MAX2 Affects Multiple Hormones to Promote Photomorphogenesis

Hui Shen, Ling Zhu, Qing-Yun Bu and Enamul Huq

Section of Molecular Cell and Developmental Biology and the Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78712, USA

ABSTRACT Ubiquitin-26S proteasome system (UPS) has been shown to play central roles in light and hormone-regulated plant growth and development. Previously, we have shown that MAX2, an F-box protein, positively regulates facets of photomorphogenic development in response to light. However, how MAX2 controls these responses is still unknown. Here, we show that MAX2 oppositely regulates GA and ABA biosynthesis to optimize seed germination in response to light. Dose–response curves showed that max2 seeds are hyposensitive to GA and hypersensitive to ABA in seed germination responses. RT–PCR assays demonstrated that the expression of GA biosynthetic genes is down-regulated, while the expression of GA catabolic genes is up-regulated in the max2 seeds compared to wild-type. Interestingly, expression of both ABA biosynthetic and catabolic genes is up-regulated in the max2 seeds compared to wild-type. Treatment with an auxin transport inhibitor, NPA, showed that increased auxin transport in max2 seedlings contributes to the long hypocotyl phenotype under light. Moreover, light-signaling phenotypes are restricted to max2, as the biosynthetic mutants in the strigolactone pathway, max1, max3, and max4, did not display any defects in seed germination and seedling de-etiolation compared to wild-type. Taken together, these data suggest that MAX2 modulates multiple hormone pathways to affect photomorphogenesis.

Key words: Arabidopsis; F-box protein; photomorphogenesis; protein degradation; strigolactone; SCF complex.

INTRODUCTION

Plants are equipped with highly sophisticated mechanisms that can integrate complex environmental signals into their endogenous programs for adaptation, as they are unable to avoid exposure to constantly changing environment. One of the most important environmental signals affecting plant growth and development throughout the lifecycle is light. Plants undergo two contrasting developmental programs: skotomorphogenesis versus photomorphogenesis, depending on the absence or presence of light, respectively. Skotomorphogenic development in the dark is characterized by a long hypocotyl, closed cotyledons, and unopened hooks, and is termed an etiolated plant. By contrast, photomorphogenic development is characterized by shortening of hypocotyls and expansion and greening of cotyledons in the presence of light, and is also termed de-etiolated plants. Plants are evolved with a suite of sensory photoreceptors: UVR8 to respond to the UV-B region of the spectrum, cryptochromes and phototropins to track the UV-A/blue region of the spectrum, and phytochromes (phys) to track the blue/red/far-red region of the spectrum (Chen et al., 2004; Bae and Choi, 2008; Rizzini et al., 2011). The multiplicity of photoreceptors is presumably required to track a number of parameters including the presence, absence, wavelength, intensity, duration of ambient light to optimize plant growth and development (Schaefer and Nagy, 2006).

The phy family of photoreceptors is encoded by a small multigene family (designated PHYA to PHYE in Arabidopsis) encoding ~125-kDa soluble proteins that form selective homo- and hetero-dimers among family members (Mathews and Sharrock, 1997; Clack et al., 2009). Monomers are composed of two major domains: an amino-terminal domain attached to a bilin chromophore responsible for sensing light signals, and a carboxy-terminal domain for dimerization. Phys also exist as two spectrally distinct forms: a red light-absorbing Pr form (biologically inactive) and a far-red light-absorbing Pfr form (biologically active). The Pr form can be converted to the Pfr form by exposing it to red light; the Pfr form can then be converted back to the inactive Pr form by exposing it to far-red light. In the dark, phys are largely cytosolic proteins. Upon

1 To whom correspondence should be addressed. E-mail huq@mail.utexas.edu, tel. 512-471-9848, fax 512-232-3402.

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light exposure, the Pfr forms migrate into the nucleus (Fankhauser and Chen, 2008) and interact with a group of transcription factors called Phytochrome Interacting Factors (PIFs) (Castillon et al., 2007; Leivar and Quail, 2011). PIFs are encoded by seven genes in Arabidopsis (PIF1, PIF3-8) (Leivar and Quail, 2011) and are able to form homo- and heterodimers (Toledo-Ortiz et al., 2003; Bu et al., 2011a). They belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors (Littlewood and Evans, 1998; Duek and Fankhauser, 2005; Castillon et al., 2007). Although PIFs are highly homologous proteins at least in the bHLH domains, monogenic pif mutants displayed distinct visible phenotypes (Castillon et al., 2007; Leivar et al., 2008b). For example, pif1, pif3-pif5, and pif7 single mutants displayed short hypocotyl phenotypes under red and/or far-red light conditions (Huq et al., 2009; Shin et al., 2009), suggesting that PIFs repress photomorphogenic development in the dark. Light signals perceived by phy induce rapid phosphorylation, poly-ubiquitylation, and 26S proteasome-mediated degradation of PIFs in a phy-dependent manner to remove this negative regulation, and thereby promote photomorphogenesis (Castillon et al., 2007; Leivar and Quail, 2011).

The ubiquitin-26S proteasome system (UPS) has been shown to play critical roles in modulating plant growth and development, including light-signaling pathways (Hua and Vierstra, 2011). This system targets specific proteins by selectively attaching a multi-ub chain by the action of the three enzymes: ubi-activating enzyme (E1), ubi-conjugation enzyme (E2), and ubi-ligase (E3). The multi-ub chain serves as a tag for specific substrates involving in photomorphogenesis for degradation. However, the positive factors (e.g. HY5, HYH, HFR1, LAF1, and possibly others) are degraded in the dark to repress photomorphogenesis, while the negatively acting factors (e.g. PIFs) are targeted for degradation in response to light to promote photomorphogenesis. Light-induced stabilization of positive factors and destabilization of negative factor synergistically promote photomorphogenesis (Huq, 2006). In addition, Casein Kinase II (CK2) phosphorylates both the positive and negatively acting transcription factors independently of light. CK2-mediated phosphorylation also stabilizes the positively acting factors and destabilizes the negatively factor to promote photomorphogenesis (Bu et al., 2011b, 2011c).

In an effort to identify new components in phy-signaling pathways, we have shown that the F-box protein MAX2 (More AXillary growth 2) positively regulates photomorphogenesis under all three (blue, red, and far-red) light conditions (Shen et al., 2007). MAX2/ORE9 has previously been shown to function in branching and senescence pathways (Woo et al., 2001; Stirmberg et al., 2002). Recently, MAX2 has also been shown to function in signaling pathways downstream of the newly discovered hormone, strigolactone (Gomez-Roldan et al., 2008; Umehara et al., 2008) and smoke-derived compounds, karrkkins (Nelson et al., 2011). However, how MAX2 regulates these diverse signaling pathways at different stages of development is still unknown. Here, we focus on MAX2’s role in the photomorphogenic pathways and show that the seed germination and seedling de-etiolation phenotypes of max2 are largely due to mis-regulation of hormone biosynthetic/signaling/transport pathways. Moreover, we show that the photomorphogenic phenotypes are specific to max2, as the strigolactone biosynthetic mutants (max1, max3, and max4) are not defective in light-signaling pathways. MAX2 might form multiple SCFMAX2 complexes potentially targeting specific substrates functioning in diverse signaling pathways at different developmental stages of the plant lifecycle.

RESULTS

max2 Shows Decreased Sensitivity to GA during Seed Germination

Seed germination is coordinately regulated by light and multiple hormones, including Gibberellin (GA) (Finkelstein et al., 2008; Seo et al., 2009). GA is absolutely required for Arabidopsis seed germination even in the presence of light (Koornneef and van der Veen, 1980). Previously, all three max2 alleles (pps, max2-1, max2-2) showed reduced seed germination in response to both R and FR light conditions (Shen et al., 2007). To investigate whether the reduced seed germination phenotype of max2 alleles was due to an altered sensitivity towards GA, seed germination was measured under increasing concentrations of GA3 using a fixed fluence of R light. GA3 was used because it is less catabolized in vivo compared to other GAs (e.g. GA4) (Nakayama et al., 1990; Oh et al.,
Results showed that all three max2 alleles were hypo-sensitive to exogenous GA₃ in R light-induced seed germination responses (Figure 1A and 1B). These data suggest that the reduced seed germination phenotype of max2 seeds in response to light is at least partly due to reduced sensitivity to GA₃.

max2 Shows Increased Sensitivity to ABA during Seed Germination

Although light and GA promote seed germination, ABA displays an antagonistic effect on seed germination by increasing the dormancy of seeds (Finkelstein et al., 2008; Seo et al., 2009). To investigate whether the seed germination phenotype of max2 alleles is also affected by ABA, we measured germination rates in the absence or presence of increasing concentrations of exogenously applied ABA under white light. We also counted the germination rate for 5 d in the presence of a fixed concentration (2 μM) of ABA, as, often, seed germination is delayed in the presence of ABA. Results show that all three max2 alleles are hypersensitive to increasing concentrations of ABA (Figure 2A and 2B). These data suggest that either the endogenous ABA level or ABA signaling is enhanced in the max2 alleles compared to wild-type seeds.

GA and ABA Biosynthetic/Catabolic Genes Are Mis-Regulated in max2 Compared to Wild-Type Seeds

To investigate whether GA and ABA biosynthetic/catabolic/signaling genes are mis-regulated in max2 compared to wild-type seeds, we performed semi-quantitative RT–PCR analyses of selected genes from seed RNA isolated from wild-type and max2 alleles. The data show that the expression of GA biosynthetic gene (GA3ox1) is down-regulated and GA catabolic gene (GA2ox2) is up-regulated in max2 compared to wild-type seeds under both FR and FR/R light exposure (Figure 3). These data suggest that max2 seeds might have a lower level of GA compared to wild-type seeds. The expression of ABA biosynthetic genes (ABA1, NCED6, and NCED9) and ABA catabolic gene (CYP707A2) is also up-regulated in max2 compared to wild-type seeds under both FR and FR/R light exposure. Because expression of both ABA biosynthetic and catabolic genes is up-regulated in max2 compared to wild-type seeds (Figure 3), it is not clear whether max2 seeds have a higher level of ABA or not. However, increased expression of both ABA biosynthetic and catabolic genes is often associated with

Figure 1. max2 Shows Decreased Sensitivity to GA₃ during Seed Germination.
(A) Schematic representation of the phyB-mediated seed germination assay as described with slight modification (Oh et al., 2004; Shen et al., 2005). Seeds of all the genotypes were surface-sterilized within 1 h, plated on MS media, and then either exposed to FR light (34 μmol m⁻² s⁻¹) for 5 min or FR light followed by R light (13 μmol m⁻² s⁻¹) for 5 min. After light exposure, the plates are kept in the dark for 5 d before being scored for seed germination. (B) max2 alleles are hyposensitive to GA₃. Seeds were treated as described above except they were plated on MS media without and with various concentrations of GA₃ as indicated. The plate without GA₃ was treated only with FR light and the plates with GA₃ were treated with FR light followed by R light as described above. Germination was counted after 5 d and plotted against different concentrations of GA₃. Experiments were repeated three times. Error bars indicate SEM.

Figure 2. Germination Rates of max2 and Wild-Type in Response to ABA.
Germination was determined in response to variable concentrations of ABA (A) or in response to a fixed concentration (2 μM) of ABA from 2–5 d (B). Seeds were sterilized and plated on MS plates with and without ABA, stratified at 4°C for 2 d and then transferred to a phytochamber at 21°C under white light. Germination was determined after 3 d (A) or at various days (B) of growth. Error bars indicate SEM.
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to examine genetic interactions. We investigated seed germi-

max2 

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phyB, exclusively regulates seed germination in response to

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max2 

shows increased auxin transport at the

Seedling De-Etiolation Stage

Previously, it was shown that increased auxin transport is nec-

nary for the branching phenotype of the max mutants

Bennett et al., 2006; Crawford et al., 2010). Because auxin

transport has been shown to be involved in hypocotyl elonga-
tion under light conditions (Jensen et al., 1998), it is possible

that the increased auxin transport in max2 seedlings contrib-
utes to the long hypocotyl phenotypes of the max2 seedlings.

To examine this possibility, we first investigated tissue-specific

expression patterns of MAX2. Results show that MAX2 is

in the dark for additional 12 h.

decreased GA level and/or increased ABA signaling (Finkelstein

et al., 2008; Seo et al., 2009). Taken together, the gene expres-
sion data are consistent with the seed germination phenotypes

of the max2 seeds.

The Seed Germination and Seedling De-Etiolation

Phenotypes of max2 Are Epistatic to pif1

Because max2 shows striking light-dependent hyposensitive

phenotypes for seed germination and seedling de-etiolation, it is possible that MAX2 mediates light-induced degradation of negatively acting factors to promote photomorphogenesis. One of these classes of proteins is called the PIFs, which are degraded in response to light (Castillon et al., 2007). Moreover, PIF1, the member with the highest affinity for both phyA and phyB, exclusively regulates seed germination in response to light by modulating GA and ABA biosynthesis as well as signaling (Oh et al., 2007). If PIF1 is more stable in max2 background, max2 mutant seeds are expected to be hyposensitive to light for both seed germination and seedling de-etiolation phenotypes. To investigate this possibility, we first created pif1pps double mutant between pps (a null allele of max2) and pif1 to examine genetic interactions. We investigated seed germination phenotypes and measured hypocotyl elongation under both dark and light. Strikingly, the data show that pps is

epistatic to pif1 in both seed germination and hypocotyl elon-
gation phenotypes (Figure 4A–4C). pif1pps double mutant

seeds failed to germinate after FRp, while the pif1 seeds ger-
minate 100% under these conditions (Figure 4A). Because the

pps 

ppps double mutant seeds displayed an intermediate germin-
ation rate in response to an increasing amount of R light com-
pared to the single mutants (Figure 4A), it is also possible that

PIF1 and MAX2 function in different pathways to regulate

seed germination and seedling de-etiolation. In addition, these data are opposite to that expected if MAX2 targets PIF1 and possibly other PIFs to promote photomorphogenesis.

To confirm whether PIF1 is degraded through the SCFMAX2 in response to light, we crossed LUC–PIF1 into pps background and selected homozygous lines. Immunoblot analyses were performed to determine protein levels of native PIF1 and LUC–PIF1 in darkness and in the light. Results show that the light-induced ubiquitylation and degradation of PIF1 are unaffected in pps background compared to wild-type seedlings (Figure 4D and 4E), suggesting that MAX2 might target other factors for degradation to promote photomorphogenesis.

max2 Suppresses cop1 Seedling De-Etiolation Phenotype

Major regulatory steps in R, FR, and B light-signaling pathways are controlled by COP1, a ring domain containing WD40 repeat protein functioning as E3 ligase that targets positively acting transcription factors to degradation by the UPS and, thus, represses photomorphogenesis in the dark (Hardtke and Deng, 2000). Because max2 is defective in light signaling (Shen et al., 2007), and to place max2 on the genetic pathways for light-signaling networks, we produced cop1max2 double mutants and examined hypocotyl elongation in response to R, FR, and B light in comparison to background lines. Results show that max2 suppresses the cop1 seedling de-etiolation phenotypes under all three light conditions (Figure 5). Conversely, the data also show that cop1 suppresses the max2 phenotypes in the dark and light conditions. These data suggest that MAX2 functions either in parallel to or down-
stream of COP1 to regulate photomorphogenesis.

max2 Shows Increased Auxin Transport at the Seedling Stage

Figure 3. Expression of GA and ABA Biosynthetic/Catabolic Genes in max2 and Wild-Type Seeds.

Semi-quantitative RT–PCR assays for the selected genes were performed using total RNA isolated from wild-type, max2-2, pps, and pif1-1 mutants. Seeds were sterilized and plated within 1 h of imbibition, exposed to either FRp (34 μmol m⁻² s⁻¹) for 5 min or FRp followed by Rp (13 μmol m⁻² s⁻¹) for 5 min before growing them in the dark for additional 12 h.

Figure 4. Seedling De-Etiolation Phenotype

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tion under light conditions (Jensen et al., 1998), it is possible

that the increased auxin transport in max2 seedlings contrib-
utes to the long hypocotyl phenotypes of the max2 seedlings.

To examine this possibility, we first investigated tissue-specific

expression patterns of MAX2 promoter driving GUS reporter
gene during seedling de-etiolated (Shen et al., 2007). Results from

4-day-old seedlings grown under R light showed that MAX2 is

mainly expressed along the vascular system (Figure 6A). These
data are consistent with previous observation that MAX2 is

expressed in the vascular bundles of both dark and
light-grown young seedlings (Shen et al., 2007), and of inflorescence stem at the adult stage (Stirnberg et al., 2007). Because alterations in auxin accumulation patterns have not been shown in max2 at the young seedling stage where we examine light-regulated seedling de-etiolation phenotypes, a DR5::GFP construct was introduced into pps mutant background. DR5::GFP functions as a reporter for auxin level and auxin signaling status (Ulmasov et al., 1997; Benková et al., 2003). Examination of GFP fluorescence showed that max2 seedlings displayed a much higher level of GFP signal compared to wild-type DR5::GFP transgenic plants at vascular bundles where MAX2 is highly expressed (Figure 6A and 6B). Data suggest that max2 seedlings might have a higher level of auxin transport and/or signaling during the seedling de-etiolation stage under light conditions similar to the adult stage, as previously shown (Bennett et al., 2006; Crawford et al., 2010).

Increased Auxin Transport Contributes to the Seedling De-Etiolation Phenotypes of max2 Mutant

To examine whether auxin transport contributes to the long hypocotyl phenotypes of the max2 seedlings, we grew seedlings with and without NPA (an inhibitor of auxin transport) in the presence and absence of light. Results show that the percent inhibition of hypocotyl elongation by NPA is much higher for all three max2 alleles compared to wild-type controls (Figure 7A and 7B). However, hypocotyl lengths of max2 seedlings were still longer compared to wild-type even in the presence of NPA. These data suggest that auxin transport contributes to the long hypocotyl phenotype of the max2 seedlings; however, max2 seedlings may have additional defects in sensing and responding to light signals.

Strigolactone Is Not Involved in Photomorphogenesis

MAX pathway has recently been shown to encode enzymes necessary to produce strigolactone in both rice and Arabidopsis (Gomez-Roldan et al., 2008; Umehara et al., 2008). MAX1, MAX3, and MAX4 encode biosynthetic enzymes for strigolactone and MAX2 encodes an F-box protein necessary for signaling (Leyser, 2008). A recent report also showed that strigolactone is involved in modulating light-signaling in the dark followed by 4 d under continuous Rc (7 μmol m⁻² s⁻¹) light. * indicates cross-reacting band showing loading control.
pathways using a chemical genetics approach (Tsuchiya et al., 2010). However, this report used a presumably non-physiological level of strigolactone analogs to assess light responses. To examine whether strigolactone is truly involved in affecting photomorphogenesis, we obtained max1-1 mutant from the ABRC stock center (Booker et al., 2005) and also isolated max3 and max4 T-DNA insertion lines from the SALK collection (Figure 8A) (Alonso et al., 2003). Phenotypic characterization confirmed that the max3 and max4 mutants display characteristic branching phenotypes (Figure 8B and 8C), as previously reported (Sorefan et al., 2003; Booker et al., 2004). We then examined the seed germination and seedling de-etiolation phenotypes of max1, max3, and max4 and compared with max2 and wild-type controls. The results showed that max1, max3, and max4 did not display any defect in hypocotyl elongation in response to increasing fluence rates of R and FR light conditions (Figure 8D–8F). Additionally, seed germination phenotypes of max1, max3, and max4 were similar to wild-type controls under an increasing fluence rate of R light, while max2 displayed characteristic reduced seed germination phenotype similar to published results (Figure 8G and 8H) (Shen et al., 2007). These data suggest that strigolactone is not involved in affecting photomorphogenesis. However, MAX2 affects photomorphogenesis under all three light conditions by an unknown mechanism.

**DISCUSSION**

Although the UPS system plays central roles in light and hormone-signaling pathways (Lau and Deng, 2010; Hua and Vierstra, 2011), the E3 ligase responsible for the light-induced degradation of PIFs is still unknown. Because MAX2, an F-box protein capable of forming an SCF\(^{\text{MAX2}}\) E3 ligase, functions positively, while PIFs function negatively in light-signaling pathways, one potential mechanism of MAX2 function would be to participate in the light-induced degradation of PIFs. Here, we provide genetic and biochemical evidence that MAX2 does not participate in the light-induced degradation of PIF1. Double mutant analysis showed that max2 is epistatic to pif1 (Figure 4A), the opposite of what is expected if PIF1 is a target of MAX2. Light-induced degradation of native PIF1 and LUC–PIF1 fusion protein as well as putative ubiquitylated forms of LUC–PIF1 is also unaltered in max2 background compared to wild-type (Figure 4D and 4E), confirming that MAX2 does not regulate PIF1 to promote photomorphogenesis.

Alternatively, the data presented here suggest that the light-regulated seed germination and seedling de-etiolation phenotypes of max2 are largely due to defects in multiple hormone biosynthesis/catabolism/transport pathways. At the seed stage, max2 seeds appear to have defects in both GA and ABA

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**Figure 5.** Phenotype of max2, cop1, and max2cop1 Seedlings under Different Light Conditions.

(A) Photographs of cop1, pps (a null allele of max2), and two independent cop1pps double mutants grown in the dark for 5 d or 1 d in the dark followed by 4 d under Rc (12 µmol m\(^{-2}\) s\(^{-1}\)), FRc (1.25 µmol m\(^{-2}\) s\(^{-1}\)), or Bc (15 µmol m\(^{-2}\) s\(^{-1}\)) light. Bar = 10 mm.

(B) Bar graph showing hypocotyl lengths for various genotypes. Seedlings were grown under the conditions described in (A). Hypocotyl lengths of the cop1pps double mutants were significantly different compared to those of cop1-6 single mutant seedlings. Error bars indicate SEM (n = 16). * indicates significant difference (P < 0.05).
pathways (Figures 1 and 2). Consistently with these data, RT-PCR analyses showed that the expression of GA biosynthetic gene (GA3ox1) is down-regulated while the GA catabolic gene (GA2ox2) is up-regulated in max2 seeds compared to wild-type. Conversely, the expression of the majority of the ABA biosynthetic genes (ABA1, NCED6, and NCED9) is up-regulated in the max2 seeds compared to wild-type. The net effect of mis-regulation of these genes might result in altered GA and ABA levels/signaling status that inhibits seed germination of max2 in response to light. Previously, PIF1 has been shown to regulate both GA and ABA biosynthetic pathways in response to light (Oh et al., 2006, 2007). However, PIF1 directly regulates GA signaling genes (e.g. RGA and GAI) as well as SOMNUS to control GA biosynthesis (Oh et al., 2007; Kim et al., 2008). MAX2 appears to affect the expression of GA biosynthetic/catabolic genes without affecting GA signaling genes, suggesting that MAX2 targets other protein(s) to regulate these pathways. Interestingly, karrikins, smoke-derived abiotic signals, have been shown to promote photomorphogenesis including seed germination in response to light (Nelson et al., 2010). In addition, MAX2 has been shown to mediate karrikin signaling in Arabidopsis (Nelson et al., 2011). It is possible that karrikins also promote seed germination in response to light through MAX2 by modulating GA and ABA levels/signaling pathways.

At the seedling stage, genetic evidence suggests that MAX2 appears to function in parallel or downstream of COP1, PIFs (e.g. PIF1) as well as phy (Figures 4 and 5) (Shen et al., 2007). Previously, it was shown that all max mutants display an increased rate of auxin transport at the adult stage due
Figure 8. Effect of Strigolactone on Photomorphogenesis.

(A) Schematic representation of the gene structure of MAX3 and MAX4. The position of T-DNA insertion in the SALK lines is indicated by triangles.

(B) Multiple branching phenotypes of adult plants. Photographs show Col-0 wild-type, max2, max3, and max4 plants.

(C) Bar graphs showing the number of axillary branches. Plants were grown in a growth room under continuous light. Number of axillary branches was counted after the plants completed flowering. Error bars indicate SEM.
to increased accumulation of PIN1 (Bennett et al., 2006; Crawford et al., 2010). The increased expression of DR5-GFP reporter confirms that max2 also has increased auxin transport and/or signaling at the young seedling de-etiolation stage (Figure 6). In addition, the higher percent inhibition of hypocotyl elongation in response to NPA (an inhibitor of polar auxin transport) for all three max2 alleles compared to wild-type (Figure 6) is consistent with previous reports that auxin transport is necessary for hypocotyl elongation under light (Jensen et al., 1998). However, max1, max3, and max4 plants also displayed increased auxin transport (Bennett et al., 2006) without showing longer hypocotyl under R and FR light conditions (Figure 8). These data suggest that increased auxin transport in max2 seedlings may contribute to the long hypocotyl phenotype of max2. However, max2 seedlings might have additional defects that contribute to its role in affecting photomorphogenesis.

Because MAX2 is the only signaling factor identified so far in Arabidopsis that is involved in strigolactone pathway, one important question is whether strigolactone is involved in promoting photomorphogenesis. Using non-physiological levels of strigolactone analogs (GR24), a recent report also showed that strigolactone pathway promotes photomorphogenesis by inducing nuclear exclusion of COP1 and thereby stabilizing HY5 that promotes photomorphogenesis (Tsuchiya et al., 2010). In addition, karrikins and GR24, two groups of structurally similar abiotic and endogenous signals, respectively, also promote seed germination and seedling de-etiolation phenotypes in response to light (Nelson et al., 2010, 2011). Another recent report showed that strigolactones also reduce thermoinhibition of seed germination in part by increasing GA biosynthesis and reducing ABA biosynthesis (Toh et al., 2012). The increased thermosensitivity of the strigolactone biosynthetic mutants can be reversed by adding GR24, but not for the sensitivity mutant max2. However, convincing genetic evidence for the involvement of strigolactone in promoting photomorphogenesis is still lacking. Here, we provide compelling genetic evidence that strigolactone is not involved in promoting photomorphogenesis. All three strigolactone biosynthetic mutants (max1-1, max3-100, and max4-100) did not display any defect in seed germination and seedling de-etiolation phenotypes in response to increasing fluence of R and FR light conditions (Figure 8). In addition, suppression of cop1 phenotype by max2 suggests that MAX2 functions either in parallel or downstream of cop1 (Figure 5). These data are consistent with previous reports that only max2 among all max mutants displayed long hypocotyl phenotype under continuous white light (Stirnberg et al., 2002) or under a fixed amount of continuous red light conditions (Nelson et al., 2011). In addition, absence of seed germination phenotype for the strigolactone biosynthetic mutants was also observed under a fixed amount of R light (Nelson et al., 2011). Because a single pulse of R light might be saturated for response like seed germination, our fluence response curves using a very low amount of R light provides strong evidence that strigolactone biosynthetic mutants are not defective in light-mediated seed germination. Previously, alleles of max3 and max4 showed a severely reduced level, if any, of strigolactone biosynthesis compared with wild-type controls (Umehara et al., 2008). One explanation for the observed results is that photomorphogenic pathways are extremely sensitive to strigolactone and, thus, even very low levels of strigolactone are sufficient to promote photomorphogenesis. Alternatively, and more likely, strigolactone might not be necessary for photomorphogenesis at all. This hypothesis would imply that MAX2 affects photomorphogenesis in a strigolactone-independent manner by modulating multiple hormone (e.g. GA, ABA, and auxin) and abiotic (e.g. karrikins and temperature) signaling pathways at the seed and seedling stages.

In summary, our data and those of others suggest that MAX2 affects photomorphogenesis by modulating multiple hormone and abiotic-signaling pathways in a strigolactone-independent manner. MAX2 also affects branching, senescence, karrikin, and temperature-signaling pathways at different developmental stages (Woo et al., 2001; Stirnberg et al., 2002; Nelson et al., 2011; Toh et al., 2012). One possibility is that MAX2 might interact with multiple ASK proteins potentially expressed in a tissue-specific manner and might form different SCF<sup>MAX2</sup> E3 ligase complexes to target many unrelated substrates that regulate diverse biological processes at different developmental stages. Alternatively, MAX2 targets a single substrate regulating these diverse biological processes at different developmental stages. Isolation and characterization of MAX2 target(s) will help distinguish these possibilities.

**METHODS**

**Plant Growth Conditions**

All the plants for each genotype were grown in a growth room under identical conditions. Seeds were harvested, dried for 2 weeks in drierite, and then stored at room temperature. Plant growth conditions and light fluence measurements were

(D, E) Fluence rate response curves of seedlings grown in the dark and under increasing fluence rates of Rc (D) and FRc (E) for 4 d. Hypocotyl lengths were measured and plotted against fluence rate of light. Error bars indicate SEM.

(F) Photographs of seedlings grown under conditions described in (D) and (E).

(G) Photographs of plates showing seed germination responses under FRp or Rp. Seeds were sterilized within 1 h of imbibition and then either exposed to FRp (10 000 μmol m<sup>-2</sup>) or FRp (10 000 μmol m<sup>-2</sup>) followed by Rp (10 000 μmol m<sup>-2</sup>) before incubation in the dark for 6 additional days.

(H) Fluence rate response curves of seed germination responses. Seeds were treated as described in (G) except that they were exposed to increasing photon fluences of Rp as indicated. Error bars indicate SEM.
as described (Shen et al., 2005). Seeds were surface-sterilized and plated on Murashige-Skoog (MS) growth medium containing 0.9% agar without sucrose (GM-Suc) as described (Shen et al., 2005). After 4–5 d of stratification at 4°C, seeds were exposed to 3-h white light at room temperature to induce germination before placing them in the dark for 21 h at 21°C. After dark incubation, seeds were exposed to R or FR light for 3 or 4 additional days at 21°C. Hypocotyl lengths of at least 30 seedlings (except for Figure 5, where 16 seedlings were used) were measured using publicly available ImageJ software (http://rsbweb.nih.gov/ij/) after taking digital photographs.

Seed Germination and Hormone Assays

Light-dependent seed germination assays were performed as described with slight modifications (Oh et al., 2004). Briefly, seeds were surface-sterilized and plated on MS growth medium within 1 h of imbibition (50–60 seeds/genotype) and then exposed to 5 min of FR light (34 μmol m⁻² s⁻¹) only or FR light followed by R light (13 μmol m⁻² s⁻¹) for 5 min or as indicated before returning to the dark for 5–6 d at 21°C. Germination was defined by emergence of the root radicals from seeds. For GA treatments, seeds were treated as described above and then plated on MS growth medium plus or minus various concentrations of GA as indicated. For ABA treatments, seeds were sterilized and plated on MS plates with and without ABA, stratified at 4°C for 2 d and then transferred to seed germination chamber at 21°C under white light. Germination was determined by the emergence of root radicals at various days of growth. Commercially available GA₃ (catalog #G7645) and (±)ABA (catalog #A1049) were obtained (Sigma-Aldrich Co., St Louis, MO). All the germination assays were repeated at least three times with independent seed batches.

**Generation of Double Mutants and LUC–PIF1 in max2 Background**

To generate max2pif1 double mutants, pif1-2 was crossed with pps plants. F₂ seedlings were selected on kanamycin plate (resistant marker for pif1-2 mutant). Two independent homozygous pif1pps double mutants (pif1pps#1 and pif1pps#2) were selected from the F₂ population based on kanamycin resistance and multiple tiller phenotypes at the adult stage (max2 adult phenotype). For cop1pps double mutants, cop1-6 was crossed with pps. Two independent homozygous cop1pps double mutants (cop1pps#9 and cop1pps#11) were selected from the F₂ population based on seedlings de-etiolation phenotype in the dark (cop1-6 phenotype) and multiple tiller phenotypes at the adult stage (max2 adult phenotype).

To generate LUC–PIF1 in max2 background, the 35S::LUC–PIF1 transgenic plant (Shen et al., 2005) was crossed with pps. The F₁ seedlings were selected on gentamycin plate (resistance marker for LUC–PIF1). Homozygous LUC–PIF1 in max2 background was selected from the F₂ population based on gentamycin resistance and multiple tiller phenotypes at the adult stage (max2 adult phenotype).

**Protein Extraction and Western Blotting**

Native PIF1 antibody preparation, protein extraction, and immunoblot analyses were performed as described (Shen et al., 2008). Detection of LUC–PIF1 is described in Shen et al. (2005). Briefly, 4-day-old dark-grown seedlings were either kept in darkness or exposed to a pulse of R light (Rp, 2 μmol m⁻² s⁻¹ for native PIF1 and 3000 μmol m⁻² for LUC–PIF1) and incubated in the dark for various times as indicated before protein extraction. Total protein was extracted from ~200-ng seedlings in 600 μl denaturing buffer as described (Shen et al., 2008). Extracts were centrifuged at 16 000 g for 10 min at 4°C, and 30-μl supernatants were loaded onto 8% SDS–PAGE gel, blotted onto PVDF membrane, and probed with anti-PIF1 or anti-LUC or anti-tubulin antibody. Peroxidase-labeled goat anti-rabbit (anti-mouse for tubulin) antibody (KPL Inc., Gaithersburg, MA) in a 1:50 000 dilution was used as secondary antibody. Membranes were developed using the KPL Protein Detector LumiGLO Reserve Western Blotting Kit (#54–13–50) (KPL Inc., Gaithersburg, MA) and visualized on an X-ray film.

**Tissue-Specific Expression of MAX2 and DR5::GFP Level in max2 Background**

The transgenic plant containing MAX2 promoter driving GUS reporter (P₃₅S::MAX2–GUS) was as described (Shen et al., 2007). For DR5::GFP in max2 background, the DR5::GFP transgenic plant (Benková et al., 2003) was crossed with pps. The F₁ seedlings were selected on sulfadiazine plate (resistance marker for DR5::GFP). Homozygous DR5::GFP in max2 background was selected from the F₂ population based on sulfadiazine resistance and multiple tiller phenotypes at the adult stage (max2 adult phenotype). Homozygous transgenic plants containing the DR5::GFP in wild-type and pps backgrounds were grown in the dark or red light (7 μmol m⁻² s⁻¹) for 4 d. GFP fluorescence was examined using a fluorescent microscope under identical settings.

**RNA Isolation and Expression Analyses by RT–PCR**

Seeds of various genotypes were surface-sterilized and plated within 1 h of imbibition as described above. The plates were exposed to either FRp (5 min, 34 μmol m⁻² s⁻¹) or FRp followed by Rp (5 min, 27 μmol m⁻² s⁻¹) light exposure before incubating at 21°C for 12 h in the dark. Seeds were then harvested for total RNA extraction using the Spectrum plant total RNA kit (Sigma-Aldrich Co., St Louis, MO) according to the manufacturer’s protocol. For RT–PCR, total RNA was treated with DNase I to remove genomic DNA. One μg of total RNA was reverse-transcribed using the RT–PCR kit from Invitrogen (Invitrogen Life Science, Carlsbad, CA), and the first-strand cDNA was used as a template for PCR amplification. For semi-quantitative gene expression, cDNAs were diluted two-fold with water and 1 μl of diluted cDNA was used for PCR amplification of GA2ox2, GA3ox1, RGA, GAI, RGL2, CYP707A2, ABA1, NCED6, NCED9, and UBQ10 fragments using gene-specific primers. The UBQ10 fragment served as a constitutive control.
For all cDNAs, the exponential range of amplification cycles for each gene was determined experimentally. Then, 28 (GA2ox2), 26 (GA3ox1), 28 (RGA), 28 (GA1), 25 (RGL2), 25 (CYP707A2), 28 (ABA1), 28 (NACD6), 28 (NACD9), and 23 (UBQ10) 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 an Alpha Innotech Imager. The RT-PCR primer sets are as follows: GA2ox2 (forward 5'-GTGTGAAAGATGGAAGTTGGGT-3'; reverse 5'-AGCGTCATGAAACGTTGAGTCAGTG-3'), GA3ox1 (forward 5'-AATAAGCAXATGTGGCCGAAAGAGATAAC-3', reverse 5'-TAGAGCGATTCAACGGGACTA-3'), RGA (forward 5'-CATTCCGGGAACCGCATTATCACG-3', reverse 5'-TACACCGTGCTTCTATGACTCCAC-3'), GA1 (forward 5'-AGGCTCATAGAAAACGTTGATCGTG-3'; reverse 5'-TGCCAACCCAACATGAGACGACG-3'), RGL2 (forward 5'-CCGACCCGAATCTGAAAACCTTAGTG-3', reverse 5'-AAAGCGCTTCGTTGAACCC-3'), CYP707A2 (forward 5'-GGCAAAAAGAAGAAGAAGACGA-3', reverse 5'-GGTAATAGCGACTC-3'), NCED9 (forward 5'-AACCGGGTTGGTTCTTTGCTAATG-3'; reverse 5'-CCCGGGTTGGTTCTATGACTCCAC-3'), ABA1 (forward 5'-GATGCAGCCAAATATGGGTCAAGG-3', reverse 5'-TGGCTTGAACAAGTGAGCTTTGCT-3'), ABA1 (forward 5'-GATGCGACCCAATATGGGTCAAGG-3', reverse 5'-GCCATTTGATGGAATACGACTC-3'), NACD6 (forward 5'-ACCGGTTCGATATAATGGTTCT-3', reverse 5'-CGGCTTACCCAAGGAGGATGT-3'), RGA (forward 5'-ATGGGGTTGCTTACATCGAGA-3'; reverse 5'- TGCTGTTAACAGTAATGTTCTG-3'), RGL2 (forward 5'-CCGACCCGAATCTGAAAACCTTAGTG-3', reverse 5'-AAAGCGCTTCGTTGAACCC-3'), CYP707A2 (forward 5'-GGCAAAAAGAAGAAGAAGACGA-3', reverse 5'-GGTAATAGCGACTC-3'), NCED9 (forward 5'-AACCGGGTTGGTTCTTTGCTAATG-3', reverse 5'-CCCGGGTTGGTTCTATGACTCCAC-3'), ABA1 (forward 5'-GATGCAGCCAAATATGGGTCAAGG-3', reverse 5'-TGGCTTGAACAAGTGAGCTTTGCT-3'), ABA1 (forward 5'-GATGCGACCCAATATGGGTCAAGG-3', reverse 5'-GCCATTTGATGGAATACGACTC-3'), NACD6 (forward 5'-ACCGGTTCGATATAATGGTTCT-3', reverse 5'-CGGCTTACCCAAGGAGGATGT-3', reverse 5'-CGACTTGTCATTAGAAAAGAGATAC-3').

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