SUMOylation of phytochrome-B negatively regulates light-induced signaling in *Arabidopsis thaliana*

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The red/far red light absorbing photoreceptor phytochrome-B (phyB) cycles between the biologically inactive (Pr, λ_{max}, 660 nm) and active (Pfr; λ_{max}, 730 nm) forms and functions as a light quality and quantity controlled switch to regulate photomorphogenesis in *Arabidopsis*. At the molecular level, phyB interacts in a conformation-dependent fashion with a battery of downstream regulatory proteins, including PHYTOCHROME INTERACTING FACTOR transcription factors, and by modulating their activity/abundance, it alters expression patterns of genes underlying photomorphogenesis. Here we report that the small ubiquitin-like modifier (SUMO) is conjugated (SUMOylation) to the C terminus of phyB; the accumulation of SUMOylated phyB is enhanced by red light and displays a diurnal pattern in plants grown under light/dark cycles. Our data demonstrate that (*i*) transgenic plants expressing the mutant phyB^{Lys996Arg}-YFP photoreceptor are hypersensitive to red light, (*ii*) light-induced SUMOylation of the mutant phyB is drastically decreased compared with phyB-YFP, and (*iii*) SUMOylation of phyB inhibits binding of PHYTOCHROME INTERACTING FACTOR 5 to phyB Pfr. In addition, we show that OVERLY TOLERANT TO SALT 1 (OTS1) de-SUMOylates phyB in vitro, it interacts with phyB in vivo, and the *ots1/ots2* mutant is hyposensitive to red light. Taken together, we conclude that SUMOylation of phyB negatively regulates light signaling and it is mediated, at least partly, by the action of OTS SUMO proteases.

photoreceptor | phytochrome | sumoylation | signaling | photomorphogenesis

Plants are sessile organisms; thus, they have to adapt to the ever-changing environment by modifying their growth and developmental programs. To respond to changes in ambient light conditions, plants evolved a battery of photoreceptors including the blue/UVA absorbing cryptochromes and phototropins (1, 2), the red/far red absorbing phytochromes (phys) (3), and the UVB-specific photoreceptor UVR8 (4). The red light/far red light (RL/FRL) absorbing phys exist as dimers, and each monomer has a covalently linked open tetrapyrrole chain as chromophore. They are synthesized in their biologically inactive Pr form (RL-absorbing state; λ_{max}, 660 nm) and converted into the biologically active Pfr (FRL-absorbing state; λ_{max}, 730 nm) by RL. Subsequent FRL treatment converts the Pfr form back into Pr. The Pr and Pfr conformers have partially overlapping absorption spectra; thus, phys cycle between their Pfr/Pr forms, and the ratio of Pr/Pfr forms is determined by the RL/FRL content of the incipient sunlight (5). In *Arabidopsis* five phys have been identified (phyA, phyB, phyC, phyD, and phyE), and among these, phyB has been shown to be especially important after seedling establishment (6).

The overwhelming majority of molecular events underlying phyB-controlled photomorphogenesis take place in the nucleus. Light in a quality- and quantity-dependent fashion induces translocation of phyB Pfr in the nuclei (7, 8), where it interacts with downstream acting regulatory proteins including PHYTOCHROME-INTERACTING-FACTORS (PIFs) (9). The very early steps of phyB signaling include (*i*) inactivation or alteration of the substrate specificity of CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) that targets proteins to degradation (10), (*ii*) degradation and/or modulation of the transcriptional activity of negative regulatory PIF TFs (11), and (*iii*) induction of transcriptional cascades that modulate the expression of 2,500–3,000 genes of the *Arabidopsis* genome (12).

The number of phyB Pfr molecules quantitatively determines physiological responses. Thus, it is evident that any molecular process that modifies (*i*) the abundance of phyB Pfr such as ubiquination of the photoreceptor followed by its subsequent degradation (13, 14), (*ii*) the accessibility (8), and (*iii*) the capacity of phyB Pfr to interact with its signaling partners alters the flux of RL-induced signaling. Very recently it was shown that the Ser86 (15) and/or Tyr104 amino acid residues (16) of phyB are phosphorylated *in planta*. These reports established that phosphorylation of S86 accelerates thermal relaxation (light-independent reversion of Pfr into Pr, also called dark reversion) of phyB, whereas light-induced phosphorylation of Tyr104 inhibits binding of PIF3 to phyB Pfr. Taken together, these results clearly demonstrated that multiple phosphorylation of phyB negatively regulates the action of this photoreceptor.

Reversible conjugation of small ubiquitin-like modifier (SUMO) to the target proteins represents an emerging posttranslational modification (17–20). Work performed in mammalian and yeast cells showed that (*i*) reversible SUMOylation of proteins regulates a

Significance

The photoreceptor phytochrome-B (phyB) cycles between its active Pfr [far red light (FRL)-absorbing state λ_{max}, 730 nm] and inactive Pr [red light (RL)-absorbing state λ_{max}, 660 nm] forms and regulates as red/far red light-activated/inactivated molecular switch plant growth and development. Here we show that conjugation of small ubiquitin-like modifier to the photoreceptor inhibits interaction of phyB Pfr with its immediate signaling partner PHYTOCHROME INTERACTING FACTOR 5 (PIF5). The impaired interaction of these proteins negatively affects photomorphogenic responses; thus, SUMOylation similar to phosphorylation plays a role in desensitizing phyB-mediated signaling. OVERLY TOLERANT TO SALT 1 and 2 (OTS1 and OTS2) are involved in regulating phyB action as these SUMO proteases mediate deconjugation of SUMO from phyB.

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broad spectrum of cellular processes and (*ii*) addition of SUMO either modifies the activity of the target protein and/or promotes interaction with specific proteins harboring the SUMO-interacting motif (21, 22). It has also been established that in plants, like in mammalian and yeast cells, a SUMOylation pathway exists for SUMOylation and de-SUMOylation of proteins (23, 24). More specifically, it was shown that in *Arabidopsis thaliana* (*i*) expression of isoforms of SUMO1, -2, -3, and -5 can be detected (25); (*ii*) conjugation of SUMO to target proteins occurs via the same sequential biochemical steps as in yeast (26, 27); (*iii*) these reactions are catalyzed by the E1 heterodimer (SAE1a/b and SAE2), a single E2 (SCE1), and two E3 (SIZ1 and MMS2/HPY2) enzymes as well as E4-type SUMO ligases (28); whereas (*iv*) de-conjugation of SUMO substrates is performed by ubiquitin-like SUMO-specific proteases including ESD4 and OTS1/2 (29–34). It appears that in plants reversible conjugation of SUMO to target proteins is particularly important in regulating responses to abiotic environmental stresses including heat shock (35), phosphate starvation (36), drought (37), and salt stress (31), but SUMOylation of specific proteins also modifies responses to ABA signaling (38) and phytopathogen infection (39) and modulates flowering time (33).

Light is arguably the most important environmental factor regulating plant growth and development, yet until now, in vitro and *in planta* studies have not indicated SUMOylation of any component of photoreceptor-controlled light signaling cascades. Here we report that (*i*) the photoreceptor phyB is reversibly SUMOylated *in planta*, (*ii*) SUMOylated phyB preferentially accumulates in light, (*iii*) binding of PIF5 to the SUMOylated phyB Pfr is significantly reduced, and (*iv*) transgenic plants expressing the mutant phyB^{Lys996Arg} photoreceptor are hypersensitive to RL. Furthermore, we show that the *ots1/ots2* mutant displays hyposensitivity to RL and enhanced phyB SUMOylation, whereas OTS1 de-SUMOylates phyB in vitro and binds to phyB *in planta*. Taken together, these data suggest that OTS1/OTS2 is involved in mediating the reversible SUMOylation of phyB and that SUMOylation of phyB desensitizes RL-induced signaling, similarly to phosphorylation of the photoreceptor.

Results

PhyB Is SUMOylated in Plants Grown in Light/Dark Cycles. Experimentally determined SUMO attachment sites contain the ΨKXE sequence, where Ψ (Psi) is a large hydrophobic amino acid and K represents the lysine that binds SUMO (40). The ever-increasing number of identified SUMOylation sites in eukaryotic cells made it possible to construct reliable sequence analysis programs to identify, in silico, sites that are potential targets for SUMOylation in vivo (41). We used the SUMOsp 2.0 software to search for potential SUMOylation site(s) in phyB of *A. thaliana* (42). This search predicted the presence of two potential SUMOylation sites, K996 (high probability) in the C terminus and Lys475 (low probability) in the N terminus of the photoreceptor in AtphyB (Table S1). Fig. 1*A* shows the sequence context for the Lys996 in *Arabidopsis* as well as in phyB sequences of other plants species. To test if phyB is indeed SUMOylated *in planta*, first we analyzed SUMOylation of AtphyB-YFP in *Nicotiana benthamiana* leaves transiently coexpressing HA-SUMO and phyB-YFP or the phyB^{Lys475Arg} or phyB^{Lys996Arg} mutants. Our data demonstrate that AtphyB is SUMOylated and that mutation of Lys996 but not that of Lys475 inhibits SUMOylation of the photoreceptor in tobacco leaves (Fig. S1). To confirm that phyB is indeed subject of SUMOylation, we grew transgenic *phyB-9 Arabidopsis* plants expressing the *35S:PHYB-YFP* transgene in light/dark (L/D) cycles, isolated the phyB-YFP fusion protein by affinity purification, and determined its SUMOylation status by using an antibody specific to AtSUMO-1. Accumulation of SUMOylated phyB-YFP reached high levels in 12 h RL/12 h D (Fig. 1*B*), and it was around the limit of detection in 12 h FRL/12 h D cycles (Fig. 1*C*). This figure also demonstrates that

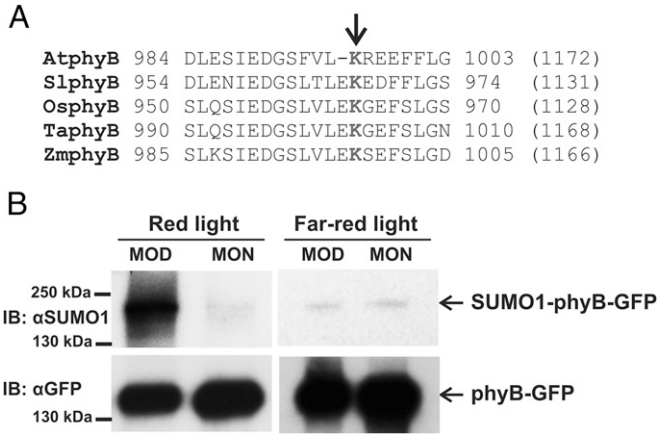

Fig. 1. The photoreceptor phyB is SUMOylated in seedlings grown in RL/D cycles. (*A*) Analysis of *A. thaliana* (At, NP_179469), *Solanum lycopersicum* (Sl, XP_010318997), *Oryza sativa* (Os, EEE58928), *Triticum aestivum* (Ta, BAP91202), and *Zea mays* (Zm, NP_001168077) phyB protein sequences by SUMOsp 2.0 software identified sequence motifs as potential targets for SUMOylation. The Lys996 amino acid of the conserved SUMOylation site for AtphyB is indicated by an arrow. Numbers at the beginning and at the end of the presented sequences indicate the corresponding amino acid positions, and numbers in brackets show the length of PHYB proteins from different species. (*B*) *phyB-9* seedlings expressing the *35S:PHYB-GFP* transgene were grown on MS medium in 12 h RL (25 μmol $m^{-2}·s^{-1}$)/12 h D cycles and 12 h FRL (10 μmol $m^{-2}·s^{-1}$)/12 h D cycles for 5 d. Samples were harvested on day 6, at MOD and MON. phyB-GFP was immunoprecipitated by using GFP-Trap agarose beads (IP:αGFP), and samples containing identical amounts of the fusion protein were analyzed. phyB-GFP was detected by anti-GFP (IB:αGFP), whereas AtSUMO1-conjugated phyB-GFP was visualized using anti-SUMO1 antibody (IB:αSUMO1).

accumulation of the SUMOylated phyB in RL/D cycles is light regulated, as it accumulates to higher levels at the middle of day (MOD) and decreases to lower levels by the middle of the night (MON). The abundance of phyB does not significantly change in the *35S:PHYB-YFP* plants grown under these conditions (Fig. S2*A*); thus, we conclude that phyB is SUMOylated in a reversible fashion in RL/D cycles. We also monitored SUMOylation of fusion proteins containing truncated forms of phyB including phyB(1–651)-YFP (43) and phyB(1–991)-YFP. The abundance of the truncated fusion proteins is similar to that of phyB-GFP, yet SUMOylation of these fusion proteins was below detection level in plants grown in RL/D cycles (Fig. S2*B*). Taken together, these data show that phyB is SUMOylated in vivo, the SUMOylated form of phyB accumulates to high levels in RL, and in harmony with data obtained by the transient SUMOylation assays, the site of reversible SUMOylation of phyB is located at the C-terminal domain of the photoreceptor.

Transgenic phyB-9 plants expressing the mutant phyB^{Lys996Arg}-YFP are hypersensitive to RL. To determine the physiological importance of the predicted SUMOylation site in phyB, we generated transgenic lines expressing, under the control of the viral *35S* promoter, the phyB-GFP and phyB^{Lys996Arg}-YFP fusion proteins at comparable levels (Fig. S2*C*, Set 1). Analysis of photomorphogenic responses in RL demonstrated that the transgenic *35S:PHYB-GFP* lines displayed characteristic overexpression phenotypes compared with wild-type (Col-0) seedlings including fluence rate-dependent inhibition of hypocotyl elongation (Fig. 2*A* and Fig. S3*A*). Importantly, we also show that the *35S:PHYB^{Lys996Arg}-YFP* lines displayed greater hypersensitivity to continuous RL not only for hypocotyl elongation (Fig. 2*A*) but cotyledon expansion (Fig. 2*C*) and cotyledon opening (Fig. 2*D*), indicating enhanced signaling by the mutant photoreceptor. In contrast, these lines did not display exaggerated responsiveness to FRL or blue light (BL) (Fig. S3 *C* and *D*). To recapitulate

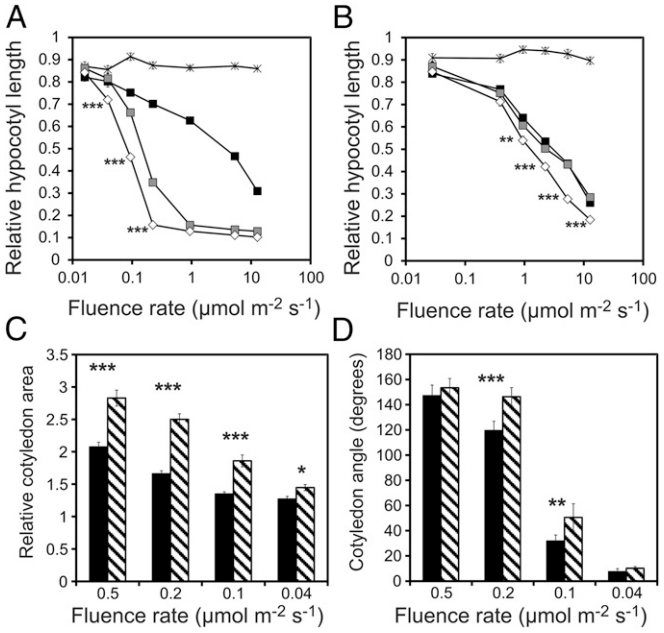

Fig. 2. Seedlings expressing the mutant phyB^{Lys996Arg}-YFP are hypersensitive to RL. Relative fluence rate-dependent inhibition of hypocotyl elongation of wild-type Col-0 (black square), *phyB-9* (star symbol), and transgenic *phyB-9* plants expressing phyB-GFP (gray square) or phyB^{Lys996Arg}-YFP (white diamond) under the control of the constitutively active (*A*) viral *35S* promoter or (*B*) the *Arabidopsis LIP1* promoter is shown. Seedlings were grown in darkness or at the indicated fluence rates of RL for 4 d. Hypocotyl lengths of irradiated seedlings were measured, and the average value of 25–30 seedlings was calculated and normalized to the hypocotyl length of D-grown plants. (*C*) Transgenic *phyB-9* seedlings expressing *35S:PHYB-GFP* (black columns) or *35S:PHYB^{Lys996Arg}-YFP* (striped columns) were grown at the indicated fluence rates of RL for 4 d. Cotyledon areas of irradiated seedlings were measured, and the average value was normalized to the cotyledon area of D-grown plants. (*D*) Cotyledon angle of D- or RL-grown seedlings analyzed in Fig. 3*C* was measured. All experiments shown in Fig. 2 were repeated three times. Error bars indicate SE. Asterisks show statistically significant differences between phyB-GFP or phyB^{Lys996Arg}-YFP expressor plants as determined by Student's *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

these data in plants containing phyB at physiological levels, we generated transgenic *phyB-9* plants that expressed phyB-YFP or phyB^{Lys996Arg}-YFP fusion proteins under the control of the moderately active *LIP1* (*LIGHT INSENSITIVE PERIOD 1*) promoter (44). Expression of the *LIP1:PHYB-YFP* transgene did not result in an overexpression phenotype but restored RL fluence rate-dependent inhibition of hypocotyl elongation similar to the wild-type (Col-0) level (Fig. 2*B*). Furthermore, we found that *LIP1:PHYB^{Lys996Arg}-YFP* seedlings are also hypersensitive to RL (Fig. 2*B* and Fig. S3*B*) compared with *LIP1-PHYB-YFP*, although the abundance of the fusion proteins is identical (Fig. S2*C*). Thus, we conclude that the mutant phyB^{Lys996Arg}-YFP, overexpressed or expressed at the physiological level, invariably displays enhanced RL-induced signaling. To test if enhanced signaling by the mutant phyB^{Lys996Arg}-YFP was brought about by increased/accelerated translocation of the mutant photoreceptor into the nucleus, we determined RL-dependent nuclear accumulation of the various phyB fusion proteins *in planta*. The data obtained indicate that the velocity of import into the nuclei and the formation as well as the size of phyB-YFP and phyB^{Lys996Arg}-YFP–associated photobodies do not differ *in planta* (Fig. S4).

The Lys^{996}Arg mutation reduces accumulation of SUMOylated phyB-YFP. To test if enhanced RL-induced signaling of the mutant phyB^{Lys996Arg}-YFP photoreceptor was indeed brought about by its altered SUMOylation, we performed two lines of experiments. First we determined whether the Lys/Arg substitution affected rates of photoconversion and/or dark reversion of phyB. Our data demonstrate that the basic photochemical properties of the mutant phyB do not significantly differ from those of phyB (Fig. S5). Next we compared the accumulation of SUMOylated phyB-GFP and phyB^{Lys996Arg}-YFP *in planta*. We found that amounts of detectable SUMOylated phyB-GFP and phyB^{Lys996Arg}-YFP are low in etiolated seedlings, whereas RL treatment elevates the level of SUMOylated phyB-GFP but not that of phyB^{Lys996Arg}-YFP (Fig. 3). Accumulation of SUMOylated phyB-GFP increases slowly during the first 3–6 h of irradiation and reaches a substantially higher level after 24 h of treatment with RL (Fig. 3), and elevated intensities of RL are more effective to induce accumulation of SUMOylated phyB-GFP (Fig. S6*A*). Exposure of etiolated seedlings to white light (WL) also differentially modulates accumulation of SUMOylated phyB-GFP and phyB^{Lys996Arg}-YFP (Fig. 4). Importantly, this figure also illustrates that the constitutively active phyB^{Tyr276His}-YFP photoreceptor is hyper-SUMOylated in a light-independent fashion in etiolated seedlings. In addition, we found that nuclear pool of phyB is more prone to SUMOylation by comparing the level of SUMOylation of phyB in phyB-GFP–, phyB-GFP-nuclear localization signal (NLS)–, and phyB-GFP-nuclear export signal (NES)–expressing transgenic lines (Fig. S6*B*). Taken together, these data suggest that SUMOylated phyB preferably accumulates in its Pfr form in the nucleus, its accumulation is RL fluence rate-dependent, and perturbation of the putative SUMOylation site significantly reduces the detectable amount of SUMOylated phyB-GFP Pfr leading to enhanced signaling.

Binding of PIF5 to phyB Pfr is inhibited by SUMOylation of the photoreceptor. PIF1 and PIF3–PIF8 represent a subgroup of the bHLH (basic-helix–loop–helix) type transcription factor family that binds to phyB Pfr (45). PIFs negatively regulate signaling by phyB (46, 47) and function as a signaling hub to integrate light and hormone-induced signaling cascades (9, 48). Binding of PIFs to phyB initiates phosphorylation and subsequent degradation (49) or inactivation of these TFs (11, 50) and results in altered transcription of hundreds of genes in seedlings exposed to RL. It has been shown that compromised binding of PIFs to phyB Pfr results in reduced sensitivity to RL (49). To test whether SUMOylation of phyB interferes with PIF5 binding to phyB Pfr, we performed pull-down assays from infiltrated *N. benthamiana* leaves transiently coexpressing phyB-YFP or phyB^{Lys996Arg}-YFP with PIF5-MYC and HA-SUMO1 proteins. Our data demonstrate that binding of PIF5 to phyB-GFP (Fig. 5, lane 1) is reduced compared with phyB^{Lys996Arg}-YFP (Fig. 5, lane 2), although the amount of phyB-YFP and phyB^{Lys996Arg}-YFP is identical. This figure also demonstrates that Lys996Arg substitution inhibits SUMOylation of the receptor. We also note that in these assays the level of SUMOylated phyB-YFP is likely to be lowered by conjugation of *N. benthamiana* SUMO1. Taken together, these results suggest that binding of PIF5 to phyB Pfr is inhibited by the SUMOylation of the photoreceptor.

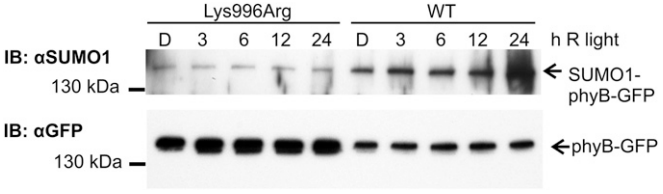

Fig. 3. RL-induced accumulation of the SUMOylated phyB^{Lys996Arg} mutant photoreceptor is reduced. *phyB-9* seedlings expressing phyB-GFP (WT) or phyB^{Lys996Arg}-YFP (Lys996Arg) fusion proteins were grown in darkness for 4 d (D) and subsequently transferred to RL (12.5 μmol $m^{-2}.s^{-1}$) for the indicated time. Sample preparation and detection of SUMOylation were performed as described in Fig. 1.

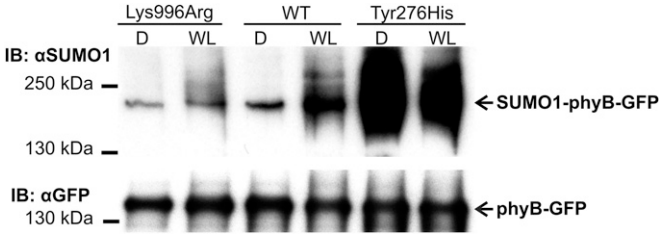

Fig. 4. The SUMOylated form of the constitutively Pfr phyB^{Tyr276His} mutant accumulates to equally high levels in darkness and light. Transgenic *phyB-9* seedlings expressing phyB-GFP (WT), phyB^{Lys996Arg}-YFP (Lys996Arg), or phyB^{Tyr276His}–YFP (Tyr276His) were grown in darkness for 4 d (D) and transferred to WL (50 μmol m^{-2}·s^{-1}) for 24 h. Sample preparation and detection of SUMOylation was performed as described in Fig. 1.

Binding of PIFs to phyB Pfr has been known to induce degradation of not only the transcription factors but also phyB (13, 45), and a more recent report described the components and molecular mechanism that mediate mutual degradation of these proteins (14). SUMOylation of phyB appears to inhibit binding of PIF5 (Fig. 5); therefore, we determined how differential binding of PIFs to phyB-YFP and phyB^{Lys996Arg}-YFP affects RL-induced degradation of these fusion proteins. Fig. S7 shows that after prolonged treatment with RL the abundance of phyB^{Lys996Arg}-YFP is moderately but significantly lowered compared with phyB-YFP, indicating that SUMOylation of phyB inhibits RL-induced, PIF-mediated degradation of the photoreceptor. Taken together, our data demonstrate that SUMOylation of phyB negatively regulates RL-induced signaling, which can be explained, at least partly, by the compromised interaction of the SUMOylated phyB Pfr with PIF5 as well as with other PIFs.

The SUMO proteases OTS1/OTS2 are involved in de-SUMOylation of phyB. SUMOylation of proteins is a reversible process mediated by enzymes that catalyze conjugation/deconjugation of SUMO to/from the target proteins. Recent research identified a number of genes encoding SUMO proteases in *Arabidopsis* (for a review, see ref. 27). We have launched a program to systematically test if any of these proteases is required for de-SUMOylation of physs, including phyB. So far we have identified two SUMO proteases, OTS1 and OTS2, that appear to play an active role in de-SUMOylation of phyB. We found that the *ots1/ots2* double mutant is hyposensitive to RL (Fig. 6*A*). Hyposensitivity of the *ots1/ots2* mutant is specific to RL, as the double mutant displayed normal responsiveness to BL and FRL (Fig. 6*B*), whereas the triple *ots1/ots2/phyB-9* mutant was completely insensitive to RL, similarly to *phyB-9* (Fig. 6*A*). To validate the involvement of OTS1/OTS2 in regulating RL-induced signaling, we determined the accumulation of SUMOylated phyB-YFP in transgenic *phyB-9* and *ots1/ots2/phyB-9* plants expressing phyB-YFP. To ensure that phyB-YFP is expressed at identical levels, we introgressed the *LIP1:PHYB-YFP* transgene from *phyB-9* (Fig. 2*B*) into an *ots1/ots2/phyB-9* background. The abundance level of OTS1/OTS2 is not regulated by light (Fig. S7 *C* and *D*), and Fig. 6*C* demonstrates that the lack of active OTS1/OTS2 proteases increased the accumulation of SUMOylated phyB-YFP. Next we performed two lines of experiments to demonstrate that OTS1 is directly involved in de-SUMOylating phyB. First, we tested if recombinant OTS1 can deconjugate SUMO from phyB in vitro, and next we investigated if phyB interacts with OTS1 *in planta*. Our data clearly demonstrate that OTS1 cleaves off SUMO from plant-derived SUMOylated phyB (Fig. 6*D*) and that OTS1 binds to phyB when transiently coexpressed in *N. benthamiana* leaves (Fig. S8). Taken together, we conclude that OTS1/OTS2 act redundantly to mediate, at least partly, reversible SUMOylation of phyB.

Discussion

PhyB regulates growth and development of plants throughout their entire life cycle. PhyB exerts it regulatory function in a RL/FRL reversible fashion via a complex signaling network consisting of an ever-growing number of components of which numerous proteins, including PIFs, directly interact with the biologically active Pfr form of the photoreceptor. Binding of the negative regulatory PIF to phyB Pfr initiates either (*i*) phosphorylation of these TFs (49) followed by mutual degradation of these proteins (14) or (*ii*) inactivation of PIFs via a not fully understood mechanism (11). These events then culminate in altered transcription of about 15% of the genome during the initial phase of light to dark transition in *Arabidopsis* (12).

Here we report that phyB is SUMOylated at its C terminus, and Lys996 is critical for the efficient conjugation of SUMO to phyB both in transient assays (Fig. S1) and in stably transformed transgenic lines. Accumulation of SUMOylated phyB-YFP follows a diurnal pattern in seedlings grown under L/D cycles (Fig. 1) and it is increased by R or WL in a fluence rate-dependent fashion (Figs. 3 and 4). In contrast, SUMOylation of phyB^{Lys996Arg}-YFP remains invariably low under all conditions tested, whereas SUMOylation of truncated phyB-YFP fusion proteins [phyB(1–651)-YFP-NLS, phyB(1–990)-GFP] lacking Lysine^{996} is below detection level *in planta* (Fig. S1). Transgenic phyB^{Lys996Arg}-YFP lines similar to phyB(1–651)-GUS-GFP-NLS (51) or phyB(1–991)-GFP (52) display hypersensitivity to RL compared with phyB-YFP. Taken together, these data indicate that reduced SUMOylation of phyB enhances RL-induced signaling; thus, SUMOylation inhibits phyB action. We note that despite its low abundance we can readily detect SUMOylated phyB^{Lys996Arg}-YFP in etiolated

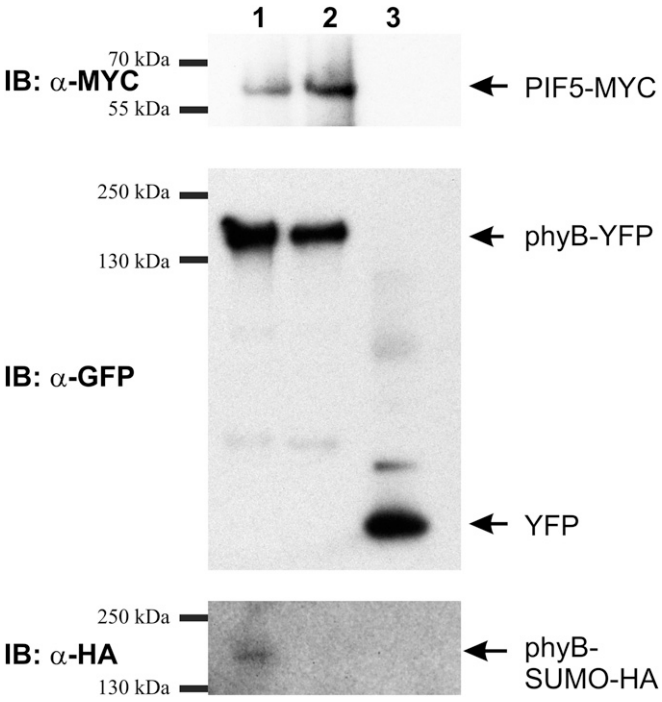

Fig. 5. SUMOylation of phyB inhibits binding of the transcription factor PIF5. The figure shows results obtained by YFP pull-down of phyB-YFP, phyB^{Lys996Arg}-YFP, and YFP proteins from *N. benthamiana* tissue coinfiltrated with PIF5-MYC and HA-SUMO1. phyB fusion proteins were immunoprecipitated using anti-GFP beads. Next phyB-YFP (lane 1), phyB^{Lys996Arg}-YFP (lane 2), and YFP (lane 3) immunoprecipitated proteins were resolved on three separate SDS/PAGE gels and blotted onto PVDF membranes to detect the presence of phyB-YFP, phyB^{Lys996Arg}-YFP, YFP, PIF5-MYC, and HA-SUMO1.

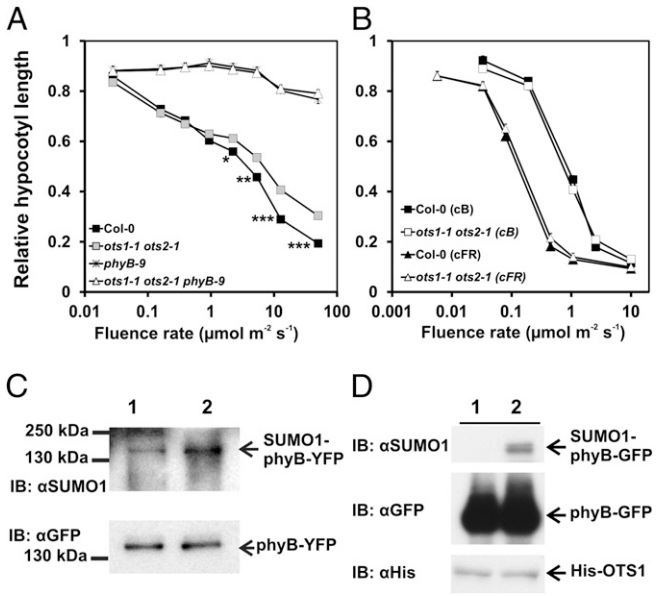

Fig. 6. The SUMO proteases OTS1 and OTS2 mediate de-SUMOylation of phyB. (*A*) The *ots1/ots2* mutant is hyposensitive to RL. Fluence rate-dependent relative inhibition of hypocotyl elongation of Col-0 (black square), *phyB-9* (star symbol), *ots1/ots2* (gray square), and *ots1/ots2/phyB-9* (white triangle) seedlings grown in RL is shown. For additional details, see the Fig. 2 legend. (*B*) The *ots1/ots2* mutant exhibits normal photomophogenesis in FRL or BL. Hypocotyl elongation inhibition in FRL-grown (Col-0, black triangle; *ots1/ots2*, white triangle) and BL-grown (Col-0, black square; *ots1/ots2*, white square) seedlings is shown. For additional details, see the Fig. 2 legend. (*C*) Accumulation of the SUMOylated form of phyB is enhanced in transgenic seedlings lacking functional OTS1/OTS2 SUMO proteases. Transgenic *phyB-9* (lane 1) and *ots1/ots2/phyB-9* (lane 2) seedlings expressing *LIP1:PHYB-YFP* were grown under 12 h L/12 h D cycles for 5 d, and samples were harvested on the sixth day at MOD. Accumulation of the SUMOylated phyB-YFP was detected as described in Fig. 1. (*D*) Recombinant OTS1 deSUMOylates phyB-GFP in vitro. SUMOylated phyB-GFP protein was affinity purified from total protein extracts with anti-GFP antibody and incubated with an identical amount of recombinant OTS1 (His:OTS1) (lane 1) or catalytically inactive OTS1 (His:OTS1^{CS}) (lane 2) proteins. After incubation (typically 4 h at room temperature), the beads were reapplied to the column [the flow-through was used to determine 6xHis-OTS1 levels with anti-Histidine tag antibodies (IB:αHis)] and washed, and bound proteins were eluted with SDS/PAGE loading buffer and separated by electrophoresis, blotted, and probed with anti-GFP (IB:αGFP) and anti-SUMO1 (IB:αSUMO1) antibodies.

seedlings (Figs. 3 and 4). At present we do not know if this is caused by conjugation of SUMO onto phyB at a second site or whether this is functionally relevant.

SUMOylation of phyB interferes with the conformation-specific interaction of phyB and the negative regulatory factor PIF5 (Fig. 5). We postulate that SUMOylation of phyB at its C-terminal domain directly inhibits interaction of these proteins. This model is supported by recent findings demonstrating that (*i*) mutations in the C-terminal domain of phyB inhibit binding of PIF3 (53) and that (*ii*) mutations that fully inactivate signaling by the N-terminal domain of phyB and binding of PIF3 in vitro only partially impair the activity of the full-length photoreceptor (54). Thus, it is plausible to assume that the interaction of PIFs with phyB Pfr is mediated by multiple binding sites and conjugation of the bulky SUMO to the C-terminal domain of phyB substantially weakens its interaction with PIFs. However, it has been recently shown that phosphorylation of phyB^{Tyr104} also inhibits binding of PIF3 to phyB Pfr (16), whereas phosphorylation of phyB^{Ser86} accelerates thermal reversion (also called D reversion) of phyB Pfr into Pr and thereby prevents interaction of these proteins (15). Thus, it is also possible that SUMOylation interferes with binding of kinases/phosphatases to phyB and thus indirectly via modifying phosphorylation status of phyB alters the interaction of phyB Pfr with PIFs. Binding of PIFs to phyB Pfr has been shown to be essential for mutual degradation of these proteins (14). Our data illustrate that extended RL treatment moderately decreases the abundance of phyB^{Lys996Arg}-YFP compared with phyB-YFP. This observation can be readily explained by compromised interaction of phyB-YFP and PIFs, which is brought about by increased SUMOylation of phyB-YFP compared with the phyB^{Lys996Arg}-YFP mutant.

We show that SUMOylation of phyB is a reversible process and propose that the OTS1 and OTS2 SUMO proteases play a significant role in deconjugating SUMO from phyB. This conclusion is supported by data demonstrating that phyB-YFP is hyper-SUMOylated in *ots1/ots2/phyB-9* compared with *phyB-9* (Fig. 6*C*), OTS1 de-SUMOylates phyB in vitro (Fig. 6*D*), OTS1 binds to phyB *in planta* (Fig. S8), and the *ots1/ots2* double mutant is hyposensitive to RL (Fig. 6*A*). However, this mutant exhibits normal BL and FRL-induced physiological responses, indicating that other photoreceptors are not targets of SUMOyltion or not deSUMOylated by OTS1/OTS2. SUMOylated phyB is hardly detectable in etiolated or FRL irradiated seedlings, whereas it accumulates to substantially higher levels in RL and WL. These data and the fact that the constitutively active phyB^{Tyr276His} mutant is highly SUMOylated both in WL and darkness indicate that SUMOylated phyB preferentially accumulates in its Pfr form. This can be explained either by conformation-specific SUMOylation and/or by de-SUMOylation of phyB. OTS1 binds to phyB Pfr in vivo (Fig. S8), removes SUMO from phyB Pfr in vitro (Fig. 6*D*), and its abundance is not regulated by light (Fig. S7 *C* and *D*). Thus, we conclude that OTS1 is able to de-SUMOylate phyB Pfr as well as phyB Pr, as the abundance of SUMOylated phyB rapidly declines during the D phase in seedlings grown under L/D cycles (Fig. 1*B*). These data suggest that light-regulated expression and/or modification of yet unknown SUMO proteases or conjugating enzymes or their conformation-specific interaction with phyB Pfr are responsible for the higher level accumulation of SUMOylated phyB Pfr compared with that of phyB Pr.

Independent of the precise mechanism mediating reversible SUMOylation of phyB, we note that SUMOylation has been shown to play a pervasive role in the regulation of stress responses including heat (35), salt (31), and ABA signaling (38). Interestingly, it was shown that OTS1/OTS2 is destabilized by salt stress, and overexpression of OTS1/OTS2 significantly alters salt tolerance of the transgenic plants (31). Because OTS1/OTS2 is involved in mediating reversible SUMOylation of the photoreceptor, we speculate that SUMOylation of phyB might play an important role in the integration of light, salt, and other stress-induced responses.

Materials and Methods

Plant Material and Growth Conditions. Col-0 and *phyB-9* (55) mutant plants were used in this work. Plants expressing PHYB-YFP or N651-GFP in *phyB-9* background have been described (7, 15, 43). The cDNA fragment corresponding to amino acid residues 1–991 of the PHYB protein was inserted as an XbaI-SmaI fragment between the *35S* promoter and the *YFP-NOS* terminator of pPCVB (47). The Lys996Arg and Tyr276His mutations were created by a site-directed mutagenesis kit (Stratagene). Plants were transformed by the Agrobacterium-mediated floral dip method (56). Basta-resistant plants were selected and grown to maturation in the greenhouse. Homozygous T3 plants grown at 22 °C were used in all assays. WL was provided by cool-white fluorescent tubes at a 25 μmol $m^{-2}{\cdot}s^{-1}$ fluence rate. RL (λ_{max}, 667 nm) or FRL (λ_{max}, 730 nm) was provided by LED light sources (Quantum Devices Inc.) at 25 or 10 μmol $m^{-2}{\cdot}s^{-1}$ fluence rates, respectively. For diurnal conditions, plants were grown in 12 h WL (25 μmol $m^{-2}{\cdot}s^{-1}$)/12 h D or 12 h RL (25 μmol $m^{-2}{\cdot}s^{-1}$)/12 h D cycles for 7 d before harvesting.

Transient Assays in *N. benthamiana*. PIF5 cDNA was cloned in pGWB17 so that expressed proteins had a C-terminal 4xMYC tag, and SUMO1 was cloned in

pEG201 so that expressed proteins had a N-terminal HA tag. Gene constructs were transiently expressed in *N. benthamiana* plants using *Agrobacterium*-mediated transformation (57). The protein samples extracted (57) were mixed with 50 μL anti-GFP (Chromotek anti-GFP beads) and incubated on ice for 15 min. The beads were centrifuged down at 10,000 × *g* for 1 h and washed three times with 1 mL of cold IP buffer. After the last wash, 50 μL of preheated (95 °C) 1× SDS loading buffer was used to elute the immuno-complex and analyzed on 8–10% SDS/PAGE using immunoblotting methods with Clontech (clone, JL-8) anti-GFP (Abcam) anti-Myc, and anti-HA antibodies.

Western Blot Analysis, Hypocotyl Length Measurement. For details, see *SI Materials and Methods*.

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