VIRUSES THREATENING STABLE PRODUCTION OF CEREAL CROPS

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VIRUSES THREATENING STABLE PRODUCTION OF CEREAL CROPS

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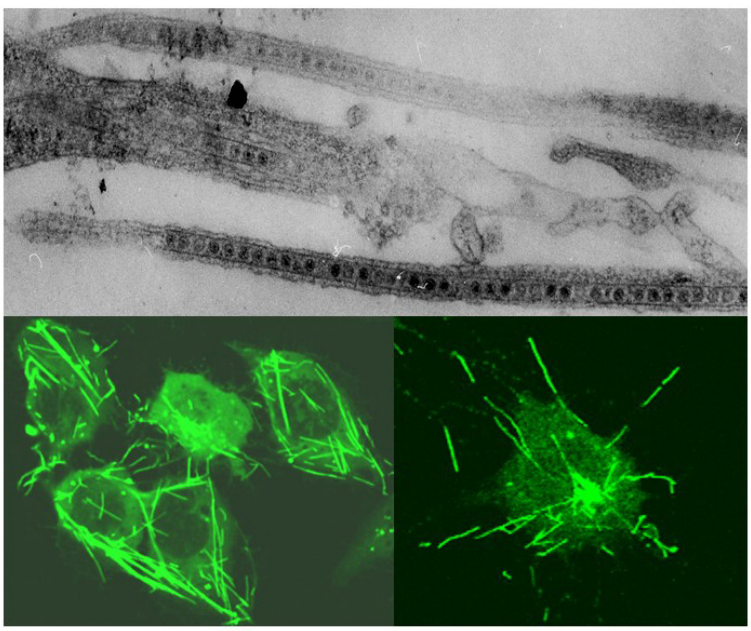

Upper: Electron micrograph of *Rice gall dwarf virus* (RGDV)-containing tubules comprising RGDV Pns11 within the cytoplasm or protruding from the surface of the cells of leafhopper *Recilia dorsalis.* Lower left: Immunofluorescence staining of RGDV Pns11 showing tubule-like structures within the cytoplasm and protruding from the surface of the cells *R. dorsalis.* Lower right: Immunofluorescence staining of RGDV Pns11 showing tubule-like structures within the cytoplasm or protruding from the surface of Sf9 cells expressing RGSV Pns11. See Chen et al. (2013).

Cereal crops such as maize, wheat, and rice account for a majority of biomass produced globally in agriculture. Continuous economic and population growth especially in developing countries accompanied more intensive production of cereal crops to meet increasing demands for them as main staple foods and livestock feeds. However, imbalance between production and consumption of cereal crops, which is inevitably reflected as their higher market prices, is becoming palpable in recent years. Stable production of cereal crops has been threatened by various abiotic and biotic stresses. One of the most threatening

constraints is virus diseases. Especially, intensification of cereal crop production is often achieved by monoculture of a popular crop variety in a wide area. Such agroecosystems with low biodiversity is usually more conducive to biotic stresses, and may result in the outbreaks of existing and emerging cereal viruses. Numerous reports on incidences of various virus diseases of cereal crops attested that viruses have been a long-standing obstacle eroding yields of cereal crops worldwide.

Despite of the evident economic losses incurred by virus disease of cereal crops, the progress in basic research on virus species causing major diseases of cereal crops lagged behind compared to that carried out for viruses that can infect dicotyledonous plants. This was partially due to the lack of ideal experimental systems to investigate the interaction between viruses and monocotyledonous crops. For example, inoculation of many viruses to cereal plants still requires tedious manipulation of vector organisms, and reverse genetic systems are not available for many cereal viruses. However, application of latest molecular biology technologies has led to significant advance in cereal virology recently; transient gene expression systems through particle bombardment and agroinfiltration have been exploited to examine the functions of cereal virus proteins. Cell culture systems of vector insects enabled to investigate the molecular interactions between cereal viruses and insect vectors. Furthermore, RNAi technologies for vector insects and monocotyledonous plants facilitated identification of specific host and viral factors involved in viral replication and transmission cycles. Also, accumulating information on the genome sequences of cereal crop species has been simplifying the roadmap to pinpoint resistance genes against cereal viruses.

The objective of this research topic is to provide and share the information which can contribute to advances in cereal virology by covering recent progresses in areas such as: 1) characterization of emerging viruses, 2) analyses of genetic and biological diversities within particular viruses, 3) development of experimental systems applicable to cereal viruses, 4) elucidation of the molecular interactions among viruses, vector organisms, and host plants, 5) identification of traits and genes linked to virus resistance in cereal crops, 6) development of novel genetic approaches for virus resistance, and 7) assessment of epidemiological factors affecting the incidences of cereal virus diseases. Synergistic integration of ideas from such areas under this research topic should help to formulate practical alternatives to the current management options for virus diseases in cereal crops.

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Editorial: Viruses threatening stable production of cereal crops

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Keywords: cereal, rice, phytoreovirus, tenuivirus, luteovirus, polerovirus, insect vector

Between 2005 and 2008 the prices of rice, wheat and maize were more than doubled (von Braun, 2008). The sudden surge in cereal prices led or was led by major uncertainties to secure staple food supplies in many developing countries, especially in Asia. Several economic and social factors collectively appear to have triggered the unstable supplies of cereal crops during the recent food crisis (Headey and Fan, 2008). Besides, lower cereal crop productivities due to unfavorable environments and outbreaks of pests and diseases may have been associated, at least in part, with the unstable supplies of cereals during the period. In fact, massive outbreaks of brown planthoppers (BPH) transmitting rice viruses were reported in many areas of Indochina Peninsula during 2006 and 2008, which resulted in a significant decrease in international trade of rice (Ta et al., 2013). This research topic consists of four original research and seven review articles covering various aspects of cereal viruses and their vector insects that are considered as current or potential threats to stable production of cereal crops. We also hope that this e-book can provide the readership an update on the recent studies on a variety of cereal viruses.

The first two articles described two cereal viruses whose genome sequences were determined recently. Southern rice black-streaked dwarf virus (SRBSDV) is a new virus found in China in 2001. SRBSDV can infect maize, rice, and other monocotyledonous plants, and quickly spread to wide areas of China and Vietnam. SRBSDV is tentatively classified in the genus *Fijivirus* of the family *Reoviridae*. SRBSDV was found to be transmitted by white-backed planthopper (WBPH), which had not been previously recognized as a vector of any viruses. Zhou et al. (2013) summarized biological and molecular characteristics of SRBSDV, and discussed key factors of its epidemiology and current options for its control. This article should serve as a basic guide to management of this newly emerged virus, which may be widely distributed in Asia.

The yellow dwarf viruses (YDVs) belonging to the *Luteoviridae* family are the most widespread group of cereal viruses worldwide. Barley yellow dwarf viruses (BYDVs) are a group of viruses included in YDVs. BYDVs were previously placed into several strains based on the differences in their biological characteristics. Genome sequence analyses revealed that the BYDV strains are classified into either the *Luteovirus* or the *Polerovirus* genera of the *Luteoviridae* family. Krueger et al. (2013) analyzed the genome sequence of one of the BYDV strains, BYDV-RMV, showed that BYDV-RMV is a polerovirus distantly related to other YDVs, and proposed to rename it Maize yellow dwarf virus-RMV. This article affirms the etiological complexity of yellow dwarf diseases of cereal crops.

A majority of cereal viruses are disseminated among plants by insect vectors. Therefore, elucidation of the mechanisms of virus-insect association is essential to understand how an insect-transmitted virus completes its life cycle. Phytoreoviruses such as rice gall dwarf virus (RGDV) and rice dwarf virus (RDV) are persistent problems in East Asian countries. RGDV and RDV propagate in their vector insects before they are transmitted to plants. Miyazaki et al. (2013) provided detailed insights into the major events underlying life cycles of RGDV and RDV in the cells of their insect vectors revealed by high-resolution images obtained with

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advanced electron microscopy (EM) techniques such as cryo-EM and tomography. The article shows how individual viral proteins of RGDV and RDV play specific roles during entry, replication, assembly and intra- and intercellular transport of viruses in the insect vector cells.

Chen et al. (2013) described the specific role of an RGDV-encoded protein Pns11 in cell-to-cell spread of RGDV among the cells of its leafhopper vector. Cytopathologic studies conducted in the cultured leafhopper cells and non-host insect cells revealed that Pns11 is the minimal viral component of tubules mediating transport of RGDV among the insect cells. The involvement of Pns11 in tubule formation was confirmed by a double-stranded (ds) RNA-induced RNAi system, demonstrating the versatility of the RNAi system in function analyses of genes from viruses that can multiply in insect cells.

Once in a plant, a virus moves from one cell to another through the host plasmodesmata. A plant virus genome generally encodes one or more movement proteins that modify the structure of plasmodesmata for the virus to spread to adjacent cells. Because of the lack of reverse genetic systems for most rice viruses, identification of candidate genes for a movement protein of rice viruses had relied mostly on the similarities of rice virus genes to known movement protein genes. However, with development of gene *trans*-complementation systems, the genes encoding a movement protein have been experimentally identified for several rice viruses. Hiraguri et al. (2014) summarized the genome structures of individual rice viruses, and discussed the characteristics and probable functions of the genes that were experimentally confirmed or predicted to be involved in cell-to-cell movement.

In an epidemiological view, revealing the migration patterns of virus-transmitting insects is critical to decipher how virus diseases are actually spread to extensive crop production areas. Three species of planthoppers, small brown planthopper (SBPH, *Laodelphax striatellus*), BPH (*Nilaparvata lugens*), and WBPH (*Sogatella furcifera*) are widely distributed in rice-growing areas of Asia. They are economically important pests since rice plants incur direct damages from feeding of the planthoppers as well as indirect damages from viruses they transmit. SBPH transmits rice stripe virus (RSV) and rice black-streaked dwarf virus (RBSDV), BPH transmits rice grassy stunt virus (RGSV) and rice ragged stunt virus, and WBPH transmits SRBSDV. Otuka (2013) provided an overview of migration patterns of these planthoppers in Asia simulated based on the results from the long-term spatio-temporal trajectory analyses of their movement. The article showed that recent outbreaks of rice virus diseases in Asia can be largely accounted for by the long-distance migration of the planthoppers, and suggests that a reliable forecasting system for rice virus diseases can be established by monitoring the migration of the vector insects.

Rapid and accurate detection of viruses in host plants and insect vectors is an essential procedure in efficient management of cereal viruses. Uehara-Ichiki et al. (2013) reviewed the current technologies available for detection of rice viruses in plants and insect vectors. Serology-based methods such as enzyme-linked immunosorbent assay and dot-immunobinding assay had been developed for rice viruses, and have been adopted mainly in places where a large number of samples needed to be processed. Since the genome sequences of most rice viruses have been determined, most rice viruses also can be identified by nucleic acid-based methods, which are generally more sensitive than serology-based methods. This article addressed the strengths, and the limitations of each detection technique and it will be helpful for researchers to consider the best option for their needs.

Exploitation of natural resistance genes against cereal viruses is one of the most practical ways to manage cereal viruses in fields. However, the sources of natural resistance genes are very limited or lacking for many viruses and the durability of some natural resistance genes is often questionable. To overcome such limitations, many attempts have been made to invent artificial resistance to cereal viruses. Sasaya et al. (2014) described development of transgenic rice plants that showed a high level of resistance to phytoreoviruses (RDV and RGDV), a fijivirus (RBSDV), and tenuiviruses (RGSV and RSV) using a viral dsRNA-induced RNAi technology. Examination of extensive sets of transgenic plants expressing a dsRNA fragment specific to one of the genes of these viruses showed that the reactions of the transgenic plants to the targeted viruses varied depending on the gene-specific dsRNA expressed. Overall, their observations suggested that target virus genes should be carefully selected to attain a high level of virus gene-derived resistance.

Accumulation of vast amounts of information on plant genomes and development of high-throughput gene expression analysis technologies have expedited research efforts aiming to reveal the diverse molecular responses of host plants to viral pathogens. This research topic includes three articles which addressed the host gene responses associated with cereal virus infection. NAC transcription factors are a large family of plant transcriptional regulators, which are involved in various development processes and tolerance responses to biotic and environmental stresses. Nuruzzaman et al. (2013) reviewed the expression patterns of NAC transcription factor genes in responses to environmental stresses and plant pathogens including cereal viruses such as RDV and RSV. They proposed the possible scenarios on how particular NAC transcription factors regulate downstream genes which were known to be involved in defense and tolerance responses to biotic and environmental stresses.

Rice tungro spherical virus (RTSV) and rice tungro bacilliform virus synergistically interact and cause a serious disease in rice, although RTSV alone rarely induces any discernible symptoms in Asian rice (*Oryza sativa*). Budot et al. (2014) showed that RTSV, which is recognized as a latent virus in Asian rice, can cause serious stunting in African rice (*O. glaberrima*), and the degree of stunting in African rice appeared to be associated with suppression of particular cell wall-related genes, which were also suppressed in Asian rice plants stunted by infection with other rice viruses.

RGSV has been a serious threat to rice cultivation in Southeast Asian countries in recent years. Typical symptoms of RGSV are stunting and excessive tillering. Satoh et al. (2013) analyzed the gene expression profile of a rice plant infected with RGSV to reveal the gene expression patterns associated with the typical

symptoms. They concluded that excessive tillering of rice infected with RGSV might be related to activation of genes involved in inactivation of gibberellin and auxin, and suppression of genes for strigolactone signaling, while stunting might be associated with suppression of genes related to synthesis of cell walls and chlorophyll.

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Southern rice black-streaked dwarf virus: a white-backed planthopper-transmitted fijivirus threatening rice production in Asia

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Southern rice black-streaked dwarf virus (SRBSDV), a non-enveloped icosahedral virus with a genome of 10 double-stranded RNA segments, is a novel species in the genus *Fijivirus* (family *Reoviridae*) first recognized in 2008. Rice plants infected with this virus exhibit symptoms similar to those caused by *Rice black-streaked dwarf virus*. Since 2009, the virus has rapidly spread and caused serious rice losses in East and Southeast Asia. Significant progress has been made in recent years in understanding this disease, especially about the functions of the viral genes, rice–virus–insect interactions, and epidemiology and control measures. The virus can be efficiently transmitted by the white-backed planthopper (WBPH, *Sogatella furcifera*) in a persistent circulative propagative manner but cannot be transmitted by the brown planthopper (*Nilaparvata lugens*) and small brown planthopper (*Laodelphax striatellus*). Rice, maize, Chinese sorghum (*Coix lacryma-jobi*) and other grass weeds can be infected via WBPH. However, only rice plays a major role in the virus infection cycle because of the vector's preference. In Southeast Asia, WBPH is a long-distance migratory rice pest. The disease cycle can be described as follows: SRBSDV and its WBPH vector overwinter in warm tropical or sub-tropical areas; viruliferous WBPH adults carry the virus from south to north via long-distance migration in early spring, transmit the virus to rice seedlings in the newly colonized areas, and lay eggs on the infected seedlings; the next generation of WBPHs propagate on infected seedlings, become viruliferous, disperse, and cause new disease outbreaks. Several molecular and serological methods have been developed to detect SRBSDV in plant tissues and individual insects. Control measures based on protection from WBPH, including seedbed coverage, chemical seed treatments, and chemical spraying of seedlings, have proven effective in China.

Keywords: Southern rice black-streaked dwarf virus, fijivirus, *Sogatella furcifera*, virus transmission, infection cycle

INTRODUCTION

Southern rice black-streaked dwarf disease (SRBSDD) was first discovered in Yangxi County, Guangdong Province, China, in 2001, and its occurrence was limited to southern China from 2002 to 2008 (Zhou et al., 2004, 2008; Wang et al., 2010a). However, by 2009, the disease had spread to 19 provinces in northern Vietnam and nine provinces in southern China, where it damaged 42,000 ha and more than 300,000 ha of rice (*Oryza sativa*) fields, respectively. In 2010, more than 60,000 ha of paddy fields in 29 provinces of Vietnam and more than 1,300,000 ha in 13 provinces of China became infected, causing crop failure in many places (Zhou et al., 2010; Hoang et al., 2011; Zhai et al., 2011). Although great efforts were undertaken to control the disease, nonetheless, it damaged more than 700,000 ha in 2011 and over 500,000 ha in 2012 in the two countries. Moreover, the disease has now been found in some areas in Japan (Matsukura et al., 2013).

SYMPTOMS AND CHARACTERISTICS OF SRBSDD

Rice is susceptible to SRBSDD during all growth periods, but the symptoms and yield losses depend on the growth stage at the time of infection. The earlier the infection occurs, the more severe the symptoms (Zhou et al., 2010). (1) Plants infected at the early seedling stage exhibit dwarfism and stiff leaves (**Figure 1A**). Most symptoms are not obvious in the seedbed; they usually appear after the rice plants have been transplanted to the paddy field. Severe stunting (shorter than one third of the normal height) and failure to elongate occur, and seriously-diseased plants may die of withering (**Figure 1B**). (2) Infection at the early tillering stage results in significant dwarfing, with excessive tillering and failure to head (**Figure 1C**). (3) Infection at the elongating stage does not stunt the plants, but they produce small spikes, barren grains, and deficient grain weight (**Figure 1D**). The diseased leaves are short, dark green, and rigid, and ruffles often appeared near the leaf base on the surfaces of upper leaves. (4) Plants infected at the booting stage show no visible symptoms, but the infection can be confirmed by enzyme-linked immunosorbent assay (ELISA) or reverse-transcription polymerase chain reaction (RT–PCR). After elongation, the plants infected before the tillering stage usually display the most common symptoms of this disease, including dark green foliage; aerial rootlets and branches on the stem nodes (**Figure 1E**); small, streaked, black or white, waxy galls 1–2 mm in size

on the stems (**Figure 1F**); and poorly-developed brown roots (**Figure 1G**).

Southern rice black-streaked dwarf disease has the following characteristics (Zhou et al., 2010): (1) it is extensively distributed in rice growing areas, but serious damage occurs only in limited regions in some years. Because the disease spreads via transmission by the white-backed planthopper (WBPH, *Sogatella furcifera*), a long-distance migratory pest, its distribution coincides with the migration and range of WBPH. However, the disease is severe only when large-scale invasion and propagation of viruliferous WBPHs take place during the seedling stage. The disease severity in a given area is thus closely related to the original sources of the virus and WBPH population, and the sowing time and growth stage of the rice, the cultivation pattern, climate conditions, and the geographic landform.

(2) Southern rice black-streaked dwarf disease affects summer rice more seriously than winter or spring rice. Its severity depends on the timing and extent of immigration of the viruliferous WBPH population. Dwarfism is observed mainly on plants infected early. Therefore, the earlier, extensive WBPH immigration causes more serious effects. Although severely-diseased winter or spring rice plants may be seen occasionally in northern Vietnam

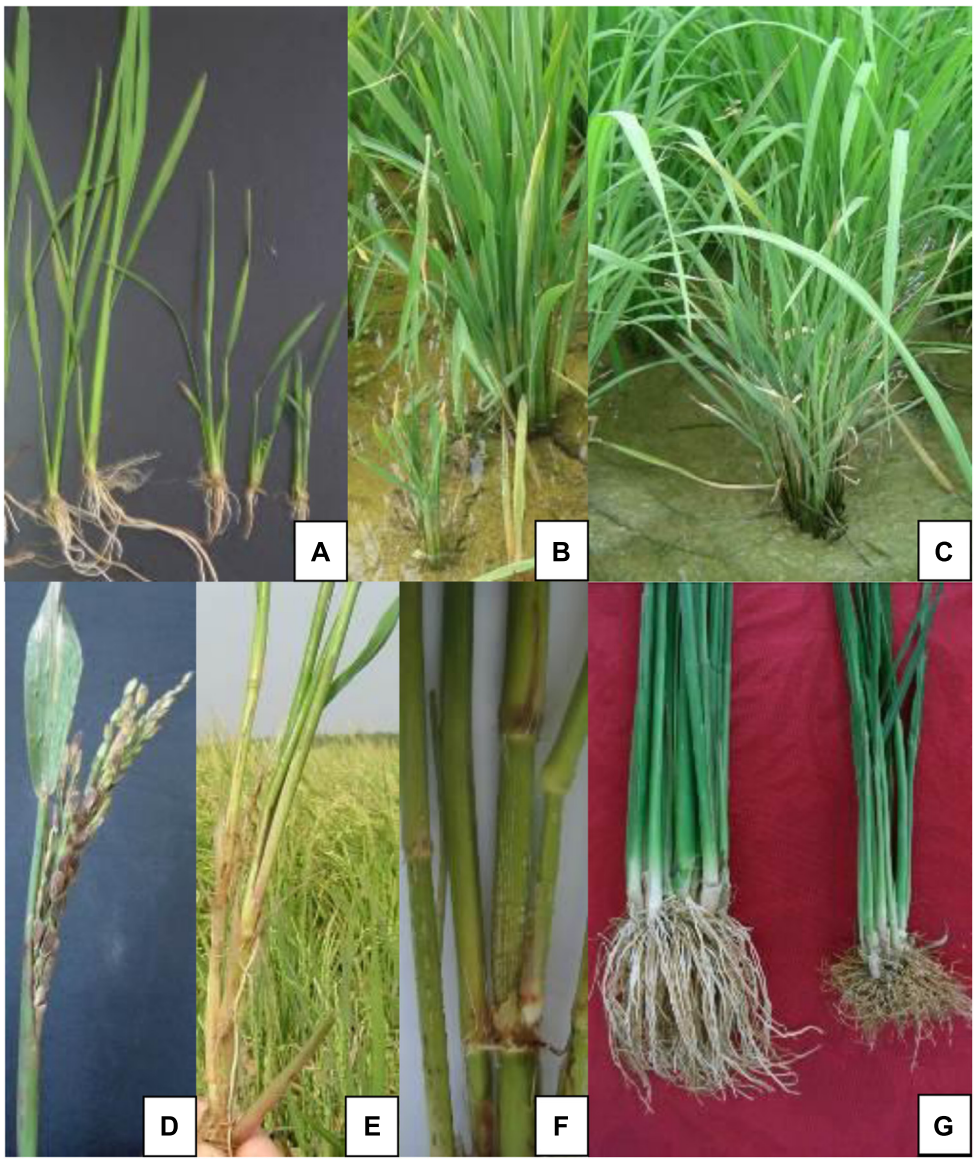

FIGURE 1 | Symptoms of southern rice black-streaked dwarf disease. **(A)** Very early infected seedlings (right) showing dwarfism and stiff leaves. **(B)** Diseased plant (front) infected at the seedling stage showing severe stunting and withering after transplanting. **(C)** Diseased plant (front) infected at the early tillering stage showing dwarfism and excessive tillering. **(D)** Diseased head showing small spikes and barren grains. **(E)** Aerial rootlets and branches on the stem nodes of an infected plant. **(F)** Small streaked white or black waxy galls on the stems of an infected plant. **(G)** Poorly developed brown roots (right) of an infected plant.

and southern Hainan Province, China, the major overwintering places for WBPH, spring rice in most rice growing areas of China is very mildly affected, with less than 1% dwarfed plants in most fields and rarely with 3–5% in several fields. However, infected spring rice plants may serve as an infection source for summer rice growing in the same or other places. In most rice growing areas in China, the infection rates steadily increase through the growing seasons, i.e., from low on spring rice to high on early and late summer rice. In southern Japan, SRBSDD is observed yearly after mid-June to mid-July, when the overseas immigration of WBPH from China or Vietnam occurs (Matsukura et al., 2013).

(3) Southern rice black-streaked dwarf disease afflicts hybrid rice more than inbred rice, as indicated by field surveys. This difference is probably due to both the higher compatibility between hybrid rice and WBPH, which may increase infection opportunities, and the single seedling transplanting of hybrid rice, which eliminates compensation from healthy plants.

(4) Normally, in fields with low SRBSDD occurrence rates (below 3%), diseased plants are dispersed and have mild symptoms, while in fields with high rates (above 10%), infected plants are clumped and have severe symptoms. These clumps are always surrounded by many plants with milder symptoms (darkened leaves and galls on stalks, but no stunting). A similar distribution pattern also occurs when a "severe disease" field is neighbored by "mild disease" fields and may result from the relatively slow dispersal (and virus transmission) of the viruliferous WBPH nymphs that develop on early-infected rice plants.

PATHOGEN AND TRANSMISSION

The viral pathogen of SRBSDD is currently a tentative species in the genus *Fijivirus*, family *Reoviridae* (Zhou et al., 2008; Wang et al., 2010a). The name Southern rice black-streaked dwarf virus (SRBSDV) was proposed because it resembles the *Rice black-streaked dwarf virus* (RBSDV) in disease symptoms, viral particles, and genome structure; the word "Southern" refers to southern China (where it was first identified) and the southern part of the Northern Hemisphere where it is currently distributed (RBSDV occurs in the northern part of that hemisphere). The virions of SRBSDV are icosahedral particles 70–75 nm in diameter that appear only in the phloem of infected plants, while the virus aggregates into a lattice structure in the cells (**Figure 2**). The viral genome contains ten linear double-stranded RNA (dsRNA) segments that range in size from approximately 4.5 to 1.8 kb and are named S1–S10 according to their molecular weights (from large to small; Zhou et al., 2008; Wang et al., 2010a). Sequence data indicate that SRBSDV is most closely related to *maize rough dwarf virus* (MRDV), followed by RBSDV and *Mal de Río Cuarto virus* (MRCV; **Table 1**; **Figure 3**).

The SRBSDV genome contains 13 open reading frames (ORFs): S5, S7, and S9 each encode two proteins (P5-1, P5-2, P7-1, etc.), while the other seven segments each encode one. Genomic comparisons have shown that S1–S4, S8, and S10 of SRBSDV encode putative structural proteins: the RNA-dependent RNA polymerase, major core structural protein, capping enzyme, outer shell B-spike protein, minor core protein, and major outer capsid protein, respectively; P6, P7-1, P7-2, P9-1, and P9-2 encode putative non-structural proteins (Zhang et al., 2008; Zhou et al., 2008; Wang et al., 2010a). Several studies have investigated the functions of these proteins. The P6 is an RNA silencing suppressor (Lu et al., 2011) and might participate in viroplasm formation by interacting with itself and P5-1 (Li et al., 2013a) and by recruiting P9-1 (Wang et al., 2011). However, Mao et al. (2013) found that the P5-1 and P9-1 can form filamentous and granular viroplasm matrices respectively in the WBPH cells without the viral infection, and suggested that the P6 was recruited by direct interaction with these two proteins. Based on their findings, a new model for the genesis and maturation of the viroplasms induced by fijiviruses in insect vector cells was proposed (Mao et al., 2013). The P6 also interacts with a rice translation elongation factor 1A (eEF-1A) to inhibit host protein synthesis (Zhang et al., 2013). The P7-1 has been reported to be the major component of the tubules; it self-interacts to form tubules in insect cells and may be a virus movement protein (Liu et al., 2011). The P9-1 is the major

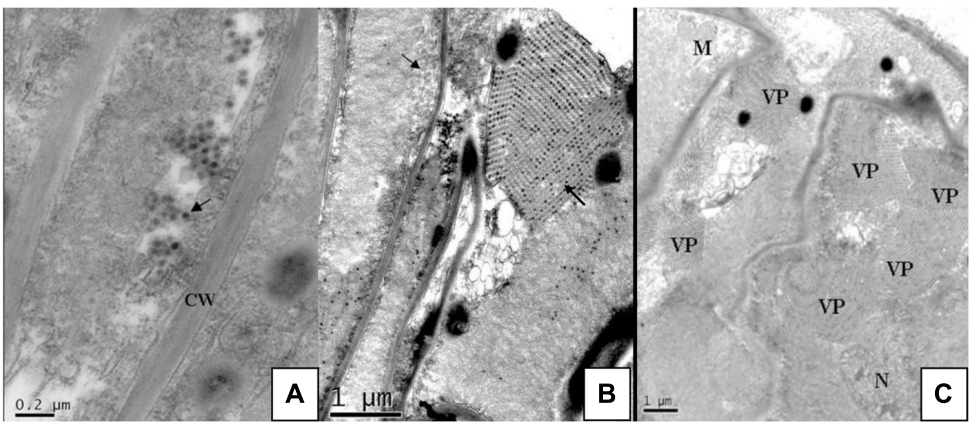

FIGURE 2 | Ultra-thin sections of rice vein cells infected with SRBSDV. From Zhou et al. (2008). (A) Separate virus particles in the cytoplasm near the cell wall (arrow). **(B)** Virus particles arrayed in rows inside a transverse tubule (arrow) and numerous virus particles aggregated into crystalline array in the viroplasms (arrow). **(C)** Viroplasms full of virus scattered in the cytoplasm. CW, cell wall; N, nucleus; VP, viroplasm; M, mitochondrion.

Table 1 | Identities (%) of nucleotide and predicted amino acid (bracketed) sequences between SRBSDV Hainan isolate and other plant-infecting fijiviruses*.

Segment	RBSDV-r-Zhj	RBSDV-m-Hub	MRCV**	MRDV	FDV	OSDV
S1	79.0 (85.8)	79.0 (86.1)	74.1 (78.6)	n/a***	67.3 (63.7)	n/a
S2	78.9 (89.0)	79.2 (89.0)	75.7 (83.1)	n/a	62.2 (59.4)	n/a
S3	73.0 (73.4)	73.1 (73.8)	65.5 (56.8)	n/a	53.8 (37.5)	n/a
S4	77.3 (84.5)	77.3 (84.5)	71.1 (72.9)	n/a	63.2 (53.6)	n/a
S5	70.6 (69.9, 61.8)	71.6 (67.7, 62.7)	66.3 (59.1, 50.5)	n/a	51.1 (30.9, –)	n/a
S6	70.6 (63.1)	70.7 (63.3)	62.4 (42.5)	n/a	11.2 (23.2)	n/a
S7	73.3 (80.7, 60.6)	73.2 (80.7, 60.6)	65.5 (61.7, 42.3)	74.2 (82.7, 61.5)	59.7 (52.5, 25.2)	22.8 (34.1, 10.2)
S8	72.6 (71.3)	72.5 (70.6)	64.2 (55.4)	72.6 (70.3)	40.3 (35.8)	15.1 (22.6)
S9	74.2 (77.4, 72.0)	74.0 (77.1, 72.0)	68.8 (67.1, 61.6)	74.9 (77.4, 73.5)	51.3 (37.1, 37.8)	40.5 (26.3, 18.0)
S10	78.5 (83.4)	78.8 (84.3)	72.5 (71.9)	79.1 (83.5)	56.4 (47.6)	45.4 (33.1)

**From Wang et al. (2010a). **MRCV, Mal de Río Cuarto virus; MRDV, maize rough dwarf virus; FDV, Fiji disease virus; OSDV, oat sterile dwarf virus. ***n/a, no sequence available*

constituent of the viroplasm and is essential for viral replication in insect vectors (Jia et al., 2012a). The functions of other SRBSDV proteins remain unknown.

The genetic diversity and evolutionary mechanisms of SRBSDV were investigated by Li et al. (2013b), who reported putative recombination events and positive selection. Through June, 2013, hundreds of SRBSDV sequences have been deposited in GenBank, including the complete genomic sequences of seven isolates. The SRBSDV genome is highly conserved, with over 97% nucleotide identities among isolates from different geographic regions, perhaps because the virus experiences large-scale transmission from limited overwintering zones via its vector's annual migration.

The virus can be transmitted highly efficiently by WBPH in a persistent circulative propagative manner (Cao et al., 2011; Pu et al., 2012). A small proportion (less than 5%) of small brown planthoppers (BPHs; *Laodelphax striatellus*) acquire SRBSDV

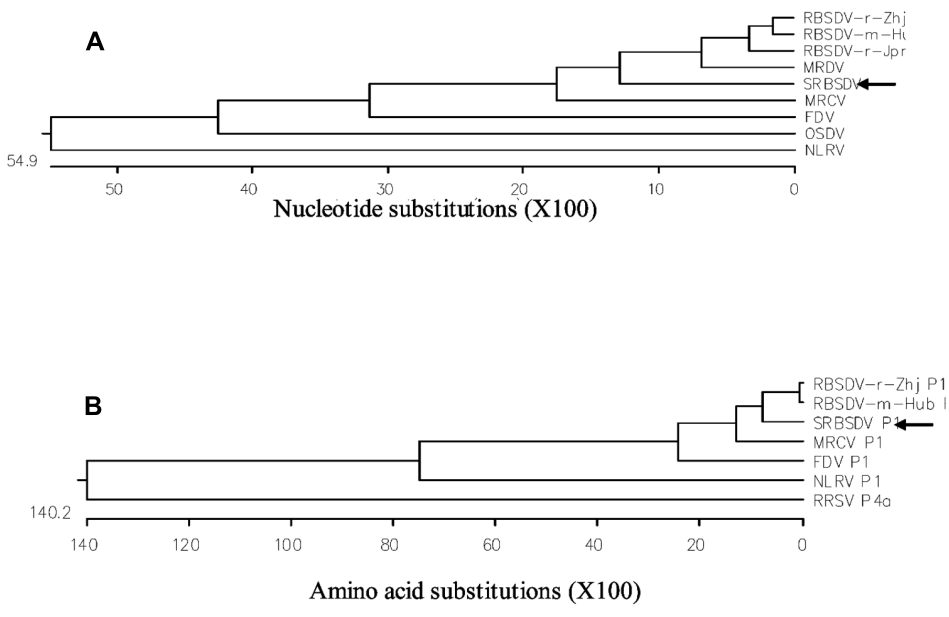

FIGURE 3 | Polygenetic trees based on S10 nucleotide sequences (A) and P1 amino acid sequences (B) of SRBSDV and other related fijiviruses. From Zhou et al. (2008) and Wang et al. (2010a). To construct the phylogenetic trees, viral nucleotide and amino acid sequences were obtained from GenBank, aligned using MegAlign, and analyzed using the neighbor-joining method (http://www.ncbi.nlm.nih.gov). S10 nucleotide sequence accession numbers are EU523360, NC_003733, D00606, AF227205, L76560, AY607586, NC_007162, AB011025, and NC-003653 for SRBSDV, RBSDV-r-Zhj, RBSDV-r-Jpn, RBSDV-m-Hub, MRDV, MRCV, FDV, OSDV, and NLRV, respectively. P1 amino acid sequence accession numbers are FN563983, NC_003729, AY144568, AF499925, NC_007159, NC_003654, and NC_003771 for SRBSDV, RBSDV-r-Zhj, RBSDV-m-Hub, MRCV, FDV, NLRV, and RRSV, respectively.

from infected plants under experimental conditions but cannot transmit it (Jia et al., 2012b; Pu et al., 2012), perhaps because the virus cannot pass from the midgut into the hemocoel or into the salivary glands of this insect (Jia et al., 2012b). The virus is not transmitted by the BPH (*Nilaparvata lugens*), leafhoppers, or rice seeds (Pu et al., 2012; Wang et al., 2010b). It propagates in WBPH and is carried by viruliferous vectors throughout their lives but is not transmitted to eggs. Both WBPH nymphs and adults can transmit the virus to plants, and nymphs acquire the virus more readily than adults. About 80% of nymphs propagated on infected rice plants may become viruliferous. The minimum virus acquisition and inoculation periods were 5 and 30 min, respectively, for both WBPH nymphs and adults. The circulative transmission periods of the virus in WBPH range from 6 to 14 days, and most viruliferous individuals have one or more intermittent periods that range from 2 to 6 days (Pu et al., 2012). A single newly-hatched, viruliferous WBPH nymph may infect 22–87 (avg. 48.3) rice seedlings with SRBSDV (Cao et al., 2011), and a single adult can transmit the virus to 8–25 rice plants within 5 days (Pu et al., 2012). WBPH can transmit SRBSDV from rice to maize (*Zea mays*) seedlings, but it rarely acquires the virus from infected maize (Li et al., 2012; Pu et al., 2012).

Southern rice black-streaked dwarf virus could be detected by RT–PCR in all tested rice plant parts except growth point. The virus titer differed significantly among plant parts; the leaf sheath contained the highest SRBSDV titer, followed by the oldest green leaf. The virus titer in the leaf sheath varied significantly with plant age and increased substantially 20 days after infection (Matsukura et al., 2013). The expression levels of most viral genes (except P1) were relatively low on day 14 after infection, rapidly increased from day 14–20, decreased by day 30, increased by day 40, and remained relatively stable by day 50. In contrast, the expression of P1 was significantly down-regulated on days 14 and 20 (He et al., 2013). The virus titers in leaf sheaths and old green leaf correlated positively with WBPH virus acquisition efficiency (Matsukura et al., 2013).

Southern rice black-streaked dwarf virus can naturally infect a number of grasses, including rice, maize, Chinese sorghum (*Coix lacryma-jobi*), *Avena fatua*, *Echinochloa crusgalli*, *Eleusine indica,* and *Pennisetum flaccidum* (Zhou et al., 2008; Zhu et al., 2012). Diseased maize plants are clearly stunted with swollen internodes; densely clumped and darkened foliage; and wider, shorter, fragile leaves with ruffles. Most infected maize cultivars produce dot-like wax lining small galls along the abaxial leaf veins, and also some produce them on their stalks. Diseased maize plants have short, small ears and always fail to fruit. On SRBSDV-infected Chinese sorghum, *A. fatua*, *Echinochloa crusgalli,* and *Eleusine indica*, dark green and ruffled leaves also can be observed (Zhou et al., 2008; Zhu et al., 2012).

DISEASE CYCLE

In most rice growing areas in China, no winter rice is grown that could host overwintering WBPH. Thus, the primary infection comes from viruliferous WBPH adults migrating northward from their overwintering sites in southern China every spring. The southwestern province of Yunnan and Hainan Island are considered the major WBPH overwintering regions in China. In recent years, the growing area of winter rice on Hainan Island is expanding, providing abundant virus and vector resources. The viruliferous WBPH may migrate into the southern parts of Guangdong and Guangxi provinces and northern Vietnam during February and March. Normally, the viruliferous macropterous adults are driven by the northeastward air flow to the Pearl River Basin (Guangdong) and Honghe Prefecture (Yunnan) in March. Then they migrate to northern Guangdong and Guangxi provinces, Southern Hunan and Jiangxi provinces, and central Guizhou and Fujian provinces in April; to the middle and lower reaches of the Yangtze River and the Huai River Plain during late May to middle or late June; and to the northern provinces and the southern parts of the northeastern provinces, even into Japan, during late June to early July. During autumn, the virus-carrying insects return to their overwintering places in late August when the monsoons shift direction. The SRBSDD cycle (Guo et al., 2010) is illustrated in **Figure 4**.

The occurrence and prevalence of SRBSDD are closely associated with the invasion period and invasion population of WBPH and the percentage of viruliferous individuals. Conditions that favor an epidemic include: a warm winter followed by a rainy early spring; typhoons and strong convection; high mountains, uplands, basins, and river mouths; rice in the seedling and tillering periods; peak immigration of WBPH; and companion planting of single- and double-cropping rice or spring and early/late summer rice.

SRBSDV–RICE–WBPH INTERACTIONS

Because SRBSDV propagates within its vector, WBPH, it can be considered both a plant virus and an insect virus. Plant viruses have been reported to affect the life parameters of their vectors (Boquel et al., 2012; Czosnek and Ghanim, 2012), and are well documented to influence the vector's behavior, including host selection, settling and feeding, and dispersal via olfactory cues (i.e., plant volatile organic compounds) that are altered by virus infection (Mauck et al., 2010; Bosque-Perez and Eigenbrode, 2011; Stafford et al., 2011). Tu et al. (2013) revealed that SRBSDV could adversely influence WBPH survival rates, adult longevity, and oviposition rates and that these effects were temperature-dependent. WBPH behavior appears to be altered directly by SRBSDV or indirectly by the infected plants, but more evidence is needed to clarify this relationship. Xu et al. (2012) found that SRBSDV infection may alter WBPH gene expression, involving primary metabolism, ubiquitin-proteasome, cell cytoskeleton organization, and immunity regulatory system pathways. He et al. (2013) revealed by RT–qPCR that all 13 SRBSDV genes showed similar expression patterns in rice, maize, and WBPH, indicating that the virus uses the same infection strategy in plant and insect hosts. Most SRBSDV genes were more abundant in WBPH than in rice and maize, confirming the virus' higher replication levels in insect hosts than in plant hosts. Across the SRBSDV genome, the mRNA expression levels of P3, P7-1, and P9-2 were highest, and the levels of P6 and P10 were lowest (He et al., 2013).

PREDICTION AND FORECAST

Similar to other rice viral diseases, SRBSDD occurs occasionally and suddenly with localized devastation and is difficult to

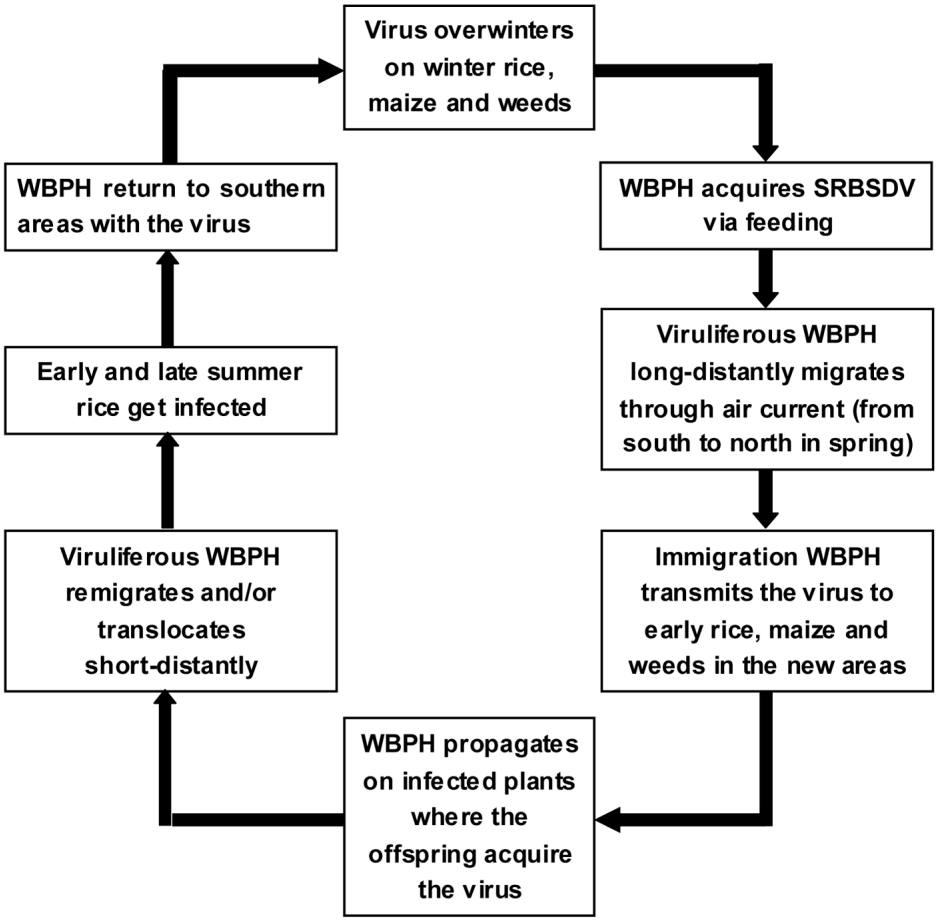

FIGURE 4 | Infection cycle of Southern rice black-streaked dwarf disease (SRBSDD).

forecast. Medium- to long-term predictions must be based on cycles of disease occurrence, disease prevalence, and long-distance WBPH migration, while short-term forecasts should consider cultivation patterns, invasion populations of WBPH, percentages of viruliferous insects, and weather conditions.

(1) Surveys of the base numbers of overwintering diseased plants and pests. Annual surveys from January to March of the infection rates and WBPH population sizes on winter rice in northern Vietnam and Hainan Island are important to predict the occurrence of SRBSDD in most rice producing areas of China, while winter surveys in Northern Burma and southwestern Yunnan province are crucial for predicting outbreaks in the southwest rice production area. Moreover, infection rates of maize growing in WBPH overwintering regions can also provide data for forecasting the disease.

(2) Surveys of the immigration period, invasion population, and percentage of viruliferous WBPH. Severe disease is highly likely when the WBPH immigration period coincides well with the rice seedling stage and where there are large viruliferous invasion populations and many immigration climaxes.

(3) Surveys of the infection rates of spring rice in mid- to late-growth stages and the WBPH population. There is a close correlation between the field infection rate of spring rice and the percentage of viruliferous insects. Viruliferous WBPHs propagated on SRBSDV-infected spring rice may be the infection source for early and late summer rice in the same or other places. Field surveys of the disease and vector populations can provide useful forecasting information.

(4) Surveys of the infection rates of early and late summer rice, the WBPH population, and the percentage of viruliferous individuals. These surveys are especially important for rice production areas in high danger, i.e., areas with past severe occurrences and those in hills, basin, or near river mouths.

(5) Methods for field sampling and virus detection. For survey and detection purposes, whole diseased plants should be collected from fields, their roots cleaned of soil and kept moist with a wet cloth or tissue, and the samples sent to a laboratory for detection. WBPHs can be captured using light traps and stored in a small container with a cotton ball or towel moistened with 70% ethanol to prevent the insects from rotting. To avoid being crushed during shipping, the insects should not be directly soaked in ethanol. Optimally, at least 100 macropterous WBPH adults should be collected for each survey. There are a number of methods for virus detection from plants and insects, such as ELISA

(Wang et al., 2012a), dot-immunobinding assay (Chen et al., 2012), Immuno-fluorescence assay (Jia et al., 2012a,b), RT–PCR (Wang et al., 2012b) and reverse transcription-loop-mediated isothermal amplification (RT-LAMP; Zhou et al., 2012). RT–PCR is a proven and highly-reliable method. For example, Wang et al. (2012b) screened 40 pairs of primers and selected two primer sets for a specific and efficient one-step duplex RT–PCR detection method that is commonly used.

DISEASE CONTROL

Thus far, no SRBSDV-resistant rice cultivar has been found, detailed disease regulation remains unclear, and disease forecast systems and control measures are still under development. Nevertheless, some effective approaches have been devised based on practical experience in disease control in recent years.

(1) Joint prevention and control. Enhanced disease and pest control in their overwintering areas may help prevent the disease from spreading widely. Managing WBPH populations in southern China (where the pest and virus propagate in early spring) may alleviate occurrences of the disease in the northern rice growing areas, while control of the disease and pest on spring rice may reduce the invading pest populations and virus infection rates on early and late summer rice, both locally and within the insect migration range.

(2) Disease prevention via pest management. This strategy focuses on managing WBPH during the pre-jointing stages of early and late summer rice. Seedling planting locations and sowing times should be properly selected. Physical protection can be used to avoid or reduce WBPH invasions. In addition, seeds can be treated before sowing with coating agents or systemic pesticides, and chemical control of WBPH after seedling transplanting may be helpful.

(3) Resistant rice cultivar screening. At present, some WBPH-resistant cultivars are available for production use, and work on screening and breeding rice cultivars with SRBSDD-resistance is ongoing.

(4) Disease prevention in cropping operations. In practice, infected seedlings should be diagnosed and discarded during the early rice growth stages, diseased tillering plants in fields with infection rates of 3–20% should be replaced with healthy tillers split from uninfected plants, and severely diseased fields should be abandoned and replanted with other crops.

TENDENCY OF OUTBREAK AND EPIDEMIC

The only currently-recognized WBPH-transmitted rice viral disease, SRBSDD has spread extensively since its discovery in 2001. After several years of viral source accumulation, a devastating disease outbreak occurred in northern Vietnam and southern China in 2009–2010, and although great efforts at disease management and vector control were made, the disease still damaged over 700,000 ha in 2011 and over 500,000 ha in 2012 in those two countries. During the past several years, the disease has spread to the rice growth region in southwestern China, and the virus has invaded the two major overwintering areas, the Gulf of Tonkin and Yunnan Province, China. It is predictable that SRBSDD will become a common major rice disease that occurs severely in limited locations and largely prevails in some years. The logic includes:

(1) The virus has an extensive area with a variety of conditions for overwintering. The virus is transmitted by WBPH in a persistent propagative manner and can be carried throughout the vector's life, so it may overwinter in WBPH and in many plant species (including some weeds). Although the availability of these susceptible overwintering plants as sources of virus infection must still be experimentally examined, the viral overwintering range is probably even larger than the vector's. Moreover, WBPH is more cold-resistant than BPH, overwinters in the vast warm areas south of 26° north latitude (i.e., southern Hainan Island and southwestern Yunnan Province), and propagates there year round. These conditions favor long-term survival of the virus, which maintains a high potential to trigger future outbreaks and epidemics of SRBSDD.

(2) The vector immigration period coincides strongly with the period of greatest rice susceptibility (the early growth stage), which may promote efficient secondary infection. As long-distance migrants, WBPH are very active and propagate rapidly; their populations can be many 10 to 100-folds within 1 month. More than 80% of the offspring feeding on infected plants will became viruliferous, and each individual can infect many plants. Obviously, even if only a small percentage of invasive WBPH are viruliferous, they might cause severe outbreaks of SRBSDD under favorable environmental conditions.

(3) The current rice cultivars and cropping systems are favorable for SRBSDD occurrence. To date, most of the main rice cultivars are susceptible to WBPH, and no SRBSDV-resistant cultivars have been found. Hybrid rice cultivars susceptible to SRBSDV and highly compatible with WBPH are widely grown in China. The seedling stage of summer rice in double-cropping regions temporally overlaps with the late growth stage of spring rice, and the two are spatially near, which is conducive to the transfer of the virus vector from spring to summer rice. Finally, the companion planting of single- and double-cropping rice in many areas with long sowing periods and the intercropping of maize also promote the translocation of SRBSDV and WBPH.

(4) Global warming might expand the overwintering region for SRBSDV and WBPH, increase the survival rate of overwintering vector, and result in more serious and widespread SRBSDV outbreaks.

(5) The disease is difficult to forecast and control. Only a dozen years have passed since SRBSDD was first recognized, and more studies are needed to better understand its epidemiology and develop monitoring technologies and control strategies. Exact forecasting of the disease is complicated by large uncertainties in the variable inter-year resources, immigration locations, invasion times and quantities, and infection rates of the migratory WBPH vector. Furthermore, because of the disease's relatively long incubation period, the early symptoms are difficult to recognize.

In general, SRBSDD is likely to be more widespread and more serious in the near future, and therefore will become a great threat to rice production in Asia. Further studies on the epidemiology of the disease, on long-term monitoring systems and management technologies, on screening and breeding virus-resistant rice cultivars, and on control technology are urgently needed to combat this devastating disease.

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The complete nucleotide sequence of the genome of Barley yellow dwarf virus-RMV reveals it to be a new *Polerovirus* distantly related to other yellow dwarf viruses

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The yellow dwarf viruses (YDVs) of the *Luteoviridae* family represent the most widespread group of cereal viruses worldwide. They include the Barley yellow dwarf viruses (BYDVs) of genus *Luteovirus*, the Cereal yellow dwarf viruses (CYDVs) and Wheat yellow dwarf virus (WYDV) of genus *Polerovirus*. All of these viruses are obligately aphid transmitted and phloem-limited. The first described YDVs (initially all called BYDV) were classified by their most efficient vector. One of these viruses, BYDV-RMV, is transmitted most efficiently by the corn leaf aphid, *Rhopalosiphum maidis*. Here we report the complete 5612 nucleotide sequence of the genomic RNA of a Montana isolate of BYDV-RMV (isolate RMV MTFE87, Genbank accession no. KC921392). The sequence revealed that BYDV-RMV is a polerovirus, but it is quite distantly related to the CYDVs or WYDV, which are very closely related to each other. Nor is BYDV-RMV closely related to any other particular polerovirus. Depending on the gene that is compared, different poleroviruses (none of them a YDV) share the most sequence similarity to BYDV-RMV. Because of its distant relationship to other YDVs, and because it commonly infects maize via its vector, *R. maidis*, we propose that BYDV-RMV be renamed Maize yellow dwarf virus-RMV (MYDV-RMV).

Keywords: Luteoviridae phylogenetics, P0, maize yellow dwarf virus, maize virus, rhopalosiphum maidis, luteovirid

INTRODUCTION

Barley yellow dwarf viruses (BYDVs), Wheat yellow dwarf virus (WYDV), and Cereal yellow dwarf viruses (CYDVs), collectively known as yellow dwarf viruses (YDVs) are among the most economically important causal agents of disease in cereal crops. YDVs have been reported in both agriculturally important cereal crops and non-crop grasses throughout the world (El-Muadhidi et al., 2001; Hawkes and Jones, 2005; Hesler et al., 2005; Kumari et al., 2006; Power et al., 2011; Siddiqui et al., 2012; Jarošová et al., 2013). YDVs vectored primarily by the bird cherry-oat aphid, *Rhopalosiphum padi*, can cause yield reductions of 15 to 25% in barley, wheat and oat (Lister and Ranieri, 1995). The presence of BYDV correlated with a reduction in yield of winter wheat (Banks et al., 1995) as well as wheat and oats (McKirdy et al., 2002). Perry et al. (Perry et al., 2000) found an average of 30% yield loss in affected winter wheat fields. Significant agricultural research efforts are aimed at reducing the impact of the YDVs on the yield of the various crop systems by (i) altering planting time and/or pesticide application regimes to avoid the accumulation of high densities of aphids, (ii) tilling practices, and (iii) developing virus-resistance crop varieties (Chain et al., 2005; Royer et al., 2005; Kennedy and Connery, 2012).

The YDVs are members of the *Luteoviridae* family. All members of the *Luteoviridae* (luteovirids) have linear, positive-sense, 5.5–6 kb RNA genomes and are obligately aphid-transmitted in a circulative, persistent manner, with the exception of Pea enation mosaic virus 1 (PEMV1), which is mechanically transmissible in the presence of the umbravirus, PEMV2. The key genes conserved in all luteovirids are the major coat protein (CP) and readthrough domain (RTD) generated by translational readthrough of the CP open reading frame (ORF) stop codon, which provides a long carboxy-terminal extension to the CP (Brault et al., 1995; Brown et al., 1996). The CP and CP-RTD proteins provide the virion structure and aphid transmission properties, and they play a role in virus movement and tissue specificity within the plant (Brault et al., 1995; Chay et al., 1996a; Van Den Heuvel et al., 1997; Peter et al., 2009). ORF 4, which is embedded in the CP ORF but in a different reading frame, is also conserved in all luteovirids, except PEMV1. The product of ORF 4 (P4) has features of a cell-to-cell movement protein (Chay et al., 1996a; Schmitz et al., 1997), which may confer the property that all Luteoviridae except PEMV1 are confined to the phloem. Because the sequence encoding CP-RTD and P4 is the only part of the genome conserved in all luteovirids with the aforementioned exception of PEMV1, we call this region the *Luteoviridae* block (Miller et al., 2002).

Luteoviridae fall into three genera: *Luteovirus, Polerovirus,* and *Enamovirus* (Domier, 2012). Outside of the *Luteoviridae* block, the viral genomes are completely different between *Polerovirus* and *Luteovirus* genera. The RNA-dependent RNA polymerase (RdRp) genes (the key gene used for virus classification) of poleroviruses and the only enamovirus (PEMV1) are similar to each other but quite distantly related to those of genus *Luteovirus*

(Miller et al., 2002; Domier, 2012). The polero/enamovirus RdRps are more similar to those of genus *Sobemovirus*, which has not been assigned to a family, than to those of genus *Luteovirus*. Moreover, outside of the *Luteoviridae* block, the genomes of genus *Luteovirus* (including the RdRp ORF) are most closely related to those of genus *Dianthovirus* in the *Tombusviridae* (Miller et al., 2002). In particular, the RdRp and translational control signals of genus *Luteovirus* resemble those of the *Tombusviridae* more than they resemble those of the *Polerovirus* or *Enamovirus* genera. Moreover, poleroviruses and the enamovirus contain a genome-linked protein (VPg), and also encode a viral suppressor of gene silencing (VSR) in ORF 0 (Pfeffer et al., 2002; Mangwende et al., 2009). Viruses in genus *Luteovirus*, like the *Tombusviridae*, have neither a VPg nor an ORF 0.

The original YDVs identified as the causal agents of yellow dwarf disease were all called BYDV and placed into five strains (now considered species) based on serotype, symptomatology and predominant aphid vector species (Rochow, 1969; Rochow and Muller, 1971). BYDV-RPV, -MAV, -SGV, and -RMV were found to be transmitted most efficiently by *R. padi, Sitobion avenae, Schizaphus graminum* and *R. maidis*, respectively. The most common virus is BYDV-PAV which is transmitted efficiently by *R. padi* and *S. avenae* (Rochow, 1969; Rochow and Muller, 1971).

Upon sequencing the complete genomes of some of the BYDV strains, it became clear that BYDV-RPV is a *Polerovirus*, renamed Cereal yellow dwarf (CYDV)-RPV, while BYDV-PAV and BYDV-MAV which have virtually identical RdRp sequences, are in genus *Luteovirus*. More recently discovered YDVs include CYDV-RPS, a CYDV-RPV-like virus that causes cork screw-shaped leaves and leaf notching in wheat; the former BYDV-GPV (now Wheat yellow dwarf virus-GPV (WYDV-GPV) (Zhang et al., 2009); and BYDV-PAS, a severe BYDV-PAV-like virus that breaks resistance in oat (Chay et al., 1996b).

In addition to the BYDVs, the genus *Luteovirus* includes Bean leafroll virus (BLRV), Soybean dwarf virus (SbDV), and (unofficially) Rose spring dwarf-associated virus (RSDaV). In addition to the CYDVs, the *Polerovirus* genus includes about two dozen viruses of diverse crops. Subsequently additional species have been identified in China, such as BYDV-GAV, and WYDV-GPV, transmitted primarily by *S. graminum* and *S. avenae*, and *S. graminum* and *R. padi*, respectively, and BYDV-PAV-CN which is transmitted efficiently by all three aphid species (Jin et al., 2004; Liu et al., 2007; Zhang et al., 2009). BYDV-GAV is very similar to BYDV-MAV (Jin et al., 2004; Zhang et al., 2009), WYDV-GPV is closely related to CYDV-RPV (Lucio-Zavaleta et al., 2001; Zhang et al., 2009), while BYDV-PAV-CN is highly diverged from other BYDVs (Liu et al., 2007). It should be noted that the vector specificity of the YDVs can vary by isolate within a virus species, by genotype of an aphid species, or under different environmental conditions (Lucio-Zavaleta et al., 2001). Thus, the YDVs are now classified by nucleotide sequence identity and genome organization, rather than by the most efficient aphid vector.

Until this report, the complete BYDV-RMV genome had not been sequenced, only the nucleotide region encoding the coat protein had been reported (Geske et al., 1996; Domier et al., 1997). RMV is the only BYDV transmitted efficiently by *R. maidis*, the corn leaf aphid. Hence it infects maize (Itnyre et al., 1999a,b). Moreover, maize serves as a reservoir for BYDV-RMV from which it can be transmitted to nearby wheat plots where stunting and yield losses ensue (Brown et al., 1984). BYDVs can reduce sweet corn yield dramatically by causing incomplete ear filling which can render entire harvests unmarketable (Beuve et al., 1999; Itnyre et al., 1999b). BYDV-RMV virions are difficult to purify, hence it has been little studied. Here, we report the first complete sequence of a BYDV-RMV genome. The genome organization and sequence of the RMV MTFE87 isolate indicates that BYDV-RMV is a member of genus *Polerovirus*, but it is not closely related to the CYDVs, WYDV or any other polerovirus. Therefore, we submit that BYDV-RMV is a unique species, with the proposed new name Maize yellow dwarf virus (MYDV).

MATERIALS AND METHODS

BIOLOGICAL CHARACTERIZATION

The RMV MTFE87 isolate was obtained from Dr. T. W. Carroll, Montana State University in 1990. It was originally collected from an infected wheat plant growing on the Fort Ellis Experiment Station of Montana State University in 1987. The isolate has been continually propagated in Coast Black oats by regular transfer to new plants using *R. maidis*. Serological and aphid transmission properties of RMV MTFE87 were determined using a collection of antibodies to each of the YDV strains (Rochow and Carmichael, 1979; Webby and Lister, 1992). Double antibody sandwich (DAS) ELISA was carried out as described previously (Brumfield et al., 1992). Five different aphid species were used to determine the vector specificity of the RMV MTFE87 isolate. *R. maidis, R. padi, S. avenae, S. graminum* were previously described (Rochow and Carmichael, 1979; Power and Gray, 1995) and have been maintained as clonal lineages in the laboratory since their collection. The *Metapalophium dirhodum* colony was a gift from Fred Gildow (Gildow, 1993) and has been maintained in the laboratory in Ithaca, NY since 1993. Fourth instar or adult apterous aphids were allowed a 36–48 h acquisition access period on detached leaves of Coast Black oat plants infected with RMV MTFE87 4–5 weeks previously. Ten aphids were subsequently transferred to each of eight plants for a 72 h inoculation access period. Plants were fumigated and grown in a greenhouse and observed for symptoms for 3–4 weeks and tested using DAS-ELISA.

VIRUS PURIFICATION AND VIRAL RNA EXTRACTION

Virions were extracted from infected Coast Black oat plants as described previously (Hammond et al., 1983; Webby and Lister, 1992). Viral RNA was extracted using the hot phenol method. All centrifugation was at 13,200 rpm (16,100 × *g*) in a 24 × 1.5 ml tube rotor in an Eppendorf 5415R centrifuge. Briefly: purified virions were added to 3 volumes of extraction buffer yielding a final concentration of 167 mM Tris base (pH 8.5), 1% SDS, and 12.5 mM EDTA (all reagents from Sigma-Aldrich, St. Louis, MO). Hot (65°C) phenol was then added equal to the total volume, and after vortexing, the solution was incubated at 65°C for 15 min. The solution was vortexed again, centrifuged, and the aqueous phase saved. The phenol phase was back extracted using the above extraction buffer lacking SDS. After vortexing and centrifugation,

the second aqueous phase was combined with the first. The combined aqueous phases were then extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1). The RNA was precipitated at −20°C after adding 1/15 volume 3M sodium acetate (pH 5.5) and 2.5 volumes 95% ethanol. After centrifugation, the pellet was washed in 70% ethanol, vacuum dried and dissolved in a small volume of nuclease-free water.

AMPLIFICATION OF GENOME DOWNSTREAM OF THE VIRAL COAT PROTEIN (CP)

The 3′ terminal region (1788 base pairs) of RMV MTFE87 was amplified and prepared for cloning using ligated-anchor PCR (LA-PCR) according to published protocols (Beckett and Miller, 2007). LA-PCR was performed on purified RMV MTFE87 viral RNA (500 ng). An anchor oligomer (5′-CTATAGTGTCACCTAAATGCGTGAAGAGCCTCCTACCAGCTGCTCCTATG-3′) was ligated directly to the 3′ end of the viral RNA. PCR amplification was conducted using an upstream primer (5′-AGATCACAAAAGTCATACTGGAGTTCATCT-3′) homologous to the viral coat protein (CP) and a downstream primer (5′-CATAGGAGCAGCTGGTAGGAGGCTCTTC-3′) complementary to the anchor.

AMPLIFICATION OF GENOME UPSTREAM OF THE VIRAL COAT PROTEIN

Overlapping genomic sections extending from the viral CP to the 5′ end of the genome were amplified and prepared for cloning using the SMART™ RACE cDNA Amplification Kit (Clontech) according to manufacturer's instructions. The following viral-specific primers were used in conjunction with the kit to walk step-by-step to the 5′ end of the genome: 5′-ATGCGAGGGTGCTGAGCTTGTTGTG-3′, 5′-GGATGTCATCCTCATCATCAGCCCAGTTTC-3′, 5′-AGCCGGAGTTGGAAGCGTTTATAGC-3′.

CLONING AND SEQUENCING OF VIRAL GENOME FRAGMENTS

PCR amplified genome fragments were cloned using the Zero Blunt®TOPO®PCR Cloning Kit (Invitrogen) according to manufacturer's instructions. Chimeric plasmids were purified from positive transformants. The cloned viral fragments were sequenced either by primer-walking or by 96-well sequencing on an ABI 3730 × l DNA Analyzer at the ISU DNA Sequencing Facility. Plasmid template was transposon tagged in preparation for sequencing using the Template Generation System™ II (Finnzymes) according to manufacturer's instructions.

SEQUENCE ASSEMBLY AND ANALYSIS

The Phred, Phrap, and Consed software programs were used in tandem to process and assemble raw sequencing reads for all clones which were sequenced in a 96-well plate format (Ewing and Green, 1998; Gordon et al., 1998). Vector NTI® (Invitrogen) was used to assemble the sequence reads from primer-walked clones. The complete nucleotide sequence of the RMV MTFE87 genome, as well as the amino acid sequences of all six major proteins, were compared with a range of other fully sequenced luteovirids (**Table 1**). The sequences were organized within the JalView alignment editor, version 2.4 (Clamp et al., 2004). The full length genomes were aligned with Clustal W (Thompson et al., 1994). The amino acid sequences were aligned using the MUSCLE algorithm (Edgar, 2004). Phylogenetic relationships were inferred from these alignments using the Neighbor-Joining method with 1000 replicates for bootstrapping in the MEGA4 (Molecular Evolutionary Genetics Analysis version 4.0) software package (Tamura et al., 2007). Sequence identity was determined via the Needleman-Wunsch global alignment in the EMBOSS Pairwise Alignment Algorithms, http://www.ebi.ac.uk/Tools/emboss/align/index.html.

Table 1 | Virus abbreviations and GenBank accession numbers for viruses used for sequence comparisons in this study.

Virus name	Abbreviation	Accession Number
Barley yellow dwarf virus—GAV	BYDV-GAV	NC_004666
Barley yellow dwarf virus—MAV	BYDV-MAV	NC_003680
Barley yellow dwarf virus—PAS	BYDV-PAS	NC_002160
Barley yellow dwarf virus—PAV	BYDV-PAV	NC_004750
Bean leafroll virus	BLRV	NC_003369
Beet chlorosis virus	BChV	NC_002766
Beet mild yellowing virus	BMYV	NC_003491
Beet western yellows virus	BWYV	NC_004756
Carrot red leaf virus	CtRLV	NC_006265
Cereal yellow dwarf virus—RPS	CYDV-RPS	NC_002198
Cereal yellow dwarf virus—RPV	CYDV-RPV	NC_004751
Chickpea chlorotic stunt virus	ChCSV	NC_008249
Cucurbit aphid-borne yellows virus	CABYV	NC_003688
Melon aphid-borne yellows virus	MABYV	NC_010809
Pea enation mosaic virus 1	PEMV1	NC_003629
Potato leafroll virus	PLRV	NC_001747
Barley yellow dwarf virus-RMV (proposed new name: Maize yellow dwarf virus-RMV)	BYDV-RMV (MYDV-RMV)	KC921392
Rose spring dwarf-associated virus	RSDaV	NC_010806
Soybean dwarf virus	SbDV	NC_003056
Sugarcane yellow leaf virus	ScYLV	NC_000874
Tobacco vein distorting virus	TVDV	NC_010732
Turnip yellows virus	TuYV	NC_003431
Wheat yellow dwarf virus—GPV	WYDV-GPV	NC_012931

RESULTS

BIOLOGICAL CHARACTERIZATION OF THE RMV MTFE87 ISOLATE

BYDV-RMV MTFE87 reacted in DAS-ELISA with antibodies to BYDV-RMV, but not to antibodies made against the other viruses (BYDV-PAV, BYDV-MAV, BYDV-SGV or CYDV-RPV). RMV MTFE87 was transmitted efficiently by *R. maidis* to eight of eight plants, to a lesser extent by *S. graminum* (4/8) and *R. padi* (1/8) and was not transmitted by *Metapalophium dirhodum* or *S. avenae* (both 0/8) FE87 was passaged six times in Coast Black oats using 10 *R. maidis* or *S. graminum* apterous adults for each passage. Transmission efficiency remained at 100% for *R. maidis* and increased to 95% for *S. graminum* by the sixth serial passage. The virus continued to react only with anti-BYDV-RMV antibodies following all of the serial passages by either aphid. Symptoms induced by RMV MTFE87 isolate were more severe on oat and wheat than the type RMV isolate from NY (RMV-NY) (Rochow

and Norman, 1961). The RMV-NY isolate rarely induces visible symptoms in wheat and many cultivars of oat. Symptoms in Coast Black oat are mild chlorosis or reddening of mature leaf tips, whereas the RMV MTFE87 isolate induced yellowing or reddening of flag leaves in wheat, noticeable stunting of the plants and incomplete filling of heads. Oat plants infected with RMV MTFE87 were severely stunted, with reddening and necrosis of leaves and incomplete formation of seed. The severe symptoms in oat and wheat are typical of RMV isolates collected in Montana (Brumfield et al., 1992) in contrast to RMV isolates collected in NY (Lucio-Zavaleta et al., 2001).

GENOME ORGANIZATION OF BYDV-RMV

The nucleotide sequence of RMV MTFE87 genomic RNA was found to be 5612 nt long, encoding six ORFs (**Figure 1**). The 5′ and 3′ untranslated regions (UTRs) are 54 and 158 nt long, respectively, and the only intergenic region spans nucleotides 3322–3515. The arrangement and sequences of ORFs resemble those of poleroviruses (**Figure 1**, **Table 2**). Based on sequence comparisons with poleroviruses, the ORFs encode a putative viral suppressor of silencing (VSR, ORF 0), serine protease and VPg (both in ORF 1), RdRp (ORF 2), coat protein (ORF 3), putative movement protein (ORF 4, which overlaps with ORF 3), and the CP readthrough domain (ORF 5) (**Figure 1**). A feature found in only three other luteovirids—Chickpea chlorotic stunt virus (ChCSV), Melon aphid-borne yellows virus (MABYV), and Cucurbit aphid-borne yellows virus (CABYV), is that ORF 4 of BYDV-RMV extends beyond the end of ORF 3, in this case by 4 nt. In all other *Luteoviridae*, the stop codon of ORF 4 is upstream of the CP ORF stop codon.

WHOLE GENOME ALIGNMENTS OF *Luteoviridae*

The full-length luteovirid genomes found in GenBank REFSEQ database were aligned by the neighbor-joining method using MEGA4 (Tamura et al., 2007) with 1000 replications (**Figure 2**). BYDV-RMV was grouped with the *Polerovirus* genus and in 100% of the replicates was closest to, but highly distinct from, Sugarcane yellow leaf virus (ScYLV). The BYDV-RMV/ScYLV branch was separated deeply from the CYDV and WYDV grouping indicating that BYDV-RMV is more closely related to other poleroviruses than it is to CYDVs and WYDV. As expected in this comparison, genus *Luteovirus* was well-separated from the *Polerovirus* and *Enamovirus* groups.

PROTEIN ALIGNMENTS AND ANALYSES OF RMV MTFE87 AND SELECTED LUTEOVIRIDS

The amino acid sequences of selected polerovirus and enamovirus proteins were aligned using the MUSCLE (Edgar, 2004) algorithm and from these alignments we created neighbor-joining trees via the MEGA4 software package (Tamura et al., 2007). P0 of RMV MTFE87 is separated readily from the CYDV/WYDV group and is most closely related to the Beet chlorosis virus (BChV)/Turnip yellows virus (TuYV) branch (**Figure 3A**). The

Table 2 | Sequence identity (%)[a] of BYDV-RMV proteins to those of other luteovirids.

Genus	Virus[a]	P0	P1	P2	P3	P4	P5
Luteovirus	BLRV	NA[b]	7	14	51	31	26
Luteovirus	BYDV-GAV	NA[b]	10	16	45	28	31
Luteovirus	BYDV-MAV	NA[b]	9	15	45	26	29
Luteovirus	BYDV-PAS	NA[b]	7	14	42	19	31
Luteovirus	BYDV-PAV	NA[b]	10	15	45	21	31
Luteovirus	RSDaV	NA[b]	2	19	32	25	31
Luteovirus	SbDV	NA[b]	7	14	51	38	26
Polerovirus	BChV	15	27	57	60	37	25
Polerovirus	BMYV	20	33	59	60	39	25
Polerovirus	BWYV	22	31	57	61	38	24
Polerovirus	CABYV	22	34	57	59	43	33
Polerovirus	ChCSV	18	30	60	59	37	29
Polerovirus	CtRLV	17	32	62	48	28	30
Polerovirus	CYDV-RPS	19	30	52	58	32	28
Polerovirus	CYDV-RPV	20	29	51	61	33	27
Polerovirus	MABYV	23	33	58	55	41	31
Polerovirus	PLRV	11	30	56	55	35	25
Polerovirus	ScYLV	18	30	56	40	29	37
Polerovirus	TuYV	21	39	64	61	38	26
Polerovirus	TVDV	23	32	62	53	31	26
Polerovirus	WYDV-GPV	20	30	53	59	35	27
Enamovirus	PEMV1	23	18	37	30	NA[b]	30

[a] *The identity of the sequences to BYDV-RMV was determined with the EMBOSS Needle global pairwise alignment algorithm. Molecular weights for the BYDV-RMV proteins were estimated using the ExPASy Server (Gasteiger et al., 2003).*

[b] *NA, not applicable.*

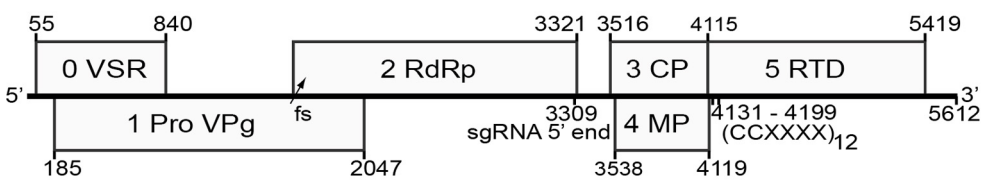

FIGURE 1 | Genome organization of BYDV-RMV. Numbers in small font indicate genomic positions of each ORF (numbered in large font) and the position of the predicted subgenomic RNA 5′ end and the readthrough sequence. VPg, viral genome-linked protein; VSR, putative viral suppressor of RNA silencing; RdRp, RNA-dependent RNA polymerase; CP, coat protein; MP, movement protein; RTD, readthrough domain; fs, site of −1 ribosomal frameshift; sgRNA 5′ end, predicted 5′ end of subgenomic RNA1 at nt 3309; $(CCXXXX)_{12}$, repeat motif required for readthrough of the CP ORF stop codon at nts 4131–4199.

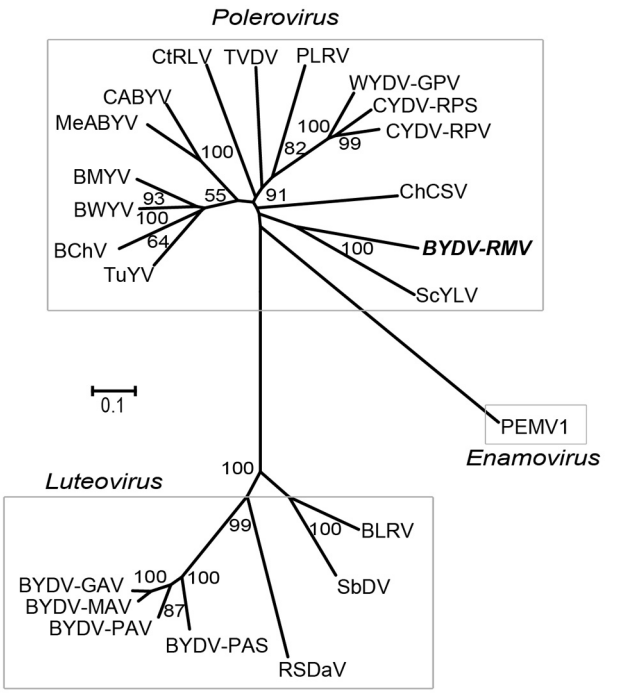

FIGURE 2 | Neighbor-joining tree of Clustal W-aligned whole genome sequences of luteovirids generated in the MEGA 4 software package. Numbers representing bootstrap values when greater than 50% for 1000 replicates are shown.

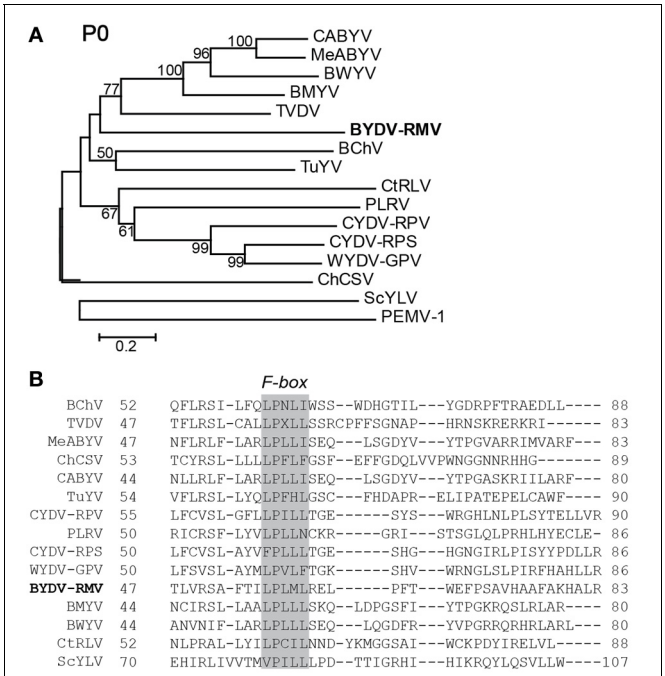

FIGURE 3 | Analysis of P0 amino acid sequences. (A) Amino acid sequences of the P0s for viruses in the *Polereovirus* and *Enamovirus* genera were aligned with the MUSCLE algorithm within JalView. The aligned sequences were used for inferring evolutionary relationships using the Neighbor-Joining method. The resultant tree, drawn to scale, is shown with the percentage (greater than 50% only) of 1000 bootstrap replicates. **(B)** A portion of the MUSCLE aligned P0 showing the conserved F-box domain LPxxL/I among the *Polerovirus* members. Accession numbers of viral genome sequences used in the alignment are in **Table 1**.

CYDV-RPS, CYDV-RPV, and WYDV group separates from the remainder of the sequences in 99% of the bootstrap replicates.

P1 of poleroviruses is a polyprotein that is cleaved by its internal protease into functional polypeptides that include the N terminus, the protease, the VPg, and a downstream RNA-binding fragment of unknown function (Prüfer et al., 1999, 2006; Li et al., 2000). The key amino acids of the catalytic triad in the protease (Li et al., 2000) are at positions 272, 306 and 373 (**Figure 4A**). Based on the known N-termini of the Potato leafroll virus (PLRV) VPg (Van Der Wilk et al., 1997b) and the enamovirus PEMV1 (Wobus et al., 1998), the N-terminus of the VPg of RMV MTFE87 is predicted to be amino acid T417 (**Figure 4A**). RMV MTFE87 P1 is most closely related to P1 of TuYV and TVDV, distinct from the CYDV/WYDV group linked in 100% of the bootstrap replicates (**Figure 4B**).

In all studied poleroviruses, P2, which encodes the active site of the RdRp, is expressed only as a fusion with P1, as it is translated by frameshifting of ribosomes from ORF 1 to ORF 2 in the region of overlap (Prüfer et al., 1992; Kujawa et al., 1993; Miller and Giedroc, 2010). The CYDV/WYDV RdRp (P2) sequences clustered tightly in 100% of the replicates, but they all were strikingly distinct from RMV MTFE87 P2, which groups rather distantly with CtRLV (**Figure 5A**).

The relationship of the RMV MTFE87 CP to that of other poleroviruses is not well resolved. The CP sequences of PEMV1 (*Enamovirus)*, and ScYLV are distinct from all of the remaining *Polerovirus* members, while 57 and 52% of bootstrap replicates support BYDV-RMV separation from TVDV and the large group of the remaining poleroviruses, respectively. But the close relatedness of the CYDV/WYDV CP to each other is present in 98% of the bootstrap replicates (**Figure 5C**).

The P4 proteins of TVDV and ScYLV are discretely separated from the remaining poleroviruses. BYDV-RMV and CtRLV form an intermediate between two larger clusters, one housing PLRV and CYDV/WYDV group while the other contains all others used in our study (**Figure 5B**). The readthrough domain, P5, is expressed as a fusion with the CP by leaky scanning through the amber stop codon of P3 (Brault et al., 1995; Brown et al., 1996). For the RTD, the *Polerovirus* and *Enamovirus* members branch into two distinct clades. RMV MTFE87 P5 groups with Sugarcane yellow leaf virus (ScYLV), CABYV, MABYV, ChCSV and PEMV1, in one clade. The remaining poleroviruses are in the other major clade including the CYDV/WYDV group present in 100% of bootstrap replicates (**Figure 5D**).

DISCUSSION

PROTEINS OF THE *Luteoviridae*

P0 of RMV MTFE87 is a putative viral suppressor of RNA silencing (VSR) because it shares homology with other poleroviruses in which P0 is a VSR. P0 of TuYV (formerly Beet western yellows virus-FL, BWYV-FL), PLRV and PEMV1 has been shown

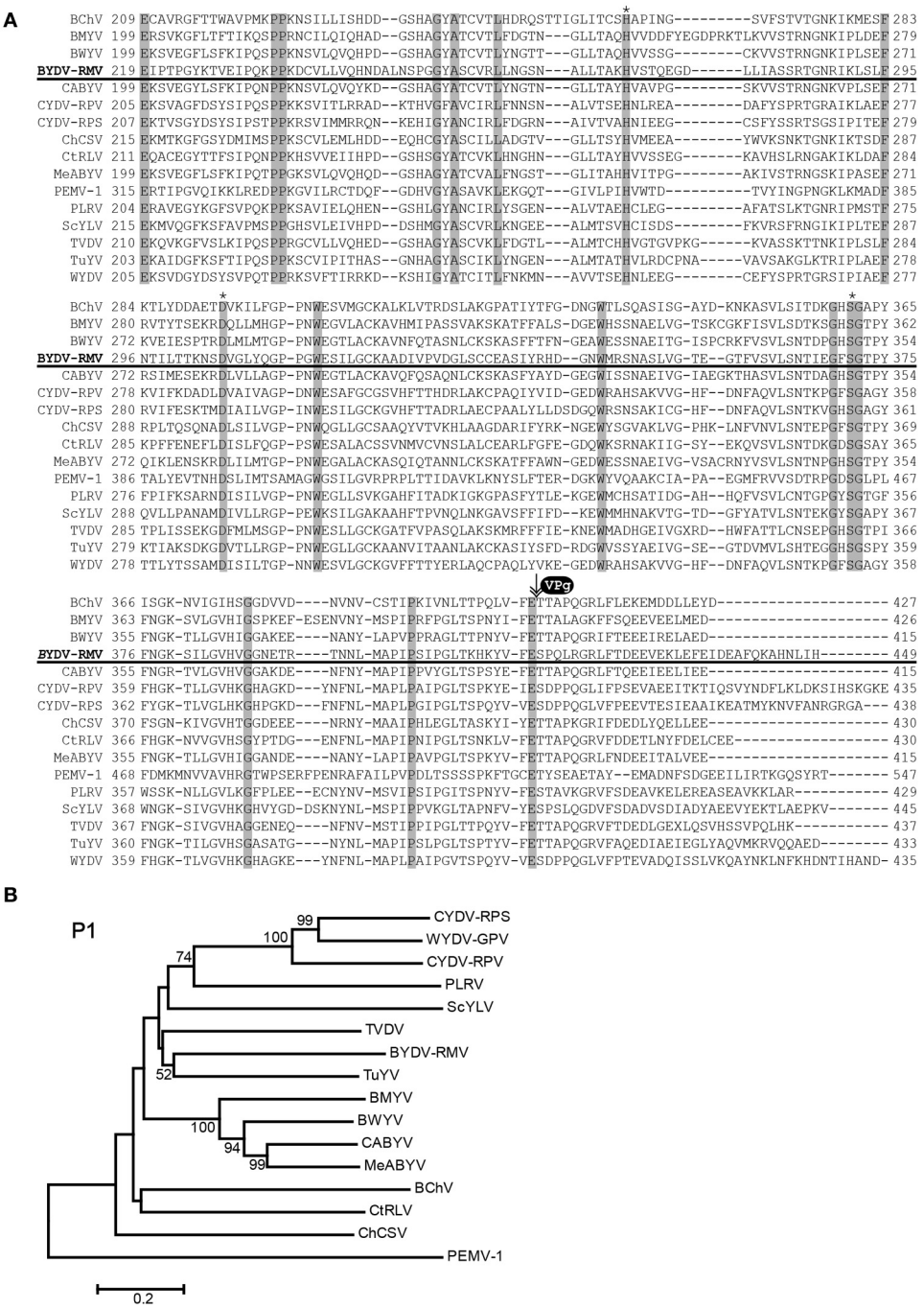

FIGURE 4 | Analysis of P1 amino acid sequences. (A) Amino acid sequences of P1 were aligned with the MUSCLE algorithm within JalView. The region with the protease domain is shown. Shaded boxes indicated conserved amino acids. Asterisks indicate amino acids of the catalytic triad in the protease active site. VPg and arrow indicate predicted proteolytic cleavage site that forms the C-terminus of the protease and the N-terminus of the VPg. **(B)** The MUSCLE aligned P1s for virus in the *Polerovirus* and *Enamovirus* genera were used for inferring evolutionary relationships using the Neighbor-Joining method. The resultant tree, drawn to scale, is shown with the percentage (greater than 50% only) of 1000 bootstrap replicates.

to suppress the host plant's defensive posttranscriptional gene silencing (PTGS) system (Pfeffer et al., 2002; Mangwende et al., 2009; Fusaro et al., 2012) by inducing the host to degrade the key *Argonaute 1* (AGO1) protein (Baumberger et al., 2007; Bortolamiol et al., 2007). The F-box domain, LPxxL/I, which is conserved in the otherwise highly variable P0 of all luteovirids including BYDV-RMV (**Figure 3B**), is required for VSR activity, as it recruits proteins to form the E3 ubiquitin ligase

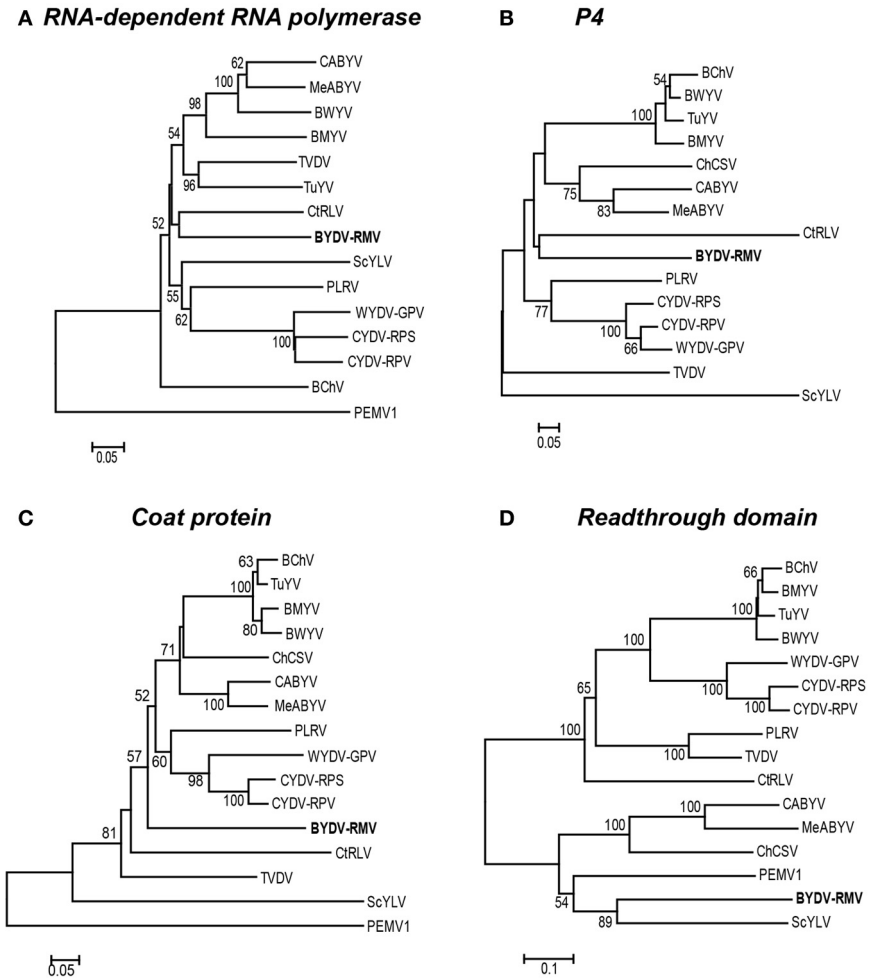

FIGURE 5 | Phylogenetic relationships of four luteoviral proteins. For each protein, the amino acid sequences were aligned using the MUSCLE algorithm in the JalView program and subsequently used to generate a Neighbor-Joining tree with the percentage of 1000 bootstrap replicates reported on a drawn-to-scale tree. Phylogenetic trees of: **(A)** RNA-dependent RNA polymerase (P2), **(B)** putative movement protein of P4, **(C)** the major coat protein (P3), and **(D)** the readthrough domain (P5) are shown.

activity that ubiquitylates AGO1, which leads to its degradation (Pazhouhandeh et al., 2006; Bortolamiol et al., 2007) by the autophagy pathway (Derrien et al., 2012). Moreover, transgenic expression of ORF0 of PLRV in potatoes, but not *Nicotiana*, is sufficient to produce virus-like symptoms (Van Der Wilk et al., 1997a). However, not all P0 proteins show VSR activity, for example those of Beet chlorosis virus (BChV) and some strains of Beet mild yellowing virus (BMYV) (Kozlowska-Makulska et al., 2010) and CYDV-RPV (Véronique Ziegler-Graff, personal communication) do not display VSR activity in standard assays. Thus, VSR function may vary depending on the virus strain–host species interaction.

The putative movement proteins of BYDV (Chay et al., 1996a,b) and PLRV (Lee et al., 2002) encoded by ORF 4 have been shown to allow host-dependent movement of the virus throughout the plant. P4 of BYDV-GAV was reported to contain an RNA-binding motif at its C-terminus with four arginine residues (Xia et al., 2008). The P4 proteins of other luteovirids were also shown to contain multiple arginine residues. In line with these observations, there are four arginine residues within the C-terminal 11 amino acids of the RMV P4 sequence.

MODULAR EVOLUTION OF *Luteoviridae*

As has been apparent since the first luteoviruses were sequenced, it is clear that luteovirid genes evolve at different rates. Note the striking lack of sequence homology among the P0 proteins (other than the F-box motif) which have around 15–23% homology to that of RMV MTFE87 (**Table 2**). This high sequence divergence of VSRs relative to other ORFs in related viruses occurs in other virus families with VSRs that act by entirely different mechanisms (Nayak et al., 2010). We speculate that VSRs are at the forefront of the evolutionary back-and-forth between virus and host immune system, which leads to rapid change, as the VSR must constantly out-evolve the host's defenses. In contrast, P2 is more conserved with 51–62% sequence identity among all of the members of the

genus *Polerovirus*. The CPs of other poleroviruses have 40–60% sequence identity to the RMV MTFE87 CP, while the overlapping P4s have significantly less similarity (**Table 2**). This suggests that base changes in the ORF 3/4 sequence that alter the meaning of ORF 4 codons are more often tolerated than those that alter the amino acid sequence of the CP. Overall, the lack of high similarity of any luteovirid ORF with those of RMV MTFE87 emphasizes its uniqueness as a virus.

CIS-ACTING SIGNALS

Cis acting sequences required for polerovirus translation, subgenomic mRNA (sgRNA) transcription and RNA synthesis have been identified in PLRV and others. The genomes of all poleroviruses begins with the sequence ACAAAA. Similarly, where known, the 5′ end of the sgRNA of the poleroviruses begins with ACAAAA (Miller and Mayo, 1991). This leads us to predict that the sgRNA required for translation of BYDV-RMV ORFs 3, 4, and 5 begins at position $_{3309}$ACAAAA$_{3314}$. This is 202 nt upstream of the CP ORF that starts at position 3516, giving an sgRNA leader sequence similar to the 212 nt leader of PLRV sgRNA1.

A 40 nt stem-loop containing a bulged adenosine, ending 3 nt upstream of the 3′ end of the genome, was shown to be required for initiation of CYDV-RPV negative strand synthesis (Osman et al., 2006). This stem-loop is conserved in CYDV-RPS and WYDV, which are very closely related to CYDV-RPV (**Figure 6A**). In contrast, RMV MTFE87 has a different predicted stem-loop that is 39 nt long with a much larger loop and a bulged guanosine (**Figure 5A**). Like all other poleroviruses (except WYDV), the two bases at the 3′ end of the RMV MTFE87 genome are GU (bold, **Figure 6A**). Thus, the first two bases incorporated by the RdRp initiating synthesis of either strand are AC. Surprisingly, WYDV is reported to contain an 11 nt A-rich tract at the 3′ end downstream of the GU (**Figure 6A**). We speculate that this is either sequence added during 3′ RACE, or sequence of a defective WYDV genome. The distinct 3′ stem-loop of RMV MTFE87 further supports that above phylogenetic comparisons about the un-CYDV-like nature of BYDV-RMV.

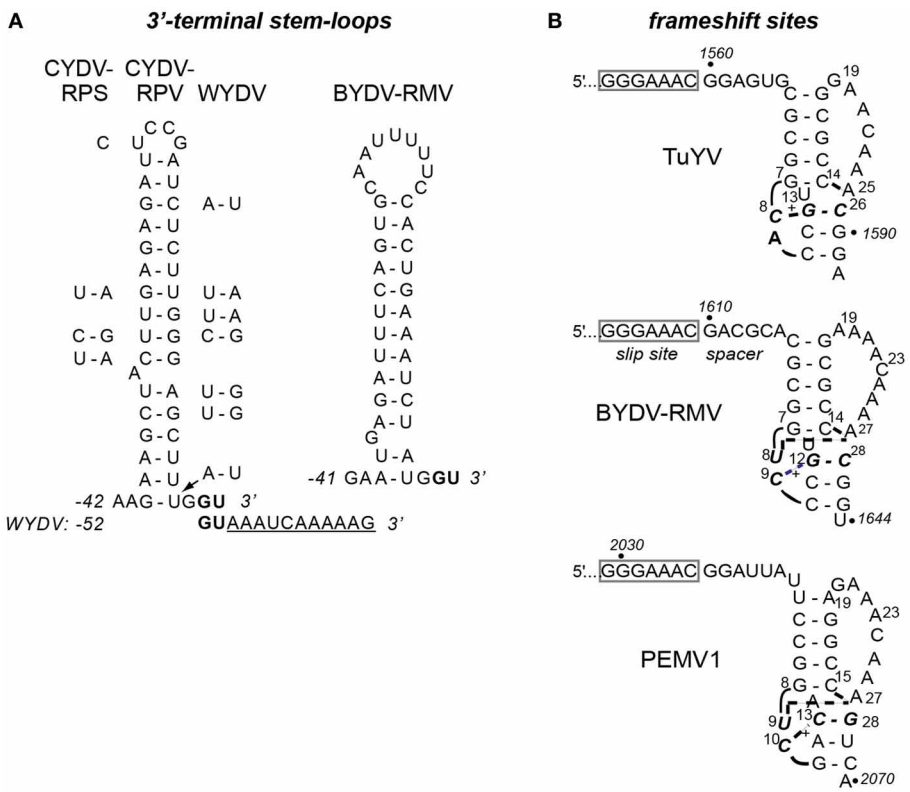

FIGURE 6 | Predicted secondary structures in BYDV-RMV RNA. (A) Stem-loop at the 3′ terminus of CYDV-RPV genome as determined by Osman et al. (2006), flanked by base differences in CYDV-RPS (left) and WYDV (right) which show covariations in base pairing that maintain secondary structure. Conserved 3′ terminal bases, GU are shown in bold. Eleven extra bases at the 3′ end of the WYDV genome, not present in any other polerovirus, are shown below the CYDV-RPV sequence in underlined text. The proposed secondary structure of the 3′ end of the BYDV-RMV genome was predicted using Mfold (Zuker, 2003). Base numbering (negative) is from the 3′ end of the genomes. **(B)** Predicted (BYDV-RMV) and known (PEMV1 and TuYV) (Su et al., 1999; Nixon et al., 2002; Miller and Giedroc, 2010) tertiary structures of pseudoknots downstream of the frameshift sites (boxed). Italics indicate base numbers in the genome. Other numbering is the position in the fragment used for nmr (except BYDV-RMV where the number allows comparison with the other structures). Short curved lines indicate phosphodiester backbone as necessary for two-dimensional rendering. Bold, dashed lines indicate non-Watson-Crick interactions between bases. $^{+}$indicated protonated cytidine that participates in base triples. Due to the recent change of the name of the BWYV isolate used in previous structural studies (Domier, 2012) it is now indicated by the new name, TuYV.

We also identified probable translational control sequences. In all *Luteoviridae*, ORF2 encoding the active site of the RdRp is translated via ribosomal frameshifting at a shifty heptanucleotide, fitting the motif XXXYYYZ (where X is any base, Y is A or U, and Z is any base except G), in the region of ORF1–ORF2 overlap. Seven nt downstream of this site, the polero- and enamovirus genomes fold into a small, compact pseudoknot that pauses the ribosome to facilitate frameshifting (Su et al., 1999; Nixon et al., 2002; Miller and Giedroc, 2010). Indeed, in the region of ORF1–ORF2 overlap in the RMV MTFE87 genome, we found the shifty heptanucleotide $_{1603}$GGGAAAC$_{1609}$, followed by a predicted pseudoknot that spans bases 1616–1643 (**Figure 6B**). This pseudoknot closely resembles those of other poleroviruses and the enamovirus, the structures of which have been determined at high resolution by NMR (Su et al., 1999; Nixon et al., 2002). The Watson–Crick helices of the RMV MTFE87 pseudoknot, CGCGG:CCGCG and CCG:CGG are identical to those in TuYV, but the helical junction region includes a predicted C+-AU triplet that resembles the junction in the PEMV1 pseudoknot (**Figure 6B**).

The sequence required for translational readthrough of the CP ORF stop codon also resembles those of other *Luteoviridae*. Readthrough of the BYDV-PAV CP ORF stop codon was shown to require at least five repeats of the sequence CCXXXX, where X is any base, beginning about 16–22 nt downstream of the stop codon (Brown et al., 1996). Indeed, in the RMV MTFE87 genome, a tract of 12 CCXXXX repeats starting at nt 4131 begins 16 nt downstream of the CP ORF stop codon (**Figure 1**). This codes for an amino acid sequence of alternating proline residues, which is a likely spacer to permit separate folding of the CP and RTD protein domains. In summary, BYDV-RMV has all the known cis-acting signals of a polerovirus to control translation of viral proteins.

PROPOSED NAME CHANGE OF BYDV-RMV TO MAIZE YELLOW DWARF VIRUS-RMV

The RMV MTFE87 sequence shows that the viruses once called BYDV are even more diverse than previously thought. The sequence also shows clearly that BYDV-RMV is not in genus *Luteovirus*, to which all other sequenced viruses currently called BYDV are assigned. Nor is it a type of CYDV or WYDV. Therefore, we propose to change the name of BYDV-RMV to Maize yellow dwarf virus-RMV (MYDV-RMV) (Miller et al., 2013). This name (1) acknowledges that the virus is clearly a new species (2) is consistent with observations that BYDV-RMV often infects maize (Brown et al., 1984; Beuve et al., 1999; Itnyre et al., 1999a,b), (3) retains the RMV notation for the predominant vector, *R. maidis* (although *S. graminum* and *R. padi* can also be efficient vectors, particularly in the western United States), and (4) retains the YDV descriptor long used for luteovirids that infect cereals.

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Life cycle of phytoreoviruses visualized by electron microscopy and tomography

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Rice dwarf virus and *Rice gall dwarf virus*, members of the genus *Phytoreovirus* in the family *Reoviridae*, are known as agents of rice disease, because their spread results in substantial economic damage in many Asian countries. These viruses are transmitted via insect vectors, and they multiply both in the plants and in the insect vectors. Structural information about the viruses and their interactions with cellular components in the life cycle are essential for understanding viral infection and replication mechanisms. The life cycle of the viruses involves various cellular events such as cell entry, synthesis of viral genome and proteins, assembly of viral components, viral egress from infected cells, and intra- and intercellular transports. This review focuses on the major events underlying the life cycle of phytoreoviruses, which has been visualized by various electron microscopy (EM) imaging techniques, including cryo-electron microscopy and tomography, and demonstrates the advantage of the advanced EM imaging techniques to investigate the viral infection and replication mechanisms.

Keywords: electron microscopy, electron tomography, virus structure, *Rice dwarf virus*, *Rice gall dwarf virus*

INTRODUCTION

The life cycle of viruses involves various cellular events including cell entry, synthesis of viral genome and proteins, assembly of viral components, and viral egress from infected cells. Structural information about viruses and their interactions with cellular components are essential for understanding viral infection and replication mechanisms. Transmission electron microscopes are commonly used to obtain two-dimensional (2D) projection images from thin sections of resin-embedded virus-infected cells, which enable visualization of viral and virus-related structures, as well as the virus localization within host cells. Thus, conventional electron microscopy (EM) has long been used to provide valuable insights into virus–host interactions and the life cycle of viruses inside host cells. However, projection causes overlap in the features, resulting in the intrinsic ambiguities in the interpretation of the 2D projection images of three-dimensional (3D) objects. Electron tomography (ET) is used to generate 3D structures from 2D EM images projected in various directions. Thus, the overlaps that appear in the 2D projection images can be resolved in the reconstructed 3D structures, thereby enabling effective and precise analysis of interactions between a virus and host cell. In this review, we describe the life cycle of phytoreoviruses as revealed by various EM imaging techniques, including cryo-electron microscopy (cryo-EM) and ET, and immunofluorescence microscopy and immunoelectron microscopy. Phytoreoviruses were chosen for this review because they are the most widely studied of all plant viruses that are currently threatening the stable production of cereal crops (Omura and Mertens, 2005).

Rice dwarf virus (RDV), *Rice gall dwarf virus* (RGDV), and *Wound tumor virus* are members of the genus *Phytoreovirus* in the family *Reoviridae*. These viruses are icosahedral double-shelled particles with an average diameter of approximately 70 nm (Takahashi et al., 1994; Miyazaki et al., 2005), and they are known as serious agents of rice diseases that cause economic damage in many Asian countries. Phytoreoviruses are transmitted to rice plants via insect vectors, and they multiply both in the plants and in the invertebrate insect vectors. RDV is the most well-characterized virus among the three phytoreoviruses. The capsid structure of RDV was previously determined at a resolution of 3.5 Å using X-ray crystallography (Nakagawa et al., 2003), and the structural organization of the capsid shell was characterized by cryo-electron single-particle analysis (Miyazaki et al., 2010). RDV has a 12-segmented, double-stranded RNA (dsRNA) genome encoding 12 viral proteins (**Table 1**), and the RDV virion is composed of seven structural proteins (P1, P2, P3, P5, P7, P8, and P9). P3 forms the inner capsid shell, which encloses the viral genome, and P1, P5, and P7 are involved in transcription (Hagiwara et al., 2003, 2004; Nakagawa et al., 2003; Miyazaki et al., 2010). The inner capsid shell is surrounded by the outer capsid shell, which is composed of P2, P8, and P9 (Omura et al., 1989; Omura and Yan, 1999; Zhong et al., 2003). Five non-structural proteins (Pns4, Pns6, Pns10, Pns11, and Pns12) are associated with the replication cycle of RDV within host cells, which involves synthesis of the viral genome and proteins, assembly of progeny viruses, and intercellular movement, among other processes. Three non-structural proteins Pns6, Pns11, and Pns12 are the constituents of viroplasms. Viroplasms are the viral inclusion bodies appearing in the cytoplasm of RDV-infected cells, which is believed to be the primary site of virus replication and assembly (Wei et al., 2006c). RDV Pns4 is a phosphoprotein localized around the viroplasms and is known to form bundles of minitubules at later stages of

Table 1 | Viral proteins of *Rice dwarf virus*.

Protein	Molecular weight (kDa)	Location	Function
P1	164	Structural, inside of the capsid shell	RNA polymerase
P2	127	Structural, outer capsid	Vector transmissibility
P3	114	Structural, inner capsid	Core capsid
Pns4	83	Non-structural	Unknown
P5	91	Structural, inside of the capsid shell	Guanylyltransferase
Pns6	56	Non-structural	Nucleic acids binding, viroplasm matrix protein in insect vector cells, cell-to-cell movement in plants
P7	55	Structural, inside of the capsid shell	RNA-binding
P8	46	Structural, outer capsid	Outer capsid
P9	39, 30	Structural, outer capsid	Unknown
Pns10	35	Non-structural	RNA silencing suppressor in plants, intercellular transport in insect vector cells
Pns11	23	Non-structural	Viroplasm matrix protein, nucleic acids binding
Pns12	34	Non-structural	Viroplasm matrix protein

infection; however, its function remains to be clarified (Wei et al., 2006b). RDV Pns10 forms tubular structures containing virus particles, and it is directly involved in the intercellular spread of RDV among insect vector cells. These observations have shown that the life cycle of RDV is tightly controlled by these non-structural proteins, which facilitates efficient virus proliferation in host cells.

VIRUS ENTRY

Initiation of a successful viral infection and replication cycle requires viral attachment to specific molecules on the surface of host cells, with subsequent entry into the host cells for delivering the viral genome and proteins required for replication. The steps involved in virus entry into host cells have recently become the most widely studied aspect of the life cycle of animal viruses, including human pathogenic viruses. Animal viruses exploit various endocytosis pathways, including clathrin-mediated endocytosis, caveola-mediated endocytosis, macropinocytosis, and phagocytosis, to enter the host cell cytoplasm. On the other hand, plant viruses are believed to enter plant cells through a wound on plants, although the cell entry pathways of plant viruses remain unclear owing to the lack of a suitable experimental system.

Rice dwarf virus is transmitted to plants by vector insects, primarily the green rice leafhopper, in a persistent-propagative manner. The vector insect acquires the virus by feeding on virus-infected plants. After ingestion by leafhoppers, the viruses first accumulate in the epithelial cells of the filter chamber in the alimentary canal of the leafhoppers, which suggests that the microvillar membrane of the filter chamber might contain abundant cellular receptors for viral attachment and entry (Chen et al., 2011). RDV proliferates in the vector insect, and the insect becomes RDV-infected after a latent period of approximately 2 weeks. Subsequently, RDV is transmitted to plants by the viruliferous vectors, most likely via a wound caused by feeding of the insects on the plants. The minor outer capsid protein P2 is essential for RDV infection to the vector insects (Yan et al., 1996; Tomaru et al., 1997; Omura et al., 1998). Viral particles containing P2 on the outer capsid layers can infect insect vectors through direct injection and by feeding insects through a membrane, whereas viral particles without P2 can infect insect vectors only through direct injection. Furthermore, non-sense mutations in the P2 gene inhibit transmission of the virus from infected plants to insect vectors (Pu et al., 2011), and the P2 protein induces membrane fusion in insect host cells (Zhou et al., 2007). These evidences suggest that P2 attaches to undefined receptors on insect vector cells and mediates viral cell entry (Omura et al., 1998).

Established monolayer cultures of leafhopper vector cells (NC24 cells), originally derived from embryonic fragments dissected from eggs of *Nephotettix cincticeps*, enable further investigation of the results of earlier studies on RDV infection and the cytopathology of infected cells. The vector cells in monolayers (VCMs) were inoculated with RDV, fixed at different post-inoculation (p.i.) times, and then examined by EM to analyze the cell entry pathway. These investigations revealed that RDV entry into the insect vector cells occurred via a clathrin-mediated endocytosis pathway (**Figure 1**; Wei et al., 2007). The cell entry process via coated pits was further confirmed by the significant reduction in RDV infectivity after the cells were treated with drugs that block receptor-mediated or clathrin-mediated endocytosis (Wei et al., 2007). The EM investigations also showed that RDV within endocytotic vesicles remained as intact double-layered particles. This process differs from that of animal reoviruses such as rotavirus, bluetongue virus, and mammalian reovirus, in which cell entry involves a series of molecular transformations in the outermost layer of proteins, which strips these proteins from the virion and delivers the inner capsid particle in a transcriptionally active form into the cytosol.

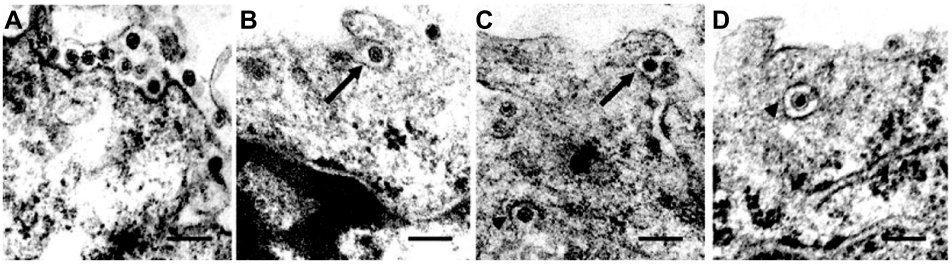

FIGURE 1 | Cell entry of *Rice dwarf virus* (RDV) via the clathrin-mediated endocytosis pathway. (A) Attachment of RDV particles to the plasma membranes of vector cell monolayers (VCMs) 30 min p.i. **(B,C)** Uptake of RDV particles in coated pits (arrows) 1 h p.i. **(C,D)** RDV particles internalized within coated pits (arrowheads) 1 h p.i. Reproduced with permission from Wei et al. (2007). Scale bars, 200 nm.

VIRUS REPLICATION AND ASSEMBLY

After cell entry, viruses initiate their replication processes. Cytoplasmic viral inclusions, known as viroplasms, viral factories, or viral inclusion bodies, commonly appear as electron-dense structures in the cytoplasm of cells infected by members of the family *Reoviridae*, and these are therefore assumed to be the site of virus replication (**Figures 2A,B**). RDV viroplasms are formed by three major constituents: the non-structural proteins Pns6, Pns11, and Pns12 (Wei et al., 2006c). Pns12 is essential for the formation of viroplasms; it was shown to possess an intrinsic ability to form aggregates (viroplasm-like structures) when expressed in the absence of any other viral proteins in *Spodoptera frugiperda* (Sf9) cells (non-hosts of RDV). On the other hand, Pns6 and Pns11 are distributed diffusely throughout the cytoplasm, and neither Pns6 nor Pns11 forms aggregates when expressed alone in Sf9 cells. Furthermore, transgenic rice plants expressing proteins that interfere with Pns12 expression were found to be strongly resistant to RDV infection (Shimizu et al., 2009). These observations strongly suggested that viroplasm matrix proteins and viroplasm formation play important roles in viral replication and morphogenesis (Isogai et al., 1998).

To date, two crystal structures (NSP2 of rotavirus in the genus *Rotavirus* and P9-1 of *Rice black streaked dwarf virus* (RBSDV) of the genus *Fijivirus*) and one cryo-EM structure (Pns9 in the genus *Phytoreovirus*) have been identified in the viroplasm matrix proteins of viruses in the family *Reoviridae* (Jayaram et al., 2002; Akita et al., 2011, 2012). The RGDV Pns9 is homologous to RDV Pns12, with only 16% identity between amino acid sequences. These matrix proteins form similar octameric structures, despite the lack of significant similarities between the respective primary and secondary structures or between domain foldings. The similarities of octamer and large aggregate formations among reoviruses imply that the structure of each octamer and its interior pore is crucial for the proper functioning of respective proteins in the viroplasm, for example, in viral morphogenesis. Furthermore, octamerization of RBSDV P9-1 is required for the formation of the matrix of viral inclusions in the cell (Akita et al., 2012). The rotavirus non-structural protein NSP2 is the best-characterized matrix protein in the family *Reoviridae*, and it forms viroplasms in the presence of another non-structural rotavirus protein, NSP5 (Fabbretti et al., 1999; Eichwald et al., 2004). Several biochemical properties of NSP2 have been identified, including RNA-binding, helix-destabilizing, nucleotide triphosphatase, 5′-RNA triphosphatase, nucleoside diphosphate kinase, and core protein-binding activities. Structural analysis by cryo-EM has further revealed that these activities are performed inside a cleft between the two domains of NSP2 and that NSP5 regulates the binding of NSP2 to RNA (Jiang et al., 2006). These findings suggest that NSP2 contributes to packaging and replication of the viral genome by relaxing the secondary structures that impede polymerase function and by facilitating the translocation of viral RNAs into progeny core particles (Jiang et al., 2006).

In addition to Pns12 of RDV, Pns6 and Pns11 are constituents of viroplasms, although neither protein has aggregate-forming ability. Pns6 has an RNA-binding motif, and although it is involved in intercellular movement at plasmodesmata in plants, its function in viroplasms remains unclear. Pns6 binds preferentially to single-stranded RNAs derived from the consensus 5′- and 3′-terminal sequences of the RNA genome as described below, and it is considered to form ribonucleoprotein complexes to transport the viral genome between plant cells. Pns11 is also a nucleic acid-binding protein (Xu et al., 1998), and newly synthesized viral RNAs accumulate within viroplasms. These RNA-binding activities and colocalization within the viroplasm suggest that Pns6 and/or Pns11 might be recruited to viral inclusions through their association with Pns12, thereby indicating that Pns6 and Pns11 might play an important role in viral RNA transport to the viroplasms or in viral RNA synthesis and replication in the viroplasms (Akita et al., 2012).

Virion components are also accumulated and sorted in and around the viroplasms, suitable for the assembly of progeny viral particles. Components for the viral core particles (P1, P3, P5, and P7 proteins) are located in the interior region of viroplasms, and progeny core particles have been observed within the viroplasms. In contrast to genomic RNAs and core component proteins, accumulation of the outer capsid proteins P2, P8, and P9 is evident in the peripheral regions of the viroplasms, and intact double-layered viral particles are commonly observed around the viroplasms, which are considered newly synthesized progeny viruses. These observations suggest that core particles are constructed inside the inclusions, whereas outer capsid proteins are assembled at the periphery of the inclusions. This hierarchical assembly model of the viral particle coincides with results by structural analysis of the capsid proteins at atomic resolutions and by

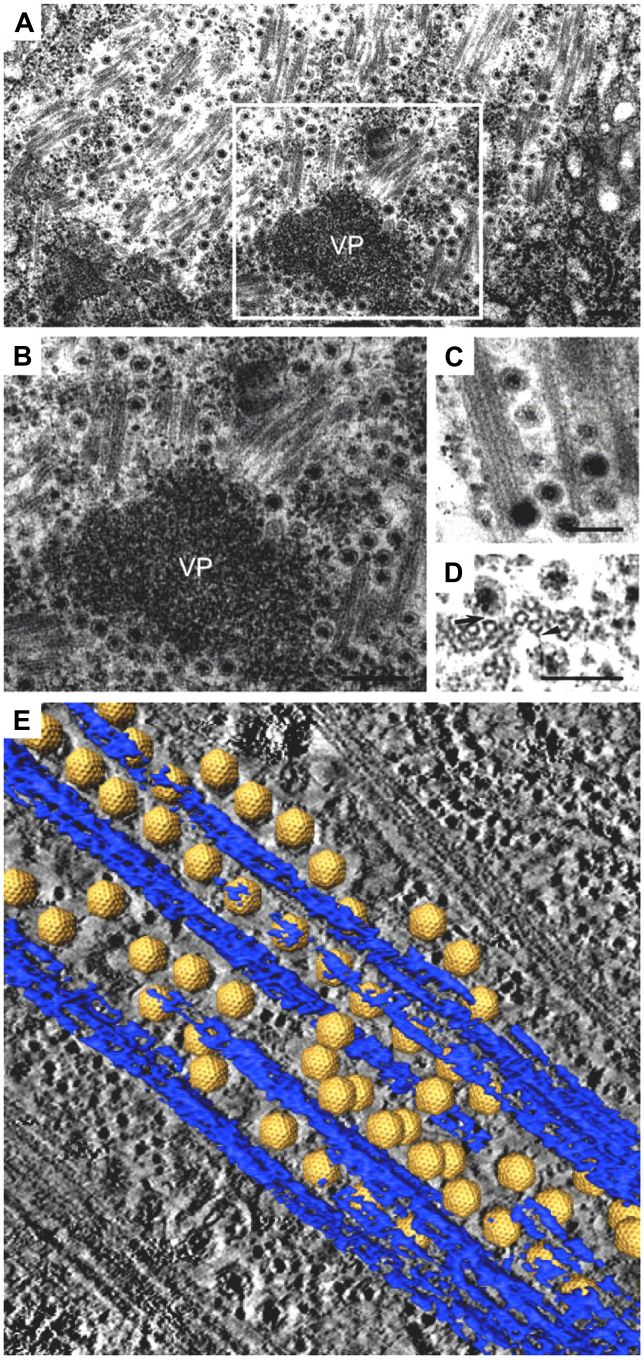

FIGURE 2 | Intracellular trafficking of *Rice gall dwarf virus* (RGDV) particles. (A) RGDV particles associated with microtubules in virus-infected VCMs 48 h p.i. Scale bar, 300 nm. Viroplasm (VP), an electron-dense inclusion, is also seen in the electron micrograph. **(B)** Enlarged view of the boxed region in **(A)**. Scale bar, 300 nm. **(C)** Viral particles along the edges of tubules, approximately 25 nm in diameter. Scale bar, 150 nm. **(D)** Transverse sections of arrays of closed circles of approximately 25 nm in diameter with viral particles directly attached to their circumference (arrow) or via a filament (arrowhead). Scale bar, 150 nm. **(E)** Three-dimensional surface-rendered model showing the association of RGDV particles (yellow) with microtubules (blue). Reproduced with permission from Wei et al. (2009b).

biochemical experiments (Nakagawa et al., 2003; Hagiwara et al., 2004; Miyazaki et al., 2005).

INTRACELLULAR TRANSPORT OF VIRIONS

After multiplication of the viruses at the replication sites, progeny viruses move to the periphery of cells to be released from viroplasms. Various animal viruses have been shown to utilize cytoskeletal motor proteins for their intracellular movements (reviewed in Sodeik, 2000; Döhner et al., 2005; Greber and Way, 2006; Radtke et al., 2006). Motor proteins mediate intracellular transport along cytoskeletal filaments (actin fibers and microtubules). Microfilament motor proteins such as myosin move along the filaments through interactions with actin molecules, whereas microtubule motor proteins such as dynein and kinesin move along the microtubules through interactions with α- and β-tubulin molecules. Dynein complexes are much larger and more complex than kinesin and myosin, and the complexes move to the minus-end of the microtubules (retrograde). Kinesins typically contain two heavy chains with motor heads that move along microtubules either toward the plus-end or toward the minus-end, depending on the specific type of kinesin involved.

The association of RGDV particles with microtubules can be seen clearly in EM images, and the 3D structures can be analyzed with ET (**Figure 2**; Wei et al., 2009b). Although RGDV particles are aligned on microtubules, they do not directly attach to the microtubules. Therefore, a gap between viral particles and microtubules is present, and a rod-like structure can be seen in the gap, which is considered a motor protein for the transportation of viral particles. Furthermore, depolymerization of microtubules using the pharmacological drugs nocodazole and colchicine resulted in decreasing the number of viruses released from the infected cells to 1/5th that released from untreated cells without significantly reducing the production of cell-associated viruses. These results suggested that the microtubule motor protein is involved in the transport of viral particles from their replication site to the cell surface for the viral egress from the infected cell, but it is not involved in transport from the cell surface to the replication site during cell entry. Based on its direction of movement to the cell surface, the rod-like structure appears to be a plus-end-directed (anterograde) kinesin motor protein. Kinesin-1 is the only anterograde microtubule motor known to be involved in the intracellular transport of viruses to date (Rietdorf et al., 2001; Jouvenet et al., 2004; Greber and Way, 2006). The size of the connecting density between RGDV particles and microtubules also supports the involvement of kinesin rather than the relatively bulky dynein complex (Iwasaki and Omura, 2010). This cytosolic transport system is similar to those of some animal viruses. For example, in the case of murine polyomavirus, EM observations revealed associations of viral particles with the free end and the lateral sides of microtubules (Sanjuan et al., 2003), which indicated that intracellular transport was mediated by the interaction between viral particles and microtubules. This was confirmed by the observation that microtubule depolymerization prevented polyomavirus migration both toward the nucleus and from the nucleus to the cell surface.

INTERCELLULAR MOVEMENT IN INSECT VECTORS

Phytoreoviruses multiply both in plants and in vector insects, but utilize different strategies for spreading in the two hosts. In insects, the non-structural protein Pns10 is involved in the intercellular movement of RDV. Pns10 forms tubular structures enclosing viral particles. RDV containing tubular structures in association with the microvilli of the midgut in viruliferous vector insects are frequently observed in EM analysis (Nasu, 1965; Chen et al., 2012). An established cell culture system and immunolabeling for viral gene products enabled further detailed analysis of the intercellular movement of RDV that exploits the unique tubular structure. In the cultured insect cells, Pns10 tubules extended from the infected cell and colocalized with actin filaments in the filopodia. Direct interaction of Pns10 with actin molecules was detected by surface plasmon resonance, and actin-depolymerizing drugs suppressed the extension of Pns10 tubules from the cell surface (Wei et al., 2006a). These results suggested that viral spread into neighboring cells occurred by direct cell-to-cell contact via filopodia, without diffusion through the extracellular environment, as evidenced in animal viruses such as African swine fever virus (Jouvenet et al., 2006) and murine leukemia virus (Lehmann et al., 2005; Sattentau, 2008). These results were further confirmed using virus-neutralizing antibodies. When virus-neutralizing antibodies were added to the medium, they captured cell-free viruses and strongly inhibited virus spreading through the extracellular environment (Wei et al., 2006a). However, RDV could spread directly from the initially infected cell to adjacent cells, even in the presence of virus-neutralizing antibodies.

The 3D structure of the association between Pns10 tubules and RDV particles in the filopodia was determined by ET (Katayama et al., 2007; **Figure 3**). The inner diameter of the Pns10 tubule corresponds very well with the maximum diameter of the RDV particle (75 nm), and the virus particles are tightly packaged inside the Pns10 tubule. The tightly packed virus particles did not appear to diffuse inside the Pns10 tubule freely. Furthermore, the tip of the short tubules protruding from the surface was always filled with virus particles. If RDV particles are loaded inside Pns10 tubules after formation of the tubular structures, empty Pns10 tubules should be observed, and packed viral particles should be more scattered toward the tip of the tubules. These observations suggest that tubule extension might be mechanically linked with virus loading. After formation of the virus-containing tubule structure, it becomes associated with the actin filaments and is then transferred to neighboring cells through interactions with actin molecules in the filopodia. When the virus-containing tubule structure surrounded by cellular membrane reaches the neighboring cells, membrane fusion between the two cells is required for the intercellular transport of RDV. Although the mechanism has been unknown, but P2 protein of RDV may be needed for membrane fusion for RDV to enter the neighboring cells, because the P2 protein is a membrane fusion protein (Zhou et al., 2007).

INTERCELLULAR MOVEMENT IN PLANTS

The intercellular movement of viruses in plants is completely different from that in animals; plant viruses move to adjacent cells via plasmodesmata. The plasmodesma is an intercellular junction unique to plants, and it directly connects the cytoplasms of adjacent cells that are separated by a rigid and thick cell wall. Plasmodesmata allow the passage of molecules with molecular mass less than ∼800 Da and mediate intercellular communication across the thick cell wall. Plant viruses exploit this intercellular communication pathway to enable their own spread within the infected plant. In order to facilitate the cell-to-cell movement through plasmodesmata, many plant viruses encode specific movement proteins that can modify the plasmodesmatal size-exclusion limit (Wolf et al., 1989) or the plasmodesmatal structure, e.g., tubule formation inside the plasmodesma (Laporte et al., 2003). Virion or viral ribonucleoprotein complexes can be transported through the modified plasmodesmata into adjacent cells. In the case of RDV in vector insects, Pns10 was found to be involved in the intercellular movement between neighboring insect cells through formation of tubular structures, as described above (Wei et al., 2006a). On the other hand, in plants, Pns10 acts as an RNA silencing suppressor (Cao et al., 2005; Ren et al., 2010; Zhou et al., 2010), and it is not essential for multiplication (Pu et al., 2011).

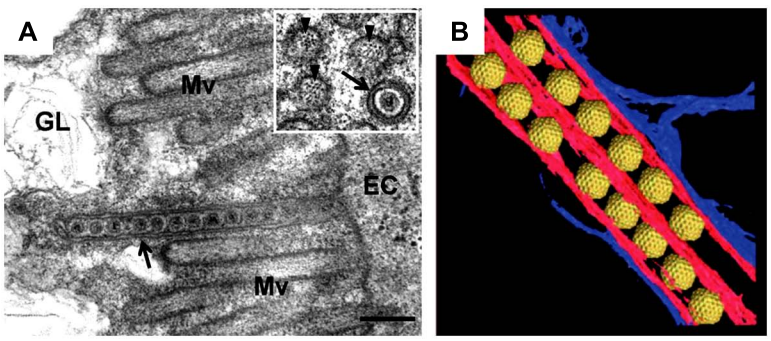

FIGURE 3 | Intercellular transport of *Rice dwarf virus* (RDV) in insect vector cells exploiting virus-induced Pns10 tubular structures. **(A)** Virus-containing Pns10 tubules associated with microvilli of anterior midgut in viruliferous leafhoppers. Inset, transverse section of microvillus containing a virus-packed Pns10 tubule approximately 100 nm in diameter. EC, epithelial cell; GL, gut lumen; Mv, microvilli. Scale bar, 200 nm. **(B)** Three-dimensional surface-rendered model showing RDV particles (yellow) surrounded by Pns10 tubules (red) and the plasma membrane (blue). Reproduced with permission from Katayama et al. (2007) and Chen et al. (2012).

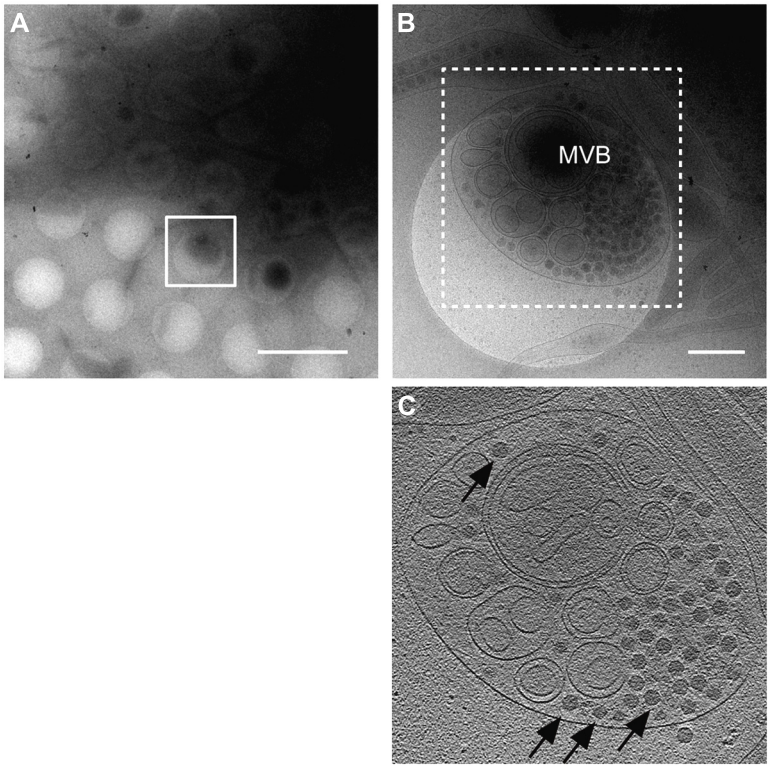

FIGURE 4 | Virus egress from infected insect cells. Cryo-electron microscopy (cryo-EM) images of a RDV-infected insect cell cultured on the EM grid. **(A)** Low magnification cryo-EM image of the RDV-infected insect cell cultured on the EM grid. Scale bar, 2 μm. **(B)** High magnification cryo-EM image of the boxed region in **(A)**. Scale bar, 400 nm. **(C)** A slice through the reconstructed tomographic volume obtained from the area highlighted in **(B)**. Black arrows indicate some of the RDV particles within the multi-vesicular body (MVB). Reproduced with permission from Miyazaki et al. (2013).

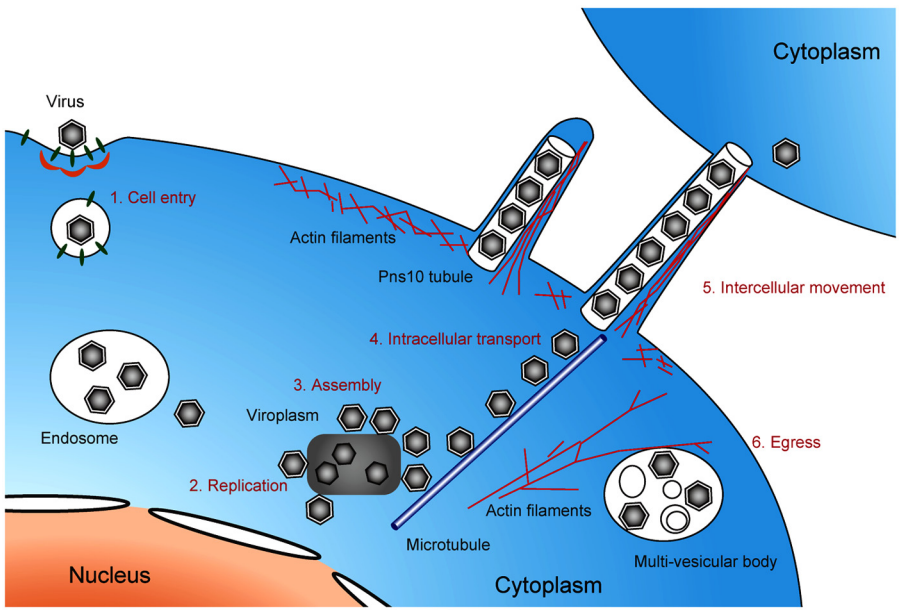

FIGURE 5 | Life cycle of pytoreoviruses in the host cell. A schematic illustration showing the life cycle of phytoreoviruses in the host insect cell, involving viral cell entry via clathrin-mediated endocytosis (1), replication of viral proteins and genome (2), assembly of progeny viruses in and around viroplasm inclusions (3), microtubule-associated intracellular transport (4), intercellular movement exploiting Pns10 tubule (5), and viral egress via multi-vesicular bodies (6).

Instead, Pns6 is primarily responsible for the movement of RDV between plant cells (Li et al., 2004; Ji et al., 2011).

Pns6 preferentially binds to single-stranded RNAs derived from the consensus 5′- and 3′-terminal sequences of the RDV genome and to non-specific dsRNA. The sense-strand RNAs from all genome segments of RDV contain the conserved 5′- and 3′-terminal sequences, 5′-GGGAAA— or 5′-GGUAAA— and —UGAU-3′ or —CGAU-3′, respectively (Kudo et al., 1991). Many other reoviruses have similar specific consensus sequences at the ends of each segment of their genomic dsRNAs, which implies functional significance and their possible biological roles, although these remain to be clarified. Pns6 is localized to the plasmodesmata, whereas the RDV virion is not found in the plasmodesmata. In addition, since the size of the RDV virion is much larger than the pore size of plasmodesmata, RDV is considered to move between plant cells in the form of viral ribonucleoprotein complexes containing Pns6 proteins. Moreover, Pns6 has ATPase activity, similar to other movement proteins found in numerous plant viruses (Peremyslov et al., 1999). The ATPase activity of the movement proteins is thought to be necessary to provide the driving force to traffic viral RNA through plasmodesmata or to suppress RNA silencing (Howard et al., 2004; Bayne et al., 2005). However, the molecular mechanism underlying the function of intercellular movement proteins remains unclear, and it should be investigated in future studies using EM imaging tools.

EGRESS

Viruses must be released from infected cells for their successful spreading. In the case of RDV, multiple pathways have been reported for viral egress from infected insect cells without cell lysis. One of the virus-release pathways involves secretory exosomes derived from multi-vesicular bodies (MVBs; Wei et al., 2008b, 2009a). Virus-containing MVBs are frequently observed in the peripheral region of the viroplasms as well as near the cytoplasmic membrane. Analyses using organelle-specific markers showed that the virus-containing vesicles are late-endosomes or lysosomes. Furthermore, actin filaments and myosin motors have been shown to affect the morphology and motility of virus-containing MVBs in infected cells (Wei et al., 2008a). Based on these results, the following RDV release pathway was proposed. Newly synthesized viral particles, which assemble at the periphery of the viroplasm, are engulfed by MVBs and moved along the cytoskeletal actin filaments to the periphery of cells by myosin motors. At the periphery, these vesicular bodies fuse with the plasma membrane in an exocytic manner to release viral particles out of the cell, thereby enabling spreading. However, further analysis is required to confirm this pathway. Virus spreading from infected insect vector cells through the virus-release pathway via exosomes and the intercellular transporting pathway using Pns10 tubular structures causes less damage to the host insect cells than virus spreading involving cell lysis does (e.g., egress of many other non-enveloped viruses). Therefore, RDV appears to use the host cell machinery to replicate itself, but it does not interfere with the vitality of the host organism, thus ensuring the survival of the vector insect. The survival of the vector insect increases the opportunity for RDV to be transmitted to plants, which widens the scope of viral spread and increases viral survival.

APPLICATIONS OF CRYO-ELECTRON TOMOGRAPHY FOR VISUALIZING VIRAL INFECTION AND REPLICATION MECHANISMS

To understand the life cycle of a virus, it is necessary to conduct a detailed investigation of the viral and virus-related structures and to analyze their molecular interactions within a cellular context. cryo-EM and cryo-electron tomography (cryo-ET) can be used to visualize fully hydrated cells in a close-to-native state at molecular resolutions, which allows analysis of molecular interactions within a cell in more detail than that using conventional EM and ET (Robinson et al., 2007). Recently, cryo-EM/ET has been applied to visualize some animal viruses and bacteriophages within their host cells. For example, morphological changes of the vaccinia virus before and after intrusion into the host cells (Cyrklaff et al., 2007) and cell entry and intracellular trafficking of the herpes simplex virus were captured by cryo-EM/ET (Maurer et al., 2008; Ibiricu et al., 2011). We also attempted to apply the cryo-EM/ET method in the RDV study (Miyazaki et al., 2013). NC24 cells were cultivated and infected with RDV on the EM grid, frozen in liquid ethane, and then embedded in vitreous ice. When the cells were examined by cryo-EM, RDV particles within multi-vesicular compartments at the edge of the cell were clearly visible (**Figure 4**). These results demonstrate the success of approaches using cryo-EM/ET techniques for observing the viral and virus-related structures within cells and for analyzing the viral life cycle around the peripheries of infected cells. In the near future, advanced cryo-EM/ET methods will be applied to not only animal viruses, but also to various plant viruses, which will extend our understanding of fundamental biological processes of viruses.

CONCLUSION

Electron microscopy imaging techniques, including the cryo-ET approach described herein, have been used for high-resolution analysis of viral and virus-related structures within cells. This review describes the major cellular events occurring in the life cycle of phytoreoviruses, the main agents of rice diseases, which were primarily elucidated through EM imaging (**Figure 5**). However, EM observations could be enhanced with other complementary methods that allow observations of dynamic events, such as confocal laser scanning microscopy, which would provide more information. Furthermore, applications of the advanced cryo-EM/ET technology to virus research have enabled more detailed investigations of viral infection and replication mechanisms. We believe that in the near future, further use of these approaches will reveal the molecular mechanisms underlying infection and replication of viruses that are currently threatening the stable production of cereal crops and will further extend our understanding of these viruses.

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Rice gall dwarf virus exploits tubules to facilitate viral spread among cultured insect vector cells derived from leafhopper *Recilia dorsalis*

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Rice gall dwarf virus (RGDV), a member of the family *Reoviridae*, causes repeated epidemics in rice fields in southern China. An RGDV isolate collected from Guangdong Province (southern China) is mainly transmitted by leafhopper vector *Recilia dorsalis* in a persistent-propagative manner. The infection by RGDV induces the formation of virus-containing tubules in the plant host and insect vector. In this study, we established continuous cell cultures of the leafhopper *R. dorsalis* to investigate the functional role of these tubules within the insect vector. Cytopathologic studies revealed that the tubules, which comprised viral non-structural protein Pns11 and contained viral particles, were able to protrude from the surface of cultured leafhopper cells. Tubule-like structures formed in non-host insect cells after the expression of Pns11 in a baculovirus system, suggesting that Pns11 was the minimal viral factor required for the formation of the tubules induced by RGDV infection. In cultured leafhopper cells, knockdown of Pns11 expression from RNA interference, induced by synthesized dsRNA from the Pns11 gene, abolished the formation of such tubules, preventing the direct cell-to-cell spread of RGDV without significant effects on viral multiplication. All these results show that RGDV exploits virus-containing tubules to facilitate viral spread among its insect vector cells.

Keywords: rice gall dwarf virus, leafhopper vector *Recilia dorsalis*, tubules, viral spread, continuous cell cultures of leafhopper

INTRODUCTION

Rice gall dwarf virus (RGDV), *Rice dwarf virus* (RDV) and *Wound tumor virus*, members of the genus *Phytoreovirus* in the family *Reoviridae*, are transmitted propagatively to plants by leafhopper vectors (Boccardo and Milne, 1984). Rice gall dwarf disease induced by RGDV was first recorded in Thailand in 1979 and was later discovered in China and Southeast Asia (Omura et al., 1980; Putta et al., 1980; Ong and Omura, 1982; Fan et al., 1983). Since 2000, the disease has repeatedly affected rice fields in southern China (Zhang et al., 2008). RGDV from Guangdong Province in southern China is mainly transmitted by a leafhopper vector, *Recilia dorsalis*, in a persistent-propagative manner (Fan et al., 1983). By 2012, the disease had spread to other areas of Guangdong Province, and the leafhopper vector *R. dorsalis* developed a higher affinity with RGDV. RGDV thus has the potential to become one of the greatest threats to rice production in these regions.

RGDV is an icosahedral double-layer particle approximately 65–70 nm in diameter, with a 12-segmented dsRNA genome (Moriyasu et al., 2000, 2007; Miyazaki et al., 2005; Zhang et al., 2008). Six segments (S1, S2, S3, S5, S6, and S8) encode structural proteins (P1, P2, P3, P5, P6, and P8), and the remainder encode non-structural proteins (Pns4, Pns7, Pns9, Pns10, Pns11, and Pns12) (Moriyasu et al., 2000, 2007; Zhang et al., 2008). Among the structural proteins, P3 is the core capsid protein, which encloses P1, P5, and P6 (Omura et al., 1985; Ichimi et al., 2002), and P2 and P8 are the minor and major outer capsid proteins, respectively (Omura et al., 1998; Miyazaki et al., 2005). Among the non-structural proteins, Pns7, Pns9, and Pns12 are the components of the viroplasm, the site for viral replication and assembly (Wei et al., 2009, 2011; Akita et al., 2011), Pns11 is a viral RNA-silencing suppressor (Shen et al., 2012), and the functions of Pns4 and Pns10 are unknown.

RGDV must replicate and spread within the insect body of the leafhopper *R. dorsalis* to be transmitted to the plant host. Previous cytopathological studies of phytoreovirus in infected plants and vector insects characterized two kinds of inclusions: the viroplasm and tubules (Fukushi et al., 1962; Omura et al., 1985). With the use of cultured monolayers of insect vector cells, the induction of the viroplasm by RGDV infection has been examined in detail (Wei et al., 2009, 2011; Akita et al., 2011); however, the mechanism underlying the genesis and maturation of the tubules is unknown. Pns11 of RGDV corresponds to Pns10 of RDV, which is the component of the tubules (Moriyasu et al., 2000; Wei et al., 2006). Pns10 of RDV has been demonstrated to facilitate viral spread among cultured insect vector cells (Wei et al., 2006, 2008) and the spread of RDV in the body of its leafhopper vector (Chen et al., 2012). Whether Pns11 of RGDV is similarly involved in tubule formation and viral spread within its insect vector is still unknown.

In the present study, we developed continuous cell cultures of leafhopper *R. dorsalis* to investigate the functional role of the tubules induced by RGDV in its insect vector. Cytopathologic results showed that viral non-structural protein Pns11 was the

minimal viral factor required for the formation of the tubules induced by RGDV infection. Such tubules protruded from the infected cell surface and attached to adjacent uninfected cells. The knockdown of Pns11 expression due to RNA interference (RNAi) induced by synthesized dsRNA from Pns11 gene abolished the formation of the tubules, preventing direct cell-to-cell spread of RGDV without significant effects on the multiplication of RGDV. All these results indicated that RGDV exploited virus-containing tubules to facilitate viral spread among insect vector cells. These results will promote our understanding of the mechanism underlying the spread of RGDV within its insect vector.

MATERIALS AND METHODS

VIRUS AND ANTIBODIES

RGDV samples, collected from rice fields from Guangdong Province, China, were maintained on rice plants via transmission by *R. dorsalis*. RGDV was purified from infected rice plants and stored at −80°C as described previously (Omura et al., 1982). Polyclonal antiserum against P8, Pns7 and Pns11 of RGDV were prepared in rabbits as described previously (Moriyasu et al., 2000, 2007; Wei et al., 2009). IgGs were purified from the antibody sample using a protein A-Sepharose affinity column and then conjugated directly to fluorescein isothiocyanate (FITC) or rhodamine according to the manufacturer's instructions (Invitrogen).

ESTABLISHMENT OF CONTINUOUS CELL CULTURES DERIVED FROM LEAFHOPPER *R. dorsalis* FOR VIRAL INFECTION

The cell line of the leafhopper *R. dorsalis* was established by adapting the protocol described by Kimura and Omura (1988). Primary cell cultures of *R. dorsalis*, originally established from the embryonic fragments dissected from eggs of *R. dorsalis*, were maintained in monolayer culture at 25°C in Kimura's insect medium as described previously (Kimura and Omura, 1988). Such vector cells in monolayers (VCMs) were then transferred into culture flasks for further subculturing.

The purifed RGDV was used to inoculate VCMs on coverslips that had been washed with 0.1 M histidine that contained 0.01 M $MgCl_2$, pH 6.2 (His-Mg), as described previously (Wei et al., 2011).

BACULOVIRUS EXPRESSION OF NON-STRUCTURAL PROTEINS OF RGDV

The baculovirus system was used to express non-structural proteins Pns11 and Pns7 of RGDV, using the protocol described by Wei et al. (2006). Recombinant baculovirus vectors containing Pns11 or Pns7 were introduced into DH10Bac (Invitrogen) for transposition into the bacmid. Sf9 cells were infected with recombinant bacmids in the presence of Cellfectin (Invitrogen).

IMMUNOFLUORESCENCE STAINING

VCMs or Sf9 cells on coverslip were fixed in 4% paraformaldehyde, immunolabeled with Pns11-specific IgG that had been conjugated to FITC (Pns11-FITC), Pns7-specific IgG that had been conjugated to FITC (Pns7-FITC), or major outer capsid protein P8-specific IgG that had been conjugated to rhodamine (P8-rhodamine) and then processed for immunofluorescence, as described previously (Wei et al., 2006, 2011). The cells were observed with a Leica TCS SP5 laser confocal microscope.

ELECTRON MICROSCOPY

VCMs or Sf9 cells on coverslips were prepared for transmission electron microscopy as described previously (Wei et al., 2006, 2009, 2011). For immunoelectron microscopy, the cell sections were immunolabeled with the Pns11-specific IgG as the primary antibody, followed by treatment with and goat anti-rabbit IgG conjugated with 15- or 10-nm gold particles, as secondary antibodies (Sigma), as described previously (Wei et al., 2006).

EXAMINATION OF RGDV SPREAD AND INFECTION OF VCMs IN THE PRESENCE OF SYNTHESIZED dsRNAs

We designed primers for PCR amplification of a 1071-bp segment of the Pns11 gene of RGDV and a 717-bp segment of a green fluorescence protein (GFP)-encoding gene as a control. The PCR products were used for dsRNA synthesis according to the protocol of the T7 RiboMAX Express RNAi System kit (Promega). For examining the effects of synthesized dsRNAs on viral spread of RGDV, VCMs were transfected with 0.5 μg/μL dsRNAs in the presence of Cellfectin (Invitrogen) for 8 h. VCMs were then inoculated with RGDV at a low multiplicity of infection (MOI) of 0.001 or a high MOI of 10 in the presence of virus-neutralizing antibodies (30 mg/mL of medium), as described previously (Wei et al., 2006). VCMs were fixed 3 h post inoculation (hpi), immunolabeled with Pns11-FITC and P8-rhodamine and visualized by fluorescence microscopy. The fluorescent cells were counted by the focus count method (Kimura, 1986). In this method, an infected cell and any adjoining infected cells were counted as one infectious unit. A minimum of four fields was counted for each sample from three or more independent experiments.

To confirm whether the synthesized dsPns11 affected multiplication of RGDV, VCMs were transfected with 0.5 μg/μL dsRNAs in the presence of Cellfectin for 8 h and then inoculated with RGDV at an MOI of 10. At 72 hpi, proteins were extracted from infected cells and further analysed by SDS-PAGE and immunoblotting with Pns11-specific or P8-specific antibodies, respectively. Insect actin was detected with actin-specific antibodies as a control to confirm loading of equal amounts of proteins in each lane.

RESULTS

TUBULES WERE FORMED BY NON-STRUCTURAL PROTEIN Pns11 OF RGDV IN INSECT VECTOR CELLS

A leafhopper cell line, originating from embryonic fragments dissected from eggs of *R. dorsalis*, was established. After 90 passages of subculturing at 7-day intervals, the dominant cell type in the established *R. dorsalis* cell line was epithelial-like and approximately 35–60 μm in diameter (**Figure 1**). Examination of the thin sections of RGDV-infected VCMs at 48 hpi by electron microscopy revealed virus-containing tubules approximately 85 nm in diameter within the cytoplasm or protruding from the surface of leafhopper cells (**Figure 2A**). These virus-containing tubules sometimes extended into the cell protrusions, namely, filopodia, from the infected cells, suggesting that RGDV particles

were accompanied by the tubules to pass through the filopodia of infected cells. The non-structural protein Pns11 of RGDV was found in numerous tubule-like structures within the cytoplasm or protruding from the cell surface (**Figure 2C**). When the subcellular localization of Pns11 of RGDV in infected VCM was examined by immunoelectron microscopy, the tubules were specifically immunolabeled with Pns11 antibodies (**Figure 2D**), confirming that Pns11 was a component of the tubules.

To determine whether Pns11 of RGDV had an inherent ability to form the tubules, we used a baculovirus system to express Pns11 in Sf9 cells. As seen by immunofluorescence microscopy, Pns11 was distributed in the tubule-like structures in the cytoplasm or protruding from the cell surface (**Figure 3A**). Immunoelectron microscopy confirmed that Pns11-specific IgG reacted specifically with the tubules approximately 85 nm in diameter (**Figure 3B**). However, Pns7 of RGDV was distributed diffusely throughout the cytoplasm (**Figure 3C**). All these results demonstrated that virus-containing tubules in infected insect vector cells were basically formed by non-structural protein Pns11 of RGDV.

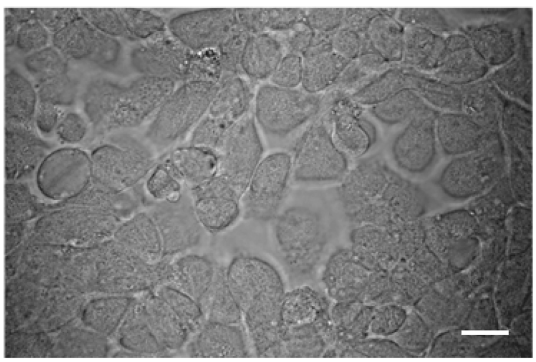

FIGURE 1 | Light micrograph of *R. dorsalis* vector cells in monolayer (VCM) after 90 passages. Bars, 50 μm.

THE TUBULES PROTRUDED FROM INITIALLY INFECTED CELLS AND ATTACHED TO ADJACENT UNINFECTED CELLS

To study the distribution of the tubules over time, we used immunofluorescence microscopy to visualize the tubules in the VCMs during infection by RGDV. VCMs were inoculated with RGDV at a low MOI of 0.001 for 2 h and then cultured in the presence of virus-neutralizing antibodies to prevent infection by free viral particles. At this low MOI, the initial and secondary infection of RGDV in VCMs could be easily monitored (data not shown).

As early as 24 hpi, small foci of one or two infected cells were visible in the presence of virus-neutralizing antibodies (**Figure 4A**). At 72 hpi, RGDV appeared to have spread from the initially infected cells to adjacent uninfected cells to form infection foci of 9–13 cells in the presence of virus-neutralizing antibodies (**Figure 4B**). The tubules were protruding from or scattered outside the infected cells, even near the surface of adjacent uninfected cells (**Figure 4**). All these results suggested that RGDV might spread from initially infected cells to adjacent uninfected cells by exploiting the tubules.

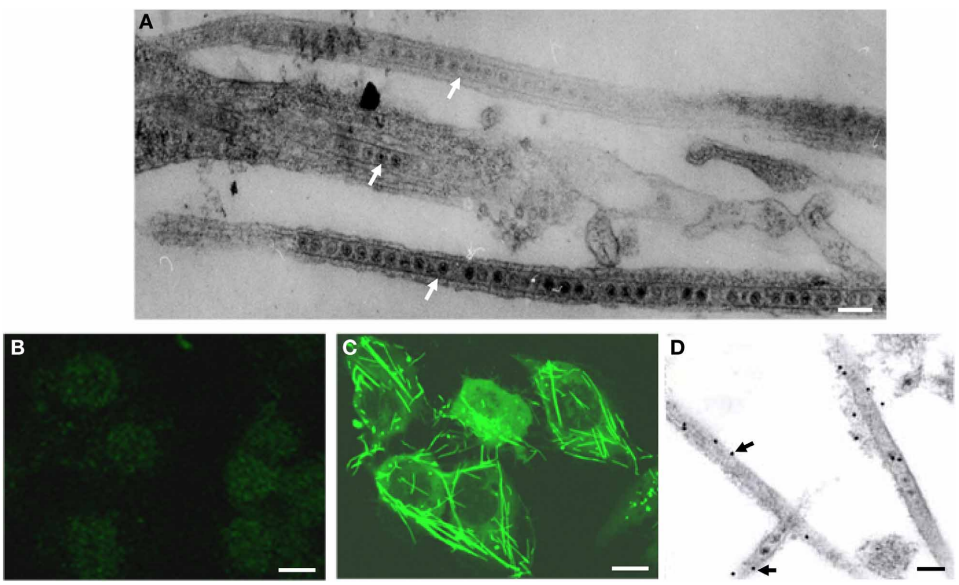

FIGURE 2 | Viral non-structural protein Pns11 is the constituent of the tubules induced by RGDV infection in VCMs at 48 hpi. (A) Electron micrograph of virus-containing tubules within the cytoplasm or protruding from the surface of infected cells. Bar, 100 nm. **(B)** Immunofluorescence staining of Pns11 showing nothing within uninfected cell. VCMs were immunostained with Pns11-FITC. Bar, 10 μm. **(C)** Immunofluorescence staining of Pns11 showing tubule-like structures within the cytoplasm and protruding from the surface of infected cell. VCMs were immunostained with Pns11-FITC. Bar, 10 μm. **(D)** Immunogold labeling of Pns11 in the tubules in RGDV-infected VCMs. Cells were immunostained with Pns11-specific IgG as primary antibodies, followed by treatment with 15-nm gold particle-conjugated goat antibodies against rabbit IgG as secondary antibodies. White arrows mark virus-containing tubules. Black arrows mark gold particles. Bar, 100 nm.

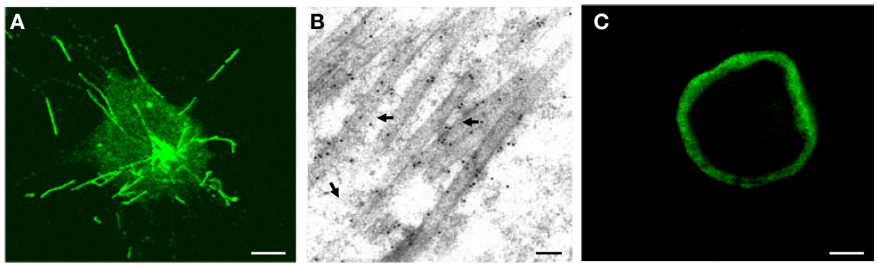

FIGURE 3 | Subcellular localization of Pns11 in recombinant baculovirus-infected Sf9 cells at 72 hpi. (A) Immunofluorescence staining of Pns11 showing tubule-like structures within the cytoplasm or protruding from the cell surface. Sf9 cells were immunostained with Pns11-FITC. Bar, 10 μm. **(B)** Immunogold labeling of Pns11 in the tubules. Cells were immunostained with Pns11-specific IgG as primary antibodies, followed by treatment with 10-nm gold particle-conjugated goat antibodies against rabbit IgG as secondary antibodies. Arrows mark gold particles. Bar, 100 nm. **(C)** Immunofluorescence staining of Pns7 showing the diffuse distribution of Pns7 in the cytoplasm of cell. Sf9 cells were immunostained with Pns7-FITC. Bar, 10 μm.

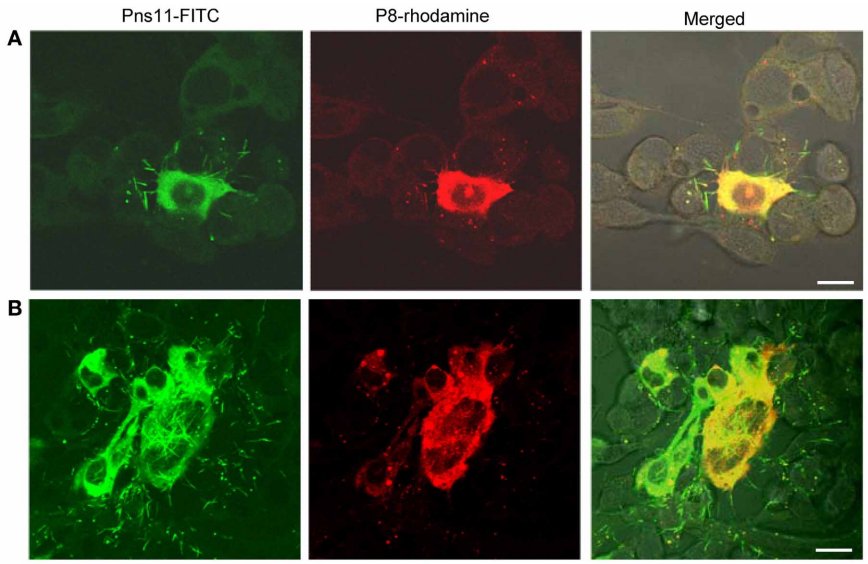

FIGURE 4 | Subcellular localization of the tubules and P8 antigens of RGDV in virus-infected VCMs viewed at 24 (A) and 72 (B) hpi. VCMs were inoculated with RGDV at an MOI of 0.001 and cultured in the presence of virus-neutralizing antibodies. VCMs were immunostained with Pns11-FITC and P8-rhodamine and viewed with confocal fluorescence microscopy. Images are representative of multiple experiments with multiple preparations. Bars, 10 μm.

RNAi INDUCED BY dsRNA FROM Pns11 GENE SIGNIFICANTLY INHIBITS FORMATION OF TUBULES AND VIRAL SPREAD AMONG INSECT VECTOR CELLS

For examining whether RNAi induced by dsRNAs affected viral multiplication in VCMs, VCMs were transfected with the synthesized dsRNAs targeting the encoding sequences of Pns11 (dsPns11) or GFP (dsGFP). At 8 h after transfection, VCMs were inoculated with RGDV at an MOI of 10, and then fixed at 72 hpi and immunolabeled with Pns11-FITC and P8-rhodamine. In VCMs transfected with dsGFP, viral infection was observed in almost 100% of cells, and abundant tubules protruded from the infected cell surface (**Figure 5A**). In VCMs transfected with dsPns11, the number of infected VCMs was not significantly reduced, but the formation of tubules was significantly inhibited (**Figure 5B**), suggesting that the expression of Pns11 had been knocked down by RNAi induced by dsPns11. To determine the effects of RNAi induced by dsPns11 on the expression of Pns11, we collected cell lysates for assay by Western blot. The expression of Pns11 and P8 under dsGFP transfection was the same as the control treatment (**Figure 5C**). However, transfection with dsPns11 resulted in a significant reduction in the level of Pns11 expression, but only weakly reduced the expression level of P8 (**Figure 5C**). Our results suggested that the inhibition of tubule formation by treatment with dsPns11 might not significantly affect viral multiplication in VCMs.

To determine whether the treatment of dsPns11 inhibited viral spread among insect vector cells, 8 h after transfection with dsRNAs, we inoculated VCMs with RGDV at a low MOI of 0.001, cultured in the presence of virus-neutralizing antibodies and then processed for immunofluorescence. At 72 hpi, viral infection was

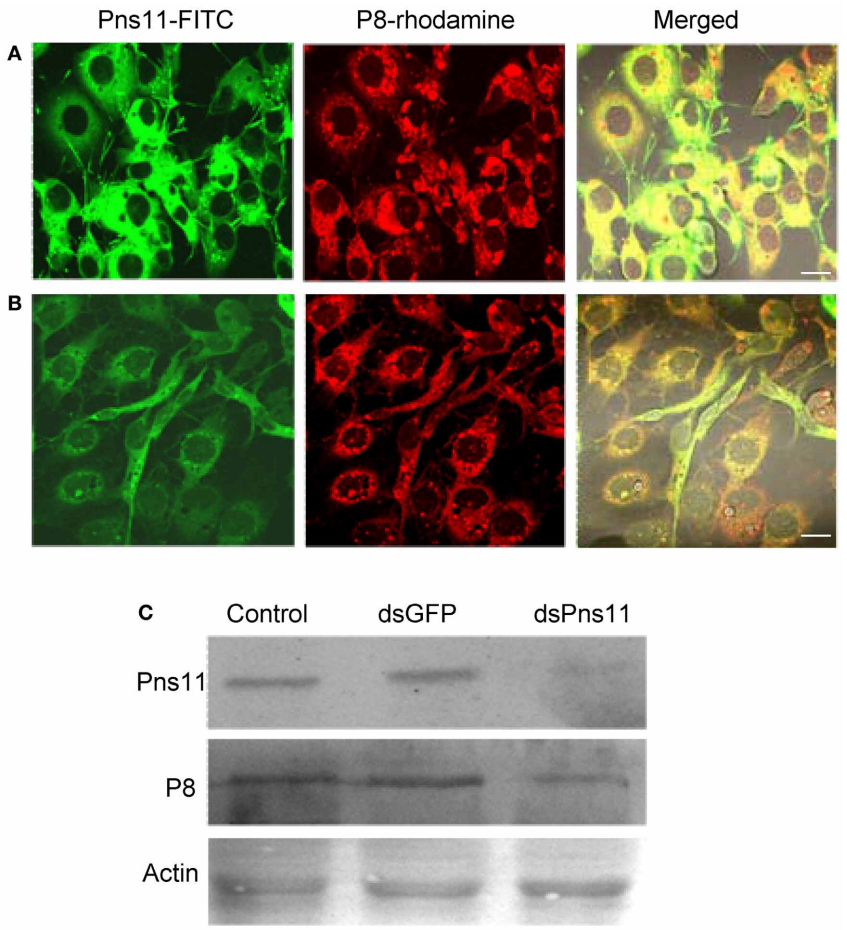

FIGURE 5 | RNAi induced by dsPns11 inhibited the formation of the tubules without significant effects on RGDV multiplication in VCMs. Twenty-four hours after transfection with dsGFP **(A)** or dsPns11 **(B)**, VCMs were inoculated with RGDV at an MOI of 10 and cultured in the presence of virus-neutralizing antibodies. At 72 hpi, cells were immunolabeled with Pns11-FITC and P8-rhodamine, then examined with confocal microscopy. Images are representative of multiple experiments with multiple preparations. Bars, 30 μm. **(C)** RNAi induced by dsPns11 significantly reduced the expression of Pns11 but not P8 of RGDV in virus-infected VCMs as shown here in Western blots. Protein extracts from cells transfected with Cellfectin, dsGFP or dsPns11 were separated by SDS-PAGE to detect Pns11 or P8 with Pns11-specific or P8-specific antibodies, respectively. Insect actin was detected with actin-specific antibodies as a control.

seen in the infection foci with approximately 9-11 infected cells in VCMs that received dsGFP and Cellfectin reagent (**Figures 6B**,**C**, data not shown). However, in VCMs that received dsPns11, viral infection was restricted to one or two cells, and the formation of tubules was inhibited (**Figures 6A,C**). Because VCMs treated with dsPns11 were able to support normal viral multiplication, we deduced that the limited infection of RGDV in VCMs is caused specifically by the inhibition of the intercellular spread of RGDV resulting from the failure of the tubules to form after treatment with dsPns11. Taken together, our results suggested that RGDV exploits the tubules to spread among insect vector cells.

DISCUSSION

During RGDV infection of the leafhopper *R. dorsalis* vector cells grown in monolayers (VCMs) in the present study, virus-containing tubules of approximately 85 nm in diameter were formed (**Figure 2**) and comprised viral non-structural protein Pns11 (**Figure 2**). When Pns11 of RGDV was expressed alone, such tubules formed in non-host Sf9 cells (**Figure 3**), suggesting that Pns11 was the minimal viral factor required for the formation of the tubules induced by RGDV infection.

As we noted earlier, Pns11 of RGDV corresponds to Pns10 of RDV, the component of the tubule induced by RDV infection (Moriyasu et al., 2000). Because the tubules constructed by Pns10 of RDV are directly involved in the intercellular spread of RDV particles among cultured leafhopper cells (Wei et al., 2006, 2008; Pu et al., 2011), here, we examined whether the tubules induced by RGDV infection also facilitate viral spread among insect vector cells. By 72 hpi, RGDV particles had spread from initially infected cells to adjacent uninfected cells, forming larger infection foci in the presence of neutralizing antibodies to avoid the infection by free viral particles (**Figure 4**), and the tubules protruded from the infected cell surface (**Figure 4**). Because viral particles were accompanied by

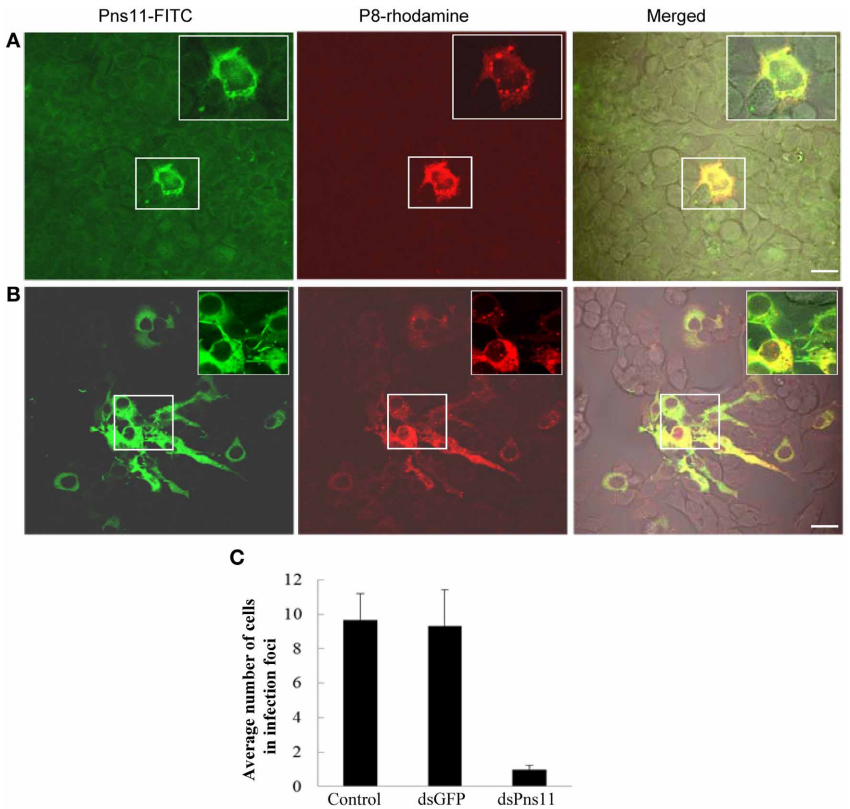

FIGURE 6 | RNAi induced by dsPns11 inhibited the spread of RGDV among insect vector cells. At 24 h after transfection with dsPns11 **(A)** or dsGFP **(B)**, VCMs were inoculated with RGDV at a low MOI (0.001) and cultured in the presence of virus-neutralizing antibodies. At 72 hpi, cells were immunolabeled with Pns11-FITC and P8-rhodamine, and then examined by confocal microscopy. Bars, 15 μm. Insets are enlarged images of boxed areas. **(C)** Mean number of RGDV-infected cells/infection focus in VCMs after treatment with dsRNAs. Error bars indicate standard deviations from three independent experiments.

the tubules, we deduced that RGDV might exploit the tubules to spread among insect vector cells. To confirm this hypothesis, we used an RNAi strategy to specifically knock down the expression of Pns11 by treatment with dsPns11; the tubules failed to assemble, and viral spread was prevented in the VCMs without significant effects on viral multiplication (**Figures 5**, **6**). Taken together, all these results indicated that the tubules facilitated viral spread among insect vector cells. Tubules were associated with the actin-based cell protrusion, namely, filopodia, in the infected cells (**Figure 2A**). We deduced that the tubules might exploit the actin-based filopodia to attach to healthy adjacent cells for viral direct intercellular spread. Recently, we also showed that such tubules could facilitate the spread of RDV in the body of its leafhopper vector (Chen et al., 2012). Whether the tubules induced by RGDV infection have similar functions in their insect vectors will be investigated in the future.

We also previously demonstrated that the transport of RGDV particles from viroplasms, the site for viral replication and assembly, to the plasma membrane and into the medium is dependent on the microtubules of the infected VCMs (Wei et al., 2009). Infection by these free viral particles in the medium was protected by the addition of neutralizing antibodies. Furthermore, we have shown that RGDV particles were accompanied by the tubules and spread from infected cells to uninfected cells in the presence of neutralizing antibodies. The model for viral direct intercellular spread presented here is thought to be advantageous over infection by cell-free virus because the virus is protected from host immune responses. Thus, RGDV evolved to exploit two pathways for the release viral particles from infected vector cells, without affecting cellular structures or morphology, corresponding to the evidence that the leafhopper cell line supports a non-cytopathic, persistent infection of RGDV. Evidently, mechanisms have evolved in the vector cells to allow efficient release of RGDV without any significant pathology.

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Recent progress in research on cell-to-cell movement of rice viruses

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To adapt to plants as hosts, plant viruses have evolutionally needed the capacity to modify the host plasmodesmata (PD) that connect adjacent cells. Plant viruses have acquired one or more genes that encode movement proteins (MPs), which facilitate the cell-to-cell movement of infectious virus entities through PD to adjacent cells. Because of the diversity in their genome organization and in their coding sequences, rice viruses may each have a distinct cell-to-cell movement strategy. The complexity of their unusual genome organizations and replication strategies has so far hampered reverse genetic research on their genome in efforts to investigate virally encoded proteins that are involved in viral movement. However, the MP of a particular virus can complement defects in cell-to-cell movement of other distantly related or even unrelated viruses. *Trans*-complementation experiments using a combination of a movement-defective virus and viral proteins of interest to identify MPs of several rice viruses have recently been successful. In this article, we reviewed recent research that has advanced our understanding of cell-to-cell movement of rice viruses.

Keywords: cell-to-cell movement, movement protein, rice, rice virus, *trans*-complementation experiment

INTRODUCTION

To transport their genome from an initially infected cell to neighboring cells, plant viruses need to pass through cytoplasmic channels, called plasmodesmata (PD) in rigid cell walls. Since the diameter of PD is smaller than virus particles, most plant viruses encode one or more movement proteins (MPs) that can localize at the PD and modify the structure of PD to allow passage of the virus particles into the next cells. During the processes involved in viral cell-to-cell movement, viral MPs would be associated with virus particles or viral RNA–protein complexes (vRNPs) to and through PD (Melcher, 1990; Oparka et al., 1997; Waigmann et al., 2004; Lucas, 2006; Benitez-Alfonso et al., 2010).

The first evidence suggesting that cell-to-cell movement for a certain plant virus is controlled by a viral MP was provided by a study on a 30-kDa protein of a temperature-sensitive mutant of tobacco mosaic virus (TMV) that replicates but is defective in movement at certain temperatures (Meshi et al., 1987). In reciprocal experiments using the infectious TMV mutants, wild-type TMV was blocked in the cell-to-cell movement when the TMV gene for the wild-type 30-kDa protein was replaced by the mutant protein. Furthermore, viral movement was completely lost when a frameshift mutation was introduced into the translation start codon of the gene for the 30-kDa protein. This research indicated that the 30-kDa protein is responsible for movement of the virus. These studies gave rise to similar ones on many other plant viruses, and it soon became clear that MPs are general features for both plant RNA and DNA viruses in different genera (Atabekov and Dorokhov, 1984; Waigmann et al., 2004; Lucas, 2006; Taliansky et al., 2008).

Over 15 viruses affect rice (*Oryza sativa* L.), one of the most important cereal crops for nearly half of the world's population, and 12 are very destructive in the major rice-producing regions, especially in Asia (Hibino, 1996). These viruses are transmitted by planthoppers, leafhoppers, and chrysomelid beetles in a persistent or semi-persistent manner, or by soil-inhabiting fungus. Although the majority of plant viruses are positive-sense RNA viruses, rice viruses encompass many types of viruses, e.g., double-stranded RNA viruses [rice black-streaked dwarf virus (RBSDV) and rice dwarf virus (RDV)], segmented negative-sense RNA viruses [rice stripe virus (RSV) and rice grassy stunt virus (RGSV)], a non-segmented negative-sense RNA virus [rice transitory yellowing virus (RTYV)], a segmented positive-sense RNA viruses [rice stripe necrosis virus (RSNV) and rice necrosis mosaic virus (RNMV)], non-segmented positive-sense RNA viruses [rice yellow mottle virus (RYMV) and rice tungro spherical virus (RTSV)], and a double-stranded DNA virus [rice tungro bacilliform virus (RTBV)].

The viral MPs are involved with viral movement without affecting virus replication in single cells. In addition, even though viral MPs can be genetically swapped between different viruses, the exchangeability and complementation of movement functions have been conserved for many plant viral MPs with viruses of different families and even with plant and insect viruses (Solovyev et al., 1996; Morozov et al., 1997; Dasgupta et al., 2001; Tamai et al., 2003). On the base of these exchangeable and

complementary characters of viral MPs, many virus-encoded proteins have been identified. Over the past 10 years, several uncharacterized proteins of rice viruses have been revealed to function in cell-to-cell movement (**Table 1**; Li et al., 2004; Huang et al., 2005; Xiong et al., 2008; Wu et al., 2010; Hiraguri et al., 2011, 2012; Zhang et al., 2012). In this review article, we summarized recent progress in research on cell-to-cell movement of rice viruses.

RICE-INFECTING REOVIRUSES

Five reoviruses, RDV and rice gall dwarf virus (RGDV) in the genus *Phytoreovirus*, rice ragged stunt virus (RRSV) in the genus *Oryzavirus*, and RBSDV and southern rice black-streaked dwarf virus (SRBSDV) in the genus *Fijivirus*, infect rice and threaten the stability of rice production in Asia (Hibino, 1996; Hoang et al., 2011). These viruses are double-shelled spherical particles, from 50 to 80 nm in diameter, and include from 10 to 12 segmented double-stranded genomic RNAs depending on the viruses (Attoui et al., 2011; **Figure 1A**). These viruses are transmitted in a persistent manner by the insects such as *Laodelphax striatellus*, *Nilaparvata lugens*, *Nephotettix cincticeps*, *Recilia dorsalis*, and *Sogatella furcifera*, and may be replicated in both plants and in their vector insects (Hibino, 1996). RDV can be distributed in vascular bundles and in parenchymatous cells of the host plants, but the other four are localized in the phloem and the lesioned tissues of the plants. RDV is transmitted transovarially to progeny at high rates by the vector insects, but the other four are not (Hibino, 1996; Hoang et al., 2011).

To identify the MP of rice-infecting reoviruses, Li et al. (2004) used *trans*-complementation experiments in leaves of *Nicotiana benthamiana* to analyze 12 proteins encoded in the segmented RDV genome for their ability to traffic movement-defective potato virus X (PVX) mutants that were tagged with β-glucuronidase (GUS) or green fluorescent protein (GFP). The cell-to-cell movement of the mutants was restored after co-bombardment with only the plasmid containing the RDV gene for the non-structural Pns6, but not for any other RDV-encoded

Table 1 | Overview of movement proteins of rice viruses.

Family/genus	Virus/abbreviation	Protein/location	Notes	Reference
Reoviridae/*Phytoreovirus*	*Rice dwarf virus*/RDV	Pns6/Segment 6	Confirmed by complementation	Li et al. (2004)
Reoviridae/*Phytoreovirus*	*Rice gall dwarf virus*/RGDV	Pns7/Segment 7	Predicted by similarity to RDV MP	Moriyasu et al. (2007)
Reoviridae/*Fijivirus*	*Rice black-streaked dwarf virus*/RBSDV	P7-1/Segment 7	Predicted by tubular structures	Isogai et al. (1998)
Reoviridae/*Fijivirus*	Southern rice black-streaked dwarf virus/ SRBSDV	P7-1/Segment 7	Predicted by tubular structures	Zhou et al. (2008)
Reoviridae/*Oryzavirus*	*Rice ragged stunt virus*/RRSV	Pns6/Segment 6	Confirmed by complementation	Wu et al. (2010)
Unassigned family/*Tenuivirus*	*Rice stripe virus*/RSV	pC4/RNA 4	Confirmed by complementation	Xiong et al. (2008)
Unassigned family/*Tenuivirus*	*Rice grassy stunt virus*/RGSV	pC6/RNA 6	Confirmed by complementation	Hiraguri et al. (2011)
Unassigned family/*Tenuivirus*	*Rice hoja blanca virus*/RHBV	pC4/RNA 4	Predicted by similarity to RSV MP	Zhang et al. (2012)
Rhabdoviridae/*Nucleorhabdovirus*	*Rice transitory yellowing virus*/RTYV	P3/gene 3	Confirmed by complementation	Huang et al. (2005)
Unassigned family/*Sobemovirus*	*Rice yellow mottle virus*/RYMV	P1/ORF 1 gene	Confirmed by mutagenesis	Bonneau et al. (1998)
Caulimoviridae/*Tungrovirus*	*Rice tungro bacilliform virus*/RTBV	Unknown/ORF 3 gene	Predicted by similarity to caulimoviral MP	Bouhida et al. (1993)
Secoviridae/*Waikavirus*	*Rice tungro spherical virus*/RTSV	Unknown/ORF 2 gene	Predicted by genome organization	Sanfacon et al. (2011)
Unassigned family/*Benyvirus*	*Rice stripe necrosis virus*/RSNV	TGB/RNA2	Predicted by triple gene blocks-like structure	Lozano and Morales (2009)
Potyviridae/*Bymovirus*	Rice necrosis mosaic virus/RNMV	P1/RNA2	Predicted by similarity to bymoviral MP	Badge et al. (1997)

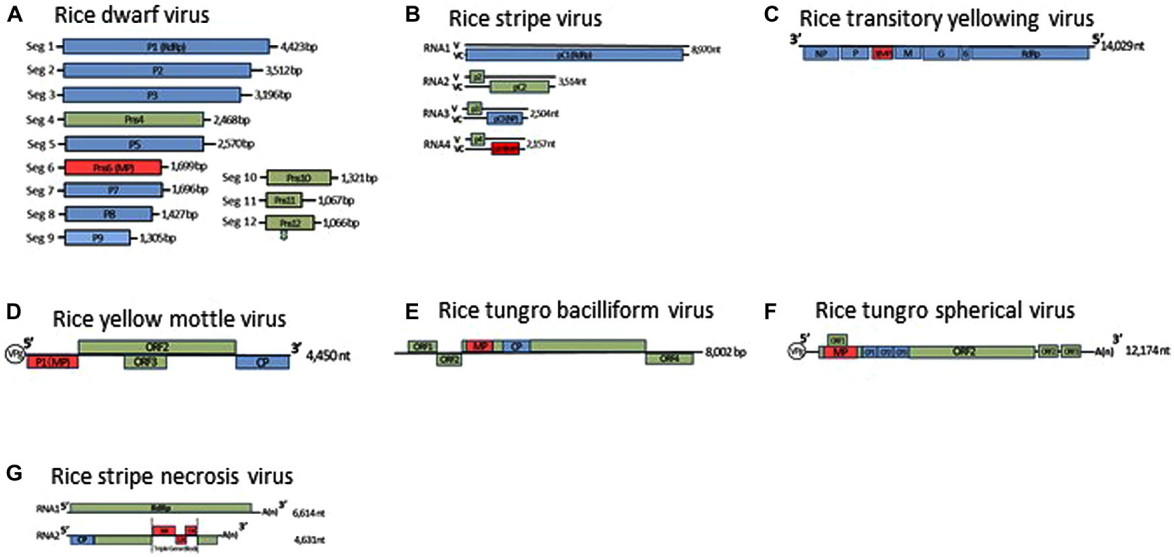

FIGURE 1 | Genome organization of rice dwarf virus (A), rice stripe virus (B), rice transitory yellowing virus (C), rice yellow mottle virus (D), rice tungro bacilliform virus (E), rice tungro spherical virus (F), and rice stripe necrosis virus (G). Lines represent the viral genomic segments. Blue boxes denote genes for structural proteins, green are for non-structural proteins, red are for movement proteins (MP). CP, Coat protein; NP, nucleocapsid protein; ORF, open reading frame; RdRp, RNA-dependent RNA polymerase; V, viral-sense strand; VC, viral-complementary-sense strand.

proteins. The complementation of viral movement was lost when the translation start codon of the gene for the Pns6 was altered from ATG to ATC. Furthermore, the GFP-fused Pns6 protein was localized near or within cell walls of epidermal cells of *Nicotiana tabacum*. Immunogold-labeling studies of thin sections from RDV-infected rice leaves using a Pns6-specific antibody showed that the Pns6 accumulated in PD of RDV-infected rice leaf cells. These results suggested that the Pns6 of RDV is the viral MP (Li et al., 2004).

Numerous studies about MPs encoded by diverse plant viruses have indicated that viral MPs have a sequence-non-specific nucleic-acid binding activity, which might represent a functional hallmark of viral MPs (Waigmann et al., 2004). The Pns6 of RDV has such sequence-non-specific binding of single- and double-stranded forms of DNAs and RNAs, but bind sequence-specifically to single-stranded forms of the viral genome, in particular, to the terminal consensus sequences of the segmented viral genome. Interestingly, the Pns6 had a stronger binding affinity to the terminal viral-sense strands than to the corresponding viral-complementary-sense strands of the RDV genome. There is a possibility that the differential binding affinity of the Pns6 may be associated with the formation of vRNPs when RDV moves through PD. In a mutagenesis analysis of Pns6, the N-terminal region of the protein was found to be responsible for the RNA-binding activities, and the conserved GKS motif, which is required for NTP binding, was also present at amino acid positions 125–127 (Ji et al., 2011). In the C-terminal region of the Pns6, the ATPase/helicase activity site, which may be involved in the unfolding of vRNPs during viral movement, was located (Lucas, 2006; Ji et al., 2011).

Pns7 of RGDV, which belongs to the same genus as RDV, is very similar to Pns6 of RDV in its amino acid sequence (Moriyasu et al., 2007). Thus, Pns7 of RGDV is assumed be functionally equivalent to Pns6 of RDV in viral movement. However, there is no direct experimental evidence to support the function of the Pns7 of RGDV as a MP.

The functioning of the non-structural Pns6 protein of RRSV in viral cell-to-cell movement has been confirmed by transient-expression experiments with the GFP-fused Pns6 protein of RRSV in epidermal cells of *Nicotiana benthamiana* and by *trans*-complementation experiments using a movement-defective TMV mutant in *Nicotiana tabacum* (Shao et al., 2004; Wu et al., 2010). Pns6 has a sequence-non-specific binding of single- and double-stranded forms of DNAs and RNAs, but binds sequence-specifically to single-stranded forms of the viral genome, and its binding domain was also determined to be located between amino acids 201 and 273 of the Pns6 of RRSV (Shao et al., 2004).

The formation of tubules that contain virus particles has been reported for many spherical viruses and considered to facilitate intercellular movement of the virus particles through the tubule structures (van Lent et al., 1991; Storms et al., 1995; Kasteel et al., 1996, 1997; Zheng et al., 1997). Similar tubular structures containing virus particles were observed in the cytoplasm of RBSDV-infected rice plants and in viruliferous vector insects (Isogai et al., 1998). Immunogold-labeled thin sections of these virus-infected rice plants and viruliferous insects indicated that the P7-1, encoded in the 5′-terminal region of RBSDV segment 7, was associated with virus-containing tubular structures (Isogai et al., 1998). Since deletion of either of two putative transmembrane domains abolishes the localization of P7-1 in the PD of

Nicotiana benthamiana and the formation of the tubular structure in the Sf9 insect cells, these putative transmembrane domains are necessary for the P7-1 proteins to form the tubular structures (Sun et al., 2013). Thus, P7-1 is considered to function as a tubule-forming MP in both the plants and the insects, and the virus may be transported within the virus-containing tubular structures in the form of virus particles. P7-1 of SRBSDV has also been reported to function similarly in tubular formation (Zhou et al., 2008; Liu et al., 2011). However, there is no direct experimental evidence that these two proteins facilitate the cell-to-cell movement of movement-defective viruses by co-expression of these putative MPs.

RICE-INFECTING TENUIVIRUSES

Tenuiviruses are non-enveloped, segmented, negative-sense RNA viruses. The genus presently comprises six species and six tentative species (Shirako et al., 2011). Virus particles are thin and filamentous, 3–10 nm in diameter, and composed of a single nucleocapsid protein. The viral genomes consist of four to six single-stranded RNA segments, which are either of negative polarity or of ambisense (**Figure 1B**). Since the putative RNA-dependent RNA polymerase is co-purified with viral ribonucleoproteins, the polymerase is thought to be associated with filamentous virus particles (Toriyama, 1986). Three tenuiviruses, RSV, RGSV, and rice hoja blanca virus (RHBV), were reported to cause serious problems for rice production in the world (Hibino, 1996). These viruses are transmitted in a persistent manner by the insects, such as *L. striatellus*, *Nilaparvata lugens*, and *Sogatodes orizicola*, and multiply in these insects as well as the plants. RSV and RHBV are transmitted transovarially to progeny at high rates by the vector insects, but RGSV is not (Hibino, 1996).

The RSV and RHBV genomes consist of four single-stranded RNA segments, designated RNAs 1–4 in order of decreasing molecular mass, and encode seven genes. The first RNA segment is of negative polarity, and the other three RNA segments are ambisense. The RGSV genome consists of six single-stranded RNA segments, all of which are ambisense, and includes 12 genes. The viral mRNAs are transcribed from each viral segment by the cap-snatching mechanism (Ramirez et al., 1995; Shimizu et al., 1996).

The pC4 is encoded by the viral-complementary-sense strand of RSV RNA4. Previous computer analysis of the secondary protein structures has predicted that the pC4 belongs to the "30K" superfamily of viral MPs (Melcher, 2000). As a result of co-expression of the GFP-fused pC4 protein with a PD marker protein, PDLP1a, both proteins localized together as punctate spots in cell walls (Yuan et al., 2011). An immunogold-labeling study of thin sections from the RSV-infected rice leaves using a pC4-specific antibody showed that the pC4 accumulated in cell walls of the RSV-infected rice leaves, confirming that the pC4 is a PD-localized protein (Xiong et al., 2008).

Similar to other viral MPs with sequence-non-specific binding to nucleic acids (Waigmann et al., 2004), pC4 showed sequence-non-specific single- and double-stranded RNA-binding properties in gel mobility shift assays (Xiong et al., 2008). *Trans*-complementation experiments using a GUS-tagged, movement-defective PVX mutant and later using GFP-tagged movement-defective mutants of tomato mosaic virus (ToMV) or TMV in *Nicotiana benthamiana* demonstrated repeatedly that pC4 complemented cell-to-cell movement of these movement-defective mutants, reinforcing the idea that pC4 functions as the viral MP (Xiong et al., 2008; Hiraguri et al., 2011; Zhang et al., 2012).

It is likely that RSV may be transported between plant cells in the movement form of a vRNP, rather than as a virus particle because yeast two-hybrid assays failed to show any interaction between the pC4 and the nucleocapsid protein of RSV (Xiong et al., 2008). Interestingly, inoculation of *Nicotiana benthamiana* with RNA transcripts of a TMV-pC4 hybrid, in which the pC4 gene was inserted under the CP subgenomic RNA promoter of the movement-defective TMV mutant, induced foliar necrosis in the upper leaves (Zhang et al., 2012). These results indicated that the pC4 supported not only the cell-to-cell movement but also long-distance movement of the TMV mutant. Similar results that showed the "30K" superfamily members of viral MPs support long-distance movement have been reported for several phylogenetically distinct viruses such as TMV (Knapp et al., 2001) and tomato spotted wilt virus (TSWV; Lewandowski and Adkins, 2005).

The pC4 protein of RSV requires an actomyosin motility system to access the PD via intracellular movement along the ER-to-Golgi secretory pathway (Yuan et al., 2011). Dominant-negative inhibitors were used to show that myosin VIII of the host plant cells was specifically required for pC4 localization to the PD. Myosin VIII was similarly shown to be associated with the localization of the viral MP to the PD in studies of a closteroviral MP, an Hsp70 homolog of beet yellows virus (BYV), although the same class of myosin was ineffective with respect to targeting the TMV MP, which required a myosin XI-2 to facilitate intracellular movement of TMV (Alzhanova et al., 2001; Avisar et al., 2008; Harries et al., 2009).

Although the pC4 of RSV accesses PD along the ER-to-Golgi secretory pathway, it does not have typical structural features of secreted proteins. Since pC4 lacks an N-terminal signal peptide, a transmembrane domain, and a short C-terminal tail that might direct the viral MP to the PD, intracellular movement of pC4 might involve the interaction of pC4 with host cellular proteins that are transported by Golgi-derived vesicles and are eventually anchored to the PD. With a yeast two-hybrid system using pC4, two chaperone proteins, which have high degrees of identity with a DnaJ, and a Hsp20 have been isolated from a rice cDNA library (Lu et al., 2009). Continuing research on the movement of TMV, BYV, TSWV, and potato virus Y (Soellick et al., 2000; Prokhnevsky et al., 2002; Qiu et al., 2006; Hofius et al., 2007; Shimizu et al., 2009) has shown that DnaJ proteins bind to their partner heat shock proteins of the host plant cells and seem to act as a key regulator for conformational change in the movement form of the vRNPs that allows them to pass through the PD. The heat shock proteins might have intrinsic ATPase activity and serve as motor proteins to facilitate the transport of the vRNPs through the PD. However, the actual roles of the two proteins from the rice cDNA library are unknown. The molecular mechanism by which the pC4 of RSV targets and modifies the PD for viral movement needs to be clarified.

Aside from pC4 of RSV, only the pC6 protein encoded by the viral-complementary-sense strand of RGSV RNA6, has been proven to function as a viral MP (Hiraguri et al., 2011) as shown by transient expression assays with a GFP-fused pC6 protein and a *trans*-complementation experiment with a movement-defective ToMV mutant. The functional similarities between the RSV pC4 and the RGSV pC6 are consistent with a previous prediction that RGSV RNA 6 may be functionally equivalent to RSV RNA 4 (Toriyama et al., 1997). The MP of RHBV has not yet been identified. However, the pC4 of PHBV shared a considerably high degree of similarity with the pC4 of RSV as well as the pC6 of RGSV, suggesting a common role for the pC4 orthologs in movement of tenuiviruses (Zhang et al., 2012).

RICE-INFECTING RHABDOVIRUSES

Rice transitory yellowing virus was first identified in Taiwan in 1965 (Chiu et al., 1965) and is identical to rice yellow stunt virus (RYSV; Hiraguri et al., 2010), which was reported at almost the same time in China (Fan et al., 1965). RTYV is a member of the genus *Nucleorhabdovirus* in the family *Rhabdoviridae* and has bullet-shaped particles 180–210 nm long and 94 nm wide and has a non-segmented, negative-sense, single-stranded RNA genome (**Figure 1C**; Dietzgen et al., 2011). The virus is transmitted in a persistent manner by the insects *Nephotettix nigropictus*, *Nephotettix cincticeps*, and *Nephotettix virescens* (Chiu et al., 1965). On the basis of an SDS-PAGE analysis of the purified virus, the virus particles were first thought to consist of five proteins: nucleocapsid protein, phosphoprotein, matrix protein, glycoprotein, and RNA-dependent RNA polymerase (Hayashi and Minobe, 1985; Chiu et al., 1990; Fang et al., 1992). In recent western blot analyses and immunogold-labeling studies of the purified virus, however, the P3 and the P6 encoded by the RTYV genes 3 and 6, respectively, has also been detected in the purified virus (Huang et al., 2003; Hiraguri et al., 2012).

The genome organizations of plant-infecting rhabdoviruses are unique in having genes in addition to the basic gene orders of rhabdoviruses (Dietzgen et al., 2011). Because the gene 3, located between the 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; Wetzel et al., 1994; Tsai et al., 2005), the proteins encoded by gene 3 were inferred to be MPs of plant-infecting rhabdoviruses. A possible MP role for the protein encoded by gene 3 was first proposed for the sc4 protein of sonchus yellow net virus (SYNV; Scholthof et al., 1994). When the predicted secondary structure of sc4 was compared with that of known viral MPs, the sc4 was categorized in the "30K" superfamily of viral MPs (Melcher, 2000). Other proteins encoded by the gene 3 of plant-infecting rhabdoviruses, such as P3 of RYSV, 4b of lettuce necrotic yellows virus (LNYV), P3 of maize mosaic virus (MMV), and P4 of maize fine streak virus (MFSV), were also predicted to form "30K" superfamily-like secondary structures and had conserved consensus motifs homologous to the LXDX50-70G motif in the "30K" superfamily members (Huang et al., 2005).

Knowledge about the MP of plant-infecting rhabdoviruses has further advanced through the studies of the P3 protein of RTYV (synonym of RYSV). The *trans*-expressed of P3 facilitated cell-to-cell movement of a movement-defective PVX mutant in *Nicotiana benthamiana* leaves (Huang et al., 2005), and also complemented a defect in movement of a movement-defective ToMV mutant (Hiraguri et al., 2012). In transient-expression experiments with the GFP-fused P3 protein of RTYV in epidermal cells of *Nicotiana benthamiana*, P3 was associated with the nucleus and the PD. Furthermore, in immunogold-labeled thin sections of the RTYV-infected rice plants, P3 was located in the cell walls. In addition, a northwestern blot of P3 indicated that it had a single-stranded RNA-binding capacity that lacks sequence specificity *in vitro* (Huang et al., 2005). Together, these data directly suggested that P3 is a rhabdovirus MP.

An interaction between P3 and the nucleocapsid protein was also revealed in a GST pull-down assay with *E. coli*-expressed recombinant proteins of RTYV (Huang et al., 2005). On the other hand, sc4 of SYNV interacts specifically with the glycoprotein and not with the nucleocapsid protein in bimolecular fluorescence complementation experiments that examined all pairwise interactions of SYNV-encoded proteins (Min et al., 2010). In addition, the Y of potato yellow dwarf virus (PYDV), which is the MP of PYDV, interacted with the matrix protein, but not the nucleocapsid protein (Bandyopadhyay et al., 2010). Since the ribonucleocapsid is the minimal infectious unit for negative-strand viruses such as the rhabdoviruses, the sc4 and Y proteins may bind directly to the glycoprotein and matrix protein, respectively, and may associate indirectly with the nucleocapsid protein to form the viral movement complex with the nucleocapsid. In contrast, P3 of RTYV may interact directly with the nucleocapsid protein and be associated with the ribonucleocapsid core of virus particles (Hiraguri et al., 2012). The presence of P3 in the virus particles may provide an advantage for rapid viral spread to neighboring cells from the initially infected cells where the enveloped virus particles are uncoated, with subsequent release of the nucleocapsid cores. In the initial infection process of rhabdoviruses, numerous virus particles are thought to be injected into plant cells via their insect vector. Translation or replication of some virus particles may begin in the initially injected cells. Others may form an intercellular movement complex of the ribonucleocapsid immediately after they enter the neighbor cells, with the assistance of the MP that had been contained in the virus particles beforehand, and could be transported more rapidly into neighboring cells. The cell-to-cell movement strategy of the RTYV-type rhabdoviruses might be distinct from those of other enveloped plant viruses.

OTHER RICE-INFECTING VIRUSES

RICE YELLOW MOTTLE VIRUS

Rice yellow mottle virus is a member of the genus *Sobemovirus* (Truve and Fargette, 2011). The virus is endemic to Africa and causes serious problems for irrigated rice. The virus is polyhedral, 30 nm in diameter, and is transmitted in a semipersistent manner by a number of chrysomelid beetles including *Sesselia pusilla*, *Chaetocnema pulla*, and *Trichispa sericea* (Hibino, 1996). The RYMV genome is composed of a positive-sense, single-stranded RNA and contains four open reading frames (ORFs; **Figure 1D**; Yassi et al., 1994; Truve and Fargette, 2011). An RYMV mutant that lacks expression of the P1 protein as a result of a point

mutation in the ORF1 initiation codon of RYMV ORF1, replicates efficiently in rice protoplasts, but is unable to systemically infect rice plants. In transgenic plants that express P1 in *trans*, the defective mutant virus recovered its ability for systemic infection (Bonneau et al., 1998). These results indicated that P1 is the RYMV MP. P1 also has silencing suppressor activity, and mutagenesis experiments using the RYMV infectious clone revealed that cysteine at amino acid position 95 of P1 is essential for viral cell-to-cell movement and that cysteine and phenylalanine at amino acids 64 and 88 are associated with the efficiency of the RNA silencing suppressor (Voinnet et al., 1999; Sire et al., 2008).

TUNGRO DISEASE-ASSOCIATED VIRUSES

Tungro disease, one of the most severe virus diseases of rice, is a significant threat to rice production in South and Southeast Asia (Hibino, 1996). Tungro disease is caused by a complex of two viruses, RTBV and RTSV. RTBV is mainly responsible for symptom expression, and RTSV assists transmission of both viruses by the insects *Nephotettix virescens*, *Nephotettix nigropictus*, *Nephotettix cincticeps*, and *Recilia dorsalis* (Hibino et al., 1978; Jones et al., 1991).

Rice tungro bacilliform virus is a member of the genus *Tungrovirus* in the family *Caulimoviridae* (Geering and Hull, 2011). The virus particles are bacilliform, 100–300 nm long and 30–35 nm wide, and its genome is circular, double-stranded DNA and contains four ORFs (**Figure 1E**). ORF3 encodes the largest polyprotein, which possesses the coat protein and the analogs of a pepsin-like aspartic protease, reverse transcriptase, and RNase H1 (Rothnie et al., 1994).

The identity of the participants and understanding of the mechanism for cell-to-cell movement of caulimoviruses has progressed through studies of the P1 protein of cauliflower mosaic virus (CaMV). P1 forms tubular structures that contains the virus particles. A mutation in the CaMV gene for the P1 abolishes viral movement but does not affect viral amplification. For these reasons, P1 of CaMV has been proposed as a viral MP (Perbal et al., 1993; Thomas et al., 1993). The N-terminal 350 amino acids of the protein encoded by the RTBV ORF3 contains a region with restricted similarity to the P1 protein of CaMV (Bouhida et al., 1993). Based on this similarity with P1, the N-terminal region of the protein has also been postulated to be a MP of RTBV, but there is no direct experimental evidence that the region is associated with movement of RTBV.

Rice tungro spherical virus is the type member of the genus *Waikavirus* in the family *Secoviridae*, characterized by positive-sense, single-stranded RNA. Virus particles are polyhedral, about 30 nm in diameter and consist of three CPs (CP1, CP2, and CP3; Sanfacon et al., 2011). The viral genome is presumably attached to the genome-linked viral protein (VPg) at the 5′-terminal and is polyadenylated at the 3′-terminus (**Figure 1F**). The viral genome encodes one large ORF encoding a viral polyprotein, which contains the regions of the three CPs, the cysteine-like protease, domains for an NTP-binding protein and an RNA-dependent RNA polymerase (Shen et al., 1993). In addition, two small ORFs near the 3′-terminal of the viral genome and one small ORF that overlaps the gene for the main viral polyprotein have been identified (Shen et al., 1993; Firth and Atkins, 2008). The gene for the MP of RTSV has not been identified yet, and there is no information on the molecular mechanism for cell-to-cell movement of waikaviruses. However, the several genes for viral MPs in the family *Secoviridae* have been identified, and a comparison of the genome organization of the waikaviruses with other members of the family *Secoviridae* led to the suggestion that the region for the MP of RTSV might be located at the N-terminal region of the large viral polyprotein and be followed by three CPs (Sanfacon et al., 2011). Further experiments such as *trans*-complementation experiments using different movement-defective virus mutants or subcellular localization experiments using these putative MPs fused with fluorescent protein, are needed to investigate the molecular mechanisms of movement of RTSV.

FUNGUS-TRANSMITTED RICE VIRUSES

Two rice viruses, RSNV and RNMV, were reported to be transmitted by a soil-inhabiting fungus, *Polymyxa graminis* (Hibino, 1996). RSNV is a tentative species of the genus *Benyvirus* (Gilmer and Ratti, 2011). The virus continues to spread in Latin America through the international trades of rice seeds produced in fields contaminated with virus-carrying fungal vectors. The virus particles have at least two rod-shaped structures that are 360 and 260 nm long, respectively, and 20 nm wide (Morales et al., 1999). The RSNV genome consists of two single-stranded RNAs, and its genome organization is nearly identical to those of beet necrotic yellow vein virus (BNYVV; **Figure 1G**; Lozano and Morales, 2009). RNA 2 of RSNV possesses three overlapping ORFs that code for polypeptides of 38.4, 12.3, and 15 kDa at amino acid positions 2194–3975, which have a typical motif of triple gene blocks (TGB). The amino acid sequences of these three overlapping ORFs of RSNV are closely related to the TGB of BNYVV, which has been revealed by site-directed mutagenesis as essential for viral movement (Gilmer et al., 1992). These polypeptides are hypothesized to be involved in movement of the virus in infected plants, but again there is no direct experimental evidence to support this hypothesis.

Rice necrosis mosaic virus is a species of the genus *Bymovirus* in the family *Potyviridae* (Adams et al., 2011). The virus particles have flexuous, filamentous structures 550 and 205 nm long and 13–14 nm wide and contain two single-stranded RNAs (Inouye and Fujii, 1977). The virus has been found in Japan and India, but is now less troublesome in these countries. RNMV is serologically related to barley yellow mosaic virus, and only the partial nucleotide sequence at the 3′-terminal of the RNMV RNA1 has so far been determined (Badge et al., 1997). By a reserve genetic system for BaYMV, the P1 protein that is encoded at the 5′-terminal of the BaYMV RNA2 has been indicated to be involved in viral movement (You and Shirako, 2010). Similarly then, the corresponding P1 encoded at the 5′-terminal of the RNMV RNA2 might also be involved in movement of RNMV. Further analyses are, however, required to determine the complete genome structure of RNMV and investigate the molecular mechanisms of its cell-to-cell movement.

CONCLUSION AND PERSPECTIVES

Viral MPs are defined experimentally; when the proteins are mutagenized, they interfere with viral cell-to-cell movement but do not affect virus replication competence in single cells. By using reverse genetic systems for plant viruses, many virus-encoded proteins have been confirmed as viral MPs. But the complexity of the unusual genome organizations and replication strategies for many rice viruses have so far prevented the development of infectious clone systems. To date, only two infectious clone systems of RTBV and RYMV have been developed (Dasgupta et al., 1991; Bonneau et al., 1998). Lack of reverse genetic systems for the rice viruses has made it impossible to use standard mutagenesis methods for functional studies of their encoded proteins that are involved in viral cell-to-cell movement. The viral MP of one particular virus can, however, complement movement not only of closely related but also of distantly related or even unrelated viruses, in spite of the striking diversity of viral movement strategies and the lack of amino acid sequence similarities among the MPs of different virus groups (Nejidat et al., 1991; Solovyev et al., 1996; Morozov et al., 1997; Morozov and Solovyev, 2003; Tamai et al., 2003). By a different experimental approach, that is, using movement-defective viruses by *trans*-complementation experiments with virus proteins of interest, the movement functions of several uncharacterized proteins of rice viruses have been determined (**Table 1**; Li et al., 2004; Huang et al., 2005; Xiong et al., 2008; Wu et al., 2010; Hiraguri et al., 2011, 2012; Zhang et al., 2012).

One of the unique features that many rice viruses are amplified in both plant and insect cells. The Pns10 protein of RDV forms tubular structures in cells of the insect vector and is thought to be associated with intercellular movement in the insect (Wei et al., 2006, 2008; Chen et al., 2012), but the protein does not facilitate movement of the movement-defective PVX mutant in plants (Li et al., 2004). The Pns6 protein of RDV functions as a viral MP in plants, but similar tubular structures are rarely found in RDV-infected rice plants (Fukushi et al., 1962; Shikata, 1969; Boccardo and Milne, 1984). These results indicated that the strategy for intercellular movement by RDV in the insect seems to differ from that in the plant. In contrast, P7-1 of RBSDV forms tubular structures containing virus particles in the RBSDV-infected rice plants and the viruliferous insects, and the protein is considered to function as the tubule-forming MP in both the host plants and the insects (Isogai et al., 1998). The differences in the strategy for movement in plants between RDV and RBSDV may be associated with their different distributions in the plants; RDV is distributed in vascular bundles and in parenchymatous cells, and RBSDV is localized only in the plant phloem and tissues with lesions. Or these unique features of the viral MPs of rice viruses may be required specifically to infect monocotyledonous rice plants. But there is little information on the molecular details for the cell-to-cell movements of rice viruses because most of well-known MPs are encoded in the viruses that infect dicotyledonous plants. To advance research on cell-to-cell movements of rice viruses, efficient experimental methods, such as complementary systems, that work in monocotyledonous plants must be developed; most experimental methods have been developed for dicotyledonous plants and their viruses rather than for monocotyledonous plants.

Plant viruses recruit host factors that facilitate viral cell-to-cell movement through PD and influence the efficiency of viral movement. Numerous studies on the interactions between viral MPs and host factor(s) have progressively revealed the molecular mechanisms by which viral MPs target and modify PD for viral movement (Chen and Citovsky, 2003; Min et al., 2010; Ueki et al., 2010; Amari et al., 2011). Many host factors involved in viral movement have been identified from dicotyledonous plants, but not as many host factors have been identified for monocotyledonous plants (Lu et al., 2009). To advance research on cell-to-cell movement of the rice viruses, we also need to learn more about the host factors that interact with viral MPs during the PD gating process. More experimental evidence is critically needed to understand the molecular details for the cell-to-cell movement of rice viruses and to elucidate the mechanisms underlying movement strategies of viruses in rice.

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Migration of rice planthoppers and their vectored re-emerging and novel rice viruses in East Asia

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This review examines recent studies of the migration of three rice planthoppers, *Laodelphax striatellus*, *Sogatella furcifera*, and *Nilaparvata lugens*, in East Asia. *Laodelphax striatellus* has recently broken out in Jiangsu province, eastern China. The population density in the province started to increase in the early 2000s and peaked in 2004. In 2005, *Rice stripe virus* (RSV) viruliferous rate of *L. striatellus* peaked at 31.3%. Since then, rice stripe disease spread severely across the whole province. Due to the migration of the RSV vectors, the rice stripe disease spread to neighboring countries Japan and Korea. An overseas migration of *L. striatellus* that occurred in 2008 was analyzed, when a slow-moving cold vortex, a type of low pressure system, reached western Japan from Jiangsu, carrying the insects into Japan. Subsequently the rice stripe diseases struck these areas in Japan severely. In Korea, similar situations occurred in 2009, 2011, and 2012. Their migration sources were also estimated to be in Jiangsu by backward trajectory analysis. *Rice black-streaked dwarf virus*, whose vector is *L. striatellus*, has recently re-emerged in eastern China, and the evidence for overseas migrations of the virus, just like the RSV's migrations, has been given. A method of predicting the overseas migration of *L. striatellus* has been developed by Japanese, Chinese, and Korean institutes. An evaluation of the prediction showed that this method properly predicted migration events that occurred in East Asia from 2008 to 2011. Southern rice black-streaked dwarf virus (SRBSDV) was first found in Guangdong province. Its vector is *S. furcifera*. An outbreak of SRBSDV occurred in southern China in 2009 and spread to Vietnam the same year. This disease and virus were also found in Japan in 2010. The epidemic triggered many migration studies to investigate concrete spring-summer migration routes in China, and the addition of migration sources for early arrivals in Guangdong and Guangxi have been proposed. *Nilaparvata lugens* is also an important insect pest of rice. Its migration situations on the Indochina peninsula and return migrations in China are discussed.

Keywords: rice planthoppers, migration, trajectory analysis, entomological radar, virus disease

INTRODUCTION

Some viruses in economic plants spread as they are carried by their invertebrate vectors (Matthews, 1991), and sometimes even migrate overseas. Rice planthoppers, major rice pests, and their vectored rice viruses are examples. Rice planthoppers consist of three species: the small brown planthopper, *Laodelphax striatellus* (Fallén); the white-backed planthopper, *Sogatella furcifera* (Horváth), and the brown planthopper, *Nilaparvata lugens* (Stål; Hemiptera: Delphacidae). Currently, rice planthoppers have been causing various problems in East Asia. An outbreak of *N. lugens* occurred in China, Korea, and Japan in 2005 (Cheng and Zhu, 2006). Major reasons for the outbreak included favorable weather conditions and insecticide resistance of the vector (Cheng and Zhu, 2006; Matsumura et al., 2008). *N. lugens* and its rice viral diseases also severely damaged rice in southern Vietnam in 2006–2007, and the Vietnamese government temporarily stopped the country's rice exports, which affected the world rice market (Chien et al., 2007). *S. furcifera* has caused a novel virus disease in southern China since the early 2000s, and the disease later spread to wide paddy areas in China, northern to central Vietnam, and Japan (Zhou et al., 2010; Hoang et al., 2011; Matsumura and Sakai, 2011). The density of *L. striatellus* in eastern China rapidly increased in the mid 2000s, and an outbreak of rice strip disease occurred (Zhou, 2010). Rice strip disease has spread to Japan and Korea from 2008 to the present (Otuka et al., 2010; Lee et al., 2012). Meanwhile, local plant protection institutes in each country conducted intensive surveys and took effective control measures against the vectors and diseases (Zhou, 2010).

Recent outbreaks of rice planthoppers and related virus diseases that occurred in East Asia were closely related to the vectors' migration. This review, therefore, examines the recent development of migration studies of rice planthoppers in East Asia, and presents a vivid image of the dispersion of viruses vectored by the insects. The review consists of three parts, covering the migration of *L. striatellus*, the migration of tropical rice planthoppers *S. furcifera* and *N. lugens*, and a discussion.

Laodelphax striatellus

This species is widely distributed in East Asia, including Japan, Korea, and China, and transmits *Rice stripe virus* (RSV) to rice

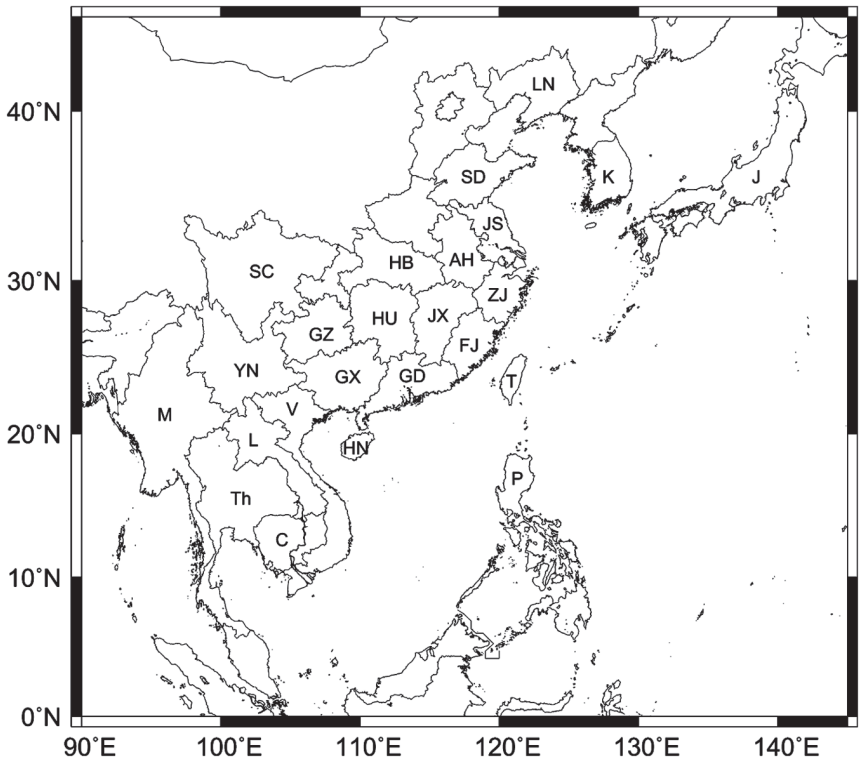

FIGURE 1 | Locations of Chinese provinces and East Asian countries of interest. Abbreviations LN, SD, JS, AH, ZJ, HB, JX, FJ, HU, GD, GZ, GX, HN, SC, T, P, V, C, L, Th, M, K, and J indicate Liaoning, Shandong, Jiangsu, Anhui, Zhejiang, Hubei, Jiangxi, Fujian, Hunan, Guangdong, Guizhou, Guangxi, Hainan, Sichuan, Taiwan, the Philippines, Vietnam, Cambodia, Laos, Thailand, Myanmar, South Korea, and Japan, respectively.

plants in persistent and transovarial manners (Falk and Tsai, 1998). Since *L. striatellus* is able to overwinter at mid-latitudes, including all areas in Japan, outbreaks of rice stripe disease in Japan had been mostly believed to be caused by domestic populations until 2008, when there was an overseas mass migration of *L. striatellus* in early June in western Japan followed by an outbreak of rice stripe disease (Otuka et al., 2010). The migration source was thought to be Jiangsu province, eastern China (**Figure 1**; Otuka et al., 2010). A set of events related to the migration, therefore, had started in China.

RECENT SITUATION IN EASTERN CHINA

The rice-wheat or -barley double-cropping system is used in Jiangsu. Indica hybrid rice was introduced there in the late 1970s in order to increase rice yield, and by the mid-1980s the area of hybrid rice accounted for a third of the total rice area (Gu et al., 2005; Sogawa, 2005). Since *L. striatellus* cannot effectively multiply on hybrid rice compared with japonica rice (Liu et al., 2007), rice stripe disease was not a problem until the end of the 1990s (Sogawa, 2005). In the late 1990s, the ratio of japonica rice started to increase in the province, because japonica varieties of good taste were more profitable in the market than indica hybrid rice varieties (Gu et al., 2005); by 2002 the area of japonica rice exceeded 80% of the total (Sogawa, 2005). However, major japonica varieties used in Jiangsu, e.g., Wuyujing 3 and Wuyunjing 7, were susceptible to both *L. striatellus* and RSV (Yang et al., 2002; Gu et al., 2005). The prevalence of susceptible varieties was the first factor for the outbreak of the insect. Secondly, early rice seeding and early transplanting became popular especially in the middle and northern parts of the province, in order to obtain high and stable yields, making the best use of high temperatures in summer (Yang et al., 2002). This resulted in the overlap of wheat harvesting and rice seedling, making it much easier for the host transfer of *L. striatellus* long-winged adults (Yang et al., 2002). Thirdly, the practice of direct seeding of wheat and barley without plowing after rice harvesting spread in Jiangsu in order to reduce labor. In these fields, the insects transferred easily from rice to wheat or barley (Yang et al., 2002). These three major factors helped the insects rapidly multiply in Jiangsu (Sogawa, 2005). The viruliferous rate of the first generation in Jiangsu peaked at 31.3% in 2005 (Zhou, 2010). Rice stripe disease consequently spread across the entire province. The occurrence area of this disease in paddy fields peaked at 1.57 million ha (79% of the total paddy area) in Jiangsu in 2004 (Zhou, 2010). The use of chemicals to control viruliferous vector insects has been one of the main measures used. An insecticide, imidacloprid or a Chinese product named *bichonglin*, was recommended to spray (Gu et al., 2005; Xi et al., 2005; Xian et al., 2005), but it was applied on average at least five times in a single summer crop (Zhou, 2010). These intensive uses resulted in the development of resistance against the insecticide (Ma et al., 2007; Otuka et al., 2010; Sanada-Morimura et al., 2011).

L. striatellus and rice stripe disease in Jiangsu have been managed as follows. One of the main methods of controlling rice

stripe disease was to introduce rice cultivars resistant to the virus (Iizuka, 1989; Zhou, 2010). When an epidemic of rice stripe disease occurred in the 1980s in the Kanto district of eastern Japan, cultivars resistant to the virus were introduced and the epidemic quickly ceased (Iizuka, 1989), indicating the effectiveness of resistance cultivars. Other methods to control the disease included chemical control of the vectors, introduction of a gap between wheat and rice cultivations (Kiritani, 1983; Wang et al., 2008; Zhou, 2010), covering of rice seedlings with an insect-proof mesh, and plowing before the seeding of wheat and barley in autumn (Zhou, 2010; Zhu, 2012). All the measures taken in Jiangsu are a standard way to control the pest, and the importance of them has been re-recognized there. With these integrated measures, the RSV viruliferous rate in Jiangsu has decreased to a low level of 3% in 2012 (Zhu, 2012).

OVERSEAS MASS MIGRATION OF *L. steriatellus*

The 2008 overseas mass migration of *L. steriatellus* occurred in western Japan under these circumstances in China. Large catches of *L. steriatellus* adults by a net trap (63 insects) or a suction trap (106) were recorded on June 5, 2008, simultaneously at two different sites 100 km apart on Kyushu Island (Otuka et al., 2010). In order to allocate the possible source of the migration, a backward trajectory analysis that traced air parcels backward from points over the trap sites was conducted, and the trajectories reached Jiangsu in about 24–36 h, suggesting Jiangsu may have been the source (Otuka et al., 2010). Immigrants were collected from rice fields in immigrated areas in Japan within 2–4 days after the migration. Insects in Jiangsu province were also collected at the end of September to early October 2008. These insects' resistance to insecticides was tested, and both the immigrants and the Jiangsu population showed resistance against imidacloprid with high LD_{50} values in the topical application method, whereas Japanese domestic populations collected in Kyushu before the 2008 migration event showed susceptibility to the same insecticide (Otuka et al., 2010). In addition, the RSV viruliferous rates of the immigrant populations were reported to be higher (9.2–11.5%) than those of the domestic populations (2.9–4.0%) by enzyme-linked immunosorbent assays (Nakagawa and Mizobe, 2010; Otuka et al., 2010), indicating supportive evidence for the overseas migration.

An outbreak of rice stripe disease subsequently occurred on the western coast of the Kyushu and Chugoku districts (Ohtsu et al., 2009; Otuka et al., 2010; Nakagawa and Mizobe, 2010). The total occurrence area of rice stripe disease in Nagasaki prefecture in 2008 was 10,720 ha, which was a 126% increase over the previous year (MAFF, 2009). All these analytical results suggested that *L. steriatellus* migrated from Jiangsu to western Japan and caused the outbreak of rice stripe disease.

IN KOREA

Responding to the mass migration to Japan, Korean scientists quickly set up a monitoring network of 13 net traps (10 m high above the ground) for *L. striatellus* along their western coast in May 2009. A similar mass immigration occurred from May 30 to June 1, 2009 (Choi et al., 2010; Otuka et al., 2012a). The catch numbers at Sinan and Taean, located along the western coast of the Korean peninsula, were 819 and 963, respectively, about 10 times larger than those in the previous Japanese case. Based on the backward trajectory analysis, a possible migration source for the Korean case was found to be Jiangsu (Otuka, 2009). The RSV viruliferous rate of *L. striatellus* was 5.3%, and the occurrence area of rice stripe disease, located along the western coastal areas, was 21,500 ha in 2009 (Lee et al., 2012). Similar migration events occurred also in Taean, Gunsan, and Buan in 2011, and in Taean in 2012 (Jeong et al., 2012; Lee et al., 2012). According to surface weather maps for the times when these migration cases occurred, low-pressure systems over Bohai Sea in 2009 and 2011, and a high pressure system over the southern Yellow Sea in 2012, caused southwesterly winds that might have carried the insects to Korea.

CHARACTERISTICS OF THE OVERSEAS MIGRATION

The frequency of possible overseas migration of *L. striatellus* into the northern Kyushu district in relation to weather conditions was analyzed (Syobu et al., 2011). The investigation covered the 10-year period from May 21, 2000, to June 10, 2009. One peak trap catch was recorded on May 27–28, 2006, and was associated with strong westerly winds at 850 and 925 hPa levels to the south of a cold vortex that passed over the southern part of the Korean peninsula. The backward trajectory analysis suggested Jiangsu, China, as a possible migration source. Another case was the event in 2008 mentioned above. No immigration was found in Japan from 2010 to 2011 (Otuka et al., 2012a). Thus, in total two overseas migrations of *L. striatellus* may have occurred in 12 years in Japan.

On the other hand, three overseas migration events may have occurred in Korea form 2009 to 2012. In addition, Lee et al. (2012) reported that severe damage to rice by rice stripe disease occurred in western areas in 2001, 2007, and 2008, but it is not certain whether these damages were caused by local *L. striatellus* populations or overseas immigrants. However, a migration was predicted in 2008 by Figure 6a in Otuka et al. (2012a), and the predicted areas in southwestern Korea matched the damaged areas: Sinan, Jindo, Haenam, and Wando (Kim et al., 2009; Lee et al., 2012). Therefore, the 2008 case may be one of the overseas migrations. Thus, the frequency of a possible overseas migration in Korea would be four times in 5 years (2008 to 2012), which is higher than that in Japan. The difference in the event frequency is attributable to the location of the destination from the source. Korea is northeast of Jiangsu, whereas Japan is west of Jiangsu. In the middle latitudes, southwesterly winds blow frequently to the south of a low pressure system. The low-level jet stream over the East China Sea carrying various insects is a typical example (Seino et al., 1987). In contrast, sustaining westerly winds by a cold vortex seems less frequent (Syobu et al., 2011). Therefore, it can be said that Korea is unfortunately located in a migration-preferred direction from the source area.

The distance between the source and the destination might have affected the number of immigrants. The distance between western Japan and the coastal line of Jiangsu province is about 750 km, whereas that between the Korean peninsula and Jiangsu is about 500 km. The catch numbers in the net trap in Korea in 2009 were more than ten times larger than those in Japan. Moreover, the distribution of the immigrated areas in Japan and Korea is of interest. All estimated immigrated areas were confined to

coastal regions (Ohtsu et al., 2009; Nakagawa and Mizobe, 2010; Lee et al., 2012). For example, in 2008 rice stripe disease occurred heavily in Nagasaki prefecture, located in the westernmost part of Kyushu Island, but no epidemic of the disease was reported in a neighboring prefecture, Saga (Ohtsu et al., 2009). In Yamaguchi prefecture, the outbreak of the disease happened mainly in the coastal areas (Nakagawa and Mizobe, 2010). Similarly, the disease's recent occurrence area in Korea was distributed along the western coastal line. These facts imply that *L. striatellus* invaded these areas by flying at low altitudes, because an outbreak of the disease could have occurred inland if the insects had flown at altitudes high enough to cross the mountains.

DOMESTIC DISPERSION AND MIGRATION IN CHINA

When the outbreak of *L. striatellus* and rice stripe disease spread to all of Jiangsu around 2004, the neighboring provinces of Zhejiang, Anhui, Shanghai, Shandong, and Hubei started to suffer similar problems (Wang et al., 2008). In Zhejiang province, the disease spread rapidly southward, from the northern to centraland eastern regions, with an increasing incidence each year from 2003 to 2006 (Wang et al., 2008). By 2006, the disease was severe in the northernmost parts of the province. The timing of the epidemic in Zhejiang province was just after the outbreak in Jiangsu. Increasing populations of RSV viruliferous vectors early in the season were clearly the primary source of the epidemic (Wang et al., 2008). Wang et al. (2008) did not discuss the source of the vectors. However, the situation in Zhejiang province and that in Jiangsu province suggest that the migration of viruliferous *L. striatellus* from Jiangsu could be, at least partly, the cause of the epidemic. More clearly, domestic migrations of the vectors were recently investigated in Zhejiang, Anhui, and Shandong provinces (Wan et al., 2011; Zhang et al., 2011; He et al., 2012). For example, a large immigration of *L. striatellus* in Jining, southern Shandong province, north of Jiangsu province, was observed by a light trap in the late night of 7 June, 2009, and backward trajectories suggested that a possible migration source was northern Jiangsu province (Zhang et al., 2011). They also studied forward trajectories for an emigration peak on 15 June, 2010, and the trajectories reached Liaoning province, suggesting a domestic overseas migration (Zhang et al., 2011). Similarly, possible migration from Jiangsu province to Liaoning province, a kind of overseas migration, has also been suggested (Zhou and Cheng, 2012).

DIVERSITY OF RSV

The vector migrates a long distance, and RSV does so as well. Therefore, the distribution of a viral population may be affected by the host's migration. The genetic diversity of the virus has been studied in East Asia (Wei et al., 2009; Jonson et al., 2009; Sakai et al., 2011). These studies included phylogenetic analyses of the nucleotide sequences of nucleocapsid protein (N) and RNA3 intergenicregion (IR3) or the whole sequence of RNA3 of RSV isolates collected in Japan (collection years 2008 and 2009), eastern China (1997–2004), and western Korea (2007–2008). The results showed that RSV isolates in China were divided two types, labeled type I and II. The isolates from eastern China including Jiangsu consisted of type I, and those from Yunnan province, southern China, formed type II. Isolates from the Kyushu district of western Japan and most of the isolates from western Korea belonged to type I. Additionally, isolates from the Kanto district, eastern Japan, and one isolate from Korea formed another subtype (J-K subtype in Sakai et al., 2011, or type II in Jonson et al., 2009). The distance between the Kyushu and Kanto districts is about 1,000 km, and the Kanto district is far from Jiangsu. These studies suggested that the RSV populations in the Kyushu district, Korea, and eastern China are indistinguishable from each other, and that the migration of *L. striatellus* from eastern China to Japan and Korea may have affected the structure of the RSV population in East Asia.

RICE BLACK-STREAKED DWARF VIRUS

Rice black-streaked dwarf disease caused by *Rice black-streaked dwarf virus* (genus *Fujivirus*; RBSDV) emerged in late *japonica* rice in Zhejiang province, eastern China in 1989, and expanded in the 1990s, having four major outbreaks in 1992, 1996, 1997 and 1998 (Wang et al., 2009). The epidemic of the disease on late *japonica* rice in Zhejiang province continued in the 2000s, and the total affected area increased from 26,000 ha in 2000 to 64,640 ha in 2005, spreading from eastern part to central and southern parts of the province (Wang et al., 2009). The virus has been detected in almost all the area of the province from 2008 to 2011(Wu et al., 2013). As the density of the vector *L. striatellus* in Jiangsu province, a northern neighboring province of Zhejiang, increased in the 2000s as described above, the occurrence of rice black-streaked dwarf disease also increased from 20,500 ha in 2007 to 33,300 ha in 2009, and the paddy area of complete yield loss in the province reached to 2,000 ha in 2008 (Lan et al., 2012). When overseas mass migrations from Jiangsu province to South Korea possibly happened, the RBSDV viruliferous rate of *L. striatellus* caught in net traps along the western coast of the Korean peninsula in early June 2009 and 2011 were found to be 3.1 and 4.4 percent, respectively (Kim, 2009; Jeong et al., 2012), indicating that migrations of both RSV and RBSDV likely occurred due to the vectors' movement over the sea. No vector immigrant with co-infection of RSV and RBSDV in Korea has been found so far (Kim, 2009; Jeong et al., 2012). Since no resistant gene of rice for RBSDV has been found, vector control by chemicals in early susceptible stages of rice plants, and the use of an insect proof net for rice seedlings are major effective measures of disease control (Lan et al., 2012).

PREDICTION OF MIGRATION OF *L. striatellus*

A method has been developed to predict the overseas migration in East Asia (Otuka et al., 2012a). The source of the migration is assumed to be Jiangsu, China. The method consists of two steps: prediction of the emergence of the first generation of *L. striatellus* (the first step), and simulation of the migration route during a predicted emigration period (the second step; Otuka et al., 2012a).

The prediction of emergence is performed by calculating the daily increase in effective accumulative temperature (EAT) for *L. striatellus* starting from the first day of each year. The EAT is calculated with daily minimum and maximum temperatures in Dongtai, a city in central Jiangsu, and the data are obtained through the Internet in real time. Growth parameters of the insect for the EAT calculation, such as low-limit temperature and growth-stop temperature, are those estimated for Japanese populations. The EAT value is updated daily, and a presumed increase is added

until the predicted value exceeds a pre-defined threshold. The threshold for the emergence was previously determined by analyzing past migration events. The migration prediction period is 9 days, starting from the predicted emergence day − 3 days (prediction error), and ending on the emergence day + 5 days (pre-emigration period of 2 days + prediction error). This is the prediction period.

Migration is then predicted during the prediction period (the second step). In the prediction model, insects take off at dusk and dawn then fly upward at a speed of 0.2 m/s for an hour to make use of upper winds favorable to migration. There is no such observation for *L. striatellus* so far, but there is a radar observation of *N. lugens* flying upward in autumn in Jiangsu (Riley et al., 1991). During migration the insects move at the velocity of the wind, which is forecasted by a weather prediction model. The migration lasts 24 h. The relative aerial density of the insects in the lowest calculation layer is calculated on the basis of the number of insects in each grid cell, and is drawn on a map. By this prediction, the areas and timing of immigration in Japan and Korea are presented. The prediction method was evaluated against the past migration events occurring in Japan and Korea, and the prediction had a 90% accuracy rate (Otuka et al., 2012a). Now the method is implemented and operational in JPP-NET (http://www.jppnet.or.jp), database service provided by the Japan Plant Protection Association, Tokyo.

Sogatella furcifera AND *Nilaparvata lugens*

EPIDEMIC OF SRBSDV AND MIGRATION OF ITS VECTOR

S. furcifera is a vector of SRBSDV that causes stunting, leaf darkening, and small enations on the stem and leaf back of rice plants (Zhou et al., 2008, 2013). This disease was first recognized in 2001 in Guangdong province, southern China, and in the next few years spread gradually to Guangxi, Hainan, Hunan, and Jiangxi provinces, but its infected hill ratio was low, 1% or less (Zhou et al., 2008,2010). In 2009, however, an epidemic of the disease occurred in Guangdong, Guangxi, Hainan, Hunan, Hubei, Jiangxi, Fujian, Zhejiang, and Anhui provinces; the total infected paddy area was about 400,000 ha, and a paddy area of 6,700 ha suffereda complete yield loss (Zhou et al., 2010; Zhao et al., 2011b). In the same year, an outbreak also occurred in Vietnam, where 19 central to northern provinces suffered from the disease (Hoang et al., 2011). In 2010, the infested area in China increased to more than 1.3 million ha in 13 provinces (Zhao et al., 2011b). In August 2010, the disease was first detected in forage rice in western Japan (Matsumura and Sakai, 2011). The virus was detected also across all of Zhejiang province by 2011 (Wu et al., 2013). The rapid spread of the virus throughout East Asia attracted scientists' renewed attention to the migration of the vector, *S. furcifera.*

EARLY IMMIGRATION IN GUANGDONG AND GUANGXI

Since Guangdong and Guangxi are possible sources of *S. furcifera* and *N. lugens* immigrants in more northern paddy areas in China (Cheng et al., 1979; NCRG, 1981), early immigrations from April to early May in these areas and their sources are important. Recent trajectory analyses with the simulation model HYSPLIT (Draxler and Hess, 1998) in these areas indicated that possible sources of the early migrations of *S. furcifera* and *N. lugens* were Hainan province, northern and central Vietnam, and southern Laos (Qi et al., 2010a, 2011; Shen et al., 2011b; Wang et al., 2011b; Jiang et al., 2012). The winter-spring rice crop along the coast of central Vietnam is earlier than that in northern Vietnam where the main emigration begins in May (Otuka et al., 2008; Zhai and Chen, 2011), and rice plants in the central region mature in April, which makes them a possible source (Shen et al., 2011b). In addition, SRBSDV in Vietnam in 2009 was distributed in the central regions as well as in the northern delta, suggesting that there was viral dispersion to central Vietnam from southern China, where SRBSDV originated (Hoang et al., 2011). Laos's border with Vietnam is mountainous, providing a possible barrier to migrations heading northeast. Chinese hybrid rice susceptible to *S. furcifera* is popular in northern Vietnam (Hoang et al., 2011), while sticky rice is popular in Laos. Information on the occurrence of rice planthoppers in spring in southern Laos is limited. Hence, it may be premature to conclude that Laos is a source of the early migration, and further investigation of rice planthoppers' migration and the occurrence of SRBSDV in Laos is necessary. It is likely that both the central and northern parts of Vietnam, as well as southern Hainan province in China, are source areas for the early immigration to Guangdong and Guangxi (Zhai et al., 2011).

It is generally believed that the East Asian populations of *S. furcifera* and *N. lugens* overwinter in Vietnam and southern Hainan province, and that in spring they migrate northeastward to eastern China, Japan, and Korea utilizing southwesterly monsoons, then migrate southward back to overwintering areas in autumn (Kisimoto, 1976; Cheng et al., 1979; NCRG, 1981). These tropical rice planthoppers of the Red River Delta in northern Vietnam have been recognized as a main source for the East Asian population, since *N. lugens* shifted from biotype 1 to biotype 2 synchronously in northern Vietnam, China, and Japan from the late 1980s to the beginning of the 1990s (Sogawa, 1992). Now most scientists who study rice insect pests believe the rice planthoppers originally migrated from northern Vietnam and southern Hainan (Zhai et al., 2011). The recent studies on the early migrations in Guangdong and Guangxi presented a modification of the initial belief about migration.

Regarding the epidemic of SRBSDV in China, migrations of *S. furcifera* in 2009 and 2010 in the northeastern paddy areas between June and July have been investigated as well (Zheng et al., 2011; Zhao et al., 2011a,b; Diao et al., 2012). Immigration sites were located in central Zhejiang, southern Anhui, and southern Jiangxi provinces. Their possible sources were estimated to be Guangdong, eastern Guangxi, southern Jiangxi, and southern Fujian provinces for the Zhejiang case; Jiangxi and Hunan provinces for the Anhui case; and mainly Guangdong province for the Jiangxi case. Additionally, source areas for *S. furcifera* immigrations in the Kyushu district, western Japan, in June and July, were estimated to be mostly Fujian (Otuka et al., 2005). On the other hand, source areas for *S. furcifera* immigrants in southern Fujian in April to May from 2007 to 2010 were estimated to be Guangdong and Hainan (Shen et al., 2011a). Possible immigration sources in April to May in western Taiwan were also estimated to be Guangdong, Hainan, and the Philippines (Huang et al., 2010).

YUNNAN PROVINCE AND INDOCHINA PENINSULA

Yunnan province is located east of Myanmar, north of Laos and Vietnam, west of Guangxi and Guizhou provinces, and south of Sichuan province **(Figure 1)**. Temperatures in winter and spring in the two-crop rice area in Yunnan are relatively high due to the low latitude, and small numbers of *N. lugens* and *S. furcifera* can overwinter on rice seedlings and ratoons (Shen, 2010). But immigrants in spring cause most of the major damage (Shen, 2010). Sources of *S. furcifera*'s large immigrations in April to early May in Yunnan were estimated to be mainly Myanmar, and immigrations in mid-May were thought to be from northern Vietnam (Shen et al., 2011c). In Myanmar, new irrigation systems have been developed recently and the winter-spring rice crop in the dry season has increased (Shen, 2010). The change in the farming system in Myanmar seems to be a cause of *S. furcifera*'s immigration to Yunnan.

In the Mekong Delta of Vietnam, in the southeastern end of the Indochina peninsula, an epidemic of *N. lugens* populations has occurred since the winter-spring rice crop in 2005–2006 (Chien et al., 2007; Hoang et al., 2011), where co-infection of two emerging viruses, *Rice grassy stunt virus* (RGSV; genus *Tenuivirus*) and *Rice ragged stunt virus* (RRSV; genus *Oryzavirus*)severely damaged more than 485,000 ha of rice production area, resulting in the loss of 828,000 tons of rice (Du et al., 2007; Cabauatan et al., 2009). The co-infection causes yellowing and light stunting of rice leaves (yellowing syndrome; Du et al., 2007). The rate of single infection with RGSV or RRSV, and the rate of co-infection with the two viruses were observed in 90, 65, and 65% of rice plants collected from 6 provinces in the delta in August 2006, respectively, whereas the rate of *N. lugens* carrying RGSV, RRSV, and both the viruses were 66, 41 and 8%, respectively (Du et al., 2007). Recently, the two genes encoded by each ambisense segment RNA3 and RNA5 of RGSV isolates from 6 provinces in southern Vietnam (5 provinces in the delta and BinhThuan province, a southeastern province near the delta) were sequenced. The results showed no relationships between the genetic diversity and the geographic distribution of the RGSV isolates, suggesting the viruliferous vector *N. lugens* migrates in southern Vietnam (Ta et al., 2013).

An escape strategy using a monitoring light trap, which shifts the timing of seeding to avoid the peak period of *N. lugens* immigration from neighboring areas, has been established in the delta to reduce viral infection (Chien et al., 2007, 2012), and campaign efforts to reduce seeding rate, fertilizer rate, and insecticide use have also decreased the density of *N. lugens* (Huan et al., 2005, 2008). Regarding the pest's resistance to insecticides, however, the *N. lugens* population in the delta is more resistant than a population in northern Vietnam (Matsumura and Sanada-Morimura, 2010). The migration of this insecticide-resistant and viruliferous population is of much interest but is not well known. The monthly mean wind direction from 1979 to 2009 over the delta was investigated recently, and it was found that easterly winds and westerly winds dominated from October to April and from June to September, respectively (Shen, 2010; Zhai et al., 2011). Southerly or southwesterly winds that could carry planthoppers to the north of the peninsula scarcely occurred. This result suggests only a small chance that *N. lugens*'s genes flowed from the delta to the northern peninsula.

Migration in Thailand is not well known. Outbreaks of *N. lugens* have occurred in irrigated areas of central Thailand since 2009 (Chaiyawat et al., 2011). A trajectory analysis was applied to light trap data obtained in central Thailand in 2009, revealing three catch peaks: March to early April, the end of July, and November (Shen, 2010). For example, the analysis suggested that emigrants from central Thailand in July could reach Laos, central Vietnam, and Cambodia under seasonal westerly winds.

DISCUSSION

FLIGHT DURATION OF TROPICAL POPULATIONS

A number of migration studies of rice planthoppers in northern Vietnam, China, Japan, and Korea have been conducted, but relatively few studies have covered the Indochina peninsula and farther-western rice-producing areas such as Bangladesh and India. The previous migration studies of *N. lugens* in Thailand and southern Vietnam used flight durations of 12–36 h (Shen, 2010; Zhai et al., 2011). However, flight duration, or the flight distances of most migrating tropical populations, might be shorter than that. In the tropics, shorter migration distances, such as a few to 30 km, have been estimated by radar observations or yellow pan water-trapping in the Philippines (Perfect and Cook, 1987; Riley et al., 1987). The flight duration of *N. lugens* macropters collected in a rice field in the Philippines in a tethered flight experiment peaked at only 3–4 h (Padgham et al., 1987). Moreover, physiological characteristics such as the pre-ovipositional period and starvation tolerance of macropters of tropical *N. lugens* populations are different from those of East Asian *N. lugens* populations (Wada et al., 2007, 2009). To unveil flight durations or distances in tropical areas of Thailand, southern Vietnam, Cambodia, etc., monitoring with a tow net trap mounted to a tall pole is recommended in combination with the standard migration analysis.

POPULATION DYNAMICS OF *S. furcifera* AND *N. lugens*

For *S. furcifera and N. lugens* in Japan, the paddy-field population is established by the overseas adult immigrants usually in July, soon after the rice transplanting (Kuno, 1968). Then *S. furcifera* generally produces two generations and the adult macropters of the second generation emigrates from the paddy fields, whereas *N. lugens* multiplies until the third generation and more, resulting in a high growth rate and causing hopperburns (Kuno, 1968). In the tropics, *S. furcifera* shows the same population dynamics as in Japan, but *N. lugens* generally produce two generations until harvest of rice, and the second generation shows a peak number (Tsurumachi, 1986). Therefore, the vector's emigration timing may differ between in the tropics and in the temperate zone. Since rice seedling and rice plants of young stage are more susceptible to infection of RRSV than those of later stages (Tsurumachi, 1986), the Vietnamese escape strategy to escape *N. lugens*'s mass immigration, hence to avoid early viral infection, is reasonable.

RADAR MONITORING AND RETURN MIGRATION

Entomological radar has a 40-year history and has provided much information about flying insects at altitudes from the ground (Chapman et al., 2011). Radar observation of rice planthoppers was conducted in the 1980s and early 1990s (Riley et al., 1987, 1991, 1994). Findings on observations, such as vertical group velocity of

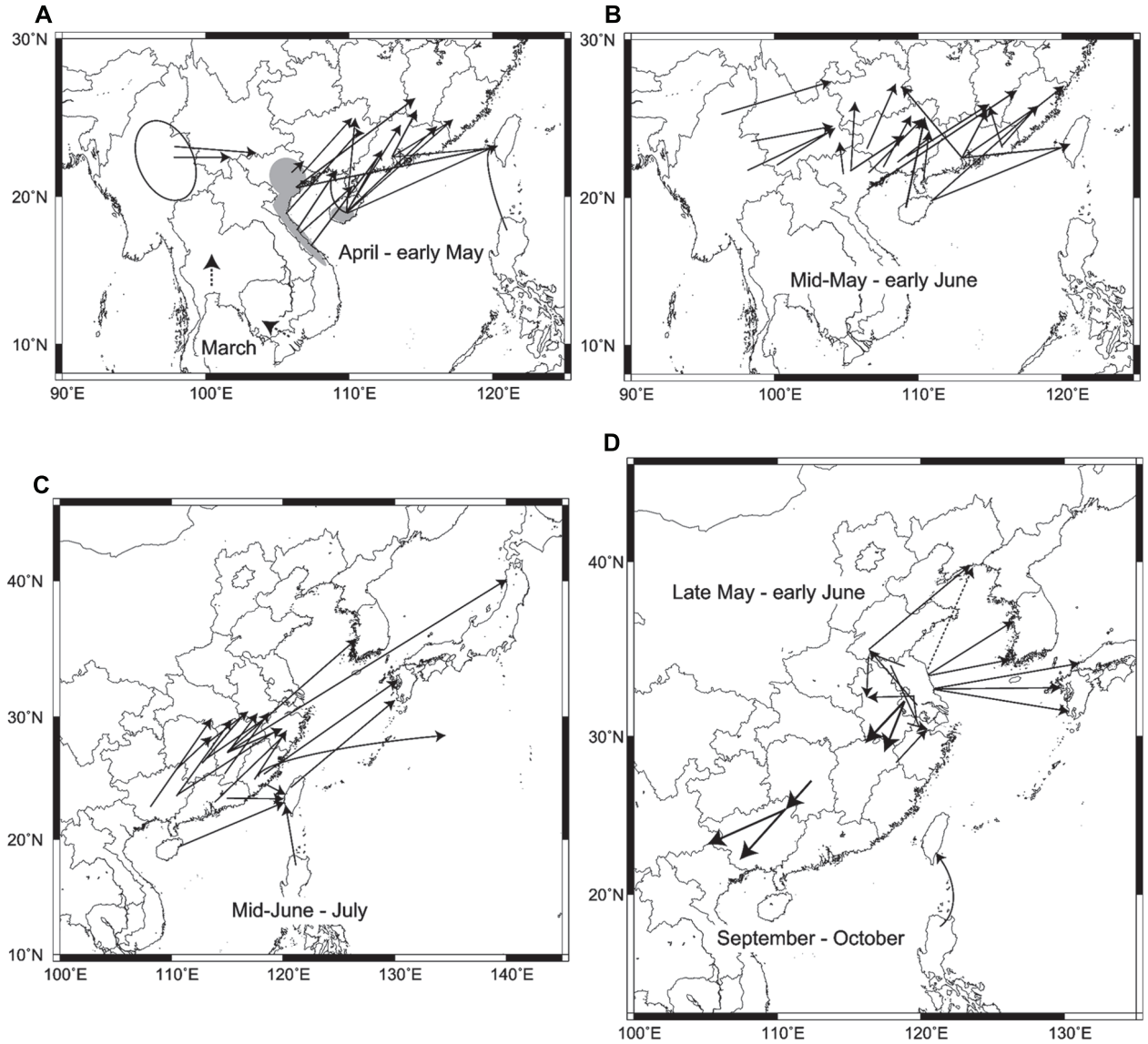

FIGURE 2 | Migration routes obtained by trajectory analyses and migration simulations: (A) routes of *Sogatella furcifera* and *Nilaparvata lugens* in early season from March–April to early May. Dashed arrows in Thailand and southern Vietnam in March are hypothetical routes based on seasonal winds. Gray areas indicate overwintering areas in Vietnam and Hainan. An ellipse shows a possible source area in Myanmar. Data from Otuka et al. (2008; 2010), Qi et al. (2010a), Shen (2010), Shen et al. (2011a,b,c), Wang et al. (2011b), Zhao et al. (2011b), Zhai et al. (2011), Jiang et al. (2012), and Wu et al. (2012), **(B)** routes of *N. lugens* and *S. furcifera* from mid-May to early June. Data from the same as in **A, (C)** routes of *N. lugens* and *S. furcifera* from mid-June to July. Data from Turner et al. (1999); Hua et al. (2002), Otuka et al. (2005); Zhao et al. (2011a), Zheng et al. (2011); Diao et al. (2012), and Otuka (2012), **(D)** routes of *Laodelphax striatellus* from late May to early June, return migration routes of *N. lugens* in September to October, and a route from the Philippines to Taiwan by a typhoon in September. The dashed arrow shows the hypothetical route based on Zhou and Cheng (2012). Data from Riley et al. (1991), Otuka (2009), Qi et al. (2010b), Wang et al. (2011a); Zhang et al. (2011), Jiang et al. (2012); He et al. (2012), Otuka et al. (2012a,b), and Zhou and Cheng (2012).

0.2 m/s after takeoff and random heading during flight (Riley et al., 1991), have been used to model a planthopper in a migration simulation model (Furuno et al., 2005). Yang et al. (2008) developed a millimetric scanning radar (8.8 mm wavelength, 10 kw peak power, 1.2 m dish) in China and observed echoes mainly from *N. lugens* and the rice leaf roller, *Cnaphalocrocismedinalis* (Lepidoptera: Pyralidae). This radar is located in Xing'an, northeastern Guangxi, under the main migration route of rice planthoppers. An autumn return migration of *N. lugens* was analyzed by the same radar, and dense echo layers were observed at altitudes between 600 and 1,100 m above ground on the night of October 1 to 2, 2009 (Qi et al., 2010b). The migration direction was toward the southwest. Another study found a sign of collective orientation in *N. lugens* autumn migration (Jiang and Cheng, 2011), which is known as a dumbbell pattern in the radar echo (Drake and Reynolds, 2012). If collective orientation

was common in *N. lugens*'s migration, the simulation model should be modified to take that into account. Further study is expected.

In summary, the major migration routes of three rice planthopper species in East Asia are illustrated in **Figure 2**. This figure, which has been made for the first time from many analytical results of trajectory analysis and migration simulation, is much more specific, concrete, and accurate, including the latest situations in East Asia, than a previous summarized figure in Cheng et al. (1979).

Early immigrants of *N. lugens* and *S. furcifera* in March and April start to arrive from central Vietnam and southern Hainan in rice seedlings or paddy fields of early rice crops in southern Guangxi, Guangdong, and southern Fujian in some years (**Figure 2A**; Shen et al., 2011a,b; Wang et al., 2011b). During the same period, *S. furcifera* arrives in Yunnan from Myanmar (Shen et al., 2011c). The dashed arrows on the map of southern Vietnam and Thailand indicate possible migration in March from winter-spring crops, with unknown migration distances. By early May, rice planthoppers reach northern Guangxi, Guangdong, southern Jiangxi, Fujian, and Taiwan from northern Vietnam, Hainan, and already invaded areas in southern Guangxi and Guangdong (**Figure 2A**; Huang et al., 2010; Qi et al., 2010a; Shen et al., 2011a; Zhao et al., 2011b). The areas of invasion gradually spread northward by early June as the monsoons penetrate northward (**Figure 2B**). *N. lugens* and *S. furcifera* migrate from southern China to paddy fields in the middle and lower reaches of the Yangtze River, western Japan, and Korea from mid-June to July (**Figure 2C**). Most of the migration routes are slanted in the same direction due to southwesterly low-level jet streams, like the diagonal belt region for early migration described in Otuka et al. (2008). It was shown that overseas migration routes appear to be longer than Chinese routes over land. An estimated flight duration of 58 h (2470 km) has been reported for a migration of *S. furcifera* to northern Japan arriving on July 11, 1987 (**Figure 2C**; Otuka, 2012). A historical migration of *S. furcifera* and *N. lugens* to South Point (29°N, 135°E) over the Pacific Ocean on July 16–17, 1967 (Asahina and Turuoka, 1968) was also analyzed, and Fujian was identified as a possible source (52 h, 1770 km; Otuka, 2012). In autumn, *N. lugens* was observed by entomological radars, and trajectory analyses showed return migrations in a southwest direction (arrows pointing to the southwest in **Figure 2D**; Riley et al., 1991; Qi et al., 2010b; Jiang et al., 2012). Overseas and Chinese domestic migrations of *L. striatellus* in late May to early June are shown around Jiangsu in **Figure 2D**.

ADDITIONAL INFORMATION TO SOURCE ESTIMATON

Trajectory analysis and migration simulation are the standard method for finding possible migration sources of rice planthoppers. Moreover, to improve this method's accuracy, different types of additional information were combined or tested, including the data on ovarian development, genetic diversity, insecticide resistance, and trace elements in the insect's body (Mun et al., 1999; Qi et al., 2010a; Otuka et al., 2010, 2012b; Peng et al., 2011; Fu et al., 2012; Matsumoto et al., 2013; Zheng et al., 2013). Ovarian grade in a light trap or paddy field can yield information on differences between emigrants (earliest grade) and immigrants (later grades). Genetic analyses of rice planthoppers' populations in Asia have shown that the internal transcribed spacer (ITS) region of the ribosomal RNA gene of *S. furcifera* populations and the mitochondrial *cox1-trnL2-cox2* regions of *N. lugens* and *S. furcifera* populations did not provide molecular markers with which to discriminate Southeast Asian and East Asian populations (Mun et al., 1999; Fu et al., 2012; Matsumoto et al., 2013). This is because (1) the ITS region of *S. furcifera* is too variable to allow discrimination of local populations, and (2) rice planthoppers that migrate for long distances have well-mixed Asian populations for a long time, and the mitochondrial genetic structures of the populations reflect genetic flow over a longer period (Matsumoto et al., 2013). Thus, there has been no report thus far of a molecular marker for Asian rice planthoppers to help to determine or suggest a migration source.

Meanwhile, the insecticide resistance of both immigrant and local populations can provide good information about differences between two populations, when the two resistance levels are different in estimated source and destination areas. Such was the case when *L. striatellus* migrated from eastern China to western Japan in 2008 (Otuka et al., 2010), and when *N. lugens* migrated from the Philippines to Taiwan under typhoon-induced windy conditions in 2010 (Otuka et al., 2012b). In both cases, differences in insecticide resistance between the source and destination populations were utilized to strengthen the identification of migration sources. Recently, trace element content in *N. lugens*'s body was studied to find regional differences. In the study by Peng et al. (2011), concentrations of 23 trace elements (Mn, Mo, Cd, and etc.) in 53 samples from seven regions in southern China were determined; the samples were successfully discriminated by region as a result. In order to apply this idea to a regular migration analysis, a basic database of regional differences in trace element contents in rice planthoppers is necessary. A scientific explanation of how the difference appears based on detailed environmental factors, such as plant, soil, water, and air quality, is also expected.

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Detection and diagnosis of rice-infecting viruses

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Rice-infecting viruses have caused serious damage to rice production in Asian, American, and African countries, where about 30 rice viruses and diseases have been reported. To control these diseases, developing accurate, quick methods to detect and diagnose the viruses in the host plants and any insect vectors of the viruses is very important. Based on an antigen–antibody reaction, serological methods such as latex agglutination reaction and enzyme-linked immunosorbent assay have advanced to detect viral particles or major proteins derived from viruses. They aid in forecasting disease and surveying disease spread and are widely used for virus detection at plant protection stations and research laboratories. From the early 2000s, based on sequence information for the target virus, several other methods such as reverse transcription-polymerase chain reaction (RT-PCR) and reverse transcription-loop-mediated isothermal amplification have been developed that are sensitive, rapid, and able to differentiate closely related viruses. Recent techniques such as real-time RT-PCR can be used to quantify the pathogen in target samples and monitor population dynamics of a virus, and metagenomic analyses using next-generation sequencing and microarrays show potential for use in the diagnosis of rice diseases.

Keywords: detection, diagnosis, rice viruses, LAR, ELISA, multiplex RT-PCR, RT-LAMP, real-time RT-PCR

INTRODUCTION

Rice, second only to corn in worldwide crop production, is produced in all Asian countries, in most of South and Central America, and in some of central and eastern Africa. More than 80% of the world's rice is grown in South, East and Southeast Asian areas where the hot, humid climate favors not only rice but also the viruses and their vectors and about 30 rice viruses diseases have been reported (Abo and Sy, 1998).

In the early years of plant virus research, detection and identification of viruses were based on symptom development on infected plants or biological indexing. However, the use of symptoms for diagnosis is not reliable because symptoms vary depending on the virus strain, the presence of any mixed viral infections, the cultivar and growth stage, growing environment, and sometimes, the resemblance of viral symptoms to those induced by environmental injury. Biological assays are one of the most widely used methods among the many diagnostic techniques for plant viruses because the assays are simple and do not require special knowledge or skill (Jones, 1993; Naidu and Hughes, 2001). However, the majority of rice viruses are only transmissible by vectors, and their host range is limited to gramineous plants (Abo and Ali Fadhila, 2001). Therefore, experimental transmission of rice viruses, e.g., with mechanical or graft inoculation of indicator plants, is inconvenient and not applicable to detect or diagnosis rice viruses.

In addition, since almost all rice viruses are vector-borne, detection methods for rice viruses in their vectors, which show no noticeable symptom, have been needed to forecast and counter disease outbreaks. For these reasons, the development of serological methods based on an antigen–antibody reaction have been active areas of research, and highly sensitive and specific methods (RT-PCR, RT-LAMP, real-time RT-PCR) based on molecular or nucleic-acid techniques have recently become available. This review introduces major methods to detect target rice viruses from the 1980s and summarizes the potential of the current technologies in contributing to diagnosis of rice diseases.

BASIC PROPERTIES OF RICE VIRUSES

The major rice viruses in Asian areas are transmitted by sucking insect vectors. Eight of these are transmitted by planthoppers or leafhoppers in a persistent manner (Hibino, 1996; Zhou et al., 2008): *Rice stripe virus* (RSV, a member of the genus *Tenuivirus*, negative sense ssRNA virus), *Rice dwarf virus* (RDV, member of *Reoviridae*, dsRNA virus), *Rice gall dwarf virus* (RGDV, a member of *Reoviridae*, dsRNA virus), *Rice ragged stunt virus* (RRSV, a member of *Reoviridae*, dsRNA virus), *Rice grassy stunt virus* (RGSV, a member of the genus *Tenuivirus*, negative sense ssRNA virus), Rice transitory yellowing virus [RTYV, same species as *Rice yellow stunt virus* (RYSV), a member of *Rhabdoviridae*, positive sense ssRNA virus; Hiraguri et al., 2010], *Rice black streaked dwarf virus* (RBSDV, a member of the *Reoviridae*, dsRNA virus) and Southern rice black streaked dwarf virus (SRBSDV, may be a member of the *Reoviridae*, dsRNA virus). Detection of these viruses in insect vectors may be easier than in rice plants, since these viruses propagate in insect bodies and the entire insect can be tested in the procedure. The tungro-disease-associated viruses, *Rice tungro bacilliform virus* (RTBV, a member of the *Caulimoviridae*, dsDNA) and *Rice tungro spherical virus* (RTSV, a member of Secoviridae, positive sense ssRNA virus) are non-propagative

and transmitted in a semipersistent manner by leafhoppers, therefore, it is difficult to detect these two viruses in the insect vector using serological methods, but more sensitive methods such as real time RT-PCR and RT-LAMP can be used (Le et al., 2010; Malathi and Mangrauthia, 2013). Detection of viruses in the insect vectors is very important to forecast outbreaks and monitor disease spread because major outbreaks of insect-borne viruses are generally associated with high densities of their respective vectors. For example, we routinely detect and diagnosis RSV in its insect vector, small brown planthopper, using a commercial DAS-ELISA detection kit and a polyclonal antibody (Japan Plant Protection Association), as described later in detail.

In the Americas, *Rice hoja blanca virus* (RHBV, a member of the genus *Tenuivirus*, negative sense ssRNA virus) is the most important causal agent of viral diseases of rice and transmitted by a planthopper in a persistent manner (Hibino, 1996).

In Africa, a few viruses have been reported to infect rice plants. Rice stripe necrosis virus (RSNV, may be a member of the genus *Benyvirus*, positive sense ssRNA virus), is transmitted by the soil-inhabiting fungal pathogen *Polymyxa graminis*. The major vectors of Maize streak virus strain A (a member of genus *Mastrevirus*, ssDNA virus) are leafhoppers (Monjane et al., 2011). *Rice yellow mottle virus* (RYMV, a member of the genus *Sobemovirus*, positive sense ssRNA virus) is transmissible by mechanical inoculation and insect vectors leaf beetles (Abo and Sy, 1998; Nwilene, 1999; Banwoa et al., 2001).

After the viruses are initially transmitted to rice plants by their vectors, the rice viruses spread from the infection foci to the entire host plant through the vascular system. RSV, RDV, and RGSV propagate in the vascular tissue and mesophyll cells, but RBSDV, RGDV and RRSV are localized in the phloem and gall tissues. RTYV and RTSV propagate in the phloem tissues, and RTBV is localized in the vascular bundles (Hibino, 1996). The localization of SRBSDV, which sometimes induces confusingly similar symptoms to those caused by RBSDV infection (Zhang et al., 2008a; Zhou et al., 2008), is presumably similar to that of RBSDV in host plants.

As is the case with many plant viruses, rice viruses are known to be distributed unevenly within the host plant. In addition, as the host plants grow and tiller, some of the tillers from one individual may be infected while others may be virus-free. Therefore, samples should be taken from several parts of the entire plant body to be certain of the diagnosis.

SEROLOGICAL DIAGNOSIS

The overview of detection methods for viruses is shown in **Table 1**. Serological methods can generally be subdivided into liquid and solid phase tests. The liquid-phase tests are the precipitin test, latex agglutination reaction (LAR), and passive hemagglutination (PHA), and solid-phase examples include the enzyme-linked immunosorbent assay (ELISA), dot-immunobinding assay (DIBA). Gel-based assay (double immunodiffusion gel assay, DIGA) have been also reported. These serological methods are used widely to detect rice viruses at pest control stations, plant protection stations, and agricultural experiment stations in Asia. Once the serological detection system is established, highly sensitive testing of a large number of samples is simpler and cheaper even though the methods are classical and

Table 1 | Overview of detection methods for viruses in rice plants and in insect vectors.

Virus/family	Vectors/transmission mode	Technique
RBSDV/Reoviridae	Planthoppers/persistent	ELISA (Wang et al., 2006; Wu et al., 2013a), RT-PCR (Yang et al., 2008; Cho et al., 2013; Wu et al., 2013b), RT-LAMP (Le et al., 2010)
RDV/Reoviridae	Leafhoppers/persistent	ELISA, PHA, LAR (Omura et al., 1984), RT-PCR (Cho et al., 2013), RT-LAMP (Le et al., 2010)
RGDV/Reoviridae	Leafhoppers/persistent	DIGA (Omura et al., 1982), RT-LAMP (Le et al., 2010)
RRSV/Reoviridae	Planthoppers/persistent	ELISA (Hibino and Kimura, 1982; Luisoni et al., 1982), RT-LAMP (Le et al., 2010)
SRBSDV/Reoviridae	Planthoppers/persistent	RT-PCR (Hoang et al., 2011; Wu et al., 2013b), RT-LAMP (Zhou et al., 2012), real-time RT-PCR (Matsukura et al., 2013; Zhang et al., 2013), DIBA (Chen et al., 2012b), ELISA (Wang et al., 2012)
RGSV/*Tenuivirus*	Planthoppers/persistent	ELISA (Hibino et al., 1985; Iwasaki et al., 1985); DIBA, ELISA, LAR (Hsu et al., 1990)
RHBV/*Tenuivirus*	Planthoppers/persistent	ELISA (Marys and Carballo, 2007)
RSV/*Tenuivirus*	Planthoppers/persistent	ELISA, LAR (Omura et al., 1986; Takahashi et al., 1987), RT-PCR (Hanada et al., 1997; Cho et al., 2013; Wu et al., 2013b), RT-LAMP (Le et al., 2010), real-time RT-PCR (Zhang et al., 2008b)
RWSV/*Tenuivirus*	Planthoppers/semipersistent	ELISA (Chen and Chiu, 1989)
RSNV/Benyvirus	*Polymyxa graminis*	Western blot (Morales et al., 1999)
RTYV/*Rhabdoviridae*	Leafhoppers/persistent	ELISA (Takahashi et al., 1988), Western blot (Chiu et al., 1990)
RYMV//*Sobemovirus*	Leaf beetles/semipersistent	ELISA (Konaté et al., 1997; Pinel et al., 2000), DIGA (Séré et al., 2005), RT-PCR (Afolabi et al., 2009)
RTSV/*Secoviridae*	Leafhoppers/semipersistent	ELISA (Bajet et al., 1985), multiplex RT-PCR (Periasamy et al., 2006), RT-LAMP (Le et al., 2010), real-time PCR (Sharma and Dasgupta, 2012)
RTBV/Caulimoviridae	Leafhoppers/semipersistent	ELISA (Bajet et al., 1985), PCR (Dasgupta et al., 1996), multiplex RT-PCR (Periasamy et al., 2006), RT-LAMP (Le et al., 2010), real-time RT-PCR (Sharma and Dasgupta, 2012)

several months are required to make practical antisera against rice viruses.

Other important considerations for serological detection and diagnostic systems are the quality of antisera and the type of epitopes recognized (sequential or linear/conformational epitopes or continuous/discontinuous epitopes). In some cases, antisera against purified virus may contain contaminating host-plant materials, which cause non-specific reactions. In addition, the antisera may cross-react with closely related viruses. For example, the antiserum against RGSV cross-reacts with the nucleocapsid protein of RSV (Hibino et al., 1985). To reduce these undesirable non-specific or cross-reactions, antisera against a viral protein or an *Escherichina coli*-expressed viral coat protein are now being used (Chen et al., 2012b).

Antisera that recognize linear epitopes instead of conformational epitopes are chosen for immunodetection of denatured proteins. For example, target proteins that are boiled or treated with β-mercaptoethanol, can be separated by SDS-PAGE and detected in a Western blot by using antisera that recognize linear epitopes. In contrast, antisera that recognize conformational epitopes are preferred for ELISA and other methods that target proteins whose structure is preserved. In an ELISA to detect RDV in rice plants, an antiserum against intact RDV particles was more sensitive than that against RDV dissociated by SDS. But for Western blot analysis, the sensitivity of antiserum against the dissociated RDV was higher than that against intact RDV particles (Chen et al., 2012).

LATEX AGGLUTINATION REACTION

Latex agglutination reaction is a classical technique for immunochemical reactions in which the antigen or the antibody is attached to the surface of red blood cells or to carrier particles, e.g., latex. The virus or antibody is simply attached to the latex particles by adsorption. In PHA, virus particles or antibodies are coupled to erythrocytes by various chemical treatments. In Japan, LAR was once the most commonly used method to detect RSV in SBPHs because it is more sensitive than other precipitin assays and requires little time and minimal facilities (**Figure 1A**). But the double antibody sandwich (DAS)-ELISA in commercially available kits has started to overtake the LAR because this technique is more sensitive, yields clear results and is inexpensive (Takahashi et al., 1991)

ELISA

Enzyme-linked immunosorbent assay is a solid-phase assay that uses antibodies labeled with enzymes that react with a substrate to yield a color change, thereby identifying the presence of a substance. We find that DAS-ELISA is easy to use for our routine diagnostic detection of RSV in insect vectors; monitoring the rate of viruliferous vector insects in early summer before rice planting season is very important to forecast rice stripe disease, an economically devastating disease, in the coming year. As shown in **Figure 1B**, up to 96 insect samples can be processed at the same time with plastic multi-sticks; for a few insect samples, we use one stick. This method is simple, and the results are robust and easy to interpret regardless of the age or sex of the insect (Uehara-Ichiki et al., 2013). Other rice viruses can also be detected with DAS-ELISA, not only in vectors but also in rice plants, and a few steps of DAS-ELISA can be skipped by grinding samples with the antibody–enzyme conjugate (Takahashi et al., 1987, 1988).

The DIBA or tissue immunoblotting assay are similar to ELISA, but in these methods, the sample extracts are spotted onto a membrane as a solid support matrix. Although false positive or false negative results are often obtained, methods to reduce the interference of chlorophyll have been reported (Srinivasan and Tolin, 1992; Chen et al., 2012a)

NUCLEIC-ACID-BASED TECHNIQUES

Detection based on viral nucleic acids is more sensitive and specific than serological methods, and any region of a viral genome can be targeted. Rice viruses, except for RTBV, are RNA viruses, and synthesis of cDNA of the viral genome by reverse transcription (RT) is necessary before the target DNA sequence is amplified. Commercial kits to extract RNA and to synthesis cDNA from several companies such as Life Technologies, QIAGEN, Takara Biotechnologies, BIO-RAD and Promega are available. The variations of RT-PCR (e.g., multiplex RT-PCR, real-time RT-PCR) and RT-loop-mediated isothermal amplification (LAMP) have been applied to detect rice viruses from plants or insects. The commercial kits for RT-PCR are sold by the companies mentioned, and the RT-LAMP kit is sold by Eiken Chemical.

MULTIPLEX RT-PCR

Multiplex RT-PCR uses multiple gene-specific primer sets within a single PCR mixture and can simultaneously detect two or more products in a single reaction. Therefore, the method is cost effective when two or more viruses are present in a single host plant (López et al., 2009). **Figure 1C** outlines the procedure and shows the resulting bands in the agarose gel after electrophoretic separation of the DNA fragments amplified by multiplex RT-PCR with the gene-specific primer sets (I and II), designed from coding sequences for the rice viral capsid or capsid-like proteins using the program FastPCR 6.0 (Institute of Biotechnology, University of Helsinki). Primer set I comprises two specific primers for rice actin as an internal control and 10 specific primers for RDV, RSV, RBSDV, RTYV, and RTSV, which have been reported in northeastern and eastern Asia. Primer set II comprises two specific primers for rice actin as an internal control and 10 specific primers for SRBSDV, RGDV, RRSV, RTBV, and RGSV, which have been reported in southeastern and southern Asia. Since the RT-PCR products derived from each virus differs in size, two viruses such as RDV/RSV, which are major rice viruses in Japan can be detected in a single reaction (**Figure 1C-c**). In rice fields of some countries where mixed infections with RRSV and RGSV, SRBSDV, and RSV and RBSDV, RSV and RBSDV, and RDV have been reported (Du et al., 2007; Cho et al., 2013; Wu et al., 2013b), multiplex RT-PCR is useful to rapidly and simultaneously identify the viruses.

RT-LAMP

The LAMP technique, developed by Notomi et al. (2000), is one of the most sensitive detection methods. This method has distinctive advantages because it is highly specific for the target sequence and can be done quickly without special equipment. The specificity of LAMP is due to the recognition of six distinct

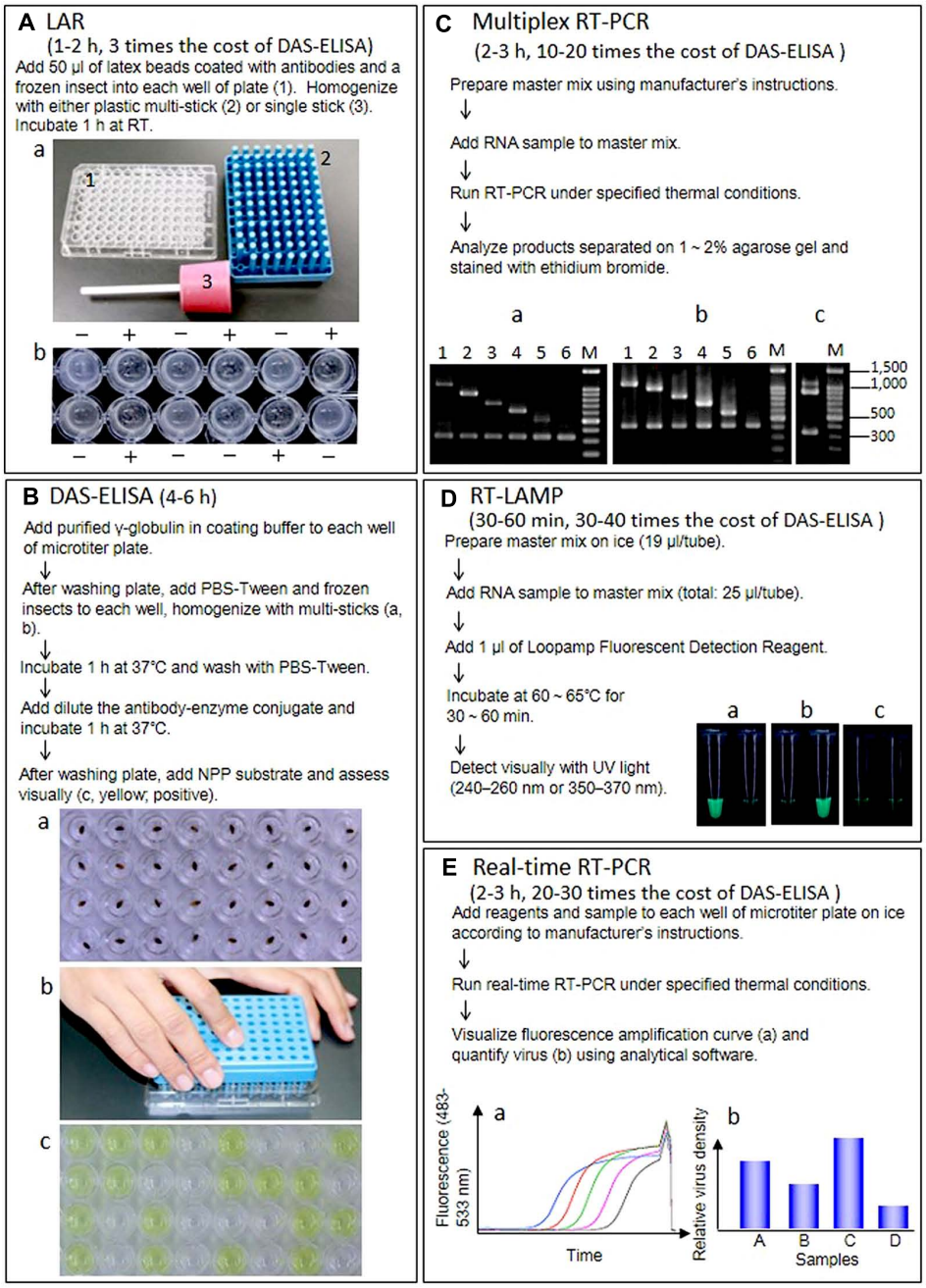

FIGURE 1 | Outline of procedure, required time, cost comparison of reagent kits per diagnosis, and results of five techniques. **(A)** Latex agglutination reaction (LAR) to detect RSV in insect vectors. RT, room temperature. **(a)** Materials for LAR and DAS-ELISA. (1) Microtiter plate, (2) multi-sticks, (3) single stick. **(b)** Results of LAR. +, positive, −, negative. **(B)** DAS-ELISA to detect RSV in insect vectors. **(a)** The frozen insects in each well. **(b)** Homogenization of insects with multi-sticks. **(c)** Visual assessment. (Yellow; positive). **(C)** Multiplex RT-PCR to detect and distinguish 10 rice viruses. **(a)** Agarose gel electrophoresis of RT-PCR products from healthy or infected rice plants using primer set I. Lane 1, infected with RDV (1106 bp); lane 2, infected with RSV (917 bp); lane 3, infected with RBSDV(734 bp); lane 4, infected with RTYV (631 bp); lane 5, infected with RTSV (504 bp); lane 6, healthy plant (339 bp). **(b)** Agarose gel electrophoresis of RT-PCR products from healthy or infected rice plants using primer sell. Lane 1, infected with SRBSDV (1097 bp); lane 2, infected with RGDV (994 bp); lane 3, infected with RRSV (834 bp); lane 4, infected with RTBV (699 bp); lane 5, infected with RGSV (574 bp); lane 6, healthy plant (419 bp). **(c)** Agarose gel electrophoresis of RT-PCR products from rice plants infected with RDV and RSV. **(D)** RT-LAMP to detect and distinguish between SRBSDV and RBSDV. **(a)** Rice plants infected with SRBSDV. **(b)** Rice plants infected with RBSDV. **(c)** Healthy rice plants. Left, SRBSDV primer sets. Right, RBSDV primer sets. **(E)** Real-time RT-PCR to quantify SRBSDV in rice and insect vectors. **(a)** Samples with a higher density of virus yield an earlier rise in the fluorescence amplification curve, which corresponds to the density of RT-PCR products. The threshold cycle (Ct), calculated from the amplification curve, is used as an indicator of virus titer in the sample (higher virus titer results in lower Ct). **(b)** Virus titer is usually given as the relative density of virus titer to expression of a housekeeping gene (e.g., actin and ubiquitin) from the host or vector.

sequences by four specifically designed primers, which partly alleviates the general problem of the background associated with all nucleic acid amplification methods. The LAMP reaction occurs at 60–65°C for 60 min in a water bath or heat block. We established a RT-LAMP detection system for nine rice viruses in Asia (Le et al., 2010). After SRBSDV was reported (Zhang et al., 2008a), we designed new primers derived using the S10 sequence of the SRBSDV genome for RT-LAMP using PrimerExplorerV4 (http://primerexplorer.jp/elamp4.0.0/index.html) and confirmed that our RT-LAMP system could distinguish between SRBSDV and RBSDV in infected plants (**Figure 1D**). Meanwhile, Zhou et al. (2012) detected and distinguished between SRBSDV and RBSDV in host plants and insect vectors with RT-LAMP targeting S9 of the SRBSDV genome.

REAL-TIME RT-PCR

In real-time PCR, amplification of DNA is monitored by the detection and quantitation of a fluorescent reporter signal, which increases in direct proportion to the amount of PCR product in the reaction. This technique, combined with RT, can be used to quantify target RNA. As noted already, RT-LAMP and conventional RT-PCR techniques offer rapid, sensitive, and accurate diagnosis, but they do not provide quantitative information like real-time PCR can. The starting amount of the target nucleic acid is quantitated, and the reaction can be monitored while in progress.

Real-time RT-PCR usually consists of four steps. After the first step, RT from RNA to cDNA, PCR is run using a specific primer set for the target region in the second step (usually 25–40 cycles). During this second step, fluorescence intensity, which corresponds to the amount of PCR product, is monitored for each cycle (**Figure 1E-a**). A cycle threshold (Ct), calculated from an amplification curve of fluorescence, is regarded as the virus titer in the sample (note that samples containing a higher density of virus have lower Ct values). In the third step, reaction reagents are incubated sequentially from 65 to 97°C at increments of ca. 0.1°C/s to obtain a melting curve for checking specific amplification of target region. The last step is cooling. Because virus titer is usually shown as the density of the virus relative to expression of a housekeeping gene in the same sample (**Figure 1E-b**), expression of the housekeeping gene (i.e., RNA titer transcribed from these genes in the sample) also should be determined by real-time RT-PCR. With the quantitative approach using a real-time RT-PCR for SRBSDV, the location of the virus was revealed and changes in viral density in the rice plant and subsequent effects on symptom appearance in rice can be monitored, as can virus acquisition by vector insects (Matsukura et al., 2013).

Since real-time PCR works better with small amplicons (the use of 50–200 bp is recommended), the cycling conditions for this method are shorter than for standard PCR, and the detection sensitivity is generally higher than for standard PCR assays and equivalent to that of RT-LAMP (Sharma and Dasgupta, 2012). Therefore, for the past few years, even though specific conditions and equipment are necessary for the real-time PCR system, techniques to detect and quantify rice viruses in plants and insects have been developed, revealing new insights into rice viral population dynamics (Zhang et al., 2008b, 2013; Sharma and Dasgupta, 2012; Matsukura et al., 2013).

CONCLUSION

Advances in the technologies to detect and diagnose plant pathogens have culminated in a variety of options for researchers in laboratory and for growers. After the application of ELISA to detect plant viruses was reported (Voller et al., 1976), ELISA and its variations were utilized widely for diagnosing plant diseases. In the case of rice viruses, some reports showed that ELISA had higher sensitivity than classical methods, such as PHA and LAR, and could detect target virus in leaf extracts diluted from 10^{-3} to 10^{-5} (Hsu et al., 1990; Takahashi et al., 1991; Kawano and Takahashi, 1997).

Since PCR was devised in the late 1980s (Mullis and Faloona, 1987), the PCR and its variations have contributed to more accurate detection and identification of plant pathogens. One of the advantages of these techniques is high sensitivity compared with serological or immunological methods. For example, the sensitivity of RT-PCR was 10^2-fold higher than dot-blot hybridization (Sharma and Dasgupta, 2012). The sensitivity of real time RT-PCR was about 10^4-fold higher than ELISA (Zhang et al., 2008b) and 10^3-fold higher than conventional RT-PCR (Sharma and Dasgupta, 2012). The sensitivity of RT-LAMP were about 10-fold higher than RT-PCR (Le et al., 2010; Zhou et al., 2012).

Current technologies, e.g., microarrays and next-generation sequencing also hold potential for use in diagnosing rice diseases. Microarrays are suitable techniques for high-throughput detection and identification, since an almost limitless number of pathogen probes can be placed on a single array (De Boer and López, 2012). The potential of array technology has not yet been realized because of various drawbacks that need to be resolved: sensitivity, cost, and lack of practical devices that can be used by non-technical personnel. But, it does allow us to detect and identify many pathogens including those not proven at one time (Boonham et al., 2007). Next-generation sequencing methods are being used to identify microbiomes within diseased plant tissue. This technique is largely dependent on software that can discriminate between host plant and viral sequences, but it provides new opportunities in the areas of viral candidate pathogen discovery and viral ecology (De Boer and López, 2012; Radford et al., 2012).

To select the most appropriate diagnostic methods, we need to focus on our objective. For large-scale analyses to evaluate incidence or for screening tests to monitor disease spread in fields, we should select methods using a user-friendly, evidence-based approach, an evaluation of cost per analysis and a calculation of post-test probability of disease (López et al., 2009; De Boer and López, 2012). Therefore, conventional serological methods such as LAR are still used, and ELISA remains one of the most widely applicable methods to forecast virus diseases of rice in the field.

The RT-LAMP and real-time RT-PCR techniques are too expensive for routine analysis in large surveys, but they do enable the differentiation of closely related viruses such as SRBSDV and RBSDV and very early detection of disease before symptoms are visible. Such features can help farmers and plant-health professionals to choose the best strategies to minimize potential damage. Particularly with real-time RT-PCR, applications such as screening for virus resistance and studying viral population dynamics, viral multiplication and virus–host interactions are feasible (Sharma and Dasgupta, 2012; Matsukura et al., 2013).

Conventional detection methods have only provided information on the presence of target pathogens. But progress in the development of technologies to diagnose plant diseases may not only contribute to the control of rice viral diseases, but also open opportunities to analyze potential pathogens/candidate pathogens and to develop a comprehensive understanding of the ecology of microorganisms in rice fields.

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Transgenic strategies to confer resistance against viruses in rice plants

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Rice (*Oryza sativa* L.) is cultivated in more than 100 countries and supports nearly half of the world's population. Developing efficient methods to control rice viruses is thus an urgent necessity because viruses cause serious losses in rice yield. Most rice viruses are transmitted by insect vectors, notably planthoppers and leafhoppers. Viruliferous insect vectors can disperse their viruses over relatively long distances, and eradication of the viruses is very difficult once they become widespread. Exploitation of natural genetic sources of resistance is one of the most effective approaches to protect crops from virus infection; however, only a few naturally occurring rice genes confer resistance against rice viruses. Many investigators are using genetic engineering of rice plants as a potential strategy to control viral diseases. Using viral genes to confer pathogen-derived resistance against crops is a well-established procedure, and the expression of various viral gene products has proved to be effective in preventing or reducing infection by various plant viruses since the 1990s. RNA interference (RNAi), also known as RNA silencing, is one of the most efficient methods to confer resistance against plant viruses on their respective crops. In this article, we review the recent progress, mainly conducted by our research group, in transgenic strategies to confer resistance against tenuiviruses and reoviruses in rice plants. Our findings also illustrate that not all RNAi constructs against viral RNAs are equally effective in preventing virus infection and that it is important to identify the viral "Achilles' heel" gene to target for RNAi attack when engineering plants.

Keywords: *Reoviridae*, *Tenuivirus*, RNA interference, transgenic rice, forage rice cultivar

INTRODUCTION

Rice (*Oryza sativa* L.), one of the most important grain crops, is grown worldwide, with more than 90% (650 million tons) in Asia, where it is consumed directly to supply 36% of the total calories consumed (Normile, 2008; Zeigler and Barclay, 2008). Of the 15 viruses damaging rice, 10 are a serious menace to rice production in Asia. In southern Vietnam during 2006–2007, more than 485,000 hectares of paddy fields were severely affected by infection with *Rice grassy stunt virus* (RGSV) or co-infection by RGSV and *Rice ragged stunt virus* (RRSV), resulting in the loss of 828,000 tons of rice valued at US$120 million and directly affecting millions of rice farmers (Cabauatan et al., 2009). The yield losses of rice caused by rice viruses are enormous. To ensure global food security for continuing population growth, controlling the various viruses that damage rice is vital.

The rice viruses encompass many types of viruses, e.g., double-stranded RNA (dsRNA) viruses such as *Rice dwarf virus* (RDV), *Rice black streaked dwarf virus* (RBSDV), and RRSV, negative-sense single-stranded RNA viruses, *Rice stripe virus* (RSV) and RGSV, a double-stranded DNA virus, *Rice tungro bacilliform virus*, and a positive-sense single-stranded RNA virus, *Rice tungro spherical virus*. Almost all these rice viruses are transmitted by leafhoppers and planthoppers, and some multiply in the insects and are transmitted transovarially, making their control more difficult (Hibino, 1996). These insect vectors are distributed widely in Asian countries and migrate long distances, even across the ocean (Kishimoto, 1971).

Recent advances in biotechnology should help solve these problems, and genetically engineering plants to have improved resistance against diseases and harmful insects is one of the most promising approaches. Based on the concept of pathogen-derived resistance, that the expression of various viral sequences is effective in preventing or reducing various plant virus infections, several strategies to confer resistance against viruses in plants have been developed over the last two decades (for reviews, see Sanford and Johnston, 1985; Baulcombe, 1996; Palukaitis and Zaitlin, 1997; Miller and Hemenway, 1998). Recently, viral RNA itself has been shown to be a potential trigger for resistance against viruses in transgenic plants, with the subsequent discovery of a novel, innate resistance in plants, known now as RNA interference (RNAi) or RNA silencing (for reviews, see Vazquez Rovere et al., 2002; Baulcombe, 2004, 2005; Voinnet, 2005; Ding and Voinnet, 2007).

RNA interference, an evolutionarily conserved process that is active in a wide variety of eukaryotic organisms, is a sequence-specific gene-silencing mechanism that is induced by dsRNA (for reviews, see Baulcombe, 2004, 2005; Voinnet, 2005). The dsRNA is diced into small interfering RNAs (siRNAs) of 21–24 nucleotides by an endonuclease called Dicer (Bernstein et al., 2001; Fusaro et al., 2006). These siRNAs are then incorporated into the RNA-induced silencing complex to guide degradation or translational repression in a sequence-specific manner. Via the expression of virus-specific dsRNA as hairpin structures, it is one of the relative easy and promising ways to render plants resistant against plant virus infection (Wang et al., 2000; Kalantidis et al., 2002; Bonfim et al., 2007).

Our successive attempts at conferring resistance against rice viruses have indicated that the RNAi constructs that target various viral genes are not equally effective in preventing virus infection; depending on the viral gene targeted, the levels of resistance have varied from complete resistance against a delay in symptom development or even an absence of resistance (Shimizu et al., 2009, 2011b). Thus, identifying the viral "Achilles' heel" gene is important for choosing an appropriate target for the RNAi attack when engineering plants that are strongly resistant against virus infection. In this article, we review recent progress, mainly conducted by our research group, in transgenic strategies to confer resistance in rice plants against the rice viruses in the family *Reoviridae* and the genus *Tenuivirus* that cause serious problems for stable rice production in Asia. In addition, we discuss our recent most attempt to develop transgenic virus-resistant forage cultivars of rice that can be cultivated in fields to control virus diseases and reduce the population of viruliferous insect vectors.

CONFERRING RESISTANCE AGAINST RICE-INFECTING REOVIRUSES

RICE-INFECTING REOVIRUSES

Four dsRNA viruses, RDV, RBSDV, *Rice gall dwarf virus* (RGDV), and RRSV, occur in rice and threaten rice production in Asia. In addition, a new virus, Southern rice black-streaked dwarf virus (SRBSDV) that was first discovered in 2001 in Guangdong, China, has rapidly spread throughout central China and Vietnam, and has recently been found in northern China and Japan (Hoang et al., 2011; Wang et al., 2012).

On the basis of virion properties, insect vector specificity, viral genome organization, and viral sequence information, these five rice-infecting reoviruses are classified into three genera, *Oryzavirus*, *Phytoreovirus,* and *Fijivirus* of the family *Reoviridae* (Attoui et al., 2011). RDV and RGDV, belonging to the genus *Phytoreovirus*, are transmitted in a persistent manner by leafhoppers (*Nephotettix cincticeps*, *N. nigropictus*, and *Recilia dorsalis*). RRSV, belonging to the genus *Oryzavirus*, is transmitted in a persistent manner by the brown planthopper (*Nilaparvata lugens*). RBSDV and SRBSDV, belonging to the genus *Fijivirus*, are mainly transmitted by the small brown planthopper (*Laodelphax striatellus*) and the white-backed planthopper (*Sogatella furcimera* Horváth), respectively. All five of these reoviruses are propagative in their vectors, and RDV and RGDV can be transmitted from female adults to their progeny via eggs. RRSV occurs mainly in the tropical regions of Asia, such as Indonesia, Malaysia, the Philippines, Vietnam, and Thailand, where it causes serious problems for rice production. The other four viruses occur mainly in the subtropical regions of Asia, such as China, Japan, Korea, and Nepal (Hibino, 1996). Outbreaks of RBSDV have created serious problems not only for rice production but sometimes also for maize production in China and Japan (Ishii and Yoshimura, 1973; Li et al., 1999).

VIRAL GENOME ORGANIZATION

The genomes of the rice-infecting reoviruses consist of 10–12 segments of linear dsRNA and encode 10–12 proteins (**Figure 1**). The viral core particle appears to be formed by viral genomic dsRNA segments and at least four proteins (major core capsid, RNA-dependent RNA polymerase, capping enzyme, and nucleic acid binding protein). The core particles are surrounded by one or two outer capsid proteins and form a double-shelled, icosahedral particle approximately 60–80 nm in diameter. In addition to these structural proteins, the viral genomes encode two to five non-structural proteins, which form cytoplasmic inclusion bodies, known as viroplasms (Isogai et al., 1998; Omura and Yan, 1999; Attoui et al., 2011).

The reoviruses form distinctive structures in the cytoplasm of infected host cells at early stages of viral infection. Depending on the genus of the family *Reoviridae*, the cytoplasmic structures are called different names: viroplasms, viral factories, and viral inclusion bodies. However, these viral structures are thought to have essentially similar functions. Since the cytoplasmic structures are formed at early stages of viral infection in the host cells and contain many viral proteins, viral genomic segments, and virus particles, these cytoplasmic structures are considered to be the sites of viral RNA replication and packaging into progeny particles. Thus, the viroplasm-associated proteins may also play important roles in viral infection and proliferation at an early stage of viral replication (McNulty et al., 1976; Sharpe et al., 1982; Eaton et al., 1987; Touris-Otero et al., 2004). In the case of the rice-infecting reoviruses, proteins Pns6, Pns11, and Pns12 encoded by RDV segments 6, 11, and 12, Pns9 encoded by RGDV segment 9, NS10 by RRSV segment 10, and P9-1s by RBSDV and SRBSDV segment 9, have been confirmed as component proteins of the viroplasm (Isogai et al., 1998; Wei et al., 2006; Akita et al., 2011; Mao et al., 2013).

Regarding the adaptation of plant reoviruses to plants as hosts, two striking differences can be discerned between the animal-infecting reoviruses and their plant-infecting counterparts. The first is the ability of a plant reovirus to modify the plasmodesmata, the cytoplasmic channel through the plant cell walls, to facilitate systemic spread of infectious viral entities throughout the plant host. Most plant viruses have one or more genes for viral movement proteins that facilitate transport of the virus genome from an initially infected cell into neighboring cells through the plasmodesmata (for reviews, see Scholthof, 2005; Lucas, 2006; Taliansky et al., 2008). The viral movement proteins tend to be localized predominantly near or in the plasmodesmata. In addition, the viral movement protein of a certain virus can often complement the cell-to-cell movement of other distantly related or even unrelated viruses, even though viral movement proteins are highly variable in their amino acid sequences (Morozov and Solovyev, 2003; Xiong et al., 2008; Hiraguri et al., 2011, 2013). In the case of the

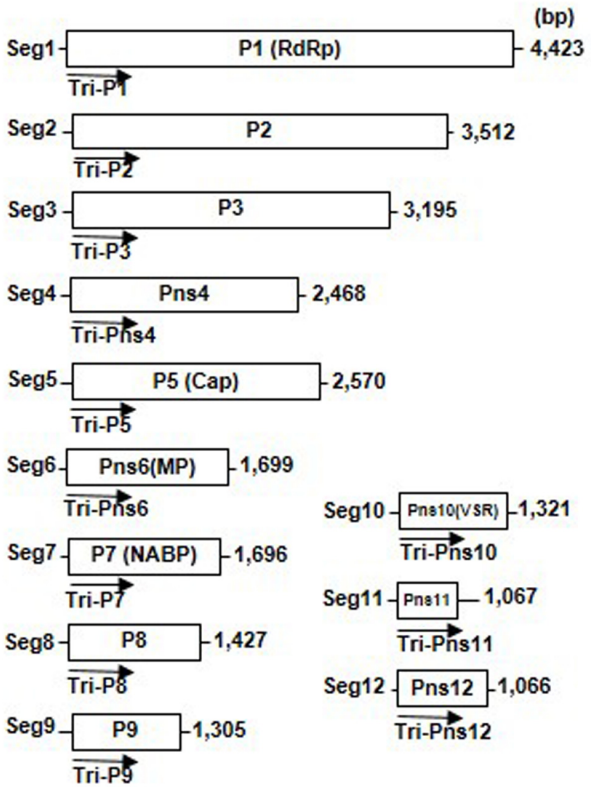

FIGURE 1 | Genome structure of *Rice dwarf virus* (RDV). Map of segments 1 through 12 of the RDV genome. Lines represent the viral genomic double-stranded RNA segments; boxes denote genes encoded by RDV genes. Arrows indicate 500-bp RNA interference (RNAi) trigger sequences that were amplified from the 5′-proximal regions of each RDV gene and cloned into the RNAi trigger plasmids used to transform the plants. RdRp, RNA-dependent RNA polymerase; Cap, capping enzyme; MP, movement protein; VSR, silencing suppressor.

rice-infecting reoviruses, Pns6 encoded by RDV segment 6 and P6 encoded by RRSV segment 6 were confirmed as a viral movement protein in experiments that localized the viral-encoded protein to the plasmodesmata and showed that the protein can restore cell-to-cell movement of a movement-defective virus (Li et al., 2004; Wu et al., 2010).

The second difference is the capacity of the plant reovirus to counteract RNAi, the innate antiviral defense system of plants and insects. RNAi involves a sequence-specific degradation that is induced by dsRNA molecules and can target transgenes as well as homologous endogenous genes. Because all RNA viruses replicate through the formation of dsRNA intermediates, these intermediates are potential targets for RNAi. To counteract the RNAi mechanism of their host, plant viruses have developed ways to evade or neutralize this response. The most common way is to encode the so-called RNA silencing suppressor proteins (VSR). A simple and elegant assay for RNAi suppressor activity that involves a combination of green fluorescent protein (GFP)-silenced reporter plants and *Agrobacterium*-based transient transformation has been developed (Voinnet et al., 2000; Bucher et al., 2003; Baulcombe, 2004).

A large number of RNA silencing suppressor proteins have been identified from numerous plant viruses (Pruss et al., 1997; Brigneti et al., 1998; Voinnet et al., 2000; Dunoyer et al., 2002; Pfeffer et al., 2002; Bucher et al., 2003; Zhang et al., 2005). In the case of rice-infecting reoviruses, Pns10 encoded by RDV segment 10 and Pns11 encoded by RGDV segment 11, have been shown to have RNA silencing suppressor activity (Cao et al., 2005; Zhang et al., 2005; Liu et al., 2008).

RESISTANCE AGAINST RICE DWARF VIRUS IN TRANSGENIC RICE

RNA interference has been an important tool to render plants resistant against virus infections. During our work to develop resistance against RDV in rice plants by introducing dsRNAs of a 500-bp fragment in the 5′-proximal regions of each viral genes as the viral target genes (**Figure 1**), the degrees of resistance that were conferred to rice differed (Shimizu et al., 2009; Sasaya et al., 2013). The degrees of resistance against RDV infection differed, depending on the viral target genes, from almost immune (i.e., no symptom development or virus amplification), moderate resistance, to no resistance (**Table 1**).

The transgenic rice plants with the introduced RNAi construct targeting the RDV gene for Pns 6 (viroplasm associated protein and movement protein), P8 (major outer capsid), and Pns12 (viroplasm associated protein) were almost immune to RDV infection. There were no apparent differences in morphology or growth, based on plant height, number of tillers, and rice grain yields between inoculated and mock-inoculated rice plants (**Figure 2A**). The transgenic plants did not contain detectable amounts of the virus through harvest as determined by ELISA. In contrast, transgenic plants with an introduced construct for P2 (outer capsid), P5 (capping enzyme), P7 (nucleic acid binding protein), P9 (outer capsid), or Pns10 (silencing suppressor) did not develop any resistance against RDV; symptoms developed at the same rate and severity as those in the susceptible non-transgenic control plants.

By analyzing the effects of potential target sequences in each of the coding genes in the RDV genome, we found transgenic plants that harbored the RNAi constructs targeting the genes for Pns6, P8, or Pns12 were completely resistant against the RDV infection, suggesting that these proteins are key components at the early stages of viral proliferation. Reiterating the functions of these proteins, P8 is a major outer capsid, and Pns6 and Pns12 are component proteins of the viroplasm, and Pns6 also functions as a viral movement protein. Thus, when the plants inhibited the expression of the viral major outer capsid, viroplasm-associated protein and viral movement protein via RNAi, they developed the strongest resistance against RDV infection.

By contrast, transgenic plants that harbored the RNAi construct specific for the genes for P2 and Pns10 were susceptible to RDV. Considering that when RDV is maintained in rice plants for a long period, non-sense mutations gradually accumulate in RDV segments 2 and 10, resulting in a decrease in the expression of these proteins and complete loss of insect-transmissibility, these proteins might not be essential for viral proliferation in rice plants but instead function in the insect vectors (Pu et al., 2011). Thus, transgenic plants with the introduced RNAi constructs targeting the

Table 1 | Degree of resistance against *Rice dwarf virus* (RDV) infection in transgenic rice plants induced by different RNAi-targets of RDV genes[a].

Target gene for	Location/putative function[b]	GenBank accession	Resistance[c]
P1	Core particle/RNA polymerase	D90198	Strong
P2	Outer particle/vector transmission	AB263418	Absent
P3	Core particle/major core capsid	X54620	Moderate
Pns4	Cytoplasmic fibril/intracellular movement	X54622	Strong
P5	Core particle/capping enzyme	D90033	Absent
Pns6	Viroplasm/movement protein	M91653	Immune
P7	Core particle/nucleic acid binding	D10218	Absent
P8	Outer particle/major outer capsid	D10219	Immune
P9	Outer particles/unknown	D10220	Absent
Pns10	Tubule structure/silencing suppressor	D10221	Absent
Pns11	Viroplasm/unknown	D10249	Strong
Pns12	Viroplasm/unknown	D90200	Immune

[a] To evaluate any resistance to RDV infection, more than 30 rice plants from three independent lines of transgenic plants were exposed to approximately 10 viruliferous RDV-carrying viruliferous leaf hopper per plant for 1 day.
[b] Suzuki et al. (1990a,b, 1991, 1992a,b), Suzuki (1993), Uyeda et al. (1994), Zhong et al. (2003), Li et al. (2004), Cao et al. (2005).
[c] Immune, no symptoms developed, and no virus was detected by ELISA in inoculated rice plants through harvest; Strong, weak symptoms developed but were delayed for 2–4 weeks, but growth was almost the same as for mock-inoculated rice plants; Moderate, typical symptoms developed but were delayed 2–4 weeks, and growth was slightly stunted after RDV infection; Absent, typical symptoms developed, as severe as those of RDV-infected non-transgenic rice plants.

RDV genes for P2 and Pns10 seem not to have induced resistance against RDV infection.

At any rate, not all RNAi constructs against RDV genes are equally effective in preventing viral infection. The genes for the major outer capsid, viroplasm associated proteins, and viral movement protein can be considered the "Achilles' heel" of the rice-infecting reoviruses and be targeted for RNAi attack for engineering resistance in plants.

CONFERRING STRONG RESISTANCE AGAINST OTHER REOVIRUSES

The results discussed in the previous section brought the idea that the transgenic rice plants inhibited the expression of other reoviral gene for the viroplasm associated protein showed complete resistance against their other reoviruses. Because RGDV Pns9 and RBSDV P9-1 are the functional orthologs of the viroplasm associated protein of RDV, the genes for Pns9 and P9-1 might be thought the appropriate targets for suppressing the proliferation of RGDV and RBSDV, respectively, in infected rice plants (Isogai et al., 1998; Akita et al., 2011). To make clear that these target genes induce complete resistance against these rice-infecting reoviruses by RNAi, the rice plants (cv Nipponbare), which have been introduced the RNAi trigger plasmids transcribed into dsRNAs of a 500-bp fragment in the 5′-proximal regions of the genes for Pns9 and P9-1 were evaluated any subsequent resistance against RGDV or RBSDV infections, respectively (Shimizu et al., 2011a, 2012).

All transgenic plants with the introduced RNAi trigger construct of the RGDV gene for Pns9 were asymptomatic and continued to be symptom-free until harvest (ca. 4 months), whereas growth of the RGDV-infected non-transgenic rice plants was severely stunted and small galls had developed along the leaf veins on the abaxial surface of leaves and on the outer surface of sheaths by 4 weeks post-inoculation. The transgenic rice plants that remained asymptomatic after challenge with RGDV, were almost immune to RGDV infection because the transgenic plants did not contain detectable amounts of the virus, as determined by ELISA. In addition, no apparent differences in morphology or growth, based on plant height, number of tillers, and rice grain yields were observed between transgenic and mock-inoculated non-transgenic rice plants (**Figure 2B**).

Similarly, the transgenic rice plants that harbored the RNAi trigger plasmid that inhibits the expression of the RBSDV gene for P9-1 were completely resistant against RBSDV infection and remained asymptomatic with no virus detectable by ELISA after challenge with RBSDV. In contrast, the RBSDV-infected non-transgenic rice plants were severely stunted with darkened leaves, twisted leaf tips, split leaf margins, and waxy white-to-black galls along the veins on the adaxial surface of leaf blades and the adaxial surface of sheaths by 4 weeks post-inoculation (**Figure 2C**).

These findings further confirmed evidence that the genes for the viroplasm associated proteins are the viral "Achilles' heel" for RNAi attack and that these genes can be targeted to confer strong resistance against plant-infecting reoviruses in transgenic rice plants. Furthermore, our strategy to interfere with the expression of viroplasm-associated proteins can induce strong resistance and should be effective for controlling other rice-infecting reoviruses of important crop plants such as RRSV and SRBSDV. The RRSV gene for NS10 and the SRBSDV gene for P9-1 are promising genes to target.

CONFERRING RESISTANCE AGAINST RICE-INFECTING TENUIVIRUSES

RICE-INFECTING TENUIVIRUSES

Three tenuiviruses, RGSV, RSV, and *Rice hoja blanca virus* (RHBV) are known to cause severe damage to rice. RSV is transmitted in

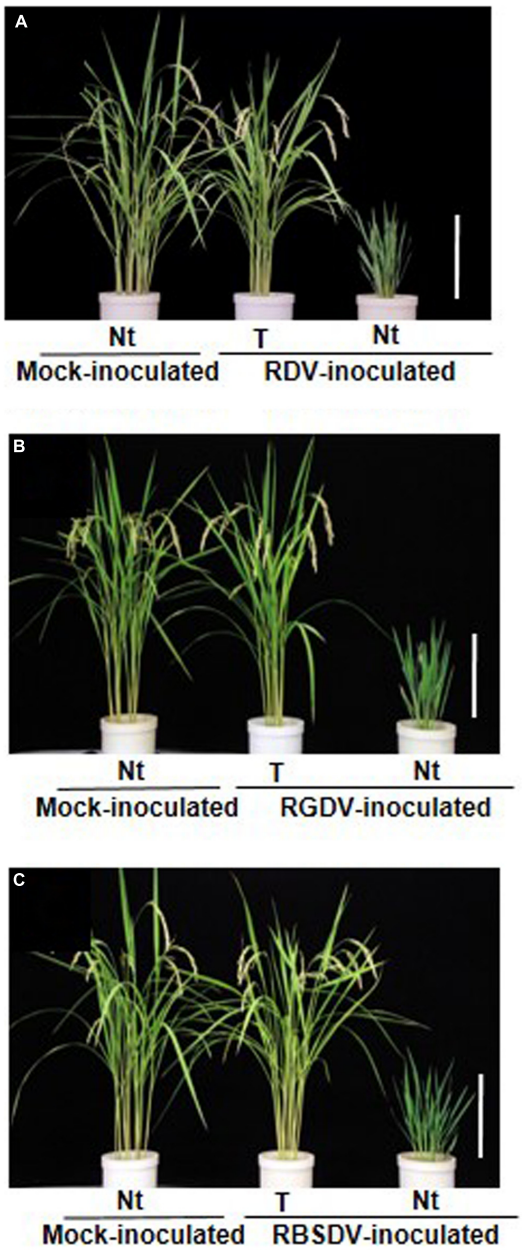

FIGURE 2 | Transgenic rice plants enhanced resistance against three rice-infecting reoviruses. (A) Phenotype of transgenic rice plants (cv. Nipponbare) that harbor the RNAi trigger sequence targeting the *Rice dwarf virus* (RDV) gene for Pns12 (from Shimizu et al., 2009). **(B)** Phenotype of transgenic rice plants that harbor the RNAi trigger sequence targeting the *Rice gall dwarf virus* (RGDV) gene for Pns9 (from Shimizu et al., 2012). **(C)** Phenotype of transgenic rice plants that harbor the RNAi trigger sequence targeting the *Rice black streaked dwarf virus* (RBSDV) gene for P9-1 (from Shimizu et al., 2011a). Ten-day-old transgenic rice seedlings were exposed to approximately 10–15 viruliferous vector insects per plant for 1 day and evaluate plant response to virus infection at 4 months after virus inoculation. Rice plants in pots from left to right: mock-inoculated non-transgenic rice plants (Nt) exposed to virus-free insect vectors, showing normal growth; virus-inoculated transgenic rice plants (T), showing healthy growth and fertility after inoculation; virus-inoculated non-transgenic rice plant (Nt), showing typical symptoms caused by RDV, RGDV or RBSDV infection. Bar, 30 cm.

a persistent manner by the small brown planthopper and induces significant economic losses in temperate regions of East Asia, especially in China, Japan, and Korea. RGSV, transmitted to rice plants in a persistent manner by the brown planthopper, has become a serious problem for rice production in South, Southeast, and East Asian countries (Hibino, 1996). RHBV is propagatively transmitted by the planthopper *Tagosodes orizicolus* and occurs in Central and South America, the Caribbean, and the southern United States (Falk and Tsai, 1998). RSV and RHBV are transmitted transovarially to progeny at high rates, but RGSV is not (Hibino, 1996; Falk and Tsai, 1998).

VIRAL GENOME ORGANIZATION

Virus particles of the tenuiviruses are thin filaments 3–10 nm in diameter and may be composed of a single nucleocapsid protein and four to six single-stranded RNA segments with positive (virion-sense) and negative (virion complementary-sense) polarities (**Figure 3**; Shirako et al., 2011). The genomes of RSV and RHBV consist of four ssRNA segments, designated RNAs 1–4 in order of decreasing molecular mass, and have seven genes. The first RNA segment has negative polarity, and the other three are ambisense. The RGSV genome consists of six ssRNA segments, all of which are ambisense, and includes 12 genes. The viral mRNAs are transcribed from each viral gene by the cap-snatching mechanism (Ramirez et al., 1995; Shimizu et al., 1996).

The unusual genome organization and replication strategy of tenuiviruses has so far prevented the development of an infectious clone system for the virus. The lack of a reverse genetics system for any viruses in this genus has been an obstacle for functional studies of virus-encoded proteins using standard mutagenesis. The functions of several virus-encoded proteins have, however, been predicted (**Table 2**). The pC1 encoded

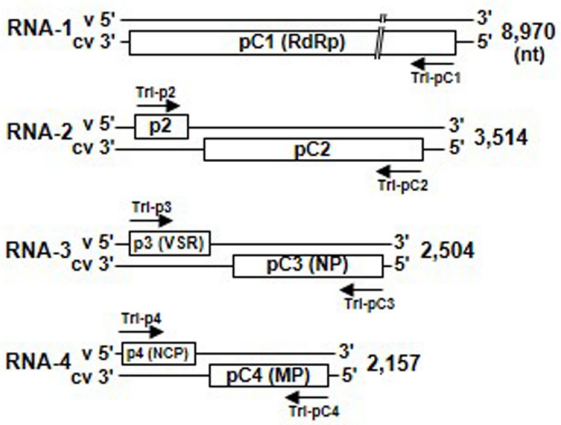

FIGURE 3 | Genome structure of *Rice stripe virus* (RSV). Map of RNAs 1 through 4 of the RSV genome. Upper and lower lines represent the virion-sense RNA segments and virion complementary-sense RNA segments, respectively, and boxes denote genes encoded by RSV genes. Arrows indicate 500-bp RNA interference (RNAi) trigger sequences that were amplified from the 5′-proximal regions of each RSV gene and cloned into the RNAi trigger plasmid for plant transformation. MP, movement protein; NCP, major non-capsid protein; NP, nucleocapsid protein; RdRp, RNA-dependent RNA polymerase; VSR, silencing suppressor.

in the first RNA segment of the *Tenuivirus* genome contains the major RNA-dependent RNA polymerase modules and RNA-dependent RNA polymerase activity (Toriyama, 1986; Toriyama et al., 1994, 1998; Nguyen et al., 1997). The pC2 is encoded in the virion-complementary sense of the second RNA segment, has stretches of weak but significant amino acid similarity with amino acids of the glycoproteins of phleboviruses and may be evolutionarily linked with the membrane-associated glycoprotein (Takahashi et al., 1993). The p3 protein encoded in the virion-sense of RNA-4 of RSV and RHBV functions as the viral silencing suppressor (Bucher et al., 2003; Hemmes et al., 2007; Schnettler et al., 2008; Xiong et al., 2009). The pC3 protein encoded in the virion-complementary sense of RNA-3 of RSV and RHBV and pC5 encoded in the virion-complementary sense of RGSV RNA-5 are nucleocapsid proteins and major components of the thin filamentous particles (Zhu et al., 1991; de Miranda et al., 1994; Toriyama et al., 1997). The p4 protein encoded in the virion-sense strand of RNA-4 of RSV and RHBV and p6 encoded by the virion-sense of the RGSV RNA-6 accumulate in large amounts and form crystalline inclusion bodies in virus-infected rice plants, but their functions are unknown (Koganezawa, 1977; Falk et al., 1987; Hayano et al., 1990; Miranda and Koganezawa, 1995). The pC4 protein encoded in the virion-complementary sense of RNA-4 of RSV and pC6 encoded in the virion-complementary sense of the RNA-6 of RGSV are viral movement proteins; their proteins accumulate close to the cell walls of infected host cells and facilitate intercellular transport of movement-defective viruses (Xiong et al., 2008; Hiraguri et al., 2011).

RESISTANCE AGAINST RICE STRIPE VIRUS IN TRANSGENIC RICE

To identify the most appropriate target genes for induction of strong resistance against tenuiviruses by RNAi, we have generated transgenic rice plants that inhibited the expression of one of the various coding genes in the RSV genome. Seven RNAi trigger plasmids that would be transcribed into dsRNAs of the 5′-proximal regions of each gene in the RSV genome were constructed and introduced into the rice plants (cv. Nipponbare; **Figure 3**) for evaluating resistance against RSV infection (Shimizu et al., 2011b).

The transgenic rice plants with the introduced RNAi trigger construct of the various individual RSV genes exhibited varying degrees of resistance against RSV infection (**Table 2**). The transgenic plants with the RNAi trigger plasmids of RSV genes for pC3 (nucleocapsid protein) and pC4 (movement protein) were conferred near immunity to RSV infection because the virus was not amplified in the transgenic rice plants through harvest (ca. 4 months). Furthermore, morphology and growth, based on plant height, number of tillers, and rice grain yields apparently did not differ between the transgenic and mock-inoculated non-transgenic rice plants (**Figure 4A**). In contrast, the transgenic plants with the introduced pC2 or p4 did not have any resistance against RSV infection; symptoms developed at the same rate and severity as in the susceptible non-transgenic control plants. Transgenic rice plants with the p2 and p3 constructs exhibited moderate resistance against RSV infection; typical symptoms were induced but their appearance was delayed for 2–4 weeks, and plant growth was moderately stunted by RSV infection.

CONFERRING STRONG RESISTANCE AGAINST RICE GRASSY STUNT VIRUS

Because the genes for the nucleocapsid protein and movement protein were appropriate targets for RNAi to confer complete resistance against RSV infection, transgenic rice plants that expressed dsRNAs of the RGSV genes for pC5 and pC6, the functional orthologs of pC3 and pC4 of RSV, respectively (Hiraguri et al., 2011) were generated with the expectation that they would be appropriate targets for completely suppressing the proliferation of RGSV in infected rice plants (Shimizu et al., 2013).

All the transgenic plants with the introduced RNAi trigger constructs for pC5 and pC6 were asymptomatic at 4 weeks post-inoculation, in contrast to the typical severe stunting of plant growth with profuse tillering of all infected non-transgenic plants,

Table 2 | Degree of resistance against *Rice stripe virus* (RSV) infection in transgenic rice plants induced by different RNAi-targets of RSV genes[a].

Target gene for	Location/putative function[b]	GenBank accession	Resistance[c]
pC1	RNA polymerase	D31879	Strong
p2	Unknown	D13176	Moderate
pC2	Glycoprotein-like	D13176	Absent
p3	Silencing suppressor	X53563	Moderate
pC3	Nucleocapsid protein	X53563	Immune
p4	Crystalline inclusion	D10979	Absent
pC4	Movement protein	D10979	Immune

[a] To evaluate any resistance to RSV infection, more than 30 rice plants from three independent lines of transgenic plants were exposed to approximately 15 viruliferous RSV-carrying viruliferous small brown hopper per plant for 1 day.

[b] Kakutani et al. (1990), Zhu et al. (1991), Takahashi et al. (1993), Toriyama et al. (1994), Xiong et al. (2008, 2009).

[c] Immune, no symptoms developed, and no virus was detected by ELISA in inoculated rice plants through harvest; Strong, weak symptoms developed but were delayed for 2–4 weeks, but growth was almost the same as for mock-inoculated rice plants; Moderate, typical symptoms developed but were delayed 2–4 weeks, and growth was slightly stunted after RSV infection; Absent, typical symptoms developed, as severe as those of RSV-infected non-transgenic rice plants.

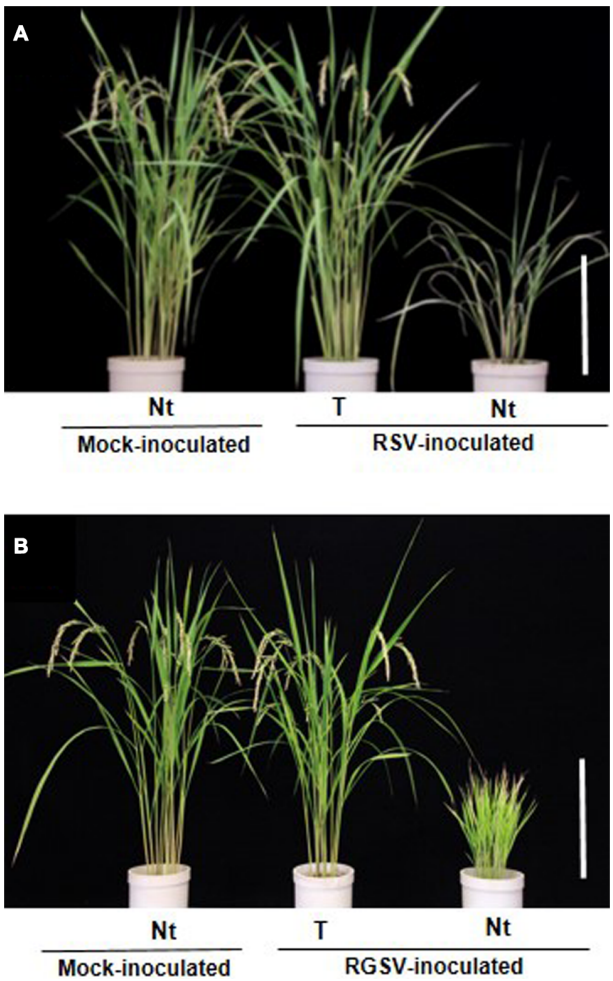

FIGURE 4 | Transgenic rice plants with enhanced resistance against two tenuiviruses. (A) Phenotype of transgenic rice plants (cv. Nipponbare) that harbored the RNAi trigger sequence targeting the *Rice stripe virus* (RSV) gene for pC3 (from Shimizu et al., 2011b). **(B)** Phenotype of transgenic rice plants that harbored the RNAi trigger sequence targeting the *Rice grassy stunt virus* (RGSV) gene for pC5 at 4 months after RGSV inoculation (from Shimizu et al., 2013). Ten-day-old transgenic rice seedlings were exposed to approximately 10–15 viruliferous vector insects per plant for 1 day and evaluate plant response to virus infection. Rice plants in pots from left to right: mock-inoculated non-transgenic rice plants (Nt) exposed to virus-free insect vectors, showing normal growth; virus-inoculated transgenic rice plants (T), showing healthy growth and fertility after inoculation; virus-inoculated non-transgenic rice plant (Nt), showing typical symptoms caused by RSV or RGSV infection. Bar, 30 cm.

and they continued to be symptom-free and with no detectable amounts of the virus until harvest (ca. 4 months). In addition, no apparent differences in morphology or growth, based on plant height, number of tillers, and rice grain yields were observed between transgenic and mock-inoculated non-transgenic rice plants (**Figure 4B**). These results indicated that the RGSV genes for the nucleocapsid protein and movement protein are the viral "Achilles' heel" to target for RNAi attack and provided further evidence that targeting these genes are effective in conferring strong resistance against tenuiviruses in transgenic rice plants. Our strategy for interfering with the expression of the nucleocapsid protein and movement protein should also be effective for controlling other tenuiviruses. For example, the genes for pC3 and pC4 in RHBV, which causes economically important rice crop plants in Central and South America, are promising candidates to confer strong resistance against RHBV infection in rice plants.

VIRUS-RESISTANT TRANSGENIC FORAGE RICE CULTIVAR: PRACTICAL APPLICATION

From the 1960s on, the production of livestock feed in Japan has decreased dramatically to only 26% of the total consumed by 2012, critically impairing the ability to maintain stable production of livestock (Annual Governmental Report about Agricultural Trends 2012, available at http://www.maff.go.jp/j/wpaper/w_maff/h23/index.html). To increase Japan's self-sufficiency for livestock feed, the production of forage rice cultivars is strongly promoted by the Japanese government, and significant progress in research on forage rice cultivars has resulted in several new rice cultivars that produce large gross weights.

As global temperatures have increased, viruliferous insects that can transmit rice viruses are also on the increase (Shiba, 2012). Damage caused by rice viruses and their insect vectors has become a serious problem for both edible and forage rice cultivars in Japan. The risk of outbreaks of rice viruses, especially RSV and RDV, which have caused serious yield loss in rice production before the 1960s (Toriyama, 2010), is increasing. The use of insecticides to control the vector insects is one of the most effective methods of protecting rice plants from virus infection. However, the high cost of insecticide application is a major burden to rice growers, and cost-prohibitive for forage rice growers, who are forced to reduce the cost of rice production as much as possible. Genetic resistance against rice viruses or their insect vectors is also one of the most effective methods of protecting rice plants from virus infection. Although several rice genes confer resistance against RSV infection, the resistance is only partial (Noda et al., 1991). Furthermore, there are no reports, to the best of our knowledge, of naturally occurring genes that confer resistance against RDV infection.

For the reasons that we have discussed, the use of RNAi to genetically engineer plants with improved resistance against these viruses has been considered one of the most promising approaches to solve these virus problems. To develop transgenic forage cultivars with strong resistance against RSV and RDV, we generated an RNAi trigger plasmid by introducing a fused chimeric gene composed of 500-bp fragments from the 5′-proximal region for the gene for RSV pC3 and for RDV Pns12. The plasmid was then introduced into two popular forage rice cultivars, Tachisugata and Tachiaoba, and the T1 generation plants were used to evaluate plant response to infection with RSV and RDV (**Figure 5**; Sasaya et al., 2013).

All the transgenic forage cultivar plants with the introduced RNAi trigger construct for RSV pC3 and the RDV Pns12 continued to be symptom-free and did not contain detectable amounts of these viruses through harvest (ca. 5 months), whereas the RSV- and the RDV-infected non-transgenic forage cultivar plants developed typical severe symptoms by 4 weeks post-inoculation.

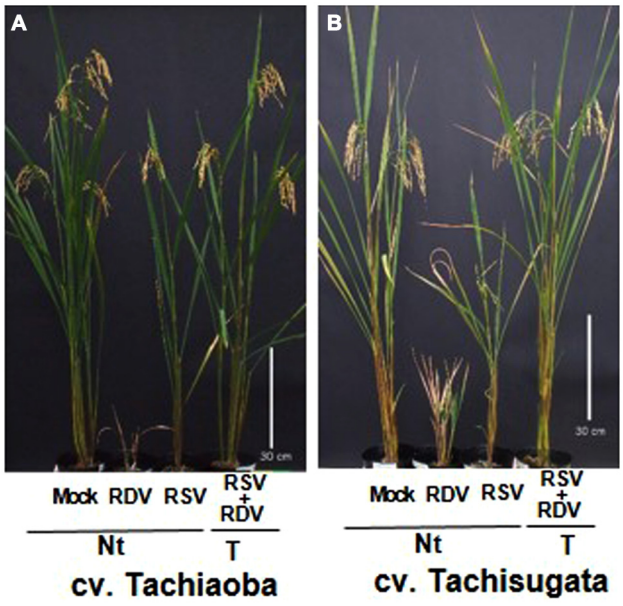

FIGURE 5 | Transgenic forage rice cultivars inoculated with *Rice stripe virus* (RSV) and *Rice dwarf virus* (RDV) (from Sasaya et al., 2013). Phenotypes of transgenic forage rice cultivars Tachiaoba **(A)** and Tachisugata **(B)** that harbored the RNAi trigger sequence targeting the RSV gene for pC3 and the RDV gene for Pns12 at 5 months after RDV and RSV inoculation. Ten-day-old transgenic forage rice seedlings were exposed to approximately 15 RSV-carrying viruliferous small brown hoppers and 10 RDV-carrying leaf hoppers per plant for each 1 day, and evaluate plant response to infection with RSV and RDV at 4 months after virus inoculation. The forage rice cultivars in pots from left to right are: mock-inoculated non-transgenic forage rice cultivars (Nt) exposed to virus-free insect vectors, showing normal growth; RDV-inoculated non-transgenic forage rice cultivars (Nt), showing typical symptoms caused by RDV infection; RSV-inoculated non-transgenic forage rice cultivars (Nt), showing typical symptoms caused by RSV infection; RDV+RSV-inoculated transgenic forage rice cultivars, showing healthy growth and fertility. Bar, 30 cm.

These transgenic forage cultivar plants were almost immune to RSV and RDV infections. In addition, no apparent differences in morphology or growth, based on plant height, number of tillers, and grain yield, were observed between transgenic and mock-inoculated non-transgenic forage cultivar plants (**Figure 5**). These results indicated that targeting the genes for the nucleocapsid protein and the viroplasm associated protein are effective in conferring strong resistance against the plant-infecting tenuivirus and reovirus, respectively, not only in the edible rice cultivar Nipponbare but also in the forage rice cultivars Tachisugata and Tachiaoba. Furthermore, simultaneously using the fused chimeric gene construct for the nucleocapsid protein and the viroplasm associated protein is effective in conferring strong resistance against both the viruses.

CONCLUSION

Rice viruses cause significant economic losses in rice production in South, Southeast, and East Asian countries. The use of insecticides against insect vectors is one of the most effective approaches to prevent damage and yield loss from rice viruses, but the high cost is a major burden on rice growers, and the continuous usage of certain insecticides is likely to result in the insect vectors developing resistance against the insecticide (Brogdon and McAllister, 1998; Wang et al., 2007). Exploitation of genetic resistance against the rice viruses is another common approach to control rice plants from viral infection. However, a few rice cultivars/lines that show resistance against the viruses have been described (Noda et al., 1991; Azzam and Chancellor, 2002; Zhang et al., 2011).

The use of RNAi is an effective, more promising method to confer strong resistance against rice viruses. When developing transgenic rice plants with strong resistance against rice viruses, it is important to target viral genes that play important roles in viral infection and proliferation at an early stage of viral replication. Furthermore, the spectrum of RNA-mediated virus resistance is generally restricted to viral strains with greater than ≈90% sequence identities with the introduced transgenes (Jones et al., 1998; Bau et al., 2003; Bucher et al., 2006; Hassani-Mehraban et al., 2009). When we compared the nucleotide sequences of the gene for the nucleocapsid protein between our strain and all the strains of RSV that are available in DDBJ/EMBL/GeneBank, the 500 bp of the RNAi trigger sequence used for cloning and transformation in our transgenic experiment, share 95.0–99.2% nucleotide sequence identities with those of other RSV strains. Thus, our transgenic plants seem to possess a potentially durable, broad-spectrum resistance against heterologous strains of RSV originating from different regions.

These transgenic techniques to confer strong resistance against virus infection are a promising approach to control viral diseases and should contribute greatly to ensuring a stable food supply in Asian countries burdened with viral disease. It is important to advance research on the development of virus-resistant transgenic rice plants, although there are many obstacles that must be overcome to actually cultivate such virus-resistant transgenic rice plants in these areas. Other than transgenic papaya resistant against papaya ring spot virus (Bau et al., 2003; Mendoza et al., 2008), no transgenic crops intended for human consumption have been widely accepted, so they are not yet cultivated in the world. The primary reason is that consumers are afraid to eat transgenic foods. Developing a virus-resistant forage rice cultivar is, however, one way to gain acceptance of the use of transgenic crops because forage rice cultivars are not edible and transgenic forage crops such as BT-corn have generally been accepted for field cultivation. These transgenic forage cultivar plants should also play a role in decreasing the viruliferous insect populations, and hence, decreasing the incidence of viral diseases not only of forage rice cultivars but also of edible rice cultivars.

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Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants

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NAC transcription factors are one of the largest families of transcriptional regulators in plants, and members of the *NAC* gene family have been suggested to play important roles in the regulation of the transcriptional reprogramming associated with plant stress responses. A phylogenetic analysis of *NAC* genes, with a focus on rice and Arabidopsis, was performed. Herein, we present an overview of the regulation of the stress responsive *NAC SNAC*/(*IX*) group of genes that are implicated in the resistance to different stresses. SNAC factors have important roles for the control of biotic and abiotic stresses tolerance and that their overexpression can improve stress tolerance via biotechnological approaches. We also review the recent progress in elucidating the roles of NAC transcription factors in plant biotic and abiotic stresses. Modification of the expression pattern of transcription factor genes and/or changes in their activity contribute to the elaboration of various signaling pathways and regulatory networks. However, a single *NAC* gene often responds to several stress factors, and their protein products may participate in the regulation of several seemingly disparate processes as negative or positive regulators. Additionally, the NAC proteins function via auto-regulation or cross-regulation is extensively found among *NAC* genes. These observations assist in the understanding of the complex mechanisms of signaling and transcriptional reprogramming controlled by NAC proteins.

Keywords: phylogenetic analysis, motif, NAC transcription factors, defense signaling pathways, biotic infections, abiotic stresses

INTRODUCTION

Biotic and abiotic stresses trigger a wide range of plant responses, from the alteration of gene expression and cellular metabolism to changes in plant growth and development and crop yields. Transcription factors (TFs) and *cis*-elements function in the promoter region of different stress-related genes, and the overexpression or suppression of these genes may improve the plant's tolerance to both types of stress. The NAC acronym is derived from three genes that were initially discovered to contain a particular domain (the NAC domain): NAM (for no apical meristem), ATAF1 and −2, and CUC2 (for cup-shaped cotyledon) (Souer et al., 1996; Aida et al., 1997). The *NAC* genes constitute one of the largest families of plant-specific TFs and are present in a wide range of species. Extensive investigation aided by the availability of several complete plant genomic sequences has identified 117 *NAC* genes in Arabidopsis, 151 in rice, 79 in grape, 26 in citrus, 163 in poplar, and 152 each in soybean and tobacco(Rushton et al., 2008; Hu et al., 2010; Nuruzzaman et al., 2010, 2012a; Le et al., 2011).

In the past decade, significant progress has been achieved in determining the molecular mechanisms of innate immune responses in rice, host recognition of pathogens, recognition-triggered early signaling events, and signaling pathways and their involvement in activating defense responses (Skamnioti and Gurr, 2009; Liu et al., 2010; Valent and Khang, 2010). To date, numerous studies have elucidated the regulatory mechanism of innate immune response in rice against blast disease, which is caused by *Magnaporthe* (M) *oryzae*. Multiple disease resistance genes (*R* genes) have been cloned and characterized (Liu et al., 2010). Similar to Arabidopsis, the salicylic acid (SA) and ethylene (ET)/jasmonic acid (JA)-mediated signaling pathways are critical in activating innate immune responses in rice and can operate in concert using some common components or biochemical events (Chern et al., 2005; Qiu et al., 2007; Yuan et al., 2007; Li et al., 2011). A number of regulatory proteins, including several TFs (e.g., OsNAC6), function in regulating defense responses against *M. grisea* (Nakashima et al., 2007). However, a complete understanding of the molecular network regulating the rice immune responses against pathogens remains unclear. Microarray profiling after biotic treatments [rice stripe virus (RSV) and rice tungro spherical virus (RTSV)] in rice seedlings has revealed six *OsNAC* genes induced by both virus infections (Nuruzzaman et al., 2010). Rice plants with a mutation in *rim1-1* are resistant to infection by dwarf virus (Yoshii et al., 2009; Satoh et al., 2011). The *StNAC* (*Solanum tuberosum*) gene is induced in response to *Phytophthora infestans* infection (Collinge and Boller, 2001). Furthermore, numerous *NAC* genes are involved in the response of plants to abiotic stresses, such as drought, salinity, cold, and submergence (Hu et al., 2006; Jeong et al., 2010; Nuruzzaman et al., 2012b).

Genes in the NAC family have been shown to regulate a wide range of developmental processes, including seed development (Sperotto et al., 2009), embryo development (Duval et al., 2002), shoot apical meristem formation (Kim et al., 2007a), fiber development (Ko et al., 2007), leaf senescence (Guo et al., 2005; Breeze et al., 2011), and cell division (Kim et al., 2006). Additionally, expression of the *AtNAC1* gene is induced by lateral root development, which in turn is regulated by the hormone auxin (Xie et al., 2000).

Regardless, few of these genes have been characterized to date. Indeed, most of the NAC family members have yet to be characterized, even though these genes are likely to play important roles in plant physiology, and substantial experimental work will be required to determine the specific biological function of each *NAC* gene. Based on phylogenetic analyses, it is apparent that this large family of TFs consists of groups that are closely related to each other (Kranz et al., 1998; Reyes et al., 2004; Tian et al., 2004). The focus of this review is the phylogeny of *NAC* genes with respect to resistance pathways. We also present an overview of the regulation of the *SNAC*/(*IX*) group of genes that are implicated in the resistance to different stresses. Furthermore, we will emphasize on the roles of *NAC* TFs genes in plant biotic and abiotic stresses.

STRUCTURAL FEATURES OF THE NAC PROTEINS

The N-terminus of NAC proteins is a highly homologous region containing the DNA-binding NAC domain. NAC proteins commonly possess a conserved NAC domain at the N-terminus that consists of approximately 150–160 amino acids and is divided into five sub-domains (A to E) (Ooka et al., 2003). The function of the NAC domain has been associated with nuclear localization, DNA binding, and the formation of homodimers or heterodimers with other NAC domain-containing proteins (Olsen et al., 2005). The structure of the DNA-binding NAC domain of Arabidopsis *ANAC019* has been solved by X-ray crystallography (Ernst et al., 2004), and the functional dimer formed by the NAC domain was identified in the structural analysis. The NAC domain structure of a rice stress-responsive NAC protein (SNAC1; STRESS-RESPONSIVE NAC 1) was also reported (Chen et al., 2011) and shares structural similarity with the NAC domain from Arabidopsis *ANAC019*. In contrast, the C-terminal regions of NAC proteins are highly divergent (Ooka et al., 2003) and are responsible for the observed regulatory differences between the transcriptional activation activity of NAC proteins (Xie et al., 2000; Yamaguchi et al., 2008; Jensen et al., 2010). The divergent C-terminal region of these proteins generally operates as a functional domain, acting as a transcriptional activator or repressor (Tran et al., 2004; Hu et al., 2006; Kim et al., 2007b). The C-terminal region is large and possesses protein-binding activity.

STRUCTURAL CONSERVATION OF SNAC GROUP

The evolutionary analysis of developmental processes of *NAC* genes through the correlation of function and phylogeny is a well-known approach in plant research (**Figure 1**; Nuruzzaman et al., 2010, 2012a). The NAC TF family has experienced extensive expansion through gene duplication events. Although NAC structural diversity has been constrained within the 60-amino acid conserved domain, which comprises a unique DNA-interacting β-sheet structure, structural conservation outside this conserved domain is extremely limited. Additional highly conserved motifs can be identified only within specific groups (e.g., SNAC, TIP, and SND), and most members in the same group share one or more motifs outside the NAC domain (Nuruzzaman et al., 2012a). A phylogeny of the SNAC group, which includes the *ANAC019* and *OsNAC6* genes, indicates the existence of multiple co-orthologs in dicots and monocots (**Figure 1**). Indeed, the SNAC group has some highly conserved motifs (**Figure 2**) within regions outside the conserved domain. A 28-amino acid (WVLCR) motif (RSARKKNSLRLDDWVLCRIYNKKGGLEK in OsNAC) is found amino-terminal to the conserved DNA-binding domain in monocots and in dicots. We first identified putative conserved motifs outside of the NAC domain in rice and compared with those of Arabidopsis and citrus. Outside of the NAC domain, rice specific conserved motifs were detected (Nuruzzaman et al., 2012a). These conserved motifs are likely to be involved in the recruitment of proteins that are involved in activating gene expression or perhaps in the control of protein stability. It is notable that only some of these motifs are conserved in both dicots and monocots, suggesting that protein function has both diverged and been conserved even within this evolutionarily conserved NAC family. Further analysis of motif function via protein-interaction analyses of TF complexes is needed.

ROLES PLAYED BY NAC TRANSCRIPTION FACTORS

Since the early research into NAC TFs, it was evident that these factors play roles in regulating several different plant processes. For convenience, some of these processes are discussed individually below. The recent data presented here provided new insight, namely, that it is common for a single NAC NF to regulate transcriptional reprogramming that is associated with multiple plant programs: the dynamic web of signaling in which NAC factors operate has multiple inputs and outputs.

NAC FUNCTION IN BIOTIC STRESS

The majority of reports concerning NAC TFs have indicated that numerous members of the multigene family play roles in the transcriptional reprogramming associated with plant immune responses. This is an active research area that has been extensively reviewed and therefore will only be briefly considered here. To date, it is clear that NAC NFs are central components of many aspects of the plant innate immune system, basal defense, and systemic acquired resistance. There are many examples in which the overexpression or knockdown of *NAC* gene expression has effects on plant defense, observations that have allowed the resolution of some components of the web of signaling pathways (**Figures 3–5**; **Table 1**) (Collinge and Boller, 2001; Delessert et al., 2005; He et al., 2005; Jensen et al., 2007, 2008, 2010).

REGULATION OF NAC TFs BY PATHOGEN INFECTION

Sun and co-workers applied Virus-induced gene silencing (VIGS) system to investigate the function of NAC TFs (ONAC122 and ONAC131) in disease resistance against *M. grisea* (Sun et al., 2013). VIGS is a useful tool for the rapid analysis of gene function in plants (Liu et al., 2002; Purkayastha and Dasgupta,

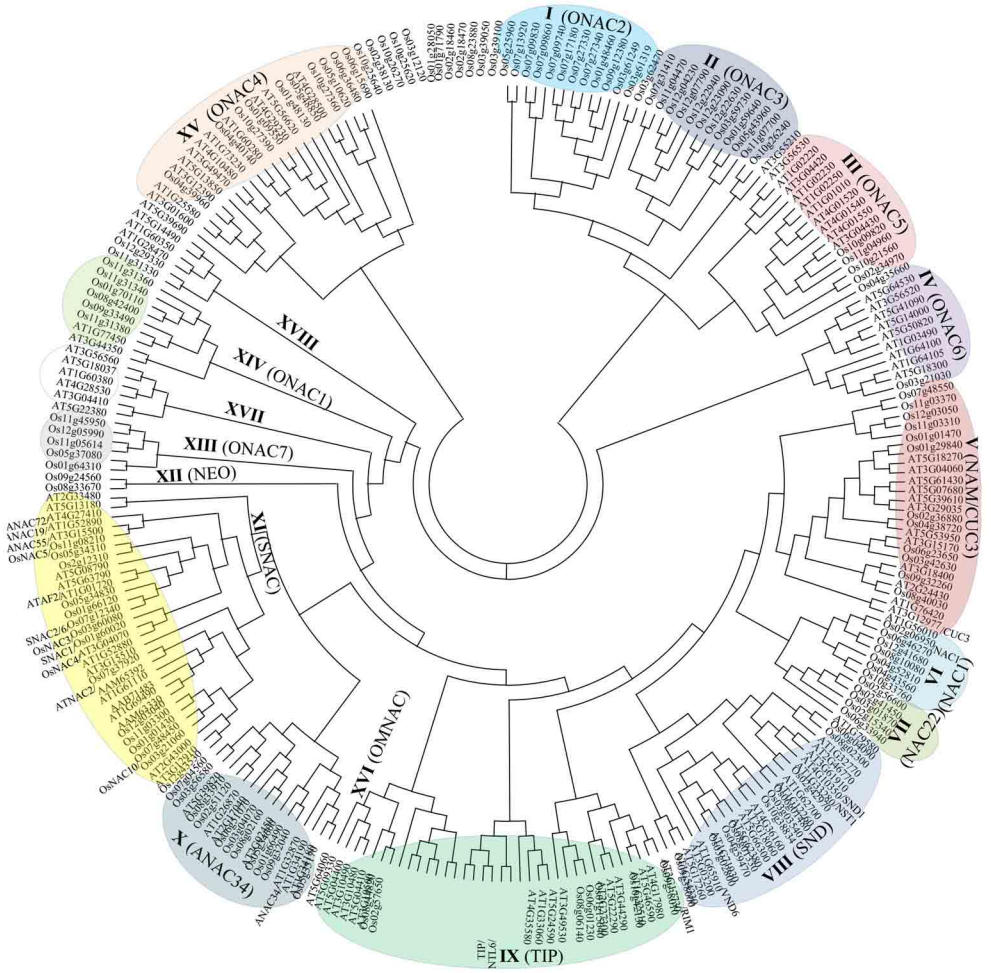

FIGURE 1 | An unrooted phylogenetic tree of the NAC transcription factors of rice and Arabidopsis. The amino acid sequences of the NAC domain of 135 rice NAC family proteins and 117 Arabidopsis NAC proteins were aligned by ClustalW, and the phylogenetic tree was constructed using MEGA 4.0 and the NJ method. Bootstrap values from 1000 replicates were used to assess the robustness of the trees. The classification by Nuruzzaman et al. (2010) is indicated in parentheses.

2009; Scofield and Nelson, 2009). Some VIGS vectors have been developed for dicotyledonous plants among which the tobacco rattle virus (TRV)-based VIGS vector is the most successful example for members of Solanaceae, such as *Nicotiana benthamiana* and *Lycopersicon esculentum* (Liu et al., 2002; Chakravarthy et al., 2010). The barley stripe mosaic virus (BSMV)-based VIGS vector was used to characterize multiple genes for their roles in disease resistance in wheat and barley (Hein et al., 2005; Scofield et al., 2005; Zhou et al., 2007; Sindhu et al., 2008). Several scientists have developed a brome mosaic virus (BMV)-based VIGS vector, and this vector was demonstrated to be a versatile tool for rapid gene function analysis in barley, rice, and maize (Ding et al., 2006; Pacak et al., 2010; van der Linde et al., 2011; Biruma et al., 2012). In rice seedlings, 19 and 13 *NAC* genes were up-regulated after RSV and RTSV infection, respectively, at different days after inoculation (Nuruzzaman et al., 2010). Several NAC proteins can either enhance or inhibit virus multiplication by directly interacting with virus-encoded proteins (**Figure 3**; Xie et al., 1999; Ren et al., 2000, 2005; Selth et al., 2005; Jeong et al., 2008; Yoshii et al., 2009), and increases in the expression level of *NAC* genes have been monitored in response to attack by viruses, several fungal elicitors, and bacteria (**Figures 3**, **4**; Xie et al., 1999; Ren et al., 2000; Collinge and Boller, 2001; Mysore et al., 2002; Hegedus et al., 2003; Oh et al., 2005; Selth et al., 2005; Jensen et al., 2007; Lin et al., 2007; Jeong et al., 2008; Wang et al., 2009a,b; Xia et al., 2010a,b). Such dual modulation in plant defense implies the association of NAC proteins with distinct regulatory complexes.

Kaneda et al. (2009) reported that *OsNAC4* is a key positive regulator of hypersensitive cell death in plants, and hypersensitive cell death is markedly decreased in response to avirulent bacterial strains in *OsNAC4*-knock-down lines. After induction by an avirulent pathogen recognition signal, *OsNAC4* is translocated into the nucleus in a phosphorylation-dependent manner. Conversely, the overexpression of *OsNAC6* does not lead to hypersensitive cell death (Kaneda et al., 2009), whereas transgenic rice plants overexpressing *OsNAC6* exhibited tolerance to

```
AAM65392          156  1.10e-28 NLSTAAKPPD LTTTRKNSLRLDDWVLCRIYKKNSSQRP TMERVLLRED
AAB71483          153  1.10e-28 NLSTAAKPPD LTTTRKNSLRLDDWVLCRIYKKNSSQRP TMERVLLRED
Os01g66120        138  2.10e-27 MHEYRLADVD RSARKKNSLRLDDWVLCRIYNKKGGLEK PPAAAVAAAG
AT1G01720         136  3.73e-27 HEYRLADVDR SVRKKKNSLRLDDWVLCRIYNKKGAERR GPPPPVVYGD
AT5G63790         179  6.51e-27 EYRLANVDRS ASTNKKNNLRLDDWVLCRIYNKKGTMEK YLPAAAEKPT
Os05g34830        147  7.81e-27 MHEYRLADVD RSARKKNTLRLDDWVLCRIYNKKGGVEK PSGGGGGERS
AT3G15510         155  1.89e-26 ENKPNNRPPG CDFGNKNSLRLDDWVLCRIYKKNNASRH VDNDKDHDMI
Os03g60080        149  7.19e-26 EYRLADAGRA AAGAKKGSLRLDDWVLCRLYNKKNEWEK MQQGKEVKEE
Os07g48450        156  4.56e-25 AAANTYKPSS SSRFRNVSMRLDDWVLCRIYKKSGQASP MMPPLAADYD
AT5G08790         137  9.38e-25 EYRLANVDRS ASVNKKNNLRLDDWVLCRIYNKKGMEKY FPADEKPRTT
AAM63330          140  1.24e-24 HEYRLHDSRK ASTKRSGSMRLDEWVLCRIYKKRGASKL LNEQEGFMDE
AT1G61110         154  3.25e-24 GNLSTAAKPP DLTTTRKSLRLDDWVLCRIYKKNSSQRP TMERVLLRED
AT1G52880         154  4.24e-24 RLTDNKPTHI CDFGNKKSLRLDDWVLCRIYKKNNSTAS RHHHHLHHIH
Os11g08210        145  1.05e-23 DVDRSAAARK LSKSSHNALRLDDWVLCRIYNKKGVIER YDTVDAGEDV
Os12g03040        151  4.63e-23 TSANNTTTTK QRRASSMTMRLDDWVLCRIHKKSNDFNS SDQHDQEPEG
Os11g03300        151  4.63e-23 TSANSTTTTK QRRASSMTMRLDDWVLCRIHKKSNDFNS SDQHDQEPEE
Os01g01430        177  4.63e-23 SNMKQLASSS SSSSSSASMRLDEWVLCRIYKKKEANQQ LQHYIDMMMD
AT1G69490         139  9.42e-23 HEYRLHDSRK ASTKRNGSMRLDEWVLCRIYKKGASKLL NEQEGFMDEV
AT3G04070         163  1.19e-22 SGGSEVNNFG DRNSKEYSMRLDDWVLCRIYKKSHASLS SPDVALVTSN
Os07g12340        149  1.19e-22 EYRLAKKGGA AAAAGAGALRLDDWVLCRLYNKKNEWEK MQSRKEEEEA
Os03g21060        152  2.10e-22 ADAHAANTYR PMKFRNTSMRLDDWVLCRIYKKSSHASP LAVPPLSDHE
Os01g60020        154  9.84e-22 HEYRLADADR APGGKKGSQKLDEWVLCRLYNKKNNWEK VKLEQQDVAS
AT1G52890         140  1.86e-21 NWIMHEYRLI EPSRRNGSTKLDDWVLCRIYKQSSAQKQ VYDNGIANAR
AT4G27410         140  2.54e-21 NWIMHEYRLI EHSRSHGSSKLDDWVLCRIYKTSGSQRQ AVTPVQACRE
Os07g37920        179  1.57e-20 TTRRPPPPIT GGSKGAVSLRLDDWVLCRIYKKTNKAGA GQRSMECEDS
Os05g34310        152  7.16e-17 GGSTASHPSL SSSTAHPSVKLDEWVLCKIFNKSPEPDN TAPPSNVVSR
AT2G33480         138  5.05e-14 NWVLHEYRLV DSQQDSLYGQNMNWVLCRVFLKKSNSNS KRKEDEKEEV
                                                    WVLCR
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FIGURE 2 | Conserved motifs outside of the NAC domain of the SNAC/(IX) group in rice and Arabidopsis.

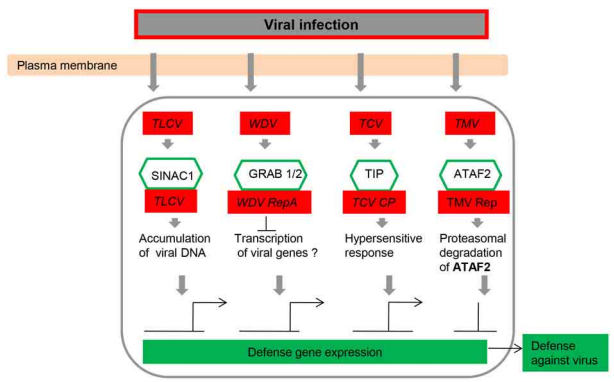

FIGURE 3 | NAC transcription factors as key components in the transcriptional regulation of gene expression during virus infection. Abbreviations: TCV, turnip crinkle virus; TIP, TCV-interacting protein; TLCV, tomato leaf curl virus; TMV, tobacco mosaic virus; WDV, wheat dwarf geminivirus.

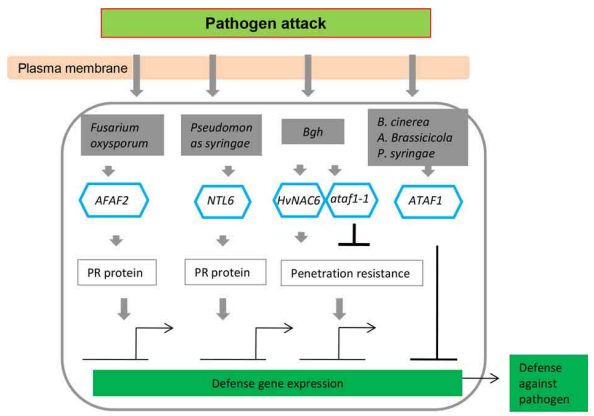

FIGURE 4 | NAC transcription factors as key components in transcriptional regulation of gene expression during pathogen attack, integrating both positive (arrows) and negative (bars) regulatory mechanisms.

blast disease (Nakashima et al., 2007). *ATAF2* overexpression resulted in increased susceptibility toward the necrotrophic fungus *Fusarium oxysporum* under sterile conditions due to the repression of pathogenesis-related (*PR*) genes (Delessert et al., 2005) but induced *PR* genes, reducing tobacco mosaic virus accumulation in a non-sterile environment (Wang et al., 2009b). RNA interference and overexpression studies have also revealed the function of NAC TFs in various plant–pathogen interactions (**Figure 4**). A number of NAC proteins may positively regulate plant defense responses by activating *PR* genes, inducing a hypersensitive response (HR), and cell death at the infection site (**Figure 4**; Jensen et al., 2007, 2008; Kaneda et al., 2009; Seo et al., 2010). *ATAF1* and its barley homolog *HvNAC6* positively regulate penetration resistance to the biotrophic fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) (Jensen et al., 2007, 2008) but attenuate the resistance to other pathogens, such as *Pseudomonas syringae*, *Botrytis cinerea*, and *Alternaria brassicicola* (Wang et al., 2009a; Wu et al., 2009). Unlike *ATAF2*, *ATAF1* and *HvNAC6* are transcriptional activators and may indirectly regulate the repression of *PR* genes via a hypothetical negative regulator (**Figure 4**). Hence, the ATAF subfamily clearly appears to have a conserved but non-redundant function in regulating the responses to different pathogens. The immune response in plants elicited upon pathogen infection is characterized by activation of multiple defense responses including expression of a large set of defense-related genes (van Loon et al., 2006), which are regulated by different types of TFs. Many TFs belonging to the NAC, ERF, and WRKY families have been identified (Eulgem and Somssich, 2007;

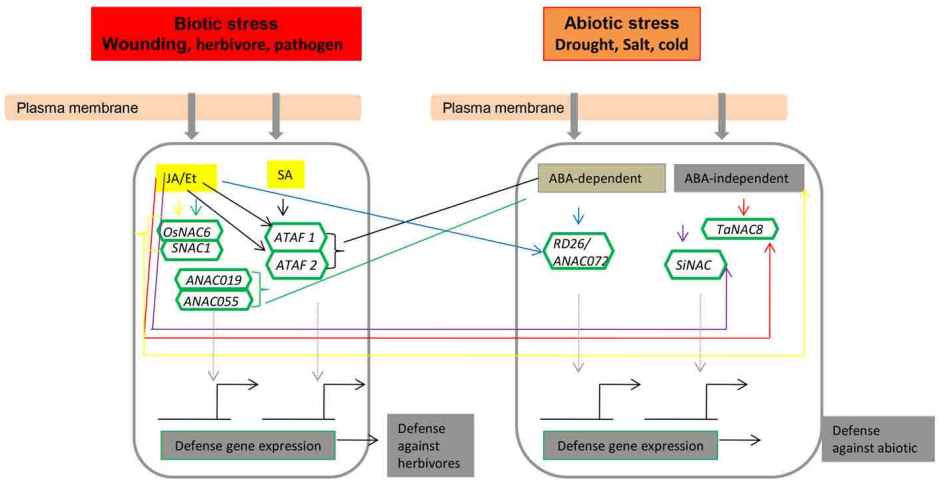

FIGURE 5 | The role of NAC transcription factors in the herbivore/biotic and abiotic response signaling pathway. Key to all colors: OsNAC6/SNAC1, yellow; ANAC019/ANAC055, green; ATAF1/ATAF2, black; TaNAC8, red; SiNAC, purple; RD26/ANAC072, blue. Abbreviations: ABA, abscisic acid; ANAC, *Arabidopsis thaliana* NAC; JA, jasmonic acid; Et, ethylene; and SA, salicylic acid.

Gutterson and Reuber, 2004) and revealed to play important roles in regulating expression of defense-related genes.

Arabidopsis stress-responsive *NAC* genes, such as *RD26*, respond to JA, a well-described phytohormone that is functionally involved in regulating wounding and biotic stress responses (Fujita et al., 2004, 2006). Hence, it is reasonable to consider that JA-responsive SNAC factors might function in both biotic and abiotic stress responses. In rice, most of the genes in the SNAC group respond to JA. Among them, *SNAC1*, *OsNAC3*, *OsNAC4*, *OsNAC5*, *OsNAC6*, and *OsNAC10* are present in the same phylogenetic SNAC/(IX) group (**Figure 1**). In particular, the SNAC group (**Figure 1**) comprises several genes that regulate disease resistance pathways, as inferred from the increased resistance to pathogens upon overexpression under the control of a constitutive promoter. Data indicate that NAC TFs also have an important role in the regulation of plant defense responses to different pathogens in addition to wounding and insect feeding (**Figure 5**). The application of exogenous phytohormones, such as JA, SA, and ET, has also been shown to induce *NAC* genes in several species (Tran et al., 2004; He et al., 2005; Hu et al., 2006; Sindhu et al., 2008; Lu et al., 2007; Nakashima et al., 2007; Yokotani et al., 2009; Zheng et al., 2009; Xia et al., 2010a,b; Yoshii et al., 2010; Nuruzzaman et al., 2012b). Hence, NAC TFs can possibly modulate the phytohormonal regulation of the biotic stress cellular network for convergent and divergent adaptive pathways.

NAC TFs IN ROS AND SENESCENCE SIGNALING PATHWAYS

Reactive oxygen species (ROS) is an active molecule in most biotic plant stress. Such ROS as H_2O_2 act as important signal transduction molecules, mediating the acquisition of tolerance to various stresses (Bhattacharjee, 2005; Davletova et al., 2005). In rice, *OsNAC6* gene is involved in both response and tolerance to biotic stress (Nakashima et al., 2007). In Arabidopsis, ATAF subfamily (*ATAF1*, *ATAF2*, and *RD26*) is also involved in biotic stress. The expression of *RD26* is induced by JA and H_2O_2, and pathogen infections (Fujita et al., 2004; Zimmermann et al., 2004). Large-scale transcriptiome analysis with both types of mutants revealed that *RD26*-regulated genes are involved in the detoxification of ROS, defense, and senescence (Fujita et al., 2004; Balazadeh et al., 2011). The role of stress-responsive NAC proteins in senescence is poorly understood. Recently, the *NTL4*, (Lee et al., 2012), *MtNAC969* (de Zélicourt et al., 2012), *Os07g37920*, wheat *GPC* (Distelfeld et al., 2012) genes were found to be induced senescence in different plants. Leaf senescence is a unique developmental process that is characterized by massive programmed cell death and nutrient recycling. Leaf senescence is induced by pathogen infection (Dhindsa et al., 1981; Buchanan-Wollaston et al., 2003; Gepstein et al., 2003). *AtNAP* gene, which belongs to the closest NAC subfamily of the ATAF subfamily, has been shown to be involved in senescence (Guo and Gan, 2006). In addition all ATAF subfamily *NAC* genes, including *ATAF1*, *ATAF2*, and *RD26*, are upregulated during senescence in Arabidopsis leaves (Guo et al., 2004). These findings suggest that *RD26* may function at the node of convergence between the pathogen defense and senescence signaling pathways. Taken together, these results support the notion that ROS and senescence may be closely related to NAC-mediated stress responses.

NAC FUNCTION IN ABIOTIC STRESS

The NAC TFs function as important components in complex signaling progresses during plant stress responses. Considering the relatively large number of NAC TFs from different plants and their unknown and diverse roles under complex environmental stimuli, it remains a considerable challenge to uncover their roles in abiotic stress. Until recently, the possible involvement of TF NAC proteins in abiotic stress responses was deduced indirectly from transcription profiling; recent functional analyses, however, have provided some direct evidence. The recent data presented here mainly summarize the function of most NAC TFs in regulating the transcriptional reprogramming associated with plant

Table 1 | Function of NAC transcription factors in biotic infections.

Genes/target genes	Functions	Method	Species	References
HvNAC6	*HvNAC6* positively regulates penetration resistant toward *Bl. gramini f.sp. hordei (Bgh)* attack	Knockdown/overexpression	*H. vulgare*	Jensen et al., 2007
ataf1-1	Loss-of-function mutants have attenuated penetration resistance toward *Bgh* attack	Knockout	*A. thaliana (At)*	Jensen et al., 2008
ATAF1, PR1	ATAF1 negatively regulates resistance to *B. cinerea*	Overexpression/*ataf1-1* and *ataf1-2*, knockout	*A. thaliana*	Wu et al., 2009
ATAF1, PR-1, PR-5, NPR1, PDF1.2	ATAF1 negatively regulates resistance to *P. syringae*, *B. cinerea*, *A. brassicicola*	Overexpression/*ataf1-2*, knockout	*A. thaliana*	Wang et al., 2009a
ATAF2, PR1, PR2, PR4, PR5, PDF1.1, PDF1.2	ATAF2 negatively regulates resistance to *F. oxysporum*, represses pathogenesis-related proteins	Overexpression/knockout	*A. thaliana*	Delessert et al., 2005
ATAF2, PR1, PR2, PDF1.2	OX = Reduced tobacco mosaic virus accumulation, increased pathogenesis-related genes	Overexpression/knockout	*A. thaliana*	Wang et al., 2009a
ATAF2, NIT2	Defense hormones, pathogen infection	Overexpression/knockout	*A. thaliana*	Huh et al., 2012
ANAC019, ANAC055	Defense disease, JA pathway	Overexpression	*A. thaliana*	Bu et al., 2008
NTL6, PR1, PR2, PR5	Positive regulator of pathogen resistance against *P. syringae*	Gene silencing/overexpression	*A. thaliana*	Seo et al., 2010
ANAC042, P450	Regulation of camalexin biosynthesis, pathogen infection	β- Glucuronidase (GUS)-reporter assays	*A. thaliana*	Saga et al., 2012
SlNAC1	Increased tomato leaf curl virus (TLCV) DNA accumulation	Transient overexpression	*N. benthamiana*	Selth et al., 2005
OsNAC4	Inducer of HR cell death upon *Acidovorax avenae* infection, loss of plasma membrane integrity, nuclear DNA fragmentation	Overexpression/knockdown	*Oryza (O) sativa*	Kaneda et al., 2009
OsNAC6, PR protein 1, Probenazoleinducible proteins (PBZ1s), DUF26- like Ser/Thr protein kinase, Thioredoxin, Peroxidase, Lipoxygenase,	Slightly increased tolerance to rice blast disease	Overexpression	*O. sativa*	Nakashima et al., 2007
rim1-1	Resistance to rice dwarf virus (RDV), susceptible to *rice* transitory yellowing virus (RTYV) and RSV	Knockout	*O. sativa*	Yoshii et al., 2009
Os02g34970, Os02g38130, Os11g03310, Os11g03370, Os11g05614, Os12g03050	RSV, RTSV infections	Microarray	*O. sativa*	Nuruzzaman et al., 2010
OsNAC19	Disease resistance	Infection	*O. sativa*	Lin et al., 2007
GRAB1, GRAB2	Inhibited wheat dwarf virus replication	Transient Overexpression	*T. monococcum*	Xie et al., 1999
ATAF2	Tobacco mosaic virus	Transgenic	*Tobaco*	Wang et al., 2009b
ONAC122 and *ONAC131* brome mosaic virus (BMV)	Defense responses against *Magnaporthe grisea*	–	*O. sativa*	Sun et al., 2013
SlNAC1	Upregulated during pseudomonas infection	Pathogen infection	*S. lycopersicum*	Huang et al., 2012
CaNAC1	Defense responses against pathogen	Infection	*C. arietinum*	Oh et al., 2005
GmNAC6	Responses to biotic signals, osmotic stress-induced	Transctiption	*G. max*	Faria et al., 2011
TLCV, SlNAC1	Enhances viral replication	Overexpression	*L. esculentum*	Selth et al., 2005
BnNAC14, BnNAC485, ATAF1 or ATAF2	Response to biotic and abiotic stresses including wounding	cDNA libraries	–	Hegedus et al., 2003
Stprx2, StNAC	Wounding and pathogen response	Transcriptome	*S. tuberosum*	Collinge and Boller, 2001

(Continued)

Table 1 | Continued

Genes/target genes	Functions	Method	Species	References
NT L4	ROS under abscisic acid, leaf senescence	Transgenic	*A. thaliana*	Lee et al., 2012
NTL9	Osmotic stress responses, leaf senescence	Overexpression/knocout	*A. thaliana*	Yoon et al., 2008
MtNAC969	Symbiotic nodule senescence	Overexpresion	*M. truncatula*	de Zélicourt et al., 2012
VNI2, OR/RD	Leaf senescence	Transcription	*A. thaliana*	Seo and Park, 2011
Os07g37920, Wheat GPC	Senescence	Overexpression/RNAi	*O. sativa, T. aestivum*	Distelfeld et al., 2012
AtNAP	Leaf senescence	Overexpression/RNAi	*A. thaliana*	Guo and Gan, 2006

abiotic responses (**Figures 5**, **6**; **Table 2**). The tight regulation and fine-tuning of *NAC* genes during plant stress responses contribute to the establishment of complex signaling webs, and the important roles of *NAC* genes in plant abiotic stress responses make them potential candidates for imparting stress tolerance.

DROUGHT, SALINITY, COLD, AND OSMOTIC STRESS

Abiotic stress triggers a wide range of plant responses, from the alteration of gene expression and cellular metabolism to changes in plant growth, development, and crop yield. Thus, understanding the complex mechanism of drought and salinity tolerance is important for agriculture production. Interestingly, many *NAC* genes have been shown to be involved in plant responses to drought and salinity stress. In transgenic rice, the *Os01g66120/OsNAC2/6* and *Os11g03300/OsNAC10* genes were found to enhance drought and salt tolerance (**Figure 5**; Nakashima et al., 2009; Jeong et al., 2010), and *Os03g60080/SNAC1* increased grain yield (21–34%) under drought stress (Hu et al., 2006). Udupa et al. (1999) reported that comparative gene expression profiling is an efficient way to identify the pathways and genes regulating a stress response under different stress conditions. The Arabidopsis *NAC* gene *ANAC092* demonstrates an intricate overlap of *ANAC092*-mediated gene regulatory networks during salt-promoted senescence and seed maturation (Balazadeh et al., 2010). Lan et al. (2005) found that a large portion of the genes regulated by dehydration are also up-regulated by fertilization; indeed, pollen is a major site of variations in the expression levels for many genes (Czechowski et al., 2005). Related conclusions have been drawn from analyses based on promoter-GUS fusions of cold-inducible *Os01g66120/SNAC2/6*, *Os11g03300/OsNAC10*, *RD29A*, *COR15A*, *KIN1*, and *COR6.6* in rice and Arabidopsis, genes that are regulated during plant development (root, leaf, and pollen) under both stress (drought and cold) and non-stress conditions (Sindhu et al., 2008; Jeong et al., 2010). You et al. (2013) reported that *OsOAT* is a direct target of the stress-responsive NAC transcription factor SNAC2, and *OsOAT* overexpression in rice resulted in significantly enhanced drought and osmotic stress tolerance. Plants overexpressing *GmNAC085* show enhanced drought tolerance (Le et al., 2011), whereas the overexpression of *GmNAC11* led to increased sensitivity to salt and mannitol stresses (Hao et al., 2011). Microarray profiling of the roots and leaves of drought-treated rice revealed the induction of 17 *NAC* genes by severe or mild drought treatment (Nuruzzaman et al., 2012b). *SiNAC* is also simultaneously induced by dehydration, salinity, ethephon, and methyl jasmonate treatments (Puranik et al., 2011). Similarly, the expression of *DgNAC1*, *TaNAC2a* and *EcNAC1* were strongly induced by NaCl and drought stresses in transgenic tobacco plants (Liu et al., 2011; Ramegowda et al., 2012; Tang et al., 2012). Several genes, such as *ZmSNAC1* (Lu

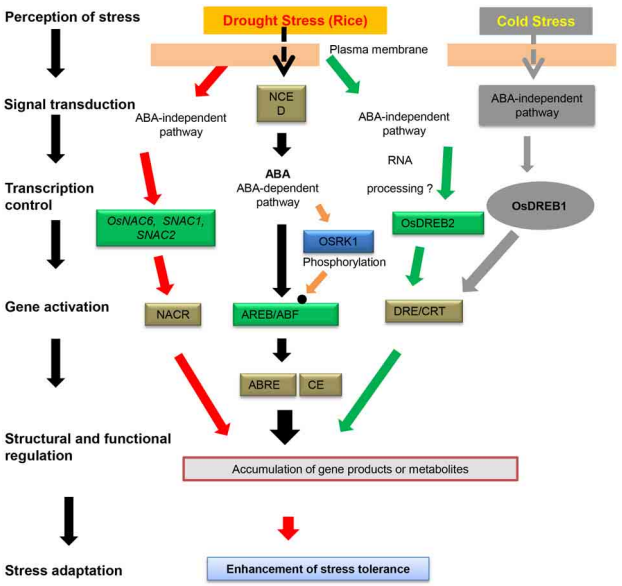

FIGURE 6 | Transcriptional regulatory networks of the *cis*-elements and NAC transcription factors involved in abiotic stress-induced gene expression in rice. The *cis*-elements involved in stress-responsive transcription are shown in white boxes. TFs controlling stress-inducible gene expression are shown in green boxes. Protein kinases involved in the phosphorylation of TFs are shown in blue boxes. The small solid black circle indicates TF modification, i.e., through phosphorylation, in response to stress signals.

Table 2 | Function of NAC transcription factors in abiotic stresses.

Genes	Functions	Method	Species	References
ANAC019/AT1G52890	Drought, high salinity, ABA signaling	Overexpression	*A. thaliana*	Tran et al., 2004
ANAC055/AT3G15500	Drought, high salinity, ABA signaling	Overexpression	*A. thaliana*	Tran et al., 2004
ANAC072/AT4G27410	Drought, high salinity, ABA signaling	Overexpression	*A. thaliana*	Tran et al., 2004
RD26, RD20, Glyoxalase, Glutathione, transferase, Aldo/keto reductase, senesence associated gene13, cinnamil-alcohol dehydrogenase	Drought, salt and ABA response	Overexpression	*A. thaliana*	Fujita et al., 2004
ANAC019, COR47, RD29b, FER1, ERD11	Cold, ABA signaling	Overexpression	*A. thaliana*	Jensen et al., 2010
anac092-1, ANAC083, ANAC041, ANAC054, ANAC084	Positive regulator of seed germination under salinity	Mutant	*A. thaliana*	Balazadeh et al., 2010
ntl8-1	Positive regulator of seed germination under salinity	Mutant	*A. thaliana*	Kim et al., 2008
ATAF1, COR47, ERD10, KIN1, RD22, RD29A	Positive regulator of drought tolerance	*knockouts (ataf1-1/2)*	*A. thaliana*	Lu et al., 2007
ATAF1, ADH1, RD29A, COR47	Positive regulator of drought tolerance	Overexpression	*A. thaliana*	Wu et al., 2009
ONAC063	Higher seed germination under high salinity and osmotic stress	Overexpression	*A. thaliana*	Yokotani et al., 2009
AhNAC2, RD29A, RD29B, RAB18, ERD1, AtMYB2, AtMYC2, COR47, COR15a, KIN1, AREB1, CBF1	Drought and salt tolerance	Overexpression	*A. thaliana*	Liu et al., 2011
GmNAC20, DREB1A/CBF3, KIN2/cor6.6, Cor15A, RD29A/cor78, ARF19, LBD12, AIR1	Salt and freezing tolerance	Overexpression	*G. max, A. thaliana*	Hao et al., 2011
NTL8	Salt tolerance, GA, and ABA pathway	Gene expression	*A. thaliana*	Kim et al., 2008
ANAC019, ANAC055	Defense disease, JA pathway	Overexpression	*A. thaliana*	Bu et al., 2008
XND1	Programmed cell death	Overexpression	*A. thaliana*	Zhao et al., 2008
LOV1	Cold response, photoperiod pathway	Overexpression	*A. thaliana*	Yoo et al., 2007
NAC1	Auxin, root development	Overexpression	*A. thaliana*	Guo et al., 2005
ZmSNAC1	Low temperature, high-salinity, drought stress, and abscisic acid (ABA)	Transgenic	*Z. mays*	Lu et al., 2012
NTL6, SnRK2.8	Drought-stress response	Overexpression/ RNAi	*A. thaliana*	Kim et al., 2012
ANAC019, ANAC055 and *ANAC072, ICS1* and *BSMT1*	Inhibits salicylic acid accumulation	Transgenic	*A. thaliana*	Zheng et al., 2012
TaNAC2	Drought, salt, and freezing stresses	Overexpression	*A. thaliana*	Mao et al., 2012
ANAC2/AT3G15510	Salt and ABA stress tolerance	Overexpression	*A. thaliana*	He et al., 2005
SNAC1/Os03g60080	Stomata close, higher seed setting	Overexpression	*O. sativa*	Hu et al., 2006
SNAC2/OsNAC6/Os01g66120	Salt, drought, disease resistance drought, salinity, cold, wounding, and abscisic acid (ABA) treatment	Overexpression	*O. sativa*	Sindhu et al., 2008
OsNAC5/ Os11g08210	ABA, salt, cold tolerance, grain filling	Overexpression	*O. sativa*	Sperotto et al., 2009
ONAC04/Os11g033005	Drought, salt, cold tolerance	Overexpression	*O. sativa*	Zheng et al., 2009
OsNAC10/Os11g03300	Root, panicle, drought, salt, ABA	Overexpression	*O. sativa*	Jeong et al., 2010
Ostil1	Shoot branching	Overexpression	*O. sativa*	Mao et al., 2007
RIM1/Os03g02800	JA pathway signaling	Mutant	*O. sativa*	Yoshii et al., 2010
Os07g04560, Os10g38834	Root, severe drought	Microarray	*O. sativa*	Nuruzzaman et al., 2012b

(Continued)

Table 2 | Continued

Genes	Functions	Method	Species	References
Os01g28050, Os01g29840	Leaf, severe drought	Microarray	*O. sativa*	Nuruzzaman et al., 2012b
Os03g12120, *Os03g59730*, *Os06g15690*, *Os08g06140*, *Os08g33670*	Panicle, severe drought	Microarray	*O. sativa*	Nuruzzaman et al., 2012b
Os12g41680, *Os07g48550*, *Os11g03300*, *Os12g03040*, *Os01g66120*, *Os05g34830*,	Cold, drought, submergence, laidown-submergnece	Microarray	*O. sativa*	Nuruzzaman et al., 2010
Os02g34970, *Os07g48450*, *Os01g01430*, Os01g48460	Drought, submergence, laidown-submergnece	Microarray	*O. sativa*	Nuruzzaman et al., 2010
OsOAT, *SNAC2*	Drought and oxidative stress tolerance	Overexpression	*O. sativa*	You et al., 2013
SNAC1, *OsSRO1c*	Oxidative stress tolerance	Overexpression	*O. sativa*	You et al., 2013
TaNAC69	PEG-induced dehydration	Overexpression	*T. aestivum*	Xue et al., 2011
GmNAC11, *DREB1A*, *ERD11*, *Cor15A*, *ERF5*, *RAB18*, *KAT2*	Salt tolerance in soybean transgenic hairy roots	Overexpression	*G. max*	Hao et al., 2011
GmNAC glycoside hydrolases, defensins and glyoxalase I family proteins	Drought stress	Soybean array GeneChip	*G. max*	Le et al., 2011
GmNAC085	Dehydration stress	Soybean Affymetrix array	*G. max*	Le et al., 2011
TaNAC2a	Drought tolerance	Overexpression	*N. tabacum*	Tang et al., 2012
DgNAC1	ABA, NaCl, drought and cold	Overexpression	*N. tabacum*	Liu et al., 2011
CarNAC3	Seed germination, drought, ethephon, ABA, IAA signaling	Transcriptome	*C. arietinum*	Peng et al., 2009
miR319, AsNAC60	Drought and salinity stress		*Agrostis stolonifera*	Zhou et al., 2013
EcNAC1	Water-deficit and salt stress	Overexpression	*N. tabacum*	Ramegowda et al., 2012
AhNAC2	Salt	Overexpression	Arachis	Liu et al., 2011
RhNAC2 or *RhEXPA4*	Dehydration tolerance	Transgenic	*R. hybrida*	Dai et al., 2012
ClNAC	Hormonal treatments including salt, drought, cold, heat, abscisic acid and salicylic acid treatments	Reverse transcriptase polymerase chain reaction	*C. lavandulifolium*	Huang et al., 2012
CsNAM	Drought, osmoticum, salt, heat and hydrogen peroxide		*Camellia sinensis*	Paul et al., 2012
Os04g0477300	Boron-toxicity tolerance	RNA interference	*O. sativa*	Ochiai et al., 2011
SiNAC	Dehydration, salinity, ethephon, and methyl jasmonate.	Transcription	*S. italica*	Puranik et al., 2011
ANAC102	Waterlogging	Overexpression	*A. thaliana*	Christianson et al., 2009
HSImyb and *HSINAC*	Gibberellin response	Transcript	*H. vulgare*	Robertson, 2004
ANAC042 it is also in biotic	Heat stress	Overexpression	*A. thaliana*	Shahnejat-Bushehri et al., 2012
TaNAC2a, *TaNAC4a*, *TaNAC6*, *TaNAC7*, *TaNAC13* and *TaNTL5*	Dehydration, salinity and low temperature	Transgenic	*T. aestivum*	Tang et al., 2012
TaNAC4	Environmental stimuli, including high salinity, wounding, and low-temperature also induced	Transcription	*T. aestivum*	Xia et al., 2010a
ONAC063	High-temperature and high-salinity	Transactivation	*O. sativa*	Yokotani et al., 2009

et al., 2012), *TaNAC69* (Xue et al., 2011), *CarNAC3* (Peng et al., 2009), *miR319, AsNAC60* (Zhou et al., 2013), *AhNAC2* (Liu et al., 2011), *RhNAC2* or *RhEXPA4* (Dai et al., 2012), *ClNAC* (Huang et al., 2012), *CsNAM* (Paul et al., 2012), *SiNAC* (Puranik et al., 2011), *HSImyb* and *HSINAC* (Robertson, 2004), and *TaNAC2a, TaNAC4a, TaNAC6*, and *TaNAC4* (Tang et al., 2012; Xia et al., 2010a), were increased by drought and NaCl (**Figure 5**; **Table 2**).

PHYTOHORMONE SIGNALING PATHWAY

The expression of members of the *OsNAC* gene family under hormone treatment requires extensive cross-talk between the response pathways, and it is likely that substantial physiological connections exist between NAC protein production and phytohormone treatment. Phytohormones are involved in influencing signaling responses by acting in conjunction with or in opposition to each other to maintain cellular homeostasis (Fujita et al., 2006; Miller et al., 2008). The NAC TFs form a complex but interesting group of important arbitrators of this process (**Figure 5**). *ANAC019* and *ANAC055* are involved in both ABA- and JA-mediated regulation (Greve et al., 2003; Bu et al., 2008, 2009; Jensen et al., 2010). The ATAF subfamily TFs are another group of NAC proteins that act at the convergence point of biotic and abiotic stress signaling (Delessert et al., 2005; He et al., 2005; Jensen et al., 2007). Because *ATAF1* alleles expedite drought perception at the cost of optimal basal defense, ATAF1 acts as a negative regulator of ABA signaling but induces JA/ET-associated defense signaling marker genes (Jensen et al., 2008). Conversely, *ATAF2* expression was induced by dehydration, JA, and SA (**Figure 5**; Delessert et al., 2005). We have proposed the participation of *SiNAC* in the ABA-independent pathway of abiotic stress and in regulating biotic stress via an antagonistic JA and SA pathway (Puranik et al., 2011). A number of *NAC* genes (e.g., *AtNAC2*) in plants are affected by auxin, ethylene (Xie et al., 2000; He et al., 2005), and ABA (e.g., *OsNAC5*; Sperotto et al., 2009). In Arabidopsis, NAC TF *NTL8* regulates GA3-mediated salt signaling in seed germination (Kim et al., 2008). ABA plays a major role in mediating the adaptation of a plant to stress, and this hormone can stimulate root growth in plants that need to increase their ability to extract water from the soil. *OsNAC5*/*ONAC009*/*ONAC071* and *OsNAC6* are homologs that are induced by abiotic stress, such as drought and high salinity, and ABA (Takasaki et al., 2010). *AtNAC1* and *AtNAC2* are induced by auxin and ABA, respectively, and *AtNAC1* mediates auxin signaling to promote lateral root development in Arabidopsis (Xie et al., 2000; He et al., 2005). ABA signaling induces the expression of genes encoding proteins that protect the cells in vegetative tissues from damage when they become dehydrated. These well-known ABA responses are less sensitive to ABA in NPX1-overexpressing plants (Kim et al., 2009). The expression of the *RD26* gene is induced by drought and also ABA and high salinity (Fujita et al., 2004). NAC TFs regulate many target genes by binding to the CATGTG motif in the promoter region of the target gene to activate transcription in the response to drought stress (Nakashima et al., 2007), a transcriptional regulatory system that is known as a regulon. ABA is produced under conditions of drought stress and plays a crucial role in drought tolerance in plants (**Figure 6**; Shinozaki et al., 2003). In addition to NAC and other regulons, *OsDREB2* responds to dehydration in rice (Dubouzet et al., 2003); the dehydration-responsive element binding protein 1 (DREB1)/C-repeat-binding factor (CBF) and DREB2 regulons function in ABA-independent gene expression, whereas the ABA-responsive element (ABRE)-binding protein (AREB)/ABRE-binding factor (ABF) regulon functions in ABA-dependent gene expression. ABA-activated OSRK1 protein kinases phosphorylate and activate AREB/ABF-type proteins in rice (**Figure 6**; Chae et al., 2007). Both ABA-independent and ABA-dependent signal transduction pathways convert the initial stress signal into cellular responses (**Figures 5**, **6**). The TF family members involved in both ABA-independent (AP2/ERF, bHLH, and NAC) and ABA-dependent (MYB, bZIP, and MYC) pathways are up-regulated in rice; the TFs belonging to this family interact with specific *cis*-elements and/or proteins, and their overexpression confers stress tolerance in heterologous systems (Fujita et al., 2004; Tran et al., 2004; Hu et al., 2006). The expression of *OsNAC6* is induced by ABA and abiotic stresses, including cold, drought, and high salinity (Nakashima et al., 2007). Together, these data provide evidence that different *NAC* genes play differential roles in the specific responses to different phytohormone treatments. Thus, gene expression profiles under both biotic and abiotic stresses to determine the vital role of *NAC* genes in plant growth and stress responses and the identification of target genes for TFs involved in stress responses are important.

TEMPERATURE STRESS

In agriculture, high or low temperature acts as a major negative factor limiting crop production. Indeed, tremendous work has been performed in the past two decades to reveal the complex molecular mechanism in the plant responses to extreme temperature, and there is increasing evidence that NAC proteins are involved in responses to both heat and cold stresses. For example, an NAC TF gene (*ONAC063*) in rice roots responds to a combination of high-temperature stress (Yokotani et al., 2009). Another example is that transgenic Arabidopsis plants overexpressing *ANAC042* show increased tolerance to heat stress when compared to the wild-type plants (Shahnejat-Bushehri et al., 2012). Moreover, the overexpression of *ZmSNAC1* enhanced the tolerance to drought and low-temperature stress compared to the control (Lu et al., 2012). The expression of *OsNAC10*, *SNAC2/OsNAC6*, *TaNAC4,NTL6, TaNAC2a, TaNAC4a, TaNAC6,TaNAC7,TaNAC13*, and *TaNTL5* is induced by low temperature in plants (Jeong et al., 2010; Xia et al., 2010a; Tang et al., 2012), and a gene expressing a CsNAM-like protein is induced by heat in tea plants (Paul et al., 2012). Northern blot and SNAC2 promoter activity analyses suggest that the *SNAC2* gene is induced by low temperature. Additionally, a microarray analysis of rice *NAC* genes has revealed that 8 of the 14 analyzed *OsNAC* genes are regulated by severe or mild drought stress (Nuruzzaman et al., 2012b), showing distinct expression patterns upon high-temperature treatment. Yoo et al. (2007) reported that the phenotype resulting from the overexpression of an NAC-domain protein gene (*At2g02450*) is related to the control of flowering time and cold responses. The importance of NAC proteins in plant development, transcription regulation, and regulatory pathways involving protein–protein interactions is being increasingly recognized. Taken together, NAC proteins

function in plants adaptions to temperature variations through the transcriptional reprogramming of downstream stress-related genes.

NUTRIENT-USE EFFICIENCY

Various nutrient elements are required for the normal growth and development of plants. Boron (B) is an essential micronutrient for higher plants, but excessive amounts of B inhibit growth (B toxicity). As the optimal range of B concentration in tissues is narrow (Blamey et al., 1997), B toxicity occurs in many plants at levels only slightly above that required for normal growth (Mengel and Kirkby, 2001). The *Os04g0477300* gene encodes an NAC-like TF, and the function of the transcript is abolished in B toxicity-tolerant cultivars. Transgenic plants in which the expression of *Os04g0477300* is abolished by RNA interference acquire a tolerance to B toxicity (Ochiai et al., 2011). In a transcriptome analysis using Arabidopsis plants under B toxicity, nine genes encoding multidrug and toxic compound extrusion transporters, a zinc-finger family TF, a heat-shock protein-like protein, an NAC-like TF, and unknown proteins were induced (Kasajima and Fujiwara, 2007), though the functions of these proteins are not yet known. A sufficient supply of inorganic phosphate (Pi) is vital to plants, and the low bioavailability of Pi in soils is often a limitation to growth and development. Consequently, plants have evolved a range of regulatory mechanisms to adapt to phosphorus-starvation to optimize the uptake and assimilation of Pi. Recently, significant progress has been achieved in elucidating these mechanisms, revealing that the coordinated expression of a large number of genes is important for many of these adaptations. These studies provide a valuable basis for the identification of new regulatory genes and promoter elements to further the understanding of Pi-dependent gene regulation. With a focus on the Arabidopsis transcriptome, Nilsson et al. (2010) reported common findings that indicate new groups of putative regulators, including the NAC, MYB, and WRKY families. With a number of new discoveries of regulatory elements, a complex regulatory network is emerging. They evaluate the contribution of the regulatory elements to P-responses and present a model comprising the factors directly or indirectly involved in transcriptional regulation. Thus, *NAC* genes appear to respond to several aspects of nutrient excess and deficiency-induced stresses, implicating their diverse functions in these signaling pathways.

ONE NAC FOR MULTIPLE PROCESSES

Numerous studies have demonstrated that a single TF may function in several seemingly disparate signaling pathways, as can be deduced from their induced expression profiles by various stress factors. *OsNAC6* was induced by JA, a plant hormone that activates defense responses against herbivores and pathogens (**Figures 5**, **6**; Ohnishi et al., 2005). Studies on an *NAC* gene (*Os04g0477300*) showed that it functions in at least three different processes, including pathogen defense, senescence, and responses to phosphate and boron deficiency (Uauy et al., 2006; Waters et al., 2009; Nilsson et al., 2010; Ochiai et al., 2011). A number of *NAC* genes (e.g., *AtNAC2*) in plants are affected by auxin, ethylene (Xie et al., 2000; He et al., 2005), and ABA (e.g., *OsNAC5*; Sperotto et al., 2009). *Os05g34830* (SNAC group, **Figure 1**) was specifically induced in the roots of a tolerant line under severe and mild drought conditions and was activated by ABA treatment (Nuruzzaman et al., 2012b). *OsNAC5*/*ONAC009*/*ONAC071* and *OsNAC6* are homologs that are induced by pathogen infection and such abiotic stresses as drought and high salinity and ABA (Takasaki et al., 2010). *AtNAC1* and *AtNAC2* are induced by auxin and ABA, respectively, and *AtNAC1* mediates auxin signaling to promote lateral root development in Arabidopsis (Xie et al., 2000; He et al., 2005). The *TaNAC4* gene functions as a transcriptional activator involved in wheat responses to abiotic and biotic stresses (Xia et al., 2010a). *SiNAC* transcripts mostly accumulate in young spikes and were strongly induced by dehydration, salinity, ethephon, and methyl jasmonate (Distelfeld et al., 2012). These data demonstrate that a single *NAC* gene can function as regulator of several different processes and may also mediate the cross-talk between different signaling pathways.

CONCLUSION

The responses to the environment are specialized through the diversification of the structure of stress-response regulators, which are involved in stress-response pathways via binding motifs (CATGTG) in their target genes. Thus, the components and regulatory structure of specific pathways must be delimited for an understanding of the evolutionary genetics of environmental stress responses. This review summarizes the current knowledge of the genes and NAC TFs that comprise a portion of this network. Interestingly, all of the SNAC sequences known to play a role in disease resistance responses are in one group of the NAC family. Much progress in NAC TF functional research has been attained over the past decade. However, most of these advances are related to the involvement of biotic stress. The identification of NAC functions in biotic and abiotic stresses will remain a substantial challenge in the coming years. To achieve a better understanding of their role during both types of stress, it is very important to identify the interacting partner of NAC proteins that cooperates in regulating the transcription of downstream target genes under a specific condition. It is also crucial to identify the key components of the signal transduction pathways with which these factors physically interact. Applying data obtained from microarrays could help to directly determine the specific NAC DNA-binding sites on a global scale under conditions of biotic and abiotic stress. Accordingly, we may then appreciate the complex mechanisms of signaling and transcriptional reprogramming controlled by NAC proteins and the plant processes in which they participate. Certainly, further molecular studies of NAC NFs under different stresses will clarify the fine-tuning mechanisms that are controlled by NAC proteins in plants, with economical benefits to agricultural production.

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Suppression of cell wall-related genes associated with stunting of *Oryza glaberrima* infected with *Rice tungro spherical virus*

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Rice tungro disease is a complex disease caused by the interaction between *Rice tungro bacilliform virus* and *Rice tungro spherical virus* (RTSV). RTSV alone does not cause recognizable symptoms in most Asian rice (*Oryza sativa*) plants, whereas some African rice (*O. glaberrima*) plants were found to become stunted by RTSV. Stunting of rice plants by virus infections usually accompanies the suppression of various cell wall-related genes. The expression of cell wall-related genes was examined in *O. glaberrima* and *O. sativa* infected with RTSV to see the relationship between the severity of stunting and the suppression of cell wall-related genes by RTSV. The heights of four accessions of *O. glaberrima* were found to decline by 14–34% at 28 days post-inoculation (dpi) with RTSV, whereas the height reduction of *O. sativa* plants by RTSV was not significant. RTSV accumulated more in *O. glaberrima* plants than in *O. sativa* plants, but the level of RTSV accumulation was not correlated with the degree of height reduction among the four accessions of *O. glaberrima*. Examination for expression of genes for cellulose synthase A5 (CESA5) and A6 (CESA6), cellulose synthase-like A9 (CSLA9) and C7, and α-expansin 1 (expansin 1) and 15 precursors in *O. glaberrima* and *O. sativa* plants between 7 and 28 dpi with RTSV showed that the genes such as those for CESA5, CESA6, CSLA9, and expansin 1were more significantly suppressed in stunted plants of *O. glaberrima* at 14 dpi with RTSV than in *O. sativa*, suggesting that stunting of *O. glaberrima* might be associated with these cell wall-related genes suppressed by RTSV. Examination for expression of these genes in *O. sativa* plants infected with other rice viruses in previous studies indicated that the suppression of the expansin 1 gene is likely to be a signature response commonly associated with virus-induced stunting of *Oryza* species. These results suggest that stunting of *O. glaberrima* by RTSV infection might be associated with the suppression of these cell wall-related genes at the early stage of infection with RTSV.

Keywords: ***Oryza glaberrima, Rice tungro spherical virus*, stunting, cell wall genes**

INTRODUCTION

Rice tungro disease (RTD) is one of the major constraints to rice production in South and Southeast Asia (Azzam and Chancellor, 2002). RTD is a composite disease caused by two taxonomically unrelated viruses, *Rice tungro bacilliform virus* (RTBV) and *Rice tungro spherical virus* (RTSV) both transmitted by green leafhoppers (GLH) (Hull, 1996). RTBV is a plant pararetrovirus belonging to the family *Caulimoviridae*, genus *Tungrovirus*, with a circular, double-stranded DNA genome encapsidated in bacilliform particles (Fauquet et al., 2005). RTSV is the type member of the *Sequiviridae* family, genus *Waikavirus*, having a single-stranded polyadenylated plus-sense RNA genome encapsidated in polyhedral particles (Choi, 2008). Asian rice (*Oryza sativa*) plants infected with both RTBV and RTSV usually show symptoms such as severe stunting, yellowing of the leaves, and reduced tillering (Azzam and Chancellor, 2002). *O. sativa* plants infected with RTBV alone exhibit recognizable stunting and yellowing of the leaves, whereas *O. sativa* plants infected with RTSV alone exhibit very mild stunting or no clear symptoms (Hibino et al., 1990).

African rice (*O. glaberrima*) is known to have many favorable traits such as weed competitiveness, drought tolerance, pest resistance, and the ability to grow under low-input conditions (Sarla and Swamy, 2005, and references therein). Efforts have been made to transfer genes conferring such useful traits from *O. glaberrima* to *O. sativa* through inter-specific hybridization (Sarla and Swamy, 2005, and references therein). However, some accessions of *O. glaberrima* were found to be hypersensitive to RTD, and to be significantly stunted even when infected with RTSV alone (Cabauatan et al., 1993).

Stunting is a common symptom of *O. sativa* infected with viruses such as *Rice dwarf virus* (RDV), *Rice grassy stunt virus*, *Rice ragged stunt virus*, *Rice stripe virus* (RSV), and RTBV

(Hibino, 1996). Genome-wide gene expression analyses in *O. sativa* plants infected with RDV (Shimizu et al., 2007; Satoh et al., 2011) and RSV (Satoh et al., 2010) revealed that many stress response-related genes were activated, whereas various development-related genes, including genes involved in cell wall synthesis, were suppressed in *O. sativa* plants infected with RDV and RSV, indicating that stunting of *O. sativa* plants by RDV and by RSV might be associated with the suppression of various development-related genes, especially cell wall-related genes. The expression of many defense- and development-related genes, including cell wall-related genes was also found to be regulated in *O. sativa* plants by infection with RTSV (Satoh et al., 2013). However, the consequences from such changes in gene expression by RTSV were uncertain since the *O. sativa* plants infected with RTSV remained asymptomatic.

In this study, we examined the relationship between the severity of stunting and the expression of six cell wall-related genes in *O. glaberrima* infected with RTSV to see whether stunting of *O. glaberrima* is associated with the capability (pathogenicity) of RTSV to suppress the expression of cell wall-related genes. The results showed that the cell wall-related genes such as those for α-expansin 1 precursor (expansin 1), cellulose synthase A5 (CESA5), cellulose synthase A6 (CESA6), and cellulose synthase-like A9 (CSLA9) were more significantly suppressed in stunted plants of *O. glaberrima* at the early stage of infection with RTSV than in *O. sativa*, suggesting that the suppression of these genes at the early infection stage is signature responses associated with stunting of *O. glaberrima* induced by RTSV infection.

MATERIALS AND METHODS

PLANT MATERIALS

Eighteen accessions of *O. glaberrima* (International Rice Germplasm Collection (IRGC) accession numbers 86741, 96717, 96718, 96790, 96793, 96864, 96868, 100139, 100153, 102556, 102569, 103437, 103477, 104545, 104589, 104914, 112576, and 115633) were obtained from the T. T. Chang Genetic Resources Center, International Rice Research Institute (IRRI). *O. sativa* cultivar Taichung Native 1 (TN1) is susceptible to RTSV. TW16 is a backcross line (BC_5) resistant to RTSV, and was developed by serial backcrosses of RTSV-resistant cultivar Utri Merah (IRGC number 16682) with TN1 (Lee et al., 2010).

INOCULATION OF RTSV

RTSV strain A (Cabauatan et al., 1995) maintained in TN1 was used as the source of inoculum. Five plants each of TN1 and TW16, and 18 *O. glaberrima* accessions were used per treatment (mock control or RTSV-inoculated) and sampling timing [7, 14, 21, 28, or 30 days post-inoculation (dpi)]. The plants were grown in a 12-cm-diameter pot with each pot containing five seedlings. GLH-mediated inoculation of RTSV to plants was done by the tube method as described by Cabauatan et al. (1995). GLH were given a 3-day acquisition access period to RTSV-infected plants and were allowed an inoculation access period of 24 h to 9-day-old plants at three insects per plant. Plants for mock control were prepared by feeding three virus-free insects per plant for 24 h. At Twenty Four hours after inoculation started, GLH were removed by insecticide. The inoculated plants were maintained in the greenhouse of IRRI.

EVALUATION FOR REACTIONS TO RTSV

The height of individual mock- and RTSV-inoculated plants was measured at 7, 14, 21, and 28 dpi) ($n = 5$ per treatment and time point in one experiment). Height reduction rates (%) due to RTSV infection were computed as 100 × [(height of mock-inoculated plant—height of RTSV-infected plant)/height of mock-inoculated plant]. After the height measurement, the second youngest fully expanded leaf was collected from each plant for RNA extraction (see below). The leaf samples were quickly frozen in liquid nitrogen and immediately kept at −80°C until RNA extraction. Subsequently, approximately 1/3 of the upper part of each plant was collected for evaluation of RTSV accumulation in the plant. One gram of plant samples collected were pulverized in liquid nitrogen, and homogenized with 10 ml of phosphate buffer saline containing Tween-20 to prepare 10 time-diluted sap samples. The diluted plant sap samples were used to estimate RTSV accumulation in plants by enzyme-linked immunosorbent assay (ELISA) using an antibody raised against purified RTSV as described by Shibata et al. (2007). The experiment for height measurement and that for estimation of RTSV accumulation were repeated three times. The differences between the heights of mock- and RTSV-inoculated plants were examined by the analysis of variance (ANOVA) and a *t*-test, and the differences in height reduction rate and RTSV accumulation (absorbance at 405 nm in ELISA) among *O. glaberrima* and *O. sativa* plants were examined by ANOVA and the least significant difference (LSD) test.

SELECTION OF CELL WALL-RELATED GENES FOR EXPRESSION ANALYSIS

Cell wall-related genes investigated for expression in *O. glaberrima* (**Table 1**) were selected based on their expression patterns in *O. sativa* TN1 and TW16 after infection with RTSV (Satoh et al., 2013). Genes orthologous between *O. sativa* and *O. glaberrima* were identified by BLAST-Like Alignment Tool (BLAT) (Kent, 2002) using the *O. sativa* japonica gene sequences (http://rice.plantbiology.msu.edu) as query and the *O. glaberrima* genome assembly (www.genome.arizona.edu) as the database. Genes were considered to be orthologous if the length of the BLAT match along the *O. glaberrima* scaffold is more than 90% of the length of the *O. sativa* japonica gene with minimal mismatches.

REAL-TIME RT-PCR FOR CELL WALL-RELATED GENES

Total RNA was isolated individually from the second fully expanded leaves of single plants using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. The equal amounts of the RNA samples from five independent plants of the same treatment were pooled prior to cDNA synthesis. First-strand cDNA molecules were synthesized from 3 μg of the pooled RNA samples using SuperScript III (Invitrogen, USA) and an oligo(dT) primer (Invitrogen, USA) in 20 μl of reaction mix according to the manufacturer's instruction. Primers specific to six cell wall-related genes were designed to target the 3′ untranslated regions of the corresponding orthologs in both *O. sativa* and

Table 1 | Cell wall-related genes examined for expression in *Oryza glaberrima* and *O. sativa* infected with *Rice tungro spherical virus*.

Genes for	Locus ID in *O. sativa*	*O. glaberrima* scaffold ID	BLAT match (%)	Primers for RT-PCR (5′–3′)		Expected amplicon size (bp)
Cellulose synthase A5 (CESA5)	LOC_Os03g62090	Oglab03_0146	99.6	Forward Reverse	ATCTGCCGTCTGGAATAGAG ACCCACTCATCTCCAGTGTT	305
Cellulose synthase A6 (CESA6)	LOC_Os07g14850	Oglab07_0051	99.8	Forward Reverse	CCATATATGCGGTGATACGA CTTCCTTCCTTCTGTCCAAA	344
Cellulose synthase-like A9 (CSLA9)	LOC_Os06g42020	Oglab06_0208	99.3	Forward Reverse	GTGTGCAAGTGCAAAGTTTC GGTGAGCATATTGAGGAACC	304
Cellulose synthase-like C7 (CSLC7)	LOC_Os05g43530	Oglab05_0141	98.5	Forward Reverse	GAAAACACTTTGCAAGCATC ACCCAAGGTAATCGATCAAA	303
α-Expansin 1 precursor	LOC_Os05g39990	Oglab05_0133	99.2	Forward Reverse	GTGTCCTTCTTCGTCTTCGT ACTCCTCCATTACACCCAAA	447
α-Expansin 15 precursor	LOC_Os02g51040	Oglab02_0356	99.3	Forward Reverse	CGTCGTGGTAGTTGCAGTAG TGCATTAATACTCCCGCATA	468
Actin	LOC_Os03g50885	Oglab03_unplaced058	99.5	Forward Reverse	TCCATCTTGGCATCTCTCAG CAGATGCCTGATGAGGGTAC	337

O. glaberrima, and to avoid amplification from the gene family of selected genes (**Table 1**). Real-time PCR was performed with the gene-specific primers using the Light Cycler 480 SYBR Green I Master Mix (Roche, USA) in a Light Cycler 480 thermal cycler (Roche, USA) according to the manufacturer's instructions. Three replicates were performed for each sample. A threshold cycle (C_T) value was obtained for each reaction. Fold changes in cell wall-related gene expression between mock-inoculated and RTSV-inoculated plants were computed using the comparative C_T method (Schmittgen et al., 2000) with an actin gene (LOC_Os03g50885 in *O. sativa* and Oglab03_unplaced058 in *O. glaberrima*) as the internal reference control (**Table 1**). The differences in fold changes in expression of the genes among *O. sativa* and *O. glaberrima* plants were examined by ANOVA and LSD test.

RESULTS

STUNTING OF *O. glaberrima* BY RTSV

Eighteen accessions of *O. glaberrima* were inoculated with RTSV to see whether RTSV induces stunting in *O. glaberrima* accessions. Most accessions of *O. glaberrima* infected with RTSV appeared to be evidently stunted at 30 dpi (**Supplementary Material 1**), and the number of tillers usually decreased in the plants stunted with RTSV. However, many plants of *O. glaberrima* accessions died or grew poorly during the initial evaluation. This was probably due to a loss of vigor after long-term storage. Therefore, among the 18 accessions of *O. glaberrima,* we selected and propagated four (96790, 96793, 102569, and 104545) for further evaluation to confirm their reactions to RTSV. They were selected because *O. glaberrima*-96793 and -102569 appeared to be severely stunted by RTSV infection in the preliminary evaluation, whereas *O. glaberrima*-96790 and -104545 seemed not stunted or stunted only slightly.

The height reduction due to RTSV infection in the four accessions of *O. glaberrima* and two genotypes of *O. sativa*, TN1 susceptible to RTSV and TW16 resistant to RTSV, was monitored until 28 dpi to characterize the stunting phenotypes of the plants. The heights of *O. sativa* TN1 and TW16 were not reduced significantly after infection with RTSV, whereas the heights of the four accessions of *O. glaberrima* were significantly reduced after RTSV infection (**Table 2**).

The height reduction rates of the *O. sativa* and *O. glaberrima* plants were compared to examine whether the severity of stunting by RTSV infection was different among the plants. The height reduction rates of *O. glaberrima*-102569 were significantly higher than those of *O. sativa* TN1 and TW16 at 14 dpi and onwards, and they were also significantly higher than those of the other three *O. glaberrima* accessions at 21 and 28 dpi (**Figure 1A**, **Supplementary Material 2**). The reduction rates of *O. glaberrima*-96790, -96793, and -104545 were significantly higher than that of *O. sativa* TN1 only at 21 dpi. (**Figure 1A**, **Supplementary Material 2**). The height reduction rates of the four accessions of *O. glaberrima* were between approximately 8 and 14% at 7 dpi (**Figure 1A**, **Supplementary Material 2**). The height reduction rates for the four accessions of *O. glaberrima* increased to approximately 18–21% at 14 dpi, indicating that stunting of plants became more evident at 14 dpi. Stunting of *O. glaberrima*-102569 became more severe after 14 dpi, showing height reduction rates of approximately 31% at 21 dpi and 34% at 28 dpi (**Figures 1A,B**, **Supplementary Material 2**). In contrast, the height reduction rates of three other *O. glaberrima* accessions (96790, 96793, and 104545) decreased from approximately 18% at 14 dpi to approximately 14–15% at 28 dpi, indicating that stunting of these three *O. glaberrima* accessions became milder (**Figures 1A,B**, **Supplementary Material 2**). The height reduction rates for *O. sativa* TN1 and TW16 were

Table 2 | Heights of mock—and *Rice tungro spherical virus* (RTSV)-inoculated plants of *Oryza sativa* and *O. glaberrima*.

Plant	Treatment	Plant height[1] (cm) at			
		7 dpi	14 dpi	21 dpi	28 dpi
O. sativa—TW16	Mock	27.9 ± 1.0	44.1 ± 1.0	55.4 ± 0.9	69.9 ± 1.4
	RTSV	27.2 ± 1.3	43.2 ± 1.2	55.5 ± 0.8	69.8 ± 1.6
O. sativa—TN1	Mock	28.3 ± 0.9	43.0 ± 0.9	54.4 ± 0.7	68.1 ± 1.7
	RTSV	26.9 ± 0.9	41.2 ± 1.0	52.2 ± 1.1	66.6 ± 2.0
O. glaberrima—96790	Mock	28.0 ± 1.0	50.6 ± 0.5a	69.4 ± 1.6a	88.0 ± 2.2a
	RTSV	25.7 ± 1.1	41.2 ± 1.5b	57.4 ± 2.1b	75.0 ± 2.6b
O. glaberrima—96793	Mock	28.1 ± 1.1a	52.3 ± 1.0a	74.4 ± 0.9a	94.9 ± 2.3a
	RTSV	24.9 ± 0.8b	42.8 ± 1.8b	61.3 ± 1.2b	81.6 ± 1.4b
O. glaberrima—102569	Mock	23.6 ± 0.7a	42.0 ± 1.4a	64.1 ± 1.6a	82.0 ± 2.5a
	RTSV	20.4 ± 1.0b	32.9 ± 1.1b	43.8 ± 1.5b	53.3 ± 0.9b
O. glaberrima—104545	Mock	28.8 ± 1.1a	48.3 ± 1.2a	68.2 ± 1.7a	87.0 ± 2.4a
	RTSV	25.2 ± 0.5b	39.6 ± 0.9b	58.3 ± 1.6b	73.5 ± 1.2b

[1] *Mean ± standard error of mean based on three independent experiments. Values for mock- and RTSV-inoculated plants of the same rice genotype at the same days post-inoculation followed by different letters are significantly different by a t-test at the 95% confidence level.*

between approximately −0.3 and 5% during the observation (**Figure 1A**, **Supplementary Material 2**), but the differences in height between the mock-inoculated *O. sativa* plants and the corresponding RTSV-infected plants were not statistically significant throughout the observation (**Table 2**). These results suggested that, unlike *O. sativa*, a majority of *O. glaberrima* accessions are vulnerable to RTSV, but the severity of stunting varies significantly among *O. glaberrima* accessions.

ACCUMULATION OF RTSV IN *O. glaberrima* AND *O. sativa*

The accumulation of RTSV in the *O. glaberrima* and *O. sativa* plants was examined to see whether it is correlated with the height reduction rate. The levels of RTSV accumulation determined by ELISA in the four accessions of *O. glaberrima* and *O. sativa* increased rapidly until 14 dpi and then were maintained at similar levels or decreased slightly until 28 dpi (**Figure 2**, **Supplementary Material 3**). The accumulation of RTSV in *O. glaberrima*-96790 (OD_{405} of 2.40 ± 0.25) was significantly higher than that in the other three *O. glaberrima* accessions (1.48 ± 0.27–1.75 ± 0.21) at 14 dpi. The levels of RTSV accumulated in *O. glaberrima*-96793, -102569, and -104545 at a time point were not significantly different from one another throughout the observation. The levels of RTSV accumulated in *O. sativa* TN1 were significantly lower than those in at least one of the *O. glaberrima* accessions-96790, -96793, and 104545 at 14 dpi and onwards. The levels of RTSV accumulated in *O. glaberrima*-102569 were not significantly different from those in *O. sativa* TN1 throughout the observation, despite the fact that their height reduction rates were significantly different at 14 dpi and onwards. Accumulation of RTSV in *O. sativa* TW16 was not detected by ELISA. However, as in the previous studies by Encabo et al. (2009) and Satoh et al. (2013) showing detection of a very low level of RTSV in TW16 by RT-PCR, we assumed that most *O. sativa* TW16 plants inoculated with RTSV were infected since most *O. sativa* TN1 plants (susceptible control) inoculated at the same time were found to be infected with RTSV. The comparison between the height reduction rates (**Figure 1**, **Supplementary Material 2**) and the RTSV accumulation (**Figure 2**, **Supplementary Material 3**) indicates that the level of RTSV accumulation in plants is not correlated with the severity of stunting.

SUPPRESSION OF CELL WALL-RELATED GENES IN *O. glaberrima* INFECTED WITH RTSV

Temporal changes in expression of six cell wall-related genes were examined in *O. glaberrima*-96793 and -102569 and *O. sativa* TN1 to find cell wall-related genes associated with stunting of *O. glaberrima* caused by RTSV. The three accessions were selected since the severity of stunting by RTSV was significantly different from one another at 21 dpi (**Figure 1**, **Supplementary Material 2**). A genome-wide gene expression analysis in *O. sativa* TN1 and TW16 had been performed previously by Satoh et al. (2013). Genes for CESA5 and cellulose synthase A6 (CESA6), CSLA9 and cellulose synthase-like C7 (CSLC7), expansin 1, and α-expansin 15 precursor (expansin 15) were selected based on their expression patterns in *O. sativa* TN1 infected with RTSV. The previous microarray analysis by Satoh et al. (2013) showed that the expression 1) was activated for CESA5 and CSLC7 genes, 2) was suppressed for CSLA9 and expansin 15 genes, and 3) was not significantly changed for CESA6 and expansin 1 genes by RTSV infection between 6 and 15 dpi (**Table 3**).

At 14 dpi, the relative expression levels (fold changes) of four (CESA5, CESA6, CSLA9, and expansin 1genes) out of the six genes examined were significantly higher in *O. sativa* TN1 than in at least either *O. glaberrima*-96793 or -102569 (**Figure 3**). The expression of the CESA5 gene was up-regulated more than 4-fold by RTSV infection in *O. sativa* TN1 at 14 dpi, whereas the expression of the gene was found to be down-regulated slightly (approximately 0.7- to 0.8-fold) by RTSV infection in *O. glaberrima*-96793 and -102569 (**Figure 3A**). The expression of the expansin 1 gene was also up-regulated approximately 1.6-fold by RTSV infection in

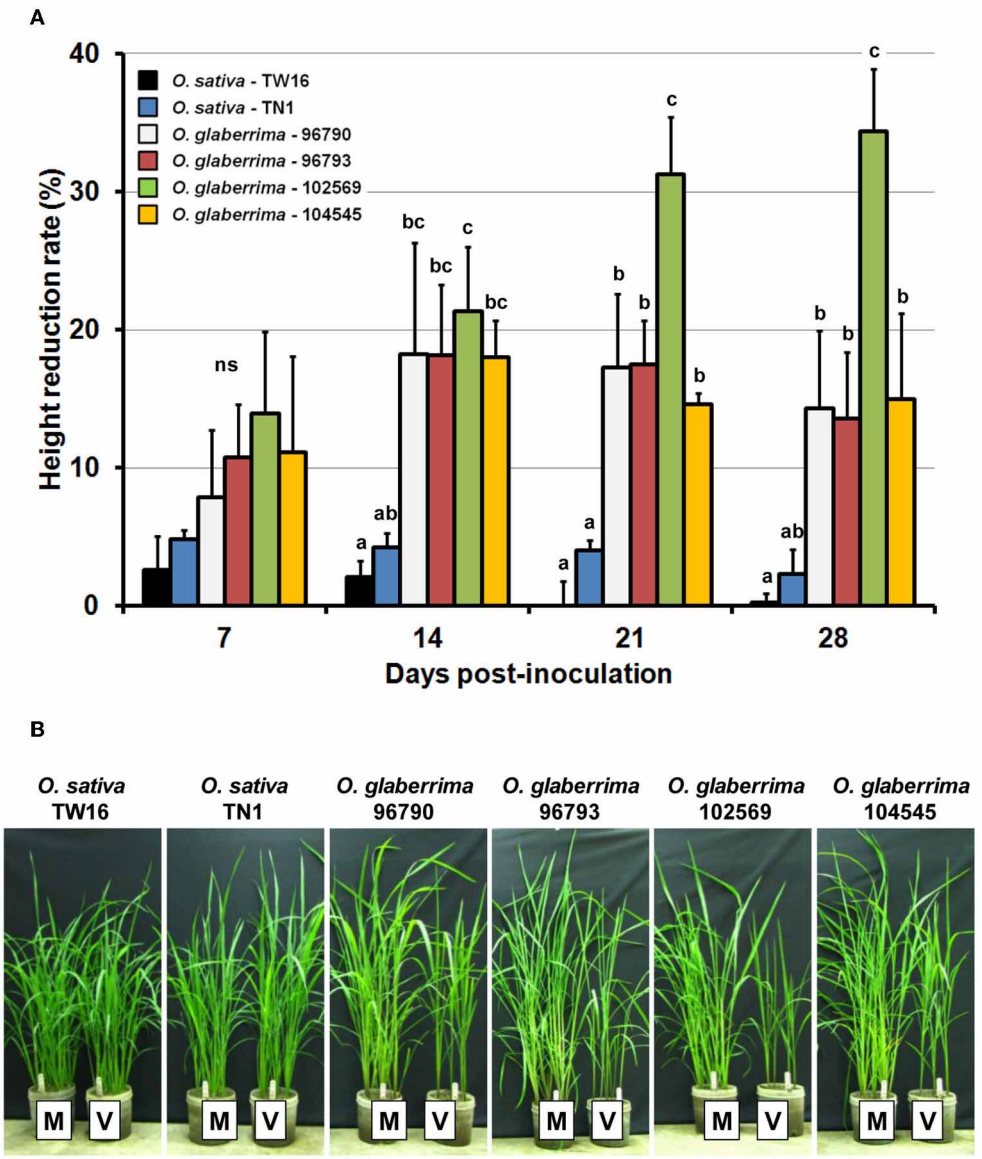

FIGURE 1 | Height reduction in *Oryza glaberrima* and *O. sativa* infected with *Rice tungro spherical virus* (RTSV). (A) Temporal changes in height reduction rates. Vertical bars and vertical lines indicate mean and standard error of mean, respectively, for the height reduction rate at the respective time points. Means and standard error of mean were based on three independent experiments ($n = 5$ per treatment and time point in one experiment). Bars at the same days post-inoculation (dpi) indicated by different letters are significantly different by the least significant difference test at the 95% confidence level. ns: not significantly different. **(B)** Plants of *O. glaberrima* and *O. sativa* at 28 dpi with RTSV. M, mock-inoculated plant; V, RTSV-inoculated plant.

O. sativa TN1 at 14 dpi, whereas the gene was down-regulated to approximately 0.2- to 0.4-fold in both *O. glaberrima* accessions (**Figure 3E**). The expression of the genes for CESA6 and CSLA9 was down-regulated more significantly by RTSV infection in *O. glaberrima*-96793 and -10569 than in *O. sativa* TN1 at 14 dpi (**Figures 3B,C**).

At 21 dpi, the relative expression levels of five genes (CESA5, CESA6, CSLA9, CSLC7, and expansin 15 genes) were significantly higher in *O. glaberrima*-96793 than in *O. sativa* TN1 (**Figure 3**). The expression of all six genes was down-regulated to approximately 0.2- to 0.8-fold in *O. sativa* TN1 at 21 dpi (**Figure 3**). The expression of the genes for CESA5 and CESA6 was up-regulated slightly (approximately 1.1-to 1.2-fold) in *O. glaberrima*-102569 at 21 dpi (**Figures 3A,B**).

At 28 dpi, the relative expression levels of five genes (CESA5, CSLA9, CSLC7, expansin 1, and expansin 15 genes) were significantly higher in *O. glaberrima*-102569 than in *O. sativa* TN1 (**Figure 3**). The relative expression levels of four genes (CSLA9, CSLC7, expansin 1, and expansin 15 genes) were significantly higher in *O. glaberrima*-102569

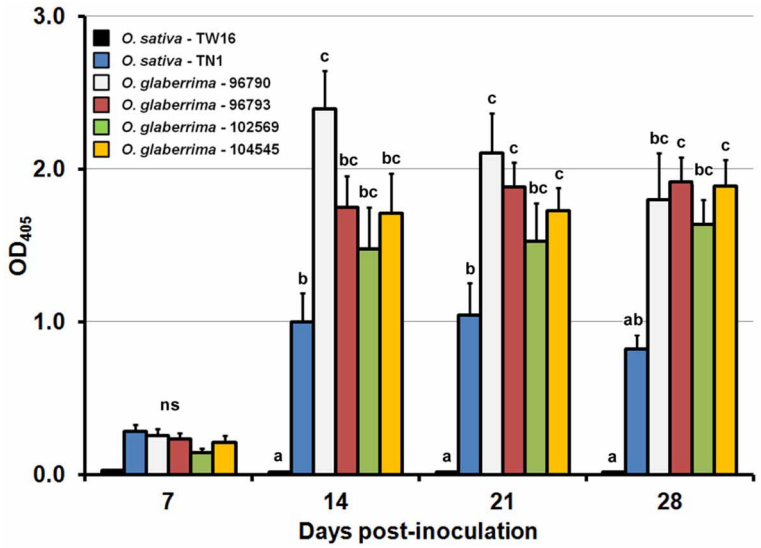

FIGURE 2 | Temporal changes in the accumulation of *Rice tungro spherical virus* in plants of *Oryza glaberrima* and *O. sativa*. Vertical bars and vertical lines indicate means and standard errors of mean, respectively, for the absorbance at 405 nm at the respective time points. Means and standard errors of mean were based on three independent experiments ($n = 5$ per treatment and time point in one experiment). Bars at the same days post-inoculation indicated by different letters are significantly different by the least significant difference test at the 95% confidence level. ns: not significantly different.

Table 3 | Changes in expression of six cell wall-related genes in rice plants infected with *Rice dwarf virus* (RDV), *Rice stripe virus* (RSV), *Rice tungro bacilliform virus* (RTBV), and *Rice tungro spherical virus* (RTSV) examined by genome-wide microarray.

Gene for	Locus ID	Fold change in gene expression in										
		O. sativa (Taichung Native 1) by							*O. sativa* (Nipponbare) by			
		RTSV[1] at			RTBV[2] at				RSV[3] at			RDV[4] at
		6 dpi	9 dpi	15 dpi	6 dpi	9 dpi	12 dpi	18 dpi	6 dpi	9 dpi	12dpi	21 dpi
Cellulose synthase A5 (CESA5)	LOC_Os03g62090	2.00	1.74	1.85	NS[5]	1.96	NS	NS	NS	NS	NS	NS
Cellulose synthase A6 (CESA6)	LOC_Os07g14850	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.62	0.76
Cellulose synthase-like A9 (CSLA9)	LOC_Os06g42020	0.95	0.43	0.65	NS	0.30	0.56	0.44	NS	NS	NS	NE[6]
Cellulose synthase-like C7 (CSLC7)	LOC_Os05g43530	1.54	1.37	1.36	NS	NS	0.86	NS	NS	NS	NS	NS
α-Expansin 1 precursor	LOC_Os05g39990	NS	NS	NS	NS	0.10	0.13	0.22	NS	0.66	0.51	0.36
α-Expansin 15 precursor	LOC_Os02g51040	0.60	0.69	0.75	NS	0.32	NS	0.41	NS	NS	NS	0.56

[1] *Data from Satoh et al. (2013).*

[2] *Unpublished data by K. Satoh.*

[3] *Data from Satoh et al. (2010).*

[4] *Data with RDV strain S from Satoh et al. (2011).*

[5] *Change in expression not significant.*

[6] *Expression not detected.*

than in *O. glaberrima*-97693 (**Figure 3**). The expression of all six genes was down-regulated in *O. sativa* TN1 (approximately 0.02- to 0.95-fold), and also in *O. glaberrima*-96793 (approximately 0.03- to 0.92-fold) at 28 dpi (**Figure 3**).

Overall, at 14 dpi, the relative expression levels of a majority of the six cell wall-related genes were significantly higher in *O. sativa* TN1 than in the two accessions of *O. glaberrima* which were stunted by RTSV infection. In contrast, at 28 dpi, the relative expression levels of a majority of the six genes were significantly higher in most severely stunted *O. glaberrima*-102569 than in *O. sativa* TN1 and *O. glaberrima*-96793.

DISCUSSION

RTSV has been considered as a latent virus since the accumulation of RTSV alone does not induce recognizable symptoms in most *O. sativa* genotypes. However, the lack of symptoms in *O. sativa* infected with RTSV might be due to defense mechanisms

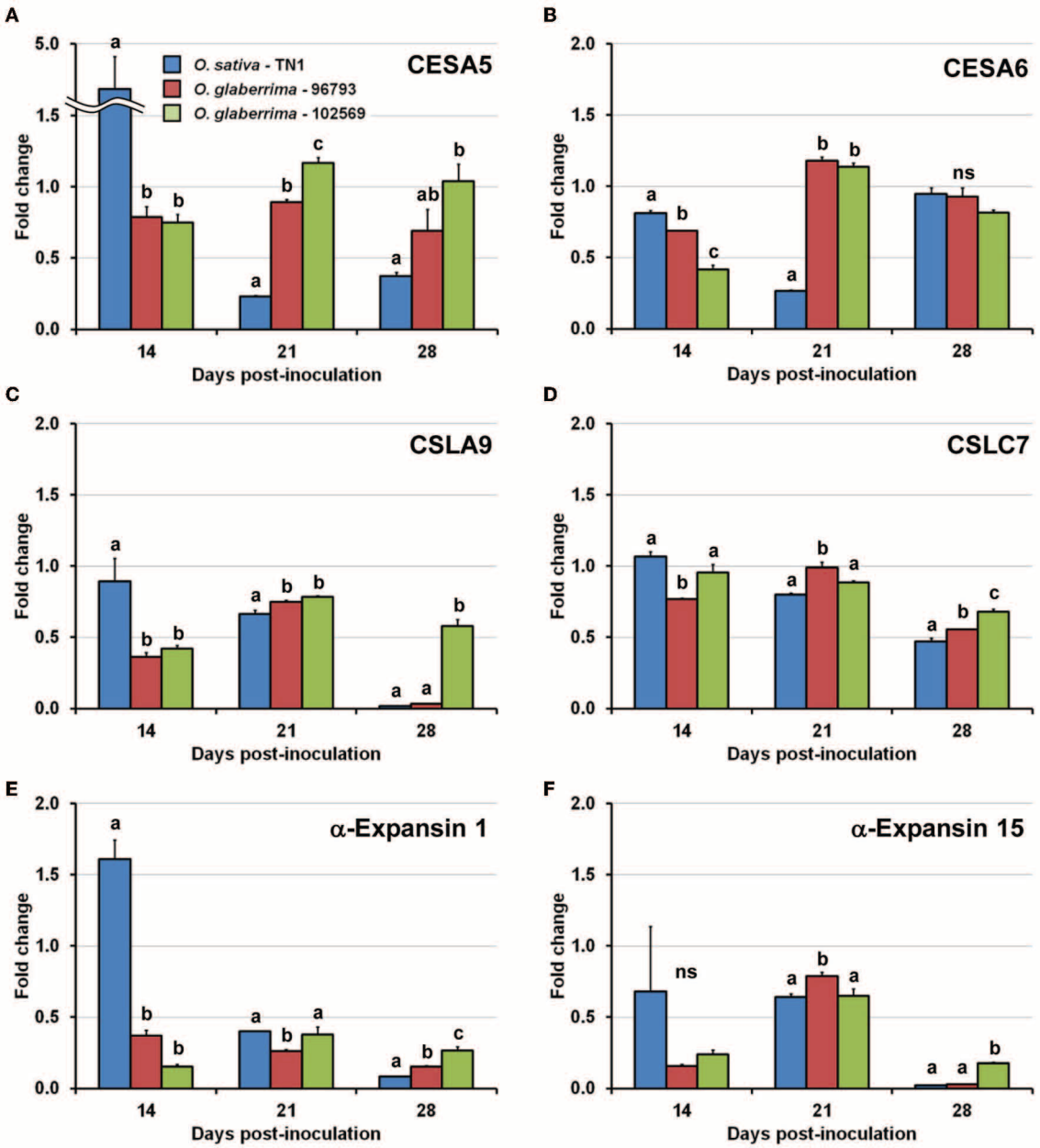

FIGURE 3 | Changes in expression of cell wall-related genes after infection with *Rice tungro spherical virus* (RTSV) in plants of *Oryza glaberrima* and *O. sativa* examined by real-time RT-PCR. Vertical bars and vertical lines indicate means and standard errors of mean, respectively, for the fold changes in expression of genes for **(A)** cellulose synthase A5 (CESA5), **(B)** cellulose synthase A6 (CESA6), **(C)** cellulose synthase-like A9 (CSLA9), **(D)** cellulose synthase-like C7 (CSLC7), **(E)** α-expansin 1 precursor (α-Expansin 1), and **(F)** α-expansin 15 precursor (α-Expansin 15) between mock and RTSV-inoculated plants at the respective time points. Means and standard errors of mean were based on three replicated reactions. Bars at the same days post-inoculation indicated by different letters are significantly different by the least significant difference test at the 95% confidence level. ns: not significantly different.

activated in *O. sativa*, not due to the lack of pathogenicity in RTSV. RTSV alone induces significant changes in the expression of diverse defense- and development-related genes in *O. sativa* (Encabo et al., 2009; Satoh et al., 2013). RTSV and RTBV synergistically enhance symptoms in *O. sativa* (Cabauatan et al., 1995). Along with these observations, the stunted growth of *O. glaberrima* plants by RTSV observed in Cabauatan et al. (1993) and this study suggests that, despite the potential pathogenicity of accumulating RTSV, most *O. sativa* genotypes may share a species-specific tolerance mechanism that prevents them from being stunted by RTSV. The accumulation of RTSV in *O. sativa* TN1 was significantly lower than that in three *O. glaberrima*

accessions-96790, -96793, and -104545 at least at one time point during the observation (**Figure 2**, **Supplementary Material 3**). Therefore, alternatively, most *O. sativa* genotypes may operate a horizontal defense mechanism to suppress the accumulation of RTSV below the threshold level necessary to trigger a significant height reduction.

The severity of stunting (**Figure 1**, **Supplementary Material 2**) and the levels of RTSV accumulation (**Figure 2**, **Supplementary Material 3**) were significantly variable among the *O. glaberrima* accessions, but a clear relationship between the severity of stunting and the accumulation of RTSV was not observed. A lack of correlation between virus accumulation and severity of stunting was also the case with the interactions between *O. sativa* cultivar Nipponbare and three RDV strains (Satoh et al., 2011). Plants infected with RDV strain S were more severely stunted than those infected with RDV strain D84, yet the accumulation of the two RDV strains in the plants was not significantly different. Thus, the severity of stunting in the *Oryza* species might be associated with a virus-induced modification of gene expression decoupled from the level of virus accumulation, as was observed in the interaction of RTSV with *O. sativa* TN1 and TW16 (Satoh et al., 2013). For example, the expression of stress response-related genes such as those for TIFY transcription factors (Ye et al., 2009) and those for glutathione S-transferases was more activated in TW16, in which RTSV accumulated significantly less than in TN1, whereas the expression of development-related genes such as the homeobox gene family was more suppressed by RTSV in TW16.

Stunting of *O. sativa* plants by infection with RDV (Shimizu et al., 2007; Satoh et al., 2011) and RSV (Satoh et al., 2010) was accompanied by the suppression of cell wall-related genes such as those for cellulose synthase (-like), expansin, and extensin. The severity of stunting in *O. sativa* cultivar Nipponbare infected with three strains of RDV was associated with the suppression of dozens of cell wall-related genes (Satoh et al., 2011). Stunting of plants and impaired leaf growth were also induced by virus-induced silencing of cellulose synthase genes in *Nicotiana benthamiana* (Burton et al., 2000), by mutations in cellulose synthase genes in rice (Tanaka et al., 2003), and by artificial repression of expansin genes in *Arabidopsis thaliana* (Goh et al., 2012), and in rice (Choi et al., 2003). These observations suggest that suppression of particular cell wall-related genes in *O. glaberrima* after RTSV infection may have led to stunting of the plants. Among the expression patterns of the six cell wall-related genes in the *O. glaberrima* and *O. sativa* plants infected with RTSV (**Figure 3**), the evident activation of the CESA5 and the expansin 1 genes in *O. sativa* TN1 and the suppression of the two genes in the two accessions of *O. glaberrima* at 14 dpi appeared to be signature responses in the plants stunted (*O. glaberrima*-97693 and -102569) by RTSV infection and the plant not stunted (*O. sativa* TN1). In fact, the expression of the expansin 1 gene was consistently suppressed in *O. sativa* plants stunted by RDV, RSV, and RTBV, but not in *O. sativa* infected but not stunted by RTSV (**Table 3**). Thus, the gene for expansin 1 is likely to be the gene commonly associated with virus-induced stunting of *Oryza* species. Expansin loosens cell walls (Cosgrove, 1998), which may make plants more vulnerable to pathogens (Ding et al., 2008). Therefore, virus-induced stunting of *O. sativa* and *O. glaberrima* accompanying the suppression of the expansin 1 gene may be a general consequence of the defense response against viruses in *Oryza* species. Together with the suppression of the expansin 1 gene, more significant suppression of the CESA5, CESA6, and CSLA9 genes in *O. glaberrima*-96793 and -102569 than in *O. sativa* TN1 might be involved in the stunting of *O. glaberrima* by RTSV.

The results of this study suggested that stunting of *O. glaberrima* might be associated with the cell wall-related genes suppressed by RTSV. Genetic factors linked to the vulnerable reaction of *O. glaberrima* to RTSV can be accidentally transferred into *O. sativa* during the inter-specific hybridization intended for the improvement of Asian rice varieties. Therefore, characterization of the genetic factors underlying the vulnerability of *O. glaberrima* to RTSV is critical to avoid the accidental introduction of such factors into *O. sativa*.

SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00026/abstract

Supplementary Material 1 | Height and RTSV accumulation in 18 accessions of *O. glaberrima* infected with RTSV—See a separate file.

Supplementary Material 2 | Temporary changes in height reduction rates in *O. sativa* (TW16, Taichung Native 1), and *O. glaberrima* (IRGC accession numbers 96790, 96793, 102569, and 104545).

Supplementary Material 3 | Temporary changes in accumulation of *Rice tungro spherical virus* in *O. sativa* (TW16, Taichung Native 1), and *O. glaberrima* (IRGC accession numbers 96790, 96793, 102569, and 104545) examined by an enzyme-linked immunosorbent assay.

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Relationship between gene responses and symptoms induced by *Rice grassy stunt virus*

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Rice grassy stunt virus (RGSV) is a serious threat to rice production in Southeast Asia. RGSV is a member of the genus *Tenuivirus*, and it induces leaf yellowing, stunting, and excess tillering on rice plants. Here we examined gene responses of rice to RGSV infection to gain insight into the gene responses which might be associated with the disease symptoms. The results indicated that (1) many genes related to cell wall synthesis and chlorophyll synthesis were predominantly suppressed by RGSV infection; (2) RGSV infection induced genes associated with tillering process; (3) RGSV activated genes involved in inactivation of gibberellic acid and indole-3-acetic acid; and (4) the genes for strigolactone signaling were suppressed by RGSV. These results suggest that these gene responses to RGSV infection account for the excess tillering specific to RGSV infection as well as other symptoms by RGSV, such as stunting and leaf chlorosis.

Keywords: *Rice grassy stunt virus*, plant hormone, stunting, tillering, transcriptome analysis

INTRODUCTION

Grassy stunt disease of rice caused by *Rice grassy stunt virus* (RGSV) is one of the severe virus diseases of rice in several Southeast Asian countries (Shikata et al., 1980; Ramirez, 2008). RGSV is a member of the genus *Tenuivirus*, and is transmitted by brown planthopper (BPH, *Nilaparvata lugens*) and by two other *Nilaparvata* spp. (Hibino, 1996). RGSV has a thin filamentous-shaped virion, and the genome is composed of six ambisense single-stranded RNA segments (RNA1—6) containing 12 open reading frames (Ramirez, 2008). RGSV RNA 1, 2, 5, and 6 correspond to the four RNA segments of the type member of *Tenuivirus*, *Rice stripe virus* (RSV). RGSV RNA 3 and 4 are unique in this genus. The phylogenic relationship among tenuiviruses, including RGSV and RSV, indicates that RGSV forms a group distinct from the other tenuiviruses (Ramirez, 2008). Typical symptoms induced by RGSV infection are leaf yellowing (chlorosis), stunting, and excess tillering (branching) (Shikata et al., 1980). Chlorosis and stunting are also observed in plants infected with other tenuiviruses, whereas excess tillering is a symptom specific to RGSV infection.

Plant disease symptoms caused by virus infection are accompanied by changes in the expression of the genes involved in morphogenesis and development (Dardick, 2007; Lu et al., 2012; Pierce and Rey, 2013). Thus, common and specific symptoms might be associated with common and specific responses of morphogenesis- and development-related genes (Dardick, 2007). *Nicotiana benthamiana* infected with either *Plum pox virus* (PPV) or *Tomato ring spot virus* (ToRSV) showed leaf chlorosis, implying that chloroplast functions might have been impaired by infection with the viruses (Dardick, 2007). Transcriptome analysis of PPV- and ToRSV-infected plants showed the suppression of genes functioning in chloroplasts, however, the genes encoding ribosomal protein functioning in chloroplasts were suppressed only in ToRSV-infected plants (Dardick, 2007). These results indicate that similarity in disease symptoms may not result from the similarity in gene responses to virus infection, and that the gene responses associated with particular symptoms may depend on the virus species.

In this study, we analyzed the gene expression profile in rice plants infected with RGSV to gain insight into RGSV-induced gene responses associated with the symptoms. The results suggested that symptoms such as stunting and leaf chlorosis caused by RGSV infection were associated with the suppression of genes related to cell wall, hormone synthesis and chlorophyll synthesis while excess tillering specific to RGSV infection is associated with the suppression of strigolactone signaling and GA metabolism.

MATERIALS AND METHODS

VIRUS, INSECT VECTOR, AND PLANT SAMPLES

RGSV was maintained in rice plants (*Oryza sativa* cv. Nipponbare) in an air-conditioned greenhouse (25–30°C) (Shimizu et al., 2013). One hundred rice seeds were sown in a pot (250 mm in diameter and 100 mm in height) that had been filled with commercial soil mixture (Bonsol; Sumitomo Chemical, Japan). Twelve-day-old rice seedlings were exposed to ∼300 viruliferous or virus-free BPH (for mock inoculation) in an

inoculation chamber (340 mm wide by 260 mm deep by 340 mm high) for 1 day. Forty-two inoculated plants were transplanted and grown in a plastic container (53 cm wide by 35 cm deep by 10 cm high) in the greenhouse. At 28 days post-inoculation (DPI), when disease symptoms such as stunting and leaf yellowing became evident, two newly developed leaves from each plant which exhibited disease symptoms were harvested, frozen in liquid nitrogen, and stored at −80°C. Sample preparations were independently repeated three times for microarray experiments. Infection with RGSV in plants was evaluated by enzyme-linked immunosorbent assay using an antiserum against RGSV.

MICROARRAY EXPERIMENT AND DATA ANALYSIS

Total RNA was extracted from leaf samples pooled from five independent RGSV-infected or mock-inoculated plants by the RNeasy Maxi kit (Qiagen, UK). A microarray experiment involving complementary RNA synthesis, hybridization, array scanning, and image processing was performed as described previously (Satoh et al., 2010). A gene was declared "expressed" if the average signal intensity of the gene was higher than 64 in plants. A significantly differentially expressed gene (DEG) between RGSV-infected and mock-inoculated plants was defined as an expressed gene with (1) a signal intensity ratio between RGSV-infected and mock-inoculated samples greater than 1.5, and (2) significant changes in gene expression between two plants ($P \leq 0.05$ by a paired t-test; permutation: all; false discovery rate correction: adjusted Bonferroni method). The gene expression profile of RGSV-infected plant (data series GSE 25217 available at NCBI GEO, **Supplementary Material 1**) was analyzed by a 4×44 K microarray system (platform number GPL7252 available at NCBI-GEO) (Edgar et al., 2002). All data are Minimum Information About a Microarray Experiment (MIAME) compliant.

SEMI-QUANTITATIVE RT-PCR

Complementary DNA (cDNA) fragments for transcripts of selected rice genes were synthesized using 1.0 μg of total RNA with 50 ng/μL of random hexamer primers by SuperScript III reverse transcriptase (Invitrogen, USA). The resultant reaction mixtures containing cDNA were diluted four times. Four μL of diluted mixture was used for PCR. Primers for selected rice genes are shown in **Supplementary Material 2**. The cycling program was initial denaturation for 2 min at 95°C, followed by 30–40 cycles of 15 s at 95°C, 15 s at variable annealing temperatures, and 45 s at 68°C, with a final extension of 1 min at 68°C. Annealing temperature was adjusted depending on the Tm of designed primers, and was between 50 and 60°C.

QUANTIFICATION OF STRIGOLACTONE

RGSV-infected and mock-inoculated rice plants were prepared as described previously and grown for 6 weeks. Then, the plants ($n = 20$) were transferred to 1 L 1/2 Hoagland hydroponic culture medium without phosfate. After 2 days of acclimatization, culture media were collected and extracted three times with an equal volume of ethyl acetate containing 2′-epi-5-deoxystrigol-d6 (200 pg) as an internal standard. The ethyl acetate solutions were combined, washed with 0.2 M K_2HPO_4 (pH 8.3), dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to produce crude root exudate samples. Crude samples dissolved with 1 mL 90% (v/v) ethyl acetate/hexane were passed through Sep-pack silica cartridges. The eluents were concentrated *in vacuo* and the residues were taken up with a small volume of acetonitrile for liquid chromatography–mass spectrometry (LC-MS)/MS analyses. Quantification of 2′-epi-5-deoxystrigol was conducted by LC-MS/MS as described previously (Yoneyama et al., 2011).

RESULTS

DISEASE SYMPTOMS CAUSED BY RGSV INFECTION

Rice plants infected with RGSV showed disease symptoms such as excessive tillering, stunting, and leaf yellowing. The symptoms became more severe after 28 DPI (**Figure 1A**). Profuse tillering became more evident in the RGSV-infected plants at 60 DPI (**Figure 1B**). The RGSV-infected plant was much shorter than the mock-inoculated plants at 60 DPI (**Figure 1B**). Leaf yellowing by RGSV infection was also more severe at 60 DPI (**Figures 1B,C**) than at 28 DPI (**Figure 1A**).

TRANSCRIPTOME RESPONSES OF RICE TO RGSV INFECTION

Changes in gene expression caused by RGSV infection were examined by direct comparison between RGSV- and mock-inoculated rice plants at 28 DPI. The numbers of expressed genes and DEGs were 24,911 and 8203, respectively (**Table 1**, **Supplementary Material 1**). The expression patterns of some DEGs were confirmed by semi-quantitative RT-PCR (**Supplementary Material 2**).

We classified the DEGs in RGSV-infected plants according to gene ontology. For many ontology categories, the ratio of the number of DEGs to the number of expressed genes was significantly higher ($P < 0.01$ by a χ^2 test) than the ratio of the total number of DEGs to the total number of expressed genes (**Table 1**). Based on categorization by cellular components, DEGs such as those categorized in "membrane" and "cell wall" were significantly overrepresented in the plants infected with RGSV, whereas the influence of RGSV infection on the expression of genes categorized in "ribosome" and "cytosol" appeared to be limited (**Table 1**). Based on categorization by

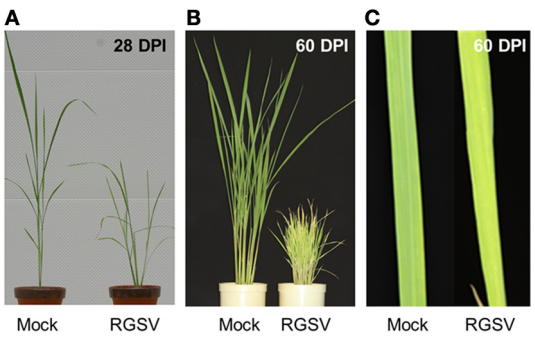


FIGURE 1 | Symptoms in rice caused by RGSV infection. (A) Rice plants at 28 days and **(B)** 60 days after RSGV inoculation and mock inoculation. **(C)** Leaf yellowing of RGSV-infected plants at 60 days after inoculation.

Table 1 | Numbers of DEGs in gene ontology groups overrepresented or underrepresented in RGSV-infected plants.

Categories	Cene ontology ID[1]	Gene ontology term[a]	Number of expressed genes	Number of DEG[b]	*P*-value[c]	Response of DEG	
						Induced	Suppressed
Component	GO:0016020	Membrane	3193	**1188**	2.53E-05	671	517
	GO:0005618	Cell wall	1645	**688**	3.25E-10	377	311
	GO:0005623	Cell	233	**100**	7.88E-03	54	46
	GO:0005622	Intracellular	627	152	1.50E-04	103	49
	GO:0005840	Ribosome	301	43	1.73E-08	18	25
	GO:0005829	Cytosol	264	56	9.08E-04	33	23
Process	GO:0009719	Response to endogenous stimulus	2619	**1072**	9.56E-13	615	457
	GO:0007582	Physiological process	2105	**836**	5.78E-08	499	337
	GO:0007165	Signal transduction	2022	**785**	3.87E-06	485	300
	GO:0006950	Response to stress	1804	**694**	4.11E-05	411	283
	GO:0009628	Response to abiotic stimulus	1527	**676**	1.14E-14	404	272
	GO:0008150	Biological process	1388	**586**	1.63E-09	357	229
	GO:0009607	Response to biotic stimulus	1348	**585**	2.12E-11	321	264
	GO:0009058	Biosynthetic process	1222	**492**	7.94E-06	214	278
	GO:0006519	Amino acid and derivative metabolic process	803	**331**	4.23E-05	152	179
	GO:0019748	Secondary metabolic process	659	**317**	1.14E-11	133	184
	GO:0006629	Lipid metabolic process	655	**272**	1.26E-04	132	140
	GO:0006118	Electron transport	481	**210**	4.12E-05	98	112
	GO: 0005975	Carbohydrate metabolic process	473	**226**	1.82E-08	107	119
	GO: 0007275	Multicellular organismal development	463	**205**	2.09E-05	128	77
	GO:0030154	Cell differentiation	426	**183**	3.10E-04	120	63
	GO:0009908	Flower development	342	**164**	1.29E-06	92	72
	GO: 00096 53	Anatomical structure morphogenesis	204	**95**	6.87E-04	61	34
	GO:0016043	Cellular component organization and biogenesis	784	196	1.09E-04	117	79
	GO:0006412	Translation	516	83	2.60E-11	39	44
	GO:0006139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	453	95	9.20E-06	59	36
			24911	8204		3896	4307

[a] Based on Rice Genome Annotation Project database Osa1 (rice.plantbiology.msu.edu).

[b] Numbers in bold (or underlined) text indicate that the corresponding ontology groups are overrepresented (or underrepresented) based on a χ^2-test between the actual ratio of DEG (the number of DEGs/the number of expressed genes belonging to the corresponding ontology group) and the expected ratio of DEG [the number of total DEGs (8204)/the number of the total expressed genes (24,911)].

[c] From the χ^2-test.

cellular processes, DEGs involved in stress responses and secondary metabolism were overrepresented in the plants infected with RGSV (**Table 1**). For DEGs categorized in stress responses, the number of induced genes was significantly greater than that of suppressed genes, whereas the number of induced genes involved in secondary metabolism was not significantly different from that of suppressed genes. On the other hand, expression of genes involved in translation process and nucleic acid metabolism was less influenced by RGSV infection than genes in other categories (**Table 1**). These results indicated that genes involved in development of cell structures, secondary metabolisms, and stress responses were noticeably affected by RGSV infection, and might be associated with RGSV-induced symptoms.

Table 2 | Genes related to cell wall components and tillering in RGSV-infected plants that are up- or down-regulated.

Classification	Locus[a]	Log_2 Ratio[b]	Response[c]
Cellulose synthase	LOC_Os0lg54620	−0.70	D
	LOC_Os05g08370	−0.68	D
	LOC_Os07g2419	−1.03	D
	LOC_Os10g32980	−1.09	D
	LOC_Os12g29300	−0.77	D
Cellulose synthase-like family A	LOC_Os02g09930	−2.20	D
	LOC_Os02g51060	−0.60	D
	LOC_Os06g12460	−1.28	D
	LOC_Os08g33740	−2.03	D
Cellulose synthase-like family C	LOC_Os01g56130	−0.68	D
	LOC_Os03g56060	1.10	U
	LOC_Os08g15420	−0.84	D
Cellulose synthase-like family E	LOC_Os09g30120	1.13	U
	LOC_Os09g30130	−1.21	D
Cellulose synthase-like family F	LOC_Os07g36610	−0.89	D
	LOC_Os07g36690	−2.80	D
	LOC_Os07g36700	−1.99	D
	LOC_Os07g36740	−1.34	D
	LOC_Os08g06380	−0.92	D
Cellulose synthase-like family H	LOC_Os04g35030	1.35	U
Fasci din-like arabinogalactan protein	LOC_Os03g57460	−1.07	D
	LOC_Os07g06680	−0.73	D
	LOC_Os08g38270	−1.03	D
	LOC_Os05g07060	−1.76	D
	LOC_Os05g48900	−0.73	D
	LOC_Os08g39270	1.38	U
	LOC_Os05g38500	−0.99	D
	LOC_Os03g03600	−1.38	D
	LOC_Os08g23180	−1.02	D
	LOC_Os09g07350	−1.39	D
	LOC_Os09g30010	1.78	U
α-Expansin	LOC_Os01g14660	−2.34	D
	LOC_Os02g51040	−1.28	D
	LOC_Os03g60720	2.28	U
	LOC_Os04g15840	−1.12	D
	LOC_Os05g39990	−0.77	D
	LOC_Os06g41700	0.90	U
	LOC_Os10g30340	−1.24	D
β-Expansin	LOC_Os03g01270	−2.06	D
	LOC_Os 10g40710	−2.26	D
Expansin-like	LOC_Os03g04020	−0.90	D
	LOC_Os06g50960	−0.66	D
	LOC_Os10g39640	−1.36	D
Extensin	LOC_Os01g67390	−2.86	D
	LOC_Os04g32850	−1.66	D
	LOC_Os11g41120	1.73	U
OsNACs/Ostiin	LOC_Os04g38720	0.58	U
RCN1	LOC_Os03g17350	2.47	U
SPL14	LOC_Os03g39890	−0.65	D

[a] Based on Rice Genome Annotation Project database Osa1 (rice.plantbiology.msu.edu).

[b] Log_2-based differential expression ratio (signal intensity in RTSV-infected plant/signal intensity in mock-inoculated plant).

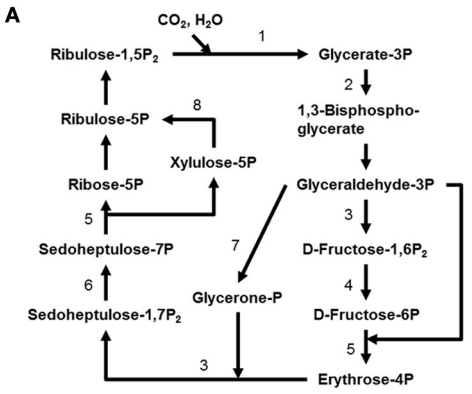
[c] U (D): Significantly induced (suppressed) by RGSV infection.

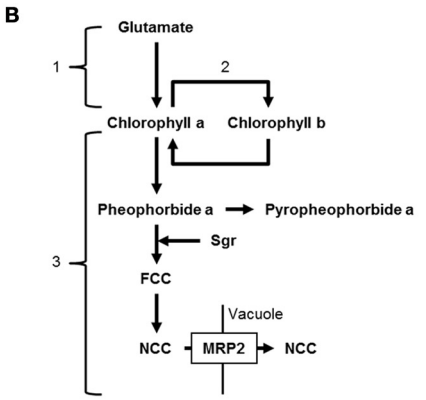
Cell wall-related genes

The expression of genes related to cell wall components was affected by RGSV infection (**Table 2**). The expression of genes for cellulose synthase (-like) and arabinogalactan proteins associated with cell wall formation was predominantly suppressed by RGSV infection (**Table 2**). The genes encoding expansin proteins involved in cell elongation by loosening cell wall components (Choi et al., 2003) were also predominantly suppressed by RGSV infection (**Table 2**). These results indicate that RGSV infection suppressed both cell wall formation and cell elongation in rice.

Genes associated with tillering

One of the major symptoms caused by RGSV infection is excessive tillering. The genes *RCN1* (*REDUCED CULM NUMBER 1*; Yasuno et al., 2009), *SPL14* (*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 14*; Miura et al., 2010), and *Ostill1* (*ORYZA SATIVA TILLERING 1*; Mao et al., 2007) were reported to promote shoot branching. RGSV infection induced the expression of *RCN1*and *Ostill1*, but suppressed that of *SPL14* (**Table 2**), suggesting that activation of *RCN1* and *Ostill1* alone may contribute to the development of excess tillers in RGSV-infected plants.





Designation	Annotaiton	Locus[1]	Log_2Ratio[2]	Response[3]
1	Ribulose-bisphosphate carboxylase large chain	LOC_Os12g10580	-0.82	D
	Ribulose-bisphosphate carboxylase small chain	LOC_Os12g17600	-0.65	D
		LOC_Os12g19381	-0.85	D
		LOC_Os12g19470	-0.91	D
2	Phosphoglycerate kinase	LOC_Os01g58610	1.07	U
		LOC_Os02g07260	0.62	U
		LOC_Os06g45710	1.67	U
3	Fructose-bisphosphate aldolase	LOC_Os01g02880	1.20	U
		LOC_Os05g33380	0.97	U
		LOC_Os06g40640	-0.64	D
		LOC_Os08g02700	0.90	U
4	Fructose-1,6-bisphosphatase	LOC_Os05g36270	0.76	U
5	Transketolase	LOC_Os04g19740	1.04	U
6	Sedoheptulose-bisphosphatase	LOC_Os04g16680	-0.65	D
7	Triosephosphate isomerase	LOC_Os03g54240	-0.61	D
8	Ribulose-phosphate 3-epimerase	LOC_Os03g07300	-0.92	D

Designation	Annotaiton	Locus[1]	Log_2Ratio[2]	Response[3]
1	Glutamyl-tRNA reductase	LOC_Os10g35840	-1.04	D
	Uroporphyrinogen-III synthase	LOC_Os03g08730	-0.66	D
	Uroporphyrinogen decarboxylase	LOC_Os01g43390	-0.74	D
		LOC_Os03g21900	-0.61	D
	Coproporphyrinogen III oxidase	LOC_Os04g52130	-1.02	D
	Magnesium chelatase subunit H	LOC_Os03g20700	-0.79	D
		LOC_Os03g59640	-0.80	D
		LOC_Os03g36540	-0.77	D
	Magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase	LOC_Os01g17170	-0.59	D
	Protochlorophyllide reductase	LOC_Os04g58200	-2.93	D
		LOC_Os10g35370	-0.81	D
2	Chlorophyllide a oxygenase	LOC_Os10g41780	-1.11	D
3	Red chlorophyll catabolite reductase	LOC_Os10g25030	1.68	U
	Stay green	LOC_Os09g36200	1.52	U

FIGURE 2 | Genes related to Calvin-Benson cycle and chlorophyll metabolisms in RGSV-infected plants that are up- or down-regulated. Responses of genes for **(A)** Calvin-Benson cycle, and **(B)** chlorophyll metabolism. Enzymes catalyzing reactions are shown in the table below:

[1]based on Rice Genome Annotation Project database Osa1 (rice.plantbiology.msu.edu), [2]log_2-based differential expression ratio (signal intensity in RTSV-infected plant/signal intensity in mock-inoculated plant), and [3]U (D): Significantly induced (suppressed) by RGSV infection.

Genes functioning in chloroplast

Expression of many genes functioning in chloroplasts was affected by RGSV infection (**Supplementary Material 1**). Eight genes functioning in the Calvin-Benson cycle were induced by RGSV infection, whereas 8 genes including those encoding ribulose bisphosfate carboxylase (Rubisco) large and small subunits were suppressed (**Figure 2A**). Among 35 genes involved in the photosynthesis system whose expression was affected by RGSV, 34 were suppressed in RGSV-infected plants (**Supplementary Material 1**). The chlorophyll is a photoreceptor in the chloroplast. Among 14 genes for chlorophyll metabolism whose expression was affected by RGSV infection, 12 genes were suppressed and 2 genes were induced (**Figure 2B**). The suppressed genes were those involved in chlorophyll synthesis and conversion. The two induced genes were *Staygreen* (*Sgr*) and the gene encoding red chlorophyll catabolite reductase, both of which are associated with chlorophyll degradation (Park et al., 2007). Thus, it is likely that RGSV infection inhibits the carbon fixation and chlorophyll synthesis, and promotes degradation of chlorophyll.

EXPRESSION OF GENES ASSOCIATED WITH PLANT HORMONE METABOLISM AND SIGNALING

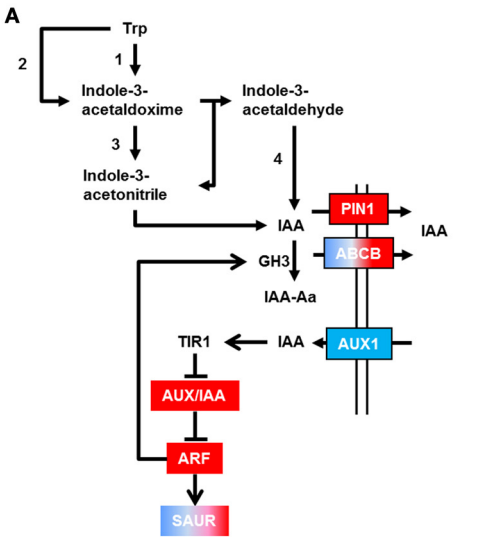
We examined the expression patterns of genes involved in plant hormone biosynthesis and signaling processes. Rice genes for hormone biosynthesis and signaling and their orthologous genes in *Arabidopsis* are described in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2010).

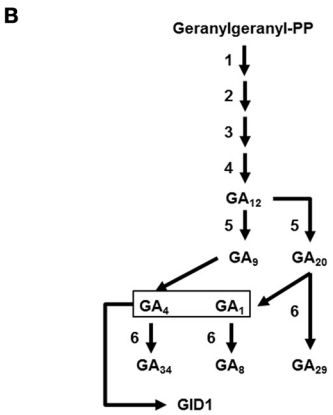
Genes related to auxin biosynthesis and signaling

GH3 converts IAA into an inactive form by conjugating IAA to amino acids (Domingo et al., 2009; Zhang et al., 2009). Four genes encoding GH3 were activated by RGSV infection (**Figure 3A**). RGSV infection also affected the expression of genes for auxin transporters such as *AUX1/LAX1*, *PIN1*, and *ABCB* (**Figure 3A**, **Supplementary Material 1**). RGSV suppressed *AUX1/LAX1* genes, but activated *OsPIN1* genes. RGSV infection induced the genes for the regulator of auxin signaling such as *AUX/IAA* and *ARF* (*AUXIN RESPONSE FACTOR*) (Lau et al., 2008) (**Figure 3A**). The expression of *SAUR* (*SMALL AUXIN-UP RNA*) genes involved in regulation of auxin synthesis and transport (Kant et al., 2009) was also affected by RGSV infection, and the number of induced *SAUR* genes was similar to that of suppressed genes (**Figure 3A**). These results suggest that RGSV infection might have inhibited the accumulation of auxin in rice, but activates IAA signaling process.

Genes related to gibberellic acid (GA) biosynthesis and signaling

Enzymes such as *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO), and gibberellin 20-oxidase (GA20ox) catabolize the





Designation	Annotaiton	Locus[1]	Log_2Ratio[2]	Response[3]
1	Cytochrome P450, family 79 subfamily B, polypeptide 3	LOC_Os04g08828	-0.70	D
2	YUCCA family monooxygenase	LOC_Os01g45760	-0.98	D
		LOC_Os01g12490	0.76	U
		LOC_Os07g25540	-0.60	D
		LOC_Os04g03980	1.61	U
3	ROOTY/SUPERROOT1	LOC_Os11g35040	-1.10	D
4	N-hydroxythioamide S-beta-glucosyltransferase	LOC_Os09g34214	-1.31	D
GH3	Auxin-responsive GH3 gene family member	LOC_Os07g38860	0.79	U
		LOC_Os01g55940	4.50	U
		LOC_Os05g05180	0.81	U
		LOC_Os07g40290	5.81	U

Designation	Annotaiton	Locus[1]	Log_2Ratio[2]	Response[3]
1	*ent*-Copalyl diphosphate synthase	LOC_Os02g17780	-1.87	D
		LOC_Os04g09900	1.70	U
2	*ent*-Kaurene synthase	LOC_Os02g36140	2.00	U
		LOC_Os02g36220	-1.38	D
		LOC_Os12g30824	3.06	U
3	*ent*-Kaurene oxidase	LOC_Os06g37224	2.04	U
4	*ent*-Kaurenoic acid hydroxylase	LOC_Os06g02019	-1.38	D
5	Gibberellin 20 oxidase 2	LOC_Os04g39980	0.59	U
		LOC_Os05g34854	-1.03	D
		LOC_Os10g39140	1.47	U
6	Gibberellin 2-oxidase	LOC_Os01g11150	4.67	U
		LOC_Os01g22910	2.12	U
		LOC_Os01g55240	1.22	U
		LOC_Os04g44150	2.63	U

FIGURE 3 | Genes related to auxin and gibberellic acid biosynthesis and signaling in RGSV-infected plants that are up- or down-regulated. Responses of genes for **(A)** auxin metabolisms and signaling, and **(B)** gibberellic acid (GA) metabolism. Enzymes catalyzing reactions are shown in the table below: [1]based on Rice Genome Annotation Project database Osa1 (rice.plantbiology.msu.edu), [2]log_2-based differential expression ratio (signal intensity in RTSV-infected plant/signal intensity in mock-inoculated plant), and [3]U (D): Significantly induced (suppressed) by RGSV infection. Boxes of red (or blue) indicate that the corresponding genes are predominantly induced (or suppressed). Boxes of red/blue indicate that the number of the corresponding genes induced was similar to that of suppressed genes. →: Reaction and translocation of substrate →: Positive signaling ⊣: Negative signaling.

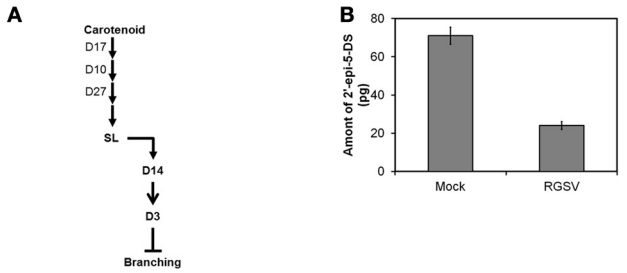
production of bioactive GA precursor (Yang and Hwa, 2008, and references therein). Five genes encoding these enzymes were induced by RGSV infection (**Figure 3B**). RGSV infection also activated the expression of four genes encoding gibberellin 2-oxidase (GA2ox), which degrades bioactive GA. The expression of genes for GA metabolism is regulated by Dwarf 62 (D62) and YABBY1 transcription factors (Dai et al., 2007; Li et al., 2010). The genes encoding D62 and YABBY1 were induced and suppressed by RGSV infection, respectively (**Supplementary Material 1**). On the other hand, the expression of genes encoding GID1, DELLA, and GID2, which are involved in GA signaling (Itoh et al., 2008), was not affected by RGSV infection (**Supplementary Material 1**). These results suggest that RGSV infection might have induced both GA biosynthesis and GA degradation.

Gene related to strigolactone (SL) biosynthesis and signaling

SL was recently classified as a plant hormone. The expression of genes involved in SL biosynthesis such as those encoding Dwarf 17 (D17), D10, and D27 was not changed by RGSV infection (**Figure 4A**). We quantified 2′-epi-5-deoxystrigol, one of the major SLs n rice, in root exudates to examine whether endogenous SL level was changed by RGSV infection. Quantification of 2′-epi-5-deoxystrigol was made on plant tissue at 42 dpi instead of 28 dpi, because the symptoms induced by RGSV infection were clearly observed at this stage. The amounts of 2′-epi-5-deoxystrigol were low in both RGSV and mock plants (**Figure 4B**), but it seemed that RGSV infection suppressed the strigolactone synthesis. *HTD2* (*HIGH TILLERING DWARF 2*) and *D3* are related to SL signaling (Umehara et al., 2010; Yamaguchi and Kyozuka, 2010). The expression of the two genes was suppressed by RGSV infection. These results suggest that SL synthesis and signaling in rice might have been suppressed by RGSV infection.

DISCUSSION

We previously reported gene responses of rice to RSV and *Rice dwarf virus* (RDV) (Shimizu et al., 2007; Satoh et al., 2010, 2011). RSV is the type member of *Tenuivirus* and RDV belongs to the genus *Phytoreovirus*. Symptoms such as leaf stripe and stunting are common to plants infected with either virus. Symptoms caused by RSV infection are shown in **Supplementary Material 3**. The transcriptome analyses of plants infected with either virus commonly showed the predominant



Annotaiton	Locus[1]	Log_2Ratio[2]	Response[3]
D17/HTD1	LOC_Os04g46470	-0.17	
D10	LOC_Os01g38580	0.35	
D27	LOC_Os11g37650	0.56	
D14/HTD2	LOC_Os03g10620	-0.97	D
D3	LOC_Os06g06050	-1.45	D

FIGURE 4 | Influence of RGSV infection on strigolactone biosynthesis and signaling. Responses of genes for **(A)** strigolactone metabolisms and signaling. Enzymes catalyzing reactions are shown in the table below: [1]Based on Rice Genome Annotation Project database Osa1 (rice.plantbiology.msu.edu), [2]Log_2-based differential expression ratio (signal intensity in RTSV-infected plant/signal intensity in mock-inoculated plant), [3]D: Significantly suppressed by RGSV infection. **(B)** Strigolactone in root exudates from mock-inoculated and RGSV-infected plants. →: Reaction and translocation of substrate → : Positive signaling ⊣: Negative signaling.

suppression of genes involved in cell wall synthesis, GA synthesis and chloroplast function, implying strong associations of such gene responses with leaf stripes and stunting. We expected that the symptoms of leaf yellowing, stunting and excessive tillering caused by RGSV are also associated with specific gene responses in RGSV-infected plants. In this study, we focused on the responses of genes involved in cell wall formation, chloroplast function, and hormone biosynthesis and signal transduction since expression of these groups of genes were also significantly affected in rice plants infected with RDV and RSV (Shimizu et al., 2007; Satoh et al., 2010, 2011).

GENE EXPRESSION RESPONSES ASSOCIATED WITH STUNTING AND LEAF YELLOWING IN RGSV INFECTED PLANTS

RGSV infection induces stunting of rice plants. Many cellulose synthase (-like) genes were suppressed in RGSV-infected plants (**Table 2**). The suppression of many cellulose synthase (-like) genes was also seen in RSV-infected plants, which exhibits stunting symptom (Satoh et al., 2010). Expansins mediate loosening of cell wall. Suppression of *OsEXP4*, one of the rice expansin genes, resulted in stunted growth (Choi et al., 2003). The expression of many expansin (-like) genes were suppressed by RGSV infection. The suppression of many expansin genes was also observed in RSV-infected plants (Satoh et al., 2010). Therefore, the suppression of cellulose synthase and expansin genes is one of the common gene responses associated with stunting of rice infected with tenuiviruses.

The expression of the Rubisco genes was suppressed by RGSV infection (**Figure 2A**). The suppression of the Rubisco genes was also observed in RSV-infected plants (Satoh et al., 2010). RGSV-infected plants show leaf yellowing (**Figure 1**), implying the chlorophyll contents may have been decreased by RGSV infection. The appearance of leaf chlorosis in RSV-infected plants is different from that in RGSV-infected plants. RSV-infected plant showed white stripes on leaves (Satoh et al., 2010). The difference in leaf chlorosis pattern may have resulted from the difference in influence by RGSV and RSV on chlorophyll synthesis and degradation processes. In RGSV-infected plants, the expression of genes involved in chlorophyll synthesis was suppressed, and the suppression of those genes was also detected in RSV-infected plants. This suppression indicates that RGSV-infected plants contain low chlorophyll content, and have lower photosynthesis activity than healthy plants. These suggest that decreased biomass accumulation such as cell wall synthesis and carbon fixation activity may account for stunting of the virus-infected plants.

The difference in leaf chlorosis pattern may be related to chlorophyll degradation process (**Figure 2B**). The degradation of chlorophyll is regulated by *Sgr* gene (Park et al., 2007). Overexpression of *Sgr* resulted in yellowing leaves which is similar to the disease symptoms of RGSV-infected plants (Park et al., 2007). The expression of *Sgr* gene was induced by RGSV infection (**Figure 2**). Thus, the activation of *Sgr* by RGSV infection may play a role to induce the leaf yellowing symptom. In RSV-infected plants, the expression of genes involved in chlorophyll degradation was also activated, but the expression of *Sgr* was not significantly changed (Satoh et al., 2010). Therefore, the difference in the appearance of leaf chlorosis between the plant infected with RGSV and that infected with RSV may be related to the activation of *Sgr*.

Cell elongation is controlled by plant hormones such as GA and IAA. The expression of genes for GA biosynthesis such as those for KS, KO, and GA20ox, and those involved in GA inactivation such as the genes for GA2ox increased in RGSV-infected plants (**Figure 3B**), whereas the expression of these genes decreased in plants infected with RSV (Satoh et al., 2010). It was reported that rice plants overexpressing *GA2ox* contained lower bioactive GA, and were shorter than the wild type (Lo et al., 2008). The expression of *GA2ox* increased in stunted rice plants after infection with RDV (Satoh et al., 2011). These observations, in total, suggest that there are multiple ways to reduce the bioactive GA content and produce stunted plants, and that for RGSV an induction of *GA2ox* may aid in producing the stunted plants. On the other hand, many genes for both GA biosynthesis and degradation were suppressed in plants infected with RSV, which may have caused a reduction in GA content. Sakamoto et al. (2004) reported that a GA-deficient mutant was stunted. Therefore, the stunting symptom of RSV-infected plants might be related to GA deficiency. These observations indicate that RGSV- and RSV-infected plants may contain low bioactive GA, though the pathways to the reduction may be different between the plant infected with RGSV and that infected with RSV.

RGSV infection suppressed the genes for IAA biosynthesis, and induced the genes for IAA inactivation (**Figure 3A**). This implies that IAA content decreases in plants infected with RGSV. In addition, RGSV infection increased the expression of *GH3* genes (**Figure 3A**). It is known that the activation of

GH3 genes inhibits plant growth by suppressing genes related to auxin biosynthesis and signaling, and those for expansin (Ding et al., 2008; Domingo et al., 2009). The activation of *GH3* genes was also observed in RSV-infected plants (Satoh et al., 2010). These observations suggest that stunting of plants infected with rice tenuiviruses is associated with a reduction in auxin content.

Overexpression of a SAUR gene resulted in various morphological changes in rice plants (Kant et al., 2009). The expression of a SAUR gene was conspicuously induced in *Arabidopsis thaliana* by *Beet curly top virus* (BCTV), and the induction of the SAUR gene was correlated with symptom development and tissue-specific accumulation of BCTV (Park et al., 2004). The expression of many SAUR genes was found to be regulated in the plants infected with RGSV (**Supplementary Material 1**, **Figure 3A**), RDV (Satoh et al., 2011), and RSV (Satoh et al., 2010). Among the regulated SAUR genes, *OsSAUR13*, *OsSAUR26,* and *OsSAUR33* were commonly activated by RGSV and RDV, whereas *OsSAUR8, OsSAUR30, OsSAUR44, OsSAUR46, OsSAUR53, OsSAUR57*, and *OsSAUR58* were commonly suppressed by RGSV, RDV and RSV. Consequences of the regulated expression of various SAUR genes in the RGSV-infected plant are not clear, but the SAUR genes commonly regulated by RGSV, RDV, and RSV may be associated with stunted growth of plants infected with these viruses.

GENE ASSOCIATED WITH EXCESS TILLERING BY RGSV INFECTION

Excess tillering (shoot branching) is RGSV-specific symptoms. Shoot branching is controlled by plant hormones (Dill and Sun, 2001; Ferguson and Beveridge, 2009; Shimizu-Sato et al., 2009). Auxin and SL inhibit shoot branching, whereas cytokinin promotes it. In RGSV-infected plants, genes related to IAA biosynthesis were suppressed and those related to IAA degradation were induced (**Figure 3A**), implying that the IAA content was decreased by RGSV infection. The suppression of genes involved in IAA synthesis genes and the induction of genes involved in IAA degradation genes were also observed in RSV-infected plants which do not show excess tillers. These indicate that the reduction in auxin content is not directly associated with the excess tillering of RGSV-infected plants. A rice plant overexpressing the *GH3-8* displayed phenotypes of dwarfism and excess tillering (Ding et al., 2008) which are similar to the symptoms of RGSV-infected plants. However, the activation of *GH3-8* was also observed in RSV-infected plants. Therefore, the induction of the *GH3* genes in RGSV-infected plants may not be related to excess tillering.

Rice genes *D10, D17/HTD1*, and *D27* are involved in SL biosynthesis, and *D3* and *D14/HTD2* are involved in SL signaling (Arite et al., 2009; Lin et al., 2009; Yamaguchi and Kyozuka, 2010). Rice mutants for these genes have more tillers than the wild type. The expression of *D3* and *D14/HTD2* was suppressed in rice plants infected with RGSV (**Figure 4**), implying that the SL signaling process is repressed by RGSV infection. In addition, endogenous SL was reduced by RGSV infection (**Figure 4**). The expression of *D17/HTD1* was also suppressed in RSV-infected plants. Thus, SL biosynthesis might have been suppressed by RSV infection. These results suggest that excessive tillering because of RGSV infection may be related to the repression of SL signaling, but not to the decrease in SL content in RGSV-infected plants.

A reduction in active GA also promotes the formation of axillary buds (Curtis et al., 2005; Lo et al., 2008). *GA2ox* is one of the key genes for GA degradation to produce inactive GA (Curtis et al., 2005; Lo et al., 2008). Overexpression of *GA2ox* in rice resulted in development of excess tillers, a decrease in bioactive GA, and an increase in inactive GA (Lo et al., 2008). RGSV infection induced *GA2ox* (**Figure 3B**). Thus, it is likely that RGSV infection decreases bioactive GA content and increase inactive GA content in rice plants. On the other hand, genes involved in biosynthesis and degradation of bioactive GA were suppressed by RSV infection (Satoh et al., 2010), indicating that GA content may have been decreased by RSV infection. Plants deficient in GA display stunted growth and reduced tillers (Margis-Pinheiro et al., 2005), which are similar to the symptoms caused by RSV. These observations suggest that excessive tillering because of RGSV infection is related to the accumulation of inactive GA, but not to the lack of bioactive GA.

Overall, excessive tillers induced specifically by RGSV infection might be a consequence from the additive actions of SL, GA, and the genes regulating shoot branching, such as *RCN1*and *Ostill1*. Examination for possible interactions between RGSV proteins and host proteins, and phenotypes of transgenic plants with RGSV genes may help to elucidate the molecular mechanism of excessive tillering caused by RGSV infection. Moreover, there could be modifications in gene expression that occur earlier than 28 dpi, and further studies will determine how such modifications influence the symptoms observed at 28 dpi.

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SUPPLEMENTARY MATERIAL

The Supplementary Materials for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2013.00313/abstract

Supplementary Material 1 | List of expressed genes and differentially expressed genes in RGSV-infected plants.

Supplementary Material 2 | Examination of expression of selected genes by RT-PCR. The numbers below DNA band images are average $\log_2$ (signal intensity of microarray data) for the corresponding genes in mock-inoculated or RGSV-infected plants (Supplementary Material 1).

Supplementary Material 3 | Symptoms in rice caused by RSV infection. Rice plants at 60 days after RSV inoculation and mock inoculation.

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