The Light-Response BTB1 and BTB2 Proteins Assemble Nuclear Ubiquitin Ligases That Modify Phytochrome B and D Signaling in Arabidopsis

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Members of the Bric-a-Brac/Tramtrack/Broad Complex (BTB) family direct the selective ubiquitylation of proteins following their assembly into Cullin3-based ubiquitin ligases. Here, we describe a subfamily of nucleus-localized BTB proteins encoded by the LIGHT-RESPONSE BTB1 (LRB1) and LRB2 loci in Arabidopsis (Arabidopsis thaliana) that strongly influences photomorphogenesis. Whereas single lrb1 and lrb2 mutants are relatively normal phenotypically, double mutants are markedly hypersensitive to red light, but not to far-red or blue light, and are compromised in multiple photomorphogenic processes, including seed germination, cotyledon opening and expansion, chlorophyll accumulation, shade avoidance, and flowering time. This red light hypersensitivity can be overcome by eliminating phytochrome B (phyB) and phyD, indicating that LRB1/2 act downstream of these two photoreceptor isoforms. Levels of phyB/D proteins but not their messenger RNAs are abnormally high in light-grown lrb1 lrb2 plants, implying that their light-dependent turnover is substantially dampened. Whereas other red light-hypersensitive mutants accumulate phyA protein similar to or higher than the wild type in light, the lrb1 lrb2 mutants accumulate less, suggesting that LRB1/2 also positively regulate phyA levels in a phyB/D-dependent manner. Together, these data show that the BTB ubiquitin ligases assembled with LRB1/2 function redundantly as negative regulators of photomorphogenesis, possibly by influencing the turnover of phyB/D.

The selective breakdown of short-lived regulatory proteins is a key feature of many signal transduction pathways, thus providing a mechanism to enhance or quench output when repressors or activators are the respective targets. In eukaryotes, much of this specific turnover is mediated by the ubiquitin/26S proteasome system (UPS; Small and Vierstra, 2004; Kerscher et al., 2006). Here, polymeric chains of ubiquitin (Ub) are covalently attached to proteins destined for degradation via an ATP-dependent, E1-E2-E3 conjugation cascade. These poly-Ub moieties are then recognized by the 26S proteasome, a 2.5-MDa protease complex that directs proteolysis of the target with the concomitant release of the Ub moieties for reuse. The specificity of the UPS resides mainly in the E3 Ub protein ligases that choose appropriate proteins for ubiquitylation. E3s recognize structural motifs in the target and then catalyze an isopeptide linkage between the C-terminal Gly of Ub and an accessible lysl ε-amino group in the target, using an E2-Ub thioester intermediate as the donor. Substantial variations in E3 sequence and organization generate the wide range of specificities needed to handle the myriad of likely UPS substrates (Vierstra, 2009). In Arabidopsis (Arabidopsis thaliana), for example, phylogenetic analyses have identified well over 1,500 possible E3 genes, with genetic studies on a few implying that many have unique substrates (Vierstra, 2009; Hua and Vierstra, 2011).

The multisubunit Cullin-RING Ligases (CRLs) are a highly polymorphic collection of E3s composed of a Cullin (CUL) backbone subunit onto which assembles the E2-Ub-docking RING Box1 (RBX1) protein and a diverse assortment of adaptors that recruit ubiquitylation substrates (Hua and Vierstra, 2011). One prominent CRL subtype encompasses the Bric-a-Brac/Tramtrack/Broad Complex (BTB) E3s. They contain RBX1, the CUL3 isoform, and a member from the large family of BTB target-recognition adaptors that docks with CUL3 via a signature approximately 95-amino acid BTB domain (Furukawa et al., 2003; Xu et al., 2003; Stogios et al., 2005). In Arabidopsis and rice (Oryza sativa ssp. japonica), 81 and 149 BTB proteins are predicted that employ an array of target recognition domains, including Meprin and TRAF Homology, NON-PHOTOTROPIC HYPOCOTYL3, ankyrin, TAZ-zinc finger, and tetracopeptide repeat domains (Dieterle et al., 2003).
BTB E3s Regulate Photomorphogenesis

In this report, we add another set of UPS components to the phy regulatory system with the discovery of a pair of nucleus-localized LIGHT-RESPONSE BTB (LRB1 and -2) proteins in Arabidopsis that negatively influence phy action. Whereas the single lrb1 and lrb2 null mutants respond normally to light, the lrb1 lrb2 double mutants have altered photomorphogenesis and are strongly hypersensitive to R but not B or FR. Genetic analyses pinpoint their activity downstream of phyB/D, with biochemical studies implicating the resulting BTBLRB1/2 E3 complexes in the control of phyB/D accumulation. The R hypersensitivity of the mutants further demonstrates the importance of phy levels to appropriate light responsiveness.

RESULTS

LRB1 and LRB2 Are Members of a Conserved Subfamily of BTB Proteins in Plants

To investigate the range of functions controlled by BTB E3s in plants, we undertook a reverse genetic analysis of representative loci from the 81-member Arabidopsis family (Dieterle et al., 2005; Figueroa et al., 2005; Gingerich et al., 2005, 2007). Initial genetic analyses with Arabidopsis have shown that several BTB subfamilies regulate physiologically important processes in plants. Examples include roles in ethylene biosynthesis (Wang et al., 2004; Christians et al., 2009), abscisic acid signaling (Lechner et al., 2011), plant defense (Sopel et al., 2009), gametophyte development (Robert et al., 2009), phototropism and auxin-regulated organogenesis (Motchoulski and Liscum, 1999; Sakai et al., 2000; Cheng et al., 2007), and leaf/flower morphogenesis (Hepworth et al., 2005).

Given the importance of light for both photosynthesis and as a measure of diurnal and seasonal cycles, plants have evolved a sophisticated set of interconnected transduction pathways to perceive and respond appropriately to light. Upstream are three main classes of photoreceptors, UVR8, cryptochromes/phototropins, and phytochromes (phys), which enable detection of the near-UV, blue (B), and red (R)/far-red (FR) light regions of the light spectrum, respectively (Schafer and Nagy, 2005; Rizzi et al., 2011). Phys are a collection of dimeric biliproteins that can assume two photointerconvertible states, a R-absorbing Pr conformer that represents the ground state, and a FR-absorbing Pfr conformer that represents the biologically active state and is generated only upon R excitation (Quail, 2002; Rockwell et al., 2006). Through their ability to phototransform repeatedly between Pr and Pfr, phys uniquely act as long-lived photo-switches in various signaling cascades. As Pr, phys are mostly localized to the cytoplasm, but after photoconversion to Pfr, they are rapidly imported into the nucleus, where they substantially alter much of the transcriptome (Quail, 2002; Bae and Choi, 2008). Ultimately, almost all aspects of a plant’s life cycle are affected, including seed germination, seedling photomorphogenesis, leaf expansion, shade avoidance, entrainment of circadian rhythms, and floral induction. Whereas processes early in the transition from etiolated to green seedlings respond acutely to the amount of Pfr, processes in more mature green plants often respond to the relative ratio of Pr to Pfr, thus providing a simple form of color vision (Quail, 2002; Rockwell et al., 2006).

Plants typically express a small family of phys that have distinct and overlapping roles in R/FR perception. For example, Arabidopsis ecotype Columbia-0 (Col-0) assemblies five isoforms, phyA to phyE (Quail, 2002; Rockwell et al., 2006). PhyA is the dominant member in etiolated seedlings early during photomorphogenesis and under FR-rich environments, whereas phyB, and to a lesser extent its paralog phyD, are the dominant members in more mature plants in white light or R-rich environments.

Although the initial signaling event(s) after Pfr photoconversion have not been completely resolved, genetic studies have implicated members of the Phytochrome-Interacting Factor (PIF) family as key intermediates, which belong to the basic helix-loop-helix (bHLH) superfamily of transcriptional regulators (Bae and Choi, 2008; Leivar and Quail, 2011). For most PIFs studied, degradation is greatly accelerated upon association with Pfr, suggesting that phys act primarily to promote PIF turnover, thus releasing their influence on photomorphogenesis (Monte et al., 2004; Al-Sady et al., 2006). PIFs typically act as negative photomorphogenic regulators (e.g. PIF1, -3, -4, -5, and -7; Huq and Quail, 2002; Leivar et al., 2008a, 2008b). However, an example where a PIF-type bHLH acts in opposition to other PIFs (Hornitschek et al., 2009) implies that the relationship between this bHLH subfamily and phy signaling is complex.

In addition to the downstream activities that process phy signals, the amount of each phy isoform can influence proper photoperception (Clough and Vierstra, 1997; Quail, 2002). Whereas decreased phy levels lead to R/FR hyposensitivity (Parks and Quail, 1993; Reed et al., 1993), even subtle increases in phy levels (e.g. 2-fold) often generate plants hypersensitive to R/FR (Boylan and Quail, 1989, 1991; Cherry et al., 1992; Wester et al., 1994; Leivar et al., 2008a, 2008b). Phy levels are delicately balanced by controls on the rates of PHY gene transcription and on the rates of photoreceptor degradation. For example, phyA levels drop dramatically in R or FR as a consequence of decreased transcription coupled with rapid breakdown of the Pfr conformer (half-life approximately 1–2 h; Somers and Quail, 1995; Clough and Vierstra, 1997). Similar but less dramatic declines in phyB to phyE levels also occur following R irradiation (Sharrock and Clack, 2002). The UPS has been implicated in phyA breakdown following photoconversion, but the exact mechanism remains unclear (Shanklin et al., 1987; Jabben et al., 1989). To date, the CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) accessory subunit of CUL4-based CRL E3s (Seo et al., 2004; Jang et al., 2010) and the CUL1 scaffold subunit in Skp1-CUL1-F-box protein (SCF)-type CRLs (Quint et al., 2005; Gillkerson et al., 2009) have been implicated in Arabidopsis.
Two genes of particular interest were At2g46260 and At5g61600 (now designated LRB1 and LRB2, respectively), based on their high amino acid sequence identity (88%) and the colinearity of their encoded proteins, both within the BTB domain and the surrounding regions, some of which we presume are involved in target recognition (Supplemental Figs. S1, S2, and S4). A third locus, At4g01160 (designated LRB3), was also identified that has the same domain organization and shares reasonable amino acid sequence similarity (55% identity). Alignments and homology searches discovered a BTB-and-C-terminal-Kelch (BACK) domain downstream of the BTB domain and a putative nuclear localization sequence (NLS) near the N terminus of all three polypeptides (Fig. 1A; Supplemental Fig. S1).

Analysis of the Genevestigator and Massively Parallel Signature Sequencing DNA expression databases revealed that LRB1 and LRB2 are broadly transcribed in a wide variety of tissues and throughout all stages of Arabidopsis development and are not dramatically up- or down-regulated by a host of environmental conditions (http://www.genevestigator.ethz.ch and http://mpss.udel.edu). The only notable difference was a higher level of LRB1 mRNA in pollen. Transcripts for LRB1 and LRB2 were also evident in the EST database (www.arabidopsis.org), with values of 57 for LRB1 and 64 for LRB2, implying a similar expression strength for the two loci. Comparable expression data were absent for LRB3 (last checked on September 27, 2011), suggesting that this gene is silent. However, we could generate sequence-verified complementary DNAs (cDNAs) for LRB3 by reverse transcription (RT)-PCR of Arabidopsis seedling mRNA, indicating that the locus is transcribed (Fig. 1B).

Confirmation that the LRB1 and LRB2 proteins assemble with CUL3 to generate BTB-type Ub ligases was provided by yeast two-hybrid (Y2H) and coimmunoprecipitation assays. Both BTB proteins interacted specifically with the CUL3a and CUL3b isoforms by Y2H, similar to another BTB family member from Arabidopsis (At4g08455), but not with CUL1, which scaffolds SCF-type CRLs (Fig. 2A). CUL1 was shown to be active by Y2H interaction with its adaptor protein ASK1. Interestingly, LRB1 and LRB2 associated with self and each other but not with the BTB protein At4g08455 or the representative F-box proteins CORONATINE-INSSENSITIVE1 (COI1) and UNUSUAL FLORAL ORGANS (UFO), which serve as target adaptors for SCF CRLs, indicating that LRB1 and LRB2 may homodimerize and heterodimerize (Fig. 2A). Such associations have been found with other CRL-based substrate adaptor proteins (Bosu and Kipreos, 2008; Hua and Vierstra, 2011), suggesting that the assembled BTB–LRB complexes work as dimers.

Using transgenic seedlings expressing LRB1 and L2 fused to the C terminus of GFP (see below), we confirmed that LRB1/2 also interact with CUL3 in planta. Immunoprecipitation of GFP-LRB1/2 from the resulting crude extracts with anti-GFP antibodies enriched

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**Figure 1.** Description of the LRB gene family and associated T-DNA insertion mutants. A, Diagrams of the LRB1, LRB2, and LRB3 genes in Arabidopsis. Boxes and lines denote exons and introns, respectively. Numbers to the right indicate amino acid (aa) length. The positions of the T-DNA insertions are indicated by triangles. The locations of the primers used for RT-PCR analysis in B are shown by the half arrows. Regions predicted to encode NLS, BTB, and BACK domains are highlighted by the shaded boxes. B, RT-PCR analysis of mRNA isolated from wild-type (WT) and homozygous lrb1-1, lrb2-1, lrb2-2, lrb2-3, and lrb3-1 seedlings. The locations of the primers used are shown in A. RT-PCR analysis of PAE2 is included as a control. C, Phylogenetic tree of LRB sequences from 11 plant species. The tree was generated by maximum likelihood (ML) analysis from a Gblocks-edited full-length sequence alignment (Supplemental Fig. S2). Asterisks at selected internal nodes indicate branches identified by maximum likelihood, maximum parsimony, and neighbor-joining analyses at 70% or greater bootstrap values. Species abbreviations are as follows: Arabidopsis (At), A. lyrata (Al), Vitis vinifera (Vv), G. max (Gm), P. trichocarpa (Pt), rice (Os), Sorghum bicolor (Sb), Brachypodium distachyon (Bd), Zea mays (Zm), S. moellendorffii (Sm), and Physcomitrella patens (Pp). Gene locus names, corresponding accession numbers, and database identifiers are listed in Supplemental Data Set S1. The scale bar reflects the number of substitutions per site.
Many species contain multiple members (four in Arabidopsis) that cluster phylogenetically with LRB1/2. In contrast, a close relative of LRB3 was found only in Arabidopsis lyrata, suggesting that the LRB3 locus appeared more recently during the emergence of the Arabidopsis lineage. LRB3 sits within a segmental duplication block on chromosome 2 that is syntenic with a region on chromosome 4 that includes LRB1, implying that LRB1 is its likely progenitor (Vision et al., 2000). Using the analysis of nonsynonymous (Ka) versus synonymous (Ks) substitution frequencies as an estimate of evolution rates, it appears that LRB3 has experienced much less purifying selection as compared with LRB1 and LRB2. Whereas the Ka/Ks values for the LRB1 and LRB2 orthologs from Arabidopsis and A. lyrata are 0.062 and 0.031, respectively, the Ka/Ks value for the LRB3 counterparts is 0.29, thus representing a 5- to 10-fold increase in diversification rate. This higher diversification rate combined with poor evidence of expression and limited species distribution strongly imply that the LRB3 locus is a recent addition to the LRB subfamily and is on the path to pseudogenization.

Figure 2. LRB1 and LRB2 interact with CUL3. A, Y2H interaction of LRB1 and LRB2 with CUL3a and CUL3b. Interactions of CUL3 with the BTB protein A4g08455, CUL1 with ASK1, and UFO with ASK1 were included as positive controls. The empty Y2H vectors pGBK7 and pGADT7 and p53 in pGBK7 were used as negative controls. B, Coimmunoprecipitation of CUL3 with GFP-LRB1/2. Extracts prepared from transgenic lines expressing GFP, GFP-LRB1, or GFP-LRB2 maintained in the dark (D) or irradiated for 12 h with R (20 μmol m⁻² s⁻¹) were immunoprecipitated with anti-GFP antibodies, and the immunoprecipitates were subjected to SDS-PAGE and immunoblot analysis with anti-CUL3a antibodies (top panel). The middle and bottom panels show the input extracts probed with anti-GFP and anti-tubulin (TUB) antibodies (control). A shorter exposure was used to show the presence of GFP in the plants expressing free GFP alone. The asterisk identifies a species cross-reacting with the anti-GFP antibodies.

To help appreciate the importance of the LRB subfamily, we queried a number of other sequenced plant genomes for relatives. Likely orthologs with identical NLS-BTB-BACK organizations, similar BTB domain sequences, and similar C-terminal regions were identified in representative seedless plants, monocots, and eudicots but not in any algae, fungi, or animals, implying that the LRB subfamily appeared early in plant evolution and thus likely influences a land plant-specific process (Fig. 1C; Supplemental Figs. S1–S5). Many species contain multiple members (four in Glycine max and Populus trichocarpa and gene pairs in many monocots and eudicots) that cluster phylogenetically with LRB1/2. In contrast, a close relative of LRB3 was found only in Arabidopsis lyrata, suggesting that the LRB3 locus appeared more recently during the emergence of the Arabidopsis lineage. LRB3 sits within a segmental duplication block on chromosome 2 that is syntenic with a region on chromosome 4 that includes LRB1, implying that LRB1 is its likely progenitor (Vision et al., 2000). Using the analysis of nonsynonymous (Ka) versus synonymous (Ks) substitution frequencies as an estimate of evolution rates, it appears that LRB3 has experienced much less purifying selection as compared with LRB1 and LRB2. Whereas the Ka/Ks values for the LRB1 and LRB2 orthologs from Arabidopsis and A. lyrata are 0.062 and 0.031, respectively, the Ka/Ks value for the LRB3 counterparts is 0.29, thus representing a 5- to 10-fold increase in diversification rate. This higher diversification rate combined with poor evidence of expression and limited species distribution strongly imply that the LRB3 locus is a recent addition to the LRB subfamily and is on the path to pseudogenization.

LRB1 and LRB2 Together Control R-Mediated Responses in Arabidopsis

Searches of the SIGNAL T-DNA insertion population generated with the Arabidopsis Col-0 ecotype identified single mutants in LRB1 (lrb1-1) and LRB3 (lrb3-1) and three mutants in LRB2 (lrb2-1, lrb2-2, and lrb2-3) that might disrupt expression of the affected genes (Fig. 1A). RT-PCR analysis of homozygous lines indicated that all five mutations block synthesis of the full-length transcript but that often partial transcripts upstream but not downstream of the insertion site accumulate (Fig. 1C). Based on the fact that mRNAs generated from the lrb1-1, lrb2-1, and lrb3-1 mutants would be missing the region encoding the BTB domain, we predict that these mutations represent protein-null alleles even if the truncated mRNAs are translated.

Initial observations of the lrb single mutant collection grown under standard growth chamber conditions (22°C under white light in long days [LDs; 16 h of light/8 h of dark] or short days [SDs; 8 h of light/16 h of dark]) did not reveal any striking phenotypic abnormalities, suggesting that the trio, or at least LRB1 and LRB2, work together in Arabidopsis (Fig. 3A). The only obvious effect was a slight compaction of rosette growth for lrb2 plants in SDs (Fig. 3B). Consequently, we generated double homozygous mutants combining lrb1-1 with the lrb2-1, lrb2-2, or lrb2-3 allele and found that they displayed dramatic photomorphogenic defects. All three lrb1 lrb2 combinations (lrb1-1 lrb2-1, lrb1-1 lrb2-2, and lrb1-1 lrb2-3) grown under white light developed dwarfed rosettes with significantly shorter petioles in LDs and SDs and flowered later, as determined by the number of leaves generated before bolting in LDs (Fig. 3). Whereas etiolated lrb1 lrb2 seedlings were phenotypically normal (Fig. 4B), those exposed to light had substantially shorter hypocotyls and increased cotyledon size, consistent with the
possibility that the lrb1 lrb2 mutants are light hypersensitive.

To identify which of the photoreceptor systems might be affected, we exposed etiolated lrb1 lrb2 seedlings to various fluence rates of continuous red light (Rc), continuous far-red light (FRc), or continuous blue light (Bc) and measured their hypocotyl lengths after 4 d. As shown in Figure 4A, the three lrb1 lrb2 combinations but not the single mutants displayed a striking hypersensitivity to Rc only. Under all FRc and Bc fluences tested, the lrb1 lrb2 mutants responded identically to the wild type, but under Rc, the mutant hypocotyls were significantly shorter (e.g. 10.4 mm for the wild type versus 1.6 mm for lrb1 lrb2 seedling exposed to 10 μmol m$^{-2}$ s$^{-1}$ Rc).

Whereas the lrb1-1 single mutant had the same R sensitivity as the wild type with respect to hypocotyl elongation, we noticed that the lrb2-1, lrb2-2, and lrb2-3 single mutants were slightly more sensitive to intermediate Rc fluence rates (1–10 μmol m$^{-2}$ s$^{-1}$; Fig. 4A). Thus, consistent with the morphology of more mature lrb2 plants in SDs, LRB2 may have more impact on photomorphogenesis as compared with LRB1. Given that LRB1 and LRB2 likely work with CUL3a and CUL3b in BTB$^{\text{LRR1/2}}$ complexes, we also tested the Rc response of the cul3a-1 and cul3b-1 null single mutants (Gingerich et al., 2005). (The cul3a-1 cul3b-1 double mutant is embryo lethal, thus precluding its analysis.) Like the lrb2-1 line, hypocotyl elongation of these cul3 mutant lines was also slightly more sensitive to Rc (Supplemental Fig. S6A). It was reported previously that Suc availability modulates R perception in Arabidopsis (Kozuka et al., 2005), suggesting that the Rc hypersensitivity seen here could be driven by Suc hypersensitivity. Repeat analysis of seedlings grown on medium without Suc indicated that this scenario is not the case for the lrb1 lrb2 mutants. Hypocotyl elongation of lrb1-1 lrb2-1 seedlings remained Rc hypersensitive as compared with the wild type and the single lrb1-1 and lrb2-1 mutant seedlings (Supplemental Fig. S6B).

Even though the accumulating genomic data implied that LRB3 is nonfunctional, it remained possible that it plays a minor role as compared with LRB1 and LRB2 or becomes important when its paralogs are absent. To test for these scenarios, we generated all homozygous mutant combinations of lrb3-1 with lrb1-1 and lrb2-1. The single lrb3-1 mutant, the double lrb1-1 lrb3-1 and lrb2-1 lrb3-1 mutant combinations, and the triple lrb1-1 lrb2-1 lrb3-1 mutant behaved similar to comparable lines wild type for LRB3, strongly suggesting that the LRB3 protein contributes little or nothing to R perception, at least in young seedlings (Fig. 5).

To confirm that disruption of LRB1 and LRB2 alone generated the photomorphogenic defects, we attempted to link the phenotypes to the lrb1-1 lrb2-1 genotype by cosegregation analysis and by complementation of the lrb1-1 lrb2-1 mutant with a LRB1 transgene. In the former approach, individual seedlings from a segregating population obtained by self-crossing a heterozygous lrb1-1 lrb2-1 plant were tested for Rc hypersensitivity and PCR genotyped for the wild-type and LRB1 type for lrb1-1 lrb2-1/+ parent were tested for Rc hypersensitivity and PCR genotyped for the wild-type and lrb1-1 lrb2-1/+ parent were tested for Rc hypersensitivity and PCR genotyped for the wild-type and lrb1-1 lrb2-1/+ parent were tested for Rc hypersensitivity and PCR genotyped for the wild-type and lrb1-1 lrb2-1/+ parent were tested for Rc hypersensitivity and PCR genotyped for the wild-type and lrb1-1 lrb2-1/+ parent were tested for Rc hypersensitivity and PCR genotyped for the wild-type. As shown in Supplemental Figure S7, we observed a tight linkage between the short-hypocotyl phenotype in Rc and homozygosity for both lrb1-1 and lrb2-1. In the latter approach, we attempted to rescue the Rc-hypersensitive phenotype of lrb1-1 lrb2-1 seedlings with the full-length LRB1 protein bearing an N-terminal Flag epitope tag and expressed under the control of the cauliflower mosaic virus 35S promoter.
A number of independent transgenic lines were identified that expressed the Flag-LRB1 mRNA and accumulated the Flag-LRB1 protein in the homozygous lrb1-1 lrb2-1 background (Fig. 4, C and D). These lines substantially rescued the R hypersensitivity of hypocotyl elongation near to that of the wild type (Fig. 4, E and F). Taken together, we conclude that LRB1 and LRB2 act redundantly in BTB E3 complexes to negatively regulate photomorphogenesis in response to R.

LRB1 and LRB2 Influence Light Responses Controlled by phyB and phyD

The R hypersensitivity of lrb1 lrb2 seedlings, the phenotype of white light-grown rosettes, and the late flowering of mature plants were all consistent with the encoded BTB proteins repressing pathway(s) initiated by phyB and possibly phyD. To further examine this possibility, we tested the reaction of lrb1 lrb2 plants to a range of responses under phyB/D control (Schafer and Nagy, 2005; Bae and Choi, 2008). For example, phyB is the principal inducer of seed germination when seeds kept in the dark are irradiated with white light via a mechanism that can be reversed by a subsequent irradiation with FR (Shinomura et al., 1996). As shown in Figure 6A, such white light irradiation can substantially improve the germination of wild-type seeds but not of seeds harboring the previously described PHYB null allele phyB-9 (Reed et al., 1993). Subsequent irradiation with a short pulse of 3 μmol m⁻² s⁻¹ FR can block this promotion, indicating that the Pfr form of phyB is required. Whereas the lrb1-1 and lrb2-1 single mutant seeds responded like...
the wild type to the light stimulus, the lrb1-1 lrb2-1 double mutant seeds were relatively immune to subsequent FR inhibition; even a high-fluence-rate pulse of FR (50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) failed to inhibit the germination of most white light-irradiated lrb1-1 lrb2-1 seeds (Fig. 6A).

phyB also plays a central role in the end-of-day (EOD) FR response of young seedlings, in which a short pulse of FR before a prolonged dark period can partially overcome the R-induced inhibition of hypocotyl elongation by converting most phyB back to Pr (Fig. 6B). Consistent with previous studies on phy mutants (Devlin et al., 1998), we found that EOD-FR did not affect hypocotyl elongation of phyB-9 seedlings. In contrast, EOD-FR strongly stimulated the elongation of lrb1-1 lrb2-1 hypocotyls (Fig. 6B). phyB promotes the unfolding and expansion of young cotyledons in response to increasing Rc fluence rates (Fankhauser and Casal, 2004). Whereas cotyledon opening and expansion were substantially blocked in phyB-9 seedlings, these processes were more robust in lrb1-1 lrb2-1 seedlings as compared with the wild type (Fig. 6D; Supplemental Fig. S8A). At the molecular level, R irradiation signaled through the Pfr form of phyB is known to stimulate the expression of various photosynthetic genes, including the nuclear locus encoding the CAB2 chlorophyll \( a/b \)-binding protein (Hamazato et al., 1997), and to enhance chlorophyll accumulation (Huq et al., 2004). Accordingly, we readily detected by real-time quantitative (q)PCR an increased amount of CAB2 transcript in wild-type seedlings within 4 h after R irradiation, which increased faster and more strongly in lrb1-1 lrb2-1 seedlings (Fig. 6C). Likewise, chlorophyll levels in seedlings exposed to Rc for 4 d were lower than normal in the phyB-9 mutant, unaltered in the lrb1-1 and lrb2-1 single mutants, and higher than normal in the lrb1-1 lrb2-1 double mutant (Supplemental Fig. S8B).
PHYB and PHYD Are Epistatic to LRB1 and LRB2

All the phenotypic defects observed here for *lrb1 lrb2* plants were consistent with the BTB-RB1/2 complexes negatively regulating signaling downstream of phyB and possibly phyD. To test for this epistasis directly, we introgressed the *lrb1-1* and *lrb2-1* mutations into the *phyB-9* and *phyD-2* null mutant backgrounds and examined how the mutant combinations responded to various light regimes detected by phyB/D (see above). The *phyB-9* allele in the Col-0 background (formally called *hy3-EMS142* [Reed et al., 1993]) contains a premature stop codon that was introduced by ethyl methanesulfonate mutagenesis; it is a null protein but still expresses low levels of the *PHYB* transcript (Supplemental Figs. S9D and S11, A and B). The *phyD-2* mutant used here was obtained from the SIGNAL mutant collection generated with the Col-0 background. It contains a T-DNA insertion within the *fi* first exon and was found by RT-PCR and by immunoblot analysis to block accumulation of the full-length *PHYD* transcript and the phyD polypeptide, respectively (Supplemental Figs. S9, A and B, and S11A). Homozygous plants harboring various combinations of the *lrb1-1*, *lrb2-1*, *phyB-9*, and *phyD-2* mutations were located by genomic PCR and, for the *phyB-9* mutation specifically, by the absence of the phyB protein as determined by immunoblot analysis of seedling extracts (Supplemental Fig. S9, C and D).

The sum of the epistasis analyses confirmed that LRB1 and LRB2 work downstream of phyB and phyD. As shown in Figure 7, A and B, the effects of Rc (10 and 125 μmol m⁻² s⁻¹) and EOD-FR on etiolated hypocotyl elongation, which were absent or weakened in homozygous *phyB-9*, *phyD-2*, and *phyB-9 phyD-2* plants and accentuated in *lrb1-1 lrb2-1* plants, were absent in the *phyB-9 lrb1-1 lrb2-1* triple mutant and the *phyB-9 phyD-2 lrb1-1 lrb2-1* quadruple mutant. In fact, the quadruple mutant was insensitive to even high fluence rates of Rc, similar to the *phyB-9 phyD-2* double mutant. Flowering time in LDs was earlier in *phyB-9* and *phyB-9 phyD-2* plants but later in *lrb1-1 lrb2-1* plants (Fig. 7C). The flowering delay for *lrb1-1 lrb2-1* plants was partially reversed by introgressing the *phyB-9* allele and more fully reversed in the quadruple mutant. Likewise, cotyledon expansion for triple and quadruple mutant seedlings when exposed to Rc resembled *phyB-9* seedlings but not *lrb1-1 lrb2-1* seedlings (Fig. 7D). For both hypocotyl elongation and chlorophyll accumulation in response to white light, introgressing both the *phyB-9* and *phyD-2* alleles similarly reversed the phenotype of the *lrb1-1 lrb2-1* mutant so that they now mirrored *phyB-9* *phyD-2* plants (Supplemental Fig. S10, A and B).

For all the responses, inactivation of PHYB in the *lrb1-1 lrb2-1* background had the greatest impact in reversing the phenotype of *lrb1-1 lrb2-1* plants, indicating that the BTB-RB1/2 complexes work primarily through phyB. However, for some of the phenotypes, we either observed subtle suppression of the *lrb1-1 lrb2-1*
phenotype when PHYD was inactivated alone or accentuated suppression when the phyB-9 and phyD-2 mutants were combined in the quadruple mutant (Figs. 7D; Supplemental Fig. S10, B and C), suggesting that these E3s also impact phyD signaling. The nearly complete suppression of the lrb1-1 lrb2-1 phenotypes in the quadruple mutant further implied that the BTB\(^{lrb1/lrb2}\) complexes do not also act through the remaining phyA, phyC, and phyE isofoms.

**phyB and phyD Protein Stability Is Controlled by LRB1 and LRB2**

Given that even small increases in phyB (and possibly phyD) levels can profoundly alter Arabidopsis photomorphogenesis (Boylan and Quail, 1989, 1991; Wester et al., 1994; Leivar et al., 2008a) and the likely role of BTB\(^{lrb1/lrb2}\) in protein ubiquitylation and turnover, we tested whether the lrb1 lrb2 phenotypes are generated by increased phyB/D accumulation as a consequence of dampened UPS-mediated breakdown. Whereas phyB and phyD levels in the lrb1-1 and lrb2-1 single mutants as well as all three combinations of lrb1 lrb2 double mutants were unaltered in dark-grown seedlings as compared with the wild type (Supplemental Fig. S11A), increased relative amounts of the two photoreceptors were obvious when the double mutants were exposed to Rc for 4 d (2- to 4-fold and 5- to 8-fold, respectively; Fig. 8A), indicating that LRB1 and LRB2 together suppress phyB/D accumulation (Fig. 8A).

qRT-PCR analyses showed that the steady-state levels of the PHYB and PHYD mRNAs were not significantly affected by the lrb1-1 lrb2-1 combination in the dark or under Rc, implying that these increases were not driven by up-regulated transcription in the absence of LRB1/2 (Supplemental Fig. S11B). Conversely, we found that LRB1 and LRB2 together likely promote light-induced turnover of the phyB/D proteins. Upon Rc irradiation of etiolated seedlings, phyB and phyD levels steadily declined in the wild type such that after 48 h most of the photoreceptors disappeared (Fig. 8B). These rates of loss were markedly slower in the lrb1-1 lrb2-1 background. Importantly, phyB breakdown was also substantially attenuated upon exposing wild-type seedlings to the proteasome inhibitor MG132, thus implicating the 26S proteasome in particular (Fig. 8C). MG132 treatment also slightly stabilized phyB levels in lrb1-1 lrb2-1 seedlings (Fig. 8C), implying that other factors besides LRB1 and LRB2 might also direct phyB/D breakdown. It should be noted that Rc appeared to have little or no impact on LRB1 and LRB2 transcript levels, indicating that the light-induced loss of phyB/D is not driven by increased expression of LRB1/2 (Supplemental Fig. S12C).

In addition to phyB/D, we discovered that LRB1/2 is turned over in Arabidopsis seedlings via a process that appears to be light independent (Supplemental Fig. S12, A and B). Treatment of etiolated seedlings with the translation inhibitor cycloheximide accelerated the loss of Flag-LRB1 both in darkness and during Rc irradiation. This loss could be slowed by MG132, suggesting that these BTB proteins may be targets of autoubiquitylation and subsequent breakdown.

**LRB1 and LRB2 Are Nucleus Localized**

Given that phyB degradation is proposed to occur in the nucleus following import as Pr and subsequent association with punctate nuclear bodies also called speckles (Jang et al., 2010), it was possible that LRB1/2 colocalize with phyB/D and/or affect their relocation. Using a previously developed phyB C-terminal-GFP reporter (Boccalandro et al., 2009) combined with fluorescence confocal microscopy, we confirmed the nuclear sequestering and aggregation of phyB under Rc. As observed previously (Yamaguchi et al., 1999; Kircher et al., 2002), we found phyB-GFP diffusively distributed throughout the cytoplasm and nucleus in etiolated hypocotyls and then more enriched in the nucleus under Rc concomitant with its concentration in nuclear speckles (Fig. 9A). The R-induced redistribution of phyB-GFP into speckles was indistinguishable in the lrb1-1 lrb2-1 background, indicating that LRB1/2 does not influence this nuclear aggregation (Fig. 9A).

To determine if LRB1 and LRB2 also localize to the nucleus, possibly via their predicted NLS motifs, and similarly coalesce in nuclear speckles, we examined their intracellular distribution in root tips using full-length, N-terminal GFP fusions expressed under the UBQ10 promoter. Following introduction of the UBQ10-GFP-LRB1 and UBQ10-GFP-LRB2 transgenes into homozygous lrb1-1 lrb2-1 plants, GFP fusion proteins of the expected mass (approximately 95 kD) were detected immunologically with anti-GFP antibodies and in association with CUL3 (Fig. 2B), demonstrating that intact and functional fusions accumulated (Supplemental Fig. S13A). Segregation analysis of selfed T1 progeny from lines hemizygous for the GFP-LRB1/2 transgenes and homozygous for the lrb1-1 and lrb2-1 mutations demonstrated that the fusion proteins also retained phenotypic functionality based on their ability to reverse the R suppression of hypocotyl elongation in lrb1-1 lrb2-1 seedlings (Supplemental Fig. S13B). When the rescued lines were examined by fluorescence confocal microscopy, we detected nuclear enrichment of both GFP-LRB1 and GFP-LRB2 in dark-grown roots as compared with those expressing free GFP (Fig. 9B; Supplemental Fig. S13C). Long-term Rc irradiation had no detectable effect on this nuclear distribution, nor did the GFP-LRB1/2 reporters coalesce in nuclear speckles, indicating that the nuclear/cytoplasmic partitioning of LRB1/2 is not under light control (Fig. 9B). Thus, while LRB1/2 and phyB/D are together in the nucleus upon R irradiation of seedlings, LRB1/2 do not appear to follow phyB/D into punctate nuclear bodies.
PHYA Expression Is Up-Regulated by LRB1/2

Based on the synergism between phyB and phyA in regulating low-fluence R responses of etiolated seedlings (Franklin and Quail, 2010) and the known ability of phyB to attenuate PHYA expression in light (Cantón and Quail, 1999), we tested whether LRB1/2 might also affect phyA accumulation even though lrb1-1 lrb2-1 seedlings are not detectably altered in phyA-mediated responses (e.g. FR inhibition of hypocotyl elongation; Fig. 4A). For dark-grown seedlings, phyA protein levels were indistinguishable in the lrb1-1 and lrb2-1 single mutants and the three combinations of lrb1 lrb2 double mutant as compared with those in the wild type and phyB-9 (Fig. 10A). However, the lrb1 lrb2 seedlings had dramatically less phyA upon exposure to R, whereas phyB-9 seedlings accumulated substantially more. The response of the lrb1 lrb2 seedlings had dramatically less phyA upon exposure to R, whereas phyB-9 seedlings accumulated substantially more. The response of the lrb1 lrb2 seedlings indicated that the resulting BTB E3s somehow enhance phyA accumulation in addition to its ability to dampen phyB/D levels. This drop in phyA was at least partially suppressed in phyB-9 lrb1-1 lrb2-1 seedlings, implying that LRB1/2 works through phyB to control phyA abundance (Fig. 10B).

Given the negative control of phyB as Pfr on PHYA transcription, one likely possibility was that the increased level of phyB in the lrb1 lrb2 background subsequently attenuated PHYA gene expression. qRT-PCR measurement of PHYA transcripts confirmed this scenario (Fig. 10C). Dark-grown lrb1-1 lrb2-1, phyB-9, and phyB-9 lrb1-1 lrb2-1 seedlings had PHYA transcript levels comparable to that in the wild type. However, when the seedlings were exposed to R, PHYA transcript abundance was significantly lower in lrb1-1 lrb2-1 seedlings but significantly higher in phyB-9 and phyB-9 lrb1-1 lrb2-1 seedlings. Collectively, it seems that LRB1 and LRB2 do not directly promote phyA protein accumulation but enhance it indirectly by inhibiting the phyB-dependent process that suppresses PHYA transcription.

Other Rc-hypersensitive mutants, including those affecting members of the PIF family and COP1, contrast the effects of lrb1 lrb2 mutants on phyA protein levels. In as much as they increase phyB levels, they either do not alter phyA levels (e.g. pif3 pif4 [Khanna et al., 2004; Leivar et al., 2008]) or increase them (e.g. cop1 [Seo et al., 2004]). Direct comparison of homozygous cop1-6, pif3-1 pif4-1, and lrb1-1 lrb2-1 plants confirmed this result and showed that LRB1/2 work differently from these other phy regulators in the control of phyA levels, even though all three mutants dampen the accumulation of the PHYA mRNA (Fig. 10, D and E).

LRB1 and LRB2 Are Involved in Many Developmental Processes Mediated by phyB/D

Recently, Qu et al. (2010) reported that the lrb1-1 lrb2-2 double mutant (proteins generically named POZ/ BTB-Containing Protein2 and -1, respectively) is mildly hypersensitive to jasmonic acid (JA), up-

Figure 8. LRB1 and LRB2 regulate phyB and phyD protein levels. phyB and phyD protein levels in crude extracts from the wild type (WT) and the indicated mutant backgrounds were assayed by immunoblot analysis with anti-phyB and anti-phyD monoclonal antibodies. Anti-PBA1, anti-RPN1a, and anti-histone H3 antibodies were used to confirm equal protein loading. A, phyB and phyD levels are increased in lrb1 lrb2 seedlings grown in R for 4 d. The numbers indicate the relative abundance (%) of each phy polypeptide as compared with the wild type, using the signal from PBA1 to normalize the phy signals. B, Loss of phyB and phyD proteins during R is slowed in the lrb1 lrb2 (lrb) mutants as compared with the wild type. C, R-induced loss of phyB in the wild type is inhibited by 100 μM MG132.
regulates more robustly the signature defense gene PDF1.2, and is more resistant to fungal pathogens, thus directing the authors to conclude that the corresponding BTBLRB1/2 E3s regulate biotic defense in Arabidopsis. However, under our growth conditions, we either failed to recapitulate the responses or found that these responses were much more subtle than those regulated by light. For example, in either dark or Rc, root elongation in the presence of JA or methyl-JA for the homozygous lrb1-1 lrb2-1, phyB-9, phyB-9 lrb1-1 lrb2-1, and the quadruple mutant was indistinguishable statistically from that of the wild type (Supplemental Fig. S14, A and B). Like wild-type seedlings, lrb1-1 lrb2-1 seedlings increased the abundance of the PDF1.2 transcript in response to JA but to a level only slightly greater than the wild type. This up-regulation of PDF1.2 mRNA by JA was attenuated when the phyB-9 mutation was combined with lrb1-1 lrb2-1 and absent in phyB-9 seedlings (Supplemental Fig. S14C), in agreement with previous studies on the control of PDF1.2 transcript abundance by phyB (Moreno et al., 2009). Given that light is known to profoundly influence plant defense responses through the circadian clock (Wang et al., 2011), we consider it likely that the responses seen previously for the lrb1 lrb2 double mutants (Qu et al., 2010) actually reflect an indirect role of LRB1/2 on JA perception and biotic defense mediated by a more direct role of LRB1/2 on phyB/D accumulation.

DISCUSSION

Extensive genetic and transcriptomic analyses of light perception in Arabidopsis have revealed an interconnected web of signaling components that regulate and respond to the phy family of R/FR-absorbing photoreceptors (Quail, 2002; Bae and Choi, 2008; Leivar and Quail, 2011). Here, we add another level of regulation to this web with the discovery of two BTB-type E3s assembled with the LRB1 and LRB2 BTB proteins that together negatively regulate many aspects of phy signaling. Whereas the single homozogyous mutants are relatively normal phenotypically, the double mutants are strongly hypersensitive to R, but not FR or B, with respect to hypocotyl elongation, and they are compromised for numerous white light or R responses at various stages of the Arabidopsis life cycle, including seed germination, cotyledon opening and expansion, chlorophyll accumulation, expression of the R-regulated CAB2 gene, the effect of EOD-FR on hypocotyl growth, petiole elongation, rosette shape, and flowering time. This phenotypic connection is firmly supported genetically by the assay of multiple alleles affecting LRB2, cosegregation analysis, and complementation with Flag- and GFP-tagged transgenes.

Most, if not all, of the phenotypic defects associated with the lrb1 lrb2 double mutants are primarily mediated through phyB (with assistance from phyD). Accordingly, we found that all the lrb1 lrb2 phenotypes could be largely abrogated by further removal of phyB and phyD, indicating that LRB1 and LRB2 together affect signaling by these two phy isoforms specifically. While the normal sensitivity of lrb1 lrb2 plants to FR rules out a role for these BTB E3s in phyA signaling, it remains possible that they affect signaling by phyC and phyE (Devlin et al., 1998; Franklin et al., 2003a, 2003b). However, the nearly complete phenotypic congruence of phyB-9 phyD-2 lrb1-1 lrb2-1 plants with phyB-9 phyD-2 plants argues that phyC and phyE signaling is not compromised or, at a minimum, is altered below the sensitivity of our phenotypic assays. Whereas a recent preliminary study attempted to link

Figure 9. Cellular localization of LRB2 and the effect of lrb1 lrb2 mutants on phyB localization. A, Fluorescence confocal microscopic images of hypocotyl cells expressing 35S:PHYB-GFP in the wild-type (WT) or lrb1-1 lrb2-1 background. Four-day-old dark-grown plants were either kept in darkness (D) or irradiated with Rc for 24 h prior to imaging. Bars = 10 μm. B, Fluorescence confocal microscopic images of root tip cells expressing UBQ10:GFP-LRB2 in the lrb1-1 lrb2-1 mutant background. Four-day-old plants were either kept in darkness or irradiated with Rc for 24 h prior to imaging. Images of 35S:GFP seedlings are shown as a control. BF, Bright-field images; Merge, superimposition of fluorescence and bright-field images. Bars = 20 μm.
LRB1/2 to JA signaling and pathogen defense, our data instead more strongly connect this BTB protein pair to light signaling. Given the negative effect of light on pathogen invasion through its control of circadian rhythms (Wang et al., 2011), we consider it likely that LRB1/2 affect plant defense indirectly, working through a more direct control on phyB/D levels and/or action.

In addition to LRB1 and LRB2, Arabidopsis encodes a third related BTB protein (LRB3) with an identical NLS-BTB-BACK domain organization. An obvious LRB3 ortholog could only be found in A. lyrata, indicating that this relative is a more recent addition to the plant BTB superfamily, possibly appearing after emergence of the Arabidopsis lineage. The low expression of LRB3, its relatively high diversification rate, and the inability of the lrb3-1 mutant to alter phyB/D signaling phenotypically, either by itself or in combination with lrb1 and lrb2 mutants, strongly suggest that this locus is inactive despite an intact coding region and is possibly on the way to pseudogenization.

Both LRB1 and LRB2 likely function as parts of BTB CRL complexes based on their specific interaction with both isoforms of CUL3 both in yeast and in planta and our observations here that mutants partially compromising CUL3 levels (cul3a-1 and cul3b-1 single mutants; Gingerich et al., 2005) also generate a R-hypersensitive phenotype. A connection between the LRB1/2-CUL3 complexes and R signaling is also supported by our observations that GFP-LRB1/2 binding to CUL3 is enhanced by R irradiating the seedlings. Interestingly, we and Qu et al. (2010) found by Y2H that LRB1 and LRB2 can homodimerize and heterodimerize, suggesting that the BTB3LRB1/2 E3s assembled with these substrate adaptors act as dimers in vivo. Such dimerization has been demonstrated for...
several other CRL-type E3s (Bosu and Kipreos, 2008; Hua and Vierstra, 2011). This association could theoretically increase the target range of individual BTB complexes combinatorially via the assembly of heterodimers or provide a mechanism to assemble poly-Ub chains on a target protein via ubiquitylation in trans across the dimer. That the R-hypersensitive phenotypes can only be seen well in the double lrb1 lrb2 mutant background is inconsistent with them acting in obligate heterodimeric BTB\(^{LRB1/2}\) complexes, but biochemical characterizations of these complexes are clearly needed to support such speculation. Qu et al. (2010) proposed that LRB1 and LRB2 dimerize through their BTB domains, suggesting that such self-association might block the assembly of LRB1/2 with CUL3 and thus negate LRB1/2 action. However, as dimerization of BTB CRL complexes and BTB-CUL3 interactions are proposed to occur via different interfaces in the BTB domain (Stogios et al., 2005), such dimerization and docking might be concurrent.

At present, the target(s) of the BTB\(^{LRB1/2}\) E3s remain to be identified, but the nuclear localization of both LRB1 and LRB2 implies that they are involved in nuclear aspects of phyB/D signaling. Clearly, possible targets are phyB and phyD themselves upon photoconversion to Pfr and subsequent import into the nucleus. This proposal is supported by our observations that (1) the R-hypersensitive lrb1 lrb2 phenotypes are consistent with the mutant plants accumulating more of these phy isoforms, (2) the hypersensitive phenotypes can be negated by removing phyB and phyD, (3) etiolated lrb1 lrb2 plants have normal levels of phyB and phyD proteins but retain higher levels of both upon prolonged exposure to Rc without effects on the corresponding mRNA levels, and (4) the turnover of at least phyB is dampened by the proteasome inhibitor MG132, thus implicating the UPS more generally. That LRB1 and LRB2 do not follow phyB into nuclear speckles upon R irradiation might indicate that these speckles are not the sites of phyB/D turnover or might simply reflect the possibility that LRB1/2 overexpression driven by the UBQ10 promoter overwhelms their normal distribution within the nuclear compartment.

Unfortunately, numerous attempts to prove that LRB1/2 directly interact with phyB/D have been unsuccessful. These approaches include communoprecipitation of phyB with Flag-LRB1 or GFP-LRB1/2 expressed in Arabidopsis, in vitro interaction assays using recombinant phyB and glutathione S-transferase-LRB1 or glutathione S-transferase-LRB2, nondirected Y2H assays, and directed Y2H assays with phyB. Challenges have included difficulties in assembling photoactive full-length phyB chromoproteins in heterologous systems and the insolubility of recombinant LRB1/2 proteins and their poor expression in plants. A further impediment might be that phyB/D turnover requires a heretofore unknown modification of the chromoproteins (e.g. phosphorylation) after photoconversion to Pfr before they can be recognized by BTB\(^{LRB1/2}\). It is also possible that phyB/D are not the direct targets of BTB\(^{LRB1/2}\). However, they must be close to phyB/D signaling and turnover in the nucleus after photoconversion to Pfr, given the nuclear localization of LRB1/2 and the strong effects of lrb1 lrb2 mutants on phyB/D signaling and abundance in R.

In addition to their negative control on phyB/D levels, LRB1/2 have a strong positive effect on phyA levels, with light-exposed lrb1 lrb2 double mutants accumulating substantially less phyA protein. Our observations that lrb1 lrb2 plants accumulate significantly less PHYA mRNA implies that this effect is an indirect consequence of increased phyB levels repressing PHYA transcription. It was also possible that LRB1/2 indirectly control phyB and phyD levels by regulating the action of COP1 and PIFs. However, the lrb1 lrb2 mutants clearly act in a different manner than cop1 or the pif mutants. First, cop1 and higher order pif mutants display a constitutive photomorphogenic phenotype in the dark (Ang et al., 1998; Leivar et al., 2008a, 2008b), whereas the lrb1 lrb2 mutants do not. This suggests that LRB1/2 action depends on light, whereas COP1 and PIFs act constitutively even in the dark. Second, we showed that the lrb1 lrb2 mutants and the cop1 and pif mutants differ in the R-dependent inhibition of phyA accumulation. Although all three mutants (cop1, pif3 pif4, lrb1 lrb2) have similarly decreased levels of PHYA mRNA (most likely caused by hyperactivation of phyB signaling), only the lrb1 lrb2 mutants have reduced levels of phyA protein (Fig. 10).

Regardless of the targets, the BTB E3s assembled with LRB1 and LRB2 likely represent a key conserved step in phyB/D signaling. Obvious orthologs based on sequence homology can be found in all land plants but not in algae, implying that their roles in light signaling appeared early in land plant evolution. With the exception of the lycophyte Selaginella moellendorfii, all land plant genomes analyzed appear to encode at least two functional LRB genes, suggesting that genetic redundancy may help underpin their functions. However, at this point, we see no functional or expression differences between Arabidopsis LRB1 and LRB2 to imply subfunctionalization.

The fact that the strong light-hypersensitive phenotype of lrb1 lrb2 plants is presumably elicited by only 2- to 4-fold more phyB (and 3- to 8-fold more of its minor partner phyD) further illustrates the importance of phy levels on phy signaling. This accumulation is regulated at multiple levels, including controls on transcription and light-dependent degradation. Whereas phyA turnover by the UPS has been well established (Shanklin et al., 1987; Jabben et al., 1989; Seo et al., 2004; Quint et al., 2005; Gilkerson et al., 2009), our studies with the BTB\(^{LRB1/2}\) E3s strongly support the involvement of the UPS in phyB/D turnover and its subsequent influence on plant photomorphogenesis. It should be emphasized that phyB/D levels drop much more slowly in R (half-life approximately 12–24 h) as compared with phyA (half-life approximately 1–2 h; Shirrock and Clack, 2002; this report). Much of this distinction likely reflects the
stronger negative feedback control of Pfr on phyA synthesis relative to phyB due to the strong repression of PHYA expression (Somers and Quail, 1995). It remains possible that the BTB CRLs assembled with LRB1/2 are not completely responsible for phyB/D turnover and that other UPS E3s are also involved. This notion is supported by the fact that lrbl1 lrbl2 mutants do not completely stabilize phyB/D levels and prior studies implicating the COP1 subunit of DWD CRLs in phyB turnover (Jang et al., 2010). Whereas increasing phy levels through transgenic expression has been offered as a mechanism to manipulate agronomically important processes controlled by these photoreceptors (e.g. seed germination and yield, shade avoidance, and flowering time; Garg et al., 2006; Boccalandro et al., 2009), we propose that manipulating the action of BTB-LRB1/2 might offer an alternative route, at least for phyB/D-mediated photoreponses.

MATERIALS AND METHODS

Sequence Alignment, Domain Predictions, and Phylogenetic Analyses

The predicted full-length LRB1 and LRB2 nucleotide and protein sequences from the Arabidopsis (Arabidopsis thaliana) Col-0 ecotype were used as queries in BLAST searches for related loci in other plant genomes available at the Phytozone (http://www.phytozone.net/), National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), Rice Genome Annotation Project (http://riceplantbiology.msu.edu/), The Arabidopsis Information Resource (http://arabidopsis.org/), Genoscope (http://www.genoscope.cns.fr/externe/English/corps_anglais.html), and Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) Web sites (for the complete list, see Supplemental Fig. S1). Methods for reannotating the LR8 gene and pseudogene models when necessary are described in Supplemental Materials and Methods S1 in the Supplemental Data. BTB and BACK domains and LNS motifs were predicted by SMART (http://smart.embl-heidelberg.de); Ptam (http://ptam.sanger.ac.uk/), and sequence alignments. Multiple sequence alignments of the predicted full-length amino acid sequences and BTB domains alone were generated independently by progressive, iterative, and consistency-based approaches. Progressive alignments were obtained with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using the default settings, iterative alignments were obtained in Multiple Alignment with Fast Fourier Transform (MAFFT; http://mafft.cbrc.jp/alignment/software/index.html) using the Fast Fourier Transform with a Normalized Similarity matrix (FFT-NS-i) iterative strategy and the default settings, and consistency-based alignments were obtained using T-Coffee (http://www.tcoffee.org/) using the default settings. A consensus alignment was generated for the full-length LRB proteins using the Combine function in T-Coffee, which then underwent minor hand editing in Jalview (http://www. jalview.org/) to produce the final alignment (Supplemental Fig. S1). Prior to phylogenetic analysis, poorly aligned and divergent positions were removed with Gblocks (http://molevol.univ.cisic.es/castresana/Gblocks.html; Supplemental Fig. S2). Evolutionary models were selected by ProTest 2.4 (Abascal et al., 2005) using the fast strategy (model parameters and branch lengths, but not tree topology, were optimized), and a Biology-Neighbour-Joining (BIONJ) tree was calculated by the program. For both the BTB domain and Gblocks-edited full-length LRB alignments, the most probable model according to the Akaake Information Criterion score was Jones-Taylor-Thornton (JTT) with γ-correction (Akaake weight [probability] in both cases = 0.73). ProTest estimated the γ-parameter at 0.83 for the Gblocks-edited full-length sequence alignment and 0.91 for the BTB domain alignment (Supplemental Fig. S4).

Distance-based neighbour-joining trees were generated with MEGA4 using the JTT substitution matrix, a fixed γ-shape parameter of 0.83 (tree generated from the Gblocks-edited full-length LRB protein alignment) or 0.91 (tree generated from the predicted LRB BTB domain alignment), pairwise deletion, and 1,000 bootstrap trials. Maximum parsimony trees from 1,000 bootstrap trials were generated with MEGA4 using the Close-Neighbor-Interchange algorithm at search level 3, in which the initial trees were obtained with the random addition of sequences (10 replicates). All alignment gaps were treated as missing data. Maximum likelihood trees were generated with PhyML (http://www.atgc-montpellier.fr/phyml/) with the JTT substitution matrix, zero invariable sites (as estimated from the data sets), four substitution rate categories, a fixed γ-shape parameter of 0.83 (tree generated from the Gblocks-edited full-length LRB protein alignment) or 0.91 (tree generated from the predicted LRB BTB domain alignment), and the initial tree generated by BIONJ. In each case, 500 bootstrap trials were performed. The three phylogenetic trees generated from the Gblocks-edited full-length sequence alignment or just the BTB domains can be found in Supplemental Figures S3 and S5. The method for calculating Ka/Ks estimations of evolution rates was determined by Nei and Gojobori (1986).

Identification of Mutant Alleles

The lrb1-1 (Salk_145146), lrb2-1 (Salk_001013), lrb2-2 (Salk_044446), lrb3-3 (Salk_100118), lrb3-1 (Salk_092868), and phyD-2 (Salk_027956) T-DNA insertion lines in the Arabidopsis Col-0 background were obtained from the SIGNAL-T-DNA insertion collection (Alonso et al., 2003) available from the Arabidopsis Biological Resource Center. The cul3a-1, cul3-b1, and piz-3 piz-4-2 T-DNA mutants were as described by Gingerich et al. (2005) and Leivar et al. (2007), respectively. The phy-A-211, phy-9 (phy-EMS142), and cop1-6 missense alleles were as described (Reed et al., 1993, 1994; McNellis et al., 1994). Genotypes of the mutants were determined by genomic PCR using gene-specific and/or T-DNA left border-specific primers. The phy-B9 allele was tracked by PCR amplification of the genomic region surrounding the point mutation followed by assessing the sensitivity of the mutated site to StyI digestion (New England Biolabs; Reed et al., 1993). All PCR primers used in this study are described in Supplemental Table S1. The primer pairs used for genotyping in combination with the T-DNA-specific Lba1 primer were as follows: P1+P2 (lrb1-1), P3+P4 (lrb2-2), P7+P8 (lrb2-2), P9+P10 (lrb3-3), P11+P12 (lb2-3-1), and P13+P14 (phyD-2). Linkage of the lrb1-1 and lrb2-1 alleles to R hypersensitivity was accomplished with segregating progeny from a self-cross heterozygous lrb1-1/lrb2-1+ plant by their hypersensitivity of hypocotyl growth inhibition to Rc (5 μmol m-2 s-1). Individual seedlings were genotyped by PCR using primers P1+P2 (lrb1-1) and P5+P6 (lb2-2).

RT-PCR and qRT-PCR Analyses

For light-specific treatments, total mRNA was extracted from seedlings grown for 4 d in darkness or in Rc (20 μmol m-2 s-1). For JA treatments, seedlings were grown on Murashige and Skoog (MS) agar and exposed to white light in LDs for 10 d prior to JA treatment. JA or methyl-JA (100 μm) Sigma or water alone was sprayed onto the plants until the leaves were coated with liquid and the tissue was then collected at 0, 1, or 3 h afterward. RNA was extracted using the RNeasy kit (Promega) and treated with RQ1 DNase (Promega). cDNA synthesis was performed with an oligo(dT)18 primer using the SuperScript III cDNA Synthesis kit (Invitrogen). For RT-PCR, equal amounts of cDNA were subjected to PCR and the products were examined by ethidium bromide staining after agarose gel electrophoresis. Primers used for RT-PCR were P15, P16, P17, P18 (LRB1), P19, P20, P21, P22, P23, P24, P25, P26, P27 (LRB3), P28, P29, P30, P31, P32 (PHYD), and P33, P34 (PAE2). qRT-PCR was performed using the MyiQ2 real-time detection system (Bio-Rad). Relative expression was calculated with the comparative deltaT method using the PP2A-2 gene (At5g13320) as the reference gene because its expression varies little in response to light (Czechowski et al., 2005). For CAB2 expression, total mRNA was extracted from 4-d-old dark-grown seedlings treated with 20 μmol m-2 s-1 Rc for the indicated times. Primers used for qRT-PCR were P35+P36 (CAB2), P37+P38 (PP2A-2), P39+P40 (PHYB), P41+P42 (PHYD), P43+P44 (PHYA), P45+P46 (LRB1), P47+P48 (LRB2), P49+P50 (PDE1.2), and P451+P452 (ACT2).

Y2H and Coimmunoprecipitation Assays

For Y2H, full-length coding regions for LRB1 and LRB2 were expressed as C-terminal fusions to the Gal4-AID in pGADT7 and the Gal4-BD in pGBK7. The primers used during PCR for the constructs in both pGADT7 and pGBK7 are P53+P54 (LRB1) and P55+P56 (LRB2). Constructs encoding CUL1, CUL3a, CUL3b, RBX1, ASK1, UFO, p53, COII, and At4g08455 were as described (Gingerich et al., 2005). All constructs were transformed into haploid yeast strains YP82a and LB414a and assayed for Y2H interactions.

BTB E3s Regulate Photomorphogenesis
by the method of Gingerich et al. (2005). For the coimmunoprecipitation assays, seedlings expressing GFP, GFP-LRB1, or GFP-LRB2 (see below) were grown in the dark for 4 d and either kept in the dark or irradiated with 20 μmol m⁻² s⁻¹ Rc for an additional 12 h. Frozen seedlings were pulverized and extracted (mL g⁻¹ fresh weight) into 100 mM Tris-HCl (pH 8.3), 140 mM ammonium sulfate, 10 mM Na₂EDTA, and 50% ethylene glycol with 3.8 mg mL⁻¹ sodium metabisulfite, 4 mM phenylmethylsulfonyl fluoride, 50 μg ml⁻¹ Selleck Chemicals), and 1× protease inhibitor cocktail (Sigma-Aldrich) added just before use. GFP proteins were immunoprecipitated from the clarified protein extracts by incubation with 3 μL of rabbit anti-GFP antibodies (Abcam) for 2 h, collection with anti-protein A/G magnetic beads (Pierce), and then elution into SDS-PAGE sample buffer. All manipulations were done in the dark under a green safelight. Immunoblot analyses were performed using anti-GFP and anti-CUL3 antibodies (Figueras et al., 2005).

Plant Growth Conditions and Morphological Measurements

Wild-type and mutant seeds were surface sterilized and plated on one-half-strength MS medium supplemented with 10 g L⁻¹ Suc and 0.7% agar (pH 5.7; MS agar). For light treatments, seeds were first cold treated for 4 d in the dark at 4°C, exposed to 23°C to white light (140 μmol m⁻² s⁻¹) for 8 h to induce germination, incubated for 16 h at room temperature in darkness, and then exposed to various light regimes. R (670 nm), FR (735 nm), and B (470 nm) was provided by SNAP-LITE light-emitting diode modules (Quantum Devices), and white light was provided by Octron 800 4100K cool-white fluorescent bulbs (Osem Sylvania Products). hypocotyl lengths, cotyledon angles, and cotyledon areas were measured and analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). Cotyledon angles represented the angle between a straight line drawn between the tip of the cotyledon and the cotyledon blade/cotyledon petiole junction and a straight line drawn through the cotyledon petiole. An angle of 180° reflected a fully folded cotyledon. For the FOD-FR response, seedlings were subjected to 8 h of R (100 μmol m⁻² s⁻¹), a 10-min pulse of FR (100 μmol m⁻² s⁻¹), and then 16 h of darkness for 4 d. Control seedlings were not exposed to FR but instead placed in darkness for an additional 10 min. For germination assays, seeds were surface sterilized, plated on MS agar, irradiated for 5 min with various fluence rates of FR 2 h after plating, and then placed in darkness (Oh et al., 2007). Germination was scored 5 d later by the emergence of the radicle. For flowering time, seedlings were first grown on MS agar, transferred to soil after 10 d, and then exposed at 21°C to white light in LDs. Flowering time was determined by the number of rosette leaves present when the inflorescence stem reached 1 cm. For petiole length measurements, seedlings were transferred from MS agar to soil after 7 d, grown for 8 d under high-fluence white light (140 μmol m⁻² s⁻¹) in LDs, and then exposed to low-fluence white light (6 μmol m⁻² s⁻¹) for an additional 7 d. For chlorophyll measurements, seedlings were grown in 80% acetone buffered with 50 mM Tris-HCl (pH 8.0), clarified at 12,000 g for 10 min, and the total amount of chlorophyll was determined by measuring the absorbance of the supernatant at 663 and 646 nm (Porra, 2002). For root elongation measurements, seedlings were grown vertically on MS agar supplemented with either JA or methyl-JA under 10 μmol m⁻² s⁻¹ Rc or under continuous white light for 7 or 10 d, respectively.

Rescuing the lrb1 lrb2 Phenotype with 35S:Flag-LRB1

The full-length coding sequence for LRB1 (using the upstream start codon) was PCR amplified from Col-0 cDNA using primers P56 and P57 and introduced in frame into the pEARLEYGATE 202 destination vector (Earley et al., 2006) to express the LRB1 protein bearing a N-terminal Flag epitope (MDYKDDEDDD) under the control of the 35S promoter. This construct was transformed into bhl1-1 bhl2-1 plants using the Agrobacterium tumefaciens floral dip method. Transformants were selected by Basta resistance and for expression of the Flag-LRB1 transcript by RT-PCR using primers P59 (Flag) and P60 (LRB1), using RT-PCR of the UBQ9 gene as the control for equal cDNA loading (primers P61 and P62). Lines homozygous for the 35S:Flag-LRB1 transgene were identified by segregation of Basta resistance in the T3 generation. The Flag-LRB1 protein was detected in crude soluble extracts by immunoblot analysis with a mouse monoclonal antibody against the Flag sequence (Sigma; F1804).

Immunoblot Analyses of phyA, phyB, and phyD

The phyA, phyB, and phyD proteins were extracted from 4-d-old seedlings grown in darkness or Rc (20 μmol m⁻² s⁻¹) using the phyB extraction buffer (Boccalandro et al., 2009) introduced into the bhl1-1 bhl2-1 double mutant. Triple homozygous 4-d-old seedlings grown in darkness or Rc (20 μmol m⁻² s⁻¹) prior to imaging. Induced concentration of phyB into nuclear speckles was observed by fluorescence confocal microscopy of the 35S:PHYB-GFP reporter (Boccalandro et al., 2009) probed with the phyA, phyB, and phyD antibodies (Shanklin et al., 1989; Hirschfeld et al., 1998).

Cycloheximide and MG132 Treatments

Seeds were sown on 24-well plates containing liquid one-half-strength MS plus 10 g L⁻¹ Suc, exposed to white light for 8 h at 23°C to induce germination, and then placed on a shaker in the dark for 4 d. For cycloheximide or MG132 exposure, the seedlings were incubated in the dark in the presence of 100 μM of the inhibitor for either 1 h (cycloheximide) or 12 h (MG132) and then either irradiated with Rc (20 μmol m⁻² s⁻¹) or kept in the dark for additional times before sample collection.

Subcellular Localization of LRB1/2 and phyB

The full-length LRB1 and LRB2 coding sequences were PCR amplified with primers P63 and P64 (LRB1) and P65 and P66 (LRB2) from Col-0 cDNA and introduced in frame into a modified pMDC99 vector to express the BTB proteins as a C-terminal fusion to GFP under the control of the UBQ10 promoter. UBQ10:GFP-LRB1/2 lrb1 lrb2 transformants generated by the floral dip method were selected by hygromycin resistance. Phenotypic rescue was assessed by hypocotyl elongation in Rc (20 μmol m⁻² s⁻¹) on T2 plants segregating for the presence of the GFP-LRB1/2 transgenes as determined by PCR with primers P69 (GFP), P67 (LRB1), and P68 (LRB2). GFP-LRB1/2 proteins were detected in crude soluble extracts by immunoblot analysis with mouse monoclonal antibody against the GFP moiety (Abcam; Ab1218). For GFP-LRB1/2 localization, segregating T2 seedlings were grown on MS agar for 4 d in darkness or Rc (20 μmol m⁻² s⁻¹) prior to imaging. Induced concentration of phyB into nuclear speckles was observed by fluorescence confocal microscopy of the 35S:PHYB-GFP reporter (Boccalandro et al., 2009) probed with the phyB antibody.

Sequence data for LRB1, LRB2, and LRB3 described in this article can be found in the GenBank/EMBL databases under accession numbers NM_130189.2 (At12g46260), NM_180402.1 (At4g01160), and other LRB genes in this study can be found in Supplemental Data Set S1. The reannotated At12g01160 sequence is also described in this data set.

Supplemental Data

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

Supplemental Figure S1. Multiple sequence alignment of full-length LRB proteins.

Supplemental Figure S2. Gblocks-edited sequence alignment of full-length LRB proteins.

Supplemental Figure S3. Neighbor-joining, maximum parsimony, and maximum likelihood phylogenetic trees from the full-length Gblocks-edited multiple sequence alignment of the LRB protein family.

Supplemental Figure S4. Multiple sequence alignment of the BTB domains from the LRB protein family.

Supplemental Figure S5. Neighbor-joining, maximum parsimony, and maximum likelihood phylogenetic trees from the multiple sequence alignment of the BTB domains from the LRB protein family.

Supplemental Figure S6. R-induced suppression of hypocotyl elongation for cul3a and cul3b mutants and bhl1 lrb2 mutants when grown without Suc.
Supplemental Figure S7. The R-hypersensitive phenotype cosegregates with the lrb1-1 lrb2-1 mutations.

Supplemental Figure S8. Additional light responses for the lrb1 lrb2 mutants.

Supplemental Figure S9. Description of the phyD-2 mutant and genotypic confirmation of quadruple homozygous phyB-9 phyD-2 lrb1-1 lrb2-1 mutants.

Supplemental Figure S10. Light responses of phyB-9, phyD-2, lrb1-1, and lrb2-1 mutant combinations.

Supplemental Figure S11. phyB and phyD protein levels in the dark and mRNA levels in Rc or dark are not regulated by LRB1 and LRB2.

Supplemental Figure S12. LRB1 and LRB2 proteins are regulated by the 26S proteasome but not by R.

Supplemental Figure S13. Rescue of the lrb1 lrb2 phenotype with GFP-LRB1/2 and fluorescence confocal microscopic localization of GFP-LRB1 in root cells.

Supplemental Figure S14. LRB1 and LRB2 may be involved in R-dependent plant defense responses.

Supplemental Table S1. Oligonucleotide primers used in this study.

Supplemental Data Set S1. LR family reannotations and sequence identifiers for the LR family proteins in various plant species.

Supplemental Materials and Methods S1.

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