The F-Box Protein MAX2 Functions as a Positive Regulator of Photomorphogenesis in Arabidopsis

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Light is vital for plant growth and development. To respond to ambient light signals, plants are equipped with an array of photoreceptors, including phytochromes that sense red (R)/far-R (FR) regions and cryptochromes and phototropins that respond to the ultraviolet-A/blue (B) region of the light spectrum, respectively. Several positively and negatively acting components in light-signaling pathways have been identified using genetic approaches; however, the pathways are not saturated. Here, we characterize a new mutant named pleiotropic photosignaling (pps), isolated from a genetic screen under continuous R light. pps has longer hypocotyls and slightly smaller cotyledons under continuous R, FR, and B light compared to that of the wild type. pps is also hyposensitive to both R and FR light-induced seed germination. Although photosynthetic marker genes are constitutively expressed in pps in the dark at high levels, the expression of early light-regulated genes is reduced in the pps seedlings compared to wild-type seedlings under R light. PPS encodes MAX2/ORE9 (for MORE AXILLARY BRANCHES2/ORESARA9), an F-box protein involved in inflorescence architecture and senescence. MAX2 is expressed ubiquitously in the seedling stage. However, its expression is restricted to vascular tissues and meristems at adult stages. MAX2 is also localized to the nucleus. As an F-box protein, MAX2 is predicted to be a component of the SCF (for SKP, Cullin, and F-box protein) complex involved in regulated proteolysis. These results suggest that SCF<sup>MAX2</sup> plays critical roles in R, FR, and B light-signaling pathways. In addition, MAX2 might regulate multiple targets at different developmental stages to optimize plant growth and development.

Proper responses to light signals are essential for sessile organisms like plants to survive and adapt to a continuously changing environment. Plants possess at least three classes of sensory photoreceptors that allow them to monitor and respond to the presence, absence, wavelength, intensity, direction, and duration of incidental light signals and modulate their growth and development accordingly. The sensory photoreceptors include cryptochromes (cry) and phototropins (for the UV-A/blue [B] region of the spectrum), phytochromes (phy; for the red [R]/far-R [FR] region of the spectrum), and unidentified UV-B receptors (Chen et al., 2004). Light-induced activation of these photoreceptors controls multiple responses in the plant life cycle, including seed germination, seedling deetiolation, phototropism, shade avoidance, circadian rhythms, and flowering time (termed as photomorphogenesis).

Among the photoreceptors, phys are the best-characterized photoreceptors. phy consist of a small multigene family (designated PHYA to PHYE in Arabidopsis [<i>Arabidopsis thaliana</i>]) encoding approximately 125-kD soluble proteins (Mathews and Sharrock, 1997). They can form selective homodimers and heterodimers among family members (Sharrock and Clack, 2004). Monomeric phy is composed of two major domains: an amino-terminal globular domain attached to a bilin chromophore responsible for sensing and transducing light signals, and a carboxy-terminal linear domain providing dimerization and nuclear localization functions (Matsushita et al., 2003; Chen et al., 2005). The photosensitivity stems from the ability of the holoprotein to absorb photons and interconvert between two light-switchable forms: a R light-absorbing photosensitive R (Pr; biologically inactive) form, which can be converted to the photosensitive FR (Pfr; biologically active) form by exposure to FR light, and a FR light-absorbing Pfr form that can be converted back to the Pr form by exposure to FR light. This interconversion between the Pr and the Pfr forms is critical for the biological functions of phys (Rockwell et al., 2006).

The mechanism of phy signaling has been extensively investigated using a variety of approaches (Quail, 2002; Huq and Quail, 2005; Spalding and Folta, 2005; Rockwell et al., 2006; Jiao et al., 2007). In recent years, regulated proteolysis both in the dark and light has been shown to be a central step in light-signaling pathways (Hoecker, 2005; Huq, 2006). The regulated proteolysis is ubiquitin mediated and mainly targets...
oppositely acting transcription factors in light-signaling pathways. The positively acting transcription factors (HY5, LAF1, and HFR1) are degraded in the dark to repress photomorphogenesis (Osterlund et al., 2000; Holm et al., 2002; Saijo et al., 2003; Seo et al., 2003; Duek et al., 2004; Jang et al., 2005; Yang et al., 2005), whereas the negatively acting transcription factors (PIF1, PIF3, PIF4, PIF5, and possibly others) are degraded in light to promote photomorphogenesis (Bauer et al., 2004; Monte et al., 2004; Shen et al., 2005; Oh et al., 2006; Castillon et al., 2007; Nozue et al., 2007). Recently, Al-Sady et al. (2006) showed that PIF3 is phosphorylated in vivo in a light-dependent manner. In PIF3 mutants that fail to interact with the Pfr forms of phyA and phyB, PIF3 phosphorylation is eliminated, suggesting that phy interaction is necessary for

**Figure 1.** PPS encodes MAX2/ORE9, which is an F-box protein. A, Gene structure at the pps locus. Two probes from the PPS gene used for the Southern blots are shown. Restriction enzymes are indicated by the vertical lines and the first letter of each of the enzymes. C, ClaI; H, HindIII; N, NcoI; X, XbaI. B, Southern blots of genomic DNA isolated from wild-type and pps seedlings probed with either probe 1 (left) or probe 2 (right). Wild-type bands from the PPS gene using the above restriction enzymes are missing in both blots. Different size low-intensity bands appeared in the pps background compared to wild type using probe 2 (right). C, PPS is not expressed in the pps background. Northern-blot analysis of the total RNA isolated from wild-type and pps seedlings grown in the dark for 4 d. Full-length PPS open reading frame was used as a probe. Ethidium bromide-stained gel showing the rRNA bands as loading controls. D (top), Diagrammatic representation of the structure of the PPS gene along with its neighboring genes shown as black/white rectangles on a thin line. The Atg numbers are shown on the top; bottom, RT-PCR analyses of PPS and five other neighboring genes from RNA isolated from wild type and pps mutants grown under Rc (8 μmol m⁻² s⁻¹) for 3 d. UBIQUITIN10 (UBQ10) is used as a control. E, pps did not complement the seedling phenotypes of max2-1 in reciprocal crosses. F1 seedlings and the parents, along with wild type, were grown under R light (8 μmol m⁻² s⁻¹) for 4 d. F, Northern blots of total RNA isolated from wild-type seedlings grown in the dark for 4 d or 4-d-old dark-grown seedlings exposed to R (3.4 μmol m⁻² s⁻¹) and FR (0.5 μmol m⁻² s⁻¹) light for the time indicated. Blots were probed with full-length MAX2 cDNA probe. rRNA bands are shown as a loading control. [See online article for color version of this figure.]
the light-dependent phosphorylation and subsequent degradation of PIF3.

In contrast to the mechanism of light-induced degradation of negatively acting factors, the mechanism of degradation of positively acting factors in the dark has been extensively investigated. Evidence of repression of photomorphogenesis in the dark was provided by the isolation of a group of mutants, named constitutively photomorphogenic (cop). One of these loci encodes COP1, a RING type of E3 ligase, which is localized in the nucleus in the dark and ubiquitinates the positively acting factors of photomorphogenesis (e.g. HY5, LAF1, HFRI, and possibly others; Osterlund et al., 2000; Holm et al., 2002; Saijo et al., 2003; Duek et al., 2004; Jang et al., 2005; Yang et al., 2005). Ubiquitination tags the target proteins for recognition and degradation by the 26S-proteasome complex. The E3 ligase activity of COP1 is modulated by SPA1, a negative regulator of phyA signaling (Hoecker et al., 1999), by an unknown mechanism (Saijo et al., 2003; Seo et al., 2003). COP1 is depleted from the nucleus by a nuclear exclusion mechanism under prolonged light conditions, which allows these target proteins to accumulate and induce photomorphogenesis (von Arnim and Deng, 1994; Osterlund and Deng, 1998; Subramanian et al., 2004). Recently, Chen et al. (2006) have shown that COP1 is complexed with CUL4, the COP9 signalosome, and the COP10, DDB1a, and DET1 (CDD) complex. All three of these complexes synergistically repress photomorphogenesis in the dark (Chen et al., 2006). Therefore, COP1, in conjunction with the COP9 signalosome/CDD/26S proteasome complex, functions as a critical modulator that controls the level of downstream transcription factors required for photomorphogenesis.

Apart from COP1, another type of E3 ligase that is involved in controlling photomorphogenesis is the SCF (for SKP, Cullin, and F-box protein) complex.

Figure 2. pps/max2 seedlings are hyposensitive to R, FR, and B light-mediated suppression of hypocotyl elongation. Fluence-rate response curves of mean hypocotyl lengths of wild-type (Col-0) and pps alleles grown for 3 d under either Rc (A and B), FRc (C and D), or Bc light (E and F). phyA-211, phyB-9, cry1, and cry2 in Col-0 backgrounds were included as controls. Photographs of seedlings grown under Rc (6 μmol m⁻² s⁻¹; B), FRc (1 μmol m⁻² s⁻¹; D), Bc (13 μmol m⁻² s⁻¹; F), or dark for 3 d. Data are presented as mean ± SEM (n > 30, three replicates). White bar, 10 mm. Wt, Wild-type Col-0. [See online article for color version of this figure.]
temperature-sensitive allele of CUL1 (axr6-3), a core subunit of the SCF complex, has higher levels of phyA, resulting in hypersensitivity to FR light (Quint et al., 2005). Moreover, another allele of CUL1 (cul1-6) is hyposensitive to R light and moderately hypersensitive to FR light as observed for axr6-3. Like axr6-3, cul1-6 is also defective in light-induced degradation of phyA (Moon et al., 2007). Several F-box proteins have also been implicated in light-signaling pathways (Dieterle et al., 2001; Harmon and Kay, 2003; Somers et al., 2004; Marrocco et al., 2006). However, in contrast to COP1, the involvement of SCF in light signaling is less well understood. Here, we report the isolation, characterization, and cloning of a new locus, PLEIOTROPIC PHOTOSIGNALING (PPS), which encodes a previously described F-box protein MAX2/ORE9 (for MORE AXILLARY BRANCHES2/ORESARA9; Woo et al., 2001; Stirnberg et al., 2002). pps shows hypo-

sensitive phenotypes under all three light conditions, suggesting that SCF\textsuperscript{MAX2/ORE9} promotes photomorphogenesis.

RESULTS

PPS Encodes MAX2/ORE9

To isolate new factors involved in light signaling, a genetic screen was conducted under continuous R (Rc) light using publicly available T-DNA insertion populations (Huq et al., 2000a, 2000b). One mutant, pps, showed hyposensitive phenotypes under Rc light. Adult pps plants displayed multiple inflorescences under normal growth conditions. It is basta sensitive, suggesting that the antibiotic resistance marker is either lost or silenced. pps has been backcrossed to wild-type
ecotype Columbia-0 (Col-0) twice before further phenotypic characterization.

We have cloned PPS using a combination of map-based cloning and candidate gene approaches. Southern-blot analysis of genomic DNA isolated from the wild-type and pps seedlings using a probe encompassing the full-length open reading frame of the PPS gene showed no signal in the pps mutant background compared to wild type (Fig. 1, A and B, left). A second probe from the 3′-untranslated region of the PPS gene showed altered size bands on the Southern blots (Fig. 1, A and B, right), demonstrating that the PPS gene is either deleted or partially replaced by the T-DNA insertion in the pps background. We have also performed extensive PCR analyses using multiple combinations of forward and reverse primers on the genomic DNA isolated from wild-type and pps plants and were not able to amplify any PCR products from the pps genomic DNA, whereas expected size products were obtained from the wild-type genomic DNA samples. These results suggest that the PPS gene might have been partially replaced by the T-DNA insertions at the pps locus (data not shown). The exact location of the T-DNA insertion in the PPS gene could not be determined because we were unable to amplify any DNA fragment using multiple T-DNA- and gene-specific primer combinations. Northern-blot analysis demonstrates that pps is a null allele because no PPS message is present in the pps mutant samples (Fig. 1C). The PPS nucleotide sequence is identical to the previously isolated gene, MAX2/ORE9, which has been shown to function in inflorescence architecture and senescence (Woo et al., 2001; Stirnberg et al., 2002).

Because pps was isolated from an activation-tagged seed pool, we performed reverse transcription (RT)-PCR analyses of PPS and its neighboring genes to eliminate the possibility that pps phenotype is due to misexpression of the neighboring genes at the pps locus. Results showed that these genes are not misexpressed in the pps background, whereas expression of PPS could not be detected in the pps mutant, suggesting that the pps phenotype is due to the loss of PPS expression (Fig. 1D). Moreover, pps did not complement seedling deetiolation phenotypes and multiple inflorescence phenotypes of max2-1 and max2-2 mutants (Fig. 1E; data not shown). These data establish that pps is a new allele of the max2 mutant and, for simplicity, we will refer to PPS/MAX2/ORE9 as MAX2. Because pps/max2/ore9 has been isolated from three independent genetic screens targeted for separate signaling pathways, it appears that MAX2 has pleiotropic functions throughout the plant life cycle.

To investigate whether MAX2 expression is regulated by light, we performed northern-blot experiments using total RNA isolated from wild-type seedlings grown under R and FR light conditions. The results show that MAX2 expression is induced approximately 2- to 3-fold after 3 h in R or FR light (Fig. 1F) and continued to increase for 72 h. The low abundance of MAX2 mRNA in the dark and subsequent induction by both R and FR light indicates that light-mediated induction of MAX2 might be involved in the MAX2-mediated control of photomorphogenesis.

**pps/max2 Is Hyposensitive to R, FR, and B Light**

The R light hyposensitivity of pps led us to investigate whether pps also displays defects under other light conditions. Fluence rate response curves show
that pps seedlings have longer hypocotyls under increasing intensities of Rc, F Rc, and Bc light conditions than wild-type seedlings (Fig. 2). However, in the dark, hypocotyl lengths were unaffected by the pps, max2-1, and max2-2 mutations (Fig. 2), suggesting that the long hypocotyl phenotype of the pps mutant is light dependent. The hyposensitivity of pps is slightly stronger under Rc than in FRc and Bc (Fig. 2). The cotyledons of pps seedlings were slightly smaller than wild-type cotyledons under all three light conditions (Fig. 3, A and B). The cotyledon angles of pps seedlings are much smaller than that of wild type under all three light conditions (Fig. 3C). Although the cotyledon angles of pps seedlings become wider with increasing light intensities, the angles remained smaller than that of wild type at all fluence rates tested (data not shown). In white light, pps seedlings displayed longer cotyledon petiole lengths than wild-type seedlings (Fig. 3D).

The hyposensitive phenotypes of pps seedlings is not due to reduced levels of phy proteins because western blots did not show any reduction in phyA and phyB levels in pps compared to wild type both in dark and light (data not shown). Longer hypocotyls, smaller cotyledons, reduced cotyledon angles, and longer petiole lengths under light are all hallmark phenotypes of hyposensitive mutants. Therefore, these data suggest that pps is a hyposensitive mutant under both phy- and cry-signaling pathways.

ppsl max2 Is Hyposensitive to Light-Induced Seed Germination

Seed germination of Arabidopsis is induced by light through the phy-signaling pathways (Botto et al., 1996; Shinomura et al., 1996; Poppe and Schafer, 1997). Recently, Oh et al. (2006, 2007) have shown that PIF1/PIL5, a phy-signaling factor, directly and indirectly regulates both the GA3 level and sensitivity to repress seed germination in Arabidopsis. Light-induced degradation of PIF1 relieves this negative regulation to promote seed germination (Shen et al., 2005; Oh et al., 2006). Because pps seedlings showed hyposensitive morphological phenotypes (Figs. 2 and 3), we investigated whether light-induced seed germination response is also reduced in the pps background. Figure 4,
showed a higher number of inflorescences and reduced senescence at maturity as previously observed in max2 and ore9 mutants (Supplemental Fig. S4, A and B; data not shown; Woo et al., 2001; Stirnberg et al., 2002). These results suggest that MAX2 has multiple functions throughout the plant life cycle (Figs. 2–5; Supplemental Figs. S1–S4; Woo et al., 2001; Stirnberg et al., 2002).

The R and FR Light Hyposensitivity of pps Seedlings

Is phy Dependent

To investigate whether the hypersensitive phenotypes of pps under R and FR light are phy dependent, we constructed ppsphyA and ppsphyB double mutants. Like phyA seedlings, ppsphyA double-mutant seedlings are insensitive to increasing intensities of FRC, suggesting that phyA is necessary for the hypersensitive phenotype observed in pps seedlings (Fig. 5, A and C). Similarly, the hypocotyl lengths of the ppsphyB double-mutant seedlings in Rc are similar to those of phyB seedlings, suggesting that phyB is necessary for the hypersensitive phenotype of the pps seedlings under Rc (Fig. 5, B and C). Moreover, the cotyledon areas of the ppsphyB double mutant and the phyB monogenic mutant are very similar (data not shown). These results suggest that phys are necessary for the hypersensitive phenotype of the pps mutant under both R and FR conditions. We have not investigated whether the hypersensitive phenotype of pps under Bc is cry dependent.

phyB has previously been shown to control inflorescence number in both Arabidopsis and sorghum (Reed et al., 1993; Kebrom et al., 2006). To understand the relationship between phyB and MAX2 in controlling the multiple-inflorescence phenotype under these conditions, we analyzed the monogenic and double mutants at the adult stage. phyB mutants have modestly reduced numbers of inflorescences compared to wild type, suggesting that phyB promotes inflorescence number in Arabidopsis as previously shown (Fig. 5D; Reed et al., 1993). phyA did not contribute to the regulation of inflorescence number under these conditions (Fig. 5D). Moreover, ppsphyB double-mutant plants showed a similar number of inflorescences as the pps monogenic plants under the same growth conditions (Fig. 5D), suggesting that pps/max2 is epistatic to phyB in controlling inflorescence number in Arabidopsis.

Light-Regulated Gene Expression Is Reduced in pps Seedlings

phy mutants, as well as phy-signaling mutants, show altered regulation of light-inducible genes in response to R and FR light (Wang et al., 2002; Hudson et al., 2003; Tepperman et al., 2006). To investigate the changes in gene expression in pps seedlings, we performed northern-blot analyses on selected marker genes involved in photosynthesis, including CHLO-
ROPHYLL a/b-BINDING (CAB) and RUBISCO SMALL SUBUNIT (RBCS; Tepperman et al., 2006). The results show that CAB and RBCS are expressed at a relatively higher level in dark-grown pps seedlings compared to wild-type seedlings (Fig. 6A). If the dark expression levels of these genes are normalized to 1, the rate at which CAB and RBCS are induced by R light appears slower in pps seedlings than in wild-type seedlings (Supplemental Fig. S5, A and B). However, the high-level expression in the dark suggests that these genes are constitutively expressed in the pps background compared to wild type.

To investigate whether expression of other light-regulated genes also shows similar patterns as the photosynthetic marker genes, we performed semi-quantitative RT-PCR assays for a number of genes regulated early under R light. Results show that the light-induced expression level of ELIP1, ELIP2, HY5, PKS1, and SPA1 is slightly less in the pps mutant compared to wild-type seedlings within 1 to 3 h of R light exposure (Fig. 6B; Supplemental Fig. 5C). Down-regulation of the two early light-suppressed genes (PHYA and IAA29) did not show significant differences between pps and wild-type seedlings under these conditions (Fig. 6B; Supplemental Fig. SSC). Because pps shows both light-dependent morphological and gene expression phenotypes (Figs. 2–6), it is also possible that MAX2 functions early in light-signaling pathways.

MAX2 IS EXPRESSED IN A TISSUE-SPECIFIC AND DEVELOPMENTAL STAGE-DEPENDENT MANNER

Because pps mutants showed pleiotropic phenotypes throughout the plant life cycle, we investigated
the temporal and spatial expression patterns of MAX2. We constructed the $P_{\text{MAX2}}$:GUS reporter construct using approximately a 2-kb promoter region upstream of the ATG start codon of the MAX2 gene and used this construct to transform wild-type Arabidopsis. These transgenic plants were used to perform histochemical GUS assays at different stages of development. Strong GUS activity was observed in the seed, hypocotyl, cotyledon, and root at the seedling stage (Fig. 7, A–F), and in leaf veins, vascular tissues, shoot apical meristems, and flower organs at the juvenile and adult stages (Fig. 7, G–O). No GUS activity was detected at the root tips of juvenile or adult plants, suggesting that MAX2 is not expressed in these tissues (Fig. 7, E, H, and I). These results suggest that MAX2 is expressed throughout seedling development; however, its expression is restricted to meristematic and vascular tissues at the juvenile and adult stages. Our results also complement a recent report that showed MAX2 expression in later developmental stages of the Arabidopsis life cycle using plants transformed with a translational fusion construct ($P_{\text{MAX2}}$:MAX2-GUS; Stirnberg et al., 2007).

MAX2 Is Localized in the Nucleus

To determine the subcellular localization of MAX2, we first examined the subcellular localization of a MAX2-GFP fusion protein in a transient transfection assay using approximately a 2-kb promoter region upstream of the ATG start codon of the MAX2 gene and used this construct to transform wild-type Arabidopsis. These transgenic plants were used to perform histochemical GUS assays at different stages of development. Strong GUS activity was observed in the seed, hypocotyl, cotyledon, and root at the seedling stage (Fig. 7, A–F), and in leaf veins, vascular tissues, shoot apical meristems, and flower organs at the juvenile and adult stages (Fig. 7, G–O). No GUS activity was detected at the root tips of juvenile or adult plants, suggesting that MAX2 is not expressed in these tissues (Fig. 7, E, H, and I). These results suggest that MAX2 is expressed throughout seedling development; however, its expression is restricted to meristematic and vascular tissues at the juvenile and adult stages. Our results also complement a recent report that showed MAX2 expression in later developmental stages of the Arabidopsis life cycle using plants transformed with a translational fusion construct ($P_{\text{MAX2}}$:MAX2-GUS; Stirnberg et al., 2007).

DISCUSSION

This study provides genetic, molecular, and physiological evidence that MAX2 is involved in promoting seedling deetiolation in Arabidopsis. Molecular cloning and genetic complementation data convincingly show that $PPS$ encodes a previously identified F-box protein MAX2/ORE9 involved in controlling inflorescence number and senescence in Arabidopsis (Figs. 1–5; Woo et al., 2001; Stirnberg et al., 2002). Therefore, our results and those of others suggest that MAX2 controls multiple pathways at distinct developmental stages of the Arabidopsis life cycle.

Evidence that MAX2 acts as a positive regulator of photomorphogenesis is severalfold. Three independently isolated $pps$/max2 alleles from separate genetic screens show longer hypocotyls than wild type under increasing fluence rates of R, FR, and B light conditions (Fig. 2). Compared to wild-type seedlings, $pps$ seedlings
also show slightly reduced cotyledon areas (Fig. 3, A and B) and strongly reduced cotyledon angles under R, FR, and B light conditions (Fig. 3C). pps seedlings have longer petiole lengths under white-light conditions compared to wild-type seedlings (Fig. 3D). pps seeds are hyposensitive to both R and FR light-induced germination compared to wild type (Fig. 4, A and B). In addition, expression of early light-regulated genes is reduced in the max2 mutant compared to wild-type seedlings (Fig. 6B; Supplemental Fig. 5C). By contrast, hypocotyl lengths, cotyledon areas, and cotyledon angles are similar to wild type in the dark, establishing that pps morphological and molecular phenotypes are light dependent.

In contrast to the morphological and early light-regulated gene expression phenotypes, photosynthetic marker genes are expressed constitutively in the dark at high levels in the pps seedlings compared to wild type (Fig. 6A; Supplemental Fig. 5, A and B). However, dark induction of the photosynthetic genes might be due to reduced GA level/signaling in the pps mutant than in wild type because GA has been previously shown to repress CAB and RBCS expression in the dark (Alabadi et al., 2004). This is also consistent with the reduced germination of pps seeds under limiting R and FR light conditions (Fig. 4, A and B). Recently, PIF1/PIL5, a phy-signaling factor, has been shown to negatively regulate seed germination by directly and indirectly controlling GA level and sensitivity in Arabidopsis (Oh et al., 2006, 2007). Therefore, both the morphological and the gene expression phenotypes of the pps mutant might be related to a deficiency in multiple light responses.

Previously, F-box proteins have been reported to be involved in light-signaling pathways. For example, EID1 is a negative regulator of FR signaling (Dieterle et al., 2001; Marrocco et al., 2006), whereas AFR is a positive regulator of FR signaling (Harmon and Kay, 2003). Two F-box proteins, ZTL and LKP2, involved in the circadian clock, also regulate photomorphogenesis in R and B light conditions (Schultz et al., 2001; Somers et al., 2004). Both ZTL and LKP2 function negatively in light-mediated suppression of hypocotyl elongation. However, the long hypocotyl phenotype of both the ZTL and LKP2 overexpression lines might be due to aberrations of circadian clock functions because the circadian clock has previously been shown to control hypocotyl cell expansion (Dowson-Day and Millar, 1999; Nozue et al., 2007). Therefore, MAX2 is the only characterized F-box protein that functions positively under all three light-signaling pathways.

The adult phenotypes of max2 are both light dependent and light independent. For example, all max2 alleles show strikingly reduced growth compared to wild type at the juvenile stage only under SD conditions (Supplemental Fig. S2). Inflorescence number is regulated by light mainly through phyB (Reed et al., 1993; Kebrom et al., 2006), whereas senescence at maturity is not regulated by light. Although phyB and MAX2 function as positive regulators of light-signaling pathways at the seedling stage, phyB and MAX2 play opposite roles in controlling inflorescence number at the adult stage. Previous reports showed that signaling factors could play stage-specific roles. For example, GIGANTEA (GI) is a positively acting component in the phyB-signaling pathway in seedling deetiolation responses; however, gi mutants flower late under LD conditions, a behavior opposite of phyB single-mutant plants (Huq et al., 2000b; Mizoguchi et al., 2005). Therefore, MAX2, like GI and possibly other factors, may have distinct functions at different developmental stages. The pleiotropic phenotypes observed in pps plants throughout development are consistent with the expression pattern of MAX2 at different developmental stages (Fig. 7).

Photomorphogenic development is not only regulated by linear light-signaling pathways, but also by interactions among light and multiple hormone-signaling pathways (Halliday and Fankhauser, 2003; Neff et al., 2006; Zhao et al., 2007). Recently, Bennett et al. (2006) showed that all max mutants have increased rates of auxin transport at the adult stage than in wild type (Bennett et al., 2006). Because auxin transport has been shown to regulate hypocotyl elongation in light (Jensen et al., 1998), it is possible that the increased auxin transport of max2 contributes to the long hypocotyl and multiple inflorescence phenotypes of max2 (Figs. 2 and 3; Supplemental Fig. S4; Bennett et al., 2006). However, despite the presence of decreased rates of auxin transport in all four max mutants (Bennett et al., 2006), only max2 shows long hypocotyl phenotype in light (Fig. 2; Stirnberg et al., 2002). Therefore, although max2 functions in hormone pathways, the increased auxin transport appears not solely responsible for the long hypocotyl phenotypes of the max2/pps mutants at the seedling stage. These data suggest that the hyposensitive phenotypes of the max2/pps seedlings are light dependent.

Although MAX2 controls multiple pathways at distinct developmental stages, the exact mechanism by which it regulates these pathways is still unknown. MAX2 interacts with Arabidopsis CUL1 and ASK1 in an F-box-dependent manner both in vitro and in vivo (Woo et al., 2001; Stirnberg et al., 2007), suggesting that MAX2 is a bona fide F-box protein. MAX2 is expressed ubiquitously at the seedling stage (Fig. 7) and is localized in the nucleus (Fig. 8; Stirnberg et al., 2007) and, therefore, it may promote ubiquitin-mediated degradation of its targets in the nucleus. Because MAX2 controls diverse unrelated pathways, it is possible that MAX2 targets multiple factors specific for each pathway to control these responses. Alternatively, it is equally possible that MAX2 plants have a more fundamental defect that is responsible for reducing the capacity of max2 seedlings to respond to light signals properly and decreasing apical dominance and delaying senescence of adult plants. Identification and characterization of MAX2 target proteins will be helpful in understanding the mechanism by which MAX2 controls these diverse phenotypes.
MAX2 Positively Regulates Photomorphogenesis

MATERIALS AND METHODS

Plant Growth Conditions and Genetic Screening

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture) under 24-h light at 24°C ± 0.5°C. Monochromatic R, FR, and B light sources are as described (Shen et al., 2005). Light fluence rates were measured using a spectroradiometer (model EPP2000; StellarNet) as described (Shen et al., 2005). T-DNA-tagged seed pools were obtained from the Arabidopsis Biological Resource Center (stock no. CS21995). These seeds were produced in an ecotype Col-0 background (Weigel et al., 2000). Seeds were surface sterilized with 70% EtOH and 0.1 M NaOCl for 6 min, then rinsed for 3 min with water, and 1 M sucrose (Suc) as described (Shen et al., 2005). After 3 to 4 d of stratification at 4°C, seeds were exposed to 1-h white light at room temperature to induce germination before placing them in the dark for 23 h at 21°C. After dark incubation, seeds were exposed to R or FR light for 3 or 4 more days.

Mapping and Cloning of pps

pps seedlings (ecotype Col-0) are sensitive to the antibiotic resistance marker Basta. For mapping purposes, pps was crossed to the Landsberg erecta (Ler) ecotype and seedlings with long hypocotyls were selected under Rč (8 μmol m⁻² s⁻¹) from F₂ progeny and grown in the greenhouse. Leaf tissue was excised from these plants and genomic DNA was isolated as described (Edwards et al., 1991). pps was mapped at the bottom half of chromosome 2 by a dense sequence length polymorphic markers (Bell and Ecker, 1994) by PCR analyses on these genomic DNA preparations. A previously described mutant, max2, showing similar multiple inflorescence phenotypes at the adult stage is also located in this region (Stirnberg et al., 2002). The candidate gene was later confirmed by RT-PCR and northern-blot analysis (see below). For Southern-blot analysis, genomic DNA was extracted using the DNeasy plant mini kit (Qiagen). Two micrograms of genomic DNA were digested with different restriction enzymes at 37°C for 14 h. The digested DNA was separated on a 1.0% agarose gel, transferred to a nylon membrane (MAGNACHARGE; GE Osmonics), and hybridized overnight at 65°C in Church’s buffer. Membranes were washed three times, exposed to a phosphor screen (Kodak) overnight, and the phosphor screen was developed using the Molecular Imager FX system (Bio-Rad Laboratories).

Construction of pppsphyA and pppsphyB Double Mutants

To generate ppsphyB double mutants, pps was crossed to phyB-211 plants (ecotype Col-0; Reed et al., 1994). F2 seeds were plated and grown under FRc (0.6 μmol m⁻² s⁻¹) for 3 d, and seedlings displaying a phyB mutant phenotype were selected. Adult plants with multiple inflorescences were selected as pppsphyA candidates. These candidates were further confirmed for ppsphyA and pps alleles by PCR. F3 seedlings were also grown under FRc to confirm their phenotypes.

RNA Isolation, RT-PCR, and Northern Blotting

Total RNA was isolated from 4-d-old pps mutant and wild-type Col-0 seedlings treated for different time periods under R (669 nm; 3.4 μmol m⁻² s⁻¹) or FR (739 nm; 0.5 μmol m⁻² s⁻¹) light using the RNeasy plant mini kit (Qiagen). For RT-PCR, total RNA was treated with DNase 1 to remove genomic DNA. One microgram of total RNA was reverse transcribed using the RT-PCR kit from Invitrogen and first-strand cDNA was used as a template for PCR amplification. For semiquantitative gene expression, cDNA was diluted to 40 μL with water and 1 μL of diluted cDNA was used for PCR amplification of ELPI, ELP2, HYS, IAA29, PHYA, PKS1, SDA1, and UBIQ10 fragments using gene-specific primers. The UBIQ10 fragment was used as a control to normalize the amount of cDNA used. For all CDNA, the exponential range of amplification cycles for each gene was determined experimentally. Then, 25 (ELP1), 26 (ELP2), 27 (HYS), 31 (AA29), 24 (PHYA), 26 (PKS1), 25 (SBA1), and 20 (UBIQ10) cycles were used for the RT-PCR experiments. Three biological repeats were carried out for each gene. PCR products were separated on ethidium bromide agarose gel, imaged under UV light with Alpha Innotech Imager. The RT-PCR primer sets are shown in Supplemental Table S1.

For northern blots, full-length MAX2 cDNA, CAB3, and RBCS probes were labeled using the random primer-labeling kit (TaKaRa). Northern blots were performed using the northern-Max-Gly kit on 10 μg total RNA according to manufacturer’s instructions (Ambion). Blots were washed for 15 min at low stringency followed by a high-stringency wash at 42°C, and then the membranes were exposed to a phosphor screen (Kodak) at room temperature for overnight. The phosphor screen was developed using the Molecular Imager FX system (Bio-Rad Laboratories).

Spatial and Temporal Analyses of MAX2 Expression

For tissue-specific and developmental expression of MAX2, a 2-kb promoter fragment was amplified by PCR using Pfu polymerase (Stratagene) and cloned into pENTR_D_Topo vector (Invitrogen). After sequence confirmation, the MAX2 promoter was recombined into pBGHPS2 gateway plasmid 5’ of the enhanced GFP and GUS gene (Karimi et al., 2005). This construct was then transformed into wild type using the Agrobacterium-mediated transformation protocol as described (Clough and Bent, 1998). Single-locus transgenic plants were selected based on Basta resistance and several homozygous lines were produced for GUS analysis. Homozygous transgenic lines were grown on GM – Suc plates for various time points as indicated, and histochemical GUS assays were performed as described (Somers and Quail, 1995; Shen et al., 2005).

Construction of Vectors and Subcellular Localization

For subcellular localization assays, a 4-kb genomic fragment, including a 2-kb PPS promoter and the genomic PPS gene, was cloned into the pBI121 vector replacing the 35S promoter 5’ of the GUS gene as a translational fusion. This construct, named Pmax::MAX2−GUS, was sequenced. The GUS gene was also replaced with GFP using restriction enzymes to construct Pmax::MAX2−GFP. This plasmid, along with a positive control (35S:GFP), was bombarded into leaf (Allium porrum) epidermal cells as described (Hug et al., 2004). After bombardment, leaf cells were incubated for 16 h in the dark before visualization under a fluorescence microscope. The Pmax::MAX2−GFP was transformed into pps mutant plants using the Agrobacterium-mediated transformation protocol as described (Clough and Bent, 1998). Homozygous transgenic plants containing the Pmax::MAX2−GFP were selected to investigate MAX2 localization in stable transgenic plants using a fluorescent microscope as well as to test complementation for both seedling and adult phenotypes. The Pmax::MAX2−GUS construct was electroporated into Agrobacterium strain GV3101 and the resulting strain containing the plasmid was then injected into Nicotiana benthamiana as described (Yang et al., 2000). The epidermal peels from these injected and uninjected plants were used for histochemical assays for GUS activity as described (Somers and Quail, 1995; Shen et al., 2005).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM129823.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. ppsmax2 plants have abnormal leaf architecture compared to wild-type plants under continuous white light.

Supplemental Figure S2. ppsmax2 has a greatly reduced growth under SD conditions.

Supplemental Figure S3. Reduced growth rate of pps inflorescences compared to wild type under LD (8-h dark and 16-h light with approximately 70 μmol m⁻² s⁻¹) and SD (16-h dark and 8-h light with approximately 80 μmol m⁻² s⁻¹) conditions.

Supplemental Figure S4. ppsmax2 has defects in apical dominance.

Supplemental Figure S5. Light-regulated gene expression is defective in pps.
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