Plants perceive subtle changes in light quality and quantity through a set of photoreceptors, including phytochromes and cryptochromes. Upon perception, these photoreceptors initiate signal transduction pathways leading to photomorphogenic changes in development. Using activation-tagging mutagenesis to identify novel light-signaling components, we have isolated a gain-of-function mutant, sob1-D (suppressor of phytochrome B-4 [phyB-4] dominant), which suppresses the long-hypocotyl phenotype of the phyB missense allele, phyB-4. The sob1-D mutant phenotype is caused by the overexpression of a Dof (DNA binding with one finger) transcription factor, OBF4 Binding Protein 3 (OBF3). A translational fusion between OBF3 and green fluorescent protein is nuclear localized in onion (Allium cepa) cells. Tissue-specific accumulation of an OBF3:OBF3-glucuronidase translational fusion is regulated by light in Arabidopsis thaliana. Hypocotyls of transgenic lines with reduced OBF3 expression are less responsive to red light. This aberrant phenotype in red light requires functional phyB, suggesting that OBF3 is a positive regulator of phyB-mediated inhibition of hypocotyl elongation. Furthermore, these partial-loss-of-function lines have larger cotyledons. This light-dependent cotyledon phenotype is most dramatic in blue light and requires functional cryptochrome 1 (cry1), indicating that OBF3 is a negative regulator of cry1-mediated cotyledon expansion. These results suggest a model where OBF3 is a component in both phyB and cry1 signaling pathways, acting as a positive and negative regulator, respectively. An alternate, though not mutually exclusive, model places OBF3 as a general inhibitor of tissue expansion with phyB and cry1, differentially modulating OBF3’s role in this response.

INTRODUCTION

Plants must respond to several environmental cues, one of the most important being light. Not only required for photosynthesis, light is used by plants to determine their location in relation to each other. Changes in light quality or quantity are perceived by a set of photoreceptors characterized by the wavelength they absorb. These include the red/far-red absorbing phytochromes, the blue/UV-A absorbing cryptochromes and phototropins, and the as yet unidentified UV-B photoreceptors (Nagy and Schaffer, 2002; Lin and Shalitin, 2003).

In Arabidopsis thaliana, there are five phytochromes (phy), at least three cryptochromes (cry), and two phototropins. The phototropins mediate responses such as phototropism, chloroplast movement, and changes in stomatal aperture (Briggs and Christie, 2002). The phytochromes and cryptochromes have both unique and redundant roles in mediating photomorphogenic responses, including hypocotyl elongation, cotyledon/leaf expansion, flowering time, and regulation of gene expression (Neff et al., 2000; Lin and Shalitin, 2003).

Photomorphogenic signaling in far-red and red light is mediated mainly by phytochrome A (phyA) and phyB, respectively. However, other photoreceptors mediate the red light response as well (Franklin et al., 2003; Monte et al., 2003). All phytochromes are synthesized, in their inactive form, in the cytoplasm and undergo red light–dependent photoconversion to their active form. A portion of the active phytochromes remain in the cytoplasm where signaling can occur through interactions with proteins such as Phytochrome Kinase Substrate 1 (Fankhauser et al., 1999). Active phytochromes can also undergo translocation into the nucleus to initiate further signal transduction. phyB is thought to initiate signal transduction through its C terminus, which is responsible for interactions with other proteins, including photoreceptors and transcription factors (Ni et al., 1998; Mas et al., 2000). However, the phyB C terminus may not be required for all phyB-mediated signaling (Matsushita et al., 2003). Many transcription factors are involved in phyB-mediated signal transduction, including Phytochrome Interacting Factor 3 (PIF3), Long Hypocotyl 5, Dof Affecting Germination 1 (DAG1), DAG2, and Cogwheel 1 (COG1) (Ni et al., 1998; Osterlund et al., 2000; Gualberti et al., 2002; Park et al., 2003).

In blue light, photomorphogenic responses are mediated primarily by cryptochrome 1 (cry1) and cry2. cry1 is responsible for seedling deetiolation in high fluence rates of light, and cry2 mediates flowering time and deetiolation in response to lower fluence rates (Koornneef et al., 1980; Ahmad and Cashmore, 1993; Lin et al., 1998). The C terminus of the cryptochromes contains domains responsible for subcellular localization, protein–protein interactions, and physiological function. There is evidence that phytochromes and cryptochromes work together, either through interactions with each other (Mas et al., 2000; Jarillo et al., 2001) or via common signaling components, such as Long Hypocotyl in Far-Red 1 (HFR1) and Short Under Blue Light 1 (SUB1) (Guo et al., 2001; Dueck and Fankhauser, 2003).
Several signaling components downstream of the phytochromes and crytochromes have been identified using a variety of approaches. We have used activation-tagging mutagenesis to identify novel components involved in light signaling. Using this approach, we have identified a new mutation, sob1-D (suppressor of phyB-4 dominant), which suppresses the long-hypocotyl phenotype of phyB-4. The sob1-D mutant phenotype is caused by the overexpression of a Dof (DNA binding with one finger) transcription factor, previously named OBF4 Binding Protein 3 (OBP3) (Kang and Singh, 2000).

OBP3 binds DNA in a sequence-specific manner and is able to activate transcription in a transient assay (Kang and Singh, 2000). OBP3 expression levels are regulated by salicylic acid and auxin (Kang and Singh, 2000). Hypermorphic alleles, overexpressing OBP3, are severe dwarfs, suggesting an involvement in plant development (Kang and Singh, 2000). However, this hypothesis has not been tested with hypomorphic alleles. Through characterization of transgenic lines with reduced OBP3 expression, we have uncovered a role for this gene in phyB-mediated inhibition of hypocotyl elongation in red light and cry1-mediated cotyledon expansion in blue light.

RESULTS

The sob1-D Mutant, Caused by the Overexpression of OBP3, Suppresses the Long-Hypocotyl Phenotype of phyB-4

The Arabidopsis phyB-4 mutation, caused by a single amino acid change near the chromophore binding site, confers a long-hypocotyl phenotype when compared with the wild type (Koornneef et al., 1980; Reed et al., 1993; Elich and Chory, 1997). In an activation-tagging mutagenesis of ~7000 primary transformants (T1), we identified and cloned the genes causing four independent, gain-of-function mutations that suppress the long-hypocotyl phenotype of phyB-4. This study focuses on one of these mutants identified in our lab, sob1-D.

The sob1-D phyB-4 double mutant had a dramatically shorter hypocotyl than the phyB-4 mutant and the wild type when grown in white light (Figure 1A). However, the double mutant elongated normally in the dark, suggesting that the gene altered in this mutant is involved in light signaling (Figure 1A). Both the sob1-D phyB-4 double mutant and sob1-D single mutant were severe dwarfs with reduced fertility as adults (Figure 1B; data not shown). DNA gel blot and segregation analysis indicated that there was one T-DNA in the sob1-D phyB-4 mutant, and it was genetically linked to the mutant phenotype.

Genomic DNA flanking the T-DNA insertion was cloned via plasmid rescue. Sequencing and subsequent BLAST analysis (Altschul et al., 1990) of a portion of the cloned DNA indicated that the T-DNA was inserted into chromosome III. The closest open reading frame is ~1 kb 5' from the left border of the T-DNA. Sequencing and PCR analysis confirmed that all four copies of the enhancers were inserted 1139 bp 3' of the stop codon of a Dof transcription factor previously named OBP3 (Figure 1C; Kang and Singh, 2000).

RT-PCR demonstrated that OBP3 is overexpressed in the sob1-D phyB-4 mutant (Figure 1D). To confirm that OBP3 overexpression causes the sob1-D mutant phenotype, the phyB-4
mutant was transformed with genomic DNA encoding OBP3 along with the four enhancers. Transgenic lines with increased OBP3 expression recapitulated the original sob1-D mutant phenotypes (Figures 1A, 1B, and 1D), indicating that the overexpression of OBP3 is responsible for suppressing the long-hypocotyl phenotype of phyB-4 in the sob1-D phyB-4 double mutant.

OBP3 Is Nuclear Localized

Based on sequence similarity, OBP3 is a putative Dof transcription factor (Kang and Singh, 2000). In addition, an OBP3 protein lacking 59 amino acids from the N terminus, though containing the Dof domain, is capable of binding DNA in vitro and activating transcription in a transient assay (Kang and Singh, 2000). However, OBP3’s subcellular localization is unknown. To determine the subcellular localization of OBP3, the same OBP3 cDNA used by Kang and Singh (2000) was translationally fused to green fluorescent protein (GFP). This construct was coinjected with a construct containing dsRed under control of the Zea mays ubiquitin (UBQ) promoter. This dsRed control localized throughout the cell, including within the nucleus (Figure 2A), in a manner similar to constitutively expressed GFP (data not shown). However, the OBP3-GFP fusion protein was only nuclear localized in these onion epidermal cells (Figures 2B and 2C). OBP3’s nuclear localization (Figures 2A to 2C), along with its ability to bind DNA and activate transcription (Kang and Singh, 2000), suggests that this gene encodes a bona fide Dof transcription factor.

Tissue-Specific Localization of OBP3 Is Regulated by Light

OBP3 mRNA levels were not altered in seedlings grown in continuous light or in the dark (Figure 1E). In addition, OBP3 transcript levels were unaffected by the fluence rate or wavelength of light or during the transition (first 24 h) from darkness to growth in the light (data not shown). However, many transcription factors involved in light signaling are regulated at the protein level (Osterlund et al., 2000; Bauer et al., 2004).

To determine the relative amount and localization of OBP3 in response to changes in light, wild-type Arabidopsis was transformed with a construct encoding an OBP3:β-glucuronidase fusion gene and the promoters were controlled by either 1.4 kb of DNA 5' of the OBP3 gene. OBP3:OBP3-GUS localization was observed in 5-d-old seedlings grown either in the dark (D) and (E) or in continuous white light (F) to (I). GUS staining was observed in the apex and cotyledons of dark-grown seedlings (D) and (E). In light-grown seedlings, GUS staining was observed in the apex (F) and (I) and in the vascular tissue of the cotyledons (F) and (G), hypocotyl (F) and (I), and roots (H).

Figure 2. Subcellular and Tissue-Specific Localization of OBP3.

(A) to (C) Onion epidermal cells were coinjected with constructs containing either UBQ:dsRed (A) or 35S:OBP3-GFP (B). The two images are merged to show the subcellular localization of OBP3 (C).

(D) to (I) Wild-type Arabidopsis was transformed with a T-DNA containing genomic DNA encoding OBP3 translationally fused to the GUS gene. Expression of this fusion gene was controlled by −1.4 kb of DNA 5' of the OBP3 gene. OBP3:OBP3-GUS localization was observed in 5-d-old seedlings grown either in the dark (D) and (E) or in continuous white light (F) to (I). GUS staining was observed in the apex and cotyledons of dark-grown seedlings (D) and (E). In light-grown seedlings, GUS staining was observed in the apex (F) and (I) and in the vascular tissue of the cotyledons (F) and (G), hypocotyl (F) and (I), and roots (H).
(GUS) translational fusion protein, consisting of the genomic DNA encoding OBP3 fused at the 3' end to GUS. This fusion was expressed using 1.4 kb of DNA 5' of the OBP3 open reading frame. Multiple homozygous lines containing a single locus insertion had varying degrees of sob1-D mutant phenotypes. These phenotypes correlated with OBP3-GUS mRNA levels, suggesting that the fusion protein is functional (see Supplemental Figure 1 online).

GUS analysis was performed on at least three independent lines grown for 5 d in the dark or in continuous white light (Figures 2D to 2I). GUS staining was present in the apex, cotyledons, and occasionally in the root apex of dark-grown seedlings (Figures 2D and 2E). In light-grown seedlings, GUS staining was present in the shoot apex (Figures 2F and 2I) and the vascular tissue of the cotyledons (Figures 2F and 2G), hypocotyl (Figures 2F and 2I), and roots (Figure 2H). Taken together, these data indicate that light causes OBP3 to accumulate in the vascular tissue of hypocotyls and roots and possibly cotyledons. This localization was independent of the fluence rate of light (data not shown). Seedlings grown in continuous red or blue light showed similar staining patterns to white light–grown seedlings (data not shown). Seedlings grown in far-red light had staining only in the apex and cotyledons, similar to dark-grown seedlings (data not shown).

OBP3-RNAi Hypocotyls Are Less Responsive to Red Light in a phyB-Dependent Manner

The sob1-D phyB-4 mutant suppressed the long-hypocotyl phenotype of phyB-4 in the light, while elongating normally in the dark, suggesting that OBP3 may be involved in light signaling (Figure 1). Because no null mutations in OBP3 exist to date, OBP3 partial-loss-of-function transgenic lines were generated using RNA interference (RNAi) (Hutvagner and Zamore, 2002) to further test the role of OBP3 in photomorphogenesis. A 475-bp region containing a portion of the final OBP3 exon and 3' untranslated region was amplified by PCR and cloned into a binary vector in a hairpin manner, under the control of the 35S promoter of Cauliflower mosaic virus. This region did not contain the highly conserved DoF domain, and BLAST analysis indicated no homology to other sequences in the Arabidopsis genome.

Some of the resulting homozygous OBP3-RNAi lines conferred larger adult stature (Figure 3). RT-PCR was performed on these plants to determine if they had lower OBP3 transcript levels. Those lines with larger adult stature had lower OBP3 expression (Figure 3). In addition, transgenic lines with normal adult stature had normal OBP3 expression levels (data not shown). One of these original lines, OBP3-RNAi1-4-3, was crossed with the sob1-D phyB-4 mutant, resulting in F1 progeny with a hypocotyl phenotype similar to the wild type (data not shown). This provides further evidence that OBP3 expression is reduced in the OBP3-RNAi lines because the RNAi phenotype is dominant with regard to the overexpression phenotype.

To determine whether OBP3 is involved in light signal transduction, fluence rate response experiments were performed using the wild type and the OBP3-RNAi1-4-3 line (Figure 4). Like the sob1-D mutant, OBP3-RNAi1-4-3 and other OBP3-RNAi lines elongated normally in the dark (Figures 1A and 4; see Supplemental Figure 2A online). In multiple fluence rates of white light, OBP3-RNAi1-4-3 conferred a subtle though statistically significant long-hypocotyl phenotype when compared with the wild type, suggesting that OBP3 is involved in light signaling (Figure 4A). Multiple OBP3-RNAi lines, including OBP3-RNAi1-4-3, were also less responsive to multiple fluence rates of red light when compared with the wild type (Figure 4B; see Supplemental Figure 2A online). Multiple OBP3-RNAi lines, including OBP3-RNAi1-4-3, responded normally to far-red light (Figure 4D; see Supplemental Figure 2A online). Though hypocotyls of the OBP3-RNAi1-4-3 line were shorter in blue light (Figure 4C), this phenotype was not conferred by other OBP3-RNAi lines (see Supplemental Figure 2A online). Together, these data indicate that OBP3 is a positive regulator of hypocotyl elongation inhibition in red and white light.

To determine if phyB is necessary for this OBP3-mediated hypocotyl response in red light, double mutants between OBP3-RNAi1-4-3 and weak and null alleles of phyB were generated. Although phyB-null mutants grown in red light are near their maximal growth rate, phyA phyB double mutants and phyB phyC phyD triple mutants are capable of elongating more than phyB-null mutants (Reed et al., 1994; Casal and Mazzella, 1998; Neff and Chory, 1998; Monte et al., 2003), suggesting that this genetic test will determine if phyB is required for the OBP3-mediated hypocotyl response. The OBP3-RNAi1-4-3 hypocotyls were less responsive to red light only when wild-type or partially functional (i.e., phyB-4) phyB was present (Figure 5). By contrast, the OBP3-RNAi1-4-3 phyB-null (phyB-9) double mutant hypocotyls responded the same way to red light as the phyB-9 mutant. This experiment was repeated in a lower fluence rate of red light and gave the same result (19 μM/m²/s; data not shown).
demonstrating that the OBP3-RNAi1-4-3 aberrant hypocotyl response to red light requires phyB. Because the sob1-D mutant was a severe dwarf as an adult (Figure 1B), it was predicted that OBP3-RNAi adult plants would be larger in stature. In addition to this larger adult stature (Figure 3), multiple OBP3-RNAi mutants had larger cotyledons when grown in white light (Figure 6; see Supplemental Figure 2B online). To determine if this increase in cotyledon size is because of cell expansion or cell division, epidermal imprints were examined and cell area was measured (Figure 6B, Table 1). The sob1-D phyB-4 mutant cotyledons had smaller epidermal cells when compared with both the phyB-4 mutant and the wild type (Figures 6A and 6B). OBP3-RNAi1-4-3 and other OBP3-RNAi lines had larger epidermal cells when compared with the wild type (Figure 6B, Table 1).

To determine if OBP3-mediated cotyledon expansion involves specific photoreceptor signaling pathways, multiple OBP3-RNAi lines, including OBP3-RNAi1-4-3, were grown in different wavelengths of light, and the cotyledon area was measured (Figure 7; see Supplemental Figure 2B online). Cotyledons of the OBP3-RNAi lines were dramatically larger than the wild type in blue light and slightly, though significantly, larger in red and far-red light (Figure 7; see Supplemental Figure 2B online). Taken together, these data demonstrate that OBP3 is a negative regulator of cotyledon expansion in multiple photomorphogenic signaling pathways.

The most striking OBP3-RNAi cotyledon phenotype was seen in blue light, suggesting a role for cry1 in this response. To

**Figure 4.** Fluence Rate Response Analysis for Hypocotyl Length of the OBP3-RNAi1-4-3 Mutant.

Five-day-old seedlings were grown in the dark or varying fluence rates of continuous white (A), red (B), blue (C), and far-red (D) light. Bars = ±1 SE. (*) equals P < 0.5, and (**) equals P < 0.001 from a Student’s unpaired two-tail t test comparing the OBP3-RNAi mutant and the wild type from the same light treatment.

OBP3 Is a Negative Regulator of cry1-Mediated Cotyledon Expansion in Response to Blue Light

The sob1-D phyB-4 double mutant had smaller, upwardly cupped cotyledons when grown in white light (Figures 1A and 6A). Because the sob1-D mutant was a severe dwarf as an adult (Figure 1B), it was predicted that OBP3-RNAi adult plants would be larger in stature. In addition to this larger adult stature (Figure 3), multiple OBP3-RNAi mutants had larger cotyledons when grown in white light (Figure 6; see Supplemental Figure 2B online). To determine if this increase in cotyledon size is because of cell expansion or cell division, epidermal imprints were examined and cell area was measured (Figure 6B, Table 1). The sob1-D phyB-4 mutant cotyledons had smaller epidermal cells when compared with both the phyB-4 mutant and the wild type (Figures 6A and 6B). OBP3-RNAi1-4-3 and other OBP3-RNAi lines had larger epidermal cells when compared with the wild type (Figure 6B, Table 1).

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The most striking OBP3-RNAi cotyledon phenotype was seen in blue light, suggesting a role for cry1 in this response. To

**Figure 5.** Photoreceptor Double Mutant Analysis for Hypocotyl Length.

Five-day-old seedlings were grown in the dark or continuous red light (29 μM/m²/s). The phyB-9 allele is a phyB-null mutant, and the phyB-4 allele is a phyB-missense mutant. The hypocotyls of the dark-grown double mutant were longer when compared with the wild type, so the data are normalized to the average dark-grown hypocotyl length for each line. Bars = ±1 SE. (*) equals P < 0.5, and (**) equals P < 0.001 from a Student’s unpaired two-tail t test comparing the mutant with its control.
determine if cry1 is necessary for the aberrant cotyledon response conferred by OBP3-RNAi1-4-3 in blue light, double mutants were generated with weak and null alleles of cry1. The OBP3-RNAi1-4-3 cry1-weak (cry1-102) double mutant had significantly larger cotyledons than cry1-102. By contrast, cotyledons of the OBP3-RNAi1-4-3 cry1-null (cry1-103) double mutant were the same size as the cry1-103 mutant. Thus, OBP3-mediated cotyledon expansion in response to blue light was observed only when wild-type or partially functional cry1 (i.e., cry1-102) was present (Figure 7A). Because CRY1 transcript accumulation was not affected by the genetic state of OBP3 (data not shown), these data indicate that OBP3 is a negative regulator of cry1-mediated cotyledon cell expansion in response to blue light.

DISCUSSION

Previous studies of transgenic plants overexpressing the Dof transcription factor, OBP3, have suggested that it is involved in plant growth and development (Kang and Singh, 2000). Here, we have identified OBP3 in an activation-tagging mutant screen as an overexpression suppressor of the long-hypocotyl phenotype of a phyB missense allele (Figure 1). Seedlings of this sob1-D mutant elongate normally in the dark, with aberrant phenotypes conferred only in light-grown plants, suggesting that OBP3 is involved in photomorphogenesis.

Earlier studies have shown that OBP3 binds specifically to the Dof recognition sequence, A/TAAAG, in vitro and can activate transcription in a transient assay (Kang and Singh, 2000). We have now shown that OBP3 is nuclear localized (Figures 2A to 2C), providing further evidence that this protein is a bona fide transcription factor. Though gene overexpression studies suggest a role for OBP3 in plant development, the true function of this gene is unclear. To determine OBP3’s role in planta, we have used an RNAi approach to identify multiple OBP3 partial-loss-of-function transgenic lines. Genetic and phenotypic analysis of these OBP3-RNAi lines has identified a complex role for OBP3 in seedling photomorphogenesis.

There is precedence for other Dof transcription factors being involved in light signaling. COG1 expression levels are induced in response to far-red and red light, and this regulation requires phyA and phyB, respectively (Park et al., 2003). In addition, transgenic lines with reduced COG1 levels are more responsive to both red and far-red light, suggesting that this gene is a negative regulator of multiple phytochrome signaling pathways. DAG1 and DAG2 have opposing affects during seed germination in response to red light, acting as a repressor and activator, respectively (Papi et al., 2000, 2002; Gualberti et al., 2002). Dof involvement in light signaling is not limited to Arabidopsis because transcript levels and the ability to bind DNA of maize DOF1 are also regulated by light (Yanagisawa and Sheen, 1998). Herein, we have shown that OBP3 represents another Dof transcription factor involved in multiple aspects of light signaling, modulating both phytochrome- and cryptochrome-mediated seedling photomorphogenesis.

New Role for the Dof Transcription Factor OBP3 in Light Signaling

Fluence rate response experiments using OBP3-RNAi lines uncover OBP3’s involvement in light-mediated inhibition of hypocotyl elongation. Though the OBP3-RNAi lines responded normally to blue and far-red light, they were less responsive to

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cotyledon Epidermal Cell Area µm² (SE)</th>
</tr>
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<tbody>
<tr>
<td>Col-0</td>
<td>3.91 (0.11)</td>
</tr>
<tr>
<td>OBP3-RNAi1-4-3</td>
<td>5.02 (0.16)</td>
</tr>
<tr>
<td>OBP3-RNAi1-5-7</td>
<td>4.79 (0.19)</td>
</tr>
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Seedlings were grown in continuous white light (35 µM/m²/s) for 5 d.
multiple fluence rates of white and red light (Figure 4; see Supplemental Figure 2A online). Double mutant analysis with one of these OBP3-RNAi lines, OBP3-RNAi1-4-3, demonstrates that the aberrant hypocotyl phenotype in red light requires a functional phyB. Taken together, these data place OBP3 in a red light signaling pathway, acting as a positive regulator of phyB-mediated inhibition of hypocotyl elongation.

In addition to causing subtle, though statistically significant, aberrant hypocotyl phenotypes, the genetic state of OBP3 has a strong affect on light-dependent cotyledon expansion (Figure 6, Table 1; see Supplemental Figure 2B online), with the most dramatic response seen in blue light (Figure 7; see Supplemental Figure 2B online). The aberrant cotyledon phenotype of the OBP3-RNAi1-4-3 transgenic line in blue light requires a functional cry1 (Figure 7A), demonstrating that OBP3 is a negative regulator of cry1-dependent cotyledon expansion in blue light.

Like OBP3, the photoreceptors, including phyA, phyB, and cry1, affect cotyledon expansion in blue light (Neff and Chory, 1998). Aside from these photoreceptors, Ser/Thr Protein Phosphatase 7 (PP7) is the only other identified signaling component involved in blue light–mediated cotyledon expansion (Moller et al., 2003). Mutations in the photoreceptors and in transgenic lines with reduced PP7 levels have smaller cotyledons in blue light, suggesting they are each positive regulators of cotyledon expansion (Neff and Chory, 1998; Moller et al., 2003). To date, OBP3 is the only signaling component identified that negatively regulates cotyledon expansion in blue light (Figure 7A).

In addition to this cry1-mediated cotyledon expansion phenotype, OBP3 acts as a positive regulator of phyB-mediated Figure 7.

(Cotyledon Area (mm²) vs. Light Wavelength)

(A) Cotyledon area from 5-d-old seedlings grown in continuous blue light (17 μM/m²/s). The cry1-102 allele is a cry1-missense mutant, and the cry1-103 allele is a cry1-null mutant.
(B) Cotyledon area from 5-d-old seedlings grown in continuous red light (29 μM/m²/s).
(C) Cotyledon area from 5-d-old seedlings grown in continuous far-red light (33 μM/m²/s). Error bars = ±1 SE. (*) equals P < 0.5, and (**) equals P < 0.001 from a Student’s unpaired two-tail t test comparing the mutant with its control.

Figure 8.

(Possible Models Describing OBP3’s Involvement in phyB and cry1 Signaling)

(A) OBP3 acts as a negative regulator of cry1-mediated cotyledon expansion in blue light and a positive regulator of phyB-mediated inhibition of hypocotyl elongation in red light.
(B) OBP3 is a negative regulator of expansion in the cotyledons and elongation in the hypocotyl. This regulation is modulated both negatively and positively by cry1 and phyB, respectively.
inhibition of hypocotyl elongation in red light. Other components have been identified that are involved in both phytochrome and cryptochrome signaling. Loss-of-function mutations in the basic helix-loop-helix transcription factor, HFR1 (also called RSF1 and REP1) cause reduced deetiolation responses in the hypocotyl when grown in far-red and blue light (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000; Duek and Fankhauser, 2003). A null mutation in the Ca²⁺ binding protein, SUB1, causes hypersensitivity of the hypocotyl to blue light and far-red light (Guo et al., 2001). Unlike HFR1 and SUB1, which are involved in the phyA and cry1 pathways, OBP3 represents a connection between phyB and cry1 signal transduction.

Current models of photomorphogenesis suggest that both early and late signal transduction pathways exist (Quail, 2002; Bauer et al., 2004). We propose that OBP3 is involved in late photomorphogenic signaling events for several reasons. First, the genetic state of OBP3 did not affect photoreceptor mRNA accumulation (data not shown), suggesting that OBP3 operates downstream of these photoreceptors. In addition, OBP3 transcript levels are unaffected by light (Figure 1E), and the changes in tissue-specific localization of an OBP3:OBP3-GUS translational fusion do not occur during the first 24 h after transition from darkness to growth in white light (data not shown). Finally, OBP3’s involvement in multiple photomorphogenic pathways (Figures 5 and 7) suggests that it acts downstream of photoreceptor signal integration.

Based on the data presented here, we propose a model where OBP3 operates as a positive regulator of phyB-mediated hypocotyl elongation inhibition in red light and a negative regulator of cry1-mediated cotyledon expansion in blue light (Figure 8A). Other photomorphogenic signaling components can also have opposite roles in different responses. For example, PIF3, a basic helix-loop-helix transcription factor, acts as both a positive and negative regulator of phytochrome signaling. PIF3 is a negative regulator of hypocotyl elongation inhibition, cotyledon opening, and expansion and a positive regulator of CHS transcript accumulation (Kim et al., 2003; Bauer et al., 2004).

An alternative model is that OBP3 acts as a general negative regulator of cell/tissue expansion, with the photoreceptors differently regulating this response (Figure 8B), because OBP3-RNAi lines are larger as both seedlings and adults (Figures 3, 4, and 6). These two models are not mutually exclusive, as an existing model of hypocotyl elongation proposes two forces controlling growth: one promoting and one opposing (Parks et al., 2001). A similar mechanism may be regulating cotyledon expansion, and the photoreceptors, acting in part through OBP3, may be modulating these forces to control growth during photomorphogenesis (Figure 8).

One mode of action through which OBP3 may function is via hormone signaling pathways. There are several hormones (e.g., auxin, brassinosteroids, and gibberellins) involved in cell expansion that are interconnected with light signaling during photomorphogenesis (Halliday and Fankhauser, 2003; Neff et al., 2005). Multiple suppressors of the long-hypocotyl phenotype of phyB mutants have been identified, which are involved in both photomorphogenesis and hormone signaling (Reed et al., 1998; Neff et al., 1999). BAS1/CYP72B1 (for phyB-4 activation-tagged suppressor 1/cytochrome P450 72B1) inactivates active brassino-steroids, acting as a modulator of photomorphogenesis through hormone inactivation (Neff et al., 1999; Turk et al., 2003). Gain-of-function mutants in the Auxin/Indole Acetic Acid Induced (Aux/IAA) gene, Suppressor of Long Hypocotyl 2 (SHY2)/IAA3, have deetiolation phenotypes in the dark and hyperresponsive hypocotyls in white light (Reed et al., 1998). In addition, this mutation in SHY2/IAA3 affects auxin-dependent root growth, lateral root formation, and the gravitropic response, suggesting that SHY2/IAA3 modulates both light and auxin signaling pathways (Tian and Reed, 1999). Interestingly, OBP3 transcript levels are dramatically induced in response to auxin and salicylic acid (Kang and Singh, 2000), suggesting that this gene may also be acting as a point of interaction between light and hormone signaling. Future experiments will explore OBP3’s possible involvement in hormone signaling as a means of modulating photomorphogenesis.

METHODS

Growth Conditions and Light Sources

Seeds were surface sterilized by shaking in 70% (v/v) ethanol containing 0.05% (v/v) Triton X-100 for 15 min, followed by treatment with 95% (v/v) ethanol for 5 min. After the ethanol washes, seeds were dried on sterile 70-mm Whatman filter paper (Maidstone, UK) in a laminar-flow hood and sown on media containing either 0.8% phytagar (w/v) (Gibco BRL, Gaithersburg, MD), half-strength MS salts (Invitrogen, Carlsbad, CA), 1.5% (w/v) sucrose with antibiotic, or 1.0% phytagel (Sigma-Aldrich, St. Louis, MO), half-strength MS salts. After incubation for 4 d at 4°C, germination was induced by treating seeds with 1 h of red light followed by 23 h of darkness. Seeds were then placed in the appropriate light condition. All incubators were at 25°C.

Plates were incubated in a white light chamber (Model E-30-B: Percival Scientific, Perry, IA) or a light emitting diode incubator (Model E-30-LED: Percival Scientific). White light was supplied by four fluorescent tubes (F17T8/TL741 17 W; Phillips, Eindhoven, The Netherlands) and two incandescent bulbs (PC, 13,487 25T10/F CD 25 W, 120 V; GE, Fairfield, CT). Red and blue lights were supplied by eight fluorescent tubes. Colored Plexiglas was placed on the plates to obtain blue and red light conditions. Far-red light was obtained by the far-red light emitting diodes from the E-30-LED incubator. These diodes were set at 10% of their maximal fluorescence rate. One to five layers of fiberglass window screen were placed over the plates to obtain the appropriate fluence rates of light.

Activation-Tagging Mutagenesis

Arabidopsis thaliana phyB-4 mutant plants, ecotype Columbia (Col-0) were transformed with the activation-tagging construct, pSKI074 (GenBank accession number AF218466) (Weigel et al., 2000) as in Neff et al. (1999). Plants were transformed via the floral dip method using Agrobacterium tumefaciens strain GV3101 (Clough and Bent, 1998). T1 seeds were grown on plates containing kanamycin (30 mg/L) in continuous white light (35 μM/m²/s) for 7 d. The sob1-D phyB-4 double mutant was identified by its shorter hypocotyl when compared with phyB-4 mutant plants. T2 seeds from self-fertilized sob1-D phyB-4 were screened on plates with and without kanamycin to confirm that the mutant phenotype is linked to the transgene. T2 seedlings segregated 3:1 kanamycin resistant:kanamycin sensitive, indicating that there was one locus containing a T-DNA. T3 seeds from self-fertilized T2 plants were screened in the same way to confirm the genetic heritability of the mutant phenotype
and linkage to the transgene. The presence of the phyB-4 mutation was confirmed in the sob1-D phyB-4 mutant as in Neff et al. (1999).

Cloning of the OBP3 Gene

DNA gel blot analysis and plasmid rescue were performed essentially the same as in Neff et al. (1999). The DNA gel blot indicated that the sob1-D phyB-4 mutant contains only one T-DNA insertion. The location of this T-DNA was determined by cloning the flanking genomic DNA via plasmid rescue. Genomic DNA from the sob1-D mutant was digested with HindIII and ligated with T4 DNA ligase (New England Biolabs, Beverly, MA), resulting in a 14.4-kb construct, pSOB1-H4. The genomic DNA flanking the T-DNA was sequenced with a primer (5′-GCTTCTCGGAGGTCGACGG-3′). BLAST analysis indicated that the genomic DNA is from chromosome III, and the closest open reading frame was OBP3 (At3g55370).

RT-PCR Analysis

RT-PCR analysis was performed on total RNA isolated, using the RNasy Plant Mini kit (Qiagen, Valencia, CA), from 5-d-old seedlings grown in continuous white light. Total cDNA was synthesized using SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). PCR was performed on dilute cDNA using Taq polymerase (Invitrogen). PCR was performed to amplify a 475-bp fragment of the OBP3 gene using the 5′-TCTTCTCGGAGGTCGACGG-3′ and 5′-GACGG-3′ primers. The ubiquitin10 (UBQ10) gene was used as an internal control. UBQ10 was amplified using the 5′-GATCATACATATCACCCACACCTC-3′ and 5′-GCAGCTTCATATTGAAAGAGACTGAAGG-3′ primers. The linear range of accuracy for the detection of each transcript was established by comparing samples run for different numbers of cycles. All final RT-PCR experiments were performed at least in duplicate.

Recapitulation of the sob1-D Mutant Phenotype

The sob1-D recapitulation construct was generated by ligating a SacI fragment from pSOB1-H4 into pPZIP212 (Hajdukiewicz et al., 1994) cut with SacI. This SacI fragment from pSOB1-H4 contains the four enhancer elements from the CaMV 35S promoter, the OBP3 open reading frame, and 4.1 kb of noncoding DNA 5′ of the OBP3 gene. The phyB-4 mutant was transformed with this construct via Particle Bombardment (Clough and Bent, 1998). Primary transformants were screened on plates containing kanamycin (30 mg/L).

Construction of the 3SS:OBP3-GFP Fusion and Particle Bombardment

The OBP3 cDNA lacking 59 N-terminal amino acids was amplified from cDNA synthesized from the sob1-D mutant using the following primers: 5′-CATGCCATGGCGAATGTTTC-3′ and 5′-ACATGCCATGGCGAATGTAAGATGGCCTGCTCGG-3′. The fragment was cut with EcoRI and Ncol and inserted into the pCAMBIA1381 (CAMBIA) vector, cut with these same enzymes. This C-terminal fusion to GUS resulted in an Ala linking the two proteins. This portion of the construct, which was created by PCR, was sequenced to confirm that there were no errors affecting the open reading frame.

To view the transformed epidermal cells, the epidermal layer was pulled from the piece of onion with forceps and placed on a microscope slide. The layer of epidermal cells was kept moist with distilled water. The cells were viewed by confocal microscopy using the Leica confocal system TCS2 (Leica Microsystems, Heidelberg, Germany) essentially the same as by Zentella et al. (2002). At least 10 independent transformation events were observed, and all showed the same localization patterns.

Construction of the OBP3:OBP3-GUS Fusion Construct and GUS Analysis

The entire OBP3 gene along with a small portion of the region upstream of the gene was amplified from pSOB1-H4 using the following primers: 5′-TGTGCTCGGACAACTGTTC-3′ and 5′-ACAGGCCCATGCGGAAATGAAGATGGCCTGCTCGG-3′. The PCR product was cut with EcoRI and Ncol and inserted into the pCAMBIA1381 (CAMBIA), cut with these same enzymes. This C-terminal fusion to GUS resulted in an Ala linking the two proteins. This portion of the construct, which was created by PCR, was sequenced to confirm that there were no errors. A 1438-bp region upstream of the OBP3 gene is used to drive the expression of this fusion gene. This region was cloned from the pSOB1-H4 vector by cutting with EcoRI and inserted into the vector containing the OBP3 open reading frame; GUS translational fusion gene, resulting in pJW8. Arabidopsis ecotype Col-0 was transformed with pJW8, and multiple homozygous, single locus insertion lines were identified by screening on plates containing hygromycin (20 mg/L).

GUS analysis was performed in triplicate on 5-d-old seedlings by incubating in GUS staining solution (1 mM 5-bromo-4-chloro-3-indolyl-b-D-glucoronic acid, cyclohexyl ammonium salt, 50 mM NaHPO4, pH 7.2, and 0.05% [v/v] Triton X-100) at 37°C for 24 h. Seedlings were photographed essentially the same as by Turk et al. (2003). Lines 8.4.7.1, 8.7.2, and 8.83.1 were used in the analysis, and all showed similar GUS staining patterns (see Supplemental Figure 1 online).

Construction of OBP3-RNAi Construct

Primers 5′-CGGGGATCCCGGGAACGGCGAATGATG-3′, 5′-CTTCTCATGATATCCTACCCACCTC-3′ and 5′-GACCTACGAGGGAAGGGAAGAGAGATGATG-3′ were used to amplify a 475-bp fragment of the OBP3 gene and 3′ untranslated region. These fragments were cloned into the pHANNIBAL gene is used to drive the expression of this fusion gene. This region was cloned from the pSOB1-H4 vector by cutting with EcoRI and inserted into the vector containing the OBP3 open reading frame; GUS translational fusion gene, resulting in pJW8. Arabidopsis ecotype Col-0 was transformed with pJW8, and multiple homozygous, single locus insertion lines were identified by screening on plates containing hygromycin (30 mg/L). A. tumefaciens strain GV3101. Arabidopsis ecotype Col-0 was transformed with pJW4, and multiple homozygous, single locus insertion lines were identified by screening on plates containing kanamycin (30 mg/L). Arthropod ecotype Col-0 was transformed with pJW4, and multiple homozygous, single locus insertion lines were identified by screening on plates containing kanamycin (30 mg/L).

Genetic Analysis

All mutant lines used in this study are Arabidopsis ecotype Col-0. The cry1-102 and cry1-103 alleles are described by Liscum and Hangarter (1991), Mockler et al. (1999), and Neff et al. (2002). The phyB-9 allele is described by Reed et al. (1998). Double mutants were obtained by crossing and growing seeds on kanamycin-containing plates. F2 seeds were grown in the appropriate light conditions to identify homozygous photoreceptor mutants. Putative double mutants were confirmed in the F3 generation by growing on kanamycin-containing plates to identify lines homozygous for the OBP3-RNAi T-DNA. The photoreceptor mutations were confirmed by PCR analysis using the following primer pairs for each mutation: cry1-102, 5′-TCTTCTCGGACAACTGTTC-3′ and 5′-CGGGGATCCCGGGAACGGCGAATGATG-3′; cry1-103, 5′-ACAGGCCCATGCGGAAATGAAGATGGCCTGCTCGG-3′; cry2-102, 5′-CATGCCATGGCGAATGTTTC-3′; cry2-103, 5′-ACAGGCCCATGCGGAAATGAAGATGGCCTGCTCGG-3′; and cry2-104, 5′-ACAGGCCCATGCGGAAATGAAGATGGCCTGCTCGG-3′.
Fluence Response and Cotyledon Area Measurements

To measure hypocotyl length, 5-d-old seedlings were removed from plates and laid on acetate sheets. The hypocotyls and cotyledons were measured using ImageJ 1.29J (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/java/1.3.1). All experiments were done in triplicate (n ≥ 30) on media with and without sucrose. The data reported is from experiments on media without sucrose.

Epidermal Imprints and Epidermal Cell Area Measurements

Five-day-old seedlings grown in continuous white light were used for epidermal imprints. Imprints were taken by coating the top of the cotyledon with clear fingernail polish. After drying, the polish was removed with transparent tape and digitized as above. The hypocotyls and cotyledons were measured using ImageJ 1.29J (National Institutes of Health).

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REFERENCES


Corrections


An incorrect column heading was published in Table 1. The incorrect heading read Cotyledon Epidermal Cell Area $\mu\text{M}^2$ (st). The correct column heading is Cotyledon Epidermal Cell Area $\mu\text{m}^2 \times 10^{-4}$ (st).

The authors of the articles listed below discovered an error in a spreadsheet developed by their laboratory. This error resulted in an incorrect conversion of radioactive counts to the appropriate molar units. The absence of a 1000-fold difference led them to report their auxin transport values as pmol instead of reporting them correctly as fmol. The following articles were affected by this conversion error.


The units of radioactive indole-3-acetic acid transport were mislabeled in Figures 3, 4A, and 4B and in Tables 4 and 5, as were the units of radiolabeled benzoic acid in Table 2. Both control and experimental values should have been reported as fmol rather than pmol. This change in units does not alter the interpretation of the data, since all experiments had internal controls.


The units of radioactive indole-3-acetic acid transport were mislabeled in Figure 8. Both control and experimental values should have been reported as fmol rather than pmol. This change in units does not alter the interpretation of the data, since all experiments had internal controls.
The Dof Transcription Factor OBP3 Modulates Phytochrome and Cryptochrome Signaling in Arabidopsis

Jason M. Ward, Carie A. Cufr, Megan A. Denzel and Michael M. Neff

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| Supplemental Data | /content/suppl/2005/01/13/tpc.104.027722.DC1.html |
| References | This article cites 50 articles, 29 of which can be accessed free at: /content/17/2/475.full.html#ref-list-1 |
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