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Pathogenesis of *Soybean mosaic virus* in soybean carrying *Rsv1* gene is associated with miRNA and siRNA pathways, and breakdown of AGO1 homeostasis

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ABSTRACT

Profiling small RNAs in soybean Williams 82 (*rsv*), susceptible to *Soybean mosaic virus* (SMV, the genus *Potyvirus*, family *Potyviridae*) strains G2 and G7, and soybean PI96983 (*Rsv1*), resistant to G2 but susceptible to G7, identified the microRNA miR168 that was highly overexpressed only in G7-infected PI96983 showing a lethal systemic hypersensitive response (LSHR). Overexpression of miR168 was in parallel with the high-level expression of *AGO1* mRNA, high-level accumulation of miR168-mediated *AGO1* mRNA cleavage products but with severely repressed AGO1 protein. In contrast, AGO1 mRNA, degradation products and protein remained without significant changes in G2- and G7-infected Williams 82. Moreover, knock-down of *SGS3*, an essential component in RNA silencing, suppressed *AGO1* siRNA, partially recovered repressed AGO1 protein, and alleviated LSHR severity in G7-infected *Rsv1* soybean. These results suggest that both miRNA and siRNA pathways are involved in G7 infection of *Rsv1* soybean, and LSHR is associated with breakdown of AGO1 homeostasis.

Introduction

To protect themselves from viral attack, plants have developed a sophisticated system termed RNA silencing against the invading virus, which represents a primitive, natural immune system of defense (Baulcombe, 1999; Bosher and Labouesse, 2000; Catalanotto et al., 2000; Matzke et al., 2001; Waterhouse et al., 2001). This innate resistance is executed by small interfering RNAs (siRNA)-directed sequence-specific degradation of complementary viral RNAs. Virus-specific siRNAs (vsiRNAs) are generated from viral double-stranded RNA products and secondary RNA structures cleaved by RNase-III ribonuclease Dicer-like (DCL) proteins (Ding and Voinnet, 2007; Molnár et al., 2005; Várallyay et al., 2010). In response, many viruses have evolved a corresponding system to counteract RNA silencing for their own survival through encoding a viral RNA silencing suppressor (VSR), the viral protein that inhibits RNA silencing efficiently *via* either preventing siRNAs generation or inhibiting/interfering the incorporation of siRNAs into the RNA-induced silencing complex (RISC) (Anandalakshmi et al., 1998; Burgyán, 2008; Pumplin and Voinnet, 2013). This reciprocal adaptation and counter-adaptation process results from a co-evolutionary arms race between the host and the virus (Obbard et al., 2009; Zhang et al., 2006).

Another RNA silencing pathway is mediated by microRNAs (miRNAs), a class of non-coding RNAs 20–24 nucleotides in size that regulate gene expression in eukaryotes by translational inhibition or cleavage of complementary mRNAs (Mallory and Bouché, 2008). miRNAs have a pivotal role in a wide variety of biological processes such as maintenance of genome integrity, development, hormone responses and feedback mechanisms as well as biotic and abiotic stress responses (Voinnet, 2009). In addition to regulating the expression of endogenous genes, miRNAs are also indispensable for the innate immune system in animals and plants. For instance, the human cellular miR-32 effectively restricts the accumulation of the retrovirus primate foamy virus type 1 (PFV-1) (Lecellier et al., 2005). miR122 is specifically expressed and highly abundant in the human liver, and

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the sequestration of miR-122 in liver cells results in a marked loss of autonomously replicating hepatitis C viral RNAs (Jopling et al., 2005). In plants, several lines of evidence suggest that virus infections are often associated with the altered levels of endogenous miRNAs and their mRNA targets. The levels of mature miR164, miR164a precursor and its target CUC1 mRNA are increased in *Arabidopsis* plants infected by *Tobacco mosaic virus* Cg (TMV-Cg) or *Oilseed rape mosaic virus* (ORMV) (Bazzini et al., 2009). miRNAs in cotton plants are misregulated by infection with *Cotton leafroll dwarf virus* (CLRDV) and some CLRDV-induced symptoms may be correlated with the deregulation of miRNA and/or epigenetic networks (Romanel et al., 2012). In rice, *Rice stripe virus* (RSV) infection induces the expression of novel phased miRNAs from several conserved miRNA precursors (Du et al., 2011). In *Nicotiana benthamiana* and *Arabidopsis*, the expression of miR168 and AGO1 mRNA is up-regulated in response to infection by several plant viruses (Havelda et al., 2008; Várallyay et al., 2010, 2013; Vaucheret et al., 2006), suggesting both miRNA and siRNA pathways are involved in virus infections. AGO1 protein is a central component of the RISC in the miRNAs/siRNAs-mediated PTGS pathways (Mallory and Vaucheret, 2009). AGO1 has been shown to be responsible for translational inhibition or cleavage of complementary target mRNAs in the miRNA pathway (Mallory and Bouché, 2008; Mallory et al., 2008). Regulation of *AGO1* by miR168 is through a feedback regulatory loop that maintains AGO1 homeostasis by action of miR168 and AGO1-derived siRNAs (Li et al., 2012; Mallory and Vaucheret, 2009; Vaucheret et al., 2004). AGO1 homeostasis is essential for two additional regulatory mechanisms, *i.e.*, transcriptional co-regulation of *MIR168* and *AGO1* genes and posttranscriptional stabilization of miR168 by AGO1 (Li et al., 2012; Vaucheret et al., 2006). Moreover, a recent work described that the enhanced expression of AGO1 mRNA is not accompanied by increased AGO1 protein accumulation in the virus-infected plants, and the p19 RNA-silencing suppressor, a viral protein of tombusviruses mediates the induction of miR168 accumulation and the down-regulation of the endogenous AGO1 protein level (Várallyay et al., 2010, 2013), suggesting that the *AGO1*-miR168 feedback regulation mechanism may play a role in the virus infection process.

Soybean mosaic virus (SMV) is a member of the genus *Potyvirus* in the *Potyviridae* family and its genome is a single-stranded positive-sense RNA. To date, numerous SMV isolates have been classified into seven distinct strains (G1 to G7) based on their differential responses on susceptible and resistant soybean cultivars in North America (Cho and Goodman, 1979). Three independent resistance loci (*Rsv1*, *Rsv3*, and *Rsv4*) with different SMV strain specificities have been identified, and these three loci are all dominant *R* genes that have been mapped to the respective molecular linkage groups F, B2, and D1b (Gore et al., 2002; Gunduz et al., 2002; Hayes et al., 2000; Jeong et al., 2002; Zheng et al., 2005). *Rsv1*, found in soybean cultivar PI96983, confers resistance to the SMV strains G1 to G6 but susceptible to the resistance breaking strain G7 (Chowda-Reddy et al., 2011; Yu et al., 1994). A lethal systemic hypersensitive response (LSHR) is induced by SMV G7 infection in PI96983 carrying *Rsv1*, which is associated with up-regulation of the PR-1 protein gene transcript (Hajimorad and Hill, 2001). To better understand SMV–soybean interactions, global gene expression changes in soybean plants infected by a G2 isolate were monitored during the course of infection using microarray (Babu et al., 2008). A number of genes involved in defense were found to be down-regulated or not affected at the early stages of infection but up-regulated at the late stages, indicating that the plant immune responses are suppressed or not activated until late in the infection. Such a delayed defense response may be critical for SMV to establish its systemic infection (Babu et al., 2008). Consistently, some miRNAs (miR160, miR393 and miR1510) have been shown to be involved in soybean resistance to SMV infection (Yin et al., 2013).

In this study, we show that the specific induction of miR168 accumulation in SMV-infected plants, which was in parallel with an increased *AGO1* mRNA expression. Infection of *Rsv1* soybean by SMV G7 was associated with the highly accumulated *AGO1* mRNA but with low AGO1 protein levels. Moreover, the enhanced accumulation of miR168 spatially overlapped with virus-occupied sectors, which was accompanied with a dramatic increase of miR168-mediated *AGO1* mRNA cleavage products. Furthermore, we show that silencing *Suppressor of Gene Silencing* 3 (*SGS3*) in *Rsv1* plants reduced the level of *AGO1* siRNAs, partially recovered the suppressed level of AGO1 protein resulting from G7 infection, and alleviated LSHR severity. These results suggest that both miRNA and siRNA pathways are involved in pathogenesis of SMV G7 in *Rsv1* soybean through disruption of AGO1 homeostasis.

Results

Identification of soybean miRNAs associated with SMV infection by deep sequencing

To study soybean innate resistance to SMV infection, we inoculated with SMV G2 and G7 strains two soybean cultivars, Williams 82 (*rsv*) carrying no resistance gene and PI96983 (*Rsv1*) carrying the resistance gene *Rsv1*. Consistent with previous publications (Chowda-Reddy et al., 2011; Hajimorad et al., 2003; Hajimorad and Hill, 2001), PI96983 was resistant to G2 but susceptible to G7 by showing a lethal systemic hypersensitive response (LSHR), whereas Williams 82 carrying no resistance gene was susceptible to both G2 and G7 strains (Fig. S1). To investigate miRNAs involved in antiviral response to SMV, six small RNA cDNA libraries from mock- and SMV-infected (G2 and G7) leaves in *rsv* and *Rsv1* soybeans were constructed and subjected to deep sequencing. The small RNA sequences were mapped to the soybean reference genome (Glyma1, Ensembl, http://plants.ensembl.org/Glycine_max/Info/Index) and the SMV genome, and aligned to the known soybean miRNAs database (miRBase 19, http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=gma). For each library, more than 50% of small RNA sequences perfectly matched to the soybean genome (Table S1). Among a large number of miRNAs that were up- or down-regulated in SMV-infected plants, the most strikingly up-regulated miRNA species was miR168 whose expression in the G7-infected *Rsv1* soybeans was up-regulated by more nearly three times ($\log_2$ FC=2.85) in reads *versus* that in the mock-inoculated *Rsv1* soybeans, but no significant difference ($\log_2$ FC=0.22) was found in expression of miR168 in the G2-infected *versus* mock-inoculated *Rsv1* soybeans (Fig. 1A). A high abundance of small RNA reads was found to derive from the region of AGO1 (Glyma16g34300) targeted by miR168 (Fig. 1B). Plant miR168 is one of the most abundant and important miRNAs in plants that regulates *ARGONAUTE1* (*AGO1*) mRNA, a critical component of miRNA/siRNA-mediated posttranscriptional gene silencing (PTGS) pathways (Gazzani et al., 2009; Várallyay et al., 2010; Zhang et al., 2011). Thus, miR168 and its target AGO1 became the focus of this study.

Induction of miR168 accumulation by SMV infection

Northern blot was performed to confirm the induction of miR168 accumulation by SMV infection. A comparative analysis of miR168 detected by northern blotting showed that miR168 accumulation in the G7-infected *Rsv1* plants was about 2.7 times as much as that in the mock-inoculated *Rsv1* plants (Fig. 2A). No significant increment of miR168 was found in G2-inoculated *Rsv1* soybeans that are resistant to G2 (Fig. 2A). miR168 expression in the G2- and G7-infected *rsv* plants was increased by 1.4- and 1.6-