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Arabidopsis is Susceptible to *Rice stripe virus* Infections

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Abstract

Rice stripe virus (RSV), a member of the genus *Tenuivirus*, causes rice stripe disease in East Asia and is one of the most economically important rice pathogens. The pathogenesis of RSV and the molecular basis of plant responses to the pathogen are poorly understood. We investigated the process of RSV infection in *Arabidopsis thaliana* which is highly susceptible to the virus. A simple inoculation method using viruliferous small brown planthoppers was developed to infect *A. thaliana* plants with RSV. The symptoms were developed within 2 weeks of inoculation. One month after inoculation, all infected plants showed stunted growth and vein chlorosis in newly emerged leaves. Forty-five days after inoculation, RSV-infected plants showed severely stunted growth and distorted flower stalks. RSV replication in *A. thaliana* was confirmed using a dot immunobinding assay, reverse transcription-polymerase chain reaction, and a protein gel blot assay. RSV infection strongly induced *PR1*, *PR2* and *GST1* but not *PDF1.2* expression, indicating that the pathogen activated salicylic acid-dependent basal defence mechanism, which is consistent with the characteristics of plant RNA virus. This *Arabidopsis*–RSV pathosystem provides an approach for analysing interactions between RSV and plants.

Introduction

Rice stripe virus (RSV) occurs mainly in East Asia and causes rice stripe disease, the most severe rice virus disease in China. Rice plants infected with RSV typically exhibit symptoms such as chlorosis, weakness, necrosis in newly emerged leaves, and stunted growth (Satoh et al. 2010). Rice stripe disease has recently spread widely in eastern China and caused significant losses (Wei et al. 2009). RSV is a member of the genus *Tenuivirus* and primarily infects rice plants, but it also infects other species, such as wheat and maize. RSV is transmitted transovarially in a circulative manner by some planthopper species (*Delphacidae* family), primarily the small brown planthopper (SBPH; *Laodelphax striatellus* Fallen) (Falk and Tsai 1998). The viral particles are thin, filamentous shape and are unenveloped. RSV is an RNA virus with four negative-sense single-stranded RNA segments, contains seven open reading frames and uses a negative and ambisense coding strategy for replication and infection in plants (Ramirez and Haenni 1994). *RNA1* encodes a protein of 337 kDa that may function as an RNA-dependent RNA polymerase (RdRP). Genes encoding gene-silencing suppressor and movement proteins have also been identified in the RSV genome (Xiong et al. 2008, 2009). However, knowledge regarding RSV, particularly its pathogenesis and the molecular basis of plant response to the pathogen, is poorly understood, partially because of difficulties in using traditional virological methods such as infectious cloning (Xiao et al. 2010).

Arabidopsis thaliana L. is an excellent model for addressing fundamental questions of viral pathogenicity and plant disease resistance response (Nishimura1 and Dangl 2010). *Arabidopsis thaliana* accessions vary significantly in their resistance and susceptibility to various plant virus pathogens. For example, most *A. thaliana* accessions are susceptible to Turnip crinkle virus, and the molecular mechanisms involved in providing resistance to this virus have been extensively studied (Kachroo et al. 2000; Cao et al. 2010). *Arabidopsis thaliana* is also an obvious host for studying virus–host interactions of the Alfalfa mosaic virus (Balasubramaniam et al. 2006). *Arabidopsis thaliana* has been used to investigate host plant signalling pathways involved in compatible host–virus interactions. Signal transduction pathways controlling plant defence systems are mediated by signalling molecules such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). In host–virus interactions of *A. thaliana* with

Cucumber mosaic virus and Oilseed rape mosaic virus, the expression of most plant defence-related genes is induced by an SA-dependent, NPR1-independent signalling pathway (Huang et al. 2005). Additionally, Cauliflower mosaic virus (CaMV) engages SA and JA/ET defence signalling pathways and activates rapid systemic generation of reactive oxygen species (ROS) in *Arabidopsis* (Love et al. 2005).

We describe here how RSV infects *A. thaliana* and causes serious disease symptoms. *Arabidopsis thaliana* infection with RSV strongly induces the expression of genes *PR1, PR2* and *GST1* but not *PDF1.2*. This compatible model system permits various molecular genetics and gene expression experiments to identify the defence signals and responses that inhibit RSV infection.

Materials and Methods

Virus isolates, vectors and plant materials

Rice plants infected with RSV were collected from Jiangsu Province in China. Young instar nymphs of SBPHs were fed RSV-infected rice plants for 2 days to acquire the virus. The virus was maintained by successive transovarial infections in SBPHs in an insect-rearing room at a temperature of 25°C. RSV-free SBPHs were used for mock inoculation. Viruliferous or virus-free SBPHs were reared on rice seedlings (*Oryza sativa* L. cv. Wuyujing No. 3) in glass vessels at 22°C under alternating photoperiods of 14 h of light and 10 h of dark. A dot immunobinding assay (DIBA) showed that 80% of adult insects were viruliferous. A virus-free planthopper population was generated using a pair of recently hatched nymphs, and nonvirulence was confirmed using reverse transcription-polymerase chain reaction (RT-PCR).

Arabidopsis thaliana (Col-0) seeds were donated by Dr. Hansong Dong (College of Plant Protection, Nanjing Agricultural University, Nanjing, China). Plants were grown in potting soil in a growth chamber at 24°C under 200 μE/m^2/s illumination and 16-h light/8-h dark cycle conditions.

RSV inoculation assay

Arabidopsis thaliana plants were inoculated with 10 viruliferous nymphs per plant (four or five leaves) and were kept in a growth chamber containing 30 plants. After incubation for 4 days at 22°C under artificial light, planthoppers were removed. Plants were maintained in a growth chamber for symptom development.

Dot immunobinding assay

Protein extracts were prepared by grinding frozen plant tissue to a fine powder and dissolving the powder in extraction buffer [sodium metabisulfite (50 mM), Tris–HCl (125 mM, pH 8.8), sodium dodecyl sulphate (SDS; 1%), glycerol (10%)]. Aliquots (2 μl) of the sample were then spotted on to the surface of nitrocellulose (NC) membranes and allowed to dry for 15 min. The loaded membrane was washed for 5 min in a large volume of TBST [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.05% (v/v) Tween 20] and then blocked for 15 min in 3% (w/v) bovine serum albumin (BSA) in TBST. After blocking, the NC membrane was incubated for 20 min at room temperature (RT) with an anti-mouse primary antibody for RSV (obtained from Dr. Xieping Zhou, Zhejiang University, Hangzhou, China). After washing in TBST (three times for 3 min each), the membrane was incubated with an alkaline phosphatase-conjugated secondary antibody (Sigma, St. Louis, MO, USA) for 1 h. After washing in TBST (three times for 3 min each), the bound secondary antibody was visualized by incubation with BCIP/NBT reagent (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions.

RT-PCR

RT-PCR was carried out to detect RSV in *A. thaliana* plants. Total RNA was isolated from leaves using the RNAiso Plus reagent (TAKARA, Dalian, China) and reverse transcribed using M-MLV Reverse Transcriptase (Promega), according to the manufacturer's instructions. The *EF1α* gene, which is highly conserved and constitutively expressed in eukaryotes (Berberich et al. 1995), was used as a loading control. *EF1α* and RSV *Ns3* and *CP* gene-specific primers are shown in Table 1. Each gene was amplified for 28 cycles. The sequences of RT-PCR products were determined by sequencing and compared using the BLAST program (http://www.ncbi.nlm.nih.gov). RT-PCR products were detected using agarose gel electrophoresis, and images were obtained using the Bio-Rad Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, USA) after staining with ethidium bromide.

Protein gel blotting

Protein extracts were prepared by grinding frozen plant tissue to a fine powder and dissolving the powder in extraction buffer [sodium metabisulfite (50 mM), Tris–HCl (125 mM, pH 8.8), SDS (1%), glycerol (10%)]. The extracts were then centrifuged for 10 min at 12 000 ***g***, and the supernatant was subjected to sodium dodecyl sulphate–polyacrylamide agarose gel electrophoresis (SDS–PAGE) either for staining or for protein gel blotting. The RSV coat protein (CP) antibody was obtained from Dr. Xieping Zhou (Zhejiang University, Hangzhou, China).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using the SsoFast EvaGreen Supermix (Bio-Rad) with the Bio-Rad iQ5 Real-Time PCR system. Each quantitative PCR was performed using 10 μl of SsoFast EvaGreen Supermix, 0.2 μM forward and reverse primers and 1 μl of cDNA in a total volume of 20 μl. All tubes were subjected to denaturation for 3 min at 95°C, followed by 40 cycles of 95°C for 10 s, and 58°C for 20 s. SYBR Green absorbance was detected at 58°C. All reactions were conducted in

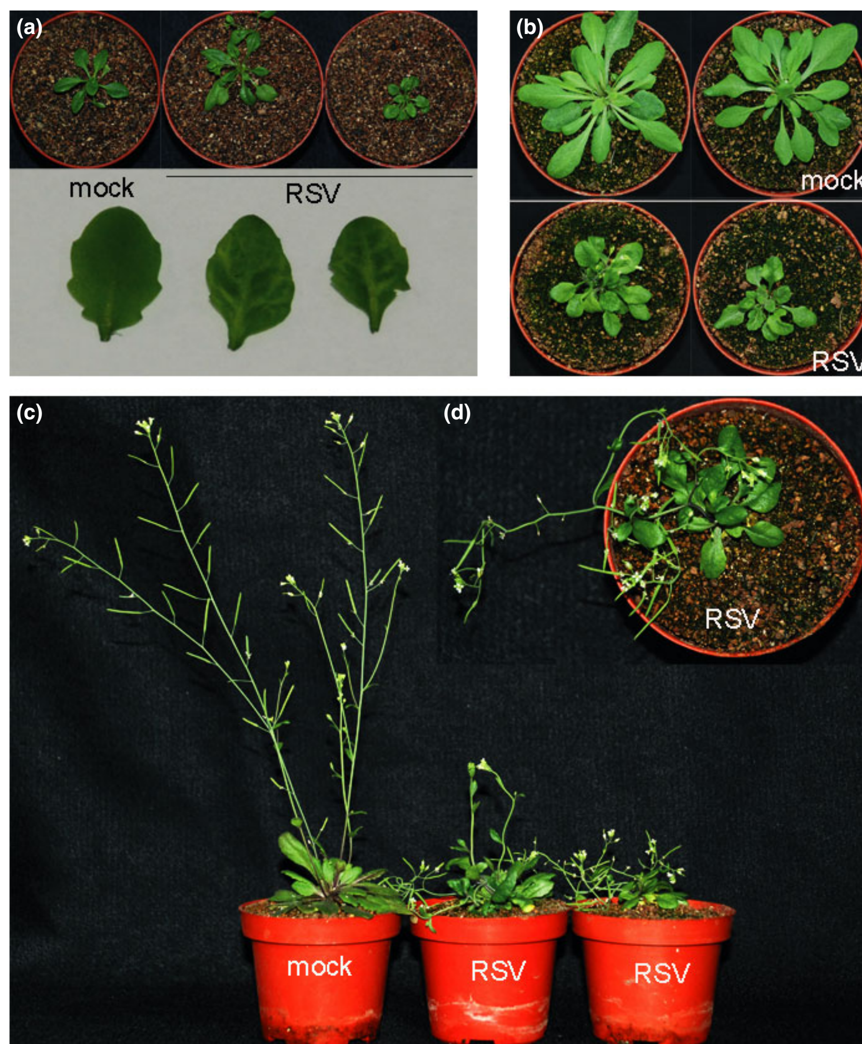

Fig. 1 *Rice stripe virus* (RSV) infection in *Arabidopsis thaliana*. (a) Initial symptoms appeared on *A. thaliana* 2 weeks after inoculation with viruliferous small brown planthoppers. Bottom, single leaf picture showed vein chlorosis. (b–d) Stunting symptoms expressed by *A. thaliana* plants 1 month (b) or forty-five days (c and d) after inoculation with RSV. (d) RSV-infected plant showed stunted growth and distorted flower stalk (c)

triplicate. Amplicon dissociation curves, i.e., melting curves, were recorded after cycle 40 by heating from 60°C to 95°C at a ramp speed of 1.9°C/min. Data were analysed using the iQ5 software (Bio-Rad). Table 1 shows information regarding additional genes and primers employed in this study.

Results

RSV infection in *Arabidopsis thaliana* plants

We characterized the phenotype of RSV infecting *A. thaliana*, which was inoculated with RSV, and the symptoms were allowed to develop under controlled environmental conditions. Thirty *A. thaliana* plants were inoculated with viruliferous (RSV) SBPHs. Two weeks after RSV inoculation, 15 infected plants showed vein chlorosis in newly emerged leaves (Fig. 1a). One month after inoculation, all infected plants showed significantly stunted growth and vein chlorosis in the newly emerged leaves (Fig. 1b). Forty-five days after inoculation, all infected plants showed severely stunted growth and distorted flower stalk (Fig. 1c, d). Infected plants also had delayed onset of flowering, and five infected plants did not produce flowers and died. In plants that bolted, the average flower stalk height was less than half of that in the mock controls.

To confirm RSV accumulation in inoculated *A. thaliana* plants, the amount of CP was measured using DIBA. Two weeks after inoculation, eight *Arabidopsis* plants showed positive results in all twelve tested infected plants, and all tested mock plants show negative results (Fig. 2a). Over the course of plant development, the time course of virus accumulation in *A. thaliana* plants that had been inoculated with RSV was tested using RT-PCR and protein gel blotting. Using *Ns3* and *CP*-specific primers, the expression levels of RSV *Ns3* and *CP* genes in *A. thaliana* plants at 0, 4, 6 and 8 days postinoculation (dpi) with RSV were analysed (Fig. 2b). *Ns3* and *CP* showed similar expression patterns, and both transcriptional levels were

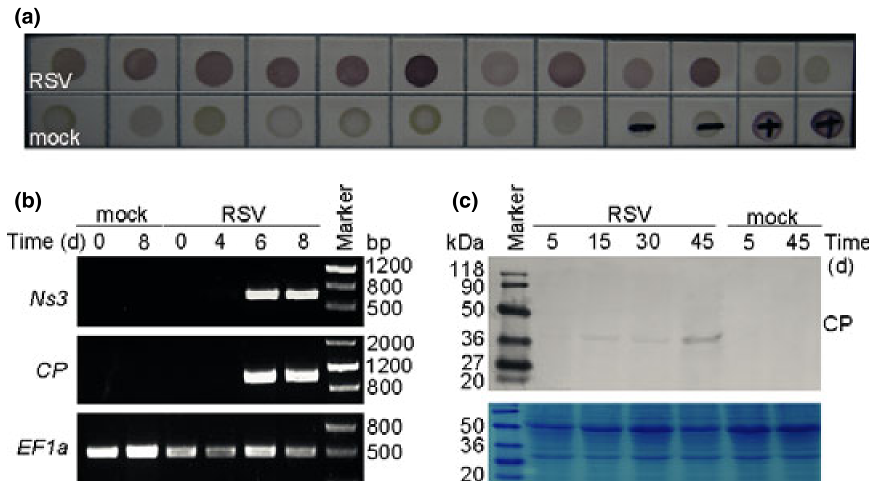

Fig. 2 Molecular detection of *Rice stripe virus* (RSV) in *Arabidopsis thaliana*. (a) Two weeks after inoculation, detection of RSV in *A. thaliana* plants using a dot immunobinding assay (DIBA). '–' indicates negative controls and '+' indicates positive controls. (b) The expression levels of RSV *Ns3* and *CP* were detected in *Arabidopsis* plants using reverse transcription-polymerase chain reaction (RT-PCR) with the *EF1a* gene as a standard. (c) Protein gel blot of proteins from *A. thaliana* plants probed with anti-CP antibody. Plants were inoculated with viruliferous (RSV) or virus-free SBPHs (mock). Inoculated plants were sampled for RNA or protein isolation at the indicated points of time given as 0, 4, 6 and 8 days postinoculation (dpi)

accumulated at 6 dpi. Using antiserum against CP, CP accumulation was detected in *A. thaliana* at 5, 15, 30 and 45 dpi (Fig. 2c). A 40-kDa protein was detected in plant that showed vein chlorosis in leaves at 15 dpi. The size of this protein was similar to that of the CP protein. RT-PCR and protein gel blotting assays indirectly indicated that RSV replication in *Arabidopsis* was due to RSV CP accumulation.

Defence pathway activation in *Arabidopsis thaliana* by RSV

In *A. thaliana*, SA- and JA⁄ET-responsive defence-related genes are induced by compatible virus infection (Whitham et al. 2006). To identify whether inoculation triggered RSV defence responses, we quantified the transcripts of four genes, *PR1* and *PR2* (encoding *β*-glucanase 2), which act as markers for SA-mediated defence, *GST1* (encoding glutathione S-transferase), which is activated by ROS (Grant et al. 2000), and *PDF1.2*, a marker for JA⁄ET-mediated defence (Yun et al. 2003). Total RNA was prepared at 0, 4, 6, 8, 10 and 12 dpi from leaves of RSV-inoculated and mock-inoculated plants, and transcript levels were estimated using qRT-PCR (Fig. 3). *PR1, PR2* and *GST1* transcript levels were clearly higher in virus-infected plants compared with those of mock-inoculated plants, whereas the *PDF1.2* gene was not induced in infected plants. The level of *PR1* mRNA in virus-inoculated plants was upregulated at 8 dpi and 100-fold higher than that in mock-inoculated plants at 12 dpi. Additionally, in virus-infected plants, *PR2* mRNA levels were upregulated at 4 dpi and 50-fold higher than in mock-inoculated plants at 12 dpi. *GST1* expression was induced in RSV-inoculated plants at 8 dpi and was 109-fold higher compared with mock-inoculated plants at 12 dpi. There was no significant induction of *PDF1.2* expression in RSV-inoculated plants than in mock-inoculated plants. These results indicate that

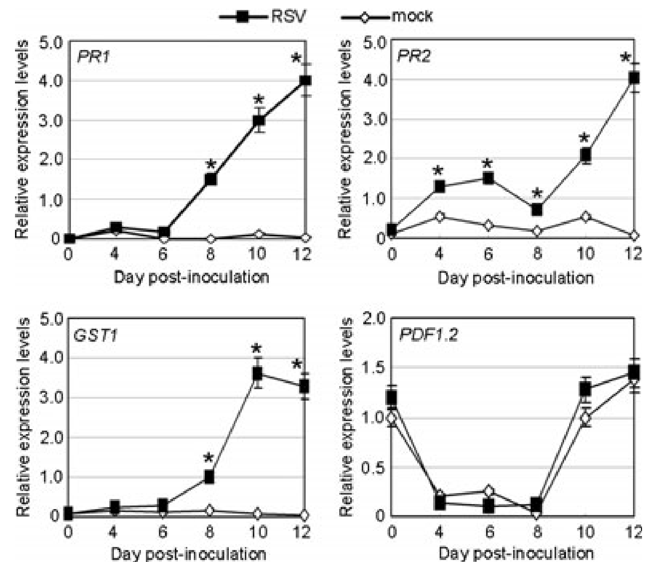

Fig. 3 The induced expression of defence genes in *A. thaliana* plants were analysed using quantitative real-time polymerase chain reaction (qRT-PCR). Plants were inoculated with viruliferous (RSV) or virus-free SBPHs (mock). Inoculated plants were sampled for RNA isolation at 0, 4, 6, 8, 10 and 12 days postinoculation (dpi). The expression levels of defence genes were normalized to those of the *EF1α* gene as internal standard gene. Asterisks indicated a significant difference ($P < 0.05$, *t*-test) between RSV and mock inoculation

RSV infection activates the SA basal defence and the ROS-dependent pathway.

Discussion

Plant multicomponent RNA virus pathogens, such as RSV and Rice black-streaked dwarf virus (RBSDV), cause many crop diseases; however, genetic approaches for controlling these diseases are very limited due to the lack of information about their pathogenesis and

Table 1
Additional genes, primers, products sizes and tests

Gene	Primers	Product size (bp)	Tests
CP	5′- ATGGGTACCAACAAGCCAG -3′ 5′-CTAGTCATCTGCACCTTCTG -3′	969	RT-PCR
Ns3	5′- ATGAACGTGTTCACATCG-3′ 5′- CTACAGCACAGCTGGAG-3′	636	RT-PCR
EF1α	5′-AGACCACCAAGTACTACTGCAC-3′ 5′-CCACCAATCTTGTACACATCC-3′	495	RT-PCR
EF1α	5′- GGCTGCTGAGATGAACAA -3′ 5′- GTGGTGGAGTCAATGATAAG -3′	225	qRT-PCR
PR1	5′- CTCTTGTAGGTGCTCTTGTTCTTCC -3′ 5′- GCGTAGTTGTAGTTAGCCTTCTCG -3′	283	qRT-PCR
PR2	5′- AATGTTGATGATTCTTCTCAGCCTT -3′ 5′- AACCCACTTGTCGGCCTCCGTTT -3′	280	qRT-PCR
GST1	5′- AACCGTTGTTGAAGAAGAAGAG -3′ 5′- GTCAGCAACCCAAGCACTCACAT -3′	204	qRT-PCR
PDF1.2	5′- CACCCTTATCTTCGCTGCTCTTG -3′ 5′- ATGATCCATGTTTGGCTCCTTCA -3′	170	qRT-PCR

interactions with plants. Because of the absence of a reverse genetic system, the interaction between RSV and plant species is not well understood. Our study describes a new pathosystem for RSV with *A. thaliana* involving simple methods of plant inoculation. This method can also be used to investigate the interaction of RBSDV with *A. thaliana*. The interaction of RSV with *A. thaliana* described here will be useful for evaluating resistance and differentiation in large numbers of *Arabidopsis* ecotypes and in mutant populations and in assessing transgene function for enhancing RSV resistance.

Symptoms caused by RSV in rice vary with the plant cultivars and developmental stage and include two types, i.e. folded leaf and unfolded leaf, with common features including chlorotic mottling and stunting (Satoh et al. 2010). Chlorotic mottling in rice leaves is typically accompanied by sparse and thick chloroplast grana or even destroyed chloroplasts (Liu et al. 2000). RSV infection in rice cells induces formation of four types of inclusion bodies (Liang et al. 2005). In this study, different symptoms and cytopathological features were observed in RSV-infected *A. thaliana*, including severely stunted growth, vein chlorosis in newly emerged leaves and distorted flower stalk (Fig. 1). The most obvious cytopathological features observed in RSV-infected *A. thaliana* plants were fibrillar amorphous-like inclusion bodies (data not showed). These different symptoms and inclusion bodies present in RSV-infected rice and *A. thaliana* cells indicate that the virus encodes proteins that interact with different host proteins. It will be interesting to further investigate whether specific *Arabidopsis* proteins are involved in RSV pathogenicity.

Previous studies have shown that RSV suppresses the transcription of genes related to host defence systems under hormone signals in rice (Satoh et al. 2010). Our results show that RSV activates *A. thaliana* basal defence responses. There are three defence pathways that plants use to respond to virus challenges (i.e. the SA-, JA-, and ET-dependent pathways). The plant DNA virus CaMV is known to induce the SA-dependent gene *PR1* and the JA- and ET-dependent gene *PDF1.2*. In contrast, *PDF1.2* is not induced in compatible interactions with RNA virus, such as Cytomegalovirus (CMV), Turnip mosaic virus (TuMV) or Oilseed Rape Mosaic Virus, but *PR1* and the ROS-dependent gene *GST1* are induced by these viruses (Huang et al. 2005). In the present study, the plant RNA virus RSV triggered SA pathways, but not the ET/JA pathway, in *A. thaliana* (Fig. 3); therefore, several mechanisms may function in host–virus interactions between *A. thaliana* and RNA virus. It is surprising that *PR1* transcription levels in RSV-infected plants were 100-fold higher than in mock inoculations. Mutant *Arabidopsis thaliana* plants, which constitutively express the *PR1* gene, show a phenotype similar to symptoms caused by RSV infection (Silva et al. 1999). Further studies are required to elucidate the function of the SA-dependent signalling pathway in RSV pathogenesis in *A. thaliana*.

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References

Balasubramaniam M, Ibrahim A, Kim BS, Sue LF. (2006) *Arabidopsis thaliana* is an asymptomatic host of Alfalfa mosaic virus. *Virus Res* **121**:215–219.

Berberich T, Sugawara K, Harada M, Kusano T. (1995) Molecular cloning, characterization and expression of an *elongation factor 1α* gene in maize. *Plant Mol Biol* **29**:611–615.

Cao M, Ye X, Willie K, Lin J, Zhang X, Redinbaugh MG, Simon AE, Morris TJ, Qu F. (2010) The capsid protein of turnip crinkle virus overcomes two separate defense barriers to facilitate systemic movement of the virus in *Arabidopsis*. *J Virol* **84**:7793–7802.

Falk BW, Tsai JH. (1998) Biology and molecular biology of viruses in the genus tenuivirus. *Annu Rev Phytopathol* **36**:139–163.

Grant JJ, Yun BW, Loake GJ. (2000) Oxidative burst and cognate redox signalling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. *Plant J* **24**:569–582.

Huang Z, Yeakley JM, Garcia EW, Holdridge JD, Fan JB, Whitham SA. (2005) Salicylic acid-dependent expression of host genes in compatible *Arabidopsis*-virus interactions. *Plant Physiol* **137**:1147–1159.

Kachroo P, Yoshioka K, Shah J, Dooner HK, Klessig DF. (2000) Resistance to turnip crinkle virus in *Arabidopsis* is regulated by two host genes and is salicylic acid dependent but NPR1, ethylene, and jasmonate independent. *Plant Cell* **12**:677–690.

Liang DL, Qu ZC, Ma XQ, Hull R. (2005) Detection and localization of Rice stripe virus gene products in vivo. *Virus Genes* **31**:211–221.

Liu L, Wu Z, Lin Q, Xie L. (2000) Cytopathological observation of rice stripe. *Acta Phytophylacica Sinica* **30**:306–311.

Love AJ, Yun BW, Laval V, Loake GJ, Milner JJ. (2005) Cauliflower mosaic virus, a compatible pathogen of *Arabidopsis*, engages three distinct defense-signaling pathways and activates rapid systemic generation of reactive oxygen species. *Plant Physiol* **139**:935–948.

Nishimural MT, Dangl JL. (2010) *Arabidopsis* and the plant immune system. *Plant J* **61**:1053–1066.

Ramirez BC, Haenni AL. (1994) Molecular biology of tenuiviruses: a remarkable group of plant viruses. *J Gen Virol* **75**:467–475.

Satoh K, Kondoh H, Sasaya T, Shimizu T, Choi IR, Omura T, Kikuchi S. (2010) Selective modification of rice (*Oryza sativa*) gene expression by rice stripe virus infection. *J Gen Virol* **91**:294–305.

Silva H, Yoshioka K, Dooner HK, Klessig DF. (1999) Characterization of a new arabidopsis mutant exhibiting enhanced disease resistance. *Mol Plant Microbe Interact* **12**:1053–1063.

Wei TY, Yang JG, Liao FL et al. (2009) Genetic diversity and population structure of rice stripe virus in China. *J Gen Virol* **90**:1025–1034.

Whitham SA, Yang C, Goodin MM. (2006) Global impact: elucidating plant responses to viral infection. *Mol Plant Microbe Interact* **19**:1207–1215.

Xiao D, Li W, Wei T, Wu Z, Xie L. (2010) Advances in the studies of Rice stripe virus. *Front Agric China* **4**:287–292.

Xiong R, Wu J, Zhou Y, Zhou X. (2008) Identification of a movement protein of the tenuivirus rice stripe virus. *J Virol* **82**:12304–12311.

Xiong R, Wu J, Zhou Y, Zhou X. (2009) Characterization and subcellular localization of an RNA silencing suppressor encoded by rice stripe tenuivirus. *Virology* **387**:29–40.

Yun BW, Atkinson HA, Gaborit C, Greenland A, Read ND, Pallas JA, Loake GJ. (2003) Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in *Arabidopsis* against wheat powdery mildew. *Plant J* **34**:768–777.