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Repression of microRNA biogenesis by silencing of *OsDCL1* activates the basal resistance to *Magnaporthe oryzae* in rice

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ABSTRACT

The RNaseIII enzyme Dicer-like 1 (DCL1) processes the microRNA biogenesis and plays a determinant role in plant development. In this study, we reported the function of *OsDCL1* in the immunity to rice blast, the devastating disease caused by the fungal pathogen, *Magnaporthe oryzae*. Expression profiling demonstrated that different *OsDCLs* responded dynamically and *OsDCL1* reduced its expression upon the challenge of rice blast pathogen. In contrast, *miR162a* predicted to target *OsDCL1* increased its expression, implying a negative feedback loop between *OsDCL1* and *miR162a* in rice. In addition to developmental defects, the *OsDCL1*-silencing mutants showed enhanced resistance to virulent rice blast strains in a non-race specific manner. Accumulation of hydrogen peroxide and cell death were observed in the contact cells with infectious hyphae, revealing that silencing of *OsDCL1* activated cellular defense responses. In *OsDCL1* RNAi lines, 12 differentially expressed miRNAs were identified, of which 5 and 7 were down- and up-regulated, respectively, indicating that miRNAs responded dynamically in the interaction between rice and rice blast. Moreover, silencing of *OsDCL1* activated the constitutive expression of defense related genes. Taken together, our results indicate that rice is capable of activating basal resistance against rice blast by perturbing *OsDCL1*-dependent miRNA biogenesis pathway.

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1. Introduction

To survive attacks by diverse pathogens, plants have evolved multiple layers of immune systems [1–5]. Upon pathogen infection, the first layer of innate immune response is the recognition of pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) by the pattern recognition receptors (PRRs), which is referred to as PAMP/MAMP-triggered immunity (PTI) [6,7]. PTI is able to resist the invasion of the majority of potential pathogens. The second layer of innate immune response is activated on recognition of variable pathogen molecules so-called avirulence (Avr) effectors by host disease resistance (*R*) gene-encoding proteins, called effector-triggered immunity (ETI) [3,4]. ETI is much faster and quantitatively stronger than PTI and is often associated with a hypersensitive response (HR) to inhibit the spread of pathogen.

In addition to the immune system manifested in protein-protein interaction, small RNAs characteristic of RNA silencing machinery is also critical for the host immunity in plants. Since the discovery of the first microRNA, *miR393* in defense against bacterial pathogens, an increasing number of small RNAs including microRNAs (miRNAs) and small interfering RNAs (siRNAs) have been characterized to function in the immunity to a wide range of pathogens [8–14]. Increasing evidence demonstrates that the components of the small RNAs biogenesis pathway are involved in host immunity in plants. For example, different Dicer like (*DCL*) genes function differentially in defense against different pathogens. All four *DCL* genes are involved in mounting an antiviral defense in *Arabidopsis* [15]. The study confirmed that *DCL2* and *DCL4* function primarily whereas

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DCL3 has a minor role in the antiviral process. On the contrary, *DCL1* plays a negative role by downregulating the expression of *DCL4* and *DCL3*. *DCL1* is also involved in the anti-bacterial and anti-Agrobacterial defenses [12,16].

Rice blast, which is caused by the fungal pathogen *Magnaporthe oryzae* and is one of the most devastating diseases, can cause huge economic losses in epidemic years [17]. Over 20 blast resistance (*R*) genes from both race and non-race specific resistance loci have been characterized thus far [18,19]. Compared to race specific resistance, which is prone to erode due to extreme variability of rice blast pathogen, non-race specific resistance performs more stable and is of importance for the durable resistance breeding in rice. Interestingly, host miRNAs are also involved in the interaction between rice and rice blast. For example, *M. oryzae* derived elicitors can induce the expression of some miRNAs in rice [20]. Most recently, Li et al. [21] identified a group of rice miRNAs showing differential expression in either compatible or incompatible interactions between rice and rice blast by small RNA profiling. Moreover, transgenic plants with overexpression of individual *miR160a* and *miR398b* display enhanced resistance to the disease. These studies indicate that miRNAs play an important role in rice immunity against *M .oryzae*.

The *OsDCL1* gene is mainly involved in the biogenesis of miRNAs and loss of function of *OsDCL1* results in the significant reduction of miRNA accumulation [22]. Moreover, impaired miRNAs production in *OsDCL1* RNAi lines displays pleiotropic developmental defects, indicating that *OsDCL1* is required for the development. Interestingly, the function of *OsDCL1* with respect to miRNA biogenesis and development shares good comparability with *DCL1* in *Arabidopsis*, indicating that these two *DCL1* genes are orthologous to each other [22,23]. It has been demonstrated that *DCL1* in *Arabidopsis* plays distinctive roles to different pathogens. It functions as a positive component in resistance to bacterial pathogen, *Pseudomonas syringae* albeit a negative one to *Agrobacterium tumefaciens* [12,24]. However, the function of *OsDCL1* in rice immunity has not been described. In this study, we found that the abundance of *OsDCL1's* transcripts was reduced upon the challenge of rice blast pathogen, indicative of its engagement in immunity to this fungal pathogen. Indeed, the resultant *OsDCL1*-silencing mutants via double strand RNAi approach showed enhanced resistance to virulent isolates, providing evidence that *OsDCL1* functions as a negative component in rice immunity against rice blast. Expression profiling of small RNA species and reporter genes was further carried out, which shed some light upon the mechanism of enhanced resistance mediated by the silencing of *OsDCL1* in rice.

2. Materials and methods

2.1. Plant materials and growth conditions

The *OsDCL1* RNAi lines in Nipponbare (NPB) were generated using pANDA vector system as described previously [25]. In brief, a portion of *OsDCL1* coding sequence (CDS) was amplified with the primer pairs: 5′ccgctcgagcgg GAGCAGAATGATGAAGGTGAA3′ and 5′cgggatcc ATGCTTTTGCGGGATCCCAA3′ [22] by RT-PCR. This fragment was then cloned into pANDA vector using Gateway cloning system (Invitrogen, USA). The derived RNAi vector, designated as pANDA-DCL1 was used for generating the *OsDCL1*-RNAi mutant lines. The *Piz-t* transgenic plants in NPB were described previously [26]. Rice seedlings were maintained in a growth chamber at 25 °C and 80% relative humidity with the cycle of 14 h light/10 h dark. For observing the root structure, rice seedlings were grown in 1/2 Murashige and Skoog (MS) medium for 3 weeks with the cycle of 12 h light/12 h dark.

2.2. Spore preparation, inoculation, and resistance quantification

The rice blast isolates used in the study were grown on complete medium (CM) for 12 days at 28 °C with the cycle of 12 h light/12 h dark. The spore suspension at the concentration of approximately 2×10^5 spores ml^{-1} was used for inoculation and the diseased plants were evaluated as described previously [27]. For punch inoculation, six-week-old rice plants were used by adopting the method described previously [28]. In brief, 10 μl of spore suspension (5×10^5 spores ml^{-1}) was added to the rice leaves which were lightly wounded by a mouse ear punch. Both sides of the inoculated area were sealed with scotch tape. For determination of biomass of *M. oryzae*, a small piece of infected rice tissue (~3 cm × 1 cm) was cut for DNA extraction. The DNA was treated with 1 μl RNase A (10 mg ml^{-1}) to remove RNA. DNA-based qPCR was performed in a CFX96 Real-Time System (Bio-Rad, USA) with three replicates. The rice genomic ubiquitin primer and *M. oryzae* Pot2 primers (Table S1) were used for calculating the relative fungal growth as a ratio (Mo-Pot2/Os-UG). The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative expression as described previously [29].

2.3. Observation of infectious growth of rice blast, determination of hydrogen peroxide accumulation, and visualization of cell death in rice sheath

To observe the infectious growth of rice blast, rice sheath penetration assay was conducted as described previously [30]. In brief, excised sheath from 4-week-old rice seedlings (NPB and *OsDCL1* RNAi lines) was inoculated with spore suspension of virulent strains (1×10^4 spores ml^{-1} for each strain). The infectious hyphae in inner leaf sheath cells were observed at 24 and 48 h post inoculation (hpi) under a microscopy. To determine the hydrogen peroxide accumulation, the infected rice sheath at 24 and 48 hpi as described above was harvested and stained with 1 mg ml^{-1} diaminobenzidine (Sigma, USA) as described previously [30]. Then sheath was cleared with acetic acid-ethanol (4:96, v/v) solution for 2 h and observed under a light microscope. To visualize the cell death, the infected rice sheaths at 30 hpi as described above were harvested and sliced for staining with trypan blue staining buffer [10 g phenol, 10 ml glycerol, 10 ml lactic acid, 10 ml distilled water, 0.02 g Trypan blue (Sigma, USA), and 60 ml ethanol (96%)] as described previously [31]. The stained rice sheaths were cleared with chloral hydrate solution and observed under a microscope.

2.4. Expression analysis of OsDCLs and defense related genes

Total RNAs from leaves samples of NPB, *OsDCL1* RNAi lines and infected ones at different time points were collected and processed for RNA isolation using Trizol reagent as indicated in the manual (Invitrogen, USA). RNAs were treated with DNase RQ1 (Promega, USA) prior to RT-PCR analysis. 2 μg total RNAs were reverse transcribed by following the protocol of the Reverse Transcription System Kit (Promega, USA). Subsequent PCR cycle was conducted for semi-quantifying the expression of *OsDCL1* with the primer pair of OsDCL1-RT-F (5′-GAGCAGAATGATGAAGGTGAA-3′) and OsDCL1-RT-R (5′-ATGCTTTTGCGGGATCCCAA-3′). Rice *actin* gene was used for the control with the primer pair of OsActin-F (5′-CCTGCTATGTACGTCGCCATC-3′) and OsActin-R (5′-CCGCAGCTTCCATTCCTATGA-3′). For quantitative analysis, the subsequent PCR cycle was carried out using SYBR premix Ex Taq (TaKaRa, China) in a CFX96 Real-Time System (Bio-Rad, USA) with three replicates. Gene-specific primers were designed using Primer3 software (http://fokker.wi.mit.edu/primer3) and their sequences were shown in Table S1. The relative expression level of each gene was calculated by comparing to the *Ubiquitin* gene (*OsUG*) using the $2^{-\Delta\Delta Ct}$ method described previously [29].

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