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Selective modification of rice (*Oryza sativa*) gene expression by rice stripe virus infection

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Rice stripe disease, caused by rice stripe virus (RSV), is one of the major virus diseases in east Asia. Rice plants infected with RSV usually show symptoms such as chlorosis, weakness, necrosis in newly emerged leaves and stunting. To reveal rice cellular systems influenced by RSV infection, temporal changes in the transcriptome of RSV-infected plants were monitored by a customized rice oligoarray system. The transcriptome changes in RSV-infected plants indicated that protein-synthesis machineries and energy production in the mitochondrion were activated by RSV infection, whereas energy production in the chloroplast and synthesis of cell-structure components were suppressed. The transcription of genes related to host-defence systems under hormone signals and those for gene silencing were not activated at the early infection phase. Together with concurrent observation of virus concentration and symptom development, such transcriptome changes in RSV-infected plants suggest that different sets of various host genes are regulated depending on the development of disease symptoms and the accumulation of RSV.

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INTRODUCTION

Rice stripe disease is the most severe virus disease of rice in east Asia. Typical symptoms are chlorosis and weakness on newly emerged leaves. The plant becomes considerably stunted when affected at the early growth stages (Ou, 1972). Rice stripe disease also causes necrosis of newly emerged leaves (Takahashi *et al.*, 1991). The causal virus is *Rice stripe virus* (RSV), which belongs to the genus *Tenuivirus* (Falk & Tsai, 1998). RSV is transmitted by small brown planthopper (SBPH; *Laodelphax striatellus*), *Terthron albovittatum*, *Unkanodes sapporonus* and *Unkanodes albifascia* (Falk & Tsai, 1998; Ou, 1972). RSV has a thin, filamentous shape and no envelope. The genome consists of four single-stranded RNA segments; RNA1 is negative-sense and RNAs 2–4 are ambisense. Viral mRNAs transcribed from viral RNA or viral cRNA by RNA-dependent RNA polymerase are released to the cytoplasm. Subsequently, a 5′-capped short ribonucleotide leader cleaved from the host mRNA is added to the viral mRNAs by cap-snatching. The 5′-capped RSV RNA is transcribed efficiently in host cells (Falk & Tsai, 1998; Shimizu *et al.*, 1996). Genes encoding a gene-silencing suppressor and movement proteins were also identified in the RSV genome (Lu *et al.*, 2009; Xiong *et al.*, 2008, 2009). Although extensive functional analysis of the RSV genome has been conducted, there have been no reports on the interaction between RSV and rice plants, which may clarify the mechanisms behind the appearance of disease symptoms.

Rice is a model cereal plant, for which many genomic and transcriptome resources and tools are already available (Liang *et al.*, 2008; Ouyang *et al.*, 2007; Rice Annotation Project, 2008; Rice Full-length cDNA Consortium, 2003). The elucidation of genome sequences and structures in diverse organisms has led to the development of various high-throughput genome and transcriptome analytical tools. Numerous transcriptome profiles in plant cells under various conditions have been characterized. Transcriptome changes in plants such as *Arabidopsis* (Espinoza *et al.*, 2007; Golem & Culver, 2003; Marathe *et al.*, 2004; Whitham *et al.*, 2003; Yang *et al.*, 2007), *Nicotiana* (Dardick, 2007; Senthil *et al.*, 2005), *Zea mays* (Uzarowska *et al.*, 2009) and *Vitis vinifera* (Espinoza *et al.*, 2007) that were infected with viruses were characterized to identify genes constituting the expression networks underlying disease symptoms and virus propagation. Although

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Three supplementary tables are available with the online version of this paper.

gene-expression profiles in rice under various stresses are accumulating continuously, limited information is available for those associated with virus infection. Shimizu *et al.* (2007) described the relationship between dwarfism and cell wall-related genes in rice plants infected with rice dwarf virus.

To characterize host responses to RSV infection at the gene-expression level, time-course transcriptome changes in RSV-infected rice plants were examined by using a 60-mer oligonucleotide microarray that covers almost all of the transcripts from the rice genome (Ouyang *et al.*, 2007; Rice Full-length cDNA Consortium, 2003; Satoh *et al.*, 2007). Time-course gene-expression analysis in rice infected with RSV and concurrent observations of virus concentration and symptom development indicate that timely expression modifications of genes involved in selective cellular processes may be associated with RSV propagation and RSV-induced symptoms.

RESULTS

Symptom development and accumulation of RSV

Symptoms of chlorotic stripes on leaves (Fig. 1a) and stunting were observed on RSV-inoculated plants as early as 6 days post-inoculation (p.i.). Most RSV-inoculated plants exhibited symptoms by 9 days p.i. Elongated and folded leaves started to appear 12 days p.i. and leaf death was first observed around 15 days p.i. We stopped harvesting samples at 18 days p.i. because leaf death was observed in many plants (Fig. 1b). Infection rates exceeded 70 % throughout the experiment. The amount of coat protein (CP) was measured by double antibody-sandwich (DAS)-ELISA. Accumulation of CP was evident by 9 days p.i. The concentration of CP increased continuously up to 15 days p.i. (Fig. 1c). The RNA of RSV CP was first detected 6 days p.i. by RT-PCR (Fig. 1d).

Changes in number of differentially expressed genes (DEGs)

The expression difference between RSV-infected and mock-inoculated rice plants was examined by direct comparison on a single custom oligomicroarray. A gene was declared as a DEG if the difference in mean intensity between RSV-infected and mock-inoculated plants was greater than 1.5 × and the significance of changes in gene expression was $P \leqslant 0.05$ by a paired *t*-test. We did not use microarray data from the samples harvested at 15 days p.i. because the expression profiles at 15 days p.i. appeared to be similar to those at 12 days p.i., and leaf necrosis due to senescence was observed in many plants at 15 days p.i.

The results of the microarray experiments revealed that 9788 genes were differentially expressed between RSV-infected and mock-inoculated plants at least at one of the time points (see Supplementary Table S1, available in JGV Online). The number of DEGs at single time points increased as the infection progressed, and the pattern of DEG increment was similar to the accumulation pattern of RSV protein and RNA (Fig. 1e). The difference in expression levels for many genes between RSV-infected and mock-inoculated rice plants also increased dramatically after around 12 days p.i. (Fig. 1e).

Response directions (up- or downregulation) of individual DEGs by RSV infection were compared among time points (Fig. 1f). More than one-third of the DEGs at 3 and/or 6 days p.i. were also differentially expressed at 12 days p.i., but the responses of one-sixth of the DEGs at 3 and/or 6 days p.i. were the opposite of their responses at 12 days p.i. On the other hand, the responses at 9 days p.i. of most genes differentially expressed at both 9 and 12 days p.i. were similar to those at 12 days p.i.

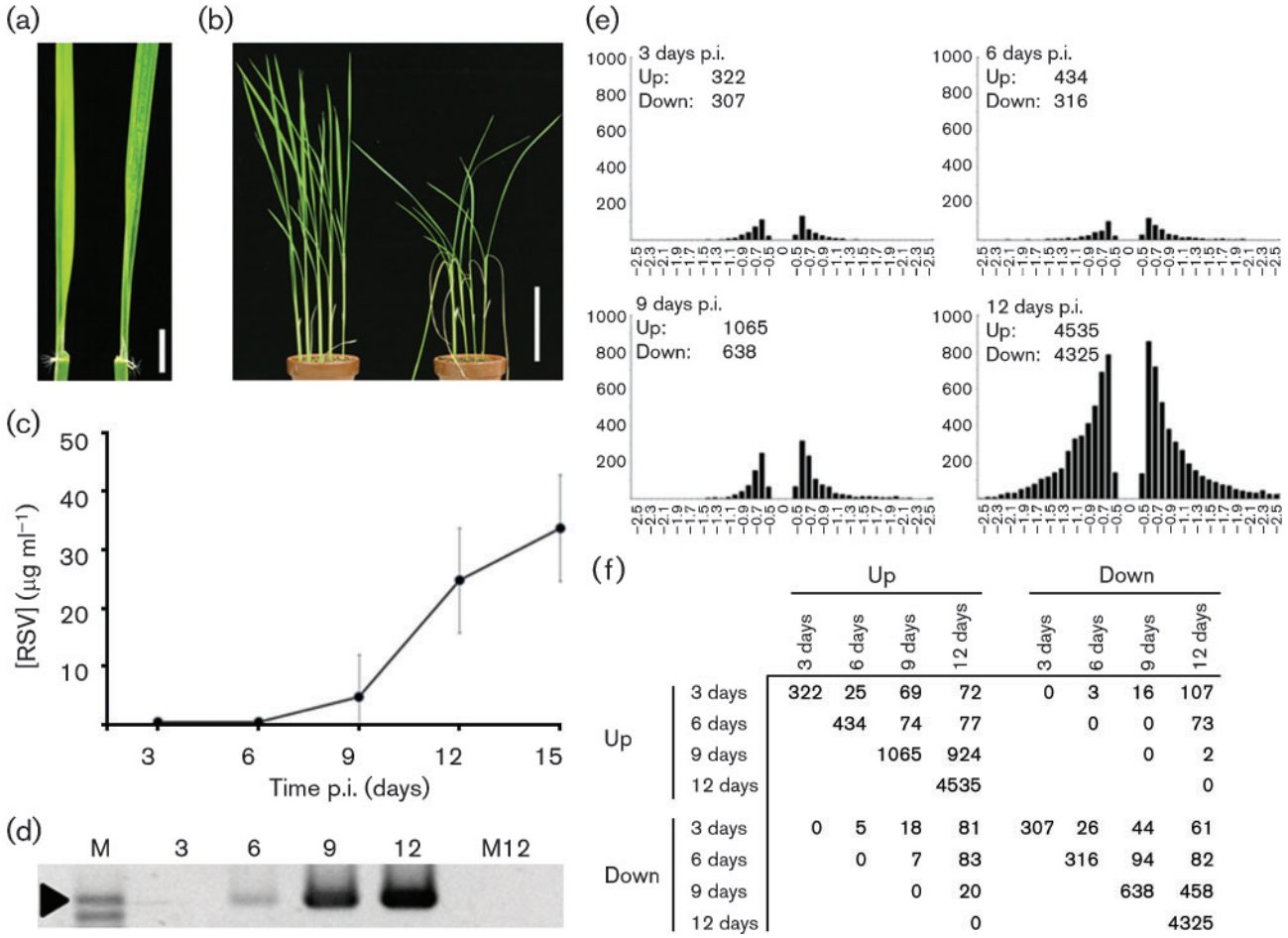
Influence of RSV infection on host cellular systems

To deduce host cellular systems influenced by RSV infection, we examined the annotation of genes evaluated by microarray on the basis of data available in various public databases and in the literature. We selected genes categorized into the following cellular functions: protein-synthesis systems, organellar function, cell structure and defence system.

Protein-synthesis systems. Many translation-machinery genes were induced after 9 days p.i. (Table 1). Ribosomal protein (RP) genes, except those for chloroplast-targeted RPs (see below), were activated at 3 days p.i., and again after around 9 days p.i. (Table 1). Many genes related to protein processing (protein maturation and degradation) were also induced after around 9 days p.i. by RSV infection (Table 1).

Protein synthesis requires amino acids and nucleotides as substrates. Nucleotides are used to generate rRNA and tRNA. Genes related to the metabolic pathways for nucleotides and amino acids were activated in most cases after around 9 days p.i. (Table 1), but some genes related to nucleotide synthesis were activated at 3 days p.i. The activated genes at 3 days p.i. were also induced after around 9 days p.i. This result indicates that RSV infection influences the transcription not only of those genes related to protein-synthesis processes, but also of those related to metabolic pathways of nucleotides and amino acids, which are substrates of protein synthesis.

Fate of nuclear-encoded organelle-targeted genes. Organelles such as mitochondria and chloroplasts produce energy to drive cellular processes. The genes related to metabolic pathways [citrate (TCA) cycle and oxidative phosphorylation] and protein synthesis (RP and translation factors) occurring in mitochondria were induced by RSV infection after around 9 days p.i. (Table 1). Moreover, many other genes whose products are



		Up				Down			
		3 days	6 days	9 days	12 days	3 days	6 days	9 days	12 days
Up	3 days	322	25	69	72	0	3	16	107
	6 days		434	74	77		0	0	73
	9 days			1065	924			0	2
	12 days				4535				0
Down	3 days	0	5	18	81	307	26	44	61
	6 days		0	7	83		316	94	82
	9 days			0	20			638	458
	12 days				0				4325

Fig. 1. Symptoms and changes in virus accumulation and number of DEGs in RSV-infected plants. (a) Chlorotic stripes on RSV-infected plant (right) at 12 days p.i. Bar, 5 mm. (b) Leaf death and stunting of RSV-infected plant at 18 days p.i. Bar, 10 cm. (c) Accumulation of RSV proteins in infected plants (error bars represent SD calculated from three biological replicates). (d) RT-PCR for RSV RNA. M, 100 bp marker (arrowhead, 1000 bp); 3–12, RSV-infected plants at 3, 6, 9 and 12 days p.i., respectively; M12, mock-inoculated plants at 12 days p.i. (e) Numbers and magnitudes of DEGs between RSV- and mock-inoculated plant at each time point. (f) Number of DEGs at each time point, and DEGs detected repeatedly at different time points.

localized in mitochondria ('Located in mitochondria' in Table 1) were also induced after around 9 days p.i. (Table 1). Acetyl-CoA is one of the substrates in the TCA cycle. The transcription of genes associated with the metabolic activity (β-oxidation) for acetyl-CoA synthesis appeared to be increased by RSV infection ('Fatty acid metabolism' in Table 1). On the other hand, the transcription of genes whose products function in the chloroplasts was suppressed by RSV infection after around 12 days p.i. (Table 1). Thus, it appeared that the effects of RSV infection on the expression of nuclear-encoded genes involved in organellar activities were dependent on the sites of localization of the gene products.

Cellular structure. The cell wall contains many types of component, and the formation of cell walls is associated with cell elongation. The transcription of many cell wall-related genes was suppressed markedly by RSV infection after around 9 days p.i. (Table 1). Cytoskeletons are structural backbones to determine cell form and size. In RSV-infected plants, genes for the construction of microtubules were suppressed markedly after around 12 days p.i., whereas only a few genes related to actin filaments were suppressed (Table 1). These results indicate that RSV infection suppressed the transcription of cell structure-related genes, although the expression of some gene families was not affected by RSV infection.

Defence systems. Plants have two key defence systems against virus infection: one is gene silencing and the other is hormone signal-related defence systems. The expression of genes related to gene-silencing systems was influenced by

RSV infection. The transcription of genes encoding the Argonautes and RNA-dependent RNA polymerase families was upregulated by RSV infection after around 9 days p.i. (Table 1).

The transcription of some genes related to hormone synthesis was influenced by RSV. The microarray data indicated that genes related to jasmonic acid synthesis were suppressed until 9 days p.i., and then induced afterwards by RSV infection (Table 1). The trend in activation of genes related to ethylene and salicylic acid synthesis was not evident from the result, because the numbers of activated and suppressed genes were similar (Table 1). Transcription of genes encoding pathogenesis-related (PR) proteins and other stress-responsive proteins in RSV-infected rice plants was also examined. Genes encoding PR and stress-responsive proteins whose transcription was affected by RSV infection were mostly suppressed until 9 days p.i. The transcription of some of these genes was found to increase after around 12 days p.i., although the activated genes were still fewer than those suppressed in many gene families (Table 1).

Some transcription factors are known to act specifically on transcriptional regulation of genes responding to biotic and abiotic stresses (Chen & Guo, 2008; Gutterson & Reuber, 2004; Nakashima *et al.*, 2007; Ryu *et al.*, 2006; Shimono *et al.*, 2007). The transcription of many genes for transcription factors was influenced by RSV infection (Table 1). The effect of RSV infection on the expression of transcription-factor genes was especially noticeable at 12 days p.i. The transcription profiles of some transcription factors responding to RSV infection looked similar (Fig. 2a): (i) many transcription-factor genes downregulated at 3 days p.i. were not downregulated at 12 days p.i.; (ii) some genes upregulated at 3 days p.i. were downregulated at 12 days p.i.; and (iii) most DEGs for transcription factors at 12 days p.i. were not differentially expressed at 3 days p.i. Moreover, the influence of RSV infection on the expression of transcription-factor genes was different, depending on the transcription-factor families. The numbers of WRKY and NAC transcription-factor genes induced at 12 days p.i. by RSV infection were greater than those of suppressed genes, whereas the numbers of induced genes in AP2, bHLH, Homeobox, MADS and Myb families were smaller than those of suppressed genes (Fig. 2a).

Specific domain structure of gene product

As described above, we examined host gene-expression responses to RSV infection according to genes categorized by cellular systems. However, the number of genes categorized by cellular systems was only about 6000. Therefore, for systematic analysis of a broader range of gene-expression profiles in RSV-infected plants, we examined the expression patterns of genes by protein domains as categorized at OSA1 (Finn *et al.*, 2008; Ouyang *et al.*, 2007).

Even at 3 days p.i., the transcription levels of 42 genes encoding a protein kinase domain (Pfam ID=PF00069) were affected in RSV-infected plants (Fig. 2b). Most genes encoding a kinase domain that were declared as a DEG at 3 days p.i. were not differentially expressed at 6 or 9 days p.i., but many of the DEGs at 3 days p.i. were differentially expressed again at 12 days p.i. However, the direction of the transcription response of many of the DEGs at 3 days p.i. was different from that at 12 days p.i. (Fig. 2b). In addition, the directions of the transcription response of 27 protein kinase domain-encoding DEGs at 6 days p.i. were also different from those of the corresponding genes at 12 days p.i. (Fig. 2b). Thus, the directions of the transcription response of genes encoding a protein kinase domain changed frequently between the early and the late infection phases.

Examination of the transcription profiles of genes categorized by protein domains suggested that the expression responses of genes in RSV-infected rice plants is associated closely with the gene families to which they belong (see Supplementary Table S2, available in JGV Online). The gene families related to signal-transduction systems (e.g. genes encoding domains such as protein kinase, PF00069; leucine-rich repeat, PF00560; EF-hand, PF00036; NB-ARC, PF00931; RAS, PF00071) and those related to DNA repair (XPG N, PF00751), transcription (RNA pol Rpb5 C/N, PF01191./PF03871), translation (EFG C, PF00679), protein processing (Mov34, PF01398; LSM, PF01423; U-box, PF04564) and mitochondrial activities (peptidase M16, PF00675; MAM33, PF02330) were activated markedly at 12 days p.i. Some genes related to translation, protein processing and mitochondrial activities were also activated at 3 days p.i. The gene families related to cell cycle (cyclin, PF00134/PF02984; kinesin, PF00225) and plastid functions (Fer2, PF00111; NPH3, PF03000) were suppressed markedly at 12 days p.i.

Confirmation of microarray data by RT-PCR

The microarray that we used detected the differential transcription of >8000 genes by RSV infection at 12 days p.i. To assess the accuracy of microarray data, we selected 10 DEGs and one non-DEG at 12 days p.i. and examined the similarity between the gene responses observed by microarray and those observed by RT-PCR. As seen in Fig. 3, most cases of up/downregulation of gene expression detected by microarray were also observed by RT-PCR, although the degrees of regulated response for some genes observed by microarray and those observed by RT-PCR were seemingly different. Only one result (replication 1 of LOC_Os02g35310; Fig. 3) by RT-PCR was inconsistent with that observed by microarray. Thus, the result suggests that the differential gene expression detected by the microarrays mostly reflected the actual transcriptome differences between RSV-infected and mock-inoculated plants.

DISCUSSION

RSV is an obligated parasite that largely depends on host cellular systems for its propagation. RSV infection causes

Table 1. Changes in transcription of genes in plants infected with RSV

Category	No. genes	3 days p.i.		6 days p.i.		9 days p.i.		12 days p.i.		Reference(s)*
		Up	Down	Up	Down	Up	Down	Up	Down	
Translation										KEGG
Aminoacyl–tRNA biosynthesis	42	0	0	0	0	3	1	9	5	
Ribosomal proteins	176	7	1	0	0	36	0	43	14	
Other translation proteins	53	0	0	1	0	1	0	12	2	
Protein processing										KEGG
Proteasome	51	1	0	0	0	0	0	19	0	
Protein export	27	0	0	0	0	3	0	7	1	
Type II secretion system	1	0	0	0	0	0	0	0	0	
Protein folding and associated processing	37	0	0	0	0	2	1	10	2	
Ubiquitin-mediated proteolysis	54	0	0	0	0	1	0	11	3	
SNARE interactions in vesicular transport	25	0	0	0	0	0	0	6	1	
Regulation of autophagy	10	0	0	0	0	0	0	1	0	
Nucleotide metabolism										KEGG
Purine metabolism	82	4	0	0	0	10	0	18	4	
Pyrimidine metabolism	72	2	0	0	0	7	0	20	3	
Amino acid metabolism										KEGG
Urea cycle and metabolism of amino groups	26	0	0	0	0	1	0	4	2	
Glutamate metabolism	39	1	1	0	0	4	1	6	4	
Alanine and aspartate metabolism	46	0	0	0	1	0	2	4	5	
Glycine, serine and threonine metabolism	42	1	0	0	0	4	0	8	4	
Methionine metabolism	29	0	0	1	0	1	1	4	3	
Cysteine metabolism	21	0	0	0	0	0	1	2	4	
Valine, leucine and isoleucine degradation	29	0	0	0	0	1	0	8	1	
Valine, leucine and isoleucine biosynthesis	26	1	0	0	1	1	0	6	1	
Lysine biosynthesis	15	1	0	0	0	0	0	1	1	
Lysine degradation	17	0	0	0	0	1	0	5	1	
Arginine and proline metabolism	18	0	0	0	0	0	0	3	0	
Histidine metabolism	17	0	0	0	0	0	0	1	0	
Tyrosine metabolism	24	0	0	0	0	0	1	4	2	
Phenylalanine metabolism	28	0	1	0	0	0	2	0	7	
Tryptophan metabolism	23	0	0	0	0	1	0	4	2	
Phenylalanine, tyrosine and tryptophan biosynthesis	37	0	1	0	0	1	1	4	3	
Mitochondria										
Citrate (TCA) cycle	47	0	0	0	1	0	0	11	2	KEGG
Oxidative phosphorylation	97	1	0	0	1	0	1	12	4	
Ribosomal proteins	10	0	0	0	0	2	0	4		KEGG, iPSORT, TargetP
Translation factors	5	0	0	0	0	1	0	3		
Located in mitochondria	1140	12	5	8	13	33	24	193	130	Gramene
Plastid										
Photosynthesis	37	0	0	0	0	0	0	1	14	KEGG
Photosynthesis – antenna proteins	8	0	0	0	0	0	0	0	4	
Carbon fixation in photosynthetic organisms	68	1	1	1	0	0	0	2	11	
Porphyrin and chlorophyll metabolism	24	1	0	0	0	0	0	1	12	
Ribosomal proteins	19	0	1	0	0	0	0	0	13	KEGG, iPSORT, TargetP
Translation factors	2	0	0	0	0	0	0	0	1	
Located in plastid	442	6	3	2	5	20	17	44	91	Gramene
Lipid metabolism										
Fatty acid biosynthesis	15	0	0	0	0	0	0	0	3	
Fatty acid metabolism	30	0	0	0	0	1	0	8	2	
Cell wall										
Cellulose synthase and synthase-like family	46	1	0	0	0	0	1	2	16	Somerville (2006)
Expansin family	52	1	0	2	2	0	5	1	11	OSA1
Extensin family	10	0	0	0	0	0	1	0	3	

Table 1. cont.

Category	No. genes	3 days p.i.		6 days p.i.		9 days p.i.		12 days p.i.		Reference(s)*
		Up	Down	Up	Down	Up	Down	Up	Down	
Cytoskeleton										KEGG
Actin filaments	17	0	1	0	0	0	1	0	2	
Microtubles	14	0	0	0	0	0	0	0	6	
Gene silencing										ChromDB
Argonaute gene family	17	0	0	0	0	2	0	8	1	
Dicer-like group	5	0	0	0	0	0	0	1	0	
Double-stranded RNA-binding protein group	5	0	0	0	0	0	0	1	2	
HUA enhancer	1	0	0	0	0	0	0	1	0	
RNA polymerase IV large subunit	2	0	0	0	0	0	0	1	0	
RNA polymerase IV small subunit	2	0	0	0	0	1	0	1	0	
RNA-dependent RNA polymerases	5	0	0	0	0	2	0	2	0	
RNA helicases	1	0	0	0	0	0	0	0	0	
Suppressor of gene silencing	2	0	0	0	0	0	0	1	0	
Pathogenesis-related proteins										
PR-1	28	0	0	0	3	0	4	2	2	Mitsuhara *et al.* (2008)
Chitinase	17	0	0	0	1	0	1	6	4	Silverstein *et al.* (2007)
Defensin	55	0	1	1	2	1	1	1	2	
Thionin	12	0	0	0	0	0	0	0	0	
β-1,3-Glucanase	64	1	0	2	3	0	3	5	19	OSA1
Thaumatin-like	21	0	0	0	4	0	0	2	3	
Proteinase-inhibitor	6	0	0	0	1	0	1	2	3	
Oxalate oxidase	4	0	0	0	0	0	0	0	1	
Other stress-responsive proteins										OSA1
Glutathione *S*-transferase	80	0	1	0	3	0	6	19	18	
Ascorbate peroxidase	11	0	0	1	0	0	2	3	2	
Superoxide dismutase	7	0	0	0	0	0	0	2	1	
Germin-like	31	1	0	0	2	0	1	6	7	
Hormone synthesis										Gramene
Ethylene	32	0	0	0	0	0	1	2	2	
Jasmonic acid	42	0	1	0	1	0	3	5	2	
Salicylic acid	9	0	1	0	0	0	1	2	3	
Gibbereric acid	16	1	0	0	0	0	0	0	3	
Transcription factor	1894	14	22	25	14	40	35	157	254	Plant TFDB

*References: KEGG (Kanehisa *et al.*, 2008); iPSORT (Bannai *et al.*, 2002); TargetP (Emanuelsson *et al.*, 2007); Gramene (Liang *et al.*, 2008); ChromDB (Gendler *et al.*, 2008); OSA1 (Ouyang *et al.*, 2007); Plant TFDB (Riano-Pachon *et al.*, 2007).

symptoms such as leaf necrosis and stunting in most rice plants. The overall results of this study showed the modification of the rice transcriptome associated with particular host cellular systems by RSV infection.

Modification of protein-synthesis processes by RSV infection

Nucleotides for viral RNA synthesis, and amino acids and synthesis machineries for viral proteins are to be supplied by host cells, although the replication and transcription of the RSV genome are carried out by the viral RNA-dependent RNA polymerase (Falk & Tsai, 1998). Our results indicate that genes related to nucleotide and amino acid synthesis were activated by RSV infection at 3 days p.i. and after around 9 days p.i. (Table 1). The activities of nucleotide and amino acid synthesis are feedback-regulated by the concentrations of nucleotides and amino acids in cells (Coruzzi & Zhou, 2001; Fafournoux *et al.*, 2000; Todeschini *et al.*, 2005; Zhang *et al.*, 1997). Thus, the activation of genes related to protein synthesis in plants infected with RSV may have been caused by the reduced concentrations of nucleotides and amino acids by RSV infection. Progenies of RSV started appearing in infected plants at 6 days p.i. (Fig. 1c, d), and it also seemed that the rate of RSV propagation increased simultaneously with the activation of nucleotide- and amino acid-synthesis systems. Therefore, the increased consumption of substrates in host

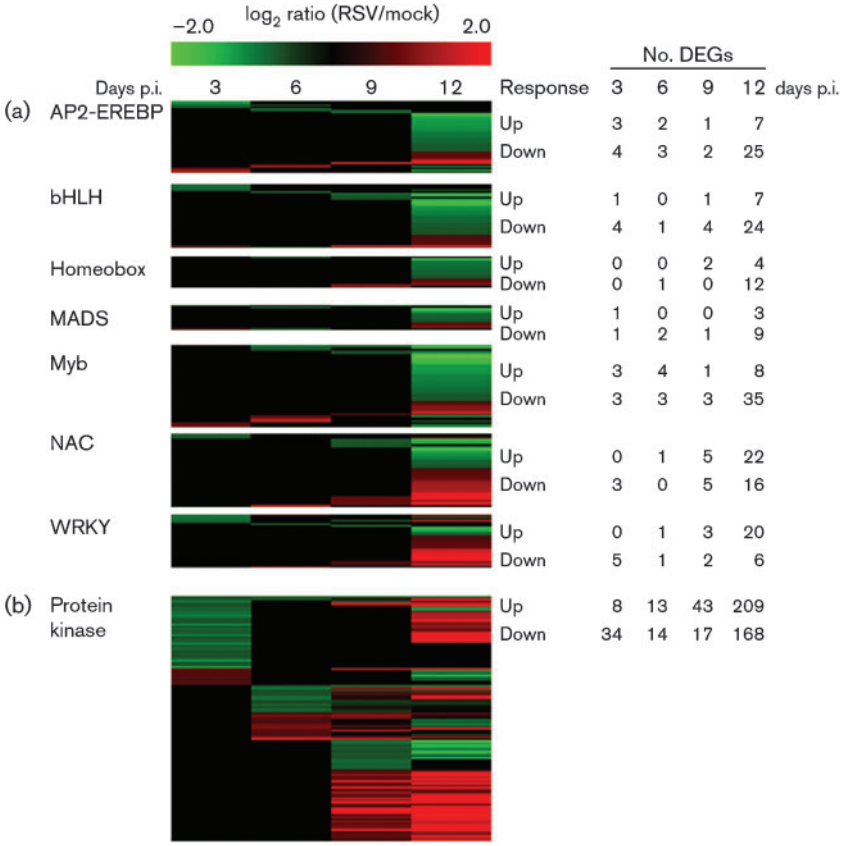


Fig. 2. Differential expression of genes in RSV-infected plants. Differential expression profiles of genes related to (a) transcription factor and (b) protein kinase (PF00069). See Supplementary Tables S2 and S3 (available in JGV Online) for details.

cells by RSV propagation may have caused the decline of nucleotides and amino acids in host cells and, subsequently, may have induced the activation of substrate synthesis after 6 days p.i.

Genes for protein synthesis were also induced in RSV-infected plants (Table 1). It was reported that hepatitis C virus (HCV) is able to control the transcription of translation-related genes (Raychaudhuri *et al.*, 2009). The NS5A protein of HCV regulates the phosphorylation of an upstream binding factor that activates the transcription of rRNA, although the NS5A protein is located in the endoplasmic reticulum. The activation of rRNA synthesis also induces the activation of protein synthesis (Raychaudhuri *et al.*, 2009). Our microarray results and previous observation imply that the protein(s) originating from the RSV genome may have been able to control the transcription of host genes for protein synthesis. The activation of protein-synthesis systems was also observed in rice plants infected with rice tungro spherical virus (RTSV) (H. Kondoh, personal communication). However, the activation in RTSV-infected rice was a temporal event, and the number of activated genes related to protein synthesis in RTSV-infected rice was only half of that observed in RSV-infected rice. Marked activation of genes for protein synthesis by RSV infection may have accelerated RSV propagation in infected rice cells.

Once plants are infected with RSV, energy products such as ATP and NADH generated by organelles are likely to be consumed not only for host cellular systems, but also for RSV propagation. Transcription of many nuclear-encoded plastid-related genes decreased in RSV-infected plants (Table 1). Such suppression of plastid-related genes by virus infection was also observed in plants infected with plum pox potyvirus, tomato ringspot nepovirus or rice dwarf virus (Dardick, 2007; Jiménez *et al.*, 2006; Shimizu *et al.*, 2007). Our results also indicate that the transcription of some nuclear genes encoding mitochondrion-targeted proteins increased in RSV-infected plants (Table 1). The activation of such gene expression seemed to occur concurrently with the activation of genes related to protein-synthesis machineries. One of the important pathways for energy production in mitochondria is the TCA cycle. Either pyruvate or acetyl-CoA can serve as the starting material of the TCA cycle. Pyruvate is produced by the carbon-fixation process in chloroplasts, whilst acetyl-CoA can be produced by β-oxidation of fatty acids. The transcription of genes related to carbon fixation was suppressed (Table 1), but the transcription activities of genes related to β-oxidation were induced in RSV-infected plants (Table 1). This may have resulted in an increase of acetyl-CoA in RSV-infected cells. Therefore, the energy supply in RSV-infected cells may be largely dependent on the mitochondria.

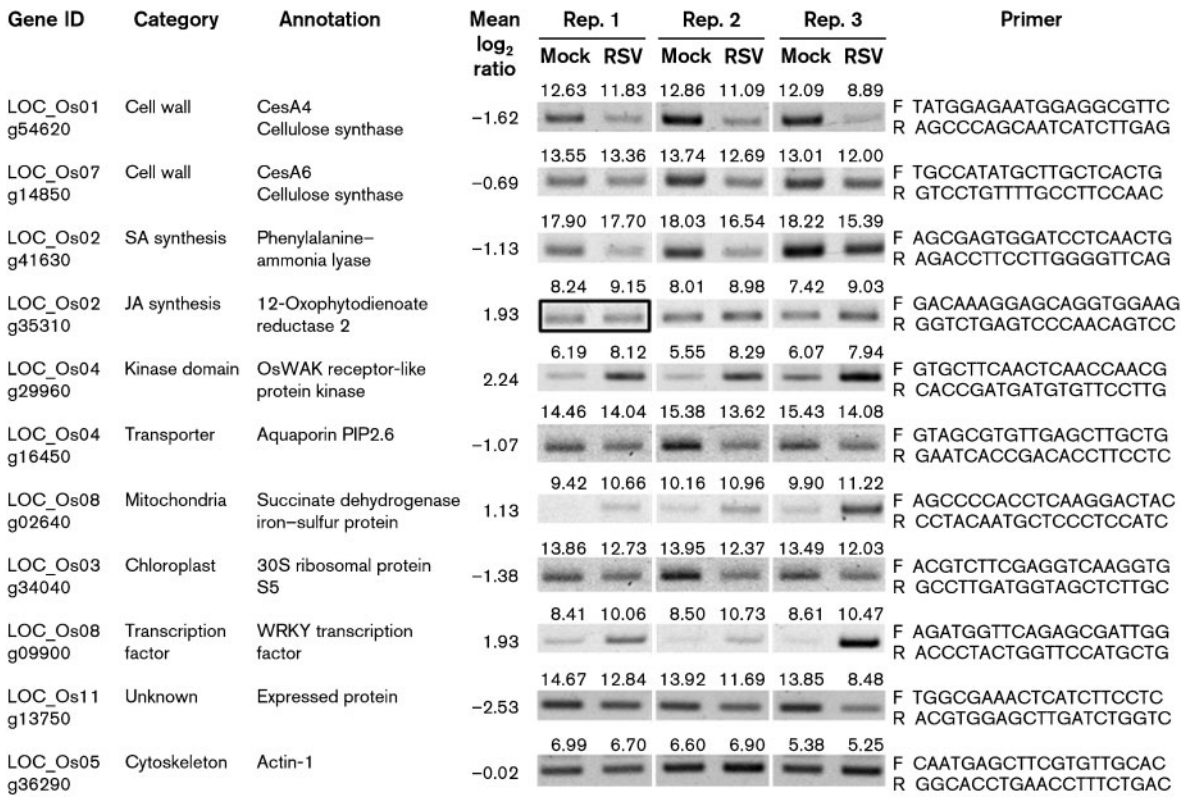


Fig. 3. Significantly differentially expressed genes evaluated by RT-PCR. Numbers above gel images are the normalized signal intensity from the microarray. Results in a black square indicate that the expression levels detected by microarray and RT-PCR were different. Abbreviations: JA, jasmonic acid; SA, salicylic acid.

Modification of cellular systems and symptom development

One of the symptoms induced by RSV infection is chlorosis, which is generally caused by the loss of chloroplasts in the cells. It was reported that proteins of tomato mosaic virus and tobacco mosaic virus interacted with proteins in the chloroplast, affecting chloroplast functions and stability (Lehto *et al.*, 2003; Zhang *et al.*, 2008a). The helper component–proteinase of sugar cane mosaic virus was reported to interact with the ferredoxin V protein of host cells, and the interaction led to the degradation of the chloroplast structure (Cheng *et al.*, 2008). Deterioration in the development of plastids was reportedly caused by the inactivation of various nuclear genome-encoded genes whose products function in plastids (Yu *et al.*, 2007). Therefore, the chlorosis observed in the leaves of RSV-infected plants may be associated with the reduced transcription of many chloroplast-related genes involved in photosystems I and II (see Supplementary Table S3, available in JGV Online), and some of these genes may mediate the interaction between chloroplast component(s) and RSV protein(s). Photosystem II was affected severely in plants infected with plum pox virus and, as a result, reactive oxygen species were generated in chloroplasts (Díaz-Vivancos *et al.*, 2008). Accumulation of reactive oxygen species can lead to necrotic cell death (Amari *et al.*, 2007; Ishiga *et al.*, 2009). Our results indicate that the transcription of genes for photosystems in the chloroplast was suppressed, whilst the genes for scavenger systems of reactive oxygen species were not affected by RSV infection. Therefore, such cellular conditions in RSV-infected rice plants may have led to a high accumulation of reactive oxygen species, causing cell death in RSV-infected plants.

The genes encoding proteins related to cell-wall synthesis started to be suppressed after around 6 days p.i. by RSV infection (Table 1). Suppression of cell wall-related genes was also reported for stunted plants infected with other viruses (Marathe *et al.*, 2004; Shimizu *et al.*, 2007). Cellulose-deficient plants showed a stunted phenotype (Burn *et al.*, 2002). Gibberellic acid is involved in cell elongation (Hedden & Phillips, 2000); the genes encoding enzymes for gibberellic acid synthesis were suppressed by RSV infection (Table 1). Suppression of genes for cell-wall and gibberellic acid synthesis was not observed in RTSV-infected plants (H. Kondoh, personal communication). Rice plants infected with RTSV usually do not show evidence of stunting (Cabauatan *et al.*, 1993; Shinkai, 1977; H. Kondoh, personal communication). Thus, the stunted growth of plants infected with RSV may be associated with

the suppression of genes for cell wall and/or gibberellic acid synthesis.

Modification of host defence systems by RSV infection

Propagation and/or movement of viruses may be inhibited by plant defence systems such as gene silencing (Kitanaga *et al.*, 2006). The results of this study suggest that gene silencing may not be activated in infected cells until 6 days p.i. (Table 1). The p3 (*NS3*) gene of RSV encodes a gene-silencing suppressor that inhibits local and systemic gene silencing (Levy *et al.*, 2008; Xiong *et al.*, 2008, 2009). Expression of the p3 protein of RSV was limited to young leaf tissues early in the infection cycle (Liang *et al.*, 2005). At 12 days p.i., the level of RSV kept increasing (Fig. 1c), although many genes involved in gene silencing were activated at 12 days p.i. (Table 1). This may indicate that the gene-silencing system in the host was not activated in a timely manner to suppress RSV replication.

Other important defence systems in plants are the salicylic acid-signalling and ethylene/jasmonic acid-signalling pathways (Love *et al.*, 2007; Radwan *et al.*, 2008; Ryu *et al.*, 2004). The hormones involved in the defence systems are synthesized in plants when these are attacked by various pathogens. The hormones subsequently activate the defence systems against the pathogens and control the expression of PR proteins (Mitsuhara *et al.*, 2008; van Loon *et al.*, 2006). The defence system against biotrophic pathogens is mediated by salicylic acid signalling, whilst ethylene/jasmonic acid signals control the defence system against necrotrophic pathogens (Loake & Grant, 2007; van Loon *et al.*, 2006; Wasternack, 2007). The two signalling systems are regulated antagonistically by each other (Loake & Grant, 2007; Wise *et al.*, 2007). The results of this study indicate that the genes related to hormone synthesis, PR proteins and other stress responses were suppressed by RSV infection until 9 days p.i. Such expression profiles imply that the RSV-infected plants were not able to activate the biotic and/or abiotic stress-responsive systems until 9 days p.i. Moreover, at 12 days p.i., when the infected plants showed distinctive symptoms, the number of upregulated DEGs involved in stress responses was nearly the same as or lower than that of downregulated DEGs. These results suggest that plant defence systems may be suppressed at the early phase of RSV infection.

Some transcription factors, such as WRKY, NAC and AP2, are known to act specifically on transcriptional regulation of genes responsive to biotic and/or abiotic stresses (Chen & Guo, 2008; Fang *et al.*, 2008; Gutterson & Reuber, 2004; Nakashima *et al.*, 2007; Ryu *et al.*, 2006; Shimono *et al.*, 2007). The transcription of many genes for transcription factors was affected after around 12 days p.i., but some down/upregulated DEGs at 12 days p.i. were also up/downregulated at 3 days p.i. (Fig. 2a). Such an expression pattern was also observed for genes encoding proteins containing protein kinase (Fig. 2b), leucine-rich, NB-ARC and EF-hand domains, which might function in signal transduction for defence systems (Kandoth *et al.*, 2007; Lee & Rudd, 2002; Li *et al.*, 2009; Syam Prakash & Jayabaskaran, 2006; Tameling & Baulcombe, 2007). These observations indicate that host defence systems associated with genes for transcription factors such as WRKY (OsWRKY45; Shimono *et al.*, 2007) and NAC (ONAC28; Fang *et al.*, 2008), and signal transduction-related genes such as those for protein kinase (CDPK) and receptor protein (NBS-LRR), were not activated at the early phase of RSV infection, but some steps of these systems were induced at the late phase, when the infected plants showed obvious symptoms. Genes associated with host defence systems in RTSV-infected plants that did not show any evident symptoms were activated at the early phase of virus infection (H. Kondoh, personal communication). Therefore, the inactivation of defence systems at the early infection phase may have accelerated the propagation of RSV and the progression of symptoms in RSV-infected rice plants.

METHODS

Virus, insect vector and plant samples. Rice plants infected with RSV were collected from Saitama Prefecture, Japan. Young-instar nymphs of SBPH were fed for 2 days on the RSV-infected rice plants to acquire the virus (Takahashi *et al.*, 1991). The virus was maintained by successive transovarial transfers in SBPH in an insect-rearing room at a temperature of 25 ± 3 °C. RSV-free SBPH was used for mock inoculation.

Fourteen seeds of *Oryza sativa* cv. Nipponbare, which is susceptible to RSV, were sown in a pot (85 mm in diameter and 75 mm in height) filled with about 250 ml commercial soil mixture (Bonsol; Sumitomo Chemical). The plants were grown under well-watered conditions in an air-conditioned greenhouse (25 ± 3 °C, natural sunlight). Fourteen seedlings at the two-leaf stage in a single pot were exposed to 70 viruliferous or virus-free (for mock inoculation) SBPH in an inoculation chamber (34 cm wide × 26 cm deep × 34 cm high) for 24 h (25 ± 3 °C, continuous light conditions). After the insects were completely removed from the plants, the inoculated seedlings were placed in an air-conditioned greenhouse (25 ± 3 °C, natural sunlight). At 3, 6, 9, 12 and 15 days p.i., the shoots of the inoculated plants (except the meristem) were cut at 3 cm above the soil surface. After measuring the fresh mass of the rice plants, the plant samples were frozen in liquid nitrogen and stored at −80 °C until the experiments for transcriptome analysis or quantification of virus. After harvest, rice seedlings were grown continuously in the same greenhouse to evaluate virus infection. Sample preparations were repeated independently three times for microarray experiments.

Detection and quantification of RSV. Infection and concentration of RSV were evaluated by DAS-ELISA using an antiserum against RSV described previously (Takahashi *et al.*, 1991). To evaluate RSV infection, pieces (about 1 cm) of leaf sheath/stem tissues were harvested from each rice seedling and subjected to DAS-ELISA. The number of RSV-infected plants was taken to calculate the RSV-infection rate. To quantify RSV in the rice plants, frozen rice samples were ground by a multibead shocker [MB501(S); Yasui Kikai] and suspended with 10-fold mass of PBS (10× extracts). The 10× extracts were further diluted serially with PBS and subjected to DAS-ELISA. The concentration of RSV protein was estimated by comparing A_{410} values of RSV-infected rice leaf saps with those of

Table 2. Sample combination for the microarray experiments in this study

	Biological replication 1					Biological replications 2 and 3			
	3 days p.i.	6 days p.i.	9 days p.i.	12 days p.i.	15 days p.i.	3 days p.i.	6 days p.i.	9 days p.i.	12 days p.i.
Cy3	RSV	RSV	RSV	RSV	RSV	RSV	RSV	RSV	RSV
Cy5	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock

purified RSV of known concentrations. Evaluation of RSV accumulation by A_{410} values was replicated three times.

RNA extraction. Prior to RNA extraction, infection of RSV in plants to be used for RNA extraction was examined by DAS-ELISA. For extraction of RNA from RSV-inoculated plants, we used only those confirmed to be infected with RSV. Total RNA samples were extracted from shoot samples pooled from five independent plants of the same treatment by using an RNeasy Maxi kit (Qiagen). The concentration and quality of microarray samples were examined by Nanodrop (Nanodrop ND-1000; Nanodrop Technologies) and BioAnalyzer (G2938A; Agilent Technologies). For the microarray experiments in this study, we prepared 30 independent RNA samples (two treatments × five time points × three replicates).

Microarray design. Gene models predicted by OSA1 (Ouyang *et al.*, 2007) and about 35 000 full-length cDNAs mapped on the rice genome pseudomolecule release 5 in OSA1 indicated that about 59 000 gene loci exist in the rice genome (Satoh *et al.*, 2007). The mRNA sequences from the 59 000 gene loci were used to design 60-mer oligonucleotide probes. The probe and array designs were executed by eArray version 4.5 supplied by Agilent Technologies (https://earray.chem.agilent.com/earray/) and 43 494 probes were selected for this custom array. Information on the probe arrangement on the array (platform no. GPL7252) is available at NCBI GEO (Barrett *et al.*, 2007). Four sets of the 43 494 probes were blotted on a glass slide (25 × 75 mm) at Agilent Technologies.

Microarray experiment. Cyanine 3 (Cy3)- or cyanine 5 (Cy5)-labelled cRNA samples were synthesized from 850 ng total RNA by using a Low Input RNA labelling kit (Agilent Technologies) according to the manufacturer's instructions. Transcriptome profiles specific to RSV-infected plants were examined by direct comparison of transcription activities between RSV-infected and mock (RSV-free SBPH)-inoculated plants on the same oligoarray (Table 2). Hybridization solution was prepared with 825 ng each of Cy3- and Cy5-labelled cRNA preparations using an *in situ* Hybridization Kit Plus (Agilent Technologies). Hybridization and washing of microarray slides were done according to the manufacturer's protocols. After washing, slide image files were produced by a DNA microarray scanner (G2505B; Agilent Technologies).

Data analysis. Signal intensities of Cy3 and Cy5 were extracted from the image files and normalized to remove the dye effect in signal intensity by rank consistency and the LOWESS method, processed by Feature Extraction version 9.5 (Agilent Technologies). Signal intensities of all samples were transformed into $\log_2$-based numbers and normalized according to the quantile method for standardization among array slides by EXPANDER version 4.1 (Shamir *et al.*, 2005). A gene was declared 'expressed' if the mean signal intensity of the gene was >6 at least at one condition; otherwise, the gene was considered not expressed. A significantly differentially expressed gene (DEG) was defined as an expressed gene with (i) a $\log_2$-based ratio (RSV-inoculated sample/mock inoculated sample) >0.585 or <−0.585, and (ii) the significance of changes in gene expression between two plants (P) ⩽0.05 by a paired t-test (permutation, all; FDR collection, adjust Bonferroni method). Data processing was performed by using MEV version 4.3 (Saeed *et al.*, 2003). The outputs of microarray analysis used in this study (series no. GSE 12681) are available at NCBI GEO (Barrett *et al.*, 2007).

RT-PCR. cDNA fragments for transcripts of selected rice genes or the RSV genome were synthesized by using 1000 ng of the corresponding RNA with 50 pmol oligo$(dT)_{20}$ or RSV genome-specific oligonucleotide primers by SuperScript III reverse transcriptase (Invitrogen), respectively. The resultant reaction mixtures containing cDNA fragments were diluted serially 10 times. The 10-fold-diluted cDNA reaction mixtures were used for PCR. To examine the expression level of rice genes, PCR was performed with 1 µl diluted cDNA reaction mixture in a final volume of 15 µl using *Taq* DNA polymerase (New England Biolabs). Gene-specific primers used for amplification of selected rice gene transcripts are listed in Fig. 3. Primers for rice genes were designed to generate PCR products of 384–720 bp in length. An actin gene (LOC_Os05g36290) whose expression levels remained nearly constant under all experimental conditions was used for the control of gene-expression analysis by RT-PCR. PCR for RSV RNA was performed with 0.1 µl 10-fold-diluted cDNA reaction mixture in a final volume of 15 µl using *Taq* polymerase. Specific primers to amplify RSV CP cDNA were designed by Zhang *et al.* (2008b) and the size of the PCR product was 979 bp. The cycling program was initial denaturation for 2 min at 94 °C, followed by 30–40 cycles of 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C, with a final extension of 2 min at 72 °C.

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