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OsDCL1a activation impairs phytoalexin biosynthesis and compromises disease resistance in rice

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• **Background and Aims** MicroRNAs (miRNAs) are small non-coding RNAs that act as post-transcriptional regulators of gene expression via sequence-specific cleavage or translational repression of target transcripts. They are transcribed as long single-stranded RNA precursors with unique stem–loop structures that are processed by a DICER-Like (DCL) ribonuclease, typically DCL1, to produce mature miRNAs. Although a plethora of miRNAs have been found to be regulated by pathogen infection in plants, the biological function of most miRNAs remains largely unknown. Here, the contribution of *OsDCL1* to rice immunity was investigated.
• **Methods** Activation-tagged *Osdcl1a* (*Osdcl1a-Ac*) rice mutants were examined for resistance to pathogen infection. mRNA and small RNA deep sequencing, quantitative real-time PCR (RT-qPCR) and stem–loop reverse tanscripion–PCR (RT–PCR) were used to examine *DCL1a*-mediated alterations in the rice transcriptome. Rice diterpene phytoalexins were quantified by liquid chromatography–tandem mass spectrometry (LC-MSMS). Accumulation of $O_2{\cdot}^-$ was determined by nitroblue tetrazolium (NBT) staining.
• **Key Results.** *dcl1a-Ac* mutants exhibit enhanced susceptibility to infection by fungal pathogens which was associated with a weaker induction of defence gene expression. Comparison of the mRNA and miRNA transcriptomes of *dcl1a-Ac* and wild-type plants revealed misregulation of genes involved in detoxification of reactive oxygen species. Consequently, *dcl1a-Ac* plants accumulated $O_2{\cdot}^-$ in their leaves and were more sensitive to methyl viologen-induced oxidative stress. Furthermore, *dcl1a-Ac* plants showed downregulation of diterpenoid phytoalexin biosynthetic genes, these genes also being weakly induced during pathogen infection. Upon pathogen challenge, *dcl1a-Ac* plants failed to accumulate major diterpenoid phytoalexins. *OsDCL1a* activation resulted in marked alterations in the rice miRNAome, including both upregulation and downregulation of miRNAs.
• **Conclusions** *OsDCL1a* activation enhances susceptibility to infection by fungal pathogens in rice. Activation of *OsDCL1a* represses the pathogen-inducible host defence response and negatively regulates diterpenoid phytoalexin production. These findings provide a basis to understand the molecular mechanisms through which *OsDCL1a* mediates rice immunity.

Key words: DICER-Like (DCL), fungal pathogen, *Fusarium fujikuroi*, innate immunity, *Magnaporthe oryzae*, methyl viologen, microRNAs, *Oryza sativa*, oxidative stress, phytoalexins, rice.

INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs that act as post-transcriptional regulators of gene expression via sequence-specific cleavage or translational repression of target transcripts in eukaryotes (Llave *et al.*, 2002; Brodersen *et al.*, 2008). MiRNA genes are transcribed by RNA polymerase II into long precursor transcripts with unique stem–loop structures (pri-miRNA) that are processed in a two-step process by a DICER-Like (DCL) ribonuclease, typically DCL1, to give rise to an miRNA-5p/miRNA-3p duplex (Kurihara and Watanabe, 2004). The miRNA duplex is exported to the cytoplasm, where one miRNA strand is selectively incorporated into an Argonaute 1 (AGO1)-containing RNA-induced silencing complex (RISC). This complex interacts with mRNA targets to direct cleavage or suppress translation.

Plant miRNAs have long been recognized as important regulators of gene expression in diverse developmental processes (Palatnik *et al.*, 2003; Mallory *et al.*, 2004; Rubio-Somoza and Weigel, 2011). They are also involved in hormone signal transduction and adaptation to abiotic and abiotic stress (Navarro *et al.*, 2006; Jagadeeswaran *et al.*, 2009; Li *et al.*, 2010; Jeong and Green, 2013; Baldrich and San Segundo, 2016; Fei *et al.*, 2016). Most of our knowledge of miRNAs involved in plant immune responses to pathogen infection is from studies of the interaction of arabidopsis plants with the bacterial pathogen *Pseudomonas syringae.*

Plants have evolved multiple defence mechanisms to defend themselves against pathogen infection, forming the innate immune system. Defence reactions are activated by the recognition of conserved pathogen-associated molecular patterns (PAMPs) by host membrane pattern recognition receptors (PRRs). This recognition triggers 'PAMP-triggered immunity' (PTI), which is effective against most pathogens (Jones and Dangl, 2006; Couto and Zipfel, 2016). PTI components include production of reactive oxygen species (ROS), reinforcement of the cell wall, activation of protein phosphorylation/dephosphorylation processes and accumulation of antimicrobial proteins, among others. The induction of *pathogenesis-related* (*PR*) genes is a ubiquitous response of plants to pathogen infection. Damage-associated molecular patterns (DAMPs) released from the plant cell wall after damage caused by the pathogen also induce plant defence responses. However, certain pathogens are able to suppress these basal resistance mechanisms by delivering effector proteins that can suppress PTI responses into the host cell. As a countermeasure, these microbial effectors are recognized by plant disease resistance proteins (R proteins), establishing 'effector-triggered immunity' (ETI). Plants also produce a variety of secondary metabolites as natural protection against microbial pathogens. Among them are phytoalexins, which are low molecular weight compounds with antimicrobial activity and structural diversity (e.g. flavonoids, terpenoids and indole phytoalexins) (Ahuja *et al.*, 2012; Schmelz *et al.*, 2014).

Rice is one of the most important crops worldwide and a primary source of food for more than half of the population. Rice blast caused by the fungus *Magnaporthe oryzae* is one of the most devastating fungal diseases of cultivated rice worldwide (Wilson and Talbot, 2009). Rice is also the model plant for research in monocotyledonous species with a sequenced genome (Goff *et al.*, 2002; Yu *et al.*, 2002). Evidence supports marked variations in the rice miRNA population during *M. oryzae* infection or treatment with *M. oryzae* elicitors (Campo *et al.*, 2013; Li *et al.*, 2014, 2016; Baldrich *et al.*, 2015). Although an important fraction of the rice miRNA transcriptome has been found to respond to *M. oryzae* infection or treatment with *M. oryzae* elicitors, a role for these pathogen-regulated miRNAs has been demonstrated for only a few of them. They are miR7695, miR160 and miR398 which function as positive regulators for rice immunity against *M. oryzae* infection, and miR169 and miR319 which negatively regulate immunity against this fungus (Campo *et al.*, 2013; Li *et al.*, 2014, 2017; Zhang *et al.*, 2018).

Regarding DCL1, a major miRNA processing component, three loci encoding DCL1 proteins are identified in the rice genome: *OsDCL1a*, *OsDCL1b* and *OsDCL1c* (Kapoor *et al.* 2008). Previous studies revealed that loss of function of *OsDCL1a* by RNA interference (RNAi; *dcl1a-IR* lines) results in abnormal shoot and root development with eventual growth arrest for the strongest RNAi lines (Liu *et al.*, 2005). Later on, silencing of *OsDCL1* was found to enhance resistance to rice blast fungus (Zhang *et al.*, 2015). In contrast, a phenotype of susceptibility to pathogen infection was observed in arabidopsis *dcl1* mutants, showing enhanced susceptibility to infection by bacterial (*Pseudomonas syringae*) and fungal (*Botrytis cinerea*) pathogens (Navarro *et al.*, 2008; Seo *et al.*, 2013; Weiberg *et al.*, 2014). *DCL1a* silencing also results in abnormal growth and development in arabidopsis plants (Gasciolli *et al.*, 2005). However, the *DCL1*-mediated mechanisms underlying these phenotypes of disease resistance or susceptibility in rice or arabidopsis remain unknown.

The goal of this research was to investigate the role of *OsDCL1* in rice immunity against fungal pathogens. To rule out the disease phenotype of *OsDCL1a* knock-down mutants being an effect of its morphological phenotype, we searched for *Osdcl1a* activation mutants. Two *OsDCL1a* activation mutants were identified and characterized (named *dcl1a-Ac* mutants). Plant growth performance of *Osdcl1a-Ac* plants was not affected. *OsDCL1a* activation enhanced susceptibility to infection by the fungal pathogens *M. oryzae* (hemibiotroph) and *Fusarium fujikuroi* (necrotroph), the causal agents of the rice blast and bakanae disease, respectively. Susceptibility to pathogen infection in *dcl1a-Ac* plants was associated with weaker induction of defence gene expression. The mRNA transcriptome and miRNAome of *dcl1a-Ac* plants were obtained and compared with those of wild-type plants. *OsDCL1a* activation had an important impact on the expression of genes involved in two processes: ROS detoxification and synthesis of diterpene phytoalexins. *dcl1a-Ac* plants featured down-regulation of genes involved in the biosynthesis of terpenoid phytoalexins. Upon pathogen infection, phytoalexin accumulation was compromised in *dcl1a-Ac* plants. Together, our results support that *OsDCL1a* plays an important role in rice immunity.

MATERIALS AND METHODS

Plant and fungal materials

Plants (*Oryza sativa*) were grown at 28 ºC/22 ºC day/night (16 h light/8 h dark cycle). Rice genotypes used were *O. sativa japonica* 'Tainung 67' (TN67), *dcl1a-Ac* mutants (M0066754, M0040827) from the Taiwan Rice Insertional Mutant collection (TRIM; http:/trim.sinica.edu.tw) and *dcl1a-IR* lines (Liu *et al.*, 2005). Genotyping of *dcl1a-Ac* mutants was carried out by PCR on genomic DNA with *DCL1a*-specific primers combined with a T-DNA-specific primer located at the left border of the T-DNA (Supplementary Data Table S1).

The fungus *M. oryzae* (strain *Guy 11*) was grown as previously described (Campos-Soriano *et al.*, 2013). The fungus *F. fujikuroi* (isolate 297) was grown for 15 d on potato dextrose agar (PDA) medium. Fungal spores were collected by adding sterile water to the surface of the mycelium and adjusted to the appropriate concentration.

Infection assays and elicitor treatment

Infection with *M. oryzae* involved spraying leaves of 3-week-old rice plants with a spore suspension (1×10^5 spores mL^{-1}). In all experiments, mock inoculations were performed. Development of disease symptoms was followed over time. Lesion area was determined by using digital imaging software (Assess 2.0, American Phytopathological Society). For infection experiments with *F. fujikuroi*, seeds were sterilized with sodium hypochlorite (30 %, for 30 min), pre-germinated for 24 h on Murashige and Skoog (MS) medium without sucrose and then inoculated with *F. fujikuroi* spores (1×10^6 spores mL^{-1}; 10 µL per seed). Fungal DNA on infected leaves was quantified by quantitative PCR (qPCR) with specific primers for the 28S DNA gene of the corresponding fungus (Qi and Yang, 2002; Jeon

et al., 2013). Primers are given in Supplementary Data Table S1. A standard curve with fungal DNA was prepared for quantification of fungal DNA in infected leaf samples. For elicitor treatment, 3-week-old wild-type plants were sprayed with an elicitor suspension obtained by autoclaving and sonicating *M. oryzae* mycelium (300 µg mL^{-1}) (Casacuberta *et al.*, 1992).

RT-qPCR and stem–loop RT–PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen). The RNA concentrations were quantified by a NanoDrop ND-2000 spectrophotometer. First-strand cDNA was synthesized from DNase-treated total RNA (1 µg) with SuperScript III reverse transcriptase (Invitrogen GmbH) and oligo(dT). Quantitative real-time PCRs (RT-qPCRs) were performed in optical 96-well plates in a Light Cycler 480 (Roche) with SYBR Green. All reactions were performed in triplicate. The average cycle threshold (Ct) values were obtained by PCR from three independent biological replicates and normalized to the average Ct values for the *cyclophilin 2* gene (Os02g02890) from the same RNA preparations to obtain the ΔCt value or normalized expression (relative expression). Primers used for RT-qPCR and stem–loop reverse transcription–PCR (RT–PCR) are listed in Supplementary Data Table S1. Analysis of variance (ANOVA) tests were used to evaluate differences in gene expression.

Quantification of rice diterpene phytoalexins

For quantification of rice phytoalexins, leaf segments were mixed with 40 vols of 80 % methanol, concentrated to dryness and resuspended in 0.5 mL of 80 % methanol.

Phytoalexins were quantified by liquid chromatography–tandem mass spectrometry (LC-MSMS) as previously described (Miyamoto *et al.*, 2016). Three biological replicates with two technical replicates each were performed. ANOVA tests were used to evaluate differences in phytoalexin accumulation.

Treatment with methyl viologen, pigment quantification and determination of the superoxide ion

Leaf segments (approx. 2 cm in length) were treated with methyl viologen (MV) solution (10 µM) at room temperature in the dark for 12 h, then incubated at 28 ºC at a 16 h/8 h photoperiod cycle for 3 d. Chlorophylls and carotenoids were extracted and quantified spectrophotometrically (Lichtenthaler and Buschmann, 2001). For histochemical detection of the superoxide ion $O_2^{\cdot -}$, leaf sections approx. 2 cm long were stained with nitroblue tetrazolium (NBT) (Campo *et al.*, 2008).

RNA-Seq and small RNA-Seq

Libraries were prepared from leaves of 3-week-old wild-type (segregated azygous) and *dcl1a-Ac* plants (two biological replicates per genotype). Indexed libraries were prepared from 1 µg of purified RNA from each sample (TruSeq Stranded mRNA Sample Prep Kit, Illumina). RNAs were quantified using the Agilent 2100 Bioanalyzer (Agilent Technologies) and pooled such that each index-tagged sample was present in equimolar amounts, with a final concentration of the pooled samples of 2 nM. The pooled samples underwent cluster generation and sequencing with the Illumina HiSeq 2500 System (Genomics4life S.R.L., Baronissi, Salerno, Italy) in a 2 × 50 single-end format at a final concentration of 8 pmol. The raw sequence files generated underwent quality control analysis with FastQC v0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimming and removal of adaptors involved use of Trimmomatic v0.33 (Bolger *et al.*, 2014) (minimum quality score 35, minimum length 25). The obtained reads were then mapped against the *O. sativa* reference genome (MSU 7.0) with STAR (v2.4.0j) (Dobin *et al.*, 2013) providing the reference gene annotation file with known transcripts (RGSP 7.0). Alignment files were filtered to remove reads with MAPQ <30. FeatureCounts v1.4.5-p1 (Liao *et al.*, 2014) was used for read summarization at the gene level, with the strand-specific option 'reversely stranded'. Statistical analysis of the read counts involved use of R 3.1.3 with the HTSFilter package to remove genes with low expression (Rau *et al.*, 2013) and the edgeR package for differential expression analysis (McCarthy *et al.*, 2012). Gene Ontology (GO) enrichment analysis of the differentially expressed genes involved use of the AgriGO webtool (http://bioinfo.cau.edu.cn/agriGO/; Du *et al.*, 2010).

For small RNA sequencing (RNA-Seq) the minimum length established was 15 bp and the quality score was 35. The high quality reads were aligned against the *O. sativa* reference genome sequence (MSU 7.0) with Bowtie (version 1.1.1, parameters 'v1', 'a'). FeatureCounts (version 1.4.5) was used together with miRBase v21 annotation to calculate gene expression values as raw read counts. Normalization was applied to the raw read counts by using the Trimmed Mean of M values (TMM) normalization. Data sets generated during the current study are available from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109307).

RESULTS

Identification and characterization of activation-tagged DCL1a *mutants*

As previously mentioned, *DCL1* silencing has a negative impact on plant growth in rice. To investigate the contribution of *DCL1* in disease resistance with no influence of intrinsic developmental cues, we searched for *DCL1* activation mutants in publicly available mutant collections. Two T-DNA tagged lines carrying the T-DNA insertion near *OsDCL1a*, lines M0066754 and M0040827, were identified in the activation/knockout TRIM collection generated in the Tainung 67 (TN67, japonica) background (Hsing *et al.*, 2007; http://trim.sinica.edu.tw) (Fig. 1A). The T-DNA insertion site in each TRIM mutant line was confirmed by PCR followed by DNA sequencing. Homozygous, hemizygous and azygous plants were identified (Supplementary Data Fig. S1A, B). The T-DNA used for generating the TRIM library contains eight tandem repeats of the *Cauliflower mosaic*

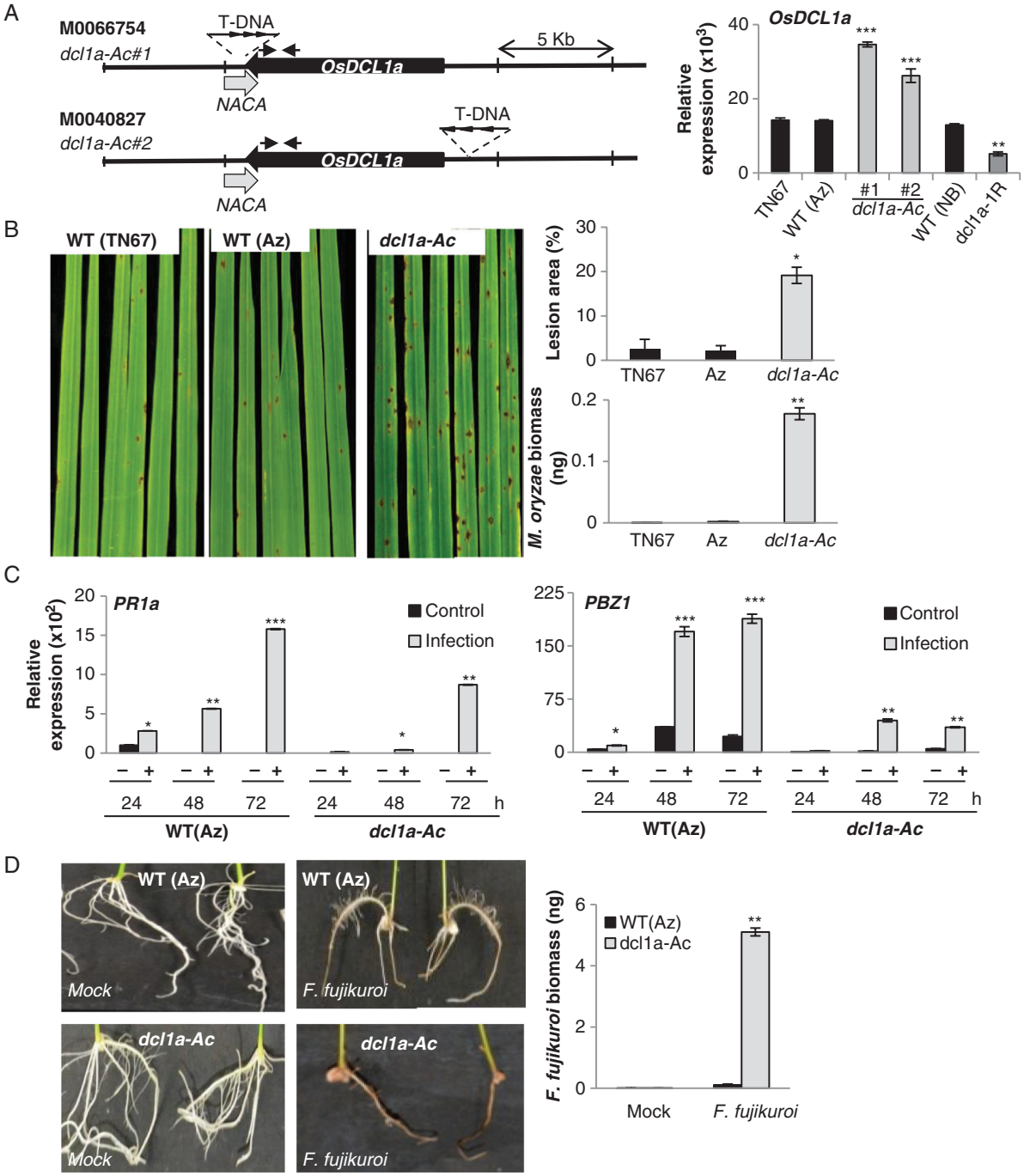

FIG. 1. Susceptibility of *OsDCL1a* activation mutants to infection by the pathogens *M. oryzae* and *F. fujikuroi*. (A) Representation of the T-DNA insertion mutants from the TRIM collection (lines M0066754 and M0040827) (left panel). Black and grey arrows represent *OsDCL1a* (Os03g02970) and the nearby genes (*Nascent polypeptide-associated complex subunit alpha*, *NACA*; Os03g02960) pointing in the direction of transcription. Arrowheads in the T-DNA represent the CaMV35S enhancer octamers. Arrows above the *OsDCL1a* gene indicate the position of primers used for RT-qPCR analysis. Right panel: *OsDCL1a* expression in leaves of 3-week-old *dcl1a-Ac* and *dcl1a-IR* plants determined by RT-qPCR. Tainung67 (TN67) and Nipponbare (NB) are the genetic backgrounds of the TRIM mutants and *dcl1a-IR* plants, respectively. (B) Susceptibility of the *dcl1a-Ac#1* mutant to *M. oryzae* infection. Three-week-old rice plants were inoculated with *M. oryzae* spores (1×10^5 spores mL^{-1}). Pictures were taken at 7 days post-inoculation (dpi). Right panels show the lesion area in infected leaves (measured by Assess 2.0) and quantification of *M. oryzae* DNA by qPCR at 7 dpi. (C) *OsPR1a* and *OsPBZ1* expression in wild-type (segregated azygous) and *dcl1a-Ac#1* plants determined by RT-qPCR at the indicated times after inoculation with *M. oryzae* spores (1×10^5 spores mL^{-1}). (D) Susceptibility of *dcl1a-Ac* plants to infection by *F. fujikuroi*. Seeds of *dcl1a-Ac#1* and wild-type (segregated azygous) plants were germinated for 24 h and inoculated with *F. fujikuroi* spores (10^6 spores mL^{-1}). Roots from *F. fujikuroi*-inoculated seedlings at 7 dpi are shown. Right panel: *F. fujikuroi* DNA in roots of wild-type and *dcl1a-Ac* plants was quantified by qPCR at 7 dpi. Three independent infection experiments with each fungus were performed (at least 24 plants per genotype in each experiment). Data are the mean ± s.d. ($n = 3$ biological replicates) (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ by ANOVA).

virus 35S promoter (*CaMV35*), which can activate the expression of genes located around the T-DNA insertion sites (Fig. 1A, left panel). *OsDCL1a* transcript levels were significantly higher in leaves from each mutant line as compared with azygous (segregated progeny) and wild-type TN67 plants (Fig. 1A, right panel), indicating that they are activation mutants for *OsDCL1a* (hereafter referred to as *dcl1a-Ac#1* and *dcl1a-Ac#2*). As expected, *OsDCL1a* expression was lower in *OsDCL1a* RNAi (*dcl1a-IR*) plants than its parental genotype (*O. sativa* 'Nipponbare'; Liu *et al.*, 2005) (Fig. 1A, right panel). qPCR revealed that each of the *dcl1a-Ac* mutants has a single copy of the T-DNA inserted in its genome (Supplementary Data Table S2). Importantly, we found no obvious phenotype differences between *dcl1a-Ac* mutant and wild-type (azygous and TN67) plants grown under controlled greenhouse conditions (Supplementary Data Fig. S1C). An examination of the genomic regions flanking *OsDCL1* identified one gene, *nascent polypeptide-associated complex subunit alpha* (*NACA*), that partially overlaps *OsDCL1a* (Fig. 1A). *OsDCL1* and *NACA* locate in opposite strands of the DNA (MSU release 7). Thus, in the *dcl1a-Ac#1* mutant, the T-DNA insertion site is found at the 3'-untranslated region (UTR) of both *NACA* and *OsDCL1a*. However, *NACA* transcripts accumulated at equivalent levels in mutant (*dcl1a-Ac#1*, *dcl1a-Ac#2*) and control plants (azygous, wild-type) (Supplementary Data Fig. S1D).

Susceptibility to infection by fungal pathogens in dcl1a-Ac *plants*

To investigate the functional relevance of *OsDCL1a* activation in disease resistance, *dcl1a-Ac* plants were examined for resistance to infection by the rice blast fungus *M. oryzae*. The *dcl1a-Ac* plants consistently exhibited higher susceptibility to *M. oryzae* infection as compared with control plants (Fig. 1B). Susceptibility of *dcl1a-Ac* to blast was confirmed by measuring the average lesion area in the infected leaves and by qPCR measurement of fungal DNA, an indicator of fungal biomass in the infected leaves (Fig. 1B, right panels). The *dcl1a-Ac#2* mutant also exhibited higher susceptibility to *M. oryzae* infection as compared with control plants (Supplementary Data Fig. S2). Increased susceptibility to blast infection in *dcl1a-Ac* plants is consistent with findings of resistance to *M. oryzae* in *dcl1a-IR* lines (Zhang *et al.*, 2015). However, *dcl1a-IR* plants showed abnormal growth, whereas *dcl1a-Ac* plants grew and developed normally.

To obtain further insights into the mechanisms underlying susceptibility to *M. oryzae* infection in *dcl1a-Ac* plants, we examined the expression pattern of the defence genes *OsPR1a* and *OsPBZ1* (a *PR10* family member) in wild-type and *dcl1a-Ac* plants. These genes are widely used as indicators of induction of rice defence responses during pathogen infection, including *M. oryzae* infection (Midoh and Iwata, 1996; Agrawal *et al.*, 2001). As expected, *OsPR1a* and *OsPBZ1* expression was induced in wild-type plants during *M. oryzae* infection (Fig. 1C). Although *OsPR1a* and *OsPBZ1* expression was also activated by fungal infection in *dcl1a-Ac* plants, their expression was induced at a much lower level in mutant plants than in wild-type plants at all times of infection. Reduced induction of defence gene expression agrees with the observed phenotype of susceptibility in *dcl1a-Ac* plants.

We also examined disease resistance of *dcl1a-Ac* plants against the necrotrophic fungus *F. fujikuroi*, the causal agent of bakanae, an important seed-borne disease of rice (Wulff *et al.*, 2010). As compared with wild-type segregated azygous plants, *dcl1a-Ac* seedlings grew poorly and their roots turned necrotic on *F. fujikuroi* inoculation (Fig. 1D). Fungal biomass was greater in roots of *dcl1a-Ac* than of wild-type plants (Fig. 1D, right panel), thus confirming that *dcl1a-Ac* plants are more susceptible to infection by *F. fujikuroi*.

Together, the results demonstrate that *OsDCL1a* activation enhances susceptibility to infection by hemibiotrophic (*M. oryzae*) and necrotrophic (*F. fujikuroi*) fungal pathogens in rice and that disease susceptibility in *dcl1a-Ac* plants is associated with weaker induction of defence gene expression during pathogen infection.

Expression of DCL *genes in* dcl1a-Ac *plants*

In plants, the *DCL* gene family typically comprises four members, *DCL1–DCL4*, which have distinct functions in miRNA and small interfering RNA (siRNA) biogenesis (Arikit *et al.*, 2013). A fifth *DCL*, *DCL3b* (also named *DCL5*), which is associated with the production of 24 nt siRNAs, appears to have evolved in monocots (Margis *et al.*, 2006; Song *et al.*, 2012; Wei *et al.*, 2014). The rice genome contains three *DCL1* genes (*OsDCL1a*, *OsDCL1b* and *OsDCL1c*). *OsDCL1a* is most closely related to *AtDCL1a* from a structural and functional point of view, and *OsDCL1a* silencing impairs miRNA biogenesis in rice (Liu *et al.*, 2005). Additionally, the rice genome has two *DCL2* paralogues with almost identical sequences (*DCL2a/b*) and unique *DCL3* (*OsDCL3a*), *DCL4* and *DCL3b* genes (Margis *et al.*, 2006; Kapoor *et al.*, 2008). *OsDCL1a*, *OsDCL2a/b* and *OsDCL3a* are ubiquitously expressed in vegetative tissues during development, but their expression is markedly reduced in the reproductive phase (Kapoor *et al.*, 2008). *DCL* genes with low expression (*OsDCL1b*, *OsDCL1c* and *OsDCL3b*) feature inflorescence-, panicle- and/or early seed-specific expression (Kapoor *et al.*, 2008). *OsDCL1a* was the most highly expressed *OsDCL1* gene in leaves of 3-week-old wild-type rice plants (Supplementary Data Fig. S3). As expected, *OsDCL1a* expression was further increased in *dcl1a-Ac* plants, with no significant difference in the expression of any of the other *DCL* genes between *dcl1a-Ac* and wild-type plants (Supplementary Data Fig. S3).

The apparently negative effect of *OsDCL1a* activation on resistance to fungal infection prompted us to investigate whether *OsDCL1a* expression itself is regulated as part of the host response to pathogen infection. This analysis revealed *OsDCL1a* expression induced in wild-type plants in response to *M. oryzae* infection [at 24, 48 and 72 h post-inoculation (hpi)] (Fig. 2A, left panel). A similar trend in *OsDCL1a* expression (i.e. upregulation) was observed after treatment with elicitors obtained by autoclaving and sonicating *M. oryzae* mycelium (Fig. 2A, right panel). Regarding other rice *DCL* genes, a different response to *M. oryzae* infection was observed depending on the family member. *OsDCL1a*, *OsDCL1b*, *OsDCL2a/b* and *OsDCL3a* expression was induced, whereas that of *OsDCL4* was repressed by *M. oryzae* infection, and *OsDCL3b* was not affected (at least at the time examined, 72 hpi) (Fig. 2B).

From these results, we conclude that pathogen infection alters the expression of rice *DCL* genes, namely *OsDCL1a*, *OsDCL1b*, *OsDCL2* and *OsDCL3a.* Knowing that these genes are involved in small RNA biogenesis pathways, this observation anticipates

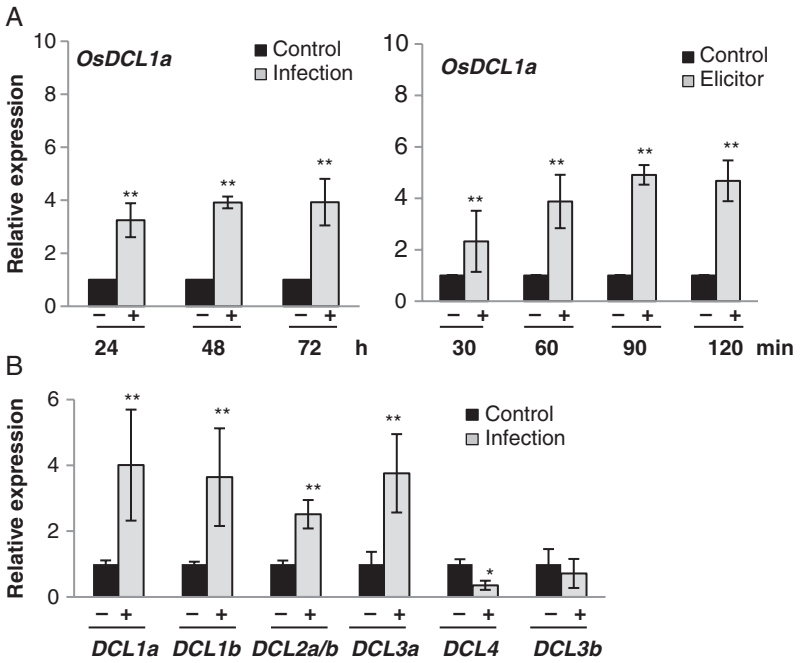

FIG. 2. Expression of *OsDCL* genes during infection with *M. oryzae* and treatment with fungal elicitors. (A) *OsDCL1a* expression at different times after inoculation with *M. oryzae* spores (1×10^5 spores mL^{-1}) (left panel) or treatment with elicitors from this fungus (300 µg mL^{-1}) (right panel) in wild-type plants. Black and red bars correspond to mock-inoculated and *M. oryzae*-inoculated (or elicitor-treated) plants, respectively. The expression level in mock-inoculated plants was set to 1.0. Three independent experiments (each with 24 plants per condition) were performed with similar results. Data are mean ± s.d. (*$P \leq 0.05$; **$P \leq 0.01$ by ANOVA). (B) Expression of *OsDCL* family members at 72 h post-inoculation (hpi) with *M. oryzae* spores.

important small RNA-mediated transcriptional reprogramming of gene expression as part of the rice response to infection by the fungal pathogen *M. oryzae*.

Transcript profiling of dcl1a-Ac *mutant plants*

To investigate *OsDCL1a*-mediated alterations in the rice transcriptome, we used RNA-Seq analysis of *dcl1a-Ac* and wild-type (segregated azygous) plants. RNA was obtained from leaves of 3-week-old plants. Illumina Solexa sequencing produced 39.6 and 31.0 million reads in wild-type and *dcl1a-Ac* plants, respectively (Supplementary Data Table S3). The processed RNA-Seq reads were mapped to the rice genome (*O. sativa* 'Nipponbare' MSU 7.0). For calling differentially expressed genes (DEGs), a fold change of 2.0 was used as a cut-off, with the false discovery rate (FDR) set to 0.05.

A total of 216 DEGs were found in *dcl1a-Ac* plants relative to wild-type plants, most downregulated in *dcl1a-Ac* plants (155 downregulated; 61 upregulated) (Fig. 3A; Supplementary Data Tables S4, S5). GO functional analysis revealed that many downregulated genes in *dcl1a-Ac* plants were in the categories 'signalling', 'metabolism', and 'biotic stress' (28, 19 and 14 %, respectively) (Fig. 3B, left panel).

The distribution of DEGs in functional categories differed greatly between upregulated and downregulated genes (i.e. genes associated with 'biotic stress' were not represented in the upregulated genes in *dcl1a-Ac* plants, whereas genes involved in oxidative stress were highly represented) (Fig. 3B, right panel). Genes involved in 'signalling' were less represented in upregulated than in downregulated genes (Fig. 3B; Supplementary Data Tables S4, S5). DEGs in *dcl1a-Ac* plants were classified according to their molecular functions by using the AgriGO tool (Du *et al.*, 2010; http://bioinfo.cau.edu.cn/agriGO/) (Supplementary Data Fig. S4). Important differences were observed in the categories of protein kinase and oxidoreductase (monooxygenase) activities. For instance, the expression of many receptor-like kinases was downregulated in *dcl1a-Ac* vs. wild-type plants (Supplementary Data Table S4). The sub-family of cell wall-associated kinase (*WAK*) genes was the most highly represented of downregulated receptor kinase genes (up to 17 *WAK* genes were downregulated in *dcl1a-Ac* plants). WAKs are involved in perception of PAMPs and DAMPs for activation of defence-associated responses, and overexpression of *WAK* genes increases resistance to *M. oryzae* in rice (Li *et al.*, 2009). Two brassinosteroid insensitive 1 receptor kinase (*BRI1*) genes were downregulated in *dcl1a-Ac* plants (Supplementary Data Table S4), these genes also being involved in recognition of PAMPs and activation of plant immune responses. Genes typically associated with disease resistance and defence mechanisms were also downregulated in *dcl1a-Ac* plants, such as several *R* genes and the *OsWRKY47* transcription factor gene. Previous studies have shown overexpression of *OsWRKY47* in rice accompanied by upregulation of *PR10* and blast resistance (Wei *et al.*, 2013). In agreement with this, *dcl1a-Ac* plants showed downregulation of both *OsWRKY47* and *PR10* expression (Supplementary Data Table S4). Among the genes downregulated in *dcl1a-Ac* were those involved in the biosynthesis of antifungal compounds, such as Agmantine hydroxycinnamoyltransferase1 (for producing antifungal hydroxycinnamoylagmantine derivatives) and strictosidine synthase (for producing alkaloids) (Supplementary Data Table S4).

Of note, genes encoding enzymes involved in oxidation–reduction reactions were highly represented among misregulated genes in *dcl1a-Ac* plants (up- and downregulated genes).

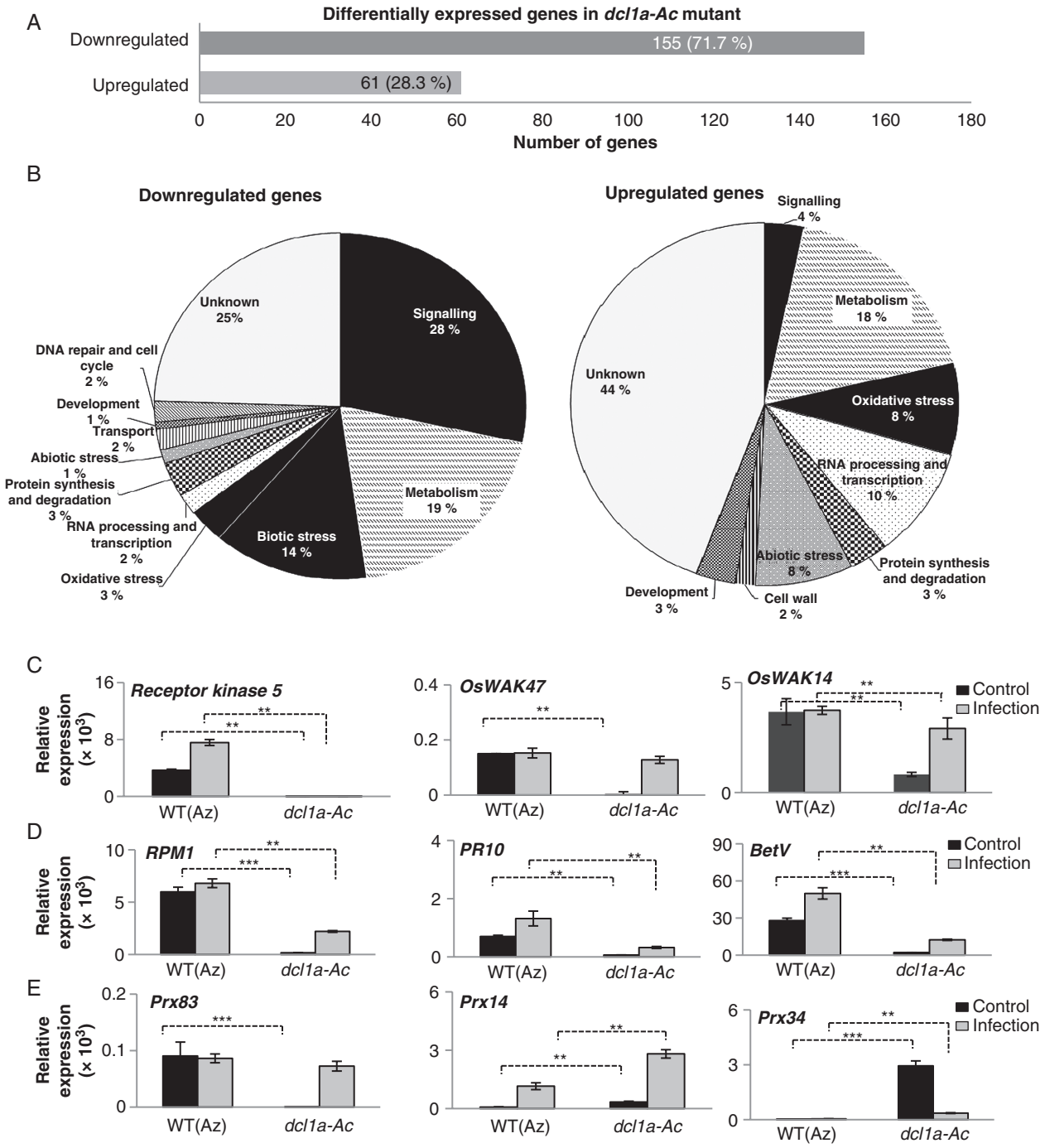

FIG. 3. Distribution and validation of differentially expressed genes in *dcl1a-Ac* plants. (A) Total number of differentially expressed genes in leaves of *dcl1a-Ac* plants compared with wild-type plants (downregulated and upregulated genes). (B) Functional categories of downregulated and upregulated genes in leaves of *dcl1a-Ac* plants. (C–E) Validation and fungal responsiveness of differentially expressed genes identified by RNA-Seq. Transcript levels were determined by RT-qPCR in leaves of control (non-infected) and *M. oryzae*-infected plants (at 72 hpi) (black and red bars, respectively). (C) *Receptor kinase 5* (Os09g37880), *OsWAK47* (Os04g30260) and *OsWAK14* (Os10g39680). (D) Disease resistance *RPM1* (Os11g12340), *PR10* (Os12g36860) and *Bet V* (PR10 family; Os12g36850). (E) *Prx83* (Os06g32990), *Prx14* (Os07g48050) and *Prx34* (Os03g02939). Four biological samples (including the same RNA samples used for RNA-Seq experiments for non-inoculated plants) and two technical replicates were examined (**$P \leq 0.01$; ***$P \leq 0.001$ by ANOVA).

They included several peroxidases and cytochrome P450 monooxygenase (CYP) genes (Supplementary Data Tables S4 and S5). CYPs catalyse the oxidation of many substrates for producing several metabolites, these enzymes being involved in the production of phytoalexins and phytohormones.

The expression of selected DEGs in *dcl1a-Ac* vs. wild-type plants was validated by RT-qPCR, including genes classified in the categories of 'signalling', 'biotic stress' and 'oxidative stress'. We further extended this analysis by determining the expression of these genes under non-infection and infection

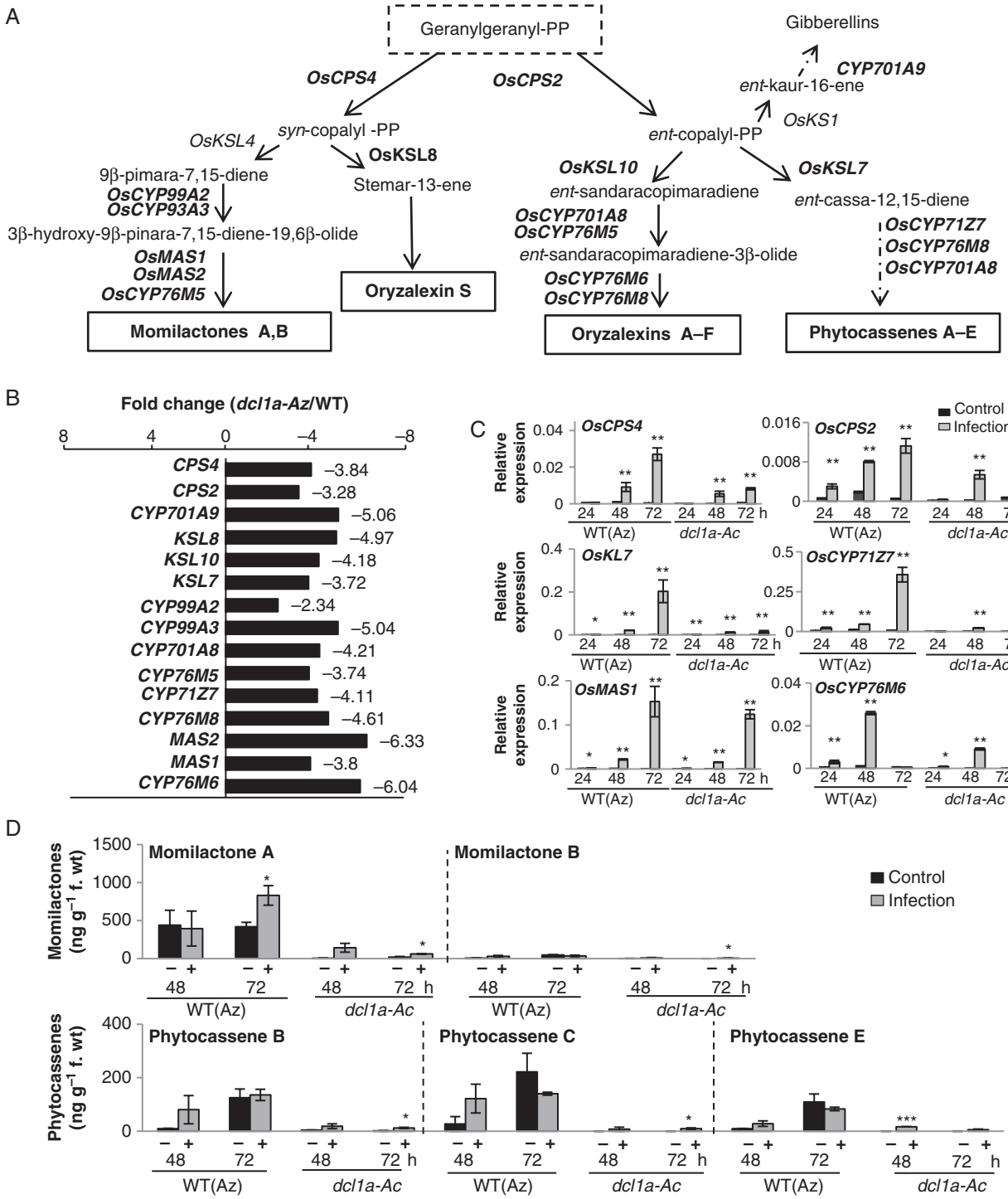

FIG. 4. Expression of genes involved in the biosynthesis of diterpenoid phytoalexins in *dcl1a-Ac* plants. (A) Biosynthetic routes of diterpenoid phytoalexins in rice. Genes with expression downregulated in *dcl1a-Ac* compared with wild-type plants are indicated in red. Diterpenoid phytoalexins are synthesized from geranylgeranyl diphosphate (geranylgeranyl-PP), which is sequentially cyclized by the diterpene synthases CPSs (copalyl diphosphate synthases) and KSLs (termed kaurene synthase-like because of their similarity to the corresponding enzyme in gibberellic acid biosynthesis), then converted to each phytoalexin by P450 monooxygenases (CYPs) and dehydrogenases. *OsCPS4* (*syn*-copalyl-diphosphate synthase 4, Os04g09900); *OsCPS2* (*ent*-copalyl diphosphate synthase 2, Os02g36210); *OsCYP93A3* (9β-pimara-7,15-diene oxidase, Os04g09920); *OsCYP99A2* (cytochrome P450, Os04g10160); *OsCYP76M5* (cytochrome P450, Os02g36030); *OsCYP701A8* (*ent*-sandaracopimaradiene 3-hydrolase, Os06g37300); *OsCYP71Z7* (*ent*-cassadiene C2-hydroxylase, Os02g36190); *OsCYP76M8* (oryzalexin D synthase, Os02g36070); *OsCYP76M6* (oryzalexin E synthase, Os02g36280); *OsCYP701A9* (*ent*-kaurene oxidase, Os06g37224); *OsKSL7* (*ent*-cassa-12-15-diene synthase, Os02g36140); *OsKSL10* (*ent*-sandaracopiramadiene synthase, Os12g30824); *OsKSL8* (stemar-13-ene synthase, Os11g28530); *OsMAS* (monilactone A synthase, *OsMAS1*, Os04g10000; and *OsMAS2*, Os04g10010). (B) Fold repression of expression (*dcl1a-Ac* vs. wild-type azygous plants) of genes involved in diterpenoid phytoalexin biosynthesis. (C) Expression of phytoalexin biosynthesis genes in wild-type (azygous) and *dcl1a-Ac* plants in response to *M. oryzae* infection (1×10^5 spores mL^{-1}) or mock inoculation (red and black bars, respectively) (*$P \le 0.05$; **$P \le 0.01$ comparing the indicated genotypes or condition by ANOVA). (D) Accumulation of diterpenoid phytoalexins is compromised in leaves of *dcl1a-Ac* plants. Three biological samples for each genotype and condition were examined (*$P \le 0.05$; ***$P \le 0.001$ by ANOVA).

conditions (e.g. 72 hpi with *M. oryzae*). In the absence of pathogen infection, the expression of receptor kinase genes (*Receptor kinase 5*, *OsWAK47* and *OsWAK14*), disease resistance (*RPM1*) and defence genes (*PR10* and *BetV*) was significantly lower in *dcl1a-Ac* than in wild-type plants (Fig. 3C, D). *Prx83* was downregulated in *dcl1a-Ac* plants, but two other peroxidase genes (*Prx14* and *Prx34*) were upregulated in the absence of pathogen infection (Fig. 3E). Together, these results indicate good correlation between RT-qPCR analysis and RNA-Seq data.

Upon pathogen challenge, the fungal responsiveness of *Receptor kinase 5* was compromised in *dcl1a-Ac* plants, whereas *WAK14*, *RPM1*, *PR10* and *BetV* reached a lower expression in *dcl1a-Ac* than in wild-type plants (Fig. 3C, D). *Prx14* and *Prx34* expression was more strongly induced by fungal infection in mutant than in wild-type plants (Fig. 3E). The lower induction of defence-related genes during pathogen infection (e.g. *Receptor kinase*, *R* and *PR* genes) and misregulation of genes involved in oxidative stress might well contribute to disease susceptibility in *dcl1a-Ac* plants.

DCL1 *activation leads to reduced expression of diterpenoid phytoalexin biosynthesis genes and compromises phytoalexin accumulation during pathogen infection*

Diterpenoid phytoalexins are the major phytoalexins in rice and are classified into five groups by the carbon skeleton: momilactones (A and B), oryzalexins (A–F), oryzalexin S, phytocassenes (A–E) and *ent*-10-oxodepressin (Ahuja *et al.*, 2012; Inoue *et al.*, 2013; Yamane, 2013). Our RNA-Seq analysis revealed the downregulation of genes involved in the biosynthesis of momilactones, oryzalexins and phytocassenes in *dcl1a-Ac* vs. wild-type plants (Fig. 4A, B; Supplementary Data Table S4). Upon pathogen challenge, diterpenoid phytoalexin biosynthetic genes were induced to a lower extent in *dcl1a-Ac* than in wild-type plants (Fig. 4C).

To investigate whether the downregulation of phytoalexin biosynthesis genes affects phytoalexin accumulation, we measured their levels in leaves of *dcl1a-Ac* and the wild type, under non-infection and infection conditions. Momilactone A and phytocassenes B, C and E accumulated at detectable levels in wild-type plants, but their accumulation was drastically reduced in *dcl1a-Ac* plants (Fig. 4D). It is of note that diterpenoid phytoalexins stayed almost at the basal level in *M. oryzae*-infected *dcl1a-Ac* plants (Fig. 4D), indicating that *DCL1a* activation, most probably, compromises diterpenoid phytoalexin production during pathogen infection. The antifungal activity of rice phytoalexins against *M. oryzae* has been described (Dillon *et al.*, 1997; Umemura *et al.*, 2003; Hasegawa *et al.*, 2010). Failure to accumulate major rice phytoalexins in *dcl1a-Ac* plants would then facilitate pathogen growth in these plants.

Reduced tolerance to oxidative stress in dcl1a-Ac *plants*

Reactive oxygen species are constantly being generated during normal plant growth and development, and an imbalance between ROS generation and safe detoxification generates oxidative stress in plants. Knowing that a substantial number of genes encoding enzymes that function in oxidation–reduction reactions (e.g. peroxidase, glutathione *S*-transferase and CYP genes) were misregulated in *dcl1a-Ac* plants (Supplementary Data Tables S4 and S5), we hypothesized that *DCL1* activation might affect ROS detoxification systems and/or ROS homeostasis. This, in turn, would affect redox-dependent cellular processes. ROS include superoxide anion ($O_2\cdot^-$), hydroxyl radical (OH·) and hydrogen peroxide (H_2O_2), with OH· being the most reactive molecule. Furthermore, $O_2\cdot^-$ and H_2O_2 can react with each other in the presence of metal ions, such as iron, to form the more reactive hydroxyl radicals OH· and OH^- via the Haber–Weiss and Fenton reactions. Hydroxyl radicals are highly reactive and interact with all biological molecules, leading to cellular damage.

To investigate whether transcriptome alterations caused by *DCL1* activation affect the host ROS detoxification system, we used the ROS-generating reagent, MV. This compound acts as an inhibitor of photosynthesis and promotes the formation of superoxide anion ($O_2\cdot^-$), which results in reduced chlorophyll content and discoloration in MV-treated leaves. Leaves of *dcl1a-Ac* plants were greatly affected by treatment with MV, and the chlorophyll content was markedly reduced in leaves of MV-treated *dcl1a-Ac* vs. MV-treated leaves of wild-type plants (Fig. 5A). Carotenoids are also able to detoxify ROS, and treatment with MV resulted in a higher reduction of carotenoid content in leaves of *dcl1a-Ac* than in those of wild-type plants (Fig. 5A, right panel). The reduction in chlorophyll content in *dcl1a-IR* plants with MV treatment was similar to that of its wild-type parental genotype Nipponbare, whereas the carotenoid level appears to be lower in *dcl1a-IR* plants than in its parental genotype (although differences in carotenoid level between *dcl1a-IR* and wild-type plants were not significant) (Supplementary Data Fig. S5A).

Finally, we examined $O_2\cdot^-$ accumulation in *dcl1a-Ac* and wild-type plants grown under controlled conditions (i.e. in the absence of pathogen infection). For detecting $O_2\cdot^-$ in rice leaves, we used NBT staining. Of note, *dcl1a-Ac* plants accumulated high levels of $O_2\cdot^-$ in leaves (Fig. 5B). As a control, leaves of wild-type plants (TN67) were treated with the ROS-generating agent H_2O_2 and examined for $O_2\cdot^-$ accumulation. In contrast to *dcl1a-Ac* plants, *dcl1a-IR* plants showed no visible alterations in $O_2\cdot^-$ accumulation (Supplementary Data Fig. S5B).

Altogether, these results indicate that *DCL1a* activation renders the plant more sensitive to oxidative stress caused by MV treatment and induces $O_2\cdot^-$ accumulation in leaves. Disturbed ROS production and/or scavenging mechanisms might interfere with the normal functioning of host antioxidant systems, which might explain, at least in part, the phenotype of disease susceptibility in *dcl1a-Ac* plants. Further studies are needed to clarify the exact biochemical mechanisms by which *DCL1a* activation stimulates $O_2\cdot^-$ accumulation and possibly alters ROS homeostasis in rice.

Characterization of the miRNAome in the dcl1a-Ac *mutant*

Knowing that the activity of DCL1 is required for processing of miRNA precursors and production of mature miRNAs, we reasoned that *DCL1a* activation might affect the rice miRNAome. Accordingly, we used small RNA sequencing for characterizing the miRNA population in leaves of wild-type and *dcl1a-Ac* plants. Two small RNA libraries, representing

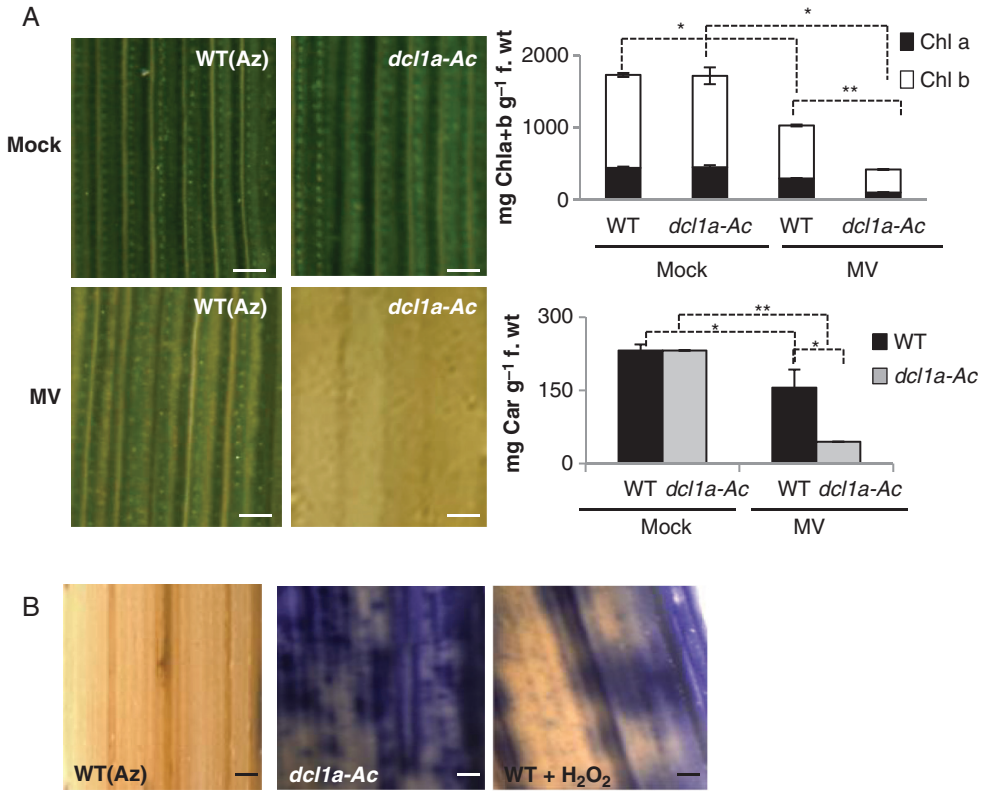

FIG. 5. Effect of treatment with methyl viologen (MV) and detection of superoxide ion in *dcl1a-Ac* plants. (A) Leaves of 3-week-old *dcl1a-Ac* (*Osdcl1a-Ac#1*) and wild-type (azygous) [WT(Az)] plants were treated with MV (10 μM). Right panels: quantification of chlorophylls (Chla+b) and carotenoids in mock-inoculated and MV-treated wild-type and *dcl1a-Ac* plants at 72 h after treatment. Data shown correspond to wild-type and *dcl1a-Ac* Plants. Scale bars = 250 μm. Data are the mean ± s.d. (**P* ≤ 0.05; ***P* ≤ 0.01 by ANOVA). (B) Detection of superoxide ion radicals ($O_2^{\cdot-}$) by nitroblue tetrazolium (NBT) staining. As a control, leaves were treated with H_2O_2 for 6 h. Three biological replicates with three technical replicates each were performed. Statistically significant differences were determined by one-way ANOVA.

independent biological replicates of each genotype, were prepared (the same biological samples as for mRNA transcript profiling). Illumina sequencing of small RNA libraries generated 36 million reads (15 and 21 million reads from wild-type and mutant plants, respectively) (Supplementary Data Table S6). After removing the adaptor sequences and sequences <15 bp, 32 million reads were obtained (14 and 18 million from wild-type and *dcl1a-Ac* plants, respectively). All unique sequences were aligned to the rice genome (Nipponbare reference genome MSU 7.0), and reads mapping to known non-coding RNA families (rRNAs, tRNAs, small nuclear RNAs and small nucleolar RNAs) were removed. The abundance of small RNAs was calculated as reads per kilobase million (RPKMs).

Consistent with the distribution of small RNA sizes typically observed in plants, the 24 nt small RNA class was the most abundant size class in both genotypes, with the 21 nt small RNAs forming a secondary peak (Fig. 6A). However, in *dcl1a-Ac* plants, the small RNA size distribution showed a substantial increase in the 21 nt small RNA class when considering both relative abundance and distinct reads (Fig. 6A). The observed increase in the 21 nt small RNA population might be due to DCL1 being involved in the production of almost all canonical 21 nt miRNAs.

A blast search against the miRNA database (miRBase release 21) allowed us to identify known miRNAs present in our small RNA sequencing data. Differentially expressed miRNAs were defined as those with changes in expression ≥1.5-fold (upregulated) or ≤0.5-fold (downregulated), and a *P*-value ≤0.05. By using these criteria, 90 miRNAs corresponding to 61 miRNA families were found to be differentially expressed in *dcl1a-Ac* plants (Supplementary Data Table S7). Although the most obvious trend that could be expected from transcriptional activation of *OsDCL1a* was an enrichment of miRNAs (which are likely to involve DCL1 in their biogenesis), differentially expressed miRNAs in *dcl1a-Ac* plants included both upregulated and downregulated miRNAs. Representative examples of differentially expressed miRNAs in *dcl1a-Ac* plants are shown in Fig. 6B. The expression of selected miRNAs was validated by stem–loop RT–PCR, including upregulated miRNAs (miR1431, miR1847, miR2865 and miR3982-3p) and downregulated miRNAs (miR393, miR396abc, miR398 and miR529b) in *dcl1a-Ac* plants (Fig. 6C, D). A concordance between the sequencing-based profiling and stem–loop RT–PCR was observed, which supports upregulation and downregulation of miRNAs in *dcl1a-Ac* plants.

According to the small RNA-Seq data and stem–loop RT–qPCR analysis, miR398 accumulation was lower in *dcl1a-Ac* than in wild-type plants (Fig. 6D), which agreed with a reduced level of miR398 precursor transcripts and increased accumulation of miR398-targeted superoxide dismutase 2 (*SOD2*) transcripts in *dcl1a-Ac* plants (Supplementary Data Fig. S6). Previous studies reported that transgenic rice lines overexpressing *MIR398* exhibit enhanced resistance to *M. oryzae* infection

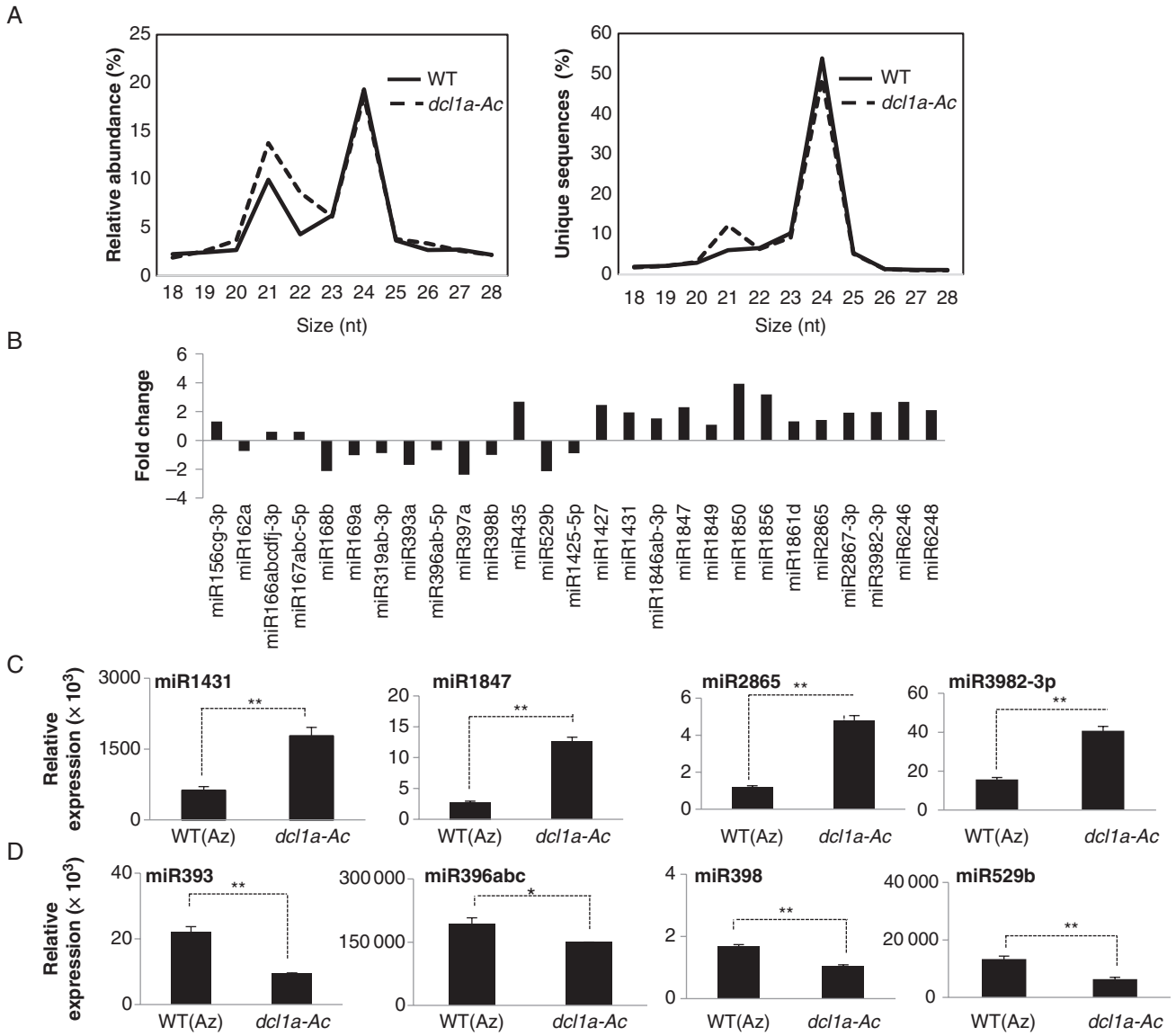

FIG. 6. Impact of *DCL1a* activation on the rice leaf miRNAome. (A) Abundance and unique small RNA sequences for each size class in leaves of wild-type (WT) and *dcl1a-Ac* plants. (B) Expression profiling of known miRNAs in *dcl1a-Ac* plants relative to wild-type plants. Representative examples are shown. Reads retrieved from the Illumina sequencing data sets for each family member were normalized to the total count of reads obtained in the corresponding library. Fold change was calculated on the basis of normalized reads (RPKM) (*dcl1a-Ac* vs. the wild type). (C, D) Stem–loop RT–PCR of miRNAs upregulated (C) and downregulated (D) in *dcl1a-Ac* plants (*$P \leq 0.05$; **$P \leq 0.01$ by ANOVA).

(Li *et al.*, 2014), which agrees with the observed phenotype of susceptibility to *M. oryzae* infection in *dcl1a-Ac* plants (with reduced miR398b accumulation as compared with the wild type).

Collectively, our results demonstrate that *DCL1a* activation results in important perturbations in the rice miRNAome. Presumably, perturbations in miRNA expression patterns might lead to altered expression of the corresponding target genes, which might contribute to susceptibility to *M. oryzae* infection in *dcl1a-Ac* plants.

DISCUSSION

In this work, we provide evidence that *OsDCL1a*, a component of the miRNA biogenesis pathway, functions as a negative regulator of the rice defence response. Several lines of evidence support this conclusion. First, mutant plants in which *OsDCL1a* expression is activated by T-DNA tagging were susceptible to infection by hemibiotrophic and necrotrophic fungal pathogens (*M. oryzae* and *F. fujikuroi*, respectively). Secondly, susceptibility to pathogen infection was accompanied by a weaker induction of defence-related marker genes (e.g. *OsPR1a* and *OsPBZ1*) during *M. oryzae* infection. Thirdly, genes involved in the production of diterpenoid phytoalexins were downregulated in *dcl1a-Ac* mutant plants. The finding that *OsDCL1a* expression itself is regulated, not only by *M. oryzae* infection but also by treatment with *M. oryzae* elicitors in wild-type plants, supports that *OsDCL1* is a component of PTI responses in rice. Also, the observation that *OsDCL1a-Ac* plants are susceptible to *M. oryzae* infection

agrees with previous results of resistance to *M. oryzae* infection in rice plants silenced for *Osdcl1a* expression by RNAi (*dcl1a-IR* plants; Zhang *et al.*, 2015). In contrast to *dcl1a-IR* plants showing developmental abnormalities (Liu *et al.*, 2005), the *Osdcl1a-Ac* mutant plants grew and developed normally.

It is of note that, whereas *DCL1a* appears to function as a negative regulator in rice immunity, this gene was reported to act as a positive regulator of immune responses in arabidopsis. Thus, arabidopsis *dcl1* mutants (*dcl1-7* and *dcl1-9* mutants) showed hypersusceptibility to infection by bacterial (*P. syringae*) and fungal (*B. cinerea*) pathogens (Navarro *et al.*, 2008; Seo *et al.*, 2013; Weiberg *et al.*, 2014). The regulatory activity of *DCL1a* in rice probably differs from its arabidopsis counterpart in determining the outcome of the plant–pathogen interaction. Alternatively, *DCL1a* might execute its regulatory role via different pathways depending on the type (fungal or bacterial pathogens) or lifestyle of the pathogen (biotrophs, hemibiotrophs or necrotrophs). Further investigation is needed to understand why altered *DCL1a* expression has a different impact on susceptibility/resistance to pathogen infection in rice and arabidopsis.

The comparison of the *dcl1a-Ac* and wild-type transcriptomes allowed us to identify *OsDCL1a*-mediated processes related to blast resistance. Under normal growth conditions, *R* genes (*RPM1* and Verticillium wilt disease resistance genes), and receptor kinase genes, including many *WAK* receptor kinase genes, were downregulated in *dcl1a-Ac* vs. wild-type plants. The involvement of these genes in resistance to pathogen infection is well documented in several plant species (Boyes *et al.*, 1998; Fradin *et al.*, 2009). In particular, WAK receptor kinases are known to regulate resistance to *M. oryzae* in rice (Li *et al.*, 2009). Downregulation of defence-related receptor kinases suggests that pathogen perception might be extensively affected in these plants, which might result in no detection of the pathogen, suppression of PAMP/DAMP-elicited defence responses or production of ineffective defence responses in *dcl1a-Ac* plants.

We also show that protective antioxidant systems do not function properly in *dcl1a-Ac* plants under normal growth conditions, as revealed by failure to alleviate MV-mediated oxidative stress. In line with this, *dcl1a-Ac* plants accumulate high levels of the superoxide ion $O_2{\cdot}^-$ in their leaves. Although $O_2{\cdot}^-$ is moderately reactive and does not cause extensive damage by itself, this radical undergoes transformation into the more reactive and toxic OH·, which is highly reactive and causes cellular damage. In the absence of infection, $O_2{\cdot}^-$ accumulation appears not to cause negative effects in plant growth. However, ROS production is also a typical response of plant tissues to pathogen attack (so-called oxidative burst). If ROS are not effectively detoxified in *dcl1a-Ac*, their overproduction during pathogen infection would facilitate oxidative damage in the host plant, which, in turn, would render the host plant more susceptible to pathogen infection. How *DCL1a* activation compromises ROS detoxification mechanisms deserve further investigation.

Even more interesting is the fact that genes involved in diterpenoid phytoalexin biosynthesis were the most predominant group of downregulated genes in *dcl1a-Ac* plants. The accumulation of momilactones and oryzalexins has been found critical to counteract *M. oryzae* infection in rice (Dillon *et al.*, 1997; Umemura *et al.*, 2003). Also, diterpenoid phytoalexin genes show faster and/or stronger induction in resistant than in susceptible rice cultivars (Hasegawa *et al.*, 2010; Bagnaresi *et al.*, 2012). The observed phenotype of disease susceptibility in *dcl1a-Ac* plants might then be attributed, at least in part, to downregulation of phytoalexin biosynthesis genes which is consistent with the observation that *dcl1a-Ac* plants fail to accumulate major diterpenoid phytoalexins also during pathogen infection. Together, these findings reinforce the notion that *OsDCL1a* is a negative regulator of immune responses in rice and also support a DCL1-mediated regulation of secondary metabolic defence pathways with relevance to pathogen resistance, most probably via regulation of miRNA accumulation.

Characterization of the miRNAome in leaves of *dcl1a-Ac* plants allowed us to identify alterations in the accumulation of specific miRNA families caused by *DCL1a* activation. The observed increase in the accumulation of miRNAs in *dcl1a-Ac* plants is consistent with targeted activation of *OsDCL1* in this mutant. Furthermore, we observed downregulation of different *MIR* genes in *dcl1a-Ac* plants, pointing to factors other than processing of miRNA precursors by *DCL1a* for the control of miRNA accumulation in rice. Several reasons can explain the otherwise paradoxical decrease in accumulation of miRNAs in *dcl1a-Ac* mutant plants. In addition to *OsDCL1a*, miRNA accumulation might be affected by the spatio-temporal expression pattern of other components of the miRNA biogenesis pathway. The abundance of mature miRNAs might be affected by precursor processing by DCL1 and also by miRNA stability (which also depends on miRNA modifications such as 3' end methylation or nucleotide addition), binding of miRNAs to AGO (which protects miRNAs from degradation) or sequestration by target mimic RNAs. As an additional complexity, evidence exists of autoregulatory feedback loops between miRNA and target genes, whereby target genes can control the level of an miRNA in addition to being regulated by it. The best known example is the transcriptional/translational interlocked feedback loop governing the miR168–AGO1 pair function, featuring miR168-guided cleavage of AGO1 and post-transcriptional stabilization of miR168 by AGO1 (Vaucheret *et al.*, 2006).

Among the miRNAs accumulating at a lower level in *dcl1a-Ac* than in wild-type plants were miR398 and miR393. A role for miR398 in protecting the plant against oxidative stress has been reported in arabidopsis and rice, and transgenic rice lines overexpressing miR398 exhibit resistance to *M. oryzae* (Jagadeeswaran *et al.*, 2009; Li *et al.*, 2014). Downregulation of miR398 in *dcl1a-Ac* plants is then consistent with a phenotype of susceptibility to *M. oryzae* in these plants. However, other studies in arabidopsis demonstrated that miR398 negatively regulates immune responses against bacterial pathogens (Li *et al.*, 2010). Regarding miR393, its overexpression in arabidopsis plants renders the host plant more resistant to biotrophic pathogens but more susceptible to the necrotrophic pathogens (Robert-Seilaniantz *et al.*, 2011). These findings indicate that certain miRNAs (e.g. miR393 and miR398) might function as positive or negative regulators of immune responses depending on the host plant and/or the pathogen lifestyle. Further investigation will reveal whether *dcl1a-Ac* plants respond in a different manner (e.g. susceptibility or resistance) to infection by pathogens other than *M. oryzae* and *F. fujikuroi*.

All these findings allowed us propose a working model for the regulation of defence responses to *M. oryzae* infection in rice plants by which *OsDCL1a* would mediate pathogen recognition processes and defence reactions (Fig. 7). According

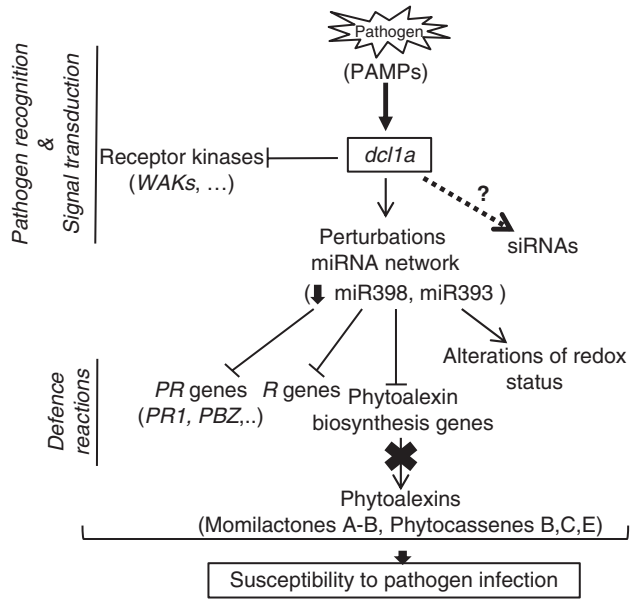

FIG. 7. Model for the role of *OsDCL1a* in disease susceptibility. In response to *M. oryzae* infection, *OsDCL1a* is activated. Pathogen-induced *OsDCL1a* expression, as well as *OsDCL1a* activation in *dcl1a-Ac* plants, would cause perturbations in the host miRNAome, which, in turn, would negatively affect pathogen recognition processes and expression of stress-responsive genes (such as *PR* genes). Additionally, *OsDCL1a* activation would negatively affect diterpenoid biosynthesis and alter ROS homeostasis, thereby compromising the ability of the host plant to mount a timely, targeted defence response.

to this model, pathogen perception would trigger *OsDCL1a* activation which in turn would have pleiotropic effects on the rice defence response. On the one hand, *OsDCL1a* activation would negatively affect PAMP recognition and signal transduction itself and, on the other hand, perturbations in the rice miRNAome caused by *Osdcl1a* activation would repress the pathogen-inducible host defence responses, such as *PR* expression and diterpene phytoalexin biosynthesis and accumulation, while altering the cellular redox status. All these factors would decrease the ability of the host plant to detect the invading pathogen and respond in a timely and appropriate manner. Moreover, even though DCL1 is predominantly involved in the production of miRNAs, we have examples of endogenous siRNAs that are generated by DCL1 activity in arabidopsis, such as certain natural antisense transcript-derived siRNAs and long siRNAs (Borsani *et al.*, 2005; Katiyar-Agarwal *et al.*, 2006). Therefore, the possibility that overexpression of *DCL1a* affects the production of other types of small RNAs which, in turn, might regulate rice defence responses should not be ruled out.

Collectively, results presented here expand our knowledge of the molecular mechanisms involved in blast resistance while providing evidence on the important role of *DCL1a* (and miRNAs) in rice immunity. In this respect, although a plethora of rice miRNAs have been found to be regulated by pathogen infection in rice, the biological function of most pathogen-regulated miRNAs remains largely unknown. Changes induced by *OsDCL1a* activation in the miRNAome are expected to cause altered expression of their corresponding target genes. To understand the impact of alterations in the miRNAome caused by *DCL1a* overexpression, and how these alterations might contribute to disease resistance in rice, a better knowledge of target genes for rice miRNAs is needed. Clearly, altered *OsDCL1a* expression and accompanying alterations in miRNA levels might affect diverse biological processes that are under miRNA regulation, which might then decrease the plant's ability to cope with pathogen infection. As *DCL1* is responsible for the majority of the miRNA processing in plants, a better understanding of the biological processes that are regulated by *DCL1a* will open up promising new avenues for the control of the rice blast disease. This is of paramount importance when considering that over half of the world's population relies on rice as the main source of calories and because the rice blast fungus *M. oryzae* has developed into a model system for the study of plant–pathogen interactions. The main challenge now is to elucidate how miRNAs function in regulating mechanisms involved in disease resistance in rice. Understanding these mechanisms will provide powerful tools for developing novel strategies to improve disease resistance in plants.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: analysis of *OsDCL1a* mutant plants. Figure S2: susceptibility of *dcl1a-Ac♯2* plants to infection by the fungal pathogen *M. oryzae*. Figure S3: expression of *OsDCL* genes in wild-type and *dcl1a-Ac* plants under normal conditions (non-infection). Figure S4: distribution of differentially expressed genes in *dcl1a-Ac* plants. Figure S5: effect of methyl viologen on chlorophylls and carotenoids, and detection of $O_2^{\cdot}$ in *dcl1a-IR* plants. Figure S6: accumulation of miR398 and *OsSOD2* transcripts in *dcl1a-Ac* plants. Table S1: sequences of oligonucleotides used. Table S2: T-DNA copy number in *dcl1a-Ac* mutants. Table S3: statistics of RNA-Seq in *dcl1a-Ac* and wild-type plants. Table S4: downregulated genes in *dcl1a-Ac* plants relative to wild-type plants sorted by functional category. Table S5: upregulated genes in *dcl1a-Ac* plants relative to wild-type plants sorted by functional category. Table S6: summary of small RNA sequencing data sets from wild-type and *dcl1a-Ac* plants. Table S7: list of miRNAs differentially accumulating in *dcl1a-Ac* plants.

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