

# Light Induces Peroxisome Proliferation in Arabidopsis Seedlings through the Photoreceptor Phytochrome A, the Transcription Factor HY5 HOMOLOG, and the Peroxisomal Protein PEROXIN11b<sup>1[C][W][OA]</sup>

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Peroxisomes are single membrane-delimited subcellular organelles that carry out numerous vital metabolic reactions in nearly all eukaryotes. Peroxisomes alter their morphology, abundance, and enzymatic constituents in response to environmental cues, yet little is known about the underlying mechanisms. In this work, we investigated the regulatory role of light in peroxisome proliferation in Arabidopsis (*Arabidopsis thaliana*). We provide evidence that light induces proliferation of peroxisomes in Arabidopsis seedlings and that the peroxisomal protein PEX11b plays an important role in mediating this process. The far-red light receptor phytochrome A (phyA) and the bZIP transcription factor HY5 HOMOLOG (HYH) are both required for the up-regulation of *PEX11b* in the light. We further demonstrate that the *phyA* and *hyh* mutants exhibit reduced peroxisome abundance, a phenotype that can be rescued by overexpressing *PEX11b* in these plants. The HYH protein is able to bind to the promoter of *PEX11b*, suggesting that the *PEX11b* gene is a direct target of HYH. We conclude that HYH and PEX11b constitute a novel branch of the phyA-mediated light signaling cascade, which promotes peroxisome proliferation during seedling photomorphogenesis.

Peroxisomes are ubiquitous eukaryotic organelles enclosed by a single membrane and devoid of DNA. They perform a variety of metabolic functions that differ depending on the species, cell type, developmental stage, and prevailing environmental conditions (Purdue and Lazarow, 2001). Plant peroxisomes can be categorized into several variants that are structurally similar but metabolically distinct, such as glyoxysomes in seeds and germinating seedlings, leaf peroxisomes, glyoxysome-related gerontosomes in senescent tissue, nodule-specific peroxisomes, and unspecialized peroxisomes. Functions attributed to plant peroxisomes include metabolism of lipids, reactive oxygen species, and nitrogen, photorespiration, plant hormone biosynthesis and metabolism, and plant pathogen interaction (Beevers, 1979; Olsen and Harada, 1995; Zolman et al., 2000; Hayashi and Nishimura, 2003; Lipka et al., 2005; McCartney et al., 2005; Nyathi and Baker, 2006; Reumann and Weber, 2006).

Although direct evidence has not been obtained from plant systems, cell biological and phylogenetic evidence suggested that peroxisomes can be formed de novo in the endoplasmic reticulum (ER) and have an ER origin in evolutionary history (Hoepfner et al., 2005; Gabaldon et al., 2006; Schluter et al., 2006; Titorenko and Mullen, 2006). Peroxisome biogenesis involves the formation of the peroxisome membrane, import of cytoplasmically synthesized proteins into the peroxisomal matrix, and multiplication and maintenance of peroxisomes, processes mediated by a group of peroxisome proteins mostly known as PEROXINS or PEX proteins (Heiland and Erdmann, 2005). Earlier studies in yeast (*Saccharomyces cerevisiae*) identified 32 PEX proteins, which have approximately 20 and 23 homologs in mammals and plants, respectively, and are referred to as PexNp in yeasts and PEXN in mammals and plants (Purdue and Lazarow, 2001; Charlton and Lopez-Huertas, 2002).

In addition to de novo formation from the ER, peroxisomes can multiply by division, constitutively or under induced conditions (the latter is also called proliferation). Yeast peroxisomes are believed to divide through a specific order of partially overlapping events, including organelle elongation, membrane constriction, and fission (Fagarasanu et al., 2007). The division process involves eight PEX proteins, which, based on sequence similarity, can be categorized into several subgroups: Pex11p, Pex25p/Pex27p, Pex28p/Pex29p, and Pex30p/Pex31p/Pex32p. The biochemical functions of these PEX proteins, all of which are associated with the peroxisomal membrane, have yet to be clearly defined (Thoms and Erdmann, 2005). The Arabidopsis

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(*Arabidopsis thaliana*) genome does not contain obvious sequence homologs to most of the aforementioned yeast PEX proteins known to specifically control peroxisome multiplication. One exception is Pex11p, which is primarily involved in peroxisome elongation/tubulation, the early stages of peroxisome division (Erdmann and Blobel, 1995; Marshall et al., 1995; Thoms and Erdmann, 2005; Fagarasanu et al., 2007). The five *Arabidopsis* PEX11 isoforms, PEX11a to PEX11e, were shown to promote peroxisome elongation and population increase with some level of specificity and redundancy (Lingard and Trelease, 2006; Orth et al., 2007). In addition to PEX proteins, the large GTPase dynamin-related protein (DRP) and FISSON1 (FIS1) proteins in yeast and mammals function in the late stages of peroxisome division, whereby the integral membrane protein FIS1 recruits the mechanochemical enzyme DRP to execute the tubulation and separation of peroxisomal membranes (Koch et al., 2003, 2005; Kobayashi et al., 2007). Interestingly, at least in yeast and mammals, the division machinery of peroxisomes and mitochondria seems to share the same DRP and FIS1 orthologs (Koch et al., 2005; Thoms and Erdmann, 2005; Schrader and Yoon, 2007). The *Arabidopsis* DRP3A protein was shown to be required for the division of both peroxisomes and mitochondria (Mano et al., 2004). Additional AtDRP proteins were also suggested to be involved in peroxisome division (Hu, 2007).

Peroxisome abundance is strongly affected by external signals. Yeast peroxisomes proliferate in response to nutritional stimuli, such as oleate and methanol and nitrogen sources, to metabolize these substrates for carbon or nitrogen utilization (Gurvitz and Rottensteiner, 2006). Likewise in mammals, when exposed to peroxisome proliferators—a pool of more than 70 chemicals ranging from hypolipidemic agents, phthalate ester plasticizers, solvents, herbicides, and dietary factors to hormones—mammalian peroxisome activity and abundance also increase (Citron, 1995; Desvergne and Wahli, 1999). Signal transduction of some of these external stimuli is mediated by transcription factors, such as the yeast Adr1p, Oaf1p, and Pip2p proteins, and the mammalian peroxisome proliferator-activated receptor- $\alpha$  and the retinoid X receptor proteins (Desvergne and Wahli, 1999; Gurvitz and Rottensteiner, 2006), all of which lack apparent orthologs in plants. Through electron microscopic analysis, ozone, herbicide, clofibrate, and high light were found to increase peroxisome abundance in plant cells with undetermined mechanisms (de Felipe et al., 1988; Ferreira et al., 1989; Palma et al., 1991; Oksanen et al., 2003).

We are particularly interested in the role of light, one of the major environmental cues, in peroxisome proliferation. Light affects many aspects of plant development, from seed germination, seedling photomorphogenesis, shade avoidance, photoperiodism, circadian clock entrainment, phototropism, chloroplast movement, stomata opening, to flowering (Jiao et al., 2007). When exposed to light, *Arabidopsis* seedlings undergo a process called photomorphogen-

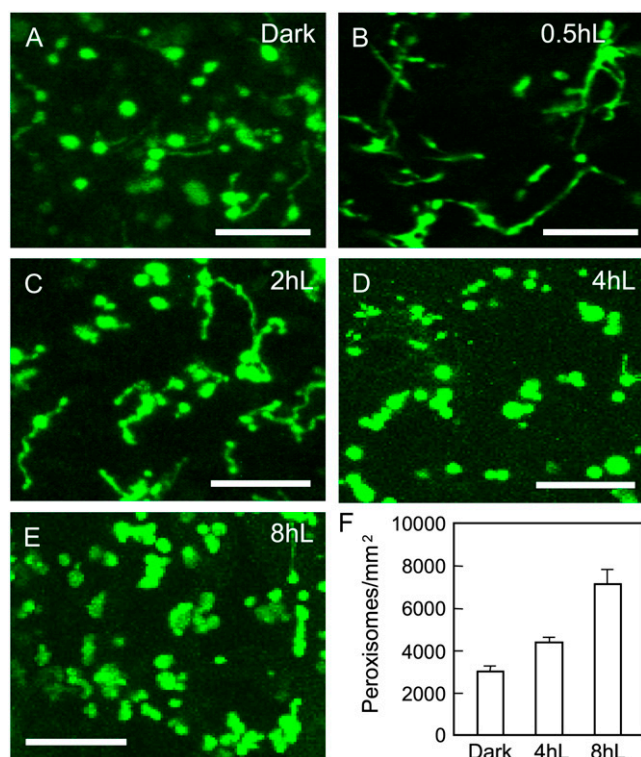
esis, in which cotyledons open, hypocotyl growth is inhibited, chloroplasts develop, and genes involved in photosynthesis and related functions are activated (Wang and Deng, 2002a). During this process, glyoxysomes are converted into leaf peroxisomes by replacing enzymes specific for lipid metabolism during germination with proteins involved in the glycolate recycling during photorespiration (Olsen and Harada, 1995). Previously, we identified a gain-of-function mutation in a peroxisomal gene (*PEX2*) in *Arabidopsis* that partially suppressed the photomorphogenic (de-etiolated) phenotype of the mutant defective in the nuclear protein DET1 (Hu et al., 2002). However, the mechanism for this suppression appears to be complex and still remains to be determined.

Here, we investigated the impact of light on peroxisome proliferation in living plant cells and identified the underlying signal transduction pathway mediating this process. We present evidence that light promotes peroxisome proliferation, at least in part, by activating the expression of the peroxisomal gene *PEX11b* through the photoreceptor phytochrome A (phyA) and the bZIP transcription factor HYH.

## RESULTS

### Light Induces Peroxisome Proliferation

To address the question of whether light plays a role in peroxisome proliferation, we analyzed transgenic *Arabidopsis* seedlings constitutively expressing a fusion of the yellow fluorescent protein (YFP) with C-terminal PEROXISOME-TARGETING SIGNAL TYPE1 (PTS1; consisting of Ser-Lys-Leu). These YFP-PTS1 plants displayed fluorescently labeled peroxisomes as numerous punctate structures in virtually all cell types (Fan et al., 2005; Orth et al., 2007). Three-day-old YFP-PTS1 seedlings grown in the dark were illuminated with  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$  continuous white light for different time intervals and scored for obvious alterations in size, shape, and abundance of peroxisomes in cotyledon cells with a confocal laser-scanning microscope. Dark-grown seedlings exhibited a morphologically heterogeneous population of peroxisomes: Most were spherical and a few were elongated (Fig. 1A). After 0.5 h of illumination, a strong increase in the population of elongated peroxisomes was evident (Fig. 1B). By 2 h of light treatment, membrane constriction and organelle fission seemed to have occurred in many elongated peroxisomes, which led to segmentation of the organelles (Fig. 1C). At 4 and 8 h, the number of elongated/segmented peroxisomes decreased, whereas the number of spherical peroxisomes, some of which were still clustered together, was markedly elevated (Fig. 1, D and E). The abundance of peroxisomes did not change significantly beyond 8 h of illumination (data not shown). During the course of this experiment, peroxisomes should be going through the transition from glyoxysomes to leaf-type peroxisomes. For



**Figure 1.** Light induces peroxisome proliferation in Arabidopsis seedlings. A to E, Confocal microscopic images of cotyledon cells from 3-d dark-grown seedlings expressing the *YFP-PTS1* peroxisomal marker. Seedlings were exposed to continuous white light at  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$  for the indicated lengths of time. Note the elongated and segmented peroxisomes appearing at 0.5 and 2 h, respectively, after light exposure. Bars =  $10 \mu\text{m}$ . F, Peroxisome quantification. Error bars indicate SD,  $n = 4$ ,  $P < 0.0002$ . [See online article for color version of this figure.]

simplicity, we use the general term peroxisomes to refer to all the organelles in this article.

The number of peroxisomes was quantified using ImageJ software, which has been used in many studies to quantitatively analyze the number and morphology of cells and subcellular structures (Soyombo et al., 2006; Shi et al., 2007). Because counting peroxisomes at 0.5 and 2 h was not feasible due to the significant degree of elongation and reticulation of peroxisomes, we only compared the number of peroxisomes at the following time points: dark, 4-, and 8-h light exposure. A significant increase in peroxisome abundance was observed after 8-h light treatment: from  $2,963.9 \pm 277/\text{mm}^2$  in etiolated seedlings to  $4,349 \pm 182.8/\text{mm}^2$  at 4 h and  $7,063.7 \pm 736.8/\text{mm}^2$  at 8 h of illumination (Fig. 1F; see "Materials and Methods" for details of counting). These data demonstrate a role of light in up-regulating the change in peroxisome abundance via a multistep process: organelle elongation, constriction, and fission.

#### PEX11b Plays a Critical Role in Light-Induced Peroxisome Proliferation

As a first step in understanding light induction of peroxisome proliferation at the mechanistic level, we

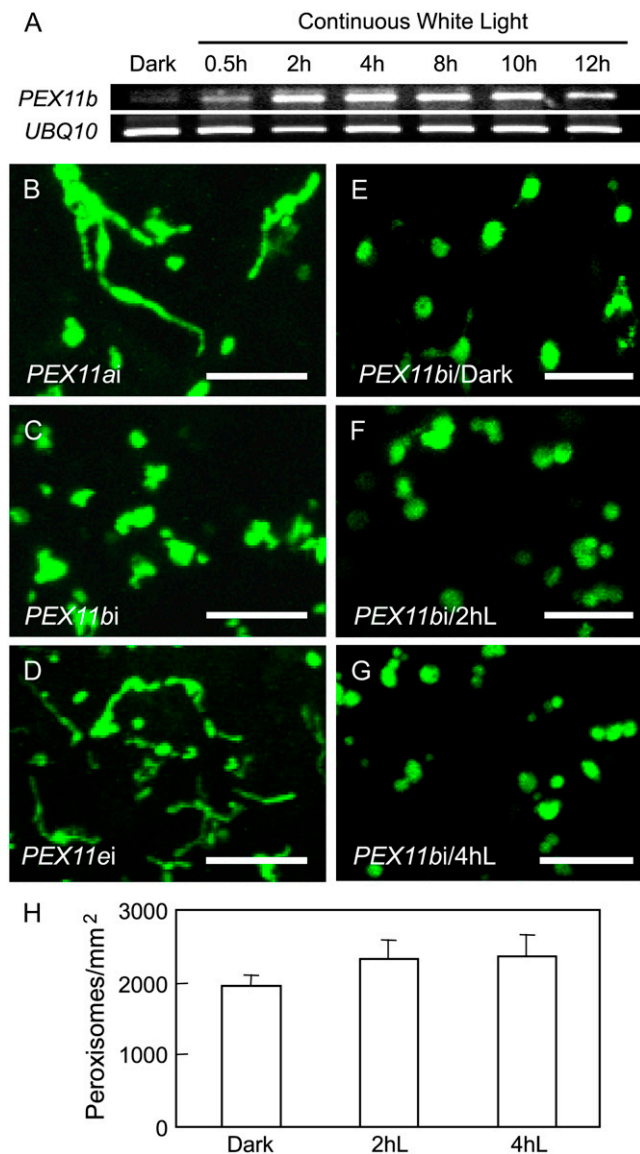
focused our attention on the PEX11 proteins, the only Arabidopsis proteins shown to date to be specifically involved in peroxisome proliferation during dark-to-light transition. Overexpression of each of the five *PEX11* isoforms led to peroxisome elongation and population increase to various degrees (Lingard and Trelease, 2006; Orth et al., 2007), whereas silencing of individual *PEX11* genes reduced peroxisomal abundance (Orth et al., 2007). *PEX11b* was the prime candidate for a mediator in this process given that it was the only Arabidopsis *PEX11* gene strongly up-regulated by light (Orth et al., 2007).

We examined the kinetics of light induction of *PEX11b* by reverse transcription (RT)-PCR with total RNA isolated from 3-d dark-grown seedlings exposed to white light for different time periods. The expression level of *PEX11b* was very low in dark-grown seedlings, but increased steadily after exposure to light, reached its maximum at 4 h, and started to drop by 12 h (Fig. 2A). The kinetics of *PEX11b* expression in light largely correlated with those of the light-dependent alterations in peroxisome morphology and abundance observed in the YFP-PTS1 plants (Fig. 1), implicating a specific role for PEX11b in light-mediated peroxisome elongation and population increase.

This correlation prompted us to examine loss-of-function *PEX11b* mutants for defects in peroxisome proliferation. To this end, we analyzed YFP-PTS1-expressing RNA interference (RNAi) plants generated in our previous study, each having a strongly reduced transcript level of *PEX11a*, *PEX11b*, or *PEX11e* (lines 1, 4, and 7 in Orth et al., 2007). These lines showed a decrease in overall peroxisome abundance, but exhibited no obvious deficiency in plant growth, lipid mobilization in germinating seedlings (evaluated by a sugar dependence assay), or photorespiration, most likely due to functional redundancy of the *PEX11* genes (Orth et al., 2007). After 2-h light treatment, significantly elongated peroxisomes were present in the *PEX11a* and *PEX11e* RNAi lines, whereas peroxisome morphology hardly changed in the *PEX11b* RNAi plant (Fig. 2, B–D). The phenotype of abnormally shaped and aggregated peroxisomes is typical for the *PEX11b* RNAi plant (Orth et al., 2007), which may reflect partial peroxisome division deficiency in this mutant. Moreover, the number of peroxisomes/ $\text{mm}^2$  in the *PEX11b* RNAi plant increased only approximately 20% from dark ( $1,966.7 \pm 141.2$ ) to 2-h ( $2,326.8 \pm 249.3$ ) or 4-h ( $2,354.5 \pm 315.7$ ) light treatment (Fig. 2, E–H), in contrast to the approximately 47% increase in the wild type (dark to 4 h light; Fig. 1F). Taken together, our data have revealed *PEX11b* as an important peroxisomal mediator in light-induced peroxisome proliferation.

#### phyA and HYH Are Required for PEX11b-Mediated Regulation of Peroxisome Proliferation

For optimal growth and development, higher plants are able to monitor the wavelength, quantity, direction,



**Figure 2.** *PEX11b* is required for light-induced peroxisome proliferation. A, RT-PCR analysis of the expression kinetics of *PEX11b* along with *UBQ10* in the light. B to G, Confocal micrographs of cotyledon cells from 3-d dark-grown seedlings expressing *YFP-PTS1*. Bars = 10  $\mu\text{m}$ . Seedlings analyzed in A to G were exposed to continuous white light at  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$  for 2 h (B–D) or as indicated (A and E–G). H, Quantification of peroxisomes in the cotyledons of the *PEX11b* RNAi plant. Error bars indicate SD,  $n = 4$ ,  $P < 0.05$ . [See online article for color version of this figure.]

and duration of light via several families of photoreceptors, transduce the signals through complex protein networks, including transcription cascades, and mount a wide array of responses (Chen et al., 2004; Jiao et al., 2007). To determine which wavelengths of the light spectrum specifically affect the expression of *PEX11b*, we searched GENEVESTIGATOR (<http://www.genevestigator.ethz.ch>), a reference expression database and analysis tool (Zimmermann et al., 2004), and obtained quantitative data of *PEX11b* expression

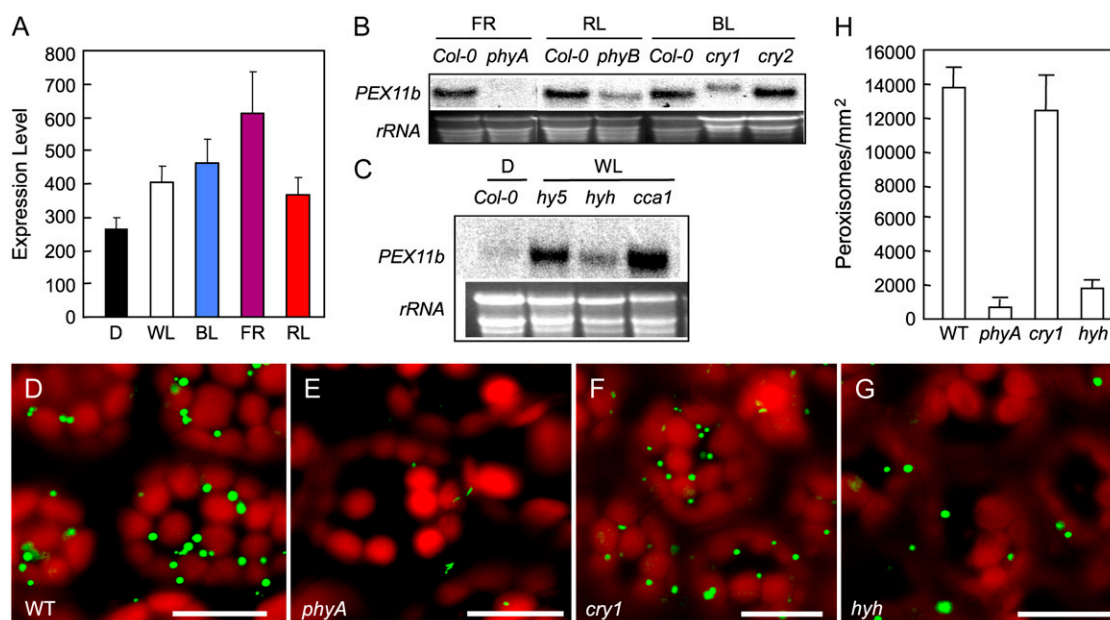
in different light conditions based on experiments performed with the Arabidopsis full genome chip (ATH1) arrays. In reference to transcript level in the dark, far-red light conferred the strongest up-regulation of *PEX11b* (2.3-fold), followed by blue (1.7-fold), white (1.5-fold), and red (1.3-fold) light (Fig. 3A), suggesting that, whereas all three wavelengths of light may activate the expression of *PEX11b*, far-red light appears to play the strongest role.

To identify components in the light signaling pathways responsible for activating *PEX11b*, we obtained null mutants of various light signaling genes and analyzed light-induced *PEX11b* expression in these lines using RNA-blot and RT-PCR analyses. First, we checked several photoreceptors known to perceive different wavelengths of light during seedling development, namely, *phyA* for far-red, *phyB* for red, and cryptochrome 1 (*cry1*) and *cry2* for blue light (lowercase letters are used in the light research field to refer to the holoproteins; Chen et al., 2004). The light induction of *PEX11b* expression was strongly decreased in *phyA* and moderately reduced in *cry1* and *phyB*, whereas no significant changes were detected in *cry2* (Fig. 3B). *PEX11b* was identified as one of the late up-regulated genes by *phyA* in previous microarray studies (Tepperman et al., 2001; Hudson and Quail, 2003). Analysis of the microarray data from Hudson and Quail (2003) revealed that the expression of *PEX11b* in wild-type seedlings was up-regulated with increasing time of far-red light irradiation, but was diminished in the *phyA* mutant under the same conditions (Supplemental Fig S1). Thus, the impact of far-red light and *phyA* on *PEX11b* expression is substantiated.

To identify *phyA* signaling intermediates involved in the regulation of *PEX11b*, we further examined null mutants of several well-characterized players in the *phyA* signaling pathway, including the novel nuclear protein FHY1, the homologous nuclear proteins FHY3 and FAR1, the GRAS family member PAT1, and the basic helix-loop-helix (bHLH) transcription factor HFR1 (Hudson et al., 1999; Bolle et al., 2000; Fairchild et al., 2000; Desnos et al., 2001; Wang and Deng, 2002b; Duek and Fankhauser, 2003). After far-red light treatment, the expression of *PEX11b* in these mutants did not differ significantly from that of the wild type, in contrast to the strongly decreased induction in *phyA* (Supplemental Fig S2). In parallel, we also analyzed mutants of several light signaling components that are downstream from multiple photoreceptors, including the key photomorphogenic transcription regulators LONG HYPOCOTYL5 (HY5; Oyama et al., 1997) and HY5 HOMOLOG (HYH; Holm et al., 2002), and CCA1—a central component of the light-entrained circadian clock oscillator (Wang and Tobin, 1998). A partial, yet significant, reduction in *PEX11b* expression was detected in the *hyh* mutant when compared with the other mutants (Fig. 3C).

Given that the light up-regulation of *PEX11b* was strongly decreased in *phyA* compared with other photoreceptor mutants, and that the light induction of *PEX11b* was relatively weak in *hyh* among all the





**Figure 3.** *phyA* and *HYH* play important roles in mediating light-induced expression of *PEX11b* and in controlling peroxisome abundance. A, GENEVESTIGATOR analysis of *PEX11b* expression level under various light conditions. Error bars indicate *SD*,  $n = 6$ ,  $P < 0.04$ . B to C, RNA-blot analyses of *PEX11b* expression in wild-type and mutant seedlings exposed to different wavelengths of light. Plants were germinated in the dark for 3 d before exposure to white ( $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), far-red ( $2 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), red ( $90 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), or blue ( $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) light for 2 h. Ethidium bromide-stained rRNA was used as a loading control. The transcript level of *PEX11b* did not vary between wild-type *Col-0*, Landsberg *erecta*, and Wassilewskija plants under different light wavelengths (data not shown); thus RNA from *Col-0* was used here as a control for all mutants. D to G, Confocal images of cotyledon cells from 14-d light-grown wild type, *phyA*, *cry1*, and *hyh* mutants each expressing *YFP-PTS1*. Bars =  $20 \mu\text{m}$ . H, Quantification of peroxisomes in the corresponding cells shown in D to G. Error bars indicate *SD*,  $n = 4$ .

downstream light signaling mutants tested, we further examined these two mutants for possible peroxisome phenotypes. To this end, the *YFP-PTS1* marker gene was individually crossed into the *phyA*, *cry1*, and *hyh* mutant background; *cry1* served as a control. Confocal microscopic analysis of the *YFP-PTS1*-expressing mutants revealed a drastic decrease in peroxisome abundance in *phyA* and *hyh*, but not in *cry1*, in both germinating seedlings (data not shown) and adult plants (Fig. 3, D–G). When quantitatively compared with the wild-type plants ( $13,850.4 \pm 1,046.4/\text{mm}^2$ ), there was a 22.5-fold decrease in peroxisome abundance in *phyA* ( $615.5 \pm 523.2/\text{mm}^2$ ;  $P < 0.001$ ), a 7.5-fold decrease in *hyh* ( $1,846.7 \pm 369.3/\text{mm}^2$ ;  $P < 0.001$ ), but no significant reduction in *cry1* ( $12,311.4 \pm 2,154.5/\text{mm}^2$ ;  $P = 0.1047$ ; Fig. 3H). These results led us to the conclusion that the far-red light receptor *phyA* and the bZIP transcription factor *HYH* may play direct roles in peroxisome proliferation through activating the expression of *PEX11b*.

We further tested the hypothesis that *phyA* and *HYH* promote peroxisome proliferation in light through *PEX11b* by overexpressing the *PEX11b* gene in the *phyA* and *hyh* mutants to see whether the peroxisomal phenotype in these mutants could be rescued. We conducted an in vivo transient assay in the leaves of the *YFP-PTS1*-expressing *phyA* and *hyh* plants by in-

roducing a *PEX11b* overexpression construct via leaf *Agrobacterium* infiltration. Two days postinoculation, we observed many elongated peroxisomes, as well as an increase in the total number of peroxisomes in the *phyA* and *hyh* mutants overexpressing *PEX11b*; this phenotype is similar to what we observed previously in wild-type plants overexpressing *PEX11b* (Orth et al., 2007). For quantification purposes, epifluorescence microscopic images exhibiting obvious increases in peroxisome abundance, but not elongation, are shown here (Fig. 4, A–D). The number of peroxisomes increased to approximately 4-fold in *phyA* ( $33.5 \pm 8.7$ – $130.7 \pm 29.3/\text{mm}^2$ ) and approximately 5-fold in *hyh* ( $29.3 \pm 5.2$ – $145.4 \pm 23.7/\text{mm}^2$ ) after *PEX11b* was expressed (Fig. 4E). The lower peroxisome abundance in these plants compared with those shown in Figure 3 may have been mainly caused by stress from agrobacterial infection. These data provide additional evidence for the notion that *PEX11b* is downstream in the *phyA*- and *HYH*-mediated pathway in light-mediated peroxisome proliferation.

#### **HYH Interacts with the Promoter of *PEX11b***

*HYH* and its homolog, *HY5*, are transcription factors that contain a bZIP DNA-binding domain and play redundant, as well as specific, roles in gene regulation during photomorphogenesis (Oyama et al., 1997;



*RBCS1A* promoter (Holm et al., 2002) as the probe. Both recombinant proteins were able to bind to this fragment; much stronger binding was observed for HY5-His (Fig. 5B). Although binding of HYH to the G box was previously shown not to be as strong as that of HY5 (Holm et al., 2002), this binding was even weaker in our assay. This is possibly due in part to the fact that the full-length HYH, instead of a truncated version (Holm et al., 2002), was used in our study.

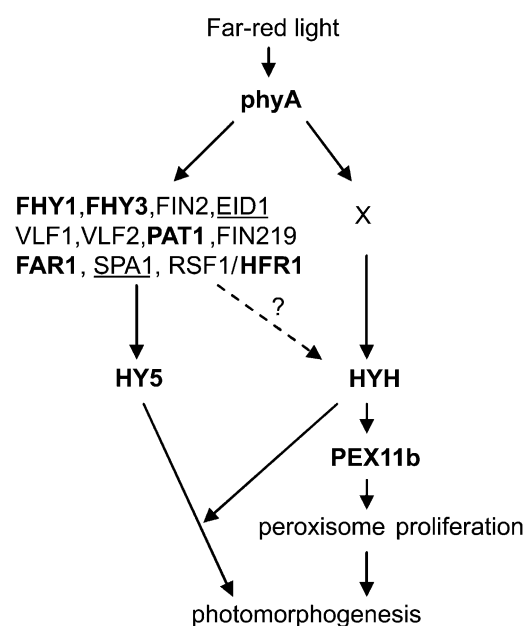
Based on these data, we conclude that HYH is able to bind specifically to the *PEX11b* promoter and therefore is most likely a direct transcription activator of *PEX11b* in the light.

## DISCUSSION

Dark-germinated *Arabidopsis* seedlings contain maximal activity of the glyoxylate cycle enzyme isocitrate lyase at 3 d, which gradually declines afterward (P. Yang and J. Hu, unpublished data). Consistent with this finding, we found more (basal) elongated peroxisomes in the dark-grown cotyledon cells than in adult tissue (observed in our previous studies, such as Fan et al. [2005] and Orth et al. [2007]). This observation suggests that peroxisomes divide at a higher rate in 3-d seedlings due to the active growth of seedlings at this stage. During photomorphogenesis, as seedlings undergo the transition from heterotrophic to autotrophic growth, organelles involved in photosynthesis and related functions (such as photorespiration) need to develop and genes required in these processes need to be activated. It was suggested that, during such a transitional stage, glyoxysomes are converted into leaf peroxisomes by replacement of glyoxysome-specific enzymes with those characteristic of leaf-type peroxisomes (Trelease et al., 1971; Olsen and Harada, 1995). In this study, we observed in *Arabidopsis* seedlings peroxisome elongation after 0.5-h light exposure, constriction and fission of these organelles at 2 h, and a sheer increase in peroxisome abundance at 4 and 8 h (Fig. 1). Therefore, we believe that, during seedling photomorphogenesis, a strong increase in peroxisome abundance occurs simultaneously with the import of leaf peroxisomal enzymes into the organelles to meet the high demand of leaf photosynthesis and photorespiration.

Given their sessile nature, plants have evolved a complex web of signaling events to perceive and respond to environmental cues, such as light (Chen et al., 2004; Jiao et al., 2007). Light-regulated plant development is one of the most extensively studied areas in plant biology. As a result, many players in light signaling have been identified through genetic and biochemical means and numerous mutants are available. To this end, we were able to take advantage of the mutant collections and employed molecular, genetic, and cell biological approaches to reveal the signal transduction pathway responsible for light-dependent peroxisome proliferation. First, *PEX11b* was strongly up-regulated by light, especially far-red light.

Second, silencing of the *PEX11b* gene caused deficiency in light-induced peroxisome proliferation. Notably, the number of peroxisomes in 3-d cotyledons from the *PEX11b* RNAi line showed approximately 33% (1,966.7 versus 2,963.9) decrease from the wild type (Figs. 1F and 2H). This reduction in basal peroxisome abundance was not as dramatic as the decrease shown in 4-week-old mesophyll cells from our previous study (approximately 75% in Orth et al., 2007), indicating possible tissue-specific functions of the *PEX11b* gene. Third, the light induction of *PEX11b* expression was significantly reduced in the *phyA* and *hyh* mutants. Fourth, *phyA* and *hyh* displayed decreased numbers of peroxisomes, a phenotype that could be rescued by overexpressing the *PEX11b* gene in these mutants. Last, HYH was able to interact with the 200-bp promoter of *PEX11b*. Based on these results, a new branch of the *phyA* pathway in seedling development has been identified in which HYH induces peroxisome proliferation by directly activating the *PEX11b* gene (Fig. 6). Although null mutants of the photoreceptors *cry1* and *phyB* also exhibited some level of reduction in the light-dependent up-regulation



**Figure 6.** A branch of the *phyA*-dependent light signaling network is controlled by HYH and *PEX11b* in *Arabidopsis* seedling photomorphogenesis. In far-red light, *phyA* promotes photomorphogenesis through multiple pathways, which contain a number of signaling intermediates. HY5 and HYH are downstream components in the *phyA* network and act as transcription factors that regulate overlapping and specific sets of *phyA*-responsive genes. The *PEX11b* gene, which encodes a peroxisomal protein directly involved in the proliferation of peroxisomes, is one of the specific target genes for HYH. Protein X could be an unidentified player acting in between *phyA* and HYH. Alternatively (dotted arrow with a question mark), some of the known signaling intermediates in the *phyA* network may also be involved in regulating *PEX11b* through HYH. Proteins analyzed in this study are in bold; negative regulators of *phyA* signaling are underlined.

of *PEX11b*, they showed no obvious peroxisome phenotypes (Fig. 3; data not shown). Thus, the influence of *cry1* and *phyB* on peroxisome proliferation through *PEX11b* might be minor compared with that of *phyA*.

In addition to the identification of *PEX11b* as one of the late up-regulated genes by *phyA* in previous microarray studies (Tepperman et al., 2001; Hudson and Quail, 2003), several early studies in mustard (*Sinapis alba*) plants also pointed to the role of *phyA* in the transformation of glyoxysomes to leaf peroxisomes. For example, transient formation of glyoxyperoxisomes (peroxisomes associated with both lipid bodies and plastids) was observed in cotyledons when mustard seedlings were exposed to continuous far-red light; formation of such functional intermediates of peroxisomes was attributed to a transitional stage from glyoxysomal to leaf peroxisomal metabolism (Schopfer et al., 1976). The activity of leaf peroxisomal enzymes, such as glycolate oxidase, urate oxidase, and allantoinase, increased in mustard cotyledons after continuous far-red light irradiation (Poucke et al., 1970; Hong and Schopfer, 1980, 1981). These reports agree well with our finding that *phyA* plays a key role in the light-dependent proliferation of peroxisomes during the transition from glyoxysomes to leaf peroxisomes in *Arabidopsis* seedlings.

A previous kinetic study of the action of *phyA* and *phyB* showed that *phyA* was responsible for initiating hypocotyl growth inhibition at the onset of continuous red light. This role of *phyA* started to decrease after 3 h of irradiation, at which point the *phyA* protein began to be degraded and the contribution of *phyB* to hypocotyl inhibition became predominant (Parks and Spalding, 1999). This finding also correlates with our results, which showed that the expression of *PEX11b* reached its maximum at 4 h of white-light exposure and declined afterward (Fig. 2), and supports the view that *phyA* plays a primary role in the up-regulation of the *PEX11b* gene during early hours of light exposure (dark-to-light transition) in *Arabidopsis* seedlings.

As a photoreceptor primarily perceiving far-red light, the *phyA* protein is mainly cytoplasmic in the dark and translocates into the nucleus after being activated by light, where it interacts with various transcriptional factors to regulate the transcription of far-red light-responsive genes (Wang and Deng, 2003). However, despite our data showing the significance of far-red light and *phyA* in regulating peroxisome abundance, we failed to detect the obvious reduction in *PEX11b* expression and any peroxisomal phenotype in several mutants of the *phyA* pathway, namely, *pat1*, *lifr1*, *flny1*, *flny3*, and *far1* (Supplemental Fig. S2; data not shown). It is possible that additional constituents of the *phyA* pathway not tested in this study are involved in regulating peroxisome proliferation through *PEX11b*. The proteins examined here may also be redundant in function with each other or with other untested proteins. In partial support of these predictions, *FHY3* and *FAR1* are closely related in sequence (Hudson et al., 1999; Wang and Deng, 2002b), *HFR1* is a member of the

bHLH family known to contain many members (such as the PIFs) with roles in phytochrome signaling (Duek and Fankhauser, 2005), and *PAT1* shares high sequence similarity with several members of the GRAS protein family (Bolle et al., 2000). Finally, despite being a less likely scenario, the possibility that *phyA* regulates *PEX11b* partially through its cytoplasmically localized protein pool cannot be completely excluded.

*HY5* and *HYH* are master regulators in the light signaling transcription cascades in photomorphogenesis, affecting the expression of numerous downstream genes (Oyama et al., 1997; Holm et al., 2002). In a recent study using in vivo chromatin immunoprecipitation combined with microarray analysis (CHIP-chip), 3,894 putative in vivo binding sites for *HY5* were mapped in the *Arabidopsis* genome. These *HY5* binding sites included G box, Z box and its variants, CG hybrid, and CA hybrid (Lee et al., 2007). Although *PEX11b* was among the putative target sites for *HY5*, we found only two G boxes and two CA hybrid motifs present in the *PEX11b* promoter, all of which are approximately 1.7 to 2.0 kb upstream from the TSS and thus absent from the promoter region analyzed in our study. Despite only minor differences in the DNA-binding domain (three exchanges over 24 amino acids; Holm et al., 2002), *PEX11b* expression was not reduced in the *hy5* null mutant (Fig. 3C) and *HY5* was unable to interact with the 200-bp promoter of *PEX11b* in our gel-shift assay (Fig. 5A). We postulate that the three unique amino acids in the DNA-binding domain, and possibly residues immediately adjacent to this domain, may facilitate the specific DNA-binding activity of *HYH* to the 200-bp promoter of *PEX11b*.

*PEX11* proteins from various organisms play mostly conserved roles in promoting peroxisome elongation and population increase (Thoms and Erdmann, 2005) and are targets for peroxisome proliferation agents. For example, *PEX11* in yeast is up-regulated by oleic acid (Erdmann and Blobel, 1995; Marshall et al., 1995). One of the three mouse *PEX11* isoforms, *PEX11 $\alpha$* , is induced by peroxisome proliferation factors, such as clofibrate and 4-phenylbutyrate (Abe et al., 1998; Li et al., 2002). Our work has revealed light to be a significant peroxisome proliferator in plants, which strongly up-regulates the expression of the *Arabidopsis PEX11b* gene. It will be important to identify the specific cis-elements in the promoter of *PEX11b* required for such activation in the light. Yeast one-hybrid screens using the promoter of *PEX11b* as bait may also be necessary to isolate additional nuclear proteins interacting with this promoter. We expect that a more complete model for light-mediated peroxisome proliferation in plants will be established in the near future.

## MATERIALS AND METHODS

### Plant Growth, Light Conditions, and Genetic Crosses

The wild-type *Arabidopsis* (*Arabidopsis thaliana*) plants used in this study were from the Columbia-0 (Col-0) ecotype. The *phyA* (SALK\_014575), *lifr1*



(SALK\_037727), *pat1* (SALK\_064220), *far1* (SALK\_031652), *fly1* (SALK\_130614), *fly3* (SALK\_002711), and *cry2-1* (Mockler et al., 2003) mutants were in the Col-0 background. The *cry1-1* (*hy4-1*) mutant was in the Landsberg *erecta* background. The *hy5-Ks50* (Oyama et al., 1997), *hyh* (Holm et al., 2002), and *cca1-1* (Alabadi et al., 2002) mutants were in the Wassilewskija background. These mutants were confirmed by their respective light phenotypes and genotyped by PCR analysis to ensure their homozygosity. RT-PCR analysis was also performed to confirm that they were true null mutants.

Seeds were surface sterilized with 20% commercial bleach and 0.025% Triton X-100, washed five times with sterile water, plated on 0.5× Murashige and Skoog medium solidified with 0.6% phytagar, stratified at 4°C for 2 d, and subject to 1-h white light treatment to induce synchronous germination. Plates were then kept for 3 d in the dark at 21°C and subsequently exposed to white (70 μmol m<sup>-2</sup> s<sup>-1</sup>), far-red (2 μmol m<sup>-2</sup> s<sup>-1</sup>), red (90 μmol m<sup>-2</sup> s<sup>-1</sup>), or blue (30 μmol m<sup>-2</sup> s<sup>-1</sup>) light for the designated length of time. After the seedling stage, plants were transferred to soil and grown in growth chambers at 21°C with 100 μmol m<sup>-2</sup> s<sup>-1</sup> white-light conditions and 16/8-h photoperiod.

Genetic crosses were made between *YFP-PTS1* plants and *phyA*, *cry1*, or *hyh*. F3 plants homozygous for *YFP-PTS1* and the mutant alleles were selected after confirmation by fluorescence microscopy and RT-PCR analysis with gene-specific primers.

## Databases Used in This Study

Databases used in this study include PLACE (<http://www.dna.affrc.go.jp/PLACE>) and PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>).

## Epifluorescence and Confocal Laser-Scanning Microscopy

Epifluorescence microscopy was performed with an Axio Imager M1 microscope (Carl Zeiss) for visualization of the YFP-PTS1 protein (excitation 500 ± 12 nm; emission 542 ± 13.5 nm). A confocal laser-scanning microscope (Zeiss LSM 510 META) was also used to obtain images of YFP-PTS1 proteins. A 488-nm argon ion laser was used for excitation of YFP and chlorophyll. Emission filters of 505- to 530-nm bandpass and 650-nm longpass were used for YFP and chlorophyll, respectively. Images were acquired at 63×.

## Peroxisome Quantification

Peroxisome quantification data were obtained from microscopic images analyzed by ImageJ software using the procedure described by Shi et al. (2007) with slight modifications. Specifically, YFP-tagged peroxisomes were imaged with a confocal (Zeiss LSM 510 META) or an epifluorescence (Zeiss Axio Imager M1) microscope. The confocal images shown in Figures 1 to 3 were each taken from four optical sections, each 0.6 μm in depth. The epifluorescence images shown in Figure 4 were obtained from six optical sections; each section was 1 μm in depth. Using ImageJ software, the following procedures were performed. First, RGB color images were converted to 8-bit black-and-white images. Second, the threshold function was used to designate the black pixels (peroxisomes) to be counted. Third, the “analyze particles” function was used to count the number of pixel groups in each image. Peroxisome quantification was performed on four to six individual images taken from different cotyledon/leaf areas of a plant. For quantification, a peroxisome having a clearly defined boundary, which means that fission has occurred, was counted as an individual organelle no matter how closely it was attached to another organelle. In contrast, a segmented peroxisome (constricted but no fission has taken place) was considered as a single organelle.

## RNA Gel-Blot and RT-PCR Analyses

Total RNA was isolated from 3-d dark-grown seedlings using the Qiagen RNeasy plant mini prep kit. Total RNA (15 μg) was loaded onto the gel and blotted to nylon membranes. The *PEX11b* probe was derived from a 0.7-kb fragment of the cDNA clone (Orth et al., 2007) and was <sup>32</sup>P-dCTP labeled by the random priming kit (Invitrogen). Hybridizations and washings were conducted according to standard protocols (Sambrook and Russell, 2001).

For RT-PCR analysis, total RNA was reverse transcribed with the Omniscript RT kit (Qiagen). *PEX11b*-specific primers At3g47430F (5'-CAGTGATCCGT-TCTTGGCC-3') and At3g47430R (5'-GGCCAGTTCCTATACCAACC-3') were

used to amplify a 0.43-kb product from *PEX11b*. *UBQ10-1* (5'-TCAATTCTCTC-TACCGTGATCAAGATGCA-3') and *UBQ10-2* (5'-GGTGTGCAACTCTCC-ACCTCAAGAGTA-3') from the *UBQ10* gene (At4g05320) were used to amplify a cDNA product (approximately 320 bp) as a loading control.

For *PEX11b* and *UBQ10* amplification, PCR was performed with the following conditions: 94°C for 3 min, 30 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 4 min.

## Constructs for Protein Expression and Purification

*P<sub>35S</sub>:PEX11b*, a *PEX11b* overexpression construct in the pCambia vector (Orth et al., 2007), was used for the transient expression experiments.

Specific oligonucleotides were synthesized for amplification of the following genes: *HYH* N terminus, 5'-CACGCCATGGGCATGCTCTCCAACG-ACC-3' and *HYH* C terminus, 5'-ATAAGAATCGGCCCGGTGATTGTCAT-CAGTTTTAGG-3' for *HYH* (At3g17609); *HY5* N terminus, 5'-CATGCCA-TGGGCATGCAGGAACAAGCGAC-3' and *HY5* C terminus, 5'-CCGCT-CGAGAAGGCTTCATCAGCATTAG-3' for *HY5* (At5g11260). Using these primer pairs, complete coding regions of *HYH* and *HY5* were PCR amplified with the *pfu* turbo enzyme (Stratagene) using Arabidopsis total cDNA from light-grown seedlings as template. The products were separately cloned into *Nco*I and *Not*I sites of the bacterial pET-28a expression vector (Novagen). Recombinant full-length *HYH* and *HY5* proteins fused to 6×-His in the pET28a+ vector (Novagen) were expressed in bacteria and purified with nickel nitrilotriacetic acid agarose (Qiagen) according to the manufacturer's protocol.

## Transient Expression Assay

The *Agrobacterium*-mediated transient expression assay was performed as described in Wieland et al. (2006). Overnight bacterial cultures of the *Agrobacterium tumefaciens* strain C58C1 containing the plasmid of interest were harvested by centrifugation. Cells were resuspended in induction buffer (10 mM MgCl<sub>2</sub> and 150 μM acetosyringone) to an OD<sub>600</sub> of 0.5, and incubated for 2 h at room temperature. *Agrobacteria* were then hand-infiltrated into 3-week-old Arabidopsis leaves with a 1-mL needleless syringe. Infiltrated plants were grown for 2 to 3 d in growth chambers before microscopic study of the YFP fusion proteins was performed.

## Electrophoretic Mobility Shift (Gel-Shift) Assay

The 200-bp promoter of *PEX11b* was PCR amplified from genomic DNA using the primer pair forward (5'-CCGCTCGAGGCACAAATCTCGGA-TTTC-3') and reverse (5'-GCTCTGTGTCTCATGTTTTGATATTCAAGC-TTGGG-3'). The amplified product was digested with *Hind*III for 2 h, gel purified, and used for labeling with <sup>32</sup>P α-dCTP.

For binding assay with the G box, oligonucleotides were synthesized for the G-box sense strand, 5'-CCGCTCGAGAATTATCTCCACGTGGCATT-ATCC-3', and antisense strand, 5'-GGAATAATGCCACGTGGAAGATAA-TTCTCGAGCGG-3', with an *Xho*I site at the 5' end of each oligonucleotide. Equal molar quantities of the two strands were annealed by incubating sequentially at 95°C for 30 s, 72°C for 2 min, 37°C for 2 min, and, finally, 25°C for 2 min. Annealed oligonucleotides were then digested overnight with *Xho*I, extracted with phenol-chloroform, and ethanol precipitated. The precipitated pellet was then washed with 70% ethanol, dried, resuspended in Tris-HCl (pH 8.0), and used for labeling with <sup>32</sup>P α-dCTP.

The DNA-binding assays were performed as described by Yadav et al. (2002) at room temperature in a final volume of 25 μL of binding buffer [15 mM HEPES, pH 7.5, 35 mM KCl, 1 mM EDTA, 6% glycerol (v/v), 1 mM dithiothreitol, 1 mM MgCl<sub>2</sub>, and 0.5 μg poly(dI-dC)]. The samples were incubated at room temperature for 15 min and later run on a 5% polyacrylamide gel at 12 to 15 mAmp. After electrophoresis, the gel was dried and autoradiographed.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NP\_190327 (*PEX11b*), NP\_850605 (*HYH*), and NP\_568246 (*HY5*).

## Supplemental Data

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

**Supplemental Figure S1.** Comparison of *PEX11b* expression at different time periods after far-red light treatment in wild-type and *phyA* mutant plants.

**Supplemental Figure S2.** RT-PCR analysis of *PEX11b* and *UBQ10* in 3-d dark-grown phyA signaling pathway mutants after exposure to far-red light.

**Supplemental Figure S3.** Analysis of the *PEX11b* promoter region.

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