Phosphorylation and negative regulation of CONSTITUTIVELY PHOTOMORPHOGENIC 1 by PINOID *in Arabidopsis*

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CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) plays crucial roles in various cellular processes via its E3 ubiquitin ligase activity in organisms, ranging from fungi to humans. As a key component in regulating various biological events, COP1 itself is precisely controlled at multiple layers. Here, we report a negative regulator of COP1, PINOID (PID), which positively mediates photomorphogenic development. Specifically, PID genetically and physically interacts with COP1 and directly phosphorylates COP1 at Ser20. As a result, this posttranslational modification serves to repress COP1 activity and promote photomorphogenesis. Our findings signify a key regulatory mechanism for precisely maintaining COP1 activity, thereby ensuring appropriate development in plants.

Arabidopsis | photomorphogenesis | phosphorylation | PID | COP1

Plants have evolved a complicated but delicate regulation system for adapting to internal and external cues throughout their life cycle. Light, one of the key environmental factors in regulating various developmental processes in plants, is perceived mainly by multiple known photoreceptors, including phytochromes, cryptochromes, phototropins, and UVR8 (1–5). In response to light, *Arabidopsis* seedlings undergo photomorphogenic development with short hypocotyls and opened cotyledons, whereas skotomorphogenic processes occur with elongated hypocotyls, closed cotyledons, and apical hooks in the absence of light (6).

During the dark to light transition, CONSTITUTIVELY PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA (COP1/DET/FUS) play critical roles and act as key repressors of photomorphogenesis (7, 8). Of these, COP1 functions as an E3 ubiquitin ligase and targets ELONGATED HYPOCOTYL 5 (HY5), HYH, LAF1, HFR1, BBX21/STH2, BBX22/STH3, and PIL1 (9–17), as well as phytocromes (phyA–phyE) (18–20) for ubiqutination and degradation, thereby repressing photomorphogenesis in darkness. Accumulating evidence shows that COP1 is also involved in flowering, the circadian clock, and root development by ubiquitinating CO, ELF3, GI, and SCAR1 (21-23).

Mammalian COP1 is a key regulator of the tumor development; it targets tumor suppressor p53 as well as the oncogene c-jun, ETS for ubiquitination and degradation (24–27). Mammalian COP1 itself is tightly controlled through multiple regulatory mechanisms. In response to DNA damage, the ataxia telangiectasia mutated (ATM) protein kinase directly targets Ser387 of COP1 for phosphorylation, subsequently promoting COP1 self-ubiquitination and degradation (28). In the meanwhile, 14-3-3σ preferentially interacts with phosphorylated COP1 and facilitates its export from the nucleus to the cytoplasm (29). Thus, it appears that phosphorylation of COP1 is a key step in the inactivation of COP1 and regulation of COP1-mediated cellular processes in mammalian cells. However, the responsible kinase and a possible role of COP1 phosphorylation in *Arabidopsis* has remained unclear.

In this study, we show that Ser/Thr kinase PINOID (PID) phosphorylates COP1 and promotes photomorphogenesis. A point mutation in *PID* that leads to a premature stop code at Arg383 completely suppresses the drastically short hypocotyls of *cop1-6* in darkness. Overexpression of *PID* transgenic seedlings exhibits a constitutively photomorphogenic phenotype with expanded cotyledons in the dark. In the light, *pid* mutant seedlings display elongated hypocotyls, whereas *PID* overexpressors show shorter hypocotyls compared with wild-type (WT) seedlings. The *YFP-COP1 S20D* transgenic plants show a larger hook unfolding angle in darkness and shorter hypocotyls in red light compared with WT, *YFP-COP1*, and *YFP-COP1 S20A* transgenic lines. Collectively, PID directly phosphorylates COP1 at Ser20 and represses its activity, which in turn serves to promote photomorphogenesis.

Results

Mutation in *CSU3* Suppresses Constitutively Photomorphogenic Phenotype of *cop1-6* in Darkness. To explore factors involved in regulating COP1 and COP1-mediated processes, in previous work we performed a forward genetic screen for suppression of *cop1-6* mutant phenotype in darkness (30). One recessive allele, *cop1 suppressor 3* (*csu3*), was recovered from this screen. The hypocotyl length of the *csu3 cop1-6* double mutant was similar to that of WT in the dark (Fig. 1 *A* and *B*). On phenotypic analysis, approximately 50% of *csu3 cop1-6* plants exhibited three cotyledons at the seedling stage, and all of the *csu3 cop1-6* adult plants displayed a *pin*-like phenotype, whereas none of the *cop1-6* plants exhibited these abnormal phenotypes (Fig. S1 *A*–*C*). Although the cotyledon angle of *csu3 cop1-6* was a little larger

Significance

As an E3 ubiquitin ligase, CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) directly ubiquitinates and triggers the degradation of various downstream targets and acts as a central repressor of light signaling. In this study, our data reveal that a Ser/Thr kinase, PINOID (PID), promotes photomorphogenic development. PID directly interacts with COP1 and phosphorylates it at the site of Ser20, thereby repressing the activity of COP1 in plants. Thus, our findings provide insight into the precise regulatory mechanism in maintaining appropriate COP1 activity in response to dynamically changing light conditions in plants.

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than that of WT, it was significantly smaller than that of the *cop1-6* single mutant (Fig. 1 *C* and *D*). Moreover, the greening rate of *csu3 cop1-6* on transfer from dark to white light was similar to that of WT, significantly higher than that of the *cop1-6* single mutant (Fig. 1 *E* and *F*). Intriguingly, *csu3* partially rescued the short hypocotyl length of the *cop1-6* mutant in the continuous white (W), blue (B), red (R), and far-red (FR) light conditions tested (Fig. S1 *D–K*). Furthermore, the *csu3 cop1-6* double mutant exhibited an intermediate adult phenotype between WT and the *cop1-6* dwarf phenotype under the long-day condition (16-h light/8-h dark) at 8 wk (Fig. S1*C*). Collectively, these genetic results suggest that *csu3* suppresses the phenotype of *cop1-6* almost completely in the dark but only partially in the light.

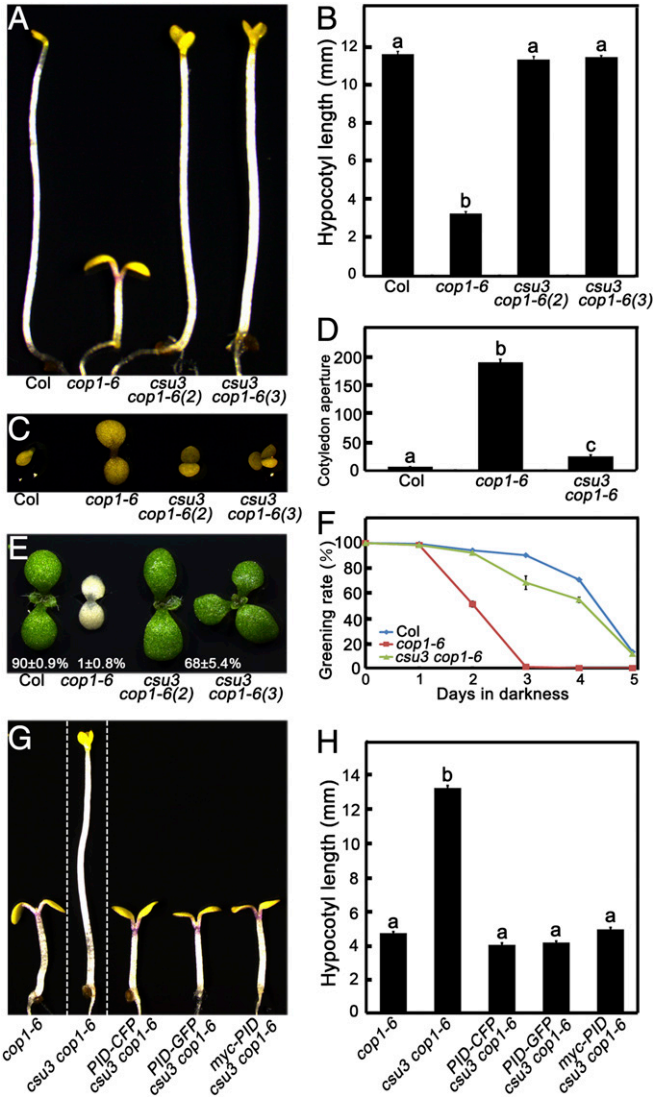

Fig. 1. *csu3* suppresses the *cop1-6* mutant in darkness. (*A* and *B*) Hypocotyl phenotypes and lengths of Col, *cop1-6*, and *csu3 cop1-6* mutant etiolated seedlings were grown in the dark for 5 d. *csu3 cop1-6 (2)* and *csu3 cop1-6 (3)* indicate *csu3 cop1-6* seedlings with two and three cotyledons, respectively. Error bars represent SE ($n \geq 20$). (*C* and *D*) Cotyledon phenotypes and angles (°) of Col, *cop1-6*, and *csu3 cop1-6* mutant etiolated seedlings grown in darkness for 5 d. Error bars represent SE ($n \geq 25$). (*E* and *F*) Cotyledon greening phenotypes and ratios of Col, *cop1-6*, and *csu3 cop1-6* mutant etiolated seedlings grown in darkness for the indicated days and then transferred to W light for an additional 2 d. Error bars represent SE ($n \geq 100$). (*G* and *H*) Complementation test, showing hypocotyl phenotypes and lengths of *cop1-6*, *csu3 cop1-6*, *PID-CFP csu3 cop1-6*, *PID-GFP csu3 cop1-6*, and *myc-PID csu3 cop1-6* seedlings grown in the dark for 5 d. *cop1-6*, *csu3 cop1-6*, and various *PID* transgenic seedlings are separated by dotted lines. Error bars represent SE ($n \geq 20$). In *B*, *D*, and *H*, letters above the bars indicate significant differences ($P < 0.05$) as determined by one-way ANOVA with Tukey's post hoc analysis.

***CSU3* Encodes Protein Kinase PINOID.** Considering that abnormal features of *csu3 cop1-6* appear at both the seedling and adult stages, and that only *pin-formed 1* (*pin1*) (31, 32), *pinoid* (*pid*) (33, 34), and *monopteros* (*mp*) (35, 36) have been reported to show these similar properties, we sequenced genomic sequences of *PIN1*, *PID*, and *MP* individually in *csu3 cop1-6* and identified a C-to-T substitution at position 1453 from the start codon in the *PID* gene, causing a premature stop codon at the site of Arg383 (Fig. S2 *A* and *B*). Fourteen independent *pid* alleles (*pid-1* to *pid-14*) have been identified and characterized previously (33, 34, 37), so *csu3*, which is a previously unidentified *pid* allele, has been designated *pid-15*.

To further confirm that the suppression of *cop1-6* was indeed caused by the disruption of PID, we individually transformed *PID-CFP*, *PID-GFP*, and *myc-PID* into *csu3 cop1-6* double mutant. *PID-CFP csu3 cop1-6*, *PID-GFP csu3 cop1-6*, and *myc-PID csu3 cop1-6* displayed phenotypes resembling *cop1-6* in the dark (Fig. 1 *G* and *H*). Moreover, the adult phenotypes of *PID-CFP csu3 cop1-6* and *PID-GFP csu3 cop1-6* plants were indistinguishable from those of *cop1-6* (Fig. S2*C*). These results suggest that a functional *PID* gene indeed complements the phenotype conferred by the *pid-15* mutation in *cop1-6*.

PID Is a Positive Regulator of Light Signaling. To illustrate the role of PID in light signaling, we isolated *pid-15* single mutant from F2 generation of *pid-15 cop1-6* crossed with WT (Col). However, the homozygous *pid-15* mutant was nearly sterile, barely producing seeds (Fig. S2*C*), indicating that *pid-15* was a strong allele. We then maintained the *pid-15* in a heterozygous state. We generated transgenic lines *PID-CFP*, *PID-GFP*, *myc-PID*, and *myc-mPID* in the Col background (Fig. S3 *A–E*). Like other *pid* alleles (33, 34), a majority of homozygous *pid-15* showed three cotyledons at the seedling stage. We measured the hypocotyl length of *pid-15* with three cotyledons and found that they developed longer hypocotyls in the W, B, R, and FR light conditions tested (Fig. 2 *A–H*). In contrast, the hypocotyls of *PID-CFP*, *PID-GFP*, and *myc-PID* transgenic lines were shorter than WT under those light conditions (Fig. 2 *A–H*). In the dark, *PID-CFP*, *PID-GFP*, and *myc-PID* transgenic seedlings did not show any significant difference in hypocotyl length compared with WT (Fig. S3 *F* and *G*); however, they displayed obviously opened cotyledons mimicking the *cop1* mutant phenotype in darkness (Fig. 2 *I* and *J*). Overexpression of *myc-mPID* (Fig. S3 *C* and *E*), which shows defects in kinase activity (38), exhibited phenotypes resembling WT (Fig. 2). Taken together, these findings suggest that PID functions as a positive regulator of photomorphogenesis in *Arabidopsis*.

PID Physically Interacts with COP1. Given that the strong genetic interaction between *PID* and *COP1*, we tested the protein–protein interaction between PID and COP1. We performed pull-down assays using recombinant His-Trigger Factor (TF)-COP1 and GST-PID expressed in *Escherichia coli* strain BL21 (DE3) host strains. Neither His-TF-COP1 nor His-TF could be pulled down by GST; however, His-TF-COP1, but not His-TF alone, was pulled down by GST-PID (Fig. 3*A*). To map the domain of COP1 required for interacting with PID, we continued the pull-down assays using recombinant GST-fused three truncation fragments of COP1 and His-PID (Fig. 3*B*). Either GST-COP1 RING or GST-COP1 coiled-coil alone could pull down His-PID, whereas GST-COP1 WD40 and GST could not (Fig. 3*C*).

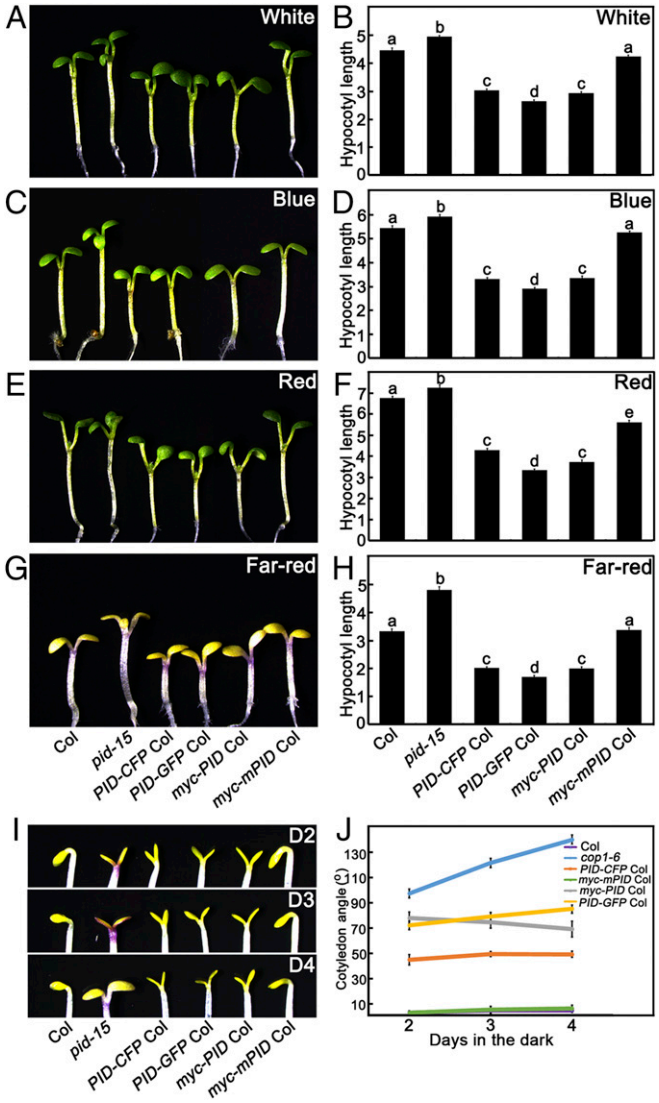

Fig. 2. Overexpression of *PID* is hypersensitive to light and results in expanded cotyledons in the dark. (*A*–*H*) Hypocotyl phenotypes and lengths of Col, *cop1-6*, *pid-15*, *PID-CFP* Col, *PID-GFP* Col and *pid-15 cop1-6* seedlings grown under white (*A* and *B*), blue (*C* and *D*), red (*E* and *F*), and far-red (*G* and *H*) light conditions for 5 d. Error bars represent SE ($n \geq 20$). (*I* and *J*) The cotyledon apertures of Col, *cop1-6*, *PID-CFP* Col, *PID-GFP* Col, *myc-PID* Col, and *myc-mPID* Col transgenic seedlings grown in darkness for 2 d (D2), 3 d (D3), or 4 d (D4). Error bars represent SE ($n \geq 20$). In *B*, *D*, *F*, and *H*, letters above the bars indicate significant differences ($P < 0.05$) as determined by one-way ANOVA with Tukey's post hoc analysis.

Next, we further verified the PID–COP1 interaction using firefly luciferase complementation imaging (LCI) assays in tobacco leaves. *Agrobacterium*-mediated transient expression with cLUC-PID and COP1-nLUC were coexpressed in *Nicotiana benthamiana* leaves, and high luciferase activity could be readily detected after the addition of luciferin (Fig. 3*D*); however, no luciferase activity was detected when coexpressed with cLUC and COP1-nLUC or cLUC-PID and nLUC (Fig. 3*D*). Moreover, in coimmunoprecipitation (co-IP) assays using transgenic seedlings carrying both *PID-CFP* and *COP1-Flag*, immunoprecipitation of PID-CFP pulled down COP1-Flag in *PID-CFP* and *COP1-Flag* transgenic lines (Fig. 3*E*), demonstrating that PID associates with COP1 in vivo. Taken together, these results suggest that PID physically interacts with COP1, and that both COP1 RING-finger and coiled-coil domains are responsible for the COP1–PID interaction.

PID Phosphorylates COP1 Directly in Vitro. As a Ser/Thr kinase, PID directly targets PIN1 on Ser231, Ser252, and Ser290 for phosphorylation and modulates its polarization activity (37). Considering that PID has kinase activity and physically interacts with COP1, we suspected that PID might target COP1 for phosphorylation. To explore this possibility, we performed in vitro phosphorylation assays using recombinant GST-PID and His-TF-COP1. Consistent with previous studies (34, 39), PID exhibited autophosphorylation activity in vitro. Phosphorylated His-TF-COP1 was detected when GST-PID was added in the reactions. As the amount of His-TF-COP1 increased, more phosphorylated His-TF-COP1 was observed. The negative control His-TF was not phosphorylated by GST-PID (Fig. 4*A*). GST-mPID, which carries an amino acid substitution (D205A) in its ATP-binding center lacking its autophosphorylation activity (38), could not phosphorylate His-TF-COP1 (Fig. 4*A*). These findings suggest that PID is able to phosphorylate COP1 in vitro.

To map the phosphorylation sites on COP1, we used COP1 truncation fragments fused with GST (Fig. 3*B*) to conduct in vitro phosphorylation assays. As shown in Fig. 4*B*, GST-PID could phosphorylate COP1 RING, but not COP1 coiled-coil and WD40, implying that the phosphorylation sites for PID reside within the COP1 RING-finger domain. To further identify the

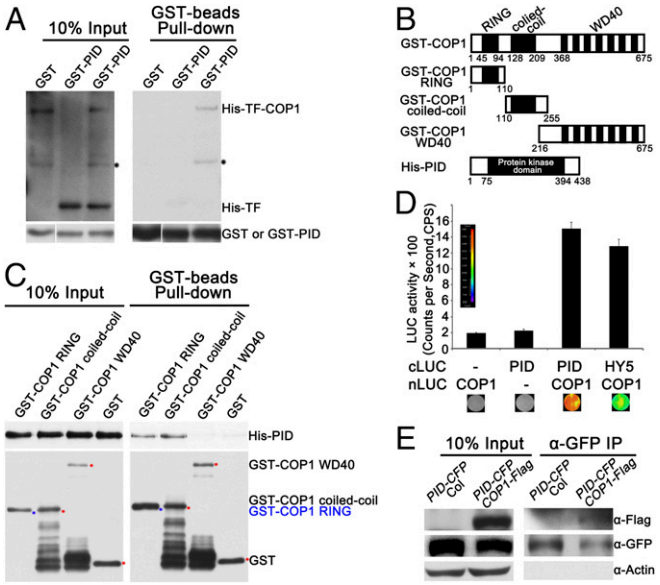

Fig. 3. PID physically interacts with COP1. (*A*) Pull-down assay showing that GST-PID pulled down His-TF-COP1, but not His-TF. Pull-down assays were performed using GST, GST-PID, His-TF, and His-TF-COP1. His-TF and His-TF-COP1 proteins were detected by immunoblotting using anti-His antibodies. GST and GST-PID proteins were detected using anti-GST antibodies. Black stars indicate nonspecific bands. (*B*) Schematics of various GST-COP1 truncated fragments and His-PID. (*C*) The RING-finger and coiled-coil domains of COP1 pulled down His-PID. The in vitro pull-down assay was performed using GST or various truncated COP1 fragments fused with GST to pull down His-PID. Bound and 10% of input His-PID fractions were detected by immunoblotting using anti-His antibodies. Immobilized GST and GST-COP1 fusion proteins were detected with anti-GST antibodies. The GST and GST-COP1 truncated protein bands are indicated by red and blue stars, respectively. (*D*) Firefly LCI assay showing COP1 interacting with PID in tobacco leaf cells. The PID-cLUC and COP1-nLUC constructs were transiently coinfiltrated in tobacco leaves, and luminescence intensity was detected using LB985 NightSHADE. HY5-cLUC and COP1-nLUC served as positive controls. (*E*) Co-IP assay showing COP1 interacting with PID in vivo. Here 4-d-old white light-grown *PID-CFP* Col and *COP1-Flag PID-CFP* transgenic seedlings were transferred to darkness for 24 h and then subjected to co-IP using anti-GFP agarose, with the immunoprecipitates detected using anti-Flag and anti-GFP antibodies, respectively. Actin served as a negative control.

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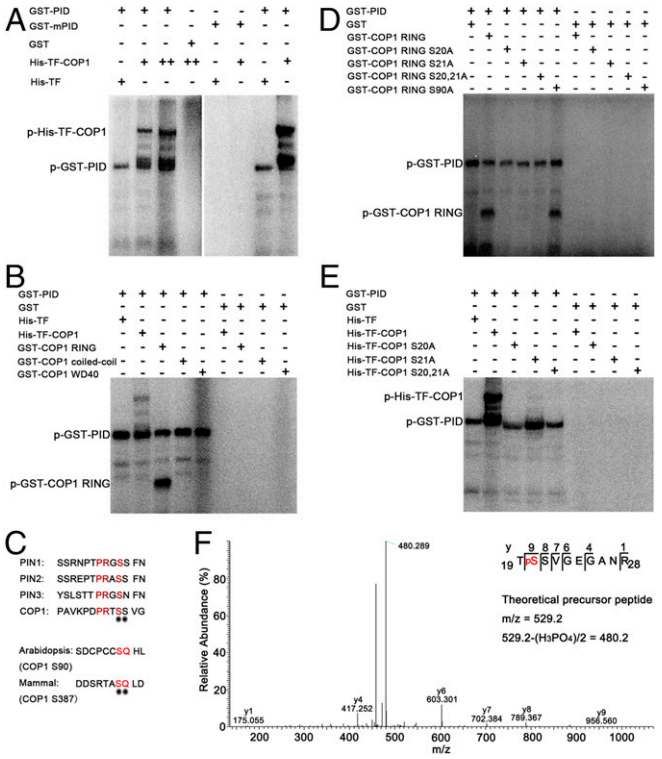

Fig. 4. PID targets Ser20 on COP1 for phosphorylation in vitro. (*A*) In vitro protein phosphorylation assay showing GST-PID efficiently phosphorylated His-TF-COP1. (*B*) PID phosphorylated the RING-finger domain of COP1. The numbers indicate the amino acid residues of COP1. (*C*) Predicted phosphorylation sites on *Arabidopsis* COP1. (*D* and *E*) PID phosphorylated the Ser20 and/or Ser21 of COP1 in an in vitro kinase assay. (*F*) Collision-induced dissociation mass spectrum clearly showing that the Ser20 on COP1 was phosphorylated by PID. The proteins were separated by SDS/PAGE and purified by PHOS-Select Iron Affinity Gel, then subjected to MS/MS spectrometric analyses.

exact phosphorylation site(s) of COP1, we analyzed the COP1 RING-finger amino acid sequence using the NetPhos program (NetPhos software prediction score >0.5) (40) and identified two potential putative phosphosites with high prediction scores, Ser20 and Ser21. It has been shown that PID phosphorylates PINs by a central serine residue within the highly conserved TPRXS (N/S) motif (37). By analyzing the COP1 RING-finger domain amino acids, we identified one conserved phosphorylation motif (DPRTSS) for PID in this region (Fig. 4*C*). In addition, the phosphorylation site on mammalian COP1 is Ser387-locating in the serine-glutamine (SQ) motif (28).

The Ser90 in *Arabidopsis* COP1 RING-finger domain is the sole potential SQ motif within this region (Fig. 4*C*); therefore, we substituted Ser-to-Ala at these three sites (S20A, S21A, and S90A) alone or together and conducted in vitro phosphorylation assays. GST-PID was not able to phosphorylate COP1 RING S20A, COP1 RING S21A, and COP1 RING S20, 21A, but could phosphorylate COP1 RING S90A in vitro (Fig. 4*D*). Consistently, GST-PID could not phosphorylate full-length COP1 carrying a substitution at either S20A or S21A (Fig. 4*E*). Either S20A or S21A substitution might affect COP1 conformation and phosphorylation by PID in vitro; thus, we performed LC-MS/MS analysis on the recombinant COP1 by adding purified GST-PID in the phosphorylation reaction to identify the exact phosphosite. A tryptic peptide (TSSVGEGANR) derived from the predicted phosphorylation motif was found in a phosphorylated state. On analysis of six other tryptic peptides, only Ser20, but not Ser21, was unambiguously identified to be phosphorylated (Fig. 4*F*). Taken together, these findings support the idea that PID targets COP1 for phosphorylation, and that the phosphorylation site on COP1 is Ser20.

PID Promotes the Degradation of PIF3. To further explore the function of PID in phosphorylating COP1, we detected the abundance of COP1 in *Arabidopsis* seedlings. The amount of COP1 was comparable in WT and *PID*-overexpressing transgenic lines grown in various light conditions (dark and W, B, FR, and R) (Fig. S4 *A–E*). In addition, COP1 was found to localize in the nucleus in both WT and *PID* transgenic lines grown in the dark (Fig. S4*F*). These results indicate that PID might have no effect on the COP1 protein level and its partitioning between the nucleus and the cytoplasm. To explore whether COP1 regulates PID, we introgressed the PID-CFP and PID-GFP into the *cop1-6* background by genetic crossing. As shown in Fig. S4*G*, the PID protein levels in the *cop1-6* background were not markedly different from those in the Col background, indicating that COP1 might not affect the PID abundance.

We next investigated whether PID affects the biochemical activity of COP1. In darkness, COP1 destabilizes HY5 (10) but stabilizes PIF3 (41). Likely owing to the already extremely low activity of HY5 and the requirement of light triggered event, HY5 protein abundance and HY5-regulated gene expression were not altered in *PID* transgenic seedlings grown in darkness (Fig. S4 *H–N*); however, PIF3 protein level was dramatically reduced in the dark-grown *PID* transgenic lines (Fig. 5*A*). In addition, PIF3-controlled gene expression was changed accordingly in the dark-grown *PID*-overexpressing transgenic lines (Fig. S5). These results suggest that PID negatively regulates the abundance of PIF3.

Phosphomimic COP1 Exhibits Reduced Biological Activity in *Arabidopsis*. To investigate the biological significance of the Ser20 of COP1 in regulating plant development, we transformed the 35S promoter-drived WT (YFP-COP1), nonphosphorylatable (YFP-COP1 S20A), and phosphomimic (YFP-COP1 S20D) forms of COP1 into a *cop1-6* mutant background. The various independent *YFP-COP1 cop1-6*, *YFP-COP1 S20A cop1-6*, and *YFP-COP1 S20D cop1-6* transgenic lines, in which WT or mutated *COP1* were overexpressed (Fig. S6 *A* and *B*), displayed an etiolated phenotype in darkness (Fig. 5), indicating that *YFP-COP1*, *YFP-COP1 S20A*, and *YFP-COP1 S20D* transgenes are largely functional and can rescue the *cop1-6* short hypocotyls in darkness. However, the hook unfolding angles of *YFP-COP1 cop1-6* and *YFP-COP1 S20A cop1-6* were significantly smaller compared with those in *YFP-COP1 S20D* transgenic plants in darkness (Fig. 5 *B* and *C*). *YFP-COP1 S20D* transgenic seedlings developed shorter hypocotyls compared with *YFP-COP1 cop1-6* and *YFP-COP1 S20A cop1-6* seedlings in the dark and R light conditions (Fig. 5 *D* and *E*), but not in the W, B, and FR light conditions (Fig. S6 *C–H*). These results indicate that phosphomimic or phosphorylated COP1 has reduced activity in regulating photomorphogenesis in planta.

Discussion

Extensive studies have demonstrated that COP1 acts as a central regulator in multiple developmental processes in plants via its E3 ubiquitin ligase activity (7, 8). Using a forward genetic screen, we have identified PID as a negative regulator of COP1. Biochemical studies showed that PID directly targets COP1 Ser20 for phosphorylation. Overexpression of *PID* in *Arabidopsis* led to shortened hypocotyls in the light and expended cotyledons in darkness. Phosphomimic COP1 exhibited lower activity in the regulation of photomorphogenesis in planta. These results suggest a critical role for PID in modulating COP1 activity and promoting photomorphogenic development.

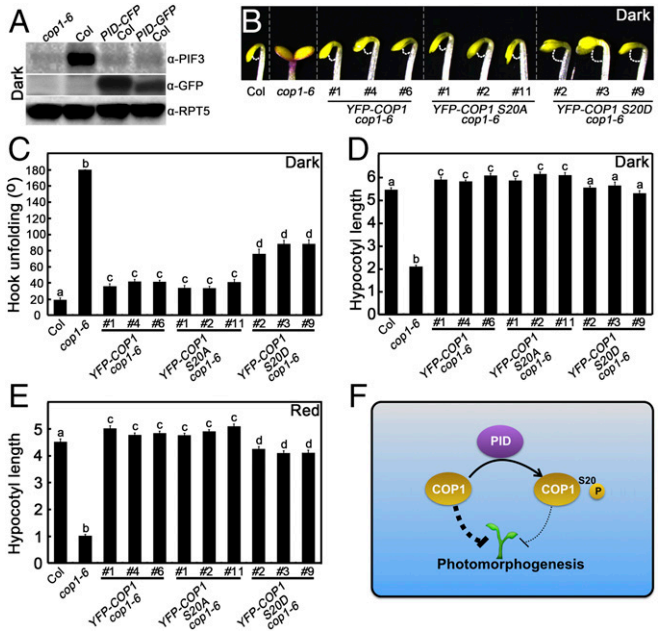

Fig. 5. Phenotypic analysis of *YFP-COP1 S20A cop1-6* and *YFP-COP1 S20D cop1-6* transgenic seedlings. (*A*) PIF3 and PID protein levels in *cop1-6*, Col, *PID-CFP* Col, and *PID-GFP* Col seedlings grown in darkness as detected by PIF3 and GFP antibodies. Plant total proteins were extracted from 5-d-old seedlings grown in the dark. *cop1-6* served as a negative control. RPT5 protein levels were used as loading controls. (*B–D*) Hook phenotypes (*B*), unfolding angles (*C*), and hypocotyl lengths (*D*) of Col, *cop1-6*, *YFP-COP1 cop1-6*, *YFP-COP1 S20A cop1-6*, and *YFP-COP1 S20D cop1-6* seedlings grown in darkness for 2 d. Error bars represent SE ($n \geq 20$). (*E*) Hypocotyl lengths of Col, *cop1-6*, *YFP-COP1 cop1-6*, *YFP-COP1 S20A cop1-6*, and *YFP-COP1 S20D cop1-6* plants grown in red light (58.7 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 4 d. Error bars represent SE ($n \geq 20$). (*F*) A working model showing how PID mediates in maintaining appropriate COP1 activity. PID directly phosphorylates COP1 on Ser20 to repress its activity, which in turn serves to precisely control COP1 activity to ensure normal plant development. In *B*, *C*, and *D*, letters above the bars indicate significant differences ($P < 0.05$) as determined by one-way ANOVA with Tukey's post hoc analysis.

cop1-6 is a weak mutant, not a null mutant, that produces a functional COP1-6 mutant protein at a lower level (30). The *pid-15* mutant might work by enhancing the activity of a functional COP1-6 to promote effective suppression. This might explain the finding that PID regulates COP1 activity through phosphorylation, but *pid-15* is epistatic to *cop1-6* (Fig. 1). The *pid-15* single mutant develops elongated hypocotyls (Fig. 2), whereas *pid-15 cop1-6* shows an intermediate phenotype in hypocotyls in various light conditions (Fig. S1), indicating that COP1 and PID may work synergistically in regulating photomorphogenesis. PID directly targets COP1 at the site of Ser20 for phosphorylation (Fig. 4), and COP1 does not regulate PID abundance (Fig. S4*G*), implying that PID-COP1 might not provide feedback regulation for PID stability.

COP1 stabilizes PIF3 in the dark, as demonstrated by the significantly lower PIF3 protein levels in the absence of COP1 (41) (Fig. 5*A*). YFP-COP1, YFP-COP1 S20A, and YFP-COP1 S20D could not rescue the PIF3 protein levels in the *cop1-6* background (Fig. S6*I*), whereas they completely complemented the constitutively photomorphogenic phenotype of the *cop1-6* mutant (Fig. 5 *A–C*), suggesting that the etiolated phenotype conferred by these transgenes in *cop1-6* is largely independent of PIF3. There are several possible explanations for these observations: (*i*) degradation of PIF3 by PID might occur in a COP1-independent manner; (*ii*) phosphorylation of COP1 by PID might affect the abundance, activity, or action of an unidentified target rather than PIF3; and (*iii*) it is possible that YFP-COP1, YFP-COP1 S20A, and YFP-COP1 S20D transgenes lack the ability to rescue PIF3 abundance in the *cop1-6* background.

Either loss or gain of COP1 function leads to abnormal phenotypes from the seedling stage to the adult stage (42–45), indicating that appropriate COP1 abundance and activity are essential for normal plant development. Recent studies have revealed that the precise abundance and activity of COP1 is under the tight control of various factors through multiple mechanisms (30, 46–48). COP1 is evolutionally conserved from plants to human. Mammalian COP1 has E3 ubiquitin ligase activity and targets various downstream substrates for ubiquitination as well. In response to DNA damage, activated ATM directly phosphorylates mammalian COP1 at the site of Ser387 (28), and subsequently triggers its self-ubiquitination and degradation as well as 14-3-3σ–mediated nucleus exportation (29). In *Arabidopsis*, PID directly targets COP1 Ser20 for phosphorylation (Fig. 4); however, unlike phosphorylated mammalian COP1, phosphorylated *Arabidopsis* COP1 likely is not required for self-ubiquitination, self-degradation, and nucleocytoplasmic repartitioning, given that overexpression of *PID* did not affect COP1 abundance or nuclear localization pattern (Fig. S4 *A–F*). PID-mediated phosphorylation of COP1 promotes photomorphogenesis, at least in part by repressing the COP1 activity. It appears that COP1 activity is precisely modulated via multiple regulatory mechanisms. PIF1, CSU2, SIZ1, and PID are all involved in the regulation of COP1 activity through distinct mechanisms (46–48). Consistently, *PID* transgenic plants mimicked the *cop1* phenotype with respect to cotyledons in darkness and exhibited shorter hypocotyls in the light (Fig. 2). YFP-COP1 S20D, which is a phosphomimic form, showed reduced activity in *Arabidopsis* (Fig. 5 *B–E*). However, it appears that PID-mediated phosphorylation is only partially responsible for the repression of COP1 activity, considering that neither *PID* nor *YFP-COP1 S20D cop1-6* transgenic seedlings fully resembled the *cop* phenotype in either the dark or the light (Figs. 2 and 5 *B–E*).

Phosphorylation of phytochromes results in the attenuation of phytochrome signaling in plants. Phosphorylation of phyA Ser598 disrupts the interaction with its downstream partners and leads to attenuating phyA signal (49). Light triggers phosphorylation of phyB Tyr104 and represses its activity (50). Similarly, PID directly phosphorylates COP1 on Ser20 and negatively regulate the ubiquitin ligase activity of COP1 to tightly modulate seedling photomorphogenesis. Phosphorylation of COP1 on Ser20 is likely a key mechanism in the precise control of COP1 E3 ubiquitin ligase activity (Fig. 5*F*). PIFs are phosphorylated at multiple sites in a light-dependent manner before their ubiquitination and degradation (51–53). Thus, it is likely that phosphorylation modification is a critical strategy in the control of appropriate photomorphogenic development in plants through distinct mechanisms.

Materials and Methods

Plant Materials and Growth Conditions. The *cop1-6* (44), *hy5-215* (54), *pid-15*, and *pid-15 cop1-6* (this study) seedlings are of the Columbia (Col) ecotype. Seeds were surface-sterilized for 10 min with 30% commercial Chlorox bleach and washed three times with sterile water, then sown on solid Murashige and Skoog medium supplemented with 0.4% Bacto-agar (Difco) and 1% sucrose for phenotypic analysis and biochemical assays. The plates were stratified at 4 °C for 3 d in darkness and then transferred to continuous W light (91 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and maintained at 22 °C for 8 h. Then the seeds were incubated in dark and in R (14.7 $\mu mol \cdot m^{-2} \cdot s^{-1}$), FR (1.205 $\mu mol \cdot m^{-2} \cdot s^{-1}$), B (1.57 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and W (15.75 $\mu mol \cdot m^{-2} \cdot s^{-1}$) light for phenotypic analysis. The light intensity was measured with a LI-COR LI-250 light meter.

In Vitro Kinase Assays. Approximately 2 μg of purified protein were added into the kinase reaction mix (30 μL total volume), containing 1× kinase buffer (25 mM Tris·HCl pH 7.5, 1 mM DTT, and 5 mM $MgCl_2$) and 1× ATP solution (100 μM $MgCl_2$/ATP and 2 μCi γ-[^{32}P]ATP). The reaction mixtures were incubated at 30 °C for 30 min, the reaction was terminated by the addition of 6 μL of 5× SDS loading buffer, and the mixtures were then boiled at 100 °C for 5 min and separated over 10% SDS/PAGE gels. The gels were dried with a gel dryer (model 583; Bio-Rad) and subsequently exposed to a phosphor screen, and signals were detected with an Amersham Typhoon FLA 7000 phosphor imager (GE Healthcare). Coomassie Brilliant Blue R 250 stain was used for SDS/PAGE.

Details of the experimental procedures for plasmid construction, genomic complementation testing, mass spectrometry, immunoblot analysis, firefly LCI analysis, in vitro pull-down analysis, measurement of hypocotyl length and cotyledon aperture, and quantitative real-time PCR assays are provided in *SI Materials and Methods*.

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