SIZ1-Mediated SUMO Modification of SEUSS Regulates Photomorphogenesis in Arabidopsis

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ABSTRACT

Small ubiquitin-like modifier (SUMO) post-translational modification (SUMOylation) plays essential roles in regulating various biological processes; however, its function and regulation in the plant light signaling pathway are largely unknown. SEUSS (SEU) is a transcriptional co-regulator that integrates light and temperature signaling pathways, thereby regulating plant growth and development in Arabidopsis thaliana. Here, we show that SEU is a substrate of SUMO1, and that substitution of four conserved lysine residues disrupts the SUMOylation of SEU, impairs its function in photo- and thermomorphogenesis, and enhances its interaction with PHYTOCHROME-INTERACTING FACTOR 4 transcription factors. Furthermore, the SUMO E3 ligase SIZ1 interacts with SEU and regulates its SUMOylation. Moreover, SEU directly interacts with phytochrome B photoreceptors, and the SUMOylation and stability of SEU are activated by light. Our study reveals a novel post-translational modification mechanism of SEU in which light regulates plant growth and development through SUMOylation-mediated protein stability.

Keywords: light signaling, phytochrome, PIF4, SUMOylation

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INTRODUCTION

As a major environmental signal, light affects the growth and development of plants throughout their life cycle. Photomorphogenesis, the light-mediated development of plants, has been extensively studied in the model plant species Arabidopsis thaliana. Seedlings grown in darkness display elongated hypocotyls and closed cotyledons without chloroplast differentiation. Light triggers photomorphogenic responses, characterized by the inhibition of hypocotyl growth and the expansion of cotyledons that subsequently form functional chloroplasts. Multiple photoreceptors, including phytochromes (phy), cryptochromes, and UVR8, perceive light signals and activate various signaling pathways that ultimately control photomorphogenesis (Gommers and Monte, 2018). Numerous downstream components in the light signaling pathway have been identified, and their activities are regulated at transcriptional and post-translational levels (Hoecker, 2005; Jiao et al., 2007).

Transcription factors and their co-regulators play fundamental roles in controlling gene expression during photomorphogenesis (Jiao et al., 2007). PHYTOCHROME-INTERACTING FACTORS (PIFs) are a group of transcription factors possessing a conserved basic helix-loop-helix domain that negatively and redundantly regulates photomorphogenesis (Leivar and Monte, 2014). Plants with simultaneous loss of PIF1, PIF3, PIF4, and PIF5 exhibit a photomorphogenic phenotype in darkness, similar to that of the constitutive photomorphogenic1 (cop1) mutant (Leivar et al., 2008; Shin et al., 2009). When plants are irradiated with red light, the bioactive Pfr form of phy translocates from the cytoplasm to the nucleus, where it interacts with PIF proteins and degrades them through the 26S proteasome pathway (Ni et al., 2014). Genome-wide transcriptomic analyses reveal that PIF proteins regulate a large number of genes either directly or indirectly (Leivar et al., 2009; Shin et al., 2009; Pfeiffer et al., 2014).

A forward genetic study led to the identification of SEUSS (SEU) as a negative transcriptional regulator of photomorphogenesis (Huai et al., 2018). SEU is a homolog of the family of LDB proteins, which couple transcription factors to form high-order activation complexes in animals (Jurata and Gill, 1997). SEU
Plant Communications

associates with the promoters of downstream cell elongation-related genes and regulates their expression by directly interacting with PIF4. The SEU–PIF4 interaction also positively regulates thermomorphogenesis (Huai et al., 2018). The short-hypocotyl phenotype of the seu knockout suggests that SEU might regulate plant growth and development at the adult stage independently of PIF4. Indeed, SEU functions as a transcriptional co-repressor with LEU1ING to regulate multiple developmental processes (Franks et al., 2002; Sridhar et al., 2006; Grigorova et al., 2011; Gong et al., 2016). Although the expression of SEU at the mRNA level is not drastically affected by light (Huai et al., 2018), its regulation at the protein level remains elusive.

The stability, activity, interaction, and/or localization of a protein are often regulated by post-translational modifications. SUMOylation is a key post-translational modification that covalently attaches small ubiquitin-like modifiers (SUMO) to certain lysine residues of target proteins (Johnson, 2004; Elrouby and Coupland, 2010). The SUMOylation machinery consists of the SUMO-activating enzyme, the SUMO-conjugating enzyme, and the SUMO E3 and E4 ligases. In Arabidopsis, SCE1 is a SUMO-conjugating enzyme, whereas SIZ1 is a SUMO E3 ligase (Park et al., 2011; Zhang et al., 2017b; Cai et al., 2017). SIZ1 mediates the conjugation of SUMO1/2 to its target proteins (Miura et al., 2005). Growing evidence indicates that SUMOylation plays critical regulatory roles in various plant developmental processes and responses to environmental cues (Miura et al., 2007, 2009; Jin et al., 2008; Park et al., 2011; Zheng et al., 2012; Conti et al., 2014; Kim et al., 2015; Crozet et al., 2016; Lin et al., 2016; Orosa et al., 2018). Two previous studies have shown that SUMOylation is involved in regulating light response. Phytochrome B (phyB) is subject to SUMOylation, which represses red light signaling partly by inhibiting the phyB–PIF5 interaction (Sadanandom et al., 2015). SIZ1 targets COP1 for SUMO modification and promotes COP1 activity, thereby negatively regulating photomorphogenesis, and COP1 in turn mediates the ubiquitination and degradation of SIZ1 (Lin et al., 2016). However, it is unknown whether and how the other components of the light signaling pathway are modified by SUMOylation.

In this study, we show that SEU undergoes SUMOylation at four conserved lysine sites and that substitution of these residues results in altered SEU activity and function. SIZ1 mediates the SUMOylation of SEU through direct interaction. In addition, phyB physically interacts with SEU and regulates its light-mediated protein stability. This study demonstrates that SEU SUMOylation plays essential roles in controlling plant growth and development in response to light.

RESULTS

SEU Is a Substrate of SUMO1

SUMO peptides are covalently linked to a SUMO consensus motif (ΨKxΕ/D; Ψ, large hydrophobic residue; K, acceptor lysine; x, any amino acid; E/D, glutamate or aspartate) in target proteins (Seeler and Dejean, 2003). To investigate the possible SUMO modification of SEU, we performed SUMOylation site prediction using three independent computational programs and found that SEU possesses four putative SUMOylation sites, K170, K200, K216, and K392, which are conserved in various plant species (Figure 1A, Supplemental Figure 1). This result suggests that SEU is likely a SUMO substrate.

To test this possibility, we performed transient in vitro and in vivo SUMOylation experiments. Firstly, we transiently coexpressed SEU–3HA with FLAG-SUMO1GG (wild type) or FLAG-SUMO1AA (a conjugation-deficient mutant) in Arabidopsis protoplasts. We then immunoprecipitated SEU–3HA with an anti-HA antibody and detected the immunoprecipitated proteins with an anti-FLAG antibody. Higher-molecular-weight SUMOylated SEU–3HA bands (SUMO1–SEU) were detected when SEU–3HA was coexpressed with FLAG–SUMO1GG, but not with FLAG–SUMO1AA (Figure 1B). Secondly, we performed a transient SUMOylation assay in Nicotiana benthamiana leaves and obtained similar results (Figure 1C). Thirdly, we generated SEUp:SEU–GFP transgenic plants, where SEU was fused with GFP and driven by its native promoter in the seu-6 background. We extracted proteins from seu-6 and seu-6/SEUp:SEU–GFP plants and immunoprecipitated them with anti-SEU and anti-SUMO1 antibodies. The anti-SUMO1 antibody detected higher-molecular-weight bands of SEU–GFP in seu-6/SEUp:SEU–GFP, but not seu-6 plants (Figure 1D). There were two higher-molecular-weight bands whose exact weights differed from those in Arabidopsis protoplast and N. benthamiana assays, indicating that SEU may be multi- or poly-SUMOylated at one or more conjugating sites in different plants. Fourthly, we incubated affinity-purified recombinant proteins MBP–SEU–FLAG (substrate), SUMO E1 (His–SAE1b and His–SAE2), and SUMO E2 (His–SCE1) with His–SUMO1GG or His–SUMO1AA in the presence or absence of SUMO E3 ligase (MBP–SIZ1–Myc). Anti-FLAG and anti-SUMO1 antibodies detected slowly migrating bands above the original SEU protein only in the reaction containing His–SUMO1GG and SIZ1, but not in the other reactions (Figure 1E), suggesting that SEU is SUMOylated in vitro and its SUMOylation requires the presence of SUMO E3 ligase. Taken collectively, these results demonstrate that SEU is SUMOylated and is a bona fide substrate of SUMO1.

SIZ1 Interacts with SEU and Regulates Its SUMOylation

As SIZ1 is a key SUMO E3 ligase, we assessed whether SEU could interact with SIZ1 using a yeast two-hybrid assay. Because GBD–SIZ1 exhibited auto-activation activity that interfered with the assay, we used a truncated version, GBD–SIZ1–Δ (amino acids 1–664 of SIZ1 fused with the GAL4 DNA-binding domain). Indeed, GAD–SEU (SEU fused with the GAL4 activation domain) strongly interacted with GBD–SIZ1–Δ (Figure 2A). GAD–SEU also interacted with GBD–SCE1 (a SUMO-conjugating enzyme), but not GBD–SUMO1 (Figure 2A).

Next, we performed a semi-in vitro pull-down assay in N. benthamiana leaves, in which MBP–SEU–FLAG was incubated with SIZ1–Δ–FLAG. Precipitation with MBP beads revealed that SEU interacts with the truncated version of SIZ1 (Figure 2B). The in vitro pull-down assay of MBP–SEU–FLAG incubated with MBP–SIZ1–Myc demonstrates that SEU directly interacts with full-length SIZ1 (Figure 2C). SEUp:SEU–GFP fully complemented the short-hypocotyl phenotype of seu-6 (see Figure 3A in detail; Huai et al., 2018). An in vivo co-immunoprecipitation (Co-IP) assay showed that the anti-GFP antibody immunoprecipitated SIZ1 from seu-6/SEUp:SEU–GFP plants, but not seu-6 mutant plants.
(Figure 2D). These results demonstrate that SEU interacts with SIZ1 in vitro and in vivo.

To investigate the functional relationship between SIZ1 and SEU, we generated a seu siz1 double mutant. The hypocotyl length of seu siz1 plants was slightly but significantly shorter than that of seu and siz1 single mutants (Supplemental Figure 2A). SEUp:SEU-GFP largely complemented the seu phenotype, but this effect was partly suppressed in the siz1 background (Supplemental Figure 2A). Similarly, at the adult stage, seu siz1 plants showed a severe dwarf phenotype, much more extreme than the phenotypes of the parental single mutants. The complementary effect of SEUp:SEU-GFP on seu-6 was largely inhibited by the siz1 mutation (Supplemental Figure 2B and 2C). These observations suggest that SEU and SIZ1 coordinate plant growth and development, and that the function of SEU largely requires SIZ1. Furthermore, the in vivo immunoblotting experiment showed that the SUMOylated SEU-GFP bands seen in seu-6/SEUp:SEU-GFP were almost abolished in siz1 seu-6/SEUp:SEU-GFP (Figure 2E). Moreover, SEU protein levels were partially reduced in the siz1 mutant in comparison with those in Col (Figure 2F), likely due to the transcriptional regulation by SIZ1 (Figure 2G). These results demonstrate that SIZ1 directly mediates the SUMOylation of SEU.

SUMOylation of SEU Is Required for Its Function

To investigate the functional importance of SEU SUMOylation, we substituted the four conserved K residues with arginine (R; non-SUMOylated form) via site-directed mutagenesis and

Figure 1. SEU Is Subject to SUMO Modification.

(A) Diagram of SEU structure and positions of four predicted SUMOylation sites. LDB, LIM-domain binding.

(B and C) Transient SUMOylation assay. FLAG-SUMOAA or FLAG-SUMOOGG together with SEU-3HA (B) or SEUp:SEU-GFP (C) were transiently expressed in Arabidopsis protoplasts and N. benthamiana leaves, respectively. Total proteins were extracted and precipitated with an anti-HA or anti-GFP antibody and then immunoblotted with different antibodies.

(D) In vivo SUMOylation of SEU. Plants were grown under light (80 μmol/m²/s) for 5 d. Proteins were precipitated with an anti-GFP monoclonal antibody conjugated to agarose beads.

(E) In vitro SUMOylation of SEU. SUMOylated MBP-SEU-FLAG was detected with an anti-SUMO1 antibody.
generated transgenic lines expressing various forms of SEU fused with GFP (SEUp:SEU(m)-GFP; m stands for various K-to-R mutations) in the seu-6 background. Multiple lines of transgenic plants expressing SEUp:SEU(K170R)-GFP, SEUp:SEU(K200R)-GFP, SEUp:SEU(K216R)-GFP, or SEUp:SEU(K392R)-GFP partly rescued the seu-6 phenotype under red, far-red, and blue light conditions, with SEUp:SEU(K392R)-GFP having the weakest effect (Figure 3A, Supplemental Figure 3), suggesting that all four lysine (K) residues contribute to the function of SEU. Quadruple mutation of all four K sites in SEUp:SEU(4KR)-GFP resulted in phenotypes similar to those resulting from the single mutations (Figure 3A, Supplemental Figure 3). Consistent with this, the expression levels of two downstream genes, INDOLE-3-ACETIC ACID INDUCIBLE6 (IAA6) and IAA19, in seu-6/SEUp:SEU(m)-GFP plants were similar to those in seu-6 plants (Figure 3B), suggesting that the SUMO modification of SEU affects its regulation of downstream genes. However, SEU protein levels of various mutant variants were comparable to that of wild-type SEU (Figure 3C).

Next, we examined the SUMOylation levels in these different transgenic mutant lines. SUMOylation expression in the K170R mutant was abolished, and its expression levels in K200R and K216R were largely reduced, whereas that in K392R was not altered compared with that in the wild type (Figure 3D). No SUMOylated band was detected in the 4KR mutant with K-to-R substitutions of all four K sites (Figure 3D). These results indicate that K170 is the major SUMOylation site. The K200R and K216R substitutions affected the efficiency of K170 SUMOylation, possibly by affecting the conformation of the region containing K170. Single or quadruple mutations did not affect nuclear localization (Supplemental Figure 4).

SEU is also involved in regulating thermomorphogenesis, and the seu mutant has reduced sensitivity to ambient warm temperature (Huai et al., 2018). SEUp:SEU-GFP completely restored the phenotype of the seu-6 plants at 28°C. Interestingly, at this temperature, the hypocotyl length of seu-6/SEUp:SEU(4KR)-GFP was indistinguishable from that of seu-6 (Figure 3E and

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**Figure 2. SIZ1 E3 Ligase Interacts with SEU and Regulates Its SUMOylation.**

(A) Yeast two-hybrid assay. GAD, GAL4 activation domain; GBD, GAL4 DNA-binding domain; SIZ1-Δ, a truncated version of SIZ1 containing amino acids 1–664. –WLHA, without Trp, Leu, His, and Ade; –WL, without Trp and Leu.

(B) Semi-in vitro pull-down assay in N. benthamiana leaves.

(C) In vitro pull-down assay.

(D) Co-IP assay. Plants were grown under light (80 μmol/m²/s) for 5 d. Proteins were precipitated with an anti-GFP antibody conjugated to agarose beads.

(E) Detection of SUMOylated proteins in vivo. Proteins were precipitated with an anti-GFP antibody conjugated to agarose beads.

(F) Immunoblot analysis of SEU. Blotting with an anti-actin antibody served as a loading control.

(G) qRT-PCR analysis of SEU expression. Relative gene expression was normalized to the level of IPP2. Data are means ± SD of three biological replicates. Different letters indicate significant differences as determined by one-way ANOVA (P < 0.01). In (E)–(G), seedlings were grown under red light (40 μmol/m²/s) for 5 d.
which may be due to the increased SUMOylation at high temperature (Rytz et al., 2018).

K-to-R Substitution of SEU Affects Its Interaction with PIF4

SEU directly interacts with the PIF4 transcription factor and the two proteins coordinately regulate target gene expression (Huai et al., 2018). We therefore examined whether SUMOylation affects the SEU–PIF4 interaction. In a yeast two-hybrid assay, SEU4KR fused with the GAL4 DNA-binding domain (GBD–SEU4KR) interacted strongly with PIF4 or PIF4-C fused with the GAL4 activation domain (GAD–PIF4, GAD–PIF4C) in the presence of 3-amino-1,2,3-triazole inhibitor, whereas the GBD–SEU control showed almost no interaction (Figure 4A). These results suggest that these K-to-R point mutations in SEU affect its interaction with PIF4. In a Co-IP assay, a greater amount of PIF4 was pulled down in seu-6/SEUp:SEU(4KR)–GFP plants compared with seu-6/SEUp:SEU–GFP plants upon precipitation with an anti-GFP antibody (Figure 4B), implying that SEU SUMOylation might reduce its interaction with PIF4. To test whether the presence of SUMO1 can affect the interaction between SEU and PIF4, we assessed SUMO1’s influence on the binding ability of SEU and PIF4 in a pull-down assay. MBP–SEU–FLAG and PIF4–GST were incubated with increasing amounts of His–SUMO1 and then pulled down with dextran Sepharose high performance (MBP beads). With increasing His–SUMO1, a decreasing amount of PIF4–GST was detected using an anti-GST antibody (Figure 4C). We excluded the possibility of SUMOylation due to the absence of necessary enzymes for SUMOylation. PIF4 has a SUMO-interaction domain in amino acids 27–31 based on site prediction with GPS–SUMO. It is likely that SUMO1 interacts with PIF4 and competes with SEU.
Next, we used the promoter of IAA19, a gene downstream of SEU in the photomorphogenesis pathway (Huai et al., 2018), to drive the luciferase reporter gene and then performed a transient luciferase reporter assay. Overexpression of either wild-type SEU or PIF4 activated IAA19p:LUC activity, and coexpression of SEU and PIF4 further increased this effect (Figure 4D). However, overexpression of SEU 4KR repressed IAA19p:LUC activity. Furthermore, the coexpression of SEU 4KR and PIF4 resulted in lower IAA19p:LUC activity than the coexpression of SEU and PIF4 (Figure 4D). Our previous study showed that SEU positively regulates the level of tri-methylation of lysine 4 of histone 3 (H3K4me3) at the chromatin region of IAA19 (Huai et al., 2018). Consistently, the levels of H3K4me3 were reduced in the chromatin region containing the G-box of IAA19 in seu-6/SEUp:SEU(4KR)-GFP compared with those in seu-6/SEUp:SEU-GFP (Supplemental Figure 5). These results imply that SEU SUMOylation promotes its transcriptional co-regulatory activity toward downstream gene expression.

**SEU Physically Interacts with phyB**

The phyB-PIF4 and SEU-PIF4 interaction prompted us to investigate whether SEU interacts with phyB directly. Firstly, we performed a semi-in vitro pull-down assay in which protein extracts from Col or 3SS:Myc-phyB plants were incubated with recombinant proteins (MBP or MBP-SEU-FLAG). The anti-Myc antibody pulled down MBP-SEU-FLAG when incubated with proteins from 3SS:Myc-phyB, but not from Col (Figure 5A). Secondly, we performed a luciferase complementation imaging assay and observed that the coexpression of SEU-nLUC (SEU fused with the N terminus of luciferase) and phyB-cLUC (phyB fused with the N terminus of LUC) generated luminescent signals in N. benthamiana leaves (Figure 5B). Thirdly, an in vivo Co-IP experiment showed that the anti-SEU antibody immunoprecipitated phyB from Col plants, but not from seu mutant plants (Figure 5C). Similarly, the anti-Myc antibody pulled down SEU from protein extracts of 3SS:Myc-phyB, but not of Col (Supplemental Figure 6A). Fourthly, a Co-IP assay further showed that SEU was pulled down by the anti-Myc antibody in 3SS:Myc-phyB seedlings (not in Col) grown under red light for 24 h, but not in the dark (Figure 5D). These results suggest that the Pfr form of phyB possibly associated with SEU more strongly than the Pr form. To test our speculation, total proteins of 3SS:Myc-phyB seedlings grown under red light for 5 d were exposed to 5 min of far-red light (for conversion to the Pr form), or 5 min of far-red light, followed immediately by 5 min of red light (for conversion back to the Pfr form). More SEU was immunoprecipitated by the anti-Myc antibody in 3SS:Myc-phyB seedlings under the far-red-to-red light transition than those under far-red light (Figure 5E). Therefore, we conclude that SEU interacts with the Pfr form of phyB.

**Light Promotes SEU Protein Accumulation and SUMOylation**

We then generated a seu phyB double mutant and found that the hypocotyl length of these plants was similar to that of the phyB-9 single mutants (Figure 6A), indicating that the phenotype of the seu mutant is dependent on phyB. An immunoblot assay further showed that SEU protein accumulation was greatly reduced in
the phyB-9 mutant compared with the wild type under red light (Figure 6B), whereas the SEU transcript level was not dramatically affected by the phyB-9 mutation (Supplemental Figure 6B), suggesting that phyB positively regulates SEU protein accumulation.

Next, we investigated how light regulates SEU SUMOylation and its protein level. We compared the levels of SEU protein over time in seu-6/SEUp:SEU-GFP and seu-6/SEUp:SEU(4KR)-GFP seedlings grown under changing light conditions. During the dark-to-light transition, SEU protein levels were gradually increased in seu-6/SEUp:SEU-GFP seedlings, whereas they were almost unaffected in seu-6/SEUp:SEU(4KR)-GFP seedlings (Figure 6C and 6D). During the light-to-dark transition, SEU protein levels were gradually decreased in seu-6/SEUp:SEU-GFP seedlings, whereas they were almost unaffected in seu-6/SEUp:SEU(4KR)-GFP seedlings (Figure 6E and 6F). Furthermore, an in vivo immunoblotting assay showed that SUMOylated SEU levels also gradually increased in seu-6/SEUp:SEU-GFP seedlings during the dark-to-light transition but decreased during the light-to-dark transition (Figure 6G and 6H). Taken collectively, these results indicate that light regulates SEU protein accumulation and SUMOylation. In addition, we tested the SUMOylation levels of SEU at different temperatures. An in vivo immunoblotting assay showed that SUMOylated SEU levels in seu-6/SEUp:SEU-GFP seedlings grown under 28°C were higher than those grown under 22°C (Supplemental Figure 7).

**DISCUSSION**

Compared with protein phosphorylation and ubiquitination, SUMO modification is less well understood in the field of plant photobiology. Previous studies have reported that phyB and COP1 are modified by SUMOylation (Sadanandom et al., 2015; Lin et al., 2016). In this study, we provide multiple lines of biochemical and genetic evidence that SEU is also regulated by SUMOylation. Firstly, a SUMOylation assay showed that SEU is a substrate of SUMO1 (Figure 1), consistent with a previous proteomic study (Miller et al., 2010). Secondly, we identified four conserved lysine residues corresponding to the
SUMOylation sites and found that mutations affecting these residues, especially K170R, K200R, and K216R, reduced or impaired SUMOylation, and altered SEU function (see discussion below). Thirdly, we showed that SIZ1, a key SUMO E3 ligase, interacts with SEU and controls its SUMOylation (Figure 2), confirming that SEU is subject to the SUMO modification. Consistently, SIZ1 was demonstrated to negatively regulate photomorphogenesis (Lin et al., 2016).

Fourthly, we determined that light regulates SEU at the post-translational level, but not the transcriptional level (Figure 7; Huai et al., 2018). SUMOylation represses or promotes the ubiquitination of its substrates, affecting protein subcellular localization, enzymatic activity, and protein–protein interactions (Geoffroy and Hay, 2009; Elrouby et al., 2013; Augustine and Vierstra, 2018). In this study, using site-directed mutagenesis and transgenic approaches, we revealed that residues K170, K200, and K216 were important for the function of SEU in photo- and thermomorphogenesis, whereas K392 played a weak role in these processes (Figure 3). The lysine-to-arginine substitutions of K200R and K216R each drastically reduced the SUMOylation level of SEU, and SUMOylation was almost undetectable in the K170R mutant, whereas it was unaffected in the K392R mutant (Figure 3C). The phenotype of the quadruple mutant carrying all four substitutions (4KR) was similar to those of K170, K200, and K216 single mutants. Thus, K170 is the predominant SUMOylation site. These mutations did not affect the protein levels or subcellular localization of SEU (Figure 3B, Supplemental Figure 4). However, the substitution of 4KR reduced the transcriptional activation activity of SEU toward IAA19p:LUC but enhanced its physical interaction with PIF4, and SUMO1 blocked the SEU-PIF4 interaction (Figure 4), indicating that SIZ1-mediated SUMOylation enhances the transcriptional activation activity of SEU but weakens its interaction with other factors. Notably, the mutations in the 4KR mutant partially complemented the photomorphogenic phenotype of the seu mutant, but fully restored its responsiveness to high temperature (Figure 3), suggesting that SEU regulates photo- and thermomorphogenesis in both overlapping and distinct ways. Interestingly, loss of SIZ1 does not affect COP1 protein accumulation (Lin et al., 2016). SEU is possibly also subject to ubiquitination, and both SUMOylation and ubiquitination of SEU may involve the same lysine residue(s). Therefore, ubiquitin-
and proteasome-mediated degradation is likely inhibited by SUMOylation. Similarly, SIZ1 stabilizes MYB30 during ABA treatment and may also promote ICE1 stability (Miura et al., 2007; Zheng et al., 2012).

Intriguingly, our data demonstrate that SEU interacts with the phyB photoreceptors, and that this interaction is greatly enhanced under red light (Figure 5), suggesting that SEU may transduce phyB signals by preferentially associating with the active Pfr form of phyB. Consequently, phyB positively regulates the accumulation of SEU protein under red light (Figure 6B). Moreover, we showed that SEU accumulates during the dark-to-light transition but gradually degrades after the light-to-dark transition; however, the protein levels were not altered when the four SUMOylated residues were mutated (Figure 6), implying that light promotes SEU SUMOylation and stability. Similarly, red light enhances the accumulation of SUMOylated phyB (Sadanandom et al., 2015). SEU and PIF4 physically interact and co-regulate downstream gene expression (Huai et al., 2018). Upon light exposure, phyB interacts with PIF4 and triggers its degradation through the 26S proteasome pathway, thus relieving the inhibitory role of PIF4 on photomorphogenesis (Huq and Quail, 2002; Zhang et al., 2017a). Therefore, the stability of PIF4 and SEU are opposite in response to light, even though both proteins are negative regulators of photomorphogenesis. SEU might act to dampen the over-suppressive effect of PIF4 on photomorphogenesis, possibly by repressing the degradation of PIF4.

In summary, we propose that light activates phyB, which interacts with PIF4 and SEU, leading to the degradation of PIF4 but the stabilization of SEU. The SUMOylation of SEU promotes its transcriptional activation activity and reduces its interaction with PIF4, thus fine-tuning the transcription of PIF4 target genes and photomorphogenesis (and thermomorphogenesis) under changing light (and temperature) conditions (Figure 7). PIF4 might also be modulated by SUMOylation (Mazur et al., 2019). The SUMO modification precisely regulates the activity of these proteins and modulates their stability and interaction with other factors. Sequence analysis revealed that the SUMOylated lysine residues in SEU are conserved in various plant species, implying that the SUMO regulation and function of SEU might be conserved during evolution in land plants.

MATERIALS AND METHODS

Plant Materials

The seu-6 (Huai et al., 2018), phyB-9 (Zhang et al., 2017c), and siz1 (Salk, 056397) (Niu et al., 2019) mutants were in the Columbia (Col-0) ecotype background. The seu siz1, seu phyB, and siz1 seu-6/ pSEU:SEU(4KR)-GFP mutants were generated by genetic crossing. All homozygous mutant and transgenic lines were verified by PCR genotyping and/or antibiotic selection. Primers are listed in Supplemental Table 1. Transgenic plants were generated by Agrobacterium tumefaciens-mediated transformation via the floral dip method (Clough and Bent, 1998) and selected on MS plates in the presence of antibiotics. Homozygous lines were used in the experiments.

Plant Growth Conditions and Phenotypic Analysis

Seeds were sown on 1 x Murashige and Skoog medium containing 1% sucrose and 0.8% agar and incubated at 4°C in the dark for 3 d. For photomorphogenic experiments, seedlings were grown in the dark or under continuous far-red (12 μmol/m²/s), red (40 μmol/m²/s), or blue (10 μmol/m²/s) light at 22°C for 5 d. For thermomorphogenic experiments, seedlings were grown under continuous white light (50 μmol/m²/s) at 22°C or 28°C for 5 d. Light was supplied by light-emitting diodes. Representative seedlings were placed on agar plates and photographed with a digital camera (Olympus). Hypocotyl length was measured and analyzed using NIH ImageJ software (http://rsbweb.nih.gov/ij/).

Plasmid Construction

The coding sequences of SEU, PIF4, SIZ1, SIZ1-J (1–1993 bp), and the promoter of IAA19 were amplified using high-fidelity Phusion DNA polymerase (Invitrogen) and cloned into the pEASY-Blunt vector (TransGen) to generate pEASY-SEU, pEASY-PIF4, pEASY-SIZ1, pEASY-SIZ1-J, and pEASY-IAA19p, respectively. The SUMOylation sites of lysine (K) in SEU were mutated to arginine (R) using the Fast Mutagenesis System kit (TransGen) to generate pEASY-SEU(R). PCR primers are listed in Supplemental Table 1.
SUMO Modification of SEU

Co-IP Assay
Co-IP assays were performed as described previously (Lin et al., 2016) to detect protein-protein interactions. Total proteins were extracted from seedlings with extraction buffer containing 50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM DTT, 0.1% TritonX-100, and 1 x complete protease inhibitor cocktail (Roche, 04693159001). Proteins were immunoprecipitated with antibody-conjugated agarose beads (20 μl) at 4°C for 2 h. Beads were washed three times with protein extraction buffer and eluted with 2 x SDS loading buffer at 95°C for 5 min. Eluted proteins were detected by immunoblotting and analyzed with an anti-PF4 (Huai et al., 2018), anti-phyB (Zhang et al., 2018), anti-SEU (Huai et al., 2018), anti-SIZ1 (Niu et al., 2019), anti-GFP, or anti-Myc antibody (TransGen, HT101).

Semi-In Vitro Pull-Down and Pull-Down Assays
MBP-, GST-, and His-fusion recombinant proteins were expressed and induced by isopropyl-beta-D-thiogalactopyranoside in the Escherichia coli BL21 (DE3) strain. Proteins were purified with dextran Sepharose high-performance beads (GE Healthcare, for MBP-fusion proteins), glutathione Sepharose 4B beads (GE Healthcare, for GST-fusion proteins), or Ni-NTA agarose (QIAGEN, for His-fusion proteins), respectively. Semi-in vitro pull-down and pull-down assays were performed as described previously (Jing et al., 2013; Yang et al., 2017). Aliquots of different purified fusion proteins were incubated at 4°C for 2 h. Beads (20 μl) were added to the samples, followed by gentle rotation at 4°C for 2 h. Input and IP proteins were detected with an anti-Myc, anti-FLAG (Sigma, F3165), anti-MBP (Abcam, ab9084), anti-GST (Abcam, ab19256), or anti-His antibody (Abcam, ab14923).

In Vivo SUMOylation Assay
To determine the SUMOylation status of SEU, proteins were extracted with SUMOylation buffer (50 mM Tris–Cl [pH 7.4], 150 mM NaCl, 1 mM EDTA [pH 8.0], 1 mM DTT, 20 mM N-ethylmaleimide [NEM, Sigma], 0.1% TritonX-100, and 1 x complete protease inhibitor mixture [Roche, 04693159001]). The protein extracts were immunoprecipitated with 20 μl anti-GFP-conjugated agarose beads (MBL, D153-8) at 4°C for 2 h. The beads were washed three times with protein extraction buffer and eluted with 2 x SDS loading for immunoblot analysis. The SUMOylated form of SEU was determined with an anti-SUMO1 antibody (Niu et al., 2019). Blotting with an anti-SEU or anti-GFP antibody was used as loading controls.

In Vitro SUMOylation Assay
The in vitro SUMOylation of SEU was examined as described previously (Lin et al., 2016). In brief, purified His-SUMO E1, His-SUMO E2, His-SUMO1, MBP-SIZ1-Myc, and MBP-SEU-FLAG recombinant proteins were purified. Proteins were then incubated in 30 μl of reaction buffer (20 mM HEPES [pH 7.5], 5 mM MgCl2 and 2 mM ATP) at 30°C for 30 min. SUMOylated MBP-SEU-FLAG was detected with anti-FLAG (Sigma, F3165) and anti-SUMO1 antibodies.

Luciferase Complementation Imaging Assay
The Agrobacterium tumefaciens GV3101 strain (OD600 = 0.8) carrying SEU-nLUC and cLUC-phyB plasmids were co-infiltrated into young N. benthamiana leaves using a needleless syringe. N. benthamiana was exposed to weak light for 2 d. Thereafter, 1 mM luciferase substrate solution was sprayed onto N. benthamiana leaves, and the fluorescence intensity was recorded using bioluminescence in vivo imaging and analysis system (NightSHADE LB985, Berthold Technologies).

Transient Luciferase Reporter Assays
Five micrograms of the IAA19p:LUC reporter plasmid containing a REN expression cassette was co-transformed with 5 μg of SEU-3HA, SEU-HKR-3HA, PIF4-3HA, SEU-3HA, and PIF4-3HA or SEU-HKR-3HA and PIF4-3HA effector plasmids into Arabidopsis protoplasts. After overnight incubation in the dark, protoplasts were lysed and resuspended with lysis buffer.
SUMO Modification of SEU

reagent (Promega). The activities of firefly (Photinus pyralis) and Renilla (Renilla reniformis) luciferases (LUC and REN, respectively) were measured with a Modulus luminometer/fluorometer (Promega). REN was used as a control to monitor the transformation efficiency. Relative LUC levels were expressed as the ratio of LUC/REN.

GFP Fluorescence

seu-6/SEUp:SEU-GFP and seu-6/SEUp:SEU(m)-GFP transgenic seedlings were grown under white light for 5 d, and fluorescence was observed with a confocal microscope (Zeiss LSM 510). All images were captured using identical settings.

ChiP–qPCR Assay

Chromatin immunoprecipitation, followed by quantitative PCR, was performed as described previously (Huai et al., 2018).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Plant Communications Online.

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AUTHOR CONTRIBUTIONS

X.Z. performed most of the experiments. J.H. constructed partial plant materials and performed ChiP and GFP fluorescence assays. S.L. generated the phyB constructs. J.J. discussed the paper and provided comments. All authors analyzed the data. X.Z. and R.L. designed the experiments. X.Z. performed most of the experiments. J.H. constructed partial plant nucleus during stress and development. Curr. Opin. Plant Biol. 45:143–154.

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