



Functional Analysis of MAX2 in Phototropins-Mediated Cotyledon Flattening in *Arabidopsis*

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Phototropins (phot1 and phot2) are blue-light receptors that control cotyledon flattening and positioning under strong light; however, their functional redundancy restricts our understanding of the specific roles of phot2. To identify the factors responsible for phot2-dependent cotyledon flattening and growth, we screened for light-insensitive mutants among mutagenized *phot1* mutants in *Arabidopsis thaliana*. The double mutant *phot1 lea1* (*leaf expansion associated 1*), which is defective in cotyledon flattening and positioning but not the phototropic response was selected. This mutant phenotype could be alleviated by constitutively expressing *MORE AXILLARY GROWTH 2* (*MAX2*), indicating that *LEA1* was allelic to *MAX2*. The *max2* mutants (*max2-2* and *max2-3*) are defective in cotyledon flattening, which is similar to that of the *phot1 phot2* mutants. Moreover, the amounts of *MAX2* transcripts are inhibited in leaves of *phot1* mutant. However, the additional disruption of *PHOT1* gene in *max2-2* or *max2-3* did not affect their phenotype, including *MAX2*-mediated inhibition of hypocotyl elongation. By contrast, phototropins-mediated hypocotyl phototropism was not regulated by *MAX2*. Together, these results suggest that cotyledon flattening was mediated by both phototropins and *MAX2* signaling, but the relationship between two pathways need further study.

Keywords: cotyledon flattening, hypocotyl elongation, MAX2, phototropin, phototropism

INTRODUCTION

Light provides not only energy but also several environmental signals important for plant growth. Plants can sense changes in light intensity, quality, and direction and alter their growth in response, including regulating parameters of the leaf morphogenesis and movement, to optimize light capture and increase photosynthetic productivity (Fankhauser and Christie, 2015). Blue light affects the formation and distribution of new leaves, regulating their thickness and area by increasing the thickness of the epidermis and mesophyll cell growth (Moreira da Silva and Debergh, 1997). The phototropins (phot1 and phot2) are blue-light receptors that detect blue light and, in response,

regulate many physiological activities, such as hypocotyl phototropism (Sakai et al., 2001), stomatal opening (Kinoshita et al., 2001), chloroplast relocation (Kagawa et al., 2001), and leaf positioning and flattening (de Carbonnel et al., 2010).

Leaves are the power houses of plants, providing energy for all organs through the process of photosynthesis (Van Dingenen et al., 2016). Leaf flattening and positioning maximizes light capture and increases photosynthetic productivity (Walters, 2005; Jiao et al., 2007). The cotyledonary petiole angle, the angle between the cotyledonary petiole and the horizontal plane, and the cotyledon angle between the petiole and cotyledonary blade (Harada et al., 2013) can be determined in different growing conditions to better elucidate the regulation of leaf positioning and flattening. Previously, these processes were found to be mainly regulated by *phot1* under weak white light, but by both *phot1* and *phot2* under moderate and strong levels of white light (Harada et al., 2013). In addition to phototropin-mediated phototropism, NPH3 (NON-PHOTOTROPIC HYPOCOTYL 3) (Inoue et al., 2008), RPT2 (ROOT PHOTOTROPISM 2) (Harada et al., 2013), and the PKSs (PHYTOCHROME KINASE SUBSTRATES) (de Carbonnel et al., 2010) are also involved in leaf positioning and flattening. Plant hormones, especially auxin, are believed to affect leaf flattening, because the distribution of auxin is altered in developing leaves that go on to have different leaf flattening phenotypes (Lumba and McCourt, 2005). CPT1 (coleoptile phototropism1) and the PKS (PHYTOCHROME KINASE SUBSTRATES) regulate the asymmetric distribution of auxin in the coleoptiles (Haga et al., 2005) and leaves (de Carbonnel et al., 2010), while *phot1* likely interacts with NPH3/RPT2 family proteins via the PKSs to regulate the activity and position of the auxin efflux carrier PIN (PIN-FORMED) 1 and the auxin influx transporter AUX (AUXIN) 1 (Blakeslee et al., 2004; Stone et al., 2008). The *phot1*-PKS-NPH3 complex therefore plays an important role in both phototropism (Fankhauser and Christie, 2015) and leaf positioning and flattening; however, the mechanism by which this protein complex functions largely unknown, although it may involve EHB1 (ENHANCED HYPOCOTYL BENDING 1) (Knauer et al., 2011). *Phot2* was reported to be involved in the control of leaf flattening and positioning independently of *phot1* and NPH3 (Inoue et al., 2008). These results demonstrated that numerous factors and pathways participate in the regulation of leaf positioning and flattening.

Although *phot2* was reported to participate in controlling leaf flattening and positioning, the functional redundancy of *phot1* and *phot2* has limited previous attempts to investigate the specific role of *phot2* in these mechanisms. To identify downstream factors of *phot2* signaling while avoiding interference by *phot1*, the *phot1* mutant was further mutagenized and used for screening defective leaf positioning and flattening, resulting in the identification of the *phot1 lea1* (*leaf expansion associated 1*) mutant. This mutant contained polymorphisms in both *MORE AXILLARY GROWTH 2* (*MAX2*) and *MAX3*; therefore, we investigated the functions of *MAX2* and *MAX3* in leaf positioning and flattening, and explored the relationships between the phototropins and the MAX proteins.

RESULTS

Isolation and Characterization of the Leaf Positioning and Flattening Defect Mutant *phot1 lea1*

Under $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light, the cotyledonary petioles of the wild type (WT), *phot1*, and *phot2* plants grew obliquely upward, resulting in flat and almost horizontal cotyledons (Figure 1A) with similar cotyledonary petiole angles and cotyledon angles (Figures 1B,C). The cotyledonary petiole of the *phot1 phot2* double mutant emerged more or less horizontally, resulting in an almost vertical cotyledon (Figures 1A,C). This suggests that *PHOT1* and *PHOT2* contribute redundantly to cotyledon positioning and flattening in *Arabidopsis thaliana* as reported previously (Harada et al., 2013).

In order to identify factors downstream of *phot2* signaling and avoid the interference of *phot1*, the *phot1* mutant was mutated using 0.3% (w/v) EMS (ethylmethane sulfonate). We identified a suspected heritable mutant with defects in leaf flattening and positioning, which was subsequently named *phot1 lea1* (Figure 1A). In comparison with the WT, *phot1*, and *phot2* plants, *phot1 lea1* exhibited a greater cotyledonary petiole angle and a smaller cotyledon angle (Figures 1A,C). Although the cotyledon angle in *phot1 lea1* (65.3°) was similar to *phot1 phot2* (74.8°), the cotyledonary petiole angle of *phot1 lea1* (67.7°) was much larger than that of *phot1 phot2* (32.2°). Both *phot1 lea1* and *phot1 phot2* had downward-pointing (epinastic) cotyledon blades (Figure 1A), although their true leaves differed; the *phot1 lea1* had shortened leaf petioles and broader, more highly lobed leaves, while *phot1 phot2* double mutant leaves were elongated with curled margins (Figures 1D,E). In addition, *phot1 lea1* formed more rosette leaves in the vegetative stage (Figure 1D) and had increased shoot branching in the flowering stage (Figure 1E). These results indicated that *LEA1* played an important role in leaf positioning and flattening.

The F_1 progeny from crosses between Col-0 and *phot1 lea1* had the WT phenotype, and the subsequent F_2 individuals had a segregating cotyledon phenotype. The phenotypes of the F_2 population segregated at a ratio of three WT to one mutant (721:226, $\chi^2 = 0.12$), suggesting that the phenotype of *phot1 lea1* resulted from a single recessive mutation (Supplementary Table 1). Crosses between *phot1 lea1* and *Landsberg erecta* resulted in the same conclusion (Supplementary Table 1) and provided material for further map-based cloning. These results indicated that the single mutation of *LEA1* caused downward curled cotyledon blades, and this phenotype is independent of *phot1* background.

Both *MAX2* and *MAX3* Have Base-Substitution Mutations in the *phot1 lea1* Mutant

The *lea1* mutation was identified using map-based cloning and sequencing of the segregating F_2 population derived from the cross between *lea1* and *Landsberg erecta*. DNA extracted from more than 100 selected seedlings was used to analyze their

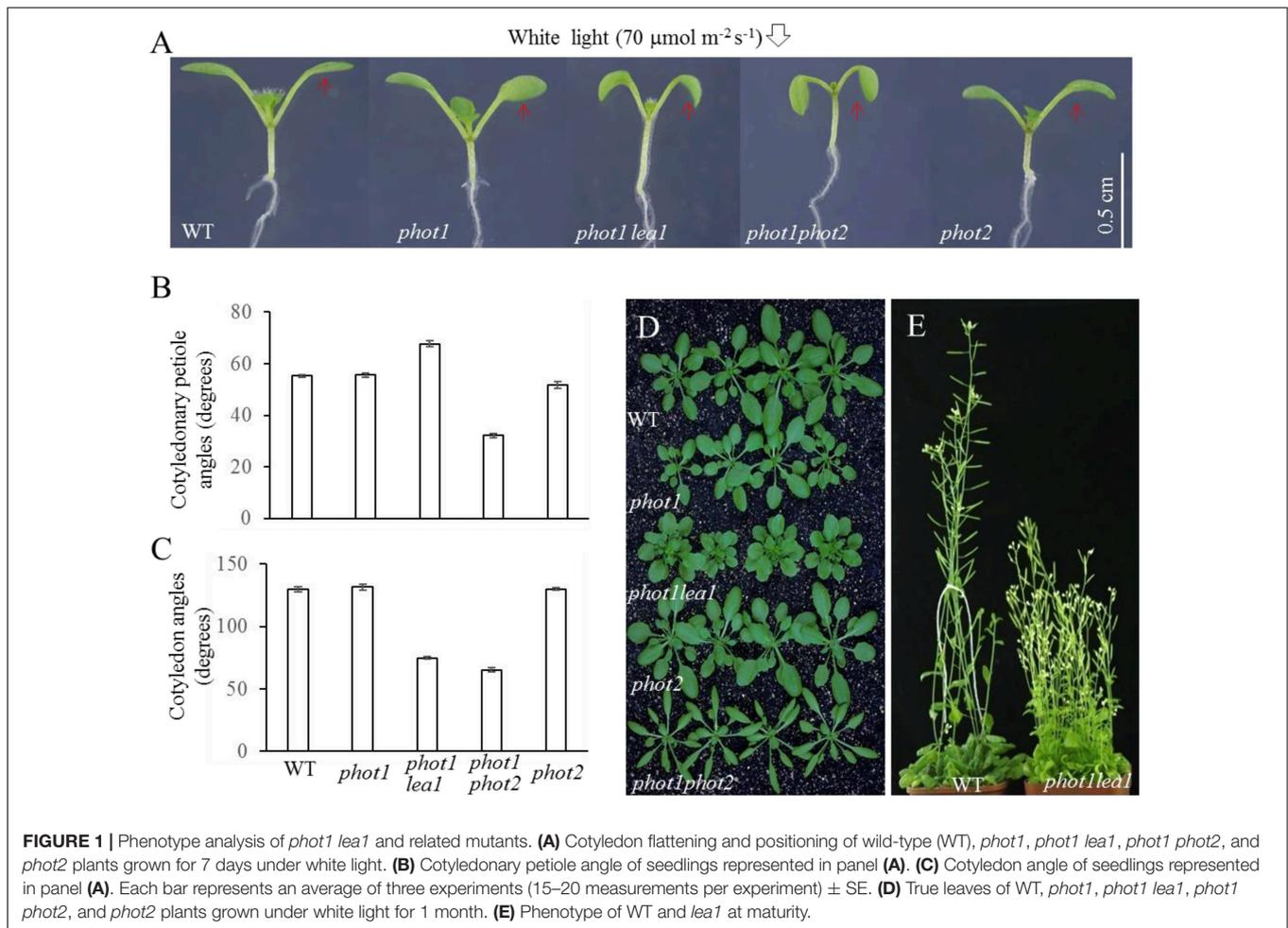


FIGURE 1 | Phenotype analysis of *phot1 lea1* and related mutants. **(A)** Cotyledon flattening and positioning of wild-type (WT), *phot1*, *phot1 lea1*, *phot1 phot2*, and *phot2* plants grown for 7 days under white light. **(B)** Cotyledonary petiole angle of seedlings represented in panel **(A)**. **(C)** Cotyledon angle of seedlings represented in panel **(A)**. Each bar represents an average of three experiments (15–20 measurements per experiment) \pm SE. **(D)** True leaves of WT, *phot1*, *phot1 lea1*, *phot1 phot2*, and *phot2* plants grown under white light for 1 month. **(E)** Phenotype of WT and *lea1* at maturity.

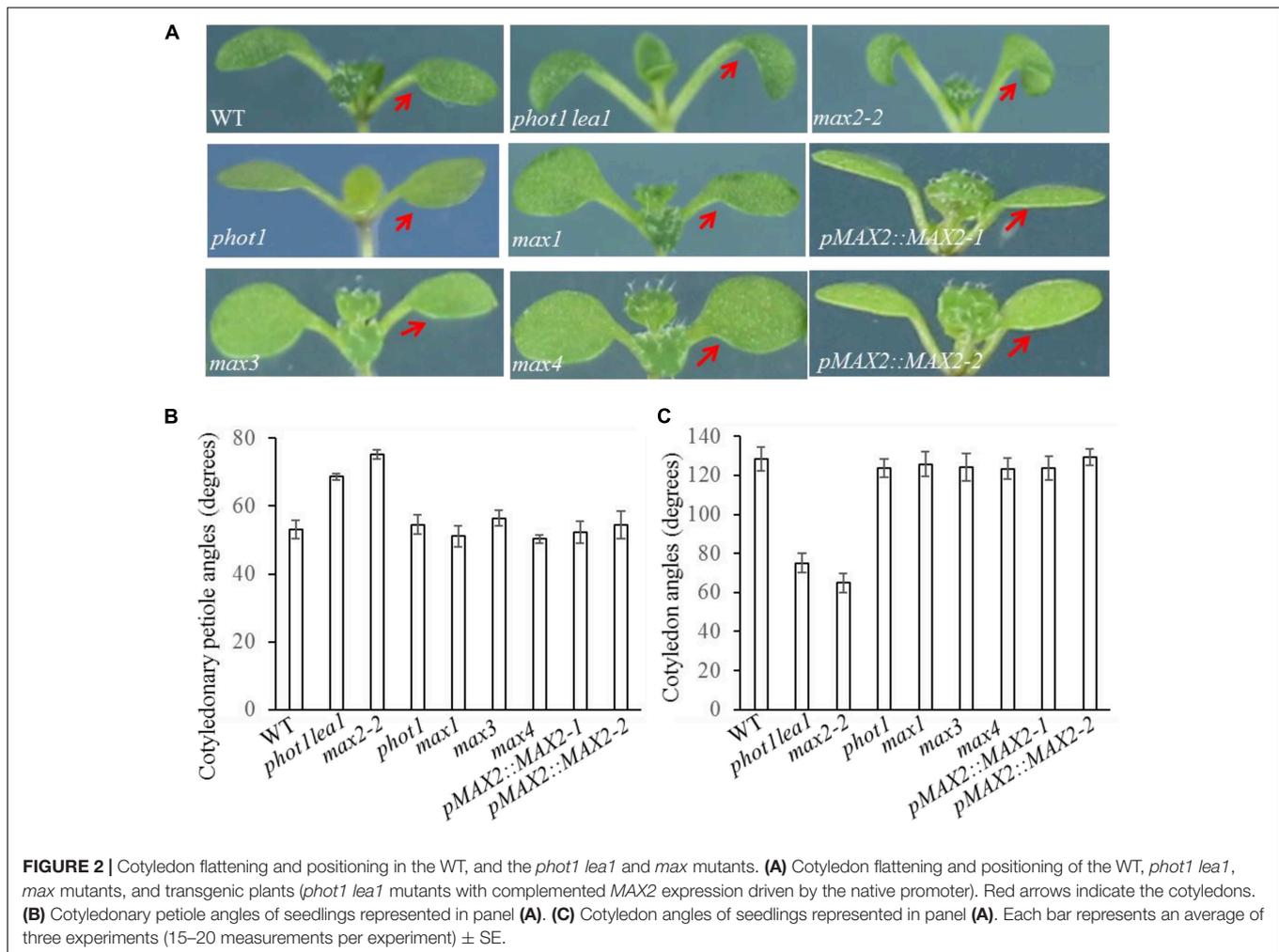
recombination frequency at nine different molecular markers. The statistical analysis results (**Supplementary Table 2**) showed that the recombination ratio was lowest at T16B24 (12.93%), indicating that the *LEA1* gene was located closest to this marker on chromosome 2. To further narrow the interval, fine mapping was performed using another seven markers located up- and downstream of the T16B24 marker. The recombination frequency was lowest at F14N22 (0.56%) (**Supplementary Table 3**). The full length of the F14N22 BAC marker sequence is approximately 96.69 kb, including a 19.39-kb overlap with F7D19 on one side and a 7.13-kb overlap with MHK10 on the other. There are 19 candidate genes located on the F14N22 BAC, including the overlap regions (**Supplementary Table 4**), and DNA sequencing of these genes indicated the presence of a C-to-T substitution mutation in *AT2G42620* (**Supplementary Figure 1A**) in the *phot1 lea1* mutant, which caused a premature stop codon (**Supplementary Figure 1B**).

AT2G42620 encodes MAX2, a member of the MAX family protein. The four MAX genes have previously been found to have redundant functions in axillary branching (Bennett et al., 2006), and strigolactone biosynthesis and signaling (Jia et al., 2014). To rule out the influence of functionally redundant MAX proteins, all MAX genes were amplified and sequenced in *lea1*. In addition

to MAX2, MAX3 (*AT2G44990*) also had a C-to-T substitution at position 1270 in *lea1* (**Supplementary Figure 1C**), which caused the premature termination of transcription (**Supplementary Figure 1D**). These results indicated that the defect of leaf positioning and flattening in *lea1* is likely mediated by both MAX2 and MAX3.

The *max2* Mutant Showed Defects in Hypocotyl Elongation, and Leaf Positioning and Flattening

As previously reported, all *max* mutants produced more rosette leaves (**Supplementary Figure 2B**), had higher levels of axillary branching (**Supplementary Figure 2A**), and formed shorter primary inflorescences (**Supplementary Figure 2A**) than the WT. The functional redundancy of the MAXs in shoot branching and the two base substitution mutations found in *phot1 lea1* suggested that cotyledon positioning and flattening may also be redundantly regulated by MAX2 and MAX3; however, only the *max2* mutants had impaired cotyledon positioning and flattening similar to *phot1 lea1* (**Figure 2**). The *max2-2* and *max2-3* allelic mutants had larger cotyledonary petiole angles and smaller cotyledon angles than the WT, and downward curled cotyledon



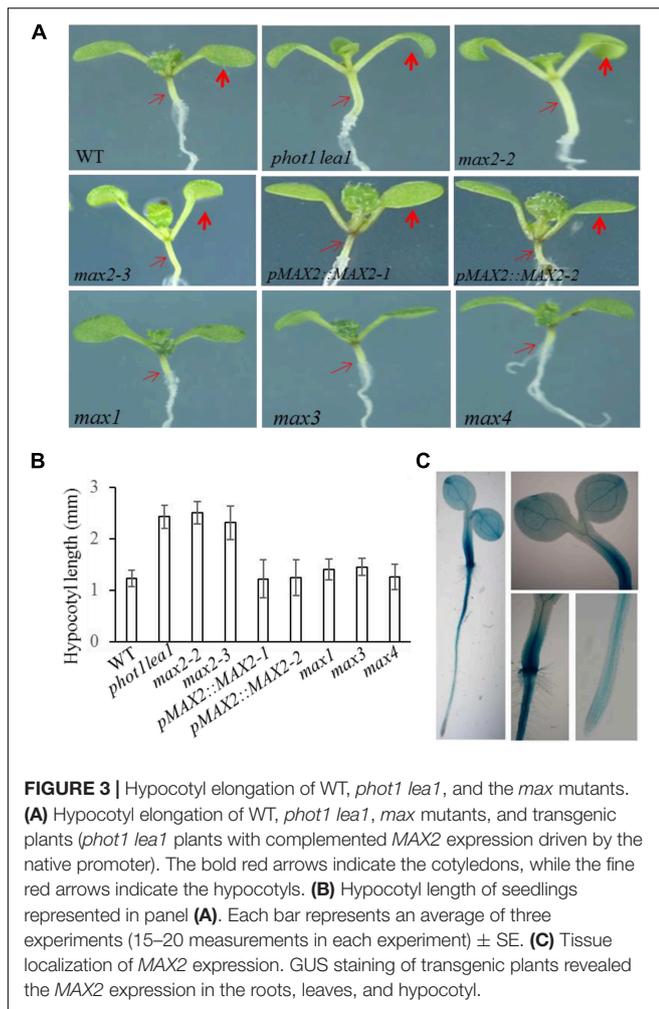
blades similar to *phot1 phot2* (Figures 2B,C). In addition, *phot1 lea1* and *max2* have other phenotypes not observed in the WT or the other *max* mutants, such as longer hypocotyls (Figures 3A,B) and flat siliques (Supplementary Figure 2C).

In order to further demonstrate that the phenotypes in *phot1 lea1* are caused by the disruption of *MAX2*, we complemented the *phot1 lea1* mutant using the *MAX2* gDNA sequence under the control of the native *MAX2* promoter (a 2.0-kb fragment upstream of the start of translation). Two independent transgenic lines (*pMAX2::MAX2-1* and *pMAX2::MAX2-2*) rescued the *phot1 lea1* leaf flattening and positioning phenotype and the hypocotyl elongation defect in T₃ homozygous plants (Figures 2, 3). The transgenic *pMAX2::MAX2-1* and *pMAX2::MAX2-2* plants in the *phot1 lea1* mutant background that the expression of *MAX2* gene has been restored (Supplementary Figure 3), had almost horizontal and flat cotyledon blades and shorter hypocotyls, as observed in the WT (Figures 2, 3). The other phenotypes, including the shape of the silique, the number of rosette leaves, and the axillary branching, were also restored (data not shown). These results indicated that *MAX2*, but not *MAX3*, regulates hypocotyl length, silique morphology, and leaf positioning and flattening, despite the mutation of both *MAX2* and *MAX3* in *lea1*.

The *MAX2* expression pattern was examined in transgenic plants expressing β -glucuronidase (*GUS*) under the control of the *MAX2* promoter. *GUS* staining revealed that *MAX2* was ubiquitously expressed in all vegetative tissues during the seedling stage (Figure 3C), as previously reported (Shen et al., 2007). The joint between the hypocotyl and the root had the strongest *GUS* activity, while the leaves and hypocotyls had higher levels of *MAX2* expression than the root (Figure 3C). These results support the developmental phenotype that *MAX2* is mainly involved in leaf flattening and positioning, hypocotyl elongation, and shoot branching regulation.

The *phot1 max2-2* and *phot1 max2-3* Double Mutants Exhibited Defects in Cotyledon Flattening

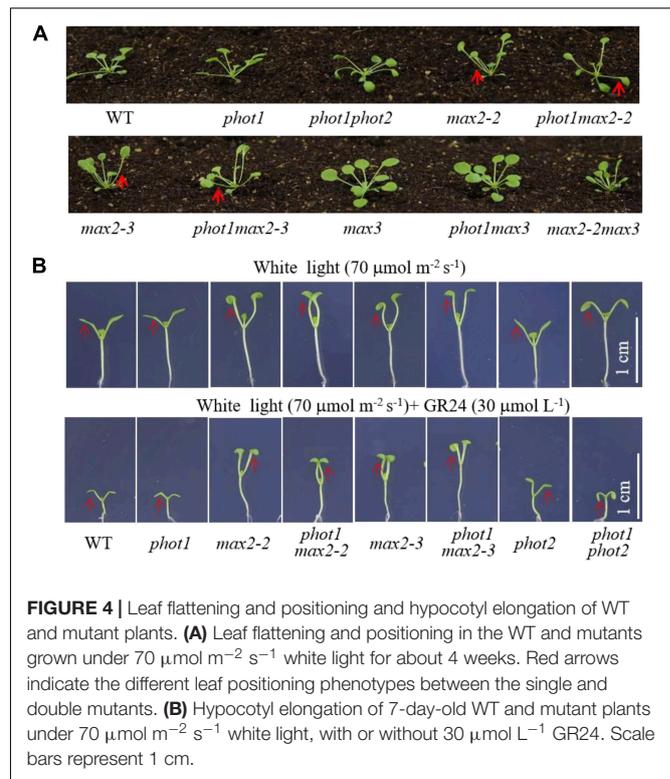
Under weak white light ($\leq 10 \mu\text{mol m}^{-2} \text{s}^{-1}$), *phot1* is known to control leaf flattening and positioning alone, while in increasing light intensities ($\geq 25 \mu\text{mol m}^{-2} \text{s}^{-1}$), both *phot1* and *phot2* regulate the responses (Harada et al., 2013). We have demonstrated that *MAX2* also plays an important role in leaf flattening and positioning; therefore, to investigate the



relationship between the two pathways, we characterized the *phot1 max2-2* and *phot1 max2-3* double mutants. With respect to leaf phenotype, *phot1 max2-2* and *phot1 max2-3* exhibited defects in leaf flattening and positioning similar to those of *max2-2* and *max2-3* (Figure 4A), and all four of these mutants had the same cotyledon flattening phenotype as *phot1 phot2* (Figure 4B). Mutating *PHOT1* in the *max3* mutant background had no effect on leaf flattening and positioning, whereas the *max2 max3* double mutant had leaf flattening and positioning defects similar to *max2-2* (Figure 4A). These results indicated that both phototropins and *MAX2* function in cotyledon flattening regulation, but their relationship need further study.

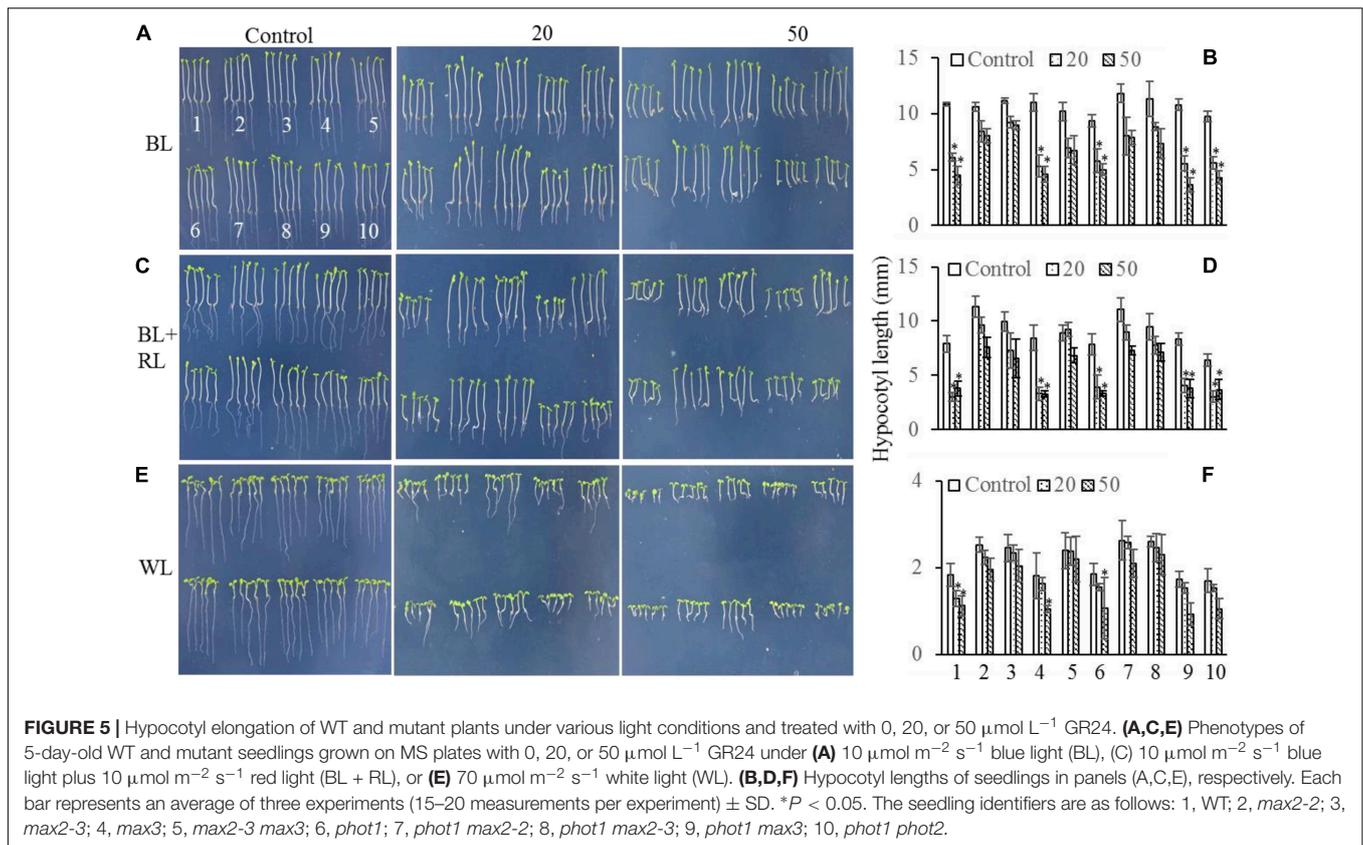
Both *phot1* and *phot2* Had Little Effect on *MAX2*-Regulated Hypocotyl Inhibition

The *phot1 max2-2* and *phot1 max2-3* double mutants produced long hypocotyls similar to those of the *max2-2* and *max2-3* single mutants, all of which were longer than those of the WT, *phot1*, *phot2*, and *phot1 phot2* plants under $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light (Figure 4B). When treated with $30 \mu\text{mol L}^{-1}$ GR24, hypocotyl elongation was reduced in all genotypes investigated, although



the reductions in *max2-2*, *max2-3*, *phot1 max2-2*, and *phot1 max2-3* were smaller than in WT, *phot1*, *phot2*, or *phot1 phot2* (Figure 4B). These results indicated that *MAX2* regulated the strigolactone-inhibited elongation of the hypocotyls, and that this was not affected by the disruption of *PHOT1*.

The mechanism controlling the *MAX2*-dependent inhibition of hypocotyl growth only functioned at low concentrations of GR24 (Jia et al., 2014), suggesting the presence of a *MAX2*-independent pathway regulating hypocotyl elongation at high concentrations of GR24. *MAX3* encodes a carotenoid cleavage dioxygenase (Sorefan et al., 2003; Booker et al., 2004) that promotes strigolactone biosynthesis. *Phot1* has also been reported to inhibit hypocotyl elongation (Folta et al., 2003); therefore, we used different concentrations of GR24 to analyze the inhibition of hypocotyl elongation in WT, *max2-2*, *max2-3*, *max3*, *max2-2 max3*, *phot1*, *phot1 max2-2*, *phot1 max2-3*, *phot1 max3*, and *phot1 phot2* plants. As previously reported, hypocotyl elongation was inhibited in all seedlings following a treatment with $50 \mu\text{mol L}^{-1}$ GR24 (Jia et al., 2014) in combination with light irradiation (Figure 5); however, when the concentration of GR24 was decreased to $20 \mu\text{mol L}^{-1}$, the inhibition of hypocotyl elongation became more complex. Under white light or a combined red and blue light, the hypocotyl elongation was reduced in all seedlings except for *max2-3 max3*, whose length showed almost no difference (Figures 5C–F). Overall, the decrease in hypocotyl elongation under blue light was greater than under the blue- and red-light combination. The *max2-2*, *max2-3*, *phot1 max2-2*, and *phot1 max2-3* plants had smaller decreases in hypocotyl length under both conditions



(Figures 5A–D). Under white light, the pattern of hypocotyl length in seedlings of the various genotypes was the same as under blue light, except that the extent of the decrease was reduced (Figures 5E,F). Taken together, these results show that MAX2 participates in strigolactone-mediated hypocotyl elongation, as previously reported (Jia et al., 2014), but that the phototropins and MAX3 have no effect on this response under the conditions observed here.

Neither MAX2 nor MAX3 Regulate Hypocotyl Phototropism

Beyond leaf flattening and positioning, *phot1* and *phot2* redundantly regulate high-intensity blue light (HBL)-induced hypocotyl phototropism (Sakai et al., 2001; Zhao et al., 2013), while MAX2 is known to participate in the regulation of hypocotyl elongation (Stirnberg et al., 2002). Hypocotyl elongation and phototropism are distinct physiological processes, but ultimately both depend on changes in cell elongation (Liscum et al., 1992); therefore, hypocotyl phototropism was analyzed in WT, *phot1*, *max2-2*, *phot1 max2-2*, *max2-3*, *phot1 max2-3*, *max3*, *phot1 max3*, *max2-2 max3*, and *phot1 phot2* plants.

Under high levels (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) of blue light, all genotypes displayed normal phototropism, except for *phot1 phot2* (Figures 6B,C), demonstrating that MAX2 has no effect on HBL-induced phototropism. Under low levels (0.01 $\mu\text{mol m}^{-2} \text{s}^{-1}$) of blue light, the phototropic responses of *max2-2*, *max2-3*, *max3*, and *max2-2 max3* resembled that

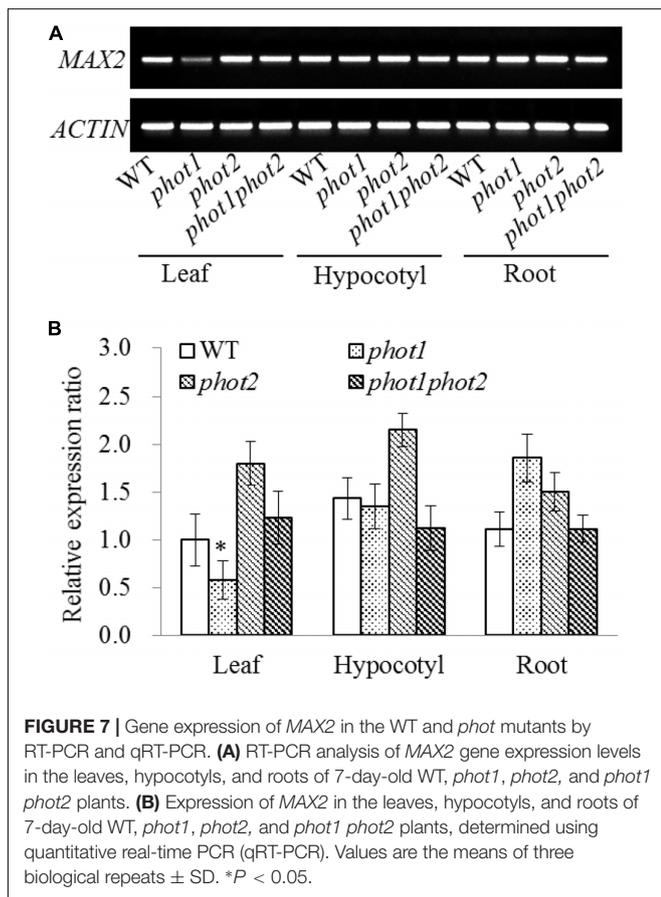
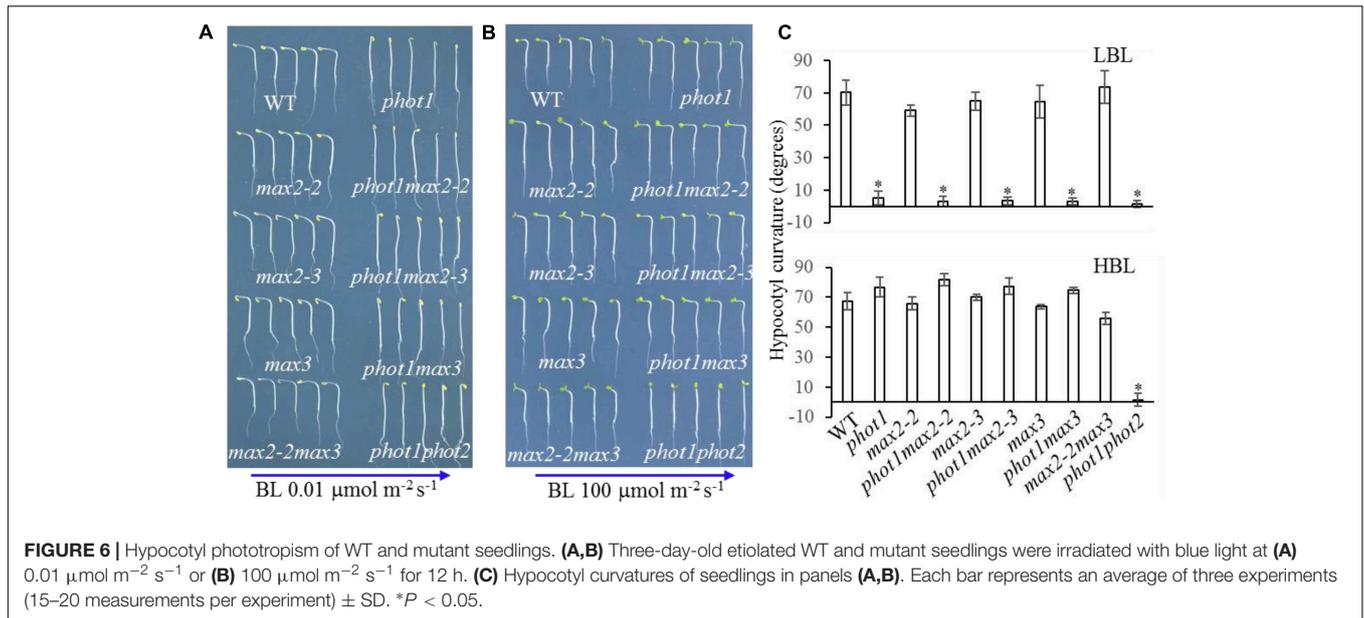
of the WT (Figures 6A,C). When *PHOT1* was disrupted in the *max2-2* and *max2-3* mutant backgrounds, the resulting double mutants showed impaired phototropism, similar to *phot1* (Figures 6A,C). These results were consistent with the previous report demonstrating that *phot1* regulates low-blue-light-induced phototropism (Briggs et al., 2001), and show that MAX2 is not required for *phot1*-dependent phototropism.

Seven-day-old WT, *phot1*, *phot2*, and *phot1 phot2* seedlings were dissected into their leaf, hypocotyl, and root tissues to investigate the expression of MAX2. The results of the RT-PCR and RT-qPCR indicated that MAX2 was almost equally expressed in all three tissues in these plants, and the mutation of *PHOT1* gene inhibited the expression of MAX2 in leaves (Figure 7).

DISCUSSION

MAX2 Is Necessary for Leaf Flattening and Positioning

Genetic studies have suggested that the phototropins (*phot1* and *phot2*) function redundantly to control leaf flattening and positioning under strong light in *Arabidopsis thaliana* (Harada et al., 2013). *Phot1* is believed to inhibit the *phot2* signaling pathway by influencing the downstream components of *phot2* (Harada et al., 2013); however, the complex functions of *phot1* and *phot2* in leaf flattening and positioning have restricted our understanding of the contribution made by *phot2*.



from *phot1*. This approach enabled the identification of the *lea1* mutant, which showed defects in leaf flattening and positioning, and had hook, epinastic cotyledon blades similar to the *phot1 phot2* double mutant (Figure 1). Subsequently, we cloned the mutated gene in *phot1 lea1* and demonstrated that *LEA1* was allelic to *MAX2*. Consistently, two null mutants of *MAX2* (*max2-2* and *max2-3*) showed the same curled cotyledon blade phenotype as *phot1 lea1* and *phot1 phot2*.

MAX2 is one of four known members of the MAX family. *MAX3* and *MAX4* encode divergent carotenoid cleavage dioxygenases (Sorefan et al., 2003; Booker et al., 2004) which promote strigolactone biosynthesis from a carotenoid-derived substance (Jia et al., 2014). *MAX1* encodes a cytochrome P450 family member that acts downstream of *MAX3* and *MAX4* (Bennett et al., 2006), while *MAX2* contains an F-box motif, a repeat sequence segment of 18 leucine residues, and a zinc-finger domain (Woo et al., 2001). The F-box protein encoded by *MAX2* is a part of the SCF complex that directs the E3-mediated protein hydrolysis process (Stirnberg et al., 2007). All *MAX* genes redundantly regulate axillary branching (Bennett et al., 2006), and strigolactone biosynthesis and signaling (Jia et al., 2014). Consistently, here we found that all *max* mutants have increased numbers of rosette leaves and higher levels of axillary branching (Supplementary Figure 2) but only *max2* seedlings have elongated hypocotyl, elevated cotyledonary petioles and downward-curved cotyledons (Figure 2). These results indicated that *MAX2* was necessary for cotyledon flattening but this signaling was not conventional *MAX3* and *MAX4*-dependent metabolism of strigolactone. Karrikins are other butenolides plant growth regulators, which is structurally similar but physiologically distinct from strigolactones (Waters et al., 2012). Karrikin and strigolactone signaling can be distinguished by *DWARF14* family members *KAI2* and *AtD14*, respectively. Although their physiological function are different, strigolactones and karrikins pathways both converge upon

Here, we attempted to identify new signaling components acting downstream of *phot2* by screening an EMS-mutagenized population in the *phot1* mutant background to avoid interference

MAX2 (Flematti et al., 2013). Activated AtD14 by strigolactones interacts with the F-box protein D3/MAX2 (D3 is the rice ortholog of *Arabidopsis* MAX2) in the SCF^{D3/MAX2}-E2 complex to regulate repression of shoot branching, promotion of senescence and secondary thickening, alteration of root growth, and enhancement of stress tolerance (Li and Tran, 2015). Similar to the action of AtD14, activated KAI2 by karrikins interacts with the F-box protein MAX2 in the SCF^{MAX2}-E2 complex, but plays a role in inhibition of hypocotyl elongation, promotion of seed germination, alteration of leaf and root hair growth, and potentially enhancement of stress tolerance (Li and Tran, 2015). As previously reported, the *Atd4-1* mutant showed increased shoot branching common to the *max2* mutant (Waters et al., 2012), while *kai2* seedlings resemble *max2* seedlings in having enlarged epinastic cotyledons (Flematti et al., 2013). These results further suggest that MAX2- and KAI2-mediated cotyledon flattening is strigolactone-independent, but the molecular mechanism still require further research.

MAX2 Might Functions Downstream of the Phototropins to Mediate Cotyledon Flattening

Given that the mutants *max2-2* and *max2-3* had similar cotyledon phenotypes to the *phot1 phot2* double mutant, we hypothesized that MAX2 may function in phototropin-dependent cotyledon flattening. In support of this, we found that the double mutants *phot1 max2-2* and *phot1 max2-3* exhibited identical epinastic cotyledon flattening to the double mutant *phot1 phot2*. The identical cotyledon flattening in the *max2* single mutants and the *phot1 max2* double mutants, as well as in *phot1 phot2*, suggests that MAX2 may function downstream of *phot1* or both phototropins to mediate phototropin-dependent cotyledon flattening.

Phot1 and *phot2* are known to redundantly regulate HBL-induced hypocotyl phototropism (Zhao et al., 2013); however, whether MAX2 regulates phototropin-mediated hypocotyl phototropism is yet to be revealed. Here, we found that the mutants *max2-2* and *max2-3* exhibit normal phototropism in response to both high and low levels of blue light, while the double mutants *phot1 max2-2* and *phot1 max2-3* showed impaired phototropism under low blue light, resembling the phenotype of *phot1*. These results indicate that MAX2 activity does not alleviate the disruption of *phot1*, and from another side proved that MAX2 might functions downstream of the phototropins to mediate cotyledon flattening. Further studies of cotyledon phenotypes of *phot2 max2* and *phot1 phot2 max2* will provide persuasive evidence.

Auxin Distribution May Be Involved in MAX2-Mediated Cotyledon Flattening

The mechanism behind the epinastic cotyledons of the *max2* and *phot1 phot2* mutants was further investigated. The expression of MAX2 was evaluated to determine its possible function in cotyledon flattening; however, the mutation of *PHOT1*, *PHOT2*, or both phototropins did not seem to affect the expression of MAX2. These results indicate

that both MAX2 and the phototropins participate in the regulation of cotyledon flattening, but the mechanism is still unknown.

The distribution of auxin plays an important role in leaf morphogenesis (Lumba and McCourt, 2005), and phototropin-mediated auxin redistribution may be responsible for leaf flattening and positioning (Hobbie and Estelle, 1995; Keller and Van Volkenburgh, 1997; Watahiki and Yamamoto, 1997). Activated *phot1* directly phosphorylates ABCB19 (ATP-BINDING CASSETTE B19) to inhibit its auxin efflux activity (Christie et al., 2011; Hohm et al., 2014), and causes the relocalization of the auxin efflux carriers, PIN1 and PIN3 (Blakeslee et al., 2004; Ding et al., 2011). Furthermore, the PKSs, signaling components in the phototropin pathways, were reported to control auxin distribution in the leaves (de Carbonnel et al., 2010; Fankhauser and Christie, 2015). MAX2 was previously reported to regulate auxin transport (Xie et al., 1998; Yu et al., 2009), and a MAX-dependent strigolactone was previously reported to regulate auxin transport (de Jong et al., 2014). This process is dependent on PIN1 activity but independent of AXR1 (AUXIN RESISTANT1)-mediated auxin signaling (Bennett et al., 2006). The further study of auxin transport will help us to better elucidate the mechanisms of MAX2 and phototropin-dependent cotyledon flattening.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The WT *Arabidopsis thaliana* ecotype used was Col-0, except for the *Landsberg erecta* used for the map-based cloning. The *phot1* (*phot1-5*), *phot2* (*phot2-1*), and *phot1 phot2* (*phot1-5 phot2-1*) lines were gifted by Ken-ichiro Shimazaki, while the *max1* (CS9564), *max2-2* (SALK_028336C), *max2-3* (SALK_092836C), *max3-12* (SALK_015785C), and *max4* (SALK_082552C), lines were purchased from The European Arabidopsis Stock Centre (NASC). The *phot1* seeds were mutagenized with 0.3% (w/v) EMS, as previously described (Zhao et al., 2017).

Isolation and Identification of *phot1 lea1* Mutants

The M₂ seeds were sown on MS medium supplemented with 0.6% (w/v) agar and 3% (w/v) sucrose. After vernalization for 3 days in the dark at 4°C, the seeds were germinated in a phytotron growth cabinet (100 μmol m⁻² s⁻¹ continuous white light, 22°C). After 7 days, the cotyledonary petiole angle and cotyledon angle were measured. Mutants were selected for defects in leaf flattening, and were grown in soil. The seeds of these plants were harvested for genetic and physiological identification.

The mutants were analyzed as described previously (Zhao et al., 2013). Steadily heritable mutants were used as the female parent in a backcross with Col-0 or a cross with *Landsberg erecta* to obtain F₁ plants, which were self-fertilized to obtain the F₂ generation. The phenotypic segregation of the F₂ plants was used to confirm the hereditary character of the mutation.

Plasmid Construction and Plant Transformation

A genomic DNA fragment containing the entire *MAX2* coding region and the approximately 2.0-kb upstream sequence was amplified using PCR and cloned into a modified pCAMBIA-1300 vector (the 35S CaMV promoter was removed). The plasmids with the correct *MAX2* DNA sequence were introduced into the *phot1 lea1* mutant an *Agrobacterium*-mediated transformation using the floral dip method. The transgenic plants were selected on a medium containing hygromycin ($25 \mu\text{L mL}^{-1}$), and the successfully transformed plants were used for further phenotypic analysis.

To generate the *MAX2-GUS* construct, the promoter sequence of *MAX2* (2.0-kb upstream sequence of *MAX2*) was amplified using PCR and cloned into the vector pCAMBIA-1391 to construct the recombinant *MAX2-GUS* plasmid. The plasmids with the correct *MAX2* promoter sequence were introduced into Col-0 in an *Agrobacterium*-mediated transformation using the floral dip method. The positive transformants were selected on a medium containing hygromycin.

Measurement of the Cotyledon Angle and the Cotyledonary Petiole Angle

The cotyledon angle and the cotyledonary petiole angle were measured as described previously (Harada et al., 2013). After vernalization for 3 days in the dark at 4°C , seeds were sown on 1.0% MS medium and placed vertically in the growth room with a white fluorescent light irradiated from above. After growth under a 16/8 h light/dark cycle at $22\text{--}24^{\circ}\text{C}$ for about 10 days, images of the seedlings were taken with a Cannon camera. The cotyledon angles, the angle between the petiole and cotyledonary blade, and the cotyledonary petiole angle, the angle between the cotyledonary petiole and the horizontal plane were measured using an e-ruler.

Histochemical Detection of GUS Activity

The transgenic plants containing the recombinant *MAX2-GUS* plasmid were analyzed after an incubation in X-gluc buffer (50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferrocyanide, and 2 mg mL^{-1} 5-bromo-4-chloro-3-indolyl β -D-glucuronide [X-gluc]) at 37°C for 12 h.

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Measurement of Hypocotyl Curvature and Length

The hypocotyl curvatures were measured as described previously (Zhao et al., 2018). Briefly, etiolated seedlings with hypocotyls 5–8 mm in length were transferred to 0.8% MS medium and placed vertically in a darkroom. After a 12-h irradiation with unilateral blue light ($100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ or $0.01 \mu\text{mol m}^{-2} \text{ s}^{-1}$), the seedlings were photographed using a Canon camera, and their hypocotyl curvatures were measured using an e-ruler. The length of the seedlings was also measured.

AUTHOR CONTRIBUTIONS

XoZ and XnZ designed the research, analyzed the data, and contributed reagents and materials. Q-PZ, X-NW, Z-YZ, S-CM, and N-NL performed the experiments. XoZ, XnZ, and Q-PZ wrote the article. All authors have read and approved this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01507/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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