

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 (Nishimura 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 PRI, 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 under artificial light, planthoppers were removed. Plants were kept in a growth chamber containing 30 plants. Total RNA was isolated from leaves using the RNAiso Plus reagent (TAKARA, Dalian, 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 EF1z gene, which is highly conserved and constitutively expressed in eukaryotes (Berberich et al. 1995), was used as a loading control. EF1z 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), 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.

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...
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
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 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...
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 (Sato 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 (Sato 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 Cucumber mosaic virus (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/3A 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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