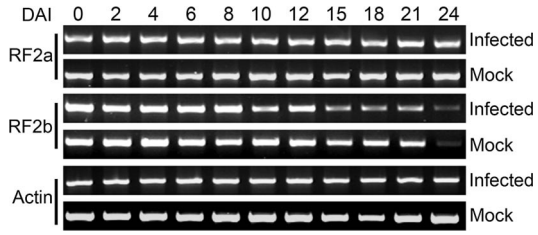

Fig. 5. RTBV infection does not cause increased expression of endogenous RF2a or RF2b in rice plants. Leaf tissues of 30 plants that were either inoculated with pRTRB1162 (infected) or pRTBV:GUS (Mock) were pooled and RNA was extracted for RT-PCR analysis using primers that amplify RF2a or RF2b transcripts. PCR products were resolved in 1% agarose gels. From top to bottom, accumulation of transcripts of RF2a in RTBV-infected plants; accumulation of RF2a in mock-inoculated plants; accumulation of RF2b in RTBV-infected plants; accumulation of RF2b in mock-inoculated plants; accumulation of actin 3 gene transcripts in RTBV-infected plants; accumulation of actin 3 gene transcripts in mock-inoculated plants. DAI, days after inoculation.

Fig. 6. Transgenic lines with elevated expression of RF2a or RF2b exhibited reduced tungro disease resistance after GLH-mediated inoculation with RTBV and RTSV. Ten-day-old plants were force-inoculated with viruliferous insects; the image was taken 28 days after inoculation. Plants in pots were (from left to right): nontransgenic TP309; LPO 345, a tungro disease susceptible variety used in the Philippines; Matatag 6, a tungro disease tolerant variety in the Philippines (resistant control); RaMT1and RaMT3, RF2a overexpression lines; RbMT6 and RbMT9, RF2b overexpression lines.

result of elevated expression of RF2a or RF2b caused by expression of the transgenes.

Resistance of Transgenic Rice Was Confirmed in a Greenhouse Trial. Virus resistance studies were conducted in greenhouses in the Philippines with plant lines RaMT1, RaMT3, RbMT6, and RbMT9. In these studies GLH were used to cotransmit RTBV and RTSV to transgenic and nontransgenic plants. The incidence of tungro disease was significantly reduced in transgenic lines compared with nontransgenic lines in these studies (Figs. 2*C* and 6). Furthermore, the level of resistance of RaMT1 and RaMT3 to RTD was similar to the level of resistance of Matatag 6, a local rice variety with resistance to insect transmission of RTD. As in the case of the studies conducted with *Agrobacterium*-mediated inoculation (Fig. 2*B*), transgenic lines RbMT6 and RbMT9 exhibited strong resistance to RTD in the GLH-based inoculation experiments, although the disease incidence index was more severe during late stages of plant development in GLH-mediated inoculation than after *Agrobacterium*-mediated inoculation (Fig. 2*C*). The number of tillers produced by transgenic plants was slightly reduced after RTBV and RTSV double inoculation with GLH, wherea sno difference was observed in plants inoculated with RTBV via *Agrobacterium*.

In greenhouse studies of noninfected plants we observed that transgenic plant lines were slightly delayed in formation of the tiller leaf compared with nontransgenic plants. It remains to be determined whether this affect is caused by expression of RF2a and/or RF2b per se or conditions in which the plants were grown.

Discussion

The major cause of symptoms of RTD replication and pathogenicity is infection by RTBV. The virus uses host transcription factors including RF2a and RF2b (and perhaps others) during replication. The transgenic plants that produce a dominant negative mutant of the factors (RF2a-3Δ) in vascular tissues exhibited phenotypes similar to RTD symptoms, i.e., plants were stunted and leaves were yellow-orange in color. We conclude from these studies that the functions of RF2a and RF2b (and perhaps other transcription factors) were negatively affected by RF2a-3Δ and propose that stunting and leaf discoloration exhibited in RTD are caused by the lack of availability of RF2a and RF2b as a consequence of infection by RTBV.

To test this hypothesis, we challenged homozygous transgenic rice lines that constitutively overexpress RF2a or RF2b with RTBV: virus infection was affected via *Agrobacterium*-mediated inoculation or transmission of both RTBV and RTSV via GLH, the insect vector for this disease. Observations of growth of inoculated plants and studies to monitor virus accumulation and virus gene expression conclusively demonstrated that transgenic plants are highly tolerant to virus infection and do not develop usual symptoms of disease. These results support the conclusion that overexpression of RF2a and/or RF2b directly or indirectly confer tolerance to infection and/or disease.

Virus infections perturb host gene expression and alter the physiological status of the host, resulting in disease symptoms. Some of these impacts are mediated by changes in genetic and biochemical pathways, including changes in expression of protein-coding and noncoding genes, which influence multiple aspects of host biology. The data presented here suggest that symptoms of RTD are caused largely by changes in activity of RF2a and RF2b that consequently affect plant development; however, much remains to be discovered to more fully describe the mechanisms of resistance.

These studies may open new avenues in the search for disease resistance genes and pathways in plants and potentially other organisms. Combining genes that cause overexpression of RF2a or RF2b with genes that confer resistance to the insect vector may create new rice varieties with durable resistance to RTD and reduce the effects of the "boom and bust cycle" of RTD (5) in vulnerable regions of the world. Furthermore, because transgenic plants contain less virus than nontransgenic plants there may be reduced acquisition/transmission of the RTD viruses by the insect vector and further reduced epidemic spread of RTD.

Materials and Methods

Plasmid Construction. Transformation plasmids were created to place the coding sequence of the bZIP domain of RF2a (designated as RF2a-3Δ) (Fig. S1) under the control of different promoters, including the PalII promoter from a phenylalanine ammonia-lyase gene of *Phaseolus vulgaris* (18), the *A. thaliana* Cab3 (chlorophyll a/b binding protein) promoter (20), the RTBV promoter (11) (which is expressed in vascular tissue, primarily in the phloem), the maize ubiquitin promoter (Ubi) (14, 21), and the maize shrunken 1 gene promoter (17). Details of plasmid construction are presented in *SI Text*.

Rice Transformation. Rice calli were induced from mature seeds of *Orizae sativa* cv. *japonica* TP309. Plasmids containing the target genes were cobombarded into rice cells with p35S:hpt, which carries a hygromycin phosphotransferase (hpt) gene that confers resistance to hygromycin. Rice transformation and plant regeneration followed previously described protocols (15). All transgenic lines used in this study were homozygous and in the T3, T4, or T5 generations. Each line contained a single copy of the target transgene.

Plant Growth. Taipei 309 (TP309) plants and transgenic TP309 were used for all studies. Rice plants were first grown in growth chambers and transferred to a greenhouse at 60 days after agro-inoculation with RTBV. Conviron PGW36 growth chambers with high-intensity discharge lighting consisting of metal halide and high-pressure sodium were used. The chambers were set at 26 °C with 70% relative humidity (RH) and 14 h/8 h of light/dark period. The greenhouse was operated at 25.6–26.7 °C and 60% (RH) with supplemental lights that came on when natural light was <400 W/m^2 for >15 min.

***Agrobacterium* Inoculation of RTBV Infectious Clone in Rice Plants and Cell Cultures.** Rice plants at 11 days after seeding were inoculated with either RTBV infectious clone pRTRB1162 (8) or pRTBV:GUS (16) through *Agrobacterium* infiltration. *A. tumefaciens* EHA105 strains carrying either of the plasmids were cultured to $OD_{600} = 0.6$ in LB medium. Cells were collected by centrifugation at $4032 \times g$ for 15 min and resuspended in a solution containing 5% sucrose (wt/vol) and 0.1% (vol/vol) of Silvet-77 to the same OD value. In the growth rate studies, *Agrobacterium* infiltration was repeated on 3 consecutive days. In the time-course studies to determine RTBV transcript, RTBV genomic DNA, and RF2a and RF2b transcription levels, *Agrobacterium* infiltration was repeated 3 times on the same day.

Measurement of Plant Growth. The virus *Agrobacterium* inoculation experiments were repeated 3 times. For each experiment, 60 plants of each transgenic line were prepared. Half of the plants were inoculated with an *Agrobacterium* strain carrying the RTBV infectious clone pRTRB1162, and the remainder were inoculated with *Agrobacterium* strain containing a control plasmid pRTBV:GUS in which the GUS reporter gene was driven by RTBV promoter. Rice plants were started in growth chambers with growth conditions as described above. Because of space constraints in the growth chambers, rice plants were transferred to greenhouse at 60 DAI where they were observed for an additional 2½ months. The height of each plant was measured twice each week.

Coinoculation of RTBV and RTSV with GLH. Forced virus inoculation experiments were performed at the Philippine Rice Research Institute using GLH (*N. virescens*), the natural insect vector of tungro viruses. The experiments were repeated 3 times with 30 plants per line.

Disease incidence ratings were recorded based on a Standard Evaluation System for Rice (developed by the International Rice Research Institute, Los Banos, Philippines): 1 = no symptom observed; 3 = 1–10% height reduction, no distinct yellowing symptoms; 5 = 11–30% height reduction, no distinct leaf symptoms; 7 = 31–50% height reduction, with distinct yellow to yellow-orange leaf coloration; and 9 = >50% height reduction, with distinct yellow to yellow orange coloration. Plant reaction of disease incidence was scored as 0–3 = resistant/tolerant, 4–6 = moderate, and 7–9 = susceptible.

RNA and DNA Isolation. Pooled leaf samples from 30 individually inoculated plants were collected at each time point for DNA and RNA isolation. DNA was isolated by using an existing protocol (15). Total RNA was isolated from 200 mg of tissue powder with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

Quantitative PCR (qPCR) and Quantitative RT-PCR (qRT-PCR). qPCR and qRT-PCR analyses were conducted to quantify accumulation of viral DNA and virus transcripts. Details are presented in *SI Text*. The C_T values reported in the figures are averages of 3 independent runs. Relative amounts of target RNA was calculated by using the comparative C_T method (PerkinElmer–Applied Biosystems). ΔC_T, the differences between the mean C_T value of RTBV reactions and the mean C_T value of *Actin 3* control reactions were calculated to normalize the DNA or RNA sample in each reaction and the efficiency of PCR or RT-PCRs. The relative DNA or RNA level of RTBV in each sample is presented after normalized with control DNA or RNA levels (22).

RT-PCR. To determine whether the amounts of RF2a or RF2b are affected by RTBV infection, a time-course study was conducted to compare the amount of transcripts of these 2 genes in plants inoculated with RTBV infectious clone pRTRB1162 or pRTBV:GUS. For each time point, leaf samples of 30 independently inoculated plants were collected and pooled for RNA isolation. RT-PCR analyses were conducted as described above. RNA samples were pretreated with DNase as the samples used in qRT-PCRs. The RT-PCR products were resolved in 1% agarose gels.

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