Osa-miR535 targets SQUAMOSA promoter binding protein-like 4 to regulate blast disease resistance in rice

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SUMMARY

Many rice microRNAs have been identified as fine-tuning factors in the regulation of agronomic traits and immunity. Among them, Osa-miR535 targets SQUAMOSA promoter binding protein-like 14 (OsSPL14) to positively regulate tillers but negatively regulate yield and immunity. Here, we uncovered that Osa-miR535 targets another SPL gene, OsSPL4, to suppress rice immunity against Magnaporthe oryzae. Overexpression of Osa-miR535 significantly decreased the accumulation of the fusion protein SPL4TBS-YFP that contains the target site of Osa-miR535 in OsSPL4. Consistently, Osa-miR535 mediated the cleavage of OsSPL4 mRNA between the 10th and 11th base pair of the predicted binding site at the 3′ untranslated region. Transgenic rice lines overexpressing OsSPL4 (OXSPL4) displayed enhanced blast disease resistance accompanied by enhanced immune responses, including increased expression of defense-relative genes and up-accumulated H2O2. By contrast, the knockout mutant osspl4 exhibited susceptibility. Moreover, OsSPL4 binds to the promoter of GH3.2, an indole-3-acetic acid-amido synthetase, and promotes its expression. Together, these data indicate that Os-miR535 targets OsSPL4 and OsSPL4-GH3.2, which may parallel the OsSPL14-WRKY45 module in rice blast disease resistance.

Keywords: Oryza sativa, Osa-miR535, transcription factor, OsSPL4, Magnaporthe oryzae, GH3.2.

INTRODUCTION

The microRNAs (miRNAs), a class of small non-coding RNAs with 20–24 nucleotides, are encoded by miRNA genes (MiRs) and mature subsequently by a series of processes including splicing and methylation (Diederichs et al., 2009; Li, Jeyakumar, et al., 2019b). The mature miRNAs are loaded to ARGONAUTES to form miRNA-induced gene silencing complexes, which target the sequence of mRNAs guided by miRNA in a sequence-complementary manner, and suppress gene expression via transcript cleavage or translational inhibition (Li, Jeyakumar, et al., 2019b; Yu et al., 2017). Subsequent to the miRNA being first identified in nematodes in 1993 (Lee et al., 1993; Wightman et al., 1993), it was widely found in most forms of life (Pfeffer et al., 2004). miRNA has been regarded as one of the essential ways for regulating plant growth, development, and immunity (Tang and Chu, 2017).

In rice, over 700 mature miRNAs have been identified in the genome (Tang and Chu, 2017) and at least 70 miRNAs respond to Magnaporthe oryzae (M. oryzae) infection, meaning that many miRNAs are involved in rice immunity (Li et al., 2014). Among them, 16 miRNAs have been characterized as fine regulators affecting rice blast resistance (Feng et al., 2021; Wang et al., 2021), as positive regulators, such as Osa-miR1320, Osa-miR160a, Osa-miR162a, Osa-miR166k-h, Osa-miR398b, and Osa-miR7695 (Li et al., 2014; Li, Jeyakumar, et al., 2019b; Quoc et al., 2019; Salvador-Guirao et al., 2018; Sánchez-Sanuy et al., 2019; Wang et al., 2021), or as negative regulators, such as Osa-miR156, Osa-miR164a, Osa-miR167d, Osa-miR168, Osa-miR169a, Osa-miR319b, Osa-miR396, Osa-miR444b.2,
Osa-miR1873, and Osa-miR535 (Chandran et al., 2019; Li et al., 2017; Wang et al., 2016, 2018, 2021; Zhang et al., 2018, 2020; Zhao et al., 2019; Zhou et al., 2019). Further investigation revealed that Osa-miR535 targets the SQUAMOSA Promoter Binding Protein Like 14 (OsSPL14) (Wang et al., 2021). OsSPL14 is also known as Ideal Plant Architecture 1 (IPA1), which promotes grain yield and blast resistance via boosting the expression of downstream transcription factor genes DEP1 and WRKY45, respectively (Jiao et al., 2010; Miura et al., 2010; Shimono et al., 2007). Consistently, over-expressing Osa-miR535 (OX535) results in compromised rice blast resistance accompanied by less filled grains, smaller panicles, and more tillers compared to wild-type (WT), indicating that the Osa-miR535-OsSPL14 module acts in the coordination of growth, development, and immunity (Wang et al., 2021).

The mature sequences of Osa-miR535 share high similarity with Osa-miR156 and Osa-miR529, although they are derived from different MIR genes (Sun et al., 2019; Wang et al., 2021). They target the same transcription factor gene family of SPL that is comprised of 19 members in rice to regulate plant growth and immunity (Sun et al., 2019; Xie et al., 2006; Wang et al., 2015; Zhang et al., 2020). OsSPL7, OsSPL14, and OsSPL17 are fine-tuned by Osa-miR156 and Osa-miR529 for an optimal panicle size of rice (Wang et al., 2015; Yue et al., 2017). In addition, Osa-miR535 is highly expressed in panicles, which leads to the suppression of SPL genes, including OsSPL7, OsSPL12, OsSPL14, and OsSPL16 (Sun et al., 2019; Wang et al., 2021). Osa-miR156 has been identified as a negative regulator in rice immunity against M. oryzae infection (Zhang et al., 2020). Over-expressing Osa-miR156 enhances the susceptibility to blast disease in rice. Conversely, over-expressing a target mimic of Osa-miR156 (MIM156) confers higher resistance compared to WT. Further investigation revealed that Osa-miR156 specifically targets the OsSPL14 gene and downregulates its expression resulting in compromised blast resistance (Zhang et al., 2020). In addition, Osa-miR156 targets OsSPL7 to regulate rice resistance to Xanthomonas oryzae pv. oryzae (Xoo) (Liu et al., 2019). However, it is unclear whether Osa-miR535 targets the other SPL genes to regulate rice immunity.

In the present study, based on the expression pattern of SPL genes in WT, OX535 and MIM535, we identified that OsSPL4 is another target of Osa-miR535 and necessary for rice blast resistance. Further investigation of the mechanism identified the pathway of OsSPL4-GH3.2 paralleling the OsSPL14-WRKY45 module regulated by Osa-miR535 in rice immunity.

**RESULTS**

Osa-miR535 negatively regulates rice immune responses

In a previous study, transcription analysis revealed that Osa-miR535 is one of the miRNAs responsive to the infection of M. oryzae in both the susceptibility accession Lijiang xin Tuan Heigu (LTH) and the resistance accession International Rice Blast Line Pyricularia-Kanto51-m-Tsuyuake (IRBLkm-Ts) (Li et al., 2014). Here, the expression pattern of Osa-miR535 was further investigated by a quantitative polymerase chain reaction with reverse transcription (RT-PCR), which confirmed that Osa-miR535 was induced by M. oryzae infection in LTH at 12 and 48 h post inoculation (h.p.i.) compared to mock treatment. However, it was suppressed in IRBLkm-Ts (Figure S1a).

Intriguingly, the Osa-miR535 level was significantly suppressed at the early stage upon chitin treatment in both LTH and IRBLkm-Ts, although its expression was still higher in LTH than that in IRBLkm-Ts (Figure S1b), indicating that miR535 may be involved in rice immunity. To confirm this hypothesis, we generated the transgenic lines overexpressing Osa-miR535 (OX535) and expressing a target mimic of Osa-miR535 (MIM535) (Figure S2), respectively. The even mixture of M. oryzae strains G28, a GFP-tagged strain Zhong 8-10-14, and 97-27-2 were used for the disease assay via spraying inoculation. Compared with WT, OX535 lines showed more susceptibility; by contrast, MIM535 lines enhanced the resistance (Figure 1a,b), suggesting that Osa-miR535 negatively regulates rice blast resistance. This is consistent with the conclusion obtained from pouching inoculation in a recent study (Wang et al., 2021).

To investigate how Osa-miR535 affects rice immunity, we examined the expression of defense-related marker genes, pathogenesis-related gene 1a (PR1a) and ENT-KAURENE synthase 4 (K54), in WT, OX535, and MIM535 lines upon M. oryzae strain G28 infection (Park et al., 2012; Zhang et al., 2015). PR1a and K54 were significantly induced by M. oryzae in all lines. Compared with WT, the induction was enhanced in MIM535 lines but suppressed in OX535 lines at 48 h.p.i. (Figure 1c). In addition, the K54 gene was significantly induced at 6 h post chitin treatment (hpt) in both WT and MIM535 lines (Figure S3a). The NAC-domain-containing protein 4 (NAC4), an earlier induced basal defense-related gene (Park et al., 2012), was also significantly upregulated in both WT and MIM535 begin at 1 hpt, which achieved a peak at 3 hpt, and then was reduced at 6 hpt. The induction of NAC4 was higher in MIM535 lines than that in WT at 1 and 3 hpt (Figure S3b). These data suggest that Osa-miR535 negatively regulates the defense-related genes at the early stage of M. oryzae infection.

Next, we examined the pathogenesis of M. oryzae strain GZ8 expressing a GFP in the sheath cells. Compared with WT, the GZ8 grown faster in OX535 but slower in MIM535 at both 24 and 48 h.p.i. (Figure 1d). Statistically, although approximately 40% germinated spores formed the invasive hyphae in the local cell (the first cell), 3% extended into the neighbor cells (the second cells) in WT at 24 h.p.i. Over
60% hyphae invaded in the first cell and 10% of them already grew in the second cells in OX535, although only 10% invaded in the first cell without second cell invasion in MIM535 (Figure 1e). This trend of G28 strain grown in different lines was continued at 48 h.p.i. Over 8% of hyphae extended into the neighbor cells of the second cells (the third cells or more) in WT, and 16% occurred in OX535, although no third or more cells invasion occurred in MIM535 (Figure 1e). Simultaneously, we examined H$_2$O$_2$ accumulation at 48 h.p.i. and found that the infected cells were stained darker brown in MIM535, but lighter in OX535, compared to WT (Figure S4). These results indicate that Osa-miR535 facilitates _M. oryzae_ invasion and suppresses H$_2$O$_2$ accumulation at the early infection stage.
The expression of several SPL genes is affected by Osa-miR535

For screening the target genes of Osa-miR535, the miRNA target prediction online tool, psRNATarget website (http://plantgrn.noble.org/psRNATarget), was used and predicted 14 potential targets with strict parameters. Among them, 12 potential target genes were members of the SPL gene family, including OsSPL2, 3, 4, 7, 11, 12, 13, 14, 16, 17, 18, and 19. Several SPL genes have been identified as targets of Osa-miR156 and Osa-miR299 that show high conservation with Osa-miR535 in the amino acid sequence (Figure S5) (Wang et al., 2015; Xie et al., 2006; Yue et al., 2017). OsSPL14 has been identified as the target of Osa-miR535 (Wang et al., 2021). Therefore, we examined the expression of these SPL genes, except OsSPL14, in WT, MIM535, and OX535, and found that the expression of OsSPL3, 4, 7, 11, 12, and 16 was largely decreased in OX535 lines and increased in MIM535 lines (Figure 2a). Among them, the expression of OsSPL4 was the greatest by Osa-miR535. Therefore, we further investigated whether OsSPL4 is another SPL gene targeted and negatively regulated by Osa-miR535.

OsSPL4 gene is targeted by Osa-miR535

It is well known that plant miRNAs are highly complementary to their binding sites in target genes for regulating the mRNA level by cleavage or inhibiting translation (Rogers et al., 2017). Therefore, a reporter assay was used to investigate whether mRNA of OsSPL4 was negatively regulated by Osa-miR535. We made a construct expressing YFP fused with the predicted binding site of Osa-miR535 in OsSPL4 (SPL4TBS-YFP) and used it in the reporter system co-expressing with or without 35S::miR535. The highest YFP intensity was in the region transient-expressing SPL4TBS-YFP alone, whereas YFP intensity was decreased as a result of the rise Osa-miR535 in co-expression. However, this trend was not observed in the control co-expressing YFP and Osa-miR535 (Figure 2b). This observation suggested that Osa-miR535 binds on the predicted site of OsSPL4 and suppresses the expression of the fusion gene. We mapped the directed cleavage site of Osa-miR535 in OsSPL4 mRNA using the 5’ RNA ligase-mediated rapid amplification of cDNA ends (5’ RLM-RACE) technique in OX535 plants. The results revealed that cleavage occurred between the 10th and 11th base pair in the predicted Osa-miR535 binding site in the 3’ untranslated region of OsSPL4 (Figure 2c), indicating that OsSPL4 can be precisely cleaved in vivo by Osa-miR535. All of these data suggest that OsSPL4 is an authentic target gene and is directly cleaved by Osa-miR535 for negatively regulating its expression.

OsSPL4 enhances rice blast disease resistance

To further investigate whether OsSPL4 is involved in Osa-miR535-mediated rice blast susceptibility, we examined OsSPL4 expression in Nipponbare leaves and found that it was significantly induced by M. oryzae inoculation (Figure 3a). Therefore, we made transgenic plants with over-expressed OsSPL4 (OXSPL4) and knocked out OsSPL4 (osspl4 mutant) via CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 gene-editing.
OsSPL4 localizes in the nucleus and exists ubiquitously in different rice tissues

To further investigate the function of OsSPL4, we first characterized the subcellular localization and expression pattern of OsSPL4. Bioinformatics analysis indicated that OsSPL4 has a putative nuclear localization signal. To validate the localization of OsSPL4, we fused OsSPL4 to the N-terminal of YFP (35S::SPL4-YFP), followed by co-expression with the construct expressing 35S::RFP-NLS, a nuclear marker, in Nicotiana benthamiana. Subcellular localization analysis showed that SPL4-YFP fusion protein localizes in the nucleus (Figure 5a).

RT-qPCR was employed to investigate the temporal and spatial expression pattern of OsSPL4. OsSPL4 had the highest expression level in the young panicle and maintained a higher expression in root, leaf, and culm, but a low expression in flowers and coleoptile (Figure 5b). These data suggested that OsSPL4 is widely expressed in different organs of rice, but with a bias in panicle, leaf, and root. In addition, we also investigated OsSPL4, a homologue gene of OsSPL4 involved in rice immunity, and their cognate miRNAs, Osa-miR535 and Osa-miR156. Osa-miR535 is highly expressed in flower, and Osa-miR156 and OsSPL4 are expressed to a lower extent in leaf and culm (Figure 5b), indicating the spatiotemporal difference between these two miRNAs and SPLs.

OsSPL4 protein directly binds the promoter of GH3.2 and enhances its expression

SPL genes encode a class of plant-specific transcription factors that have important roles in development and
immunity in Arabidopsis and rice (Birkenbihl et al., 2005; Cardon et al., 1999; Liu et al., 2019; Wang et al., 2018). To further identify the downstream component regulated by Osa-miR535-OsSPL4 module, we performed the prediction for screening the binding targets of OsSPL4 via the online tool PLACE (https://www.dna.affrc.go.jp/PLACE) and found that GH3.2 is one of the candidate genes. GH3.2 encodes an indole-3-acetic acid (IAA)-amido synthetase, which acts in IAA homeostasis and enhances rice immunity against M. oryzae (Fu et al., 2011). In addition, bioinformatic analysis revealed that there are several GTAC motifs in the GH3.2 promoter (Figure S8). The GTAC motif was reported as a site recognized and bonding specifically by the SQUA promoter-binding (SBP)-domain of SPL proteins (Birkenbihl et al., 2005). We first examined the GH3.2 expression pattern in LTH and IRBLkm-Ts upon M. oryzae inoculation. The expression of GH3.2 was significantly induced in both LTH and IRBLkm-Ts at 12 and 24 h.p.i., and it was always expressed to a greater extent in IRBLkm-Ts compared to LTH (Figure 6a). In addition, GH3.2 was significantly upregulated in both MIM535 and OXSPL4 compared to WT (Figure 6b). Other GH3 members did not show an apparent difference in WT, OXSPL4, and osspl4 (Figure S9). These data suggest that the GH3.2 might function downstream of OsSPL4.

To further investigate whether OsSPL4 bind to the GH3.2 promoter, we used a yeast-one-hybrid (Y1H) assay and a Dual-Luciferase Reporter Gene Assay Kit (Beyotime, Shanghai, China) to assess the binding stability of OsSPL4 protein on the GH3.2 promoter. As shown in Figure 6c,
OsSPL4 could bind to the GH3.2 promoter in yeast cells. Moreover, OsSPL4 was identified to enhance the expression of the luciferase gene that was expressed by the GH3.2 promoter in N. benthamiana (Figure 6d). However, the binding was disrupted in the mutated OsSPL4 protein that lacked its C-terminal containing the SBP domain (SPL4\textsuperscript{D\textsubscript{C}}) or the mutated GH3.2 promoter missing the GTAC core motif (GH3.2 m) (Figure 6c), indicating that OsSPL4 binds to the GH3.2 promoter, which is dependent on the recognition between the SBP domain and the GTAC motif.

Then, we performed an electrophoretic mobility shift assay (EMSA) to investigate the direct recognition between OsSPL4 and the GH3.2 promoter. The SBP domain of OsSPL4 was fused with GST at the N-terminal (GST-SPL4SBP) and the fusion protein was expressed and purified from prokaryotic protein expression system. The EMSA showed that GST-SPL4 SBP bound to the probe covering GTAC core motifs of the GH3.2 promoter (Figure 6e). Such binding was largely blocked by the unlabeled GH3.2 pro, but not by mutated GH3.2 promoter, which lacked a GTAC motif (GH3.2 pro-mutant) (Figure 6e), indicating that the GTAC motif in the GH3.2 promoter is essential for OsSPL4 binding. Intriguingly, OsSPL4 could not bind to the WRKY45 promoter that was bonded by OsSPL14 (Figure 6e), although it positively regulated the expression of WRKY45 (Figure S10).

The EXPa genes encode cell wall-loosening expansin proteins, resulting in the cell wall (i.e. the physical barrier of plant cell) being vulnerable to pathogens (Ding et al., 2008; Fu et al., 2011). The EXPa genes are downstream components of the IAA signal pathway and negatively regulated by GH3.2 (Fu et al., 2011). Therefore, we considered whether the EXPa genes are regulated by OsSPL4 that functions upstream of GH3.2. The expression of EXPa family genes was investigated in OsSPL4 transgenic lines, and EXPa1 and EXPa5 were significantly decreased in OXSPL4 lines with increasing GH3.2 (Figure 6b and Figure S11). In conclusion, these data suggest that GH3.2 is regulated by OsSPL4, in parallel with the OsSPL14-WRKY45 module targeted by Osa-miR535 for regulating rice immunity against M. oryzae (Figure 7).

**DISCUSSION**

miRNA-mediated gene silencing plays a vital role in plant immunity against pathogen infection. Several miRNAs are reported to act positively or negatively in rice immunity (Li, Jeyakumar, et al., 2019b). Osa-miR535 has been identified to compromise rice blast resistance by suppressing the expression of OsSPL14 gene (Wang et al., 2018). In the present study, we found that Osa-miR535 also suppresses OsSPL4, which in turn regulates the expression of GH3.2, a key regulator for immunity and IAA homeostasis (Figure 7). Our discovery highlights that Osa-miR535 employs two SPL genes to manipulate, in parallel, rice immunity against M. oryzae.

SPL family genes encode plant-specific transcription factors (Hua et al., 2019) that bind with the DNA sequence carrying a GTAC core motif via their conserved SBP domain (Birkenbihl et al., 2005). In Arabidopsis, SPL7 binds to the GTAC motif in the promoter of miR398, which was reported to elevate the expression of miR398 and downregulate the expression of copper/zinc superoxide dismutase.
dismutase in copper deficiency conditions (Yamasaki et al., 2009). In rice, OsSPL14 binds the GTAC motif in the promoter of \textit{DEP1} to regulate tiller development. In the present study, we used this theory to predict the candidate target genes of SPL4. It is fast and specific with respect to identifying that OsSPL4 binds the GTAC motif in the GTAC motif in the promoter of GH3.2 to regulate its expression (a) The expression of GH3.2 was induced by Magnaporthe oryzae infection in LTH and IRBLkm-Ts. (b) The expression of GH3.2 was enhanced in MIM535 and OXSPL4. (c) OsSPL4 binds to the GH3.2 promoter in a Y1H assay. (d) OsSPL4 enhanced the activity of the GH3.2 promoter in the dual-LUC assay. The Firefly/Renilla ratio indicates the relative luciferase activity. Error bars indicate the SD (n = 3). A double asterisk (**) indicates a significant difference (P < 0.01) via Student’s t test compared to the control. (e) SBP domain of OsSPL4 binds to the region harboring GTAC motif in the GH3.2 promoter in EMSA assay.

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its expression is induced by resistance to pathogens of bacterial blight and fungal blast. Finding out the target genes of SPL family proteins.

GH3.2 – Figure 6c

GH3.2 promoter of Figure 7.

Figure 7. A working model for Osa-miR535–OsSPL4–GH3.2 module in rice immunity. OsSPL4 is one of target genes of Osa-miR535. OsSPL4 protein binds to the GTAC motif in the GH3.2 promoter via its SPB domain. GH3.2 is an IAA-amido synthetase that catalyzes free IAA to IAA-amino acid as an inactive form to suppress IAA signaling. Upon Magnaporthe oryzae infection, Osa-miR535 is suppressed, which releases the suppression of OsSPL4 by Osa-miR535. The increased OsSPL4 promotes the expression of GH3.2, which breaks the homeostasis of IAA, causing a decrease in free IAA. Subsequently, the IAA signal pathway is finally suppressed to enhance rice blast resistance.

promoter of GH3.2 and enhances its expression (Figure 6c–e), suggesting the GTAC motif is a key pointer for finding out the target genes of SPL family proteins.

GH3.2 has been identified to confer broad-spectrum resistance to pathogens of bacterial blight and fungal blast. Its expression is induced by M. oryzae infection. Overexpressing GH3.2 enhanced (but suppressing it via RNAi reduced) the resistance to rice blast (Fu et al., 2011). GH3.2 encodes an IAA-amido synthetase to affect IAA homeostasis, which catalyzes free IAA conjugating with amino acids in the storage pool. It is still unknown whether the IAA homeostasis affected by GH3.2 is only a biological phenomenon or is also the reason for GH3.2 mediated rice immunity. Some studies have identified that IAA biosynthesized by pathogen or plants can facilitate pathogenesis with compromised plant immune responses (Naseem et al., 2015). The extent of, or the increase, in plant immunity modulated by IAA remains unknown. Intriguingly, IAA-ASP treatment increases the pathogen progress of Botrytis cinerea and Pst DC3000 in Arabidopsis (González-Lamothe et al., 2012), suggesting that IAA-ASP negatively regulates plant immunity. Nevertheless, overexpressing GH3.2 enhances rice blast resistance accompanied by increased IAA-ASP, suggesting that IAA homeostasis may simply be a biological phenomenon. GH3.2 could employ another pathway, such as increased expansins (Figure 3b and Figure S11), but not IAA homeostasis to boost rice immunity against M. oryzae. Therefore, further studies are required to dissect the GH3.2 conferred plant immunity and finally reveal the mechanism underlying Osa-miR535-SPL4-GH3.2 module mediated rice blast resistance.

Osa-miR535 has been characterized as targeting OsSPL14 for regulating rice immunity against M. oryzae positively (Zhang et al., 2020; Wang et al., 2021). The emerging evidence supports the idea that one miRNA may target different genes to perform its function redundantly. Accordingly, Osa-miR535 downregulates OsSPL7, OsSPL12, and OsSPL16 in panicles (Sun et al., 2019). Consequently, we further screened the target gene of Osa-miR535 in the SPL gene family. As expected, we successfully identified that OsSPL4 is targeted by Osa-miR535, as well as its homologous gene OsSPL14, to fine-tune rice immunity against M. oryzae (Figure 7). OsSPL14 binds on the promoter of WRKY45 to boost its expression for enhancing rice blast resistance (Wang et al., 2018). In the present study, OsSPL4 binds on the promoter of GH3.2 (Figure 6), which acts in IAA homeostasis and enhances rice immunity against M. oryzae (Fu et al., 2011), suggesting that Os-miR535 targets two parallel pathways of SPL14-WRKY45 and SPL4-GH3.2 to manipulate rice blast resistance. In addition, OsSPL4 also promotes the expression of WRKY45 (Figure S10), even though it cannot bind with the promoter of WRKY45, similar to OsSPL14 (Figure 6e), indicating that it may exist cooperatively downstream of SPL genes to regulate plant immunity. Upstream of SPL genes, OsSPL14 is also targeted by Os-miR165 and Osa-miR535 to control rice immunity (Zhang et al., 2020; Wang et al., 2021). Moreover, OsSPL7 is targeted by Os-miR156, Osa-miR529, and Osa-miR535, three highly conserved miRNAs (Figure S2) (Sun et al., 2019; Wang et al., 2015; Yue et al., 2017). OsSPL14 and OsSPL17 are target genes of Osa-miR156 and Osa-miR529 (Wang et al., 2015; Yue et al., 2017). These data suggest that there may be a complex regulatory network consisting of the three miRNAs (Osa-miR535, Osa-miR156, and Osa-miR529) and SPL genes to modulate immunity in rice. Furthermore, Osa-miR156 targets OsSPL14 to define the ideal plant architecture (Jiao et al., 2010; Miura et al., 2010) and targets OsSPL3 to regulate crown root development and cold tolerance (Shao et al., 2019; Zhou and Tang, 2018). Osa-miR529 spatiotemporally regulates OsSPL2, OsSPL7, OsSPL14, OsSPL16, OsSPL17, and OsSPL18 in different life
stages to control several important agronomic traits, including plant height, tiller number, panicle architecture, and grain size (Yan et al., 2021). Therefore, this complex network may be extensively involved in rice growth and abiotic stresses.

Spatio-temporal expression is an important feature of these three miRNAs and SPL genes. Osa-miR529 is ubiquitously expressed in leaf, root, and panicle, but more preferentially in panicle (Jeong et al., 2011). Osa-miR156 can be detected in coleoptile, root, leaf, culm, and panicle, and flower, although its expression is low in leaf, culm, and panicle (Figure 5b) (Wang et al., 2015). In addition, Osa-miR535 is ubiquitously expressed in all of these tissues and more preferentially in flower (Figure 5b). Compared with Osa-miR156, Osa-miR535 is ubiquitously expressed in panicle, leaf, culm, and root, although its expression is remarkably expressed in panicle but rarely detected in leaf tissue (Figure 5b) (Wang et al., 2015). In addition, OsSPL4 and OsSPL5 are required for rice blast resistance in leaves (Figure 3) (Wang et al., 2015). Taken together, those findings provide a research foundation for dissecting the mechanisms underlying the network consisting of Osa-miR156, Osa-miR529, and Osa-miR535, as well as their targeted SPL genes, in rice growth, development, and immunity.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The rice (Oryza sativa) accessions used in the present study included the susceptible accession Lijiangxian tuan Heigu (LTH) and International Rice Blast Line Pyricularia-Kanto 51-m-Tsuyuake (IRBLkm-Ts) (Lin et al., 2001; Taunematsu et al., 2000). Nipponbare was used as WT to generate transgenic plants. All rice plants were grown in the greenhouse under a 14:10 h light/dark photoperiod with 70% relative humidity at 26°C. Nicotiana benthamiana was planted in the greenhouse under a 16:8 h light/dark photocyte at 24°C.

Genetic transformation plasmid construct and mutant screening

To generate transgenic plants expressing artificial target mimic of Osa-miR535, target mimicry sequences of Osa-miR535-5p were used to replace the miR399 target site in IPS1 by PCR application with primers miR535-IPS1-F/R and miR535 mimic-F/R as described previously (Franco-Zorrilla et al., 2007; Wang et al., 2021) and were then cloned into the KpnI–BclI sites of the binary vector pCAMBIA1300-35S with hygromycin selection (Table S1). To achieve overexpression of Osa-miR535 transgenic plants, a fragment containing 431 bp upstream and 495 bp downstream of Osa-miR535 was amplified from Nipponbare with primers OXMIR535-F/R (Table S1). The fragment was cloned into the KpnI–SalI sites of the binary vector pCAMBIA1300-35S with hygromycin selection (Table S1). To generate transgenic line and the primers for the SPL4-detect-F/R flanking designed target site (300–500 bp) were used for PCR amplification (Table S1). The products were sequenced directly by Sangon Bio-tech (Shanghai, China) and blast searched with respect to the WT genome sequence to identify the mutation sites (Figure S7).

M. oryzae inoculation and pathogen infection

The method of M. oryzae inoculation was performed as described previously (Li et al., 2017). In brief, the punch-wound leaves detached from three-leaf old seedlings were drop-inoculated with the spores of M. oryzae (1 × 10^6 to 5 × 10^6 spores per ml) and cultured in H2O containing 0.1% 6-benzylaminopurine. Alternately, three-leaf old seedlings were spray-inoculated with the spores (1 × 10^6 to 5 × 10^6 spores per ml) and incubated in darkness for 24 h under 100% humidity in the growth room. Then the inoculated samples were continually incubated under a 12:12 h light/dark photocyte with 100% humidity in the growth room. The disease phenotypes were imaged at 5 days post inoculation (d.p.i.). For rice-sheath inoculation assays, 5-cm leaf sheaths were detached from four-leaf stage seedlings and injected with M. oryzae strain of GZ8 spore suspension (1 × 10^6 spores per ml). The injected sheaths were incubated using the same conditions as for spraying inoculation. The infection process of GZ8 in the epidermal layer of the inoculated leaf sheaths was observed by laser scanning confocal microscopy (model A1; Nikon, Tokyo, Japan) at 24 and 36 h.p.i. Simultaneously, the infected rice sheathes were stained by DAB (0.5 mg ml^-1) to examine H2O2 accumulation.

RT-qPCR assay

Total RNA was extracted from rice samples using TRizol reagent (Invitrogen, Carlsbad, CA, USA). Then, 1 μg of total RNA was reverse transcribed into cDNA using Primerscript RT reagent Kit in accordance with the manufacturer’s instructions (TaKaRa Biotechnology (Dalian) Co. Ltd, Shiga, Japan). In addition, the cDNA required for detection of Osa-miR535 was synthesized with a specific stem-loop primer named miR535-stemloop. Stem-loop RT-qPCR was used to detect and quantify the mature Osa-miR535 with primers miR535-STF-RF and Universal-RT-RF. snRNA U6 (U6-F/R) and OsUbiquitin (Ubi-F/R) were used as the internal reference for miRNAs and gene quantification, respectively. RT-qPCR was performed using a SYBR Green PCR Kit (Bimake, Houston, TX, USA). The primers for RT-qPCR in the present study are listed in Table S1.

Transient expression assay in N. benthamiana

YFP detection and accumulation was assayed as reported previously. To generate Osa-miR535 target-site reporter fusions, we fused the YFP with the target site of OsaSPL4 at the N-terminus (35S::SPL4TS-YFP), with 35S::YFP as a control. The fragments of SPL4TS were synthesized by annealing gene specific primers
SPL4SBP-F/R (Table S1) and inserted into the KpnI site of binary vector pCAMBIA1300-3SS. The constructs were transiently expressed in *N. benthamiana* mediated by Agrobacterium strain GV3101. In brief, Agrobacterium strain GV3101 harboring the respective expression constructs (35S::SPL4SBP-YFP, 35S::mirR535, 35S::YFP) was incubated at 28°C for 16 h in LB media containing kanamycin (50 mg ml⁻¹) on a table with shaking at 250 r.p.m. The Agrobacterium were collected at 1000 g for 4 min and resuspended in MMA buffer [10 mM 2-(N-morpholino)-ethanesulphonic acid, 10 mM MgCl₂, and 10 mM acetylsyringone]. The mixture was allowed to stand at room temperature for 2 h, then infiltrated into *N. benthamiana* leaves. The transient expression assay was performed as described previously (Li et al., 2017). Images were taken using a fluorescence microscope (Axio Imager A2; Carl Zeiss, Jena, Germany) at 48 h.p.i. In western blotting analysis, total proteins were extracted using the extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and 1 mM DTT) with 1% protease inhibitor cocktail (04693116001; Roche, Basel, Switzerland). The fusion proteins were detected by anti-GFP (#D110008-0025; Sangon Biotech) with 1% BSA. EMSA assay was conducted with the Chemiluminescent ECL Western Blot Kit (Clarity™ Western Blot Substrate; Bio-Rad, Hercules, CA, USA). The probe primers were listed in Table S1. The Inner Primer PCR fragments were gel purified and ligated into pEASY-Blunt Simple cloning Vector (CB111; TransGen Biotech) for sequencing to manufacturer’s instructions. Total RNA was extracted from 2-week-old leaves. The transient expression assay was performed using a Dual-Lumi™ li Luciferase Reporter Gene Assay Kit (Beyotime) in accordance with the manufacturer’s instructions.

### EMSA

5' RLM-RACE

5' RLM-RACE was conducted with the First Choice™ RLM-RACE Kit (Ambion, Austin, TX, USA) in accordance with the manufacturer’s instructions. Total RNA was extracted from 2-week-old seedlings using TRIzol reagent (Invitrogen) in accordance with the manufacturer’s instructions. Nested gene-specific downstream primer sequences are shown in Table S1. The Inner Primer PCR fragments were gel purified and ligated into pEASY-Blunt Simple cloning Vector (CB111; TransGen Biotech) for sequencing to examine the cleavage site.

### Y1H assay

The fragment of the GH3.2 promoter from 724 to 1620-bp containing the TATA box was cloned into the p038 vector to generate the construct pGH3.2-GH3.2 with primers GH3.2-Y1H-F/R1. The region of the GH3.2 promoter from 1 to 744-bp without TATA motifs was also cloned into the p038 vector to generate the negative control pHIS2-GH3.2 m with primers GH3.2-Y1H-F2/R2. The full-length cDNA sequences of OsSPL4 (SPL4SBP) and the OsSPL4 mutant without SBP domain (SPL4SBP) were amplified by PCR and cloned into the pGAD77 vector (Clontech, Mountain View, CA, USA), respectively. Sequences of the primers are listed in Table S1. The fusion constructs were co-transformed into the yeast strain Y187 yeast cells (Clontech) with the reporter vector pHIS2-GH3.2, pHIS2-GH3.2 m, and grown on SD-Trp-Leu-His medium containing 3-amino-triazole. The yeast one-hybrid assays were conducted with Yeastmaker™ Yeast Transformation System 2 (Clontech) in accordance with the manufacturer’s instructions. Images were captured using a digital camera (Canon, Tokyo, Japan).

### Dual-luciferase assay

The 1619-bp promoter of GH3.2 was amplified from Nipponbare with primers GH3.2pro-F/R (Table S1) and cloned into the pCAMBIA1300-LUC vector to drive the *firefly luciferase gene* (LUC) as a reporter construct. Renilla luciferase (REN) was driven by the CaMV 3SS promoter in the same construct as an internal control. The construct pCAMBIA1300-SPL4 was used as an effector. Then, these constructs were introduced into the agrobacterium strain GV3101. Agrobacteria harboring the reporter and effector constructs were collected by centrifugation, resuspended in MS medium [10 mM 2-(N-morpholino)-ethanesulphonic acid, 10 mM MgCl₂ and 200 μM acetylsyringone] until OD₆₀₀ = 0.8 and then incubated at room temperature for 2 h. The mixture of Agrobacterium suspension was infiltrated in *N. benthamiana* leaves. After 3 days under 14:10 h day/night photocycle conditions, the leaves were infiltrated with 150 μg ml⁻¹ luciferin solution (Luciferin Detection Reagent; V8920; Promega, Madison, WI, USA) and kept in the dark for 10 min to quench the fluorescence. Images were captured using a charge-coupled device camera (ChemiDoc XR5; Bio-Rad). Quantification analysis was performed using a Dual-Lumi™ li Luciferase Reporter Gene Assay Kit (Beyotime) in accordance with the manufacturer’s instructions.

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We thank Dr Cai-Lin Lei (Institute of Crop Science, Chinese Academy of Agricultural Sciences) for providing the oligonucleotide probes containing GTAC motives were synthesized and labelled (GH3.2pro-EMSA-Biotin/F/R) with biotin at the 5' end, or without biotin (GH3.2pro-EMSA-F/R), by Sangon Biotech. The probes were purified via glutathione sepharose 4B beads (GE Healthcare, Chicago, IL, USA). Oligonucleotide probes containing GTAC motives were synthesized and labelled (GH3.2pro-EMSA-Biotin/F/R) with biotin at the 5' end, or without biotin (GH3.2pro-EMSA-F/R), by Sangon Biotech. The probe primers were listed in Table S1. The binding reaction contained purified GST-SPL4SBP, EMSA/Gel-Shift binding buffer and probes. GST protein alone was used as a negative control. EMSA assay was conducted with the Chemiluminescent EMSA Kit (Beyotime) in accordance with the manufacturer’s instructions. The detailed EMSA procedure also followed the manufacturer’s instructions. Images were captured using a charge-coupled device camera (ChemiDoc XR5; Bio-Rad).

### AUTHOR CONTRIBUTIONS

W-MW and YL conceived the project. L-LZ, Y-YH and Y-PZ performed most of the experiments with support from X-XL, S-XZ, X-MY, S-LL, J-LL, S-LZ, HW, Y-PJ, J-WZ, MP, Z-XZ and JF. W-MW, Y-YH and L-LZ contributed to manuscript writing and editing.

### CONFLICT OF INTERESTS

The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT

All data generated or analyzed during the present study can be found within the manuscript and its supporting materials.
SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Osa-miR535 responds to Magnaporthe oryzae and chitin treatment. (a) The expression of Osa-miR535 was examined by stem-loop RT-qPCR upon M. oryzae infection in both LTH and IRBLkm-Ts accessions in 4-week-old seedlings. (b) The expression of Osa-miR535 in both LTH and IRBLkm-Ts accessions upon chitin treatment in 4-week-old seedlings. Error bars indicate the SD (n = 3). Different letters above the bars indicate significant differences (P < 0.05), which are determined by one-way ANOVA analysis followed by post-hoc Tukey’s HSD analysis.

Figure S2. Sequence alignment of Osa-miR535 and its target mimic MIM535.

Figure S3. The expression pattern of defense-related genes upon chitin treatment. Defense-related genes, K54 (a) and NAC4 (b), were analyzed by qRT-PCR in 4-week-old seedlings of wild-type (WT) and MIM535 lines at the indicated time points upon chitin and mock treatments. Hpt, hours post treatment. Error bars indicate the SD (n = 3). Different letters above the bars indicate significant differences (P < 0.05), which are determined by one-way ANOVA analysis followed by post-hoc Tukey’s HSD analysis.

Figure S4. Osa-miR535 suppresses the H2O2 accumulation upon Magnaporthe oryzae inoculation. The leaf sheathes of the indicated lines were subjected to DAB staining at 48 h.p.i. Scale bars = 20 μm. These experiments were repeated twice with similar results.

Figure S5. Sequence alignment of Osa-miR535, Osa-miR156a-1, Osa-miR156f, Osa-miR529a and Osa-miR529b.

Figure S6. The expression levels of OsSPL4 in 10 OXSL4 transgenic lines. Four-week-old seedlings of WT and OXSL4 lines were analyzed by qRT-PCR. WT is Nipponbare used for generating OXSL4 transgenic plants. 1–8, different individual lines of OXSL4.

Figure S7. The genotype of osspi4 mutants. (a) Sequence alignment of the qRNA site of OsSPL4 gene in osspi4 mutants and WT. The mutant sites are highlighted in red, - indicates the deletion. (b) The sequence peaks of osspi4 mutants. (c) Protein sequence of OsSPL4 encoded in osspi4 mutants.

Figure S8. Schematic and the sequence of the GH3.2 promoter. Orange marked bars and sequences indicate the positions of GTAC motifs.

Figure S9. The expression of GH3 family genes in WT, OXSL4 and osspi4. Four-week-old seedlings used for examining gene expression by qRT-PCR. Error bars indicate the SD (n = 3). N.D. indicates that the expression of the gene was not detected.

Figure S10. The expression of WRKY45 is positively regulated by OsSPL4. Four-week-old seedlings used for examining gene expression by qRT-PCR. Error bars indicate the SD (n = 3). Different letters above the bars indicate significant differences (P < 0.05), which are determined by one-way ANOVA analysis followed by post-hoc Tukey’s HSD analysis.

Figure S11. The expression of expansin (EXPA) genes. Four-week-old seedlings of the indicated rice plants were used to examine gene expression by qRT-PCR. Error bars indicate the SD (n = 3). A double asterisk (**) indicates significant differences (P < 0.01) via Student’s t test compared to WT.

Table S1. The primers used in the present study.

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