MicroRNA844-Guided Downregulation of Cytidinephosphate Diacylglycerol Synthase3 (CDS3) mRNA Affects the Response of *Arabidopsis thaliana* to Bacteria and Fungi

Hwa Jung Lee,¹ Young Ju Park,¹ Kyung Jin Kwak,¹ Donghyun Kim,² June Hyun Park,² Jae Yun Lim,² Chanseok Shin,² Kwang-Yeol Yang,¹ and Hunseung Kang¹

¹Department of Plant Biotechnology, College of Agriculture and Life Sciences, Chonnam National University, 300 Yongbong-dong, Buk-gu, Gwangju 500-757, Republic of Korea; ²Department of Agricultural Biotechnology, Seoul National University, Gwanak-ro, Gwanak-gu, Seoul 151-921, Republic of Korea

Submitted 11 February 2015. Accepted 12 March 2015.

Despite the fact that a large number of miRNA sequences have been determined in diverse plant species, reports demonstrating the functional roles of miRNAs in the plant response to pathogens are severely limited. Here, *Arabidopsis thaliana* miRNA844 (miR844) was investigated for its functional role in the defense response to diverse pathogens. Transgenic *Arabidopsis* plants overexpressing miR844 (35S::miR844) displayed much more severe disease symptoms than the wild-type plants when challenged with the bacterium *Pseudomonas syringae* pv. tomato DC3000 or the fungus *Botrytis cinerea*. By contrast, a loss-of-function *mir844* mutant showed an enhanced resistance against the pathogens. Although no cleavage was observed at the predicted cleavage site of the putative target mRNA, *cytidinephosphate diacylglycerol synthase3* (*CDS3*), cleavage was observed at 6, 12, 21, or 52 bases upstream of the predicted cleavage site of *CDS3* mRNA, and the level of *CDS3* mRNA was downregulated by the overexpression of miR844, implying that miR844 influences *CDS3* transcript level. To further confirm that the miR844-mediated defense response was due to the decrease in *CDS3* mRNA level, the disease response of a *CDS3* loss-of-function mutant was analyzed upon pathogen challenge. Increased susceptibility of both *cds3* mutant and 35S::miR844 plants to pathogens confirmed that miR844 affected the defense response by downregulating *CDS3* mRNA. The expression of *miR844* was decreased, and the *CDS3* transcript level increased upon pathogen challenge. Taken together, these results provide evidence that downregulation of *miR844* and a concomitant increase in *CDS3* expression is a defensive response of *Arabidopsis* to bacteria and fungi.

MicroRNAs (miRNAs) are one of the noncoding RNAs that lack protein-coding capacity. Plant miRNAs are endogenous RNAs of 20 to 24 nucleotides (nt) in length, which play important regulatory roles by targeting mRNAs for cleavage or translational

repression (Bartel 2009; Voinnet 2009). Many reports have demonstrated that miRNAs play fundamental roles in the growth, development, and stress responses of plants (Jeong and Green 2013; Khraiwesh et al. 2012; Rubio-Somoza and Weigel 2011; Sunkar et al. 2012; Voinnet 2009). Many studies have shown the functional roles of miRNAs in the abiotic stress response of plants, including miR399 in the phosphate-starvation response in *Arabidopsis thaliana* (Chiou et al. 2006; Fujii et al. 2005; Lin et al. 2008; Pant et al. 2008), miR398 in oxidative stress tolerance (Jagadeeswaran et al. 2009; Sunkar et al. 2006), miR395 in the response of plants to sulfate starvation conditions (Jones-Rhoades and Bartel 2004; Kawashima et al. 2009), miR395 in the salt and dehydration stress response (Kim et al. 2010a), and miR402 in the dehydration and cold stress response (Kim et al. 2010b).

Although our understanding of the functional roles of miRNAs in the biotic stress response is far behind compared with their identification in diverse plant species, increasing numbers of reports have demonstrated the importance of miRNAs in biotic stress responses in plants (Jin 2008; Katiyar-Agarwal and Jin 2010; Navarro et al. 2006; Padmanabhan et al. 2009; Ruiz-Ferrer and Voinnet 2009). *Arabidopsis* miR393 has been identified to play a role in pathogen-associated molecular pattern (PAMP)-triggered immunity by regulating auxin signaling (Navarro et al. 2006). It was demonstrated that miR160a positively regulates PAMP-induced callose deposition, whereas miR398b and miR773 negatively regulate PAMP-induced callose deposition and disease resistance to *Pseudomonas syringae* DC3000 (Li et al. 2010). The transcript level of *miR398* was downregulated in *Arabidopsis* leaves in response to *P. syringae* DC3000, and the mRNA level of its target gene, Cu/Zn superoxide dismutase1 (*CSD1*), was negatively correlated with miR398 level during biotic stress (Jagadeeswaran et al. 2009). The expression of several miRNAs was either up- or down-regulated in *Arabidopsis* leaves challenged with *P. syringae* DC3000 (Zhang et al. 2011). Various miRNA families were differentially regulated by fungal infection: miR156 and miR160 were repressed in pine stems infected with the rust fungus (Lu et al. 2007); miR156, miR159, miR164, miR171, and miR396 were downregulated, while miR393, miR444, and miR827 were upregulated in wheat (*Triticum aestivum*) by powdery mildew infection (Xin et al. 2010). Several miRNAs, including miR156, miR158, miR160, miR164, and miR1885, were regulated by viral infection (He et al. 2008; Navarro et al. 2008). All of these reports clearly indicate that diverse miRNAs play a prominent role in plant defense responses. However, the

Hwa Jung Lee and Young Ju Park contributed equally to this work.

Corresponding author: Hunseung Kang; Telephone: +1 82-62-530-2181; Fax: +1 82-62-530-2079; E-mail: hskang@jnu.ac.kr

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures and one supplementary table are published online.

functional role and RNA targets of a majority of miRNAs during defense responses in plants remain to be determined.

In this study, the functional roles of miR844 (miR844) in the defense response to the pathogenic bacterium *P. syringae* DC3000 and to the pathogenic fungus *Botrytis cinerea* were investigated. MiR844 was first identified by Rajagopalan et al. (2006) with two mature products named miR844-5' and miR844-3', and Fahlgren et al. (2007) confirmed miR844-5' as a mature miR844. MiR844 was reported to be marginally downregulated in *Arabidopsis* 3 h after *P. syringae* DC3000 infection (Fahlgren et al. 2007). As the putative target genes of miR844 include a disease resistance protein (At1g51480), cytidinephosphate diacylglycerol synthase3 (CDS3, At4g26770), an unknown protein (At1g05280), and protein kinase (At5g51270), it was hypothesized that miR844 may be involved in the plant defense response to pathogens. Here, we show that miR844 affects the defense response of *Arabidopsis* to *P. syringae* DC3000 and *B. cinerea* by decreasing *CDS3* mRNA level.

RESULTS

Response of miR844-overexpressing transgenic plants to *P. syringae* DC3000 or *B. cinerea*.

To understand the functional role of miR844 in the response of *Arabidopsis* to pathogen infection, transgenic *Arabidopsis*

plants overexpressing miR844 (400 nt) under control of the *Cauliflower mosaic virus* 35S promoter (35S::miR844) were generated, and their phenotypes were analyzed upon pathogen infection. Overexpression of both premiR844 (400 nt) and mature miR844 (21 nt) was verified via reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analyses (Fig. 1A and B). The miR844-overexpressing transgenic plants showed no obvious phenotype under normal growth conditions (data not shown).

To determine whether miR844 plays a role in the defense response against pathogenic bacteria, 4-week-old 35S::miR844 plants and wild-type plants were infected with the pathogenic bacterium *P. syringae* DC3000, and disease severity and the number of bacteria in the leaves were evaluated at 5 days after inoculation (DAI) (Fig. 1C). It was evident that the growth of *P. syringae* in transgenic plants was higher than that in wild-type plants. These results show that miR844-overexpressing transgenic plants are more susceptible to *P. syringae* than wild-type plants.

We next determined whether miR844 plays a role in the defense response against pathogenic fungi. Four-week-old 35S::miR844 plants and wild-type plants were infected with the necrotrophic fungus *B. cinerea*, and disease severity in the leaves of the plants was evaluated at 5 DAI (Fig. 2). The

Fig. 1. Defense response of miR844-overexpressing *Arabidopsis* plants to *Pseudomonas syringae* DC3000. **A**, Overexpression of precursor-miR844 in transgenic plants (OX-1, OX-2, and OX-3) was confirmed by reverse transcription-polymerase chain reaction analysis and **B**, overexpression of 21-nt-long mature miR844 was confirmed by Northern blot analysis. **C**, Four-week-old wild type (Col-0) and miR844-overexpressing plants (OX-1, OX-2, and OX-3) were infected with *P. syringae* DC3000. The photographs of the leaves arranged in the order of age (old to new) were taken at 0 and 5 days after inoculation (DAI). The numbers of CFU were measured 5 DAI, and the data are mean $\pm$ standard error of three replicates, seven leaves per replicate.

average area of necrotic lesions in the leaves of transgenic plants was significantly larger than that in the leaves of wild-type plants. The density of *B. cinerea* in each leaf was quantitatively determined by measuring the amount of *B. cinerea tubulin* mRNA by RT-PCR analysis. The RT-PCR results also showed that the density of *B. cinerea* was much higher in 35S::miR844 plants than that in wild-type plants. These results show that miR844-overexpressing transgenic plants are more susceptible to *B. cinerea* than wild-type plants.

Identification of *CDS3* as a miR844 target gene.

As it was evident that overexpression of miR844 resulted in increased susceptibility of *Arabidopsis* plants to pathogenic bacteria and fungi, we next investigated whether miR844 exerts its function by cleaving target genes. We first predicted the putative target genes of miR844 using web-based target prediction programs. We initially used miRNA Target Finder and later used psRNA Target Finder. When three mismatches were allowed between miR844 and the target mRNA, several putative target genes were predicted, including a disease resistance protein (At1g51480), *CDS3* (At4g26770), an unknown protein (At1g05280), and protein kinase (At5g51270) (Fig. 3A). Among these putative target genes, only *CDS3* did not contain a base mismatch with miR844, whereas the other putative target genes contained two or three mismatches with miR844 (Fig. 3A). Since the protein kinase (At5g51270) was predicted as a putative target of miR844 (Fahlgren et al. 2007), we carefully analyzed its transcript level as well. When the transcript levels of these predicted target mRNAs were analyzed in miR844-overexpressing transgenic plants with the primer pairs spanning the predicted cleavage site of the target gene (Fig. 3B, F1 and R1), the *CDS3* transcript levels were

decreased significantly, but the levels of the three other putative targets, At1g51480, At1g05280 and At5g51270, remained unchanged in transgenic plants (Fig. 3C; Supplementary Fig. S1). With the observation that the transcript levels of *CDS3* mRNA were decreased in the miR844-overexpressing transgenic plants when analyzed with the primer pairs spanning the predicted cleavage site of *CDS3* mRNA (Fig. 3B and C), we further analyzed the *CDS3* transcript levels in the miR844-overexpressing transgenic plants with the primer pairs complementary to the upstream region of the cleavage site (Fig. 3B, F2 and R2). It was evident that the *CDS3* transcript levels were significantly decreased in the miR844-overexpressing transgenic plants (Fig. 3D).

To confirm whether the decrease in target mRNA level was due to the miR844-guided cleavage of target gene, the 5' end sequences of the cleavage products of *CDS3* and At5g51270 gene were analyzed by 5' RACE (rapid amplification of C-DNA ends). We extensively analyzed the 5' end sequences of the target cleavage products using the RNAs extracted from stems, leaves, roots, and flowers of 35S::miR844 transgenic plants. Although no cleavage was observed at the predicted cleavage site of the putative target mRNAs, including *CDS3* and the protein kinase (At5g51270) genes, cleavage was observed at 6, 12, 21, or 52 bases upstream of the predicted cleavage site of *CDS3* mRNA (Fig. 3E). By contrast, no cleavage was observed at any sites of the protein kinase gene. Although we do not know, at present, how *CDS3* mRNA was cleaved upstream of the predicted cleavage site, these results indicate that miR844 downregulates specifically *CDS3* mRNA.

Response of *cds3* mutants to *P. syringae* DC3000 or *B. cinerea*.

To ascertain that the disease-susceptible phenotypes of 35S::miR844 plants were due to the downregulation of *CDS3* mRNA in transgenic plants, a loss-of-function mutant of *CDS3* (CS915999) was obtained from the Arabidopsis Biological Resource Center, and the responses of the mutant to *P. syringae* DC3000 and *B. cinerea* were analyzed. The absence of *CDS3* expression in the mutant line was confirmed by RT-PCR analysis (Fig. 4A). Because only one mutant line was analyzed in this study, a single T-DNA insertion was verified via Southern blot analysis using the β -glucuronidase (*GUS*) gene as a probe. Genomic DNAs were digested with the enzymes, including *EcoRI* and *HindIII*, which could not cut the *GUS* gene, and *EcoRV*, which could cut the *GUS* gene. One band was detected in the DNAs digested with *EcoRI* or *HindIII* and two bands were detected in the DNAs digested with *EcoRV* (Fig. 4B), verifying that T-DNA was inserted at a single site. The *cds3* knockout mutant displayed no obvious phenotype under normal growth conditions (data not shown), which is similar to the previous report, demonstrating that *CDS4* or *CDS5* knockout mutants grew and developed like wild-type plants (Haselier et al. 2010). Four-week-old *cds3* mutant and wild-type plants were infected with *P. syringae* DC3000, and disease severity and the number of bacteria in the leaves were evaluated at 5 DAI (Fig. 4C). It was evident that the growth of *P. syringae* in the mutant was significantly higher than that in wild-type plants. When the mutant and wild-type plants were infected with *B. cinerea*, the area of necrotic lesions on the leaves of the mutant was significantly larger and the density of the fungi in the leaves of the mutant was significantly higher than those of wild-type plants (Fig. 4D). These results clearly show that the *cds3* mutant was more susceptible to *P. syringae* and *B. cinerea* than wild-type plants, similar to miR844-overexpressing transgenic plants.

Fig. 2. Defense response of miR844-overexpressing *Arabidopsis* plants to *Botrytis cinerea*. Four-week-old wild type (Col-0) and miR844-overexpressing plants (OX-1, OX-2, and OX-3) were infected with *B. cinerea* and photographs were taken at 5 days after inoculation (DAI). Lesion size in each leaf was measured at 5 DAI, and the data are mean $\pm$ SE of three replicates, four leaves per replicate. The density of *B. cinerea* on the leaves was quantitatively determined by measuring the amounts of *B. cinerea tubulin* by reverse transcription-polymerase chain reaction analysis. *Arabidopsis actin* was used to show equal amounts of RNAs in each sample.

Response of *mir844* mutant to *P. syringae* DC3000 or *B. cinerea*.

To further confirm that *CDS3* is the target of *miR844* and *miR844*-mediated downregulation of *CDS3* mRNA is related to the observed pathogen response, a T-DNA tagged mutant line (SALK_116570) in which the T-DNA is inserted at the promoter region of *miR844* gene (Fig. 5A) was obtained from the Arabidopsis Biological Resource Center, and the responses of the mutant to *P. syringae* DC3000 and *B. cinerea* were analyzed. It was evident that *miR844* was not detected in the *mir844* mutant and the *CDS3* transcript level increased in the mutant, whereas the levels of the three other putative targets, At1g51480, At1g05280 and At5g51270, remained unchanged in *mir844* mutant plants (Fig. 5A; Supplementary Fig. S2), which further supports that *CDS3* is a target of *miR844*. In addition, 5' RACE analysis revealed that no cleavage products of *CDS3* mRNA were detected in *mir844* mutant at the positions in which cleavages were detected in the *miR844*-overexpressing plants, which reinforces the proposition that *miR844* guides destabilization of *CDS3* mRNA. The *mir844* mutants displayed no obvious phenotype under normal growth conditions. Four-week-old *mir844* mutant and wild-type plants were infected with *P. syringae* DC3000, and disease severity and the number of bacteria in the leaves were evaluated at 5 DAI (Fig. 5B). The results showed that the growth of *P. syringae* in the mutant was much lower than that in wild-type plants. When the mutant and wild-type plants were infected with *B. cinerea*, the area of necrotic lesions on the leaves of the mutant was significantly smaller and the density of the fungi in the leaves of the mutant was much lower than those of wild-type plants (Fig. 5C). These results clearly show that *mir844*

mutant is more resistant to *P. syringae* and *B. cinerea* than wild-type plants, which is in contrast to the *miR844*-overexpressing transgenic plants and *cds3* mutant plants.

Regulation of *miR844* and *CDS3* expression upon *P. syringae* DC3000 or *B. cinerea* challenge.

To determine the *miR844*-guided regulation of *CDS3* in the defense response of plants, the expression patterns of *miR844* and *CDS3* were analyzed in *Arabidopsis* challenged with *P. syringae* DC3000 or *B. cinerea* via real-time RT-PCR analysis (Fig. 6; Supplementary Fig. S3A). It was evident that *miR844* expression was noticeably downregulated, whereas *CDS3* expression was highly upregulated after *P. syringae* infection (Fig. 6A). Although the changes in the transcript levels of *miR844* and *CDS3* were less significant in plants infected with *B. cinerea* than those infected with *P. syringae*, the transcript level of *miR844* was downregulated, whereas *CDS3* transcript level was upregulated after *B. cinerea* infection (Fig. 6A). Notably, the transcript level of *CDS3* was much higher in *mir844* mutant than the wild-type plant throughout pathogen infection. These results suggest that downregulation of *miR844* and a concomitant increase in *CDS3* transcripts affect *Arabidopsis* response to pathogens. Transcript levels of *PR1* and *PDF1-2*, which are generally used as marker genes for the response of plants to *P. syringae* DC3000 or *B. cinerea*, respectively, were markedly upregulated in the wild-type, *miR844*-overexpressing transgenic plants, *cds3* mutant, and *mir844* mutant upon pathogen challenge (Fig. 6B), suggesting that the plants were successfully infected with and responded to *P. syringae* DC3000 or *B. cinerea*. However, no significant differences in *PR1* and *PDF1-2* levels were observed in the

Fig. 3. Identification of the *miR844* target gene. **A**, *miR844* sequences and prediction of *miR844* target genes. Two *miR844* sequences were reported by Rajagopalan et al. (2006) and Fahlgren et al. (2007). The putative targets of *miR844* were predicted using a web-based prediction program (*miRNA Target Finder*). The bases in mismatches are indicated by bold type and the bases in G•U wobble base pairs are indicated with underlines. **B**, Schematic presentation of the predicted *miR844* cleavage site of *CDS3* mRNA and the primer pairs used for real time reverse transcription-polymerase chain reaction (RT-PCR) analysis. **C**, The expression levels of the *miR844* target gene *CDS3* were measured in 2-week-old transgenic *Arabidopsis* plants by real-time RT-PCR, using the primer pairs spanning the putative cleavage site or **D**, the primer pairs complementary to the upstream region of the cleavage site. **E**, The sequences between *miR844* and *CDS3* are aligned, and the predicted cleavage site is indicated by an arrow with an asterisk. Positions of dominant 5' RACE (rapid amplification of C-DNA ends) products (number of 5' ends at position/total number of 5' ends sequenced) are indicated by arrows.

wild type, transgenic plants, and mutant plants upon pathogen challenge (Fig. 6B).

DISCUSSION

The data presented here provide clear evidence for a role of miR844 in the defense response through the regulation of *CDS3* mRNA in *Arabidopsis*. Increased susceptibility of miR844-overexpressing transgenic plants and the *cds3* knockout mutant to *P. syringae* DC3000 and *B. cinerea* clearly indicates that downregulation of *CDS3* by miR844 negatively affect the defense response in *Arabidopsis* (Figs. 1, 2, and 4). These findings were further supported by the observation that the *mir844* mutant showed an enhanced resistance to the pathogens (Fig. 5). The expression of *miR844* and its target mRNA was inversely regulated upon pathogen challenge, such that the *miR844* level was downregulated and the *CDS3* transcript level was upregulated upon pathogen challenge (Fig. 6). It is clear that the downregulation of *miR844* and a concomitant increase in *CDS3* expression after *P. syringae* DC3000 or *B. cinerea* infection is a defense response of *Arabidopsis* to bacteria and fungi.

Although the transcript level of *CDS3* mRNA in the miR844-overexpressing transgenic plants was decreased compared with that in the wild-type plants, which clearly shows the inverse relationship between miR844 expression and its target mRNA level, we were not able to confirm the cleavage of *CDS3* mRNA

or the protein kinase (At5g51270) that was determined as a target of miR844 (Fahlgren et al. 2007) (Fig. 3). However, it was emphasized in the paper that evidence for miR844-guided cleavage of the protein kinase should be interpreted cautiously, as targets were not cleaved at the canonical position (Fahlgren et al. 2007). In our analysis, no cleavage was observed in the predicted cleavage sites of putative target genes. However, when the transcript levels of these predicted target mRNAs were analyzed in miR844-overexpressing transgenic plants, it was evident that only *CDS3* mRNA level was significantly decreased by the overexpression of miR844 (Fig. 3). In addition, *CDS3* transcript levels increased in the loss-of-function *miR844* mutant (Fig. 5A), which further supports the idea that *CDS3* is a target of miR844. Moreover, the *CDS3* transcript levels were significantly decreased when analyzed with the primer pairs either spanning the predicted cleavage site of the target gene or complementary to the upstream region of the cleavage site (Fig. 3). Our 5' RACE analysis showed that, although no cleavage was observed at the predicted cleavage sites of putative target genes, cleavage was observed at 6, 12, 21, and 52 bases upstream of the predicted cleavage site of *CDS3* mRNA (Fig. 3E). Although we do not know at present how *CDS3* mRNA is cleaved at these sites, it is clear that miR844 influences *CDS3* mRNA levels. It is possible that miR844 downregulates the transcript level of the *CDS3* gene via an mRNA destabilization mechanism, as demonstrated in animals and *Caenorhabditis elegans*, in which the mRNA stabilities of

Fig. 4. Defense response of *cds3* mutant to *Pseudomonas syringae* DC3000 and *Botrytis cinerea*. **A**, The T-DNA insertion site in *cds3* mutant is shown, and the absence of *CDS3* expression in the T-DNA insertion mutant was verified by reverse transcription-polymerase chain reaction (RT-PCR) analysis. **B**, Southern blot analysis to verify single T-DNA insertion. Genomic DNA (20 µg) digested with enzymes, including *EcoRI* and *HindIII*, which could not cut the β-glucuronidase (GUS) gene, and *EcoRV*, which could cut the GUS gene, was size-fractionated on a 0.7% agarose gel, was blotted, and was hybridized with the GUS probe. **C**, Four-week-old wild type (Col-0) and the *cds3* knockout mutant were infected with *P. syringae* DC3000. Photographs of the leaves, arranged in the order of age (old to new), were taken at 5 days after inoculation (DAI). Numbers of CFU were measured at 5 DAI, and the data are mean ± standard error (SE) of three replicates, seven leaves per replicate. **D**, Four-week-old wild type (Col-0) and the *cds3* mutant were infected with *B. cinerea*, and photographs were taken at 5 DAI. Lesion size in each leaf was measured at 5 DAI, and the density of *B. cinerea* tubulin by RT-PCR analysis. The data are mean ± SE of three replicates, four leaves per replicate.

protein-coding genes significantly decrease in response to miRNA expression (Bagga et al. 2005; Fabian et al. 2010). It has also been shown in the analysis of a global view of mRNA decay in *Arabidopsis thaliana* using microarrays that the transcripts that are miRNA targets were generally determined to have short half-lives (Narsai et al. 2007). It would be of keen interest to further determine how miR844 influences cleavage of *CDS3* mRNA at 6, 12, 21, and 52 bases upstream of the predicted cleavage site.

Exactly how the miR844-mediated downregulation of *CDS3* affects disease resistance of *Arabidopsis* to bacteria and fungi is currently unclear. Five genes encoding CDS have been identified in *Arabidopsis* (Beisson et al. 2003; Haselier et al. 2010). CDS is located in the plastids, mitochondria, and microsomes of plant cells and catalyzes the formation of cytidinediphosphate diacylglycerol (CDP-DAG), the precursor of phosphoglycerolipids

which are indispensable components for essential cellular functions (Babychuk et al. 2003; Dowhan 1997; Houtkooper et al. 2009; Lykidis and Jackowski 2001; Xue et al. 2009). Analysis of *Arabidopsis cds4* and *cds5* double mutants provided evidence that phosphatidylglycerol is important for the structure and function of thylakoid membranes and for photoautotrophic growth in plants (Haselier et al. 2010). It was also demonstrated by mutant analysis that phosphatidylglycerol is essential for the development of thylakoid membranes in *Arabidopsis* (Hagio et al. 2002) and for the maintenance of photosynthetic machinery in the cyanobacterium *Synechocystis* (Domonkos et al. 2004; Sakurai et al. 2003). In addition to structural roles in cell membranes and functional roles in photosynthesis, phospholipids also play a role as important signaling molecules in plant defense (Laxalt and Munnik 2002; Mosblech et al. 2008; Murphy et al. 2008; Vossen et al. 2010; Xue et al. 2009). Several

Fig. 5. Defense response of *mir844* mutant to *Pseudomonas syringae* DC3000 and *Botrytis cinerea*. **A**, The T-DNA insertion site in a *mir844* mutant is shown, and the absence of *miR844* expression and increased levels of *CDS3* mRNA in the T-DNA insertion mutant was verified by reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR analyses. **B**, Four-week-old wild-type (Col-0) and *mir844* mutant were infected with *P. syringae* DC3000, and photographs were taken at 5 days after inoculation (DAI). The numbers of cCFU were measured at 5 DAI, and the data are mean $\pm$ standard error (SE) of three replicates, eight leaves per replicate. **C**, Four-week-old wild type (Col-0) and a *mir844* mutant were infected with *B. cinerea*. Photographs were taken and lesion size in each leaf was measured at 4 DAI, and the density of *B. cinerea* in the leaves was quantitatively determined by measuring the amounts of *B. cinerea* tubulin by RT-PCR analysis. The data are mean $\pm$ SE of three replicates, four leaves per replicate.

lipids and lipid-related molecules, including fatty acids, glycerolipids, sphingolipids, oxylipins, and sterols, play important roles in the regulation of plant defense responses (Kachroo and Kachroo 2009; Shah 2005). All of these results clearly point to the importance of lipids and lipid-related molecules in plant defense responses. Although it was not demonstrated at present how miR844-mediated downregulation of *CDS3* affects the disease resistance of *Arabidopsis* to pathogens, it is likely that miR844-mediated downregulation of *CDS3* results in decreases in the formation of CDP-DAG and phosphatidylglycerol, which negatively affects disease resistance of *Arabidopsis* against bacteria and fungi. It would be of interest to analyze the composition of phosphoglycerolipids in wild-type, miR844-overexpressing transgenic plants, and the *cds3* mutant, which would be a valuable step to better understand the link between lipid metabolism and defense responses in plants. Notably, transcript levels of *PR1* and *PDF1-2* were markedly upregulated upon pathogen challenge, but no significant differences in *PR1*

and *PDF1-2* levels were observed in the wild-type plants, miR844-overexpressing transgenic plants, *cds3* mutant plants, and *mir844* mutant plants upon pathogen challenge (Fig. 6B). Although *PR1* and *PDF1-2* are generally used as markers for the induction of salicylic acid- and ethylene- or jasmonic acid-dependent defense pathways, respectively, many studies have demonstrated that hormone crosstalk exists in plant defense response and that *PR1* and *PDF1-2* may not be sufficient to provide full plant defense and other defense responses are involved in plant defense against pathogens (Ferrari et al. 2007; Mengiste et al. 2003; Murray et al. 2005; Robert-Seilantantz et al. 2011). Because *CDS3* catalyzes the formation of CDP-DAG, the precursor of phosphoglycerolipids, it would be interesting to further analyze the role of *CDS3* in lipid metabolism and the link between lipid metabolism and defense responses in plants.

In conclusion, it is clear that miR844-mediated downregulation of *CDS3* renders *Arabidopsis* more susceptible to pathogenic bacteria and fungi. Considering that CDS are involved in the formation of CDP-DAG and phosphoglycerolipids, which are indispensable components for cellular functions and defense responses, our current findings suggest that miRNA is an important component in the regulation of lipid metabolism in cells during defense responses. More research is needed to better understand the significance of miRNA-mediated regulation of lipid metabolism in the response of plants to diverse pathogens.

MATERIALS AND METHODS

Plant material and growth conditions.

Arabidopsis thaliana Col-0 ecotype was grown in a growth chamber at 23°C under a 16 h-light and 8-h-dark photocycle. To generate miR844-overexpressing transgenic *Arabidopsis* plants, the coding region of 400-nt-long premiR844 was cloned under control of the *Cauliflower mosaic virus* 35S promoter in the pBI121 vector. The T3 homozygous lines were used for phenotype investigation. Overexpression of both premiR844 and mature miR844 in the transgenic plants was confirmed by RT-PCR and Northern blot analyses. The gene-specific primers used in the RT-PCR analysis are listed in Supplementary Table S1. For the Northern blot analysis, total RNAs (20 µg) were size-fractionated by electrophoresis on 15% acrylamide gel, were blotted, and were cross-linked to a Hybond-nylon membrane. The ³²P-labeled 21-nt miR844 was used as a probe. *Arabidopsis cds3* mutant seeds (CS915999) were obtained from the Arabidopsis Biological Resource Center, and homozygote T₃ seeds were utilized for subsequent analyses. Southern blot analysis was employed to verify single T-DNA insertion. Genomic DNA (20 µg) was digested with the restriction enzymes, was size-fractionated on a 0.7% agarose gel, and was blotted to Hybond-NX nylon membranes (Amersham Biosciences, Piscataway, NJ, U.S.A.), using the capillary transfer method. The presence of T-DNA was detected using the GUS gene as a probe as described (Nishal et al. 2005). The cross-linked membrane was hybridized overnight at 45°C with the 1.2-kb-long [³²P]-labeled GUS gene fragment amplified by PCR using the gene-specific primers: forward primer, 5'-GGATCAACAGG TGGTTGCAACTGG-3' and reverse primer, 5'-TCATTGT TTGCTCCCTGCTGCGG-3'.

RT-PCR, real-time RT-PCR, and 5' RACE analysis.

Total RNAs were extracted from sample tissues using the Plant RNeasy kit (Qiagen, Valencia, CA, U.S.A.) and were quantified by spectrophotometer. Either RT-PCR or real-time RT-PCR using the One-step RT-PCR kit (Qiagen) or QuantiTect SYBR green RT-PCR kit (Qiagen), respectively, were used to analyze the target gene expression level. Two hundred nanograms

Fig. 6. Transcript levels of *miR844*, *CDS3*, *PR1*, and *PDF1-2* in *Arabidopsis* plants challenged with *Pseudomonas syringae* DC3000 or *Botrytis cinerea*. **A**, Four-week-old *Arabidopsis* plants were infected with *P. syringae* DC3000 or *B. cinerea*, and the transcript levels of mature *miR844* and *CDS3* were analyzed via real-time reverse transcription-polymerase chain reaction (RT-PCR) and are presented as the relative expression (fold) of mock-treated controls. Asterisks above the columns indicate values that are statistically different from the control values ($P \leq 0.05$). **B**, Transcript levels of *PR1* and *PDF1-2* were analyzed via real-time RT-PCR in the wild type (Col-0), miR844-overexpressing plants (OX2), *cds3* mutant plants, and *mir844* mutant plants infected with *P. syringae* DC3000 (psy) or *B. cinerea* (Bci), respectively, and are presented as the relative expression (fold) of the controls (Col-0 at 0 h). The data are mean $\pm$ standard error obtained from three independent experiments.

of total RNA were used in the real-time RT-PCR reaction, as described previously (Kim et al. 2005; 2008), together with gene-specific primers. To verify cleavage of the miR844 target gene in *Arabidopsis* plants, total RNAs were extracted from 2-week-old wild-type and miR844-overexpressing transgenic *Arabidopsis* plants, and the levels of the target mRNA transcript were quantified via real-time RT-PCR analysis. The transcript levels of mature *miR844* were quantified using EXPRESS SYBR GreenER miRNA qRT-PCR kit (Invitrogen, Carlsbad, CA, U.S.A.). Cleavage of predicted targets was analyzed with the 5' RACE procedure, using both the GeneRacer kit (Invitrogen) and the SMARTer RACE kit (Clontech, Mountain View, CA, U.S.A.), according to the manufacturer's instruction.

Pathogen infection and disease symptom analysis.

Bacterial and fungal strains used in this study were *P. syringae* pv. tomato DC3000 and *B. cinerea*. Bacteria were grown overnight in King's B solid medium and were used to infect 4-week-old *Arabidopsis* plants by the dipping method, using 0.02% Silwet L-77. *P. syringae* DC3000 was used at a suspension of 3×10^8 CFU ml⁻¹, and bacterial growth in leaves was measured at 5 DAI. Twenty leaves per replicate were used and the experiments were performed in triplicate. *B. cinerea* was cultured on 24 g of potato dextrose broth per liter at 25°C for 2 weeks and was used to infect 4-week-old *Arabidopsis* plants by placing two 5 µl droplets (3×10^6 conidia ml⁻¹) on four rosette leaves per plant. The lesion size on the rosette leaves was measured at 5 DAI. Fifteen leaves per replicate were used, and the experiments were performed in triplicate.

Statistical analysis.

Data were square root-transformed prior to analysis, and differences between the samples were compared by *t* test ($P \leq 0.05$; SIGMAPLOT software; Systat Software, Inc., San Jose, CA, U.S.A.).

ACKNOWLEDGMENTS

We thank the Arabidopsis Biological Resource Center for the T-DNA mutant line. This work was supported, in part, by a grant from the Next-Generation BioGreen21 Program (PJ01103601), Rural Development Administration, and by the Mid-Career Researcher Program (2011-0017357) through the National Research Foundation of Korea grant funded by the Ministry of Education, Science and Technology, Republic of Korea.

LITERATURE CITED

Babychuk, E., Müller, F., Eubel, H., Braun, H. P., Frentzen, M., and Kushnir, S. 2003. *Arabidopsis* phosphatidylglycerophosphate synthase 1 is essential for chloroplast differentiation, but is dispensable for mitochondrial function. *Plant J.* 33:899-909.

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., and Pasquinelli, A. E. 2005. Regulation by *let-7* and *lin-4* miRNAs results in target mRNA degradation. *Cell* 122:553-563.

Bartel, D. P. 2009. MicroRNAs: Target recognition and regulatory functions. *Cell* 136:215-233.

Beisson, F., Koo, A. J. K., Ruuska, S., Schwender, J., Pollard, M., Thelen, J. J., Paddock, T., Salas, J. J., Savage, L., Milcamps, A., Mhaske, V. B., Cho, Y., and Ohlrogge, J. B. 2003. Arabidopsis genes involved in acyl lipid metabolism. A 2003 census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database. *Plant Physiol.* 132:681-697.

Chiou, T.-J., Aung, K., Lin, S.-I., Wu, C.-C., Chiang, S.-F., and Su, C.-L. 2006. Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. *Plant Cell* 18:412-421.

Domonkos, I., Malec, P., Sallai, A., Kovács, L., Itoh, K., Shen, G., Ughy, B., Bogos, B., Sakurai, I., Kis, M., Strzalka, K., Wada, H., Itoh, S., Farkas, T., and Gombos, Z. 2004. Phosphatidylglycerol is essential for oligomerization of photosystem I reaction center. *Plant Physiol.* 134:1471-1478.

Dowhan, W. 1997. CDP-diacylglycerol synthase of microorganisms. *Biochim. Biophys. Acta* 1348:157-165.

Fabian, M. R., Sonenberg, N., and Filipowicz, W. 2010. Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.* 79:351-379.

Fahlgren, N., Howell, M. D., Kasschau, K. D., Chapman, E. J., Sullivan, C. M., Cumbie, J. S., Givan, S. A., Law, T. F., Grant, S. R., Dangel, J. L., and Carrington, J. C. 2007. High-throughput sequencing of *Arabidopsis* microRNAs: evidence for frequent birth and death of *MIRNA* genes. *PLoS ONE* 2:e219.

Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F. M., and Dewdney, J. 2007. Resistance to *Botrytis cinerea* induced in *Arabidopsis* by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. *Plant Physiol.* 144:367-379.

Fujii, H., Chiou, T.-J., Lin, S.-I., Aung, K., and Zhu, J.-K. 2005. A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Curr. Biol.* 15:2038-2043.

Hagio, M., Sakurai, I., Sato, S., Kato, T., Tabata, S., and Wada, H. 2002. Phosphatidylglycerol is essential for the development of thylakoid membranes in *Arabidopsis thaliana*. *Plant Cell Physiol.* 43:1456-1464.

Haselier, A., Akbari, H., Weth, A., Baumgartner, W., and Frentzen, M. 2010. Two closely related genes of *Arabidopsis* encode plastidial cytidinediphosphate diacylglycerol synthases essential for photoautotrophic growth. *Plant Physiol.* 153:1372-1384.

He, X.-F., Fang, Y.-Y., Feng, L., and Guo, H.-S. 2008. Characterization of conserved and novel microRNAs and their targets, including a TuMV-induced TIR-NBS-LRR class R gene-derived novel miRNA in *Brassica*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 582:2445-2452.

Houtkooper, R. H., Turkenburg, M., Poll-The, B. T., Karall, D., Pérez-Cerdá, C., Morrone, A., Malvagia, S., Wanders, R. J., Kulik, W., and Vaz, F. M. 2009. The enigmatic role of tafazzin in cardiolipin metabolism. *Biochim. Biophys. Acta* 1788:2003-2014.

Jagadeeswaran, G., Saini, A., and Sunkar, R. 2009. Biotic and abiotic stress down-regulate miR398 expression in *Arabidopsis*. *Planta* 229:1009-1014.

Jeong, D.-H., and Green, P. J. 2013. The role of rice microRNAs in abiotic stress responses. *J. Plant Biol.* 56:187-197.

Jin, H. 2008. Endogenous small RNAs and antibacterial immunity in plants. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 582:2679-2684.

Jones-Rhoades, M. W., and Bartel, D. P. 2004. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell* 14:787-799.

Kachroo, A., and Kachroo, P. 2009. Fatty acid-derived signals in plant defense. *Annu. Rev. Phytopathol.* 47:153-176.

Katiyar-Agarwal, S., and Jin, H. 2010. Role of small RNAs in host-microbe interactions. *Annu. Rev. Phytopathol.* 48:225-246.

Kawashima, C. G., Yoshimoto, N., Maruyama-Nakashita, A., Tsuchiya, Y. N., Saito, K., Takahashi, H., and Dalmay, T. 2009. Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. *Plant J.* 57:313-321.

Khraiwesh, B., Zhu, J. K., and Zhu, J. 2012. Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochim. Biophys. Acta* 1819:137-148.

Kim, J. S., Jung, H. J., Lee, H. J., Kim, K. A., Goh, C. H., Woo, Y., Oh, S. H., Han, Y. S., and Kang, H. 2008. Glycine-rich RNA-binding protein 7 affects abiotic stress responses by regulating stomata opening and closing in *Arabidopsis thaliana*. *Plant J.* 55:455-466.

Kim, J. Y., Kwak, K. J., Jung, H. J., Lee, H. J., and Kang, H. 2010b. MicroRNA402 affects seed germination of *Arabidopsis thaliana* under stress conditions via targeting DEMETER-LIKE Protein3 mRNA. *Plant Cell Physiol.* 51:1079-1083.

Kim, J. Y., Lee, H. J., Jung, H. J., Maruyama, K., Suzuki, N., and Kang, H. 2010a. Overexpression of microRNA395c or 395e affects differently the seed germination of *Arabidopsis thaliana* under stress conditions. *Planta* 232:1447-1454.

Kim, Y.-O., Kim, J. S., and Kang, H. 2005. Cold-inducible zinc finger-containing glycine-rich RNA-binding protein contributes to the enhancement of freezing tolerance in *Arabidopsis thaliana*. *Plant J.* 42:890-900.

Laxalt, A. M., and Munnik, T. 2002. Phospholipid signalling in plant defence. *Curr. Opin. Plant Biol.* 5:332-338.

Li, Y., Zhang, Q., Zhang, J., Wu, L., Qi, Y., and Zhou, J. M. 2010. Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. *Plant Physiol.* 152:2222-2231.

Lin, S. I., Chiang, S. F., Lin, W. Y., Chen, J.-W., Tseng, C.-Y., Wu, P.-C., and Chiou, T.-J. 2008. Regulatory network of microRNA399 and *PHO2* by systemic signaling. *Plant Physiol.* 147:732-746.

Lu, S., Sun, Y.-H., Amerson, H., and Chiang, V. L. 2007. MicroRNAs in loblolly pine (*Pinus taeda* L.) and their association with fusiform rust gall development. *Plant J.* 51:1077-1098.

- Lykidis, A., and Jackowski, S. 2001. Regulation of mammalian cell membrane biosynthesis. *Prog. Nucleic Acid Res. Mol. Biol.* 65:361-393.
- Mengiste, T., Chen, X., Salmeron, J., and Dietrich, R. 2003. The *BOTRYTIS SUSCEPTIBLE1* gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. *Plant Cell* 15:2551-2565.
- Mosblech, A., König, S., Stenzel, I., Grzegarek, P., Feussner, I., and Heilmann, I. 2008. Phosphoinositide and inositolpolyphosphate signaling in defense responses of *Arabidopsis thaliana* challenged by mechanical wounding. *Mol. Plant* 1:249-261.
- Murphy, A. M., Otto, B., Brearley, C. A., Carr, J. P., and Hanke, D. E. 2008. A role for inositol hexakisphosphate in the maintenance of basal resistance to plant pathogens. *Plant J.* 56:638-652.
- Murray, S. L., Adams, N., Kliebenstein, D. J., Loake, G. J., and Denby, K. J. 2005. A constitutive *PR-1:luciferase* expression screen identifies *Arabidopsis* mutants with differential disease resistance to both biotrophic and necrotrophic pathogens. *Mol. Plant Pathol.* 6:31-41.
- Narsai, R., Howell, K. A., Millar, A. H., O'Toole, N., Small, I., and Whelan, J. 2007. Genome-wide analysis of mRNA decay rates and their determinants in *Arabidopsis thaliana*. *Plant Cell* 19:3418-3436.
- Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J. D. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312:436-439.
- Navarro, L., Jay, F., Nomura, K., He, S. Y., and Voinnet, O. 2008. Suppression of the microRNA pathway by bacterial effector proteins. *Science* 321:964-967.
- Nishal, B., Tantikanjana, T., and Sundaresan, V. 2005. An inducible targeted tagging system for localized saturation mutagenesis in *Arabidopsis*. *Plant Physiol.* 137:3-12.
- Padmanabhan, C., Zhang, X., and Jin, H. 2009. Host small RNAs are big contributors to plant innate immunity. *Curr. Opin. Plant Biol.* 12:465-472.
- Pant, B. D., Buhtz, A., Kehr, J., and Scheible, W. R. 2008. MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. *Plant J.* 53:731-738.
- Rajagopalan, R., Vaucheret, H., Trejo, J., and Bartel, D. P. 2006. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes Dev.* 20:3407-3425.
- Robert-Seilaniantz, A., Grant, M., and Jones, J. D. 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49:317-343.
- Rubio-Somoza, I., and Weigel, D. 2011. MicroRNA networks and developmental plasticity in plants. *Trends Plant Sci.* 16:258-264.
- Ruiz-Ferrer, V., and Voinnet, O. 2009. Roles of plant small RNAs in biotic stress responses. *Annu. Rev. Plant Biol.* 60:485-510.
- Sakurai, I., Hagio, M., Gombos, Z., Tyystjärvi, T., Paakkari, V., Aro, E.-M., and Wada, H. 2003. Requirement of phosphatidylglycerol for maintenance of photosynthetic machinery. *Plant Physiol.* 133:1376-1384.
- Shah, J. 2005. Lipids, lipases, and lipid-modifying enzymes in plant disease resistance. *Annu. Rev. Phytopathol.* 43:229-260.
- Sunkar, R., Kapoor, A., and Zhu, J. K. 2006. Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* 18:2051-2065.
- Sunkar, R., Li, Y.-F., and Jagadeeswaran, G. 2012. Functions of microRNAs in plant stress responses. *Trends Plant Sci.* 17:196-203.
- Voinnet, O. 2009. Origin, biogenesis, and activity of plant microRNAs. *Cell* 136:669-687.
- Vossen, J. H., Abd-El-Halim, A., Fradin, E. F., van den Berg, G. C., Ekengren, S. K., Meijer, H. J., Seifi, A., Bai, Y., ten Have, A., Munnik, T., Thomma, B. P., and Joosten, M. H. 2010. Identification of tomato phosphatidylinositol-specific phospholipase-C (PI-PLC) family members and the role of PLC4 and PLC6 in HR and disease resistance. *Plant J.* 62:224-239.
- Xin, M., Wang, Y., Yao, Y., Xie, C., Peng, H., Ni, Z., and Sun, Q. 2010. Diverse set of microRNAs are responsive to powdery mildew infection and heat stress in wheat (*Triticum aestivum* L.). *BMC Plant Biol.* 10:123.
- Xue, H.-W., Chen, X., and Mei, Y. 2009. Function and regulation of phospholipid signalling in plants. *Biochem. J.* 421:145-156.
- Zhang, W., Gao, S., Zhou, X., Chellappan, P., Chen, Z., Zhou, X., Zhang, X., Fromuth, N., Coutino, G., Coffey, M., and Jin, H. 2011. Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks. *Plant Mol. Biol.* 75:93-105.

AUTHOR-RECOMMENDED INTERNET RESOURCES

miRNA Target Finder: <http://bioinfo3.noble.org/miRNA>
 psRNA Target Finder: <http://plantgrn.noble.org/psRNATarget>