PHYTOCHROME-INTERACTING FACTOR-LIKE14 and SLENDER RICE1 Interaction Controls Seedling Growth under Salt Stress

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Early seedling development and emergence from the soil, which are critical for plant growth and important for crop production, are controlled by internal factors, such as phytohormones, and external factors, such as light and salt. However, little is known about how light and salt signals are integrated with endogenous cues in controlling plant physiological processes. Here, we show that overexpression of rice (Oryza sativa) PHYTOCHROME-INTERACTING FACTOR-LIKE14 (OsPIL14) or loss of function of the DELLA protein SLENDER RICE1 (SLR1) promotes mesocotyl and root growth, specifically in the dark and under salt stress. Furthermore, salt induces OsPIL14 turnover but enhances SLR1 accumulation. OsPIL14 directly binds to the promoter of cell elongation-related genes and regulates their expression. SLR1 physically interacts with OsPIL14 and negatively regulates its function. Our study reveals a mechanism by which the OsPIL14-SLR1 transcriptional module integrates light and gibberellin signals to fine-tune seedling growth under salt stress, enhancing understanding about how crops adapt to saline environments.

In the monocot rice (Oryza sativa), the coleoptile protects the plumule, while the mesocotyl develops in the dark to push the coleoptile toward the soil surface. Extensive studies in Arabidopsis have demonstrated that upon light activation, the bioactive (Pr) form of phytochromes translocates into the nucleus and interact with PHYTOCHROME-INTERACTING FACTORS (PIFs), resulting in the rapid phosphorylation and subsequent degradation of PIF proteins via the 26S proteasome pathway (Shen et al., 2007, 2008; Ni et al., 2014). PIF proteins are a small subfamily of basic helix-loop-helix transcription factors that accumulate in the dark and directly control the expression of thousands of genes (Leivar and Monte, 2014; Paik et al., 2017). Loss of PIF1, PIF3, PIF4, and PIF5 leads to short hypocotyls and open cotyledons in the dark, showing that these PIFs negatively control photomorphogenesis (Leivar et al., 2008; Shin et al., 2009). Correlative evidence reveals that PIFs play essential roles in the integration of light and other signaling pathways, such as GA, ethylene, auxin, brassinosteroid, reactive oxygen species, or temperature (de Lucas et al., 2008; Feng et al., 2008; Franklin et al., 2011; Bai et al., 2012; Lee and Thomashow, 2012; Chen et al., 2013; Zhong et al., 2014; Paik et al., 2017).

Rice has three phytochrome members, phytochrome A (OsphyA), phytochrome B (OsphyB), and phytochrome C (OsphyC), which are involved in regulating seedling de-etiolation, plant architecture, and cotyledon opening (Jiao et al., 2007). In the monocot rice (Oryza sativa), the coleoptile protects the plumule, while the mesocotyl develops in the dark to push the coleoptile toward the soil surface. Extensive studies in Arabidopsis have demonstrated that upon light activation, the bioactive (Pr) form of phytochromes translocates into the nucleus and interact with PHYTOCHROME-INTERACTING FACTORS (PIFs), resulting in the rapid phosphorylation and subsequent degradation of PIF proteins via the 26S proteasome pathway (Shen et al., 2007, 2008; Ni et al., 2014). PIF proteins are a small subfamily of basic helix-loop-helix transcription factors that accumulate in the dark and directly control the expression of thousands of target genes (Leivar and Monte, 2014; Paik et al., 2017). Loss of PIF1, PIF3, PIF4, and PIF5 leads to short hypocotyls and open cotyledons in the dark, showing that these PIFs negatively control photomorphogenesis (Leivar et al., 2008; Shin et al., 2009). Correlative evidence reveals that PIFs play essential roles in the integration of light and other signaling pathways, such as GA, ethylene, auxin, brassinosteroid, reactive oxygen species, or temperature (de Lucas et al., 2008; Feng et al., 2008; Franklin et al., 2011; Bai et al., 2012; Lee and Thomashow, 2012; Chen et al., 2013; Zhong et al., 2014; Paik et al., 2017).

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heading time (Takano et al., 2009). Six PHYTOCHROME-INTERACTING FACTOR-LIKE (PIL) genes, designated OsPIL11 to OsPIL16, were identified in the rice genome (Nakamura et al., 2007). Overexpression of OsPIL13/OsPIL1 in rice promotes internode growth and confers drought tolerance, whereas overexpression of OsPIL15 represses seedling growth in the dark (Todaka et al., 2012; Zhou et al., 2014). Nevertheless, the biological function and regulatory mechanism of PILs in monocots are largely unknown.

Salt stress negatively affects many aspects of plant growth and development, particularly during seed germination and seedling growth (Park et al., 2016). Hormonal signaling pathways are involved in salt stress responses. For example, bioactive GA levels are reduced upon salt treatment, and the Arabidopsis ga1-3 mutant, which is defective in GA biosynthesis, is tolerant to salt (Achard et al., 2006). DELLA proteins (characterized by the presence of a conserved Asp-Glu-Leu-Leu-Ala motif) act as central GA signaling repressors that restrain the expression of GA-responsive genes without GA. There are five DELLA members in Arabidopsis, but only a single DELLA protein, SLENDER RICE1 (SLR1), in rice (Itoh et al., 2002; Locascio et al., 2013). Upon GA binding, the GIBBERELLIN INSENSITIVE DWARF1 (GID1) receptor interacts with SLR1, which is then recognized by the F-box protein GID2 and degraded via the 26S proteasome pathway, leading to the activation of GA responses (Itoh et al., 2002; Sasaki et al., 2003; Murase et al., 2008). Root growth of a quadruple Arabidopsis della mutant lacking GA-INSENSITIVE (GAI), REPRESSOR OF GA (RGA), RGA-LIKE1 (RGL1), and RGL2 is more sensitive to salt treatment than that of the wild type (Achard et al., 2006). However, the mechanism underlying the cross talk among GA, light, and salt in regulating plant growth remains unclear.

In this study, we show that overexpression of OsPIL14 caused an elongated mesocotyl in the dark and reduced sensitivity to NaCl-mediated inhibition of seedling growth in rice. A mutant lacking SLR1 displayed a similar response to salt stress. Strikingly, salt-promoted OsPIL14 degradation but SLR1 accumulation. OsPIL14 physically interacted with SLR1 in vitro and in vivo. OsPIL14 directly regulates downstream gene expression and SLR1 negatively regulates this process by interfering with the binding and transcriptional activity of OsPIL14. Our findings reveal that OsPIL14-SLR1 interaction integrates light and GA signals to precisely control seedling growth under salt stress.

RESULTS

OsPIL14 Promotes Seedling Growth in Darkness

To study the function of these OsPIL genes, we generated transgenic rice lines overexpressing OsPIL11, OsPIL12, OsPIL14, OsPIL15, and OsPIL16 fused with GFP and driven by the OsUBQ5 promoter (UBQp:OsPIL-GFP) in the japonica cv Zhonghua 11 (cv ZH11) background (Li et al., 2019). When grown in darkness, the cv ZH11 seedlings developed only coleoptiles and...
roots. Surprisingly, all of the etiolated *UBQp:OsPIL14-GFP* transgenic lines exhibited elongated mesocotyls compared to cv ZH11 (Supplemental Fig. S1). In particular, overexpression of *UBQp:OsPIL14-GFP* (*OsPIL14OE*, lines 5 and 22 shown) caused much longer mesocotyls than overexpression of the other *OsPIL* genes (Fig. 1, A–C; Supplemental Fig. S1, B and C). Based on its strong effect, we then focused on the role of *OsPIL14* in this study.

The etiolated *OsPIL14OE* seedlings also had significantly longer primary roots but slightly shorter coleoptiles than cv ZH11 (Fig. 1, A–C). When grown under white light, however, the *OsPIL14OE* plants did not develop mesocotyls and were indistinguishable from the cv ZH11 wild type in terms of coleoptile and root length (Fig. 1, D and E). We also used clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9 genome editing to generate *OsPIL14* mutations. In one allele (*ospil14*), a 375-bp fragment was deleted in the second exon of *OsPIL14*, resulting in the truncation of amino acids 95 to 219 (Supplemental Fig. S2). The *ospil14* mutants did not produce abnormal mesocotyls but had slightly longer roots than cv ZH11 in the dark. It did not differ from cv ZH11 when grown under light (Fig. 1), likely due to functional redundancy with other *OsPIL* members. These observations suggest that *OsPIL14* and the other *OsPIL* members promote skotomorphogenesis and root growth in darkness.

GA promotes cell elongation (Heck et al., 2016). We found that supplementation with bioactive GA3 (100 μM) in the medium significantly promoted mesocotyl, coleoptile, and root growth of wild-type and *ospil14* mutant seedlings and induced mesocotyl and coleoptile length of the *OsPIL14OE* seedlings compared to the mock control (Supplemental Fig. S3). However, GA3 did not further induce primary root growth in *OsPIL14OE* lines (Supplemental Fig. S3).
Overexpression of OsPIL14 Enhances Seedling Growth under Salt Stress

Next, we investigated the effect of salt treatment on plant growth in the dark. Supplementation with salt drastically inhibited coleoptile, mesocotyl, and root growth in OsPIL14OE and ZH11 seedlings, at especially 0.2 M NaCl (Fig. 2, A and B). OsPIL14OE lines exhibited longer shoot and root than cv ZH11 under salt stress (Fig. 2, A and B). Mesocotyl elongation of the OsPIL14OE lines was greatly inhibited by salt and, to a lesser degree, in the presence of additional 100 μM GA3 (Supplemental Fig. S3, A and B). When plants were treated with 0.1 M NaCl and 120 μM paclobutrazol (PAC; a GA biosynthesis inhibitor), the coleoptile and root elongation of ospil14 and OsPIL14OE and mesocotyl elongation of OsPIL14OE were more inhibited than those treated with 0.1 M NaCl alone (Supplemental Fig. S3). These results indicate that overexpression of OsPIL14 enhances plant growth under salts stress.

When seedlings were grown under a 16-h light/8-h dark photoperiod, the fresh and dry weight, shoot length, and root length of OsPIL14OE and ZH11 were similar (Fig. 2, C–G). Although supplementation with 0.1 M NaCl inhibited seedling growth, the fresh and dry weight, shoot length, and root length of OsPIL14OE plants were significantly larger than those of ZH11 (Fig. 2, C–G). Furthermore, the chlorophyll contents and photosynthesis efficiency, including Pn (net photosynthesis) and Fv/Fm (efficiency of PSII), of OsPIL14OE plants were significantly higher than those of cv ZH11 when treated with 0.1 M NaCl (Fig. 2, H–J), indicating that OsPIL14 enhances photosynthesis under salt stress.

To mimic seedling emergence in nature and to investigate whether overexpressing OsPIL14 promoted seedling emergence from deep soil of rice, we placed the germinated seeds in pots and covered them with 1 or 3 cm of soil followed by irrigating with 75 mM NaCl or water. As shown in Figure 2K, shoot growth of the OsPIL14OE plants did not differ from that of ZH11 when seeds were covered with 1 cm of soil regardless of NaCl treatment. However, when the seeds were covered with 3 cm of soil, 70% of the OsPIL14OE seedlings but 50% of the ZH11 seedlings emerged from soil. Moreover, under 75 mM NaCl condition, 40% of OsPIL14OE could emerge, whereas all of ZH11 failed to penetrate the soil (Fig. 2K). These results suggest that OsPIL14 promotes seedlings etiolation and emerging from soil under salt stress.

Salt Promotes OsPIL14 Degradation

We then investigated whether salt regulates OsPIL14 transcription and/or OsPIL14 protein accumulation. Reverse transcription followed by quantitative PCR...
(RT-qPCR) showed that 0.3 M NaCl greatly increased OsPIL14 expression in dark-grown ZH11 seedlings (Fig. 3A). However, the OsPIL14-GFP levels were markedly decreased in the OsPIL14OE seedlings using anti-GFP antibody after 12 h of salt treatment compared to the mock control, and the reduction was greatly inhibited in the presence of 50 μM MG132, a 26S proteasome inhibitor (Fig. 3B). Furthermore, OsPIL14-GFP levels gradually decreased in seedlings incubated with increasing concentrations of NaCl (Fig. 3C).

During the dark-to-light transition in the NaCl-treated seedlings, OsPIL14 expression in shoots increased and peaked at 6 h, whereas OsPIL14 expression in roots rapidly peaked after 3 h of treatment and dropped afterward (Supplemental Fig. S4A). We found that OsPIL14-GFP levels rapidly decreased when the OsPIL14OE seedlings were transferred from darkness to light and were further reduced with the addition of NaCl treatment (Supplemental Fig. S4B), suggesting that light induces OsPIL14 degradation, similar as PIF proteins in Arabidopsis (Castillon et al., 2007).

Moreover, we examined OsPIL14 protein stability of seedlings grown under a 12-h light/12-h dark photoperiod. OsPIL14-GFP accumulated to high levels after a 12-h dark period compared to the end of the light period; however, 0.3 M NaCl prevented the accumulation of OsPIL14-GFP (Fig. 3D). Strikingly, OsPIL14 turnover was largely inhibited with the addition of MG132 (Fig. 3D). We also investigated the effects of exogenous GA3 and PAC together with NaCl on OsPIL14 accumulation. The PAC treatment reduced OsPIL14-GFP levels, and the GA3 treatment increased OsPIL14-GFP levels. OsPIL14-GFP further decreased in the presence of both PAC and NaCl, whereas NaCl antagonized the effect of GA3 (Fig. 3E). These results confirm that GA promotes OsPIL14 accumulation, whereas salt induces OsPIL14 degradation likely through the 26S proteasome pathway.

**Salt Induces SLR1 Accumulation**

Salt induces RGA (a DELLA protein) accumulation in Arabidopsis roots (Achard et al., 2006). SLR1 is the only DELLA protein in rice (Itoh et al., 2002). We then explored whether salt regulates SLR1 protein stability. The SLR1 transcript level was slightly induced in cv ZH11 seedlings after 3 h of 0.3 M NaCl treatment (Fig. 4A). The SLR1 protein level was slightly increased by NaCl treatment in ZH11 (Fig. 4B). Although SLR1 was largely degraded in response to 100 μM GA3 treatment, as previously reported (Sasaki et al., 2003), NaCl could partly inhibit GA3-induced SLR1 degradation (Fig. 4B). Furthermore, the SLR1 level increased along with the increasing concentrations of NaCl (Fig. 4C). Moreover, when seedlings were transferred to medium supplemented with 0.3 M NaCl, SLR1 accumulated and peaked after 24 h of NaCl treatment (Fig. 4D). These results suggest that salt promotes SLR1 accumulation and slightly inhibits GA-induced SLR1 degradation. Next, we investigated whether SLR1 is involved in regulating mesocotyl growth in response to salt.
We obtained a slr1-6 mutant allele, which has a point mutation that produces L246P amino acid substitution and results in a constitutive GA response (Huang et al., 2015). Ten-day-old dark-grown slr1-6 seedlings developed elongated mesocotyls and had much longer coleoptiles and primary roots than the cv ZH11 control plants (Fig. 4, E–G). These results indicate that SLR1 inhibits seedling growth under salt stress.

OsPIL14 Physically Interacts with SLR1

We next performed multiple experiments to examine whether OsPIL14 interacts with SLR1. First, a yeast two-hybrid assay showed that OsPIL14 fused with the GAL4 DNA binding domain (GBD), while SLR1 and its N or C terminus were fused with the GAL4 DNA activation domain (GAD). ~WL, Without Trp and Leu; ~HAWL, without His, Ade, Trp, and Leu. C, Pull-down assay. GST-OsPIL14 (or GST alone) was incubated with MBP-SLR1 and protein mixtures were immunoprecipitated (IP) with anti-GST antibody. IB, Immunoblotting. D, Bimolecular fluorescence complementation assay in N. benthamiana leaves. OsPIL14 was fused with N-terminal YFP, and SLR1 was fused with C-terminal YFP. BF, Bright field. Bar = 50 μm. A white triangle indicates YFP fluorescence in the nucleus. E, Coimmunoprecipitation assay. Proteins were expressed in N. benthamiana leaves as shown in D and were immunoprecipitated with anti-HA antibody.

OsPIL14 Directly Regulates the Expression of Cell Elongation-Related Genes, and SLR1 Interferes with OsPIL14 Activity

To investigate how OsPIL14 regulates rice seedling growth, we grew cv ZH11 and OsPIL14OE plants in darkness for 7 d and analyzed the global transcriptome changes using RNA sequencing. Genes with more than 2-fold changes (P < 0.05) were considered differentially expressed genes. We found that 918 genes were up-regulated, whereas 818 genes were down-regulated in OsPIL14OE versus cv ZH11 plants (Supplemental Figure 5).
Dataset S1). Gene Ontology (GO) analysis revealed that the up-regulated genes by OsPIL14 overexpression are mostly involved in oxidation reduction and catabolic and metabolic processes, whereas the down-regulated genes largely participate in photosynthesis (Supplemental Fig. S5). Promoter analysis showed that 93% of the induced genes contain at least one G-box or PBE motif (putative cis-elements for binding of PIF proteins; Pfeiffer et al., 2014), and 60% and 90% of the repressed genes have G-box or PBE motif, respectively, within their 2-kb promoter regions (Fig. 6A), suggesting that OsPIL14 likely associates with these genes.

Among the up-regulated genes, we picked Expansin A4 (OsEXPA4), which is involved in cell elongation (Choi et al., 2003; Todaka et al., 2012), and Os08g25710, which might be involved in cellulose synthesis, for further analysis. The expression of OsEXPA4 and Os08g25710 was increased in the OsPIL14OE plants, and this induction was largely inhibited by salt treatment (Fig. 6B). Their expression was also increased in the slr1-6 mutant after GA3 application (Fig. 6B). A yeast one-hybrid assay showed that OsPIL14 fused with the B42 activation domain (AD-OsPIL14) could bind and activate the expression of LacZ reporter driven by the OsEXPA4 and Os08g25710 promoter fragments (Fig. 6C). Furthermore, an EMSA showed that GST-OsPIL14, but not GST alone, bound to an OsEXPA4 fragment containing a G-box labeled with biotin, and the amount of the shifted bands was reduced by addition of recombinant MBP-SLR1 (Fig. 6D), suggesting that SLR1 inhibits the binding ability of OsPIL14 on its target.

Next, we performed a chromatin immunoprecipitation coupled with qPCR (ChIP-qPCR) analysis and found that the promoter regions of OsEXPA4 and Os08g25710 were significantly enriched in OsPIL14OE samples compared to ZH11; this enrichment was drastically reduced in OsPIL14OE plants treated with NaCl (Fig. 6E). We then carried out a transient LUC expression assay. The LUC reporter gene was driven by

Figure 6. OsPIL14 regulates downstream gene expression, and SLR1 inhibits OsPIL14 activity. A, Percentage of genes containing G-box or PBE motifs in their promoter among the genes differentially regulated by OsPIL14. B, RT-qPCR. Plants of ZH11, OsPIL14OE (line 5 and 22), and slr1-6 were grown in the dark for 7 d and treated with various chemicals for 12 h. Error bars represent so of three biological replicates. C, Yeast one-hybrid assay. The LacZ reporter gene was driven by the promoter of Os08g25710 or OsEXPA4. D, Electrophoretic mobility shift assay (EMSA). Various recombinant proteins were incubated with biotin-labeled DNA probes of OsEXPA4 for 1 h. E, ChIP-qPCR assay. Error bars represent so of three biological replicates. Asterisks indicate significant difference from cv ZH11 using a Student’s t-test (**P < 0.01). Diagrams of the promoter structure and regions for PCR were shown. F, Transient LUC assay. Different plasmid combinations were coinfiltrated into N. benthamiana leaves and incubated for 2 d. Representative LUC luminescence images are shown on the right. Control means vectors without expressing OsPIL14 and SLR1. Error bars represent so of three biological replicates. Different letters indicate significant difference using one-way ANOVA (P < 0.05). Bars = 1 cm.
either the Os08g25710 or OsEXPA4 promoter and cotransformed with 35S:OsPIL14 and/or 35S:OsSLR1 into N. benthamiana leaves. LUC luminescence in both reporter constructs was greatly induced by OsPIL14; however, LUC expression was inhibited by cotransformation with SLR1 (Fig. S1). These results indicate that OsPIL14 directly regulates the expression of cell elongation-related genes and that SLR1 interferes with OsPIL14 activity.

**DISCUSSION**

The functions of PIF and DELLLA proteins and their interactions in controlling plant growth and development have been studied extensively in Arabidopsis (Leivar and Monte, 2014). However, the roles of these factors in monocot crops, especially in response to salinity stress, are largely unknown. In this study, we showed that overexpressing OsPIL14 (and the other OsPILs) led to elongated mesocotyls in rice in the dark but not under light conditions (Fig. 1; Supplemental Fig. S1), suggesting that OsPILs promote skotomorphogenesis in rice. Consistent with this, OsPIL14 regulates the expression of cell elongation-related genes (Fig. 6). The ospi14 mutant did not show mesocotyl phenotype under the conditions we tested, likely due to functional redundancy with the other OsPIL genes. A previous study reported that heterologous expression of OsPIL11-15 represses photomorphogenesis in Arabidopsis (Nakamura et al., 2007). Transgenic rice overexpressing OsPIL13 had increased internode growth (Todaka et al., 2012). Surprisingly, overexpression of OsPIL15 represses seedling growth in the dark (Zhou et al., 2014). These studies suggest that the roles of OsPIL members could differ. Generation of single and higher order ospi mutants should help clarify this issue. However, loss of four PIF genes (PIF1, PIF3, PIF4, and PIF5) in Arabidopsis causes a constitutive light response in darkness, whereas overexpression of PIF3 results in reduced photomorphogenesis under light but does not alter phenotypes in darkness (Leivar et al., 2008; Shin et al., 2009; Chen et al., 2013). Interestingly, OsPIL14 also promotes root elongation in the dark (Fig. 1), which is likely mediated by the GA pathway, as GA3-treated cv ZH11 seedlings exhibited similar root length as the OsPIL14OE plants without GA treatment (Supplemental Fig. S3). Thus, the PIF/PIL regulators may differentially mediate seedling growth in Arabidopsis and rice in response to light signals; these regulatory mechanisms remain to be investigated in future studies. Since wild-type rice (such as cv ZH11) seedlings normally do not exhibit obvious mesocotyl elongation in the dark or under light conditions, overexpression of OsPIL14 may provide a unique approach for studying mesocotyl formation and regulation in rice.

We reveal that overexpression of OsPIL14 enhances seedling growth under salt stress and that salt induces OsPIL14 turnover via 26S proteasome-mediated degradation (Figs. 2 and 3). The function of OsPILs in salt stress response is likely conserved in monocot plants as heterologous expression of maize (Zea mays) PIF3 can enhance salt tolerance in rice (Gao et al., 2015). However, recent studies showed that PIF4 reduces salt stress tolerance, and salt does not affect PIF4/PIF5 protein accumulation in Arabidopsis (Sakuraba et al., 2017; Hayes et al., 2019); therefore, distinct mechanisms of PIFs might also be involved in different plant species. Nevertheless, the relationship between the PIF proteins and salt stress requires further investigation. Determining the mechanism by which salt triggers OsPIL14 degradation is an objective for future studies, e.g. identification of the E3 ubiquitin ligase. Moreover, photo-activated phyB interacts with PIFs, resulting in their degradation and consequently releasing the inhibitory effect on photomorphogenesis (Castillon et al., 2007). OsPIL14 interacts with rice phyB, and deficiency of OsPHYB improves salt tolerance (Cordeiro et al., 2016; Kwon et al., 2018). Therefore, PIF proteins play crucial roles in linking light and salt stress.

We show that salt promotes protein accumulation/stability of SLR1, which negatively regulates rice growth under salt stress (Fig. 4). Salt-induced SLR1 accumulation could further inhibit the function of OsPILs. Thus, OsPIL14 acts as a central transcription factor that is negatively regulated by salt in three ways: protein degradation and SLR1-mediated sequestration and degradation. An early study reported that loss of DELLA conferred salt tolerance under low salt concentration; however, overexpressing DELLA enhanced...
plants survive under high salt stress (Achard et al., 2006). This indicates that plants sacrifice growth rate for survival in saline environments via a DELLA-dependent manner. The OsPIL14-overexpressing line accumulated more SLR1 than wild type under both mock and salt stress (Supplemental Fig. S6) and exhibited a faster growth rate under saline soil (Fig. 2K), suggesting that the OsPIL14 confers salt resistance with sacrifice of less growth rate. In Arabidopsis, DELLA proteins physically interact with PIFs and block their activities by sequestering PIFs from binding to their targets (de Lucas et al., 2008; Feng et al., 2008; Leivar and Monte, 2014). Similarly, our results show that a single DELLA protein, SLR1, inhibits the binding activity of OsPIL14 in regulating the expression of target genes, such as OsEXPA4 and Os08g25710 in rice (Fig. 6). Moreover, PAC, which prevents DELLA degradation, negatively regulates OsPIL14 abundance, especially when combined with salt stress, whereas GA3 stimulates OsPIL14 accumulation (Fig. 3), suggesting that SLR1 might have an effect on OsPIL14 stability. In agreement with this, a recent study showed that DELLA also promote PIF3 degradation through the ubiquitin-proteasome system in regulating hypocotyl elongation in Arabidopsis (Li et al., 2016). An earlier study reported that salt slows plant growth by means of reduced accumulation of bioactive GAs and that Arabidopsis plants lacking four DELLA genes are salt sensitive, whereas stabilized DELLA proteins enhance salt tolerance (Achard et al., 2006). Salt-induced SLR1 accumulation could further inhibit the function of OsPILs. It was proposed that both sequestration and degradation of transcription factors by DELLA might be a general mechanism in plants (Li et al., 2016). Thus, OsPIL14 acts as a central transcription factor that is negatively regulated by salt through protein degradation and SLR1-mediated sequestration. PIF and DELLA proteins are reciprocally regulated, probably via posttranslational modification.

In summary, our study reveals a mechanism by which the OsPIL14-SLR1 transcriptional module integrates light and GA signaling to control seedling growth and fitness under changing environments. More than 6% of the world’s total land is affected by excess salts, which harm plant growth and development and hinder agricultural production (Ismail and Horie, 2017). Successful shoot elongation and penetration through the soil at early developmental stages are crucial for plant growth, particularly in saline soils. Our finding that overexpressing OsPIL14 promotes mesocotyl elongation and seedling emergence from soil, providing a suitable target gene and strategies for crop genetic modification with enhanced salt tolerance.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The UBQ::OsPIL1a-GFP transgenic plants and slrT-6 mutants (Huang et al., 2015) are in the rice (Oryza sativa ‘Zhonghua 11’ [cv ZH11]) background. Seeds were germinated for 3 d and placed in a germination bag containing modified Kimura B solution (Wang et al., 2018) and grown under cold fluorescent light (~300 μmol m⁻² s⁻¹) or in darkness at 28°C for 7 d. For chemical treatments, seedlings were grown in or transferred to solution containing different concentrations of NaCl, GA₃ (0.4% [v/v] ethanol as the mock control), PAC, or MG132 (0.5% [v/v] dimethyl sulfoxide as the mock control) as stated in the text. For light transition, seedlings were grown in darkness for 7 d and then exposed to light (~300 μmol m⁻² s⁻¹) in the absence or presence of NaCl for up to 12 h. Representative images were taken using a digital camera (Olympus), and physiological phenotypes were determined. For soil experiments, germinated seeds were covered with 1 or 3 cm of soil in the absence or presence of 75 mM NaCl and seedlings were grown under a 16-h light/8-h dark photoperiod at 28°C for 14 d.

Chlorophyll Measurement

Rice leaves were sampled before or after NaCl treatment, and chlorophylls were extracted as described previously (Li et al., 2019). Total chlorophyll contents were determined in a spectrophotometer and the amount was calculated using the equation chlorophyll = 22.12 OD₅₆₅ + 2.71 OD₆₄₅.

Photosynthetic Parameter Determination

To obtain the open reading frame (ORF) of OsPIL14 and SLR1, total RNA was extracted from cv ZH11 using the RNAprep pure plant kit (TIANGEN) and the first-strand cDNA was reverse transcribed using the GoldScript cDNA synthesis kit (Invitrogen). The ORFs and fragments of OsPIL14 and SLR1 were amplified using high-fidelity KOD DNA polymerase (TOYOBO) and cloned into the pEASY vector (TransGen), pEASY-OsPIL14, pEASY-EXPA4, pEASY-OsEXPA4p:LacZ, and pEASY-OsEXPA4p:LUC, respectively. Primers are listed in Supplemental Table S1.

To generate constructs for the yeast two-hybrid assay, the full-length and N-/C-terminal fragments of OsPIL14 were released from pEASY-OsPIL14 into pEASY-OsPIL14/N into pEASY-OsPIL14/C with EcoRI and Xhol and ligated into the EcoRI-Xhol sites of the pGBKT7 vector (Clontech), resulting in GAD-OsPIL14, GAD-OsPIL14N, and GAD-OsPIL14C, respectively. The pEASY-SLR1/L1/D2/L1D3 plasmids were cut with EcoRI and Smal, and the released fragments were inserted into the EcoRI-Smal sites of the pGBKTK7 vector (Clontech), to generate GBD-SLR1, GBD-SLR1/D1, GBD-SLR1/D2, and GBD-SLR1/D3, respectively.

The OsPIL14 ORF from pEASY-OsPIL14 (cut with EcoRI and Xhol) was cloned into the pGEX-SX-1 vector (GE Healthcare) to generate GST-OsPIL14. The SLR1 ORF from pEASY-SLR1 (cut with EcoRI and Smal) was cloned into pMAL-c5x (NEB, catalog no. N8108S) to generate MBP-SLR1. The OsPIL14 ORF from pEASY-OsPIL14 (cut with Xhol) was ligated into the Xhol site of the pRSET-A vector (cloning grade), to generate pRSET-A-OsPIL14. The SLR1 ORF from pEASY-SLR1 (cut with Xhol) was ligated into the Xhol site of the pRSET-A vector, to generate pRSET-A-SLR1. The OsPIL14 ORF from pEASY-OsPIL14 (cut with EcoRI and Xhol) was ligated into the EcoRI-Xhol sites of pB42AD (Clontech) to generate AD-OsPIL14. The promoter fragments (~2 kb upstream of the ATG start site) of OsEXPA4 and Os08g25710 were PCR amplified from cv ZH11 genomic DNA and cloned into pEASY to generate pEASY-OsEXPA4p:Os08g25710p, respectively. Their corresponding fragments from pEASY-OsEXPA4p (cut with Xhol and SalI) and pEASY-Os08g25710p (cut with KpnI and Xhol) were inserted into pLaZa2 (Lin et al., 2007; cut with KpnI and SalI) or p3102-LUC (Zhang et al., 2017; cut with EcoRI and SalI) to generate OsEXPA4p:LaZC, Os08g25710p:LaZC, OsEXPA4p:LLUC, and Os08g25710p:LLUC, respectively.

Plasmid Construction

For the yeast two-hybrid assay, the full-length and N-/C-terminal fragments of OsPIL14 were released from pEASY-OsPIL14 into pEASY-OsPIL14/N into pEASY-OsPIL14/C with EcoRI and Xhol and ligated into the EcoRI-Xhol sites of the pGBKT7 vector (Clontech), to generate GAD-OsPIL14, GAD-OsPIL14N, and GAD-OsPIL14C, respectively. The pEASY-SLR1/L1/D2/L1D3 plasmids were cut with EcoRI and Smal, and the released fragments were inserted into the EcoRI-Smal sites of the pGBKTK7 vector (Clontech), to generate GBD-SLR1, GBD-SLR1/D1, GBD-SLR1/D2, and GBD-SLR1/D3, respectively.

The OsPIL14 ORF from pEASY-OsPIL14 (cut with EcoRI and Xhol) was cloned into the pGEX-SX-1 vector (GE Healthcare) to generate GST-OsPIL14. The SLR1 ORF from pEASY-SLR1 (cut with EcoRI and Smal) was cloned into pMAL-c5x (NEB, catalog no. N8108S) to generate MBP-SLR1. The OsPIL14 ORF from pEASY-OsPIL14 (cut with EcoRI and Xhol) was cloned into the Xhol site of the pRSET-A vector (cloning grade), to generate pRSET-A-OsPIL14. The SLR1 ORF from pEASY-SLR1 (cut with KpnI and Xhol) was ligated into the Xhol site of the pRSET-A vector, to generate pRSET-A-SLR1. The OsPIL14 ORF from pEASY-OsPIL14 (cut with EcoRI and Xhol) was ligated into the EcoRI-Xhol sites of pB42AD (Clontech) to generate AD-OsPIL14. The promoter fragments (~2 kb upstream of the ATG start site) of OsEXPA4 and Os08g25710 were PCR amplified from cv ZH11 genomic DNA and cloned into pEASY to generate pEASY-OsEXPA4p:Os08g25710p, respectively. Their corresponding fragments from pEASY-OsEXPA4p (cut with KpnI and SalI) and pEASY-Os08g25710p (cut with KpnI and Xhol) were inserted into pLaZa2 (Lin et al., 2007; cut with KpnI and SalI) or p3102-LUC (Zhang et al., 2017; cut with EcoRI and SalI) to generate OsEXPA4p:LaZC, Os08g25710p:LaZC, OsEXPA4p:LLUC, and Os08g25710p:LLUC, respectively.
Generation of Overexpression and Mutant Lines

The ORFs of OsPIL11, OsPIL12, OsPIL14, OsPIL15, and OsPIL16 were fused in-frame with GFP and driven by the OsUBQ5 promoter, generating UBQ5:OsPIL-GFP. The corresponding vectors were transferred into cv ZH11 wild type via the Agrobacterium tumefaciens-mediated method. The osPIL14 mutant was generated using CRISPR/Cas9-associated protein9i by Towin Biotechnology. All overexpression and mutant lines were confirmed by antibiotic selection, immunoblotting, or sequencing and homologous lines were used in all experiments.

Real-Time RT-qPCR and RNA Sequencing

Plants were grown and treated with chemicals as indicated in the text. Total RNA was isolated from seedlings using the RNAprep pure plant kit (Tiangen). First-strand cDNA was synthesized using the Goldscript cDNA synthesis kit (Invitrogen). qPCR was performed using SYBR premix Ex Taq (Takara) in a 96-well plate (C1000; Bio-Rad). The qPCR primers were listed in Supplemental Table S1.

For the yeast one-hybrid assay, various AD fusion constructs were cotransformed into Y2HGold yeast strain, and the interaction was tested on the synthetic dextrose/-Trp-Leu-His-GBD and GAD fusion constructs were cotransformed into Y2HGold yeast strain EGY48. Transformants were grown on synthetic dextrose/-Trp-Ura-Ade dropout plate.

ChIP-qPCR Assay

The ChIP-qPCR experiments were performed as described previously (Saleh et al., 2008). In brief, 2 g of OsPIL4-OE and ZH11 seedlings were harvested and fixed in 1% (v/v) formaldehyde under a vacuum for 30 min. The cross linking was quenched with the addition of 0.125 mM Gly for 5 min. The tissues were ground, and the chromatin was isolated and sonicated to produce DNA fragments around 200 bp. The chromatin samples were incubated with anti-GFP antibody overnight, and the immunoprecipitated complexes were collected. Pre-cipitated DNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in water. The DNA fragments of the promoter regions were amplified by qPCR. Relative enrichment of target sequences after anti-GFP pull-down was sequentially normalized to levels in the input and in cv ZH11 mock treatment. OsUBQ5 was served as an internal control. Primer pairs are listed in Supplemental Table S1.

Yeast One- and Two-Hybrid Assays

For the yeast one-hybrid assay, various AD fusion constructs were cotransformed with LexAop:LucZ (Clontech) reporter plasmids into yeast strain EGY48. Transformants were grown on synthetic dextrose/-Trp-Ura dropout plates, and β-galactosidase activity was visualized with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. For the yeast two-hybrid assay, the GBD and GAD fusion constructs were cotransformed into Y2HGGold yeast strain, and the interaction was tested on the synthetic dextrose/-Trp-Leu-His-Ade dropout plate.

Protein Extraction and Immunoblotting

Seedlings were grown and treated with chemicals as indicated in the text. Seedling samples were ground to a fine powder in liquid nitrogen. About 100 μl powder was solubilized with an equal volume of extraction buffer (500 mM Tris-HCl [pH 7.4], 150 mM NaCl, 150 mM KCl, 5 mM MgCl2, 5 mM EDTA [pH 8.0], 1% [v/v] Triton X-100, 10% [v/v] glycerol, and protease inhibitor cocktail [Roche]). The supernatant was collected after centrifuging at 15,000 g for 15 min at 4°C. Protein concentration was quantified using the Bradford assay. The total protein extracts were separated on a 10% (w/v) SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% (w/v) skim milk TBS (0.1% [v/v] Tween 20 in 2 mM Tris-HCl, pH 7.4, and 13.7 mM NaCl) for 60 min and incubated with anti-GFP (TransGen, HT801), anti-SLR1 (Huang et al., 2015), anti-actin (Cwbio, CW0264M), or anti-TUB (homemade) primary antibodies for 40 min followed by blotted with horse-radish peroxidase-conjugated secondary antibody (Cwbio, CW0102S). The immunoblotting signals were captured with a chemiluminescence imaging system (Biostep).

Pull-Down Assay

The procedures for the pull-down assay were modified from a previous report (Li et al., 2011). Approximately 2 μg of purified recombinant bait proteins (GST-OsPIL14 or GST) was incubated with 2 μg of prey protein (MBP-SLR1) in 1 mL binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.6% [v/v] Triton X-100) at 4°C for 2.5 h. Following the addition of glutathione sepharose 4B beads (GE Healthcare), the samples were further incubated for 1 h. After washing with binding buffer, the precipitated proteins were eluted by heating the beads in 2× SDS-loading buffer at 95°C for 10 min and then separated on a 10% (w/v) SDS-PAGE gel. The proteins were analyzed by immunoblotting with anti-GST (Abcam, ab19256) and anti-MBP (Abcam, ab9084) primary antibodies.

Commmunoprecipitation Assay

The 35S:SLR1-HA-cyFP construct was cotransformed with 35S:nYFP or 35S:OsPIL14-Myc-nYFP and transiently expressed in N. benthamiana leaves. The infiltrated leaves were harvested and ground in liquid nitrogen and then homogenized in extraction buffer (500 mM Tris-HCl [pH 7.4], 150 mM NaCl, 150 mM KCl, 5 mM MgCl2, 5 mM EDTA, pH 8.0, 1% [v/v] Triton X-100, 10% [v/v] glycerol, and protease inhibitor cocktail [Roche]) on ice. The samples were incubated with anti-HA (TransGen, HT301) antibody for 2 to 3 h at 4°C. Protein A-sepharose beads (Roche) were added, and the reaction was incubated for a further 2 h. Precipitated proteins were eluted and analyzed by immunoblotting with anti-HA and anti-Myc (TransGen, HT101) antibodies.

Bimolecular Fluorescence Complementation Assay

The corresponding plasmids were integrated into A. tumefaciens strain GV3101. A. tumefaciens carrying the constructs of interest was infiltrated into the abaxial surface of 4-week-old N. benthamiana leaves, and the plants were grown for 2 d before examination. The YFP signals were observed with a confocal laser-scanning microscope (TCS SP5; Leica).

EMSA

GST-OsPIL14 and MBP-SLR1 recombinant proteins were expressed in the Rosetta-gami (DE3) strain of Escherichia coli. The soluble fusion proteins were purified using glutathione sepharose 4B beads (GE Healthcare) for GST fusions and dextrin sepharose high performance (GE Healthcare; for MBP fusions). The EMSA experiment was carried out using the LightShift chemiluminescent EMSA kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Two complementary oligonucleotides were synthesized and biotinylated using the biotin DNA labeling kit (Thermo Fisher Scientific). The labeled oligonucleotides were incubated with MBP-SLR1, GST-OsPIL14, or both. Free DNA and the protein-probe complexes were separated on a 6% (w/v) native polyacrylamide gel in 0.5x TBE and transferred to a nylon membrane (Millipore). Biotin-labeled DNA was captured according to the manufacturer’s instructions (Thermo Fisher Scientific) with a chemiluminescence imaging system (Biostep). The oligonucleotide sequences are shown in Supplemental Table S1.

Sequence Analysis

The amino acid sequences (PIF1, PIF3, PIF4, PIF5, PIF6, and PIF7 in Arabidopsis; OsPIL11-16 in O. sativa) were aligned with DNAMAN. The same program was used to analyze protein phylogeny by the neighbor-joining method as previously reported (Li et al., 2019).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers OsPIL14 (LOC_Os07g05010), SLR1
Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Overexpression of OsPIFs causes elongated mesocotyl.

Supplemental Figure S2. Sequencing analysis showing OsPIL14 deletion in the ospl14 mutants.

Supplemental Figure S3. Seedling phenotypes under conditions of different chemical treatments.

Supplemental Figure S4. OsPIL14 expression pattern during dark-to-light transition.

Supplemental Figure S5. GO analysis of OsPIL14-regulated genes.

Supplemental Figure S6. OsPIL14 regulates SLR1 expression and protein accumulation.

Supplemental Table S1. List of primers used in this study.

Supplemental Dataset S1. A list of differentially regulated genes by OsPIL14.

ACKNOWLEDGMENTS

We thank Dr. Yihua Zhou (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for providing the slr1-6 mutant and anti-SLR1 antibody. The authors declare no conflict of interest.

Received April 15, 2020; accepted June 15, 2020; published June 24, 2020.

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