Regulation of de-etiolation and stomatal opening responses by
HYPOSENSITIVE TO LIGHT and HYPERSENSITIVE TO RED AND BLUE 1

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Xiaodong Sun

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Dedication

This dissertation is dedicated to my parents, my wife and my friends.
Abstract

Light is an important environmental cue and regulates plant development processes including de-etiolation, phototropism, shade avoidance, photoperiodic flowering, stomatal movement and so on. In Chapter II, we reported cloning of an Arabidopsis light-signaling component, HYPOSENSITIVE TO LIGHT or HTL. *htl* mutants displayed a long hypocotyl phenotype under red, far-red, and blue light, as well as other photomorphogenic defects. HTL belongs to an alpha/beta fold protein family and is localized predominantly in the nucleus. ELONGATED HYPOCOTYL5 (HY5), a B-Zip transcription factor, directly binds to both C/G-box and G-box in *HTL* promoter and regulates *HTL* expression. Evidences suggest that HTL represents a new signaling step downstream of HY5 in de-etiolation responses. In Chapter III, we discovered the role of HYPERSENSITIVE TO RED AND BLUE1 (HRB1) and PHOSPHATE7 (PP7) in light-induced stomatal movement response. Besides its short hypocotyl phenotype upon isolation, *hrb1* mutant also showed reduced water loss and stomatal aperture phenotypes under blue light. We identified PP7 as an HRB1 interacting protein. PP7 de-phosphorylated HRB1 in vivo and HRB1 required a functional PP7 for both its stomatal opening and hypocotyl elongation responses. HRB1 was found in a protein complex of 193 kDa in the dark and blue light induced a size shift to 285 kDa. However, the size shift of HRB1 protein complex was largely impaired and HRB1 was predominately phosphorylated in *pp7* mutant. We propose that a modification of HRB1 by PP7 under blue light is essential to acquire a proper conformation of the HRB1 protein complex or to bring in a new component for the assembly of a functional HRB1 protein complex.
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Chapter I. Literature review
Photomorphogenesis

The growth and development of plants in response to light are collectively referred to as photomorphogenesis or de-etiolation. At young seedling stage, the de-etiolation responses include the inhibition of hypocotyl elongation, the opening and expansion of cotyledons, the disappearance of the hypocotyl hook, and the maturation of chloroplasts (Casal, 2000). Genetic studies in the model plant *Arabidopsis thaliana* have helped identify three groups of major photoreceptors: the red/far-red light perceiving phytochromes (phy), and blue light perceiving cryptochromes (cry) and phototropins (phot).

Phytochromes are photo-reversible chromoproteins, and there are five phytochromes in *Arabidopsis*, phyA to phyE (Chen et al., 2004). Among them, phyA and phyB are the major photoreceptors for de-etiolation responses under far-red and red light, respectively. Generally, phytochromes have an amino-terminal photosensory domain and a carboxy-terminal kinase domain (Yeh et al., 1998). The N-terminus of phyB alone in the nucleus is sufficient to trigger red light response while the C-terminus is crucial for light induced nuclear translocation (Matsushita et al., 2003). Upon red or far-red light induction, phyB or phyA undergo rapid nuclear translocation and form nuclear speckles (Fankhauser et al., 2008). Two protein chaperons, FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) and FHY LIKE (FHL), facilitate nuclear translocation of phyA (Genoud et al., 2008). Expression of *FHY1* and *FHL* is under the regulation of two transposase-derived transcription factors, FAR-RED ELONGATED HYPOCOTYL 3 (FHY3) and FAR-RED IMPAIRED RESPONSE1 (FAR1). Expression of FHY3 and
FAR1 is negatively regulated by phyA (Lin et al., 2007). Thus, these components form a feedback loop within the phyA pathway (Lin et al., 2007). Formation of phyB nuclear speckles requires HEMERA (HMR), a ubiquitination-related protein (Chen et al., 2010). phyA and phyB nuclear translocation leads to rapid phosphorylation and degradation of negative transcription regulators of photomorphogenesis such as PHYTOCHROME INTERACTING FACTOR (PIF) 1, 3, 4, and 5 (Shen et al., 2005; Al-Sady et al., 2006; Shen et al., 2007). Removal of the function of four PIFs released genome-wide transcription suppression and promoted photomorphogenesis even in the dark (Leivar et al., 2009; Shin et al., 2009).

Cryptochromes are flavoproteins with a sequence similarity to photolyase but have an additional plant-specific extension at C-terminus. Overexpression of cryptochrome C-termini caused a constitutive photomorphogenic phenotype (Yang et al., 2000; Rosenfeldt et al., 2008). cry1 is the primary photoreceptor for de-etiolation responses under high-intensity blue light, whereas cry2 mainly mediates de-etiolation responses under low-intensity blue light (Li and Yang, 2007). cry1 undergoes light-dependent nucleus/cytoplasm shuffling and cry2 completes its lifecycle in the nucleus (Yang et al., 2001; Yu et al., 2007). CIB1 (cryptochrome-interacting basic-helix-loop-helix) has been demonstrated to directly interact with cry2 in a blue light-specific manner and regulates flowering time by directly activating expression of FLOWERING LOCUS T in Arabidopsis (Liu et al., 2008).

Cryptochromes may regulate photomorphogenesis through the CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) mediated 26S proteasome degradation pathway.
COP1 is an E3 ubiquitin ligase that targets various signaling molecules for ubiquitination and degradation in the dark (Yi and Deng, 2005). The proteins targeted by COP1 in de-etiolation signaling and flowering include phyA, cry2, ELONGATED HYOCOTYL 5 (HY5), HY5 HOMOLOG (HYH), LONG HYOCOTYL IN FAR-RED (HFR1), and LONG AFTER FAR-RED LIGHT 1 (LAF1) (Yi and Deng, 2005). HY5 is a b-ZIP transcription factor and plays a key role in photomorphogenesis-related gene expression (Lee et al., 2007). HY5 remains mostly unphosphorylated in the dark and is highly phosphorylated under light. The unphosphorylated form interacts more strongly with COP1 (Hardtke et al., 2000). Under stringent regulation of COP1, overexpression of HY5 did not cause any significant de-etiolation phenotype unless the interaction between COP1 and HY5 was abolished (Ang et al., 1998). Similarly, light-induced phosphorylation of HFR1 also reduces its COP1-mediated degradation rate (Park et al., 2008). Previously cry1 and cry2 were reported to negatively regulate COP1 activity through a direct interaction (Wang et al., 2001). Recently cry1 was shown to interact with SUPPRESSOR OF PHYTOCHROME A (SPA1) in a blue light dependent manner. This interaction can suppress the SPA1-COP1 interaction and HY5 degradation, thus promoting photomorphogenesis (Liu et al., 2011; Lian et al., 2011). cry2 acts in a similar way to suppress the COP1-mediated degradation of CONSTANS (CO), a key photoperiodic flowering component (Zuo et al., 2011).

Genome-wide expression analysis showed that different photoreceptors activate a set of highly overlapping genes (Ma et al., 2001; Tepperman et al., 2004; Yang et al., 2008b; Hu et al., 2009), indicating the convergence of light signals of different
wavelengths. The integration of light signals may operate at the photoreceptor level, and cry1 was phosphorylated in vitro by a phyA-associated kinase activity (Ahmad et al., 1998). phyB interacts with cry2 in specific nuclear speckles in Arabidopsis (Mas et al., 2000). Downstream of photoreceptors, various signaling components may also serve to integrate different light signals. As mentioned above, the hy5 mutant displayed long hypocotyl under red, far-red, or blue light (Ang and Deng, 1994). Besides direct transcription activity, HY5 also physically interacts with FHY3 and FAR1 and negatively regulates their activity (Li et al., 2010). HFR1 is a bHLH transcription factor in phyA and cry1-mediated light signaling pathways (Duek and Fankhauser, 2003; Fairchild et al., 2000) FHY1 and FHL interact with HFR1 to assemble photoreceptor/transcription factor complexes for phyA signaling (Yang et al., 2009). short under blue light (sub1) mutant exhibited short hypocotyl under blue and far-red light. SUB1 is a cytosolic calcium binding protein and suppresses accumulation of HY5 protein. (Guo et al., 2001). short hypocotyl under blue 1 (shb1) has a short hypocotyl under blue light, but its gain-of-function allele shows a long hypocotyl phenotype under red, far-red, and blue light (Kang and Ni, 2006). SHB1 may function as a transcription co-activator in a signaling protein complex (Zhou and Ni, 2010).
Light induced stomatal movement response

Drought is one of the major limiting factors for agricultural production. Plants achieve drought resistance through three mechanisms including drought escape, drought avoidance and drought tolerance (Levitt, 1972). Drought escape is the ability of a plant to shorten its life cycle during serious water deficiency (early flowering, early fruiting, etc.). Drought avoidance is the ability of plants to maintain relatively high tissue water potential during drought stress (root elongation, stomata movement etc.). Drought tolerance is the maintenance of turgor pressure through osmotic adjustment (e.g. increased solute concentration). Among them, the closure of stomatal pore is able to directly reduce water loss, which confers drought resistance.

The stomatal pores are located on the plant epidermis and regulate CO$_2$ uptake for photosynthesis and the loss of water by transpiration. Stomatal apertures therefore affect photosynthesis, water use efficiency, and yields of agricultural crops (Kim et al., 2010). Light is the dominant environmental signal that controls stomatal movements in leaves of well-watered plants in a natural environment. Stomatal apertures vary over diurnal cycles, and stomata tend to be open during the day in response to blue light and tend to be closed at night (Tallman, 2004). Blue light alone is necessary and sufficient to trigger the stomatal opening response (Shimazaki et al., 2007). However, supplementing with red light can significantly enhance this blue light response (Kinoshita et al., 2001; Mao et al., 2005). Blue light activates the guard cell plasma membrane H+-ATPase, causing plasma membrane hyperpolarization and water/K+ uptake into guard cells. These physiological changes will drive the opening of stomatal apertures (Schroeder et al, 2001).
Both phot1 and phot2 are the photoreceptors for the stomatal aperture response. In guard cells, phot1 and phot2 contribute equally to blue light-induced stomatal opening (Kinoshita et al., 2001). phot1/phot2 double mutants do not respond to blue light although single mutants may be phenotypically normal or have a weak phenotype (Kinoshita et al., 2001). Similar to phot1/phot2, stomata of the cry1/cry2 double mutant also show a reduced blue light response while those of CRY1-overexpressing lines show a hypersensitive response to blue light (Mao et al., 2005). Recent research, however, also indicates that phyB proteins are positive regulators of stomata opening when red light is supplemented (Wang et al., 2010).

Both phot1 and phot2 have repeated motifs designated LOV1 (light, oxygen, or voltage-sensing domain 1) and LOV2 in their N-terminus and a serine/threonine kinase domain located within their C-terminus (Christie 2007). Upon blue light, phototropins autophosphorylate themselves and thus become active (Inoue et al., 2008). A dephosphorylation process that inactivates phot2 is catalyzed by PP2A (Tseng and Briggs, 2010). ROOT CURLING IN N-NAPHTHYLPHTHALAMIC ACID 1 (RCN1) encodes a regulatory subunit of PP2A. In rcn1 mutant, de-phosphorylation of phot2 was suppressed and the mutant has bigger stomata apertures (Tseng and Briggs, 2010).

Another phosphatase, Protein Phosphatase 1 (PP1), has been proved to function downstream of phototropins and upstream of the H+-ATPase in guard cells of Vicia faba (broad bean) (Takemiya 2006). RPT2 (ROOT PHOTOTROPISM2), which is closely related to phot1-interacting NONPHOTOTROPIC HYPOCOTYL 3 (NPH3), functions in
phot1-mediated stomatal opening response by interacting with phot1 in vivo (Inada et al., 2004).

In photomorphogenesis, cryptochrome signaling may be transmitted through COP1 by directly interaction (Deng et al., 1992; Wang et al., 2001; Yang et al., 2001) or by regulation of SPA1 (Liu et al., 2011; Lian et al., 2011). Stomata of the cop1 mutant are constitutively open in darkness. COP1 is a negative regulator of the stomatal opening and likely to act downstream of crys and interestingly, phots (Mao et al., 2005).

AtMYB60, an R2R3-MYB protein, is a positive regulator of the stomatal opening response to blue light and diurnal cues (Cominelli et al., 2005). It is specifically expressed in guard cells and its expression is negatively regulated by drought (Cominelli et al., 2005). A null mutation results in constitutive reduction of stomatal opening and reduced wilting under water stress. Expression of MYB60 is regulated by crys, phyA, phyB, and COP1 (Wang et al., 2010). AtMYB61 is another member of the Arabidopsis R2R3-MYB gene family (Liang et al., 2005). Gain-of-function AtMYB61 causes a reduction in stomatal aperture in response to light signals and diurnal cues (Liang et al., 2005).

Recent studies have shown that the quality and quantity of light also regulate stomatal development. The formation of stomatal cells is inhibited in cry1/cry2, phyA or phyB mutants under the corresponding light conditions (Casson et al., 2009; Kang et al., 2009).
Chapter II. HYPOSENSITIVE TO LIGHT, an alpha/beta fold protein, acts downstream of ELONGATED HYPOCOTYL 5 to regulate seedling de-etiolation.
Ambient light, perceived by red and far-red light-absorbing phytochromes and blue and UV-A light-absorbing cryptochromes, has profound effects on early seedling de-etiolation. Subsequent integration of various light signal transduction pathways leads to changes in gene expression and morphogenic responses. Here, we report the isolation of a new Arabidopsis light-signaling component, HYPOSENSITIVE TO LIGHT or HTL. Loss of HTL function mutants displayed a long hypocotyl phenotype under red, far-red, and blue light, whereas overexpression of HTL caused a short hypocotyl phenotype under similar light conditions. The mutants also showed other photomorphogenic defects such as elongated petioles, retarded cotyledon and leaf expansion, reduced accumulation of chlorophyll and anthocyanin pigments, and attenuated expression of light-responsive CHLOROPHYLL A/B BINDING PROTEIN 3 and CHALCONE SYNTHASE genes. HTL belongs to an alpha/beta fold protein family and is localized predominantly in the nucleus and with a small amount in the cytosol. The expression of HTL was strongly induced by light of various wavelengths and this light induction was impaired in elongated hypocotyl 5 mutant. HY5 directly bound to both a C/G-box and a G-box in the HTL promoter but with a greater affinity toward the C/G-box. HTL, therefore, represents a new signaling step downstream of HY5 in phy- and cry-mediated de-etiolation responses.
**Introduction**

Light is one of the important environment cues and regulates different aspects of plant growth and development. In Arabidopsis, light signals are perceived through three different types of photoreceptors: red/far-red light-absorbing phytochromes (phys), UV A/blue light-absorbing cryptochromes (crys), and UV A/blue light-absorbing phototropins (phots) (Kami et al., 2010). Upon light activation, these photoreceptors undergo conformational change and trigger a series of downstream molecular and physiological events (Kami et al., 2010).

Phytochromes and cryptochromes are the major photoreceptors regulating the de-etiolation responses (Franklin and Quail, 2010; Li and Yang, 2007). Although some signaling components downstream of the photo-perception act under specific light wavelength, many others function in response to multiple light wavelengths. For example, LONG HYPOCOTYL IN FAR-RED 1 (HFR1), a bHLH transcription factor, and SHORT UNDER BLUE 1, a calcium binding protein, function in both blue and far-red light signaling pathways (Duek and Fankhauser, 2003; Guo et al., 2001). The gain-of-function allele of SHORT HYPOCOTYL UNDER BLUE 1 (SHB1) shows a long hypocotyl phenotype under red, far-red, and blue light (Kang and Ni, 2006).

In this research, we have identified a long hypocotyl mutant under red, far-red, and blue light, and named this mutant as *hyposensitive to light-1* (*htl-1*) based on its long hypocotyl phenotype. Besides its hypocotyl phenotype, *htl-1* also has smaller cotyledons, longer petioles, and reduced accumulation of chlorophyll and anthocyanin pigments. *HTL* gene encodes an alpha/beta fold protein. HTL was largely found in the nucleus and
fraction of HTL can also be detected in the cytosol. The expression of HTL expression is up-regulated by light and HY5 directly binds to C/G-box and G-box in the HTL promoter and regulates the expression of HTL. HTL clearly defines a new locus of light signaling integration and offers new insights into the function of the alpha/beta fold protein family.
Materials and Methods

Plant Materials and Photomorphogenic Experiments

The *Arabidopsis thaliana* ecotype Wassilewskija was used as wild-type for most experiments except for htl-2, which is in the *Landsberg erecta* background. Characterization of htl mutants was conducted on 0.8% agar (w/v) solidified Murashige and Skoog medium minus sucrose. Monochromatic red, far-red, or blue light was generated with an LED SNAP-LITE (Quantum Devices, Barnereld, WI). Light intensity and peak wavelength were measured with a SPEC-UV/PAR spectroradiometer (Apogee Instruments, Logan, UT). Plant images were taken by using an Olympus digital Camedia C-700 (Tokyo, Japan). Hypocotyl length, petiole length, and cotyledon area were measured from the images by using ImageJ software. Petiole length was measured for the first fully expanded true leaf. The chlorophyll and anthocyanin contents were measured as previously described (Kang and Ni, 2006).

Mapping of the htl-1 Mutation and Molecular Cloning of HTL

The htl-1 mutant was crossed to *Landsberg erecta* and long hypocotyl individuals under red light from the F2 population were chosen for the mapping population. Primers used for mapping include: AP22 (TTGGGAAAATCAAATCAAACA and TCGGATCTTTTCCTTCTTCT), CIW7 (AATTTGGAGATTAGCTGGAAT and CCATGTTTGATGATAAGCACA), DHS1 (GAGCTTTGTAAATCAACAACC and GATATTTTCAGGCAGCTGGGAAG), F19F18 (GGATTCTAACTACATTGGGA and AAGACGACGAAGATTGCT), F20D10 (TAATAAAAATCACTGCGCA and
The primers used to characterize the \textit{htl-1} mutation were F1 (ATGGGTGTGGTAGAAGAA), F2 (CATAGCAATGTCATTACGA), M1 (TTTGTGGCCACTCTGT), and M2 (TATGTCGGGACGCATATT). The primers used to characterize the \textit{htl-2} mutation were F1, F2, M1, and M3 (ACCTCGGAGAAGCAGAGA). The primers used to amplify HTL cDNA from \textit{htl-1} plants and to sequence the deletion region were LP (CGTAACACTCTAAAAACACACA) and F2 or M3.

\textbf{Complementation and Overexpression Studies}

The genomic DNA fragment that spans the HTL promoter and coding sequence was PCR-amplified and cloned into pMDC107 vector. The vector was transformed into \textit{htl-1} plants for complementation analysis by using vacuum infiltration (Bent et al., 1994). The HTL genomic DNA was also PCR-purified and cloned into pMDC83 vector to generate overexpression lines that express it under the control of a CaMV 35S promoter.

\textbf{RT-PCR and Real-Time RT-PCR}

Plant mRNA was extracted with an SV total RNA extraction kit (Promega). Reverse transcription reaction was performed by using the SuperScriptIII Reverse transcriptase kit with oligo(dT) primer (Invitrogen). Quantitative RT–PCR was
performed with the SYBR premix ExTaq (Takara) on an Applied Biosystem 7500 real-time PCR machine. Primers used for RT-PCR are: ACTIN2: TATTGTGCTGGATTCTGGT and AGCAAAAGTAAGACTAATAA, CAB3: TACGGATCCGACCAGT and TTCCGTTCTTGAGCTCCT, CHS: ACAAGTCAAAATTCGGA and GTTTAGAGAGGAACGCTGT, HTL: M1 and M3, and UBQ10: AGGTACAGCGAGAGAAAAAGTAGCA and TAGGCATAGCGGCGAGGCGT. The data analysis was performed as previously described (Zhou and Ni, 2010).

Sub-Cellular Localization and Tissue Expression Analysis

Transgenic 35S::HTL::GFP seedlings were germinated in darkness for 3 day, and then were either maintained in darkness or treated with red light (5 µmol m⁻² s⁻¹) for 3 hours. The treated seedlings were stained with 10 µg ml⁻¹ propidium iodide for 10 min (Mateos et al., 2006). Images were acquired with a Nikon C1si Laser Scanning Confocal Microscope equipped with a three-channel PMT detector. The sample was excited at 488 nm for GFP fluorescence and 561 nm for PI florescence. The emission fluorescence emission was captured in 10 nm bandwidth. For GUS histochemical staining assay, the promoter region of HTL was cloned into pMDC163 vector and used for plant transformation (Brand et al., 2006). GUS assays were performed for transgenic Arabidopsis plants by vacuum infiltration of GUS staining solution (1 mM 5-bromo-4-chloro-3-indolyl-D-Gluc, 100 mM sodium phosphate, pH7.0, 1 mM EDTA, 1 mM
K₃Fe(CN)₆, 1 mM K₄Fe(CN)₆, and 0.1% Triton X-100). Samples were incubated overnight at 37°C and cleared in 70% ethanol at 37°C.

**Esterase Assay**

The HTL coding sequence was cloned into pDEST17 vector. A pDEST17 empty vector was used as control. The vectors were transformed into *E. coli* strain BL21(DE3) to produce recombinant HTL protein. Total protein extracts from *E. coli* cells were prepared in solution that contains 50 mM Na-phosphate buffer (pH 7.0) and 150 mM NaCl. Para-nitrophenyl acetate (pNPA) and para-nitrophenyl myristate (pNPM) (MP Biomedicals) were first dissolved in acetonitrile to a final concentration of 8 mM. One ml standard reaction mixture includes 40 mM Na-phosphate buffer (pH 7.0), 0.2% Triton X-100, 0.8 mM pNPA or pNPM, and 50 µg total protein. The reaction was performed at 23°C for 30 min and lipase activity was measured at 410 nm with a Beckman DU-64 spectrophotometer.

**Electrophoretic Mobility Shift Assay**

The HY5 coding sequence was PCR-amplified and cloned into pDEST15 vector. A pDEST15 empty vector was used as control. Total soluble protein extract was prepared from *E. coli* in binding buffer that contains 25 mM Hepes-KOH pH 7.2, 40 mM KCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, and a complete set of protease inhibitors. The three DNA fragments from the HTL promoter were PCR labeled with alpha-35S dATP. Primer pairs used were CGGAAGTGGAAAGAGGAT and
GCGTGAAATCGAGAGACC for G-box, GGTCTCTGATTTGACGC and GGAATGCAATGCTAGTGA for C/G-box 1, and ATTTGCTGCCACTCTTTGA and CAAACATCGCTATTCGTGA for C/G-box 2. The reaction was initiated by mixing 2 µg poly-dIdC, 3 ng radiolabeled probe (300 cpm ng⁻¹), various amounts of cold competitor, and 0.5 µg total protein in 12 µl binding buffer. The reaction mixture was incubated at room temperature for 30 min. DNA–protein complex and free DNA probe were separated by running over 6% polyacrylamide gel in 0.25xTBE buffer. The gel was air-dried and exposed to BioMax MS film and intensifying screen (Kodak) at -80°C. Site-directed mutagenesis was performed by using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The DNA fragments that contain G-box and C/G-box 1 were first cloned to pCR8/GW/TOPO vector (Invitrogen). The primer pairs that introduce mutations were GCCGATGACACGAGGGTCTCTCGAT and ATCGAGAGACCCTCGTGTCATCGGC for G-box, and CGATTTCACGCCCTCGTATCGGCG for C/G-box 1.

Chromatin Immuno-Precipitation Assay

The ChIP experiment was performed as described (Bowler et al., 2004) with a few modifications. Arabidopsis plants were grown on MS agar under white light for 17 d. Four grams of seedlings were harvested and fixed in 1% formaldehyde for 20 min under vacuum. Chromatin DNA was sonicated to shear to 0.3-2 kb fragments. During the immuno-precipitation step, 6 µl of anti-HA monoclonal antibody (Abgent) was added to
the sonicated chromatin solution and BSA used as a mock control. For the real-time PCR experiment, the primers used to amplify the various chromatin fragments were the same as those described for EMSA.

Accession Number

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under identification code: *HTL* (At4G37470), *HY5* (At5g11260), *CAB3* (At1G29910), *CHS* (At5G13930), *ACTIN2* (At3G18780), and *UBQ10* (At4G05320).
Results

htl-1 Displayed a Long Hypocotyl Phenotype under Red, Far-Red, and Blue Light

To identify mutants defective in light signaling transduction, we have screened a collection of 40000 Wisconsin lines and a collection of 30000 SALK lines containing T-DNA insertions (Arabidopsis Biological Resource Center) for aberrant de-etiolation responses. We have identified a recessive mutant that displayed long hypocotyl phenotype under red, far-red, or blue light, but not in darkness (Figure 1A). Based on its hypocotyl phenotype, we named the mutant as hyposensitive to light (htl-1). To determine how htl-1 mutant responds to various fluence rates, we examined the hypocotyl phenotype of htl-1 mutant under a range of red, far-red, and blue light intensities. Compared to Wassilewskija (Ws) wild-type, htl-1 mutant showed altered response at low intensities and remains hyposensitive to red, far-red, or blue light at higher intensities (Figure 1B).

Beside its long hypocotyl phenotype, htl-1 mutant also showed other light-related morphological defects. Compared to Ws wild-type, the cotyledon area of 5-day-old htl-1 seedlings was much smaller under red, far-red, or blue light (Figure 2A). The petiole length and the leaf area of 17-day-old htl-1 mutant plants were also significantly longer and smaller compared to that of wild-type under white light (Figure 2B). We did not observe any other visible developmental defects in htl-1 mutant at the adult stage. In addition, the accumulation of either chlorophyll or anthocyanin pigments in htl-1 was only 60-70% of that of wild-type under red, far-red, or blue light (Figure 3A and 3B). The expression of CAB3, a gene encoding a chlorophyll a/b binding protein, and CHS, a
gene encoding an enzyme involved in anthocyanin biosynthesis, was also significantly compromised in htl-1 seedlings grown in the dark for 4 d and then treated with red, far-red, or blue light for 2.5 h compared to wild-type (Figure 3C and 3D). The level of CHS and CAB3 transcripts was reduced at least by five to six-fold in the htl-1 mutant.

**Positional Cloning of HTL Gene**

Although htl-1 mutant was initially isolated from a T-DNA insertion collection, the mutant did not carry kanamycin resistance conferred by the T-DNA. We also failed to recover any genomic sequences adjacent to a possible T-DNA by chromosome walking from the T-DNA left or right borders. We therefore adopted a map-based cloning strategy to identify the mutation. HTL was initially mapped in the middle of chromosome 4 and then narrowed down to an 80 kb region, which contains 19 Open Reading Frames (Figure 4A). We sequenced several candidate genes in this region and found a 221-bp deletion that has removed the 5'-UTR and the first exon of At4g37470 (Figure 4B). We were still able to detect a transcript at a normal level in htl-1 mutant with primers derived from the region immediately downstream of the 221-bp deletion (Figure 4C). We then sequenced a RT-PCR product from the htl-1 mutant and found that a partial transcript was still generated with a new ATG codon, 305 bp downstream from the original ATG start codon that has already been deleted in htl-1. As a result, the protein made from this transcript contains only the C-terminus 172 amino acids, whereas the full-length protein contains 270 amino acids (Figure 5A). We next obtained an Arabidopsis line (CS100282) in Lansberg erecta background with a Ds transposon inserted in the first or the only
intron of \textit{HTL} from the \textit{Arabidopsis} Biological Resource Center (Figure 4B; Sundaresan et al., 1995). In the second \textit{htl} allele or \textit{htl-2}, a transcript from the first exon of \textit{HTL} was detected, and the transposon insertion resulted in a truncated protein that only contains the N-terminus 132 amino acids (Figures 4C and 5A).

\textit{htl-1} and \textit{htl-2} Are Allelic

The transposon insertion line CS100282 also showed a long hypocotyl phenotype similar to the originally identified \textit{htl-1} mutant under red, far-red, or blue light (Figure 6A). We performed an allelic test and all F1 seedlings from the cross between the original \textit{htl-1} allele and the new \textit{htl-2} allele showed a long hypocotyl phenotype under red, far-red, or blue light (Figure 6B), suggesting that these two mutants are allelic to each other. We also generated an \textit{HTL::HTL:GFP} construct and introduced this construct into the \textit{htl-1} mutant background. Of 14 independent transgenic lines, six lines showed a complete complementation of the mutant hypocotyl phenotype (Figure 6D). When the \textit{HTL:GFP} fusion gene was driven by CaMV 35S promoter in wild-type background, the transgenic plants exhibited a short hypocotyl phenotype under red, far-red, or blue light (Figure 6D).

\textit{HTL} Encodes an Alpha/Beta Fold Protein and \textit{HTL} Is Localized to the Nucleus

\textit{HTL} gene encodes a protein of 30 kD that belongs to an alpha/beta fold protein family (Figure 5A). The topology of HTL protein was predicted by 3Djigsaw based on the crystal structure of a \textit{Bacillus subtilis} protein RsbQ, which shares 39% overall
identity to the HTL protein (Contreras-Moreira and Bates, 2002; Kaneko et al., 2005). Psi-BLAST search results suggest that HTL protein may belong to an esterase/lipase superfamily. Therefore, we produced recombinant HTL protein in E. coli and examined its activity against two synthetic esterase/lipase substrates. The recombinant HTL protein did not show any detectable activity towards either para-nitrophenyl acetate (pNPA) or para-nitrophenyl myristate (pNPM). In general, HTL has no obvious similarity to any other proteins with a known biochemical function in plants. There are two closely related homologs in the *Arabidopsis* genome (AT3g03990 and AT3G24420), and the *HTL* gene also has homologous genes in other higher plants, including Medicago truncatula and Oryza sativa (Figure 7).

Protein sequence analysis did not reveal that HTL has any trans-membrane domain, nuclear localization signal, or other signaling peptides. We examined the sub-cellular localization of the HTL:GFP fusion protein in the hypocotyl cells of the *Arabidopsis* HTL:GFP overexpression lines. The transgenic plants were grown in the dark for 3 d and were either treated with red light or remained in the dark for 3 h. The seedlings were stained with Propidium Iodide (PI) and examined under a confocal microscope. PI specifically stains cell wall and nucleus in plant tissues. In these seedlings, the GFP signals were detected most intensively in the nucleus and some weak signals were also observed in the cytosol, especially under red light (Figure 5B). Overall, we did not observe any significant difference in HTL sub-cellular localization in seedlings grown in the dark or treated with red light. We also generated *HTL::GUS* transgenic plants and determined where the *HTL* gene was expressed on a whole-plant
scale. In young seedlings, the strongest histochemical staining for GUS activity was detected in hypocotyl and root. In adult plants, GUS activity was shown in rosette leaves, sepals of flower, and silique peduncles and tips (Figure 5C).

**HTL Expression Is Induced by Light and HY5 Is Required for this Light Induction**

Both *htl* mutants displayed defects in light-regulated de-etiolation responses, and we further examined whether the expression of *HTL* is regulated by light. In darkness, we detected a basal level of *HTL* expression, but after 2.5 h of red, far-red, or blue light treatment, the expression of *HTL* gene was enhanced six to eight times through real-time RT-PCR analysis (Figure 8A). As shown in a previous study, the genomic binding sites of HY5 were characterized over 3800 possible genes in *Arabidopsis*, and one of the genes was *HTL* (Lee et al., 2007). *htl-1* had a very similar seedling hypocotyl phenotype to *hy5-ks50*, a *hy5* loss-of-function allele, under red, far-red, or blue light (Figure 9). The light induction of *HTL* expression was notably impaired in *hy5-ks50* mutant compared to that in wild-type (Figure 8B). These results suggest that HY5 might directly control HTL expression.

**HY5 Directly Binds to the Promoter Region of *HTL***

We initially identified three possible HY5 binding sites: two C/G-box (GACGTG) motifs (Song et al., 2008) and one G-box (CACGTG) motif over the 800-bp *HTL* promoter region (Figure 9A). We examined the affinity of a recombinant HY5 protein to three genomic DNA fragments that contain the corresponding motifs by electrophoretic
mobility shift assay (EMSA). HY5 bound to DNA fragments that contain either the first C/G-box or the G-box, but barely to the second C/G box (Figure 9C and Figure 10). HY5 showed a greater affinity to the first C/G-box over the G-box (Figure 9C). To confirm that HY5 indeed binds to either C/G box or G-box in the DNA fragments, we mutated the core ACGT sequence to ACGA in the first C/G-box and the G-box and performed EMSA competition analysis (Figure 9B). The EMSA fragments that contain either the mutated C/G box or the G-box failed to compete effectively with the radio-labeled wild-type EMSA cognate fragments (Figure 9C).

The association of HY5 to the HTL promoter was also examined in vivo through a chromatin immunoprecipitation assay with 35S::HA:HY5 transgenic plants and anti-HA antibody. The enrichment of chromatin fragments was analyzed by real-time PCR. The amplicons that contain the G-box and first C/G-box were enriched more than five-fold compared to ChIP sample minus anti-HA antibody. There was also a three-fold enrichment for the amplicon that contains the second C/G-box (Figure 11).
Discussion

HTL Encodes an Alpha/Beta Fold Protein

We have identified a recessive Arabidopsis mutant htl-1 based on its long hypocotyl phenotype under red, far-red, and blue light, but not in the dark (Figure 1). htl-1 young seedlings also exhibited other photomorphogenic defects such as smaller cotyledon and longer petioles (Figure 2). A second allele htl-2 was subsequently identified from a transposon insertional population and showed a very similar phenotypic spectrum to htl-1 (Figures 4 and 6).

HTL gene encodes a protein belonging to the alpha/beta fold hydrolase superfamily. Proteins of this family are structurally featured by eight beta-sheets surrounded by alpha helixes on both sides and HTL is fairly diverged from the typical structure of this superfamily (Figure 5A). A catalytic triad consisting of a nucleophile, an acidic residue, and a histidine residue is highly conserved across the superfamily and is crucial for the hydrolase activity (Nardini and Dijkstra, 1999). In htl-1, the truncated HTL protein lost its N-terminal 98 amino acid, including four beta sheets, two alpha helixes, and the first amino acid of the triad (Figure 5A). The truncated HTL protein lost its C-terminal 138 amino acids in htl-2, including six beta sheets, two alpha helixes, and the last two amino acids of the triad (Figure 5A). htl-1 and htl-2 had very similar hypocotyl phenotype, and both mutants are not likely of partial loss-of-function but knockout (Figure 6). The structure integrity is therefore important for HTL function.

Alpha/beta fold hydrolase proteins may have very diversified functions and play various roles in different pathways despite their sequence similarities. In some cases, they
may function as enzymes such as proteases, esterase, or peroxidases (Nardini and Dijkstra, 1999). For example, tobacco SA-binding protein 2 (SABP2) and its Arabidopsis homolog Methyl Esterase 17 (AtMES17) belong to alpha/beta fold esterase. SABP2 is capable of converting methyl-salicylate to salicylic acid and AtMES17 functions to release indole-3-acetic acid (IAA) from its methyl ester form (Forouhar et al., 2005; Yang et al., 2008a). SABP2 was able to in vitro hydrolyze pNPM (Kumar and Klessig, 2003) and AtMES17 could hydrolyze pNPA (Yang et al., 2008a). Since HTL protein has sequence similarity to the esterase/lipase family, we tested whether recombinant HTL protein has catalytic activity over either pNPM or pNPA. We failed to observe such an esterase activity for HTL over these two substrates, suggesting that HTL hydrolyzes neither short-chain nor long-chain esters. DWARF14, a closest rice homolog of the HTL gene, was involved in strigolactone response, although the mechanism remains largely unknown (Arite et al., 2009). We also found no defects in strigolactone response for both htl mutants.

In several other cases, alpha/beta family proteins may act as signaling molecules without a need for their catalytic functions. For example, GIBBERELLIN INSENSITIVE DWARF1 (GID1) was identified as a receptor for gibberellin in rice and belongs to the alpha/beta hydrolase superfamily (Ueguchi-Tanaka et al., 2005). RsbQ is another member of the alpha/beta hydrolase family protein in Bacillus subtilis. When a bacterial cell is under stress, RsbQ specifically binds to RsbP, a PAS (Per-ARNT-Sim) domain-containing phosphatase, and initiates a downstream signaling cascade (Brody et al., 2001). Interestingly, the HTL:GFP fusion protein is localized preferentially in the nucleus
of hypocotyl cells under a confocal microscope. GID1 was also preferentially localized in the nucleus (Ueguchi-Tanaka et al., 2005). Therefore, HTL may function as a light-signaling molecule in the nucleus.

**HY5 Directly Regulates HTL Expression**

HY5 plays a critical role in photomorphogenesis downstream of phys and crys. In the hy5-ks50 mutant, the expression level of HTL was greatly reduced under red, far-red, and blue light (Figure 8). In light-grown young seedlings, HY5 expression was strong in hypocotyl and primary root, with a preference in vasculature tissue, but was weak in cotyledons (Sibout et al., 2006). Therefore, the pattern of HY5 expression largely overlaps with that of HTL expression (Figure 5C). HTL was one of the HY5 targets identified through a chromatin immunoprecipitation-chip experiment in Arabidopsis genome (Lee et al., 2007). In a gel-mobility shift assay, we showed that HY5 directly binds to a G-box and the first C/G-box in the promoter of HTL gene (Figure 9). Similarly to previous reports, HY5 presented a greater affinity to C/G-box over G-box (Song et al., 2008). However, HY5 did not specifically bind to the second C/G-box in the HTL promoter. Although the two C/G boxes share the same GACGTG core sequence, the sequence flanking the C/G-boxes is quite different and might influence the recognition of HY5 to its target DNA sequences. Similarly, in ChIP-quantitative PCR (qPCR) experiments, the amplicons that contain G-box and the first C/G-box were significantly enriched. However, a considerable level of enrichment was also observed for amplicon that spans the second C/G-box. Since the DNA was sheared with 0.3-2kb fragments,
ChIP experiments may not have enough resolution like EMSA to distinguish the affinity of HY5 towards its three potential binding sites.

As shown in previous ChIP and microarray studies, HY5 regulates the expression of many genes and a subset of the genes, such as HTL, are involved in the control of hypocotyl elongation (Lee et al., 2007; Sibout et al., 2006). In our current studies, HY5 is required for the light-induced expression of HTL, and HY5 recognizes a G/C box and a G-box in the HTL promoter both in vitro and in vivo (Figures 9 and 11). Since hy5-ks50 and htl-1 have a very similar hypocotyl phenotype, HTL is clearly one of the HY5 primary targets and may mediate HY5 action in the regulation of hypocotyl elongation (Figure 12). However, there was still certain amount of HTL transcripts accumulated in the hy5-ks50 mutant under various light conditions compared to that in the dark, and additional transcription factors may be required for the proper expression of HTL (Figure 8).

The expression of CHS is induced by light of various wavelengths, and HY5 has been shown to directly bind to the promoter of either CHS or a few members of the CAB gene family (Ang et al., 1998; Lee et al., 2007). We found that HY5 also mediates the light-induced expression of HTL and may directly bind to the HTL promoter. Although both CHS and HTL are primary targets of HY5, HTL clearly acts upstream of CHS, since the light-induced expression of CHS was also largely impaired in the htl-1 mutant and the htl-1 mutant indeed accumulated much less anthocyanin pigments (Figure 4). How HTL regulates the expression of CHS in relation to the function of HY5 remains unknown. We propose that the function of HY5 at the CHS promoter may involve HTL, a nuclear
protein, or HTL may work together with HY5 and other proteins to control the expression of *CHS*. Alternatively, the regulation of *CHS* expression by HY5 may involve other signaling components that HTL may impact.
Figure 1. *htl-1* Has a Hyposensitive Hypocotyl Response to Red, Far-Red, and Blue Light.

(A) Hypocotyl length of 4-day-old Ws and *htl-1* mutant in darkness and under red (5 μmol m\(^{-2}\) s\(^{-1}\)), far-red (0.05 μmol m\(^{-2}\) s\(^{-1}\)), or blue light (6 μmol m\(^{-2}\) s\(^{-1}\)).

(B) Hypocotyl growth responses of 4-day-old Ws and *htl-1* seedlings to various fluences of red light (left), far-red light (middle), and blue light (right). The means plus or minus...
the standard errors were calculated from at least 25 seedlings per replicate and three biological replicates total in the current and subsequent figures.
Figure 2. *htl-1* Has Smaller Cotyledons and Leaves and Longer Petioles.

(A) Cotyledon area of Ws and *htl-1* seedlings measured at 4 d after germination and grown under red, far-red, or blue light on plate at intensities as indicated in Figure 1A.

(B) Petiole length (left panel) and leaf area (right panel) of Ws and *htl-1* plants at 17 d after germination and grown under white light in soil.
(C) Image showing Ws and htl-1 grown under white light in soil for 17 d after germination. The plants were transferred to an agar plate and photographed. Bar = 10 mm.
Figure 3, *htl-1* Accumulates Less Chlorophyll and Anthocyanin Pigments and HTL Influences the Expression of *CAB3* and *CHS*.

Contents of chlorophyll (A) and anthocyanin (B), A535–A650 pigments in 5-day-old Ws and *htl-1* seedlings grown under continuous of red, far-red, or blue light at intensities indicated in Figure 1A.
(C, D) Real-time RT–PCR analysis of the expression of \textit{CAB3} and \textit{CHS} in Ws and \textit{htl-1}.

Total RNA was isolated from 4-day-old dark-grown seedlings without light treatment or being treated for 2.5 h with red, far-red, or blue light at intensities indicated in Figure 1A. The expression level of \textit{CAB3} or \textit{CHS} was normalized to that of UBQ10.
Figure 4. *HTL* Is Identified by Mapping-Based Cloning.

(A) The *HTL* locus was mapped to Chromosome 4 between the markers F6G17 and F19F18. N indicates the size of the mapping population.

(B) Schematic diagram showing the deletion of 220 bp in *htl-1* and the T-DNA insertion in *htl-2*. The deletion of the *HTL* gene in *htl-1* starts from 45 bp upstream of the ATG start codon and ends at 175 bp after the ATG start codon. A transposon is
inserted at 400 bp downstream of the ATG start codon in *htl-2*. Rectangle indicates 5' and 3' UTR, filled bar indicates exon, and line indicates intron. The numbers below indicate genomic nucleotide sequence. Arrows define various PCR primers used in (C).

(C) Expression of *HTL* in wild-type (either Ws or Ler), *htl-1*, and *htl-2* through RT–PCR analysis. *Ubiquitin 10* was used as a positive control.
Figure 5. *HTL* encodes an alpha/beta fold protein and HTL is enriched in the nucleus.

(A) HTL amino acid sequence showing the alpha helix (green), beta sheet (blue), and a possible reaction triad (red). Open arrowhead indicates where the protein translation starts in *htl-1*. Filled arrowhead indicates where the protein translation ends in *htl-2*. 
(B) Fluorescent images showing the sub-cellular localization of HTL in hypocotyl cells of 3-day-old 35S::HTL::GFP transgenic Arabidopsis seedlings grown either in darkness or in darkness followed by 3-h red light treatment (5 µmol m⁻² s⁻¹). Propidium Iodide (PI) Fluorescence is shown on the left and GFP fluorescence is shown on the right. Bar = 10 µm.

(C) GUS expression in HTL::GUS transgenic Arabidopsis plants. sd stands for 10-day-old young seedlings, rl for rosette leaf, in for inflorescence, si for silique, and fl for flowers of 40-day-old plants.
Figure 6. *htl-1* and *htl-2* Are Allelic.

(A) Hypocotyl length of 4-day-old Ws, *htl-1*, Ler, and *htl-2* in the dark or under red, far-red, or blue light at intensities indicated in Figure 1A.

(B) Hypocotyl length of 4-day-old F1 seedlings derived from a cross of Ws to Ler and a cross of *htl-1* to *htl-2* in the dark or under red, far-red, or blue light at intensities indicated in Figure 1A.

(C) Expression of *HTL* gene in 4-day-old seedlings of Ws, complementation line (C14),
and overexpression lines (OE8, OE9, and OE16) under white light (55 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). Actin was used as a positive control in the RT–PCR analysis. For both HTL and ACTIN, PCR were run for 25 cycles.

(D) Hypocotyl length of 4-day-old seedlings of Ws, htl-1, complementation line C14, and overexpression lines OE8, OE9, and OE16 in darkness or under red, far-red, or blue light at intensities indicated in Figure 1A.
| AT4G37470 | -------------- | -------------- | -------------- | -------------- |
| AT3G24420 | -------------- | -------------- | -------------- | -------------- |
| AT3G83900 | -------------- | -------------- | -------------- | -------------- |
| Ricinus communis | -------------- | -------------- | -------------- | -------------- |
| Populus trichocarpa | -------------- | -------------- | -------------- | -------------- |
| Medicago truncatula | -------------- | -------------- | -------------- | -------------- |
| Zea mays | -------------- | -------------- | -------------- | -------------- |
| AT4G37470 | -------------- | -------------- | -------------- | -------------- |
| AT3G24420 | -------------- | -------------- | -------------- | -------------- |
| AT3G83900 | -------------- | -------------- | -------------- | -------------- |
| Ricinus communis | -------------- | -------------- | -------------- | -------------- |
| Populus trichocarpa | -------------- | -------------- | -------------- | -------------- |
| Medicago truncatula | -------------- | -------------- | -------------- | -------------- |
| Oryza sativa | -------------- | -------------- | -------------- | -------------- |
| Zea mays | -------------- | -------------- | -------------- | -------------- |
| AT4G37470 | -------------- | -------------- | -------------- | -------------- |
| AT3G24420 | -------------- | -------------- | -------------- | -------------- |
| AT3G83900 | -------------- | -------------- | -------------- | -------------- |
| Ricinus communis | -------------- | -------------- | -------------- | -------------- |
| Populus trichocarpa | -------------- | -------------- | -------------- | -------------- |
| Medicago truncatula | -------------- | -------------- | -------------- | -------------- |
| Oryza sativa | -------------- | -------------- | -------------- | -------------- |
| Zea mays | -------------- | -------------- | -------------- | -------------- |
| AT4G37470 | -------------- | -------------- | -------------- | -------------- |
| AT3G24420 | -------------- | -------------- | -------------- | -------------- |
| AT3G83900 | -------------- | -------------- | -------------- | -------------- |
| Ricinus communis | -------------- | -------------- | -------------- | -------------- |
| Populus trichocarpa | -------------- | -------------- | -------------- | -------------- |
| Medicago truncatula | -------------- | -------------- | -------------- | -------------- |
| Oryza sativa | -------------- | -------------- | -------------- | -------------- |
| Zea mays | -------------- | -------------- | -------------- | -------------- |

Note: The table contains sequences of nucleotides or amino acids from various species, possibly part of a larger study or database.
Figure 7. *HTL* has homologs from other plant species

Protein sequence alignment of *HTL* gene (At4G37470) with its homologs from *Arabidopsis* (At3G24420 and At3G03990), Ricinus *communis* (RCOM_1155240), *Populus trichocarpa* (POPTRDRAFT_646169), *Medicago truncatula* (BT052459), *Oryza sativa* (Os03g0437600), and *Zea mays* (LOC100194211). The alignment was done using T-Coffee (http://tcoffee.vital-it.ch/)
Figure 8. The Expression of *HTL* Is Induced by Light and HY5 Is Required for this Induction.

(A) Expression of *HTL* in Ws seedlings after red, far-red, or blue light treatment. *HTL* expression was examined through either RT-PCR (top) or real-time RT-PCR analysis (bottom). The expression level of *HTL* was normalized to that of *UBQ10*. 
(B) Real-time RT-PCR analysis of *HTL* expression in Ws and *hy5-ks50* in darkness or after red, far-red, or blue light treatment. Total RNA was isolated from 4-day-old dark-grown seedlings without light treatment or being treated for 2.5 h with red, far-red, or blue light at intensities indicated in Figure 1A. The expression level of HTL was normalized to that of *ACTIN2*. 
Figure 9. HY5 Directly Binds HTL Promoter In vitro.

(A) Diagram showing the distribution of three amplicons that contain C/G-box (C/G1, C/G2) or G-box (G) in the HTL promoter. Rectangles represent the HTL gene and an adjacent gene At4G37480. Arrows indicate the direction of transcription. The numbers indicate genomic nucleotide sequence coordination.

(B) The nucleotide sequences of G-box, mutated G-box (G mut), first C/G-box (C/G-
box 1), and mutated first C/G-box (C/G mut). The core sequences are shaded in black and the mutated nucleotides are shaded in gray.

(C) EMSA analysis for the binding of HY5 to G-box (top panel) or C/G-box 1 (lower panel). Total protein from *E. Coli* expressing empty vector was used as control. Cold G-box and C/G-box 1 competitors were used at a molar excess of 5×, 10×, or 50×. Cold G mut and C/G mut competitors were used at a molar excess of 10×, 50×, or 100×.
Figure 10. HY5 had an extremely low affinity to C/G-box 2 in the HTL promoter.

(A) Nucleotide sequence of the second C/G-box (C/G-box 2). The core sequences are shaded in black.

(B) EMSA analysis for the binding of HY5 to C/G-box 2. Cold C/G-box 2 competitors were used at a molar excess of 5x, 10x, or 50x.
Figure 11. HY5 Associates with the HTL Promoter In vivo.

The chromatin fragments that contain G-box, C/G-box 1, or C/G-box 2 were enriched in ChIP experiments with 17-day-old 35S::HA:HY5 plants in the presence of anti-HA antibody. The Relative Enrichment was first calculated by normalizing the abundance of the target amplicons to UBQ10 amplicon. The data were then presented as a ratio of the normalized amplicon abundance in the presence of anti-HA antibody over that in the absence of anti-HA antibody.
Figure 12. *htl* has a similar hypocotyl phenotype to *hy5*

Hypocotyl length of 4-d-old Ws, *htl-1* and *hy5-ks50* mutant seedlings grown in darkness or under red, far-red or blue light at intensities indicated in Figure 1A.
Chapter III. PROTEIN PHOSPHATASE 7 activity is required for the assembly of a functional HYPERSENSITIVE TO RED AND BLUE 1 protein complex
We have previously isolated an *Arabidopsis* hypersensitive to red and blue 1 or
*hrb1* mutant for its short hypocotyl phenotype. The *hrb1* mutant also showed reduced
water loss and stomatal aperture phenotypes under blue light. HRB1 contains a ZZ-type
zinc finger motif likely involved in protein-protein interactions. We identified an HRB1-
interacting phosphatase 7 or PP7, a previously defined blue light signaling component in
the nucleus. HRB1 interacted with PP7 through its N-terminal zinc finger motif and was
phosphorylated *in vivo*. PP7 de-phosphorylated HRB1 *in vivo* and HRB1 required a
functional PP7 for both its stomatal opening and hypocotyl elongation responses. HRB1
was found in a protein complex of 188 kDa in the dark mostly as its de-phosphorylated
form and blue light induced a size increase to 315 kDa. By contrast, PP7 migrated at its
monomeric size. However, the size shift of HRB1 protein complex was largely impaired
and HRB1 was predominately phosphorylated in *pp7* mutant. We propose that a de-
phosphorylation of HRB1 by PP7 under blue light is essential to acquire a proper
conformation of the HRB1 protein complex or to bring in a new component for the
assembly of a functional HRB1 protein complex.
Introduction

The *hypersensitive to red and blue 1* (*hrb1*) mutant has a short hypocotyl phenotype under red or blue light and a late flowering phenotype (Kang et al., 2005; Kang et al., 2007). *HRB1* belongs to a plant specific drought-induced Di19 gene family (Milla et al., 2006). HRB1 is a small nuclear protein of 23 kD and its biochemical function remains unknown (Kang et al., 2005). Its N-terminal ZZ-type zinc finger motif is likely involved in protein-protein interaction as suggested by studies from mammalian systems (Hnia et al., 2007).

The *phosphatase 7* (*pp7*) knockdown plants have a long hypocotyl under blue light (Møller et al., 2003). PP7 indeed has an intrinsic phosphatase activity (Kutuzov et al., 1998). Based on their structures and substrate amino acid specificities, phosphatases are classified into four different families: serine/threonine-specific phosphoprotein phosphatase (PPP), metal ion-dependent protein phosphatase (PPM), phosphotyrosine phosphatase (PTP), and dual specificity phosphatase (DSP) (Farkas et al., 2007). PP7 belongs to a large family of serine/threonine protein phosphatases in Arabidopsis. This family was further divided into seven clusters, PP1 to PP7, based on the amino acid sequences of their catalytic subunits (Farkas et al., 2007). The members of the serine/threonine protein phosphatase family are involved in the regulation of enzyme activities (Leivar et al., 2011), ion channel activities (Lee et al., 2009), protein stability (Skottke et al., 2011) and protein-protein interactions (Tang et al., 2011). The substrate of PP7 remains unknown.
In this study, we reveal that both HRB1 and PP7 are players in the regulation of stomatal aperture under blue light. HRB1 interacts with PP7 through its N-terminal ZZ-type zinc finger domain. PP7 co-localizes with HRB1 in the nucleus and de-phosphorylates HRB1 in vivo. HRB1 is present in a large protein complex mostly in its de-phosphorylated form, Blue light induces an increase in the size of the protein complex, and this size shift requires a functional PP7.
Materials and Methods

Plant growth and light condition

The Arabidopsis thaliana ecotypes Wassilewskija and Columbia were used as wild type. Monochromatic red, far-red, or blue light was generated with an LED SNAP-LITE (Quantum Devices, Barnereld, WI). Light intensity and peak wavelength were measured with a SPEC-UV/PAR spectroradiometer (Apogee Instruments, Logan, UT).

Yeast two-hybrid screen and β-galactosidase assay

Yeast two-hybrid library screen was conducted as described previously with Matchmaker GAL4 two-hybrid system 3 (Clontech; Ni et al., 1998). The baits in the pGBKT7 vector and the preys in the pGADT7 vector were introduced into yeast stains by the PEG transformation method (Yeast Protocols Handbook, Clontech). Transformants were selected on minimal synthetic dropout (SD) medium lacking Trp and Leu and β-galactosidase activity assay was performed as described (Yeast Protocols Handbook, Clontech). Candidate interactions with more than 3 folds β-galactosidase activity compared to background were selected for further analysis.

In vitro immuno-precipitation assay

A PCR fragment that contains HRB1 codons and GAL4 BD sequence were subcloned into to pRSETB vector and transformed into E. Coli strain BL21(DE3) for protein expression. Total protein extract was prepared from E. Coli cells in pull-down buffer (1x PBS buffer, 0.1% NP40, and full set of proteinase inhibitors). About 50 µg of
total E. Coli protein was mixed with 2.5 µl anti-GAL4 BD antibody (Santa Cruz Biotechnology), 10 µl protein A/G plus agarose beads (Santa Cruz Biotechnology) in 500 µl cold pull-down buffer plus 0.05% BSA. The mixture was incubated at 4°C for 2 hours, washed 3 times with 500 µl cold pull-down buffer minus BSA, added with 4 µl of *in vitro* translated PP7, and incubated at 4°C for 2 hours. For *in vitro* translation, 40 µl TNT *in vitro* translation master mix (Promega) were mixed with 1 µg pRSETB-PP7 DNA and 2 µl 35S-labeled methionine (MP Biomedicals), and incubated at 30°C for 1 hours. The *in vitro* binding mixture was washed 3 times with cold pull-down buffer, added with 4 µl 5x SDS loading buffer, boiled for 3 minutes, and loaded onto a 12% SDS-PAGE gel. After electrophoresis, the gel was air-dried and exposed to BioMax MS film (Kodak) with intensifying screen at -80°C.

**Plant protein extraction and western blot**

Plant tissues were frozen in liquid nitrogen, ground into fine powder, and resuspended in plant protein extraction buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and full set of proteinase inhibitors) at a ratio of 0.5 ml per gram fresh weight. The extracts were centrifuged at 20,000 g at 4°C for 30 minutes and the supernatant was recovered. About 80 µg of total protein was loaded onto a 12% SDS-PAGE gel and blotted to Immobilon P membrane. The membrane blots were probed with anti-GFP or anti-Myc antibodies purchased from Santa Cruz Biotechnology and anti-mouse secondary antibody purchased from Sigma-Aldrich.
**In vivo affinity-precipitation and co-localization**

PCR-amplified HRB1 genomic DNA and PP7 cDNA were cloned into pCR8/GW/TOPO vectors. The forward and reverse primers for HRB1 are ATGGATTCGAATTCATGG and TCCCCCCGGGAACTTGTCTTCAAGCATGG. The forward and reverse primers for PP7 are ATGGAAACTGTTCACCAGATG and GCTATTTGGTGTTCGGTT. HRB1 genomic sequence or PP7 cDNA on pCR8/GW/TOPO vectors were recombined into pMDC83 and pMDC203 vectors through LR reaction (Invitrogen), respectively (Curtis and Grossniklaus, 2003).

One hundred µl of overnight agrobacterium (GV3101) culture was diluted to 4 ml LB medium and grown at 30°C for 16 hours. Cells were collected at 8,000 rpm for 2 minutes and resuspended in MES buffer (10 mM MES pH 5.6, 10 mM MgCl2, and 150 µM Acetosyringone). The cell suspension was then incubated at 30°C with gently shaking for 2 hours. Agrobacteria that carry pMDC83-control or pMDC83-HRB1 vector, pDMC203-PP7 vector, and pBin61-P19 at a ratio of 1:1:1 were co-infiltrated into well-watered nicotiana benthamiana leaves of 6 week-old. P19 encodes a suppressor of gene silencing and thus significantly increase the amount of protein produced in this transient expression system (Voinnet et al., 2003). Leaf tissues were harvested 3 days (without P19 co-infiltration) or 5 days (with P19 co-infiltration) after infiltration (Wydro et al., 2006).

About 12 grams of Nicotiana leaves were grounded in liquid nitrogen and resuspended in 10 ml cold co-precipitation buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% NP40, 10 mM Imidazole, and full set of proteinase inhibitors). After centrifugation at 20,000 g and 4°C for 30 minutes, the supernatant was added with 200 µl
pre-equilibrium nickel-Agarose beads (Qiagen), and incubated at 4°C for 2 hours (Tang et al., 2011). The incubation mixture was then washed 5 times with cold co-precipitation buffer and the proteins attached to the column were eluted with 100 µl cold co-precipitation buffer that contains 200 mM Imidazole. About 50 µl elution was mixed with 10 µl 5x SDS loading buffer and loaded onto SDS-PAGE gel. After blotting to membrane, PP7 was detected with anti-Myc antibody (Santa Cruz Biotechnology).

The HRB1 genomic DNA and PP7 cDNA in pCR8/GW/TOPO vectors were also recombined into pEarleyGate102 and pEarleyGate104 vectors (Earley et al., 2006). Agrobacteria that carry pEarleyGate102-HRB1:CFP and pEarleyGate104-PP7:YFP were co-infiltreted into Nicotiana leaves. Images were taken with a Nikon C1si Laser Scanning Confocal Microscope equipped with a three-channel PMT detector. The exciting wavelength for CFP was 458 nm and for YFP was 514 nm. The pEarleyGate102-HRB1:CFP and pEarleyGate104-PP7:YFP constructs were also introduced to Arabidopsis to generate stable transformants by Agrobacterium-mediated vacuum infiltration method (Bent et al., 1994). Rosetta leaves of two-week-old transgenic plants were soaked in 95% ethanol at 30°C for 1 hour and stained with Propidium Iodide (PI) for 10 minutes. The stomatal images were taken as described above with a 561 nm exciting wavelength for PI. Two-week-old 35S::HRB1:GFP transgenic plants were kept in dark for 3 days before images were taken directly or after 2 hours blue light treatment. HRB1:GFP fluorescence in guard cells were taken with an exciting wavelength of 488 nm.
Stomatal aperture and water loss measurements

Stomatal aperture was measured according to Mao et al (2005). *Arabidopsis* plants of 3 to 4 week-old were grown in darkness for 72 hours, and their epidermal layers were attached to adhesive tape and peeled off from the abaxial side of the leaf under dim green light. The epidermal strips were then floated in 10 ml of basal reaction buffer (5 mM MES pH 6.5, 50 mM KCl, and 0.1 mM CaCl$_2$) and kept in the dark for 1 hour. The epidermal strips were subsequently illuminated with blue light superimposed on the background with 25 µmol/m$^2$/s red light for 2 hours. Images were acquired by using a Lomo Epi-fluorescent microscope with a Spot Insight 4 MP CCD camera and analyzed with ImageJ software.

For water loss experiments, *Arabidopsis* leaves were detached and kept under blue light plus 25 µmol/m$^2$/s red light at 25% humidity. The detached leaves were weighed every 30 min and the rate of water loss was calculated as the percentage of their initial fresh weight (Leung et al., 1997; Mao at al., 2005).

PP7 RNA interference

A 365 bp DNA fragment from PP7 cDNA sequence was PCR-amplified and cloned into pCR8/GW/TOPO vector. The forward and reverse primers are CACCACCGTCGGGTAGTCTTCTTCT and GCATCTGGACCTTCATGT. This DNA fragment was then recombined into pAGRIKOLA vector (Hilson et al., 2004). This construct was transformed into *Arabidopsis* by Agrobacterium-mediated vacuum infiltration method (Bent et al., 1994).
Phosphatase assay and gel filtration analysis

Total protein was extracted from 0.5 gram of pMDC83-HRB1:GFP transgenic plants in 200 µl cold 2x PPass reaction buffer (the final 1x buffer contains 50mM HEPES pH 7.5, 100 mM NaCl, 10% glycerol, 0.5% Triton X-100, and full set of proteinase inhibitors). Eighty µg total protein was then incubated with 200 units of lambda protein phosphatase (New England Biolab) and 1 mM MnCl₂ at 30°C for 30 minutes. Activation of Na₃VO₄ was performed according to Gordon (1991), and activated Na₃VO₄ was added to a final concentration of 20 mM.

PP7:GFP:His protein was partially purified as described in the section of in vivo affinity co-precipitation. Agrobacteria that carry pMDC83-control or pMDC83-PP7, and pBin61-P19 were co-infiltrated into Nicotiana leaves. Tissues about 12 grams were harvested and PP7:GFP:His was purified through nickel-column (Tang et al., 2011). The His-tagged PP7:GFP was then eluted in 200 µl plant protein extraction buffer with 200 mM Imidazole and dialyzed against plant protein extraction buffer without Imidazole. Similarly, 80 µg total protein from pMDC83-HRB1 transgenic plants was incubated with 10 µg PP7:GFP:His or GFP:His protein and 5 mM MnCl₂ at 30°C for 30 minutes. For both assays, the reaction was stopped by adding 5x SDS loading buffer and boiling for 5 min. Samples were then loaded onto a 12% SDS-PAGE. HRB1:GFP was detected on western blot with anti-GFP antibody (Santa Cruz Biotechnology). The gel filtration profiles for HRB1:Myc or HRB1:GFP and PP7:Myc were performed as previously described (Zhou and Ni, 2010).
**Accession number**

Sequence of genes in this paper can be found at *Arabidopsis* Genome Initiative database by the following identification code: HRB1 (At5G49230), PP7 (At5G63870), Di19 (At1G56280) and UBQ10 (At4G05320).
**Results**

**HRB1 interacts with PP7 *in vitro***

To explore the biochemical function of HRB1 in light signaling, we conducted a yeast two-hybrid library screen with HRB1 as bait and identified PP7 as a potential HRB1 interacting protein. Since PP7 was previously identified as a blue light signaling component, we decided to further pursue this interaction. To determine if the ZZ-type zinc finger motif in HRB1 is involved in this interaction, we split HRB1 into its N-terminal half and C-terminal half, each fused to the GAL4 DNA binding domain (Figure 13A). The N-terminal HRB1, or most likely the ZZ-type zinc finger motif located within it, but not its C-terminal part interacted with full-length PP7 fused to GAL4 activation domain in a quantitative yeast two-hybrid assay (Figure 13B). The majority of the PP7 protein sequence is part of its catalytic domain and the most likely nature of this interaction may involve an enzyme-substrate relationship. For this reason, we only deleted the first 50 amino acids at its N-terminus as well as the last 70 amino acids at its C-terminus, leaving its catalytic domain intact in both cases (Figure 13A). The full-length or the truncated PP7s interacted with HRB1 and the interaction between HRB1 and PP7 C-terminal truncation was even stronger than that between HRB1 and full-length PP7 (Figure 13B). We subsequently performed an *in vitro* immuno-precipitation (IP) assay to verify this interaction (Figure 13C). In this assay, HRB1 protein, tagged with GAL4 DNA binding (BD) domain and produced in *E. Coli*, interacted strongly with PP7 produced in an *in vitro* transcription-translation system and radiolabeled with $^{35}$S-
methionine. By contrast, the BD domain alone did not interact with PP7 significantly (Figure 13C).

**PP7 interacts with HRB1 in vivo**

In order to have this interaction to occur, HRB1 and PP7 have to be transcribed and translated in the same cells at various stages of development. We searched eFP Gene expression database (Winter et al., 2007) and found that both *HRB1* and *PP7* are expressed in young seedlings, rosette leaves and guard cells (Figure 14A). We have learned previously that the expression of HRB1 was induced by light of various wavelengths (Kang et al., 2005). Following a dark-to-light transition, the *HRB1* mRNA accumulated, and the mRNA level declined after a light-to-dark transition (Figure 15A). By contrast, the expression of *PP7* was suppressed by light and resumed following a dark-to-light transition (Figure 15B). The accumulation of HRB1 protein was rapidly induced by one-hour treatment of blue light and the accumulation of PP7 was also rapidly induced by three-hour treatment of blue light (Figure 14B). However, the amount of either protein gradually declined with prolonged light treatment up to 9 hours (Figure 14B). Based on the information on the accumulation peak of both proteins, we designed an *in vivo* affinity-pull-down experiment in the dark and after blue light treatment for two hours. As shown in Figure 14C, HRB1:GFP:His but not GFP:His was able to pull down Myc:PP7 from plant extracts either in darkness or under blue light, suggesting that their interaction does not require light.
We next transiently expressed HRB1:CFP and PP7:YFP in Nicotiana leaves and demonstrated that both proteins co-localized to the nucleus in the epidermal cells (Figure 14D, upper panel). HRB1 and PP7 also co-localized in the nucleus of guard cells in stable transgenic Arabidopsis plants that carry HRB1:CFP and PP7:YFP (Figure 14D, lower panel). In conclusion, both proteins are normally expressed in the same cells.

**HRB1 and PP7 interact to regulate stomatal aperture under blue light**

hrb1 mutant has a defective light response under both blue and red light (Kang et al., 2005). The stomatal opening response is regulated by blue light and enhanced by red light, and we examined the stomatal aperture of the hrb1 mutant. hrb1 had much smaller stomatal aperture compared to wild type under weak to intermediate blue light (Figure 16A and 16B). The original studies on PP7 were performed in PP7 knock-down lines and we acquired SALK line 089764 that carries a T-DNA insertion in the second intron of the PP7 gene from Arabidopsis Biological Resource Center (Møller et al, 2003, Figure 17A). We verified the T-DNA insertion by PCR on genomic DNA prepared from wild type and this SALK line (Figure 17B). Reverse transcription PCR performed with two different primers that amplify sequence located upstream of the T-DNA insertion failed to detect any PP7 transcript in this SALK line (Figure 17C). Consistent with the previous studies, this pp7 knock-out SALK line also showed a long hypocotyl phenotype specifically under blue light (Figure 17D). The pp7 mutant also had a reduced stomatal aperture cross a relatively broad intensities of blue light compared to Columbia (Col) wild type (Figure 16C).
The *hrb1* and *pp7* mutants are in different ecotypes, Wassilewskija (Ws) versus Col, and both genes are located closely on chromosome 5. We therefore generated *PP7* RNAi lines in Ws and *hrb1* mutant background (Figure 18C). The stomatal opening response of the *hrb1*/PP7 RNAi double mutant was similar to that of *hrb1*, suggesting that HRB1 functions downstream of PP7 (Figure 18A). The hypocotyl phenotype of *hrb1* was also partially epistatic to that of *PP7* RNAi lines (Figure 18B). To better study their genetic interaction, we over-expressed HRB1 in *pp7* mutant since *hrb1* and *pp7* has a very similar phenotype of stomatal aperture. Compared to Col, over-expression of HRB1 caused a larger stomatal aperture and this phenotype was suppressed by the *pp7* mutation back to that of wild type (Figure 16D).

We performed quantitative analysis of water loss experiments and detached leaves of *hrb1* mutant lost less water under low to intermediate intensities of blue light compared to Ws wild type (Figure 19A). Over-expression of PP7 did not create any visible phenotype in either Ws or *hrb1* background and the transgenic lines showed a very similar water-loss response as either Ws or *hrb1* plants (Figure 19B). By contrast, the loss-of-function *pp7* mutant lost much less water compared to Col over a broad range of blue light intensities (Figure 19C). Over-expression of HRB1 in Col background promoted the rate of water loss presumably due to a larger stomatal aperture but this promotion was partially suppressed by the *pp7* mutation (Figures 16D and 19C).

**PP7 de-phosphorylates HRB1 in vivo**
HRB1:GFP migrated in two bands on SDS-PAGE gel (Figure 20A). Treatment with a non-specific Lambda protein phosphatase eliminated the upper band, presumably the phosphorylated isoform of HRB1 (Figure 20A). However, addition of Na$_3$VO$_4$, a phosphatase inhibitor, blocked the activity of the lambda phosphatase and HRB1:GFP showed the pattern of two bands (Figure 20A). To further examine if PP7 could also de-phosphorylate HRB1, we purified PP7:GFP:His or GFP:His fusion proteins from plant extracts by Nickle column, and mixed them with total protein extracts prepared from HRB1:GFP transgenic Arabidopsis plants. As shown in Figure 21A, PP7 de-phosphorylated HRB1:GFP protein directly and this activity is inhibited by Na$_3$VO$_4$. PP7 that carries a mutation in its catalytic domain (116D to A) failed to dephosphorylate HRB1 (Figure 21A). 116D is conserved in all protein serine/threonine phosphatases (Milla et al., 2006a). This aspartate residue in PP1 is critical for the conformation of its catalytic center and a D to A change reduced its phosphatase activity by 1000-fold (Huang et al., 1997). Compared to that in wild type, the level of HRB1:Myc protein accumulation in 35S::HRB1:Myc transgenic plants was not significantly altered in the pp7 mutant background (Figure 20B). The pp7 mutation also did not affect the nuclear localization of HRB1:GFP protein (Figure 20C).

**PP7 activity is required to assemble a functional HRB1 protein complex**

When plants were grown in darkness or under blue light, PP7:Myc protein was detected in gel filtration peak fraction 16, with a molecular mass of its monomer (Figure 21B). By contrast, HRB1:Myc protein was detected in peak fraction 12 and a protein
complex of 194 kDa in the dark (Figure 21C). After blue light treatment of plants for 2 hours, the peak of HRB1:Myc protein complex was shifted to fraction 11 with a molecular mass of 285 kDa (Figure 21C). Occasionally, we were also able to detect HRB1:Myc in various fractions of smaller molecular mass, ranging from its monomer or larger. The pattern of the blue light-induced size shift of the HRB1 protein complex remained exactly the same in either cry1/cry2 or phot1/phot2 double mutant, suggesting that either pair of the blue light receptors is required for this light-induced response. However, the light-induced size shift of the HRB1 protein complex was compromised in pp7 mutant background (Figure 21D). First, the peak of the HRB1 protein complex was in fraction 12 either in the dark or under blue light in the pp7 mutant background (Figure 21D). Second, HRB1:Myc was predominately de-phosphorylated in pp7 mutant plants either in the dark or under blue light (Figure 21D).
**Discussion**

**Both HRB1 and PP7 controls stomatal aperture response**

Blue light is one of the major environmental cues to regulate stomatal aperture, and both phots and crys are the photoreceptors for this response. Both *hrb1* and *pp7* mutant were isolated as hypocotyl mutants under red and blue light or blue light (Møller et al, 2003; Kang et al., 2005). We reported their defects in light-induced stomatal opening response in this study (Figure 16). In leaves, PP7 is mainly expressed in guard cells (Andreeva et al., 1999), and the regulation of PP7 on the stomatal aperture may be partially mediated through its interaction with HRB1. The wider stomatal aperture created by HRB1 overexpression was partially suppressed by the *pp7* mutation (Figure 16D). Another protein phosphatase, type 1 protein phosphatase or PP1, has also been reported as a positive regulator in the blue light-mediated stomatal opening response, downstream of phototropins but upstream of the H⁺-ATPase (Takemiya et al., 2006).

**HRB1 physically interacts with PP7**

We demonstrated that HRB1 and PP7 interacted in yeast two-hybrid system, *in vitro*, and *in vivo* (Figures 13 and 14). PP7 is a relatively large protein with the majority of its sequence as its catalytic domain and 50 to 60 amino acid extensions on each end. We found that the N-terminal ZZ-type zinc finger motif of HRB1 and the catalytic domain of PP7 mediated their interaction (Figure 13B). We have tested the hypothesis if HRB1 regulates the activity of PP7 through an *in vitro* phosphatase activity assay. Addition of recombinant HRB1 protein did not alter the phosphatase activity of PP7.
towards its synthetic peptide substrate. Our genetic analysis also did not support the idea that HRB1 acts upstream of PP7 (Figures 16 and 19).

**PP7 de-phosphorylates HRB1**

HRB1 protein migrated as two bands in SDS gel and this size difference is usually observed for posttranslational modification such as phosphorylation or ubiquitination. Either lambda phosphatase or PP7 treatment eliminated the upper band of HRB1:GFP or the phosphorylated form of HRB1 (Figures 20A and 21A). Full-length PP7 protein produced in *E. Coli* tends to form inclusion bodies and requires complicated denature-refolding treatment to recover its phosphatase activity (Kutuzov et al., 1998). A truncated form of PP7 with part of its catalytic domain deleted could be easily purified and had stronger activity toward an artificial substrate (Kutuzov et al., 1998). We found that this truncated form of PP7 did not de-phosphorylate HRB1 efficiently. We therefore produced PP7:GFP:His protein in transgenic plants and partially purified it through nickel column. Similar method has been successfully used to purify plant protein while retaining its activity (Tang et al., 2011).

Several Di19 family proteins were phosphorylated by Calcium-dependent protein kinases CPK3 and CPK11 (Milla et al., 2006a and 2006b). Interestingly, CPK4 and CPK11 regulate ABA-mediated stomatal aperture responses (Milla et al., 2006a and 2006b; Zhu et al., 2007). NetPhos software predicted 10 serine and 3 threonine sites being the potential phosphorylation site of HRB1 (Blom et al., 1999). Studies with mass spectrometry have identified at least two phosphorylation sites, Thr105 and Ser107 in
Di19 protein (Milla et al., 2006b). Thr105 is conserved in HRB1 but not Ser107, and HRB1 likely acquires new phosphorylation sites. Phosphorylation at Ser-Pro and Thr-Pro site also changes the electrophoretic mobility of a protein (Yu and Summers, 1994). We identified two Ser-Pro sites in HRB1 by NetPhos program and these sites may be involved in phosphorylation and de-phosphorylation of HRB1. In vivo identification of the true phosphorylation sites remains challenging and will be the focus of our future studies. The kinases for HRB1 remain unknown.

**PP7 activity is important for the formation of HRB1 protein complex**

Neither HRB1 stability nor its subcellular localization were altered in the *pp7* mutant. HRB1 exists in a protein complex mostly in its dephosphorylated form, and the size of this complex is about 194 kDa in the dark. Blue light caused a size increase of the protein complex (Figure 21C). This light-induced size shift was not altered in either *pho1/pho2* or *cry1/cry2* double mutant. Therefore, both pairs of photoreceptors appear to mediate this response. Attempts to introduce HRB1:Myc to quadruple *cry/phot* mutant have been unsuccessful since the quadruple mutant was weak and difficulty to transform. The size shift to a large protein complex induced by blue light was compromised in the *pp7* mutant, suggesting that the proper modification of HRB1 is required to bring in new components to the protein complex. Alternatively, HRB1 in the protein complex may adopt a different conformation upon de-phosphorylation by PP7.

PP7 protein migrated in a fraction of its monomeric size or larger (Figure 21B). Occasionally, HRB1 was also detected in the same fractions of smaller molecular weight
in either phosphorylated or de-phosphorylated form. The gel filtration fractions may contain interacting HRB1 and PP7 and/or partially assembled HRB1 protein complex. Interestingly, a fraction of HRB1 was still dephosphorylated in the *pp7* mutant background. HRB1 may be phosphorylated at multiple sites and that the crucial sites for the assembly of the complex are not the same sites responsible for the mobility shift of HRB1 on SDS-PAGE gel. Moreover, PP7 may be important to de-phosphorylate those residues that are critical for the assembly of HRB1 protein complex. In addition, a few other protein phosphatases may also potentially de-phosphorylate HRB1 at various sites to shape the final mobility pattern of HRB1 protein on SDS-PAGE gel, especially when cell structures were ruptured during the protein isolation process.
Figure 13. HRB1 interacts with PP7 in vitro.

(A) Yeast two-hybrid bait and prey constructs. Full-length and truncated forms of HRB1 proteins were fused to GAL4 DNA binding domain (BD) in pGBK7 vector. Full-length and truncated forms of PP7 proteins were fused to GAL4 activation domain (AD) in pGADT7 vector.
(B) Yeast two-hybrid assay of BD-HRB1 fusions with AD-PP7 fusions. PP7-N50 or PP7-C70 represents a N-terminal deletion of 50 amino acids or a C-terminal deletion of 70 amino acids. N-HRB1 or C-HRB1 represents N-terminus or C-terminus of HRB1.

(C) A pull-down assay using anti-GBD antibody, GBD or GBD:HRB1 bait produced in *E. coli* and *35*S-labeled PP7 prey produced in a TnT *in vitro* transcription and translation system.
Figure 14. HRB1 interacts with PP7 in vivo.

(A) Data acquired from eFP browser (bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) show the expression of HRB1 and PP7 in young seedling, rosetta leaf and guard cell. The color scales in the heat maps indicate normalized microarray expression values. The left color
scale is for seedling and rosetta leaf expression and the right color scale is for the guard cell.

(B) Accumulation of HRB1 and PP7 in leaves of 4 week-old HRB1:Myc and PP7:Myc transgenic plants in the dark and in response to blue light for 1, 3, 6 and 9 hours. Western blots were probed with anti-Myc antibody.

(C) His affinity-pull-down of PP7:Myc with GFP:His or HRB1:GFP:His in the dark and under blue light for 2 hours in transiently transformed Nicotiana leaves.

(D) Co-localization of HRB1:CFP and PP7:YFP in Nicotiana leaf epidermal cells (upper panel) under blue light for 2 hours. Co-localization of HRB1:CFP and PP7:YFP in 2 week-old Arabidopsis leaf guard cells (lower panel) under blue light for 2 hours. Propidium Iodide (PI) fluorescence is shown in pseudo color to illustrate cell shape. The blue light intensity for experiments in this figure is 4.93 µmol/m²/s.
Figure 15. Expression of HRB1 and PP7 in response to dark-to-light or light-to-dark transition.

Real-time RT-PCR analysis of the relative expression level of HRB1 (A) and PP7 (B) in transition from dark (Light 0 hr) to white light for 1, 2 and 4 hours and in transition from white light (Dark 0 hr) to dark for 1, 2 and 4 hours. Ws plants were grown in long day condition (16 hour light, 8 hour dark) for 4 weeks with 40 μmol/m²/s white light.
Figure 16. Genetic interaction of HRB1 with PP7 in stomatal aperture response.

(A) Images show the stomatal aperture of 4 week-old Ws and hrbl plants under 2 hour blue light (2 µmol/m²/s) plus 25 µmol/m²/s red light.
(B) and (C) Fluence response of stomatal opening in 4 week-old Ws and hrb1 or 4 week-old Col and pp7 plants. Plants were treated under the indicated blue light intensities plus 25\(\mu\)mol/m\(^2\)/s red light for 2 hours.

(D) Stomatal aperture of 4 week-old Col, pp7, 35S::HRB1::MYC in Col (HRB1OE) and 35S::HRB1::MYC in pp7 (HRB1OE pp7) transgenic plants in the dark or under blue light (0.33 \(\mu\)mol/m\(^2\)/s) plus 25 \(\mu\)mol/m\(^2\)/s red light for 2 hours.
Figure 17. Characterization of a PP7 knockout mutant.

(A) Schematic diagram shows T-DNA insertion in the PP7 gene (SALK_089764). Filled bar indicates 5’ and 3’ UTR, rectangle indicates exon, and line indicates intron. Numbers indicate the beginning and end of each exon. Primers used and their relative positions were shown in arrows.
(B) PCR verification of the T-DNA insertion in *pp7*. Full-length PP7 coding region was amplified with primers PP7-5 and PP7-3 from genomic DNA preparation. The T-DNA insertion allele was detected with primers lb5 and PP7-3.

(C) RT-PCR analysis of *PP7* expression in Col and *pp7* with two pairs of primers, PP7-5/PP7-851 and PP7-5/PP7-629. *ACTIN* and *UBQ10* were used as controls, respectively.

(D) Hypocotyl length of 4 day-old Col and *pp7* seedlings in the dark or under 5 μmol/m²/s red, 0.05 μmol/m²/s far-red or 1 or 6 μmol/m²/s blue light.
Figure 18. Genetic interaction of HRB1 with PP7 in hypocotyl elongation.

(A) Stomatal aperture in 4 week-old Ws, hrb1, PP7 RNAi in Ws (RNAi Ws) and PP7 RNAi in hrb1 (RNAi hrb1) plants in the dark or under 0.33 μmol/m²/s blue light plus 25 μmol/m²/s red light for 2 hours.

(B) Hypocotyl length of 4 day-old Ws, hrb1, PP7 RNAi in Ws (RNAi Ws) and PP7 RNAi in hrb1 (RNAi hrb1) seedlings in the dark and under 4.8 μmol/m²/s red or 1 and 5 μmol/m²/s blue light.
(C) Semi-quantitive RT-PCR analysis shows the expression level of PP7 in Ws or hrb1 mutant, and PP7 RNAi lines in Ws or hrb1 mutant background.
Figure 19. Genetic interaction of HRB1 with PP7 in water loss.

Fluence response of water loss in detached leaves from 4 week-old Ws and hrbl plants (A), Col, pp7, 35S::HRB1:MYC in Col (HRB1OE Col) and 35S::HRB1:MYC in pp7 (HRB1OE pp7) transgenic plants (B), and Ws, hrbl, 35S::PP7:MYC in Ws (PP7OE Ws) and 35S::PP7:MYC in hrbl (PP7OE hrbl) transgenic plants (C) under indicated blue
light intensities plus 25 µmol/m²/s red light treatment for 2 hours. Water loss is calculated as the percentage of their initial fresh weight.
Figure 20. *pp7* mutation does not affect HRB1 stability or subcellular localization.

(A) Phosphorylation status of HRB1:GFP from *35S::HRB1::GFP* transgenic plants treated with or without lambda phosphatase or Na$_2$VO$_4$.

(B) Accumulation of HRB1:Myc in Col (HRB1:Myc Col) or *pp7* (HRB1:Myc *pp7*) plant leaves in the dark or under 4.93 $\mu$mol/m$^2$/s blue light for 1 and 2 hours.

(C) Real-time RT-PCR analysis shows the relative expression of *HRB1:MYC* in Col (*HRB1:MYC* Col) or *pp7* (*HRB1:MYC* *pp7*) mutant background.
(D) Subcellular localization of HRB1:GFP in guard cells of Col or pp7 in the dark or under 4.93 µmol/m²/s blue light for 2 hours. Propidium Iodide (PI) fluorescence shows cell shape.

Figure 21. PP7 dephosphorylates HRB1 and affects the gel filtration profile of HRB1 protein complex.

(A) Phosphorylation status of HRB1:GFP from 35S::HRB1:GFP transgenic plants treated without or with GFP, with partially purified PP7::GFP (PP7G) or mutated

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PP7:GFP (PP7mG), and with PP7:GFP (PP7G) plus Na$_3$VO$_4$ (upper panel). The lower panel shows the partially purified PP7G, PP7mG, and GFP proteins.

(B) Gel filtration profile of PP7:Myc from leaves of 4 week-old Col transgenic plants in the dark (top) or under blue light for 3 hours (bottom).

(C) Gel filtration profile of HRB1:Myc from leaves of 4 week-old Col transgenic plants in the dark (top) or under blue light for 3 hours (bottom).

(D) Gel filtration profile of HRB1:Myc from leaves of 4-week-old $pp7$ transgenic plants in the dark (top) or under blue light for 3 hours (bottom). The blue light intensity for experiments in this figure is 4.93 µmol/m$^2$/s.
Chapter IV: Perspectives on future research
At current stage, the two projects have been finished. However, there are still important questions to be answered.

In HTL project, we will continue to elucidate how HTL regulates de-etiolation response as well as the biochemical function of HTL protein. We will overexpress HTL in hy5-ks50 mutant background. If over-accumulation of HTL could rescue hypocotyl phenotype of hy5 mutant, we would have a stronger argument that HTL is a key component in HY5 signaling pathway in photomorphogenesis.

htl-1 has a low germination rate. Therefore we have initiated several hormone sensitivity assays on germination. htl-1 is hypersensitive to ABA and Paclobutrazol (PAC), a GA biosynthetic inhibitor (unpublished data). However, hypocotyl elongation rate was equally suppressed by PAC compared to wildtype (unpublished data). Therefore, it is unlikely that GA biosynthesis or signaling was altered in htl-1 during de-etiolation.

HTL expression is highly enriched in vasculature tissues in root, hypocotyls and stem. In htl-1 mutant, vasculature bundles are not well developed with significantly less vein structures (unpublished data). Auxin related gene, for example, IAA6, is expressed at higher level in htl-1 mutant. Correspondingly, root elongation of htl-1 is less sensitive to 2,4-D inhibition (unpublished data). Using DR5::GUS as a reporter, we observed more GUS signal in cotyledons, but less in root tip in htl-1, suggesting that more auxin is accumulated in cotyledons (unpublished data). These results may indicate that htl-1 has defects in auxin distribution.

D14, HTL homolog in rice, was proposed a component in strigolactone biosynthesis or signaling pathways (Arite et al., 2009). MORE AXILLARY GROWTH2 (MAX2) is an
F-box protein involved in strigolactone signaling and max2 mutant also displays a long hypocotyl phenotype (Shen et al., 2007, Stirnberg et al., 2007). Interestingly, phyb mutant has reduced bud outgrowth capacity and this phyB function requires MAX2 (Finayson et al., 2010). Considering that htl-1 mutant has auxin distribution defects and auxin distribution is tightly related to strigolactione response, we believe that HTL may also participate in strigolactone signaling pathways.

To make our argument stronger, we may design different control set in current experiments for HRB1 and PP7 interaction. For example, we did not observe a difference in HRB1 protein accumulation in wild-type or pp7 mutant. Although it is most likely that the stability of HRB1 protein in pp7 mutant background did not change, we could not rule out the possibility that both de novo protein synthesis rate and degradation rate of HRB1 is changed. Using protein synthesis inhibitor Geneticin or G418 in this experiment could help distinguish which hypothesis describes the true situation. In gel-filtration experiment, sometimes we did not see a clear pattern of phosphorylated or de-phosphorylated HRB1 protein. We could treat protein samples with lambda phasphatase and check whether there is a change in HRB1 gel mobility. This experiment will help us determine the phosphorylation status of HRB1 in each fraction.

In this project, we mainly studied the interaction between PP7 and HRB1, a phosphatase and its substrate. However, we still do not know what the function of HRB1 itself is. Since we have already proved that HRB1 is involved in a protein complex and the size of this complex changes in response to light, it is very important to know what else are involved in this protein complex and the dynamics of different components in response to
light. We proposed to do an affinity and immune-purification of HRB1 containing protein complex and subsequently using GC-MS analysis to identify different components in HRB1 protein complex.
Bibliography


binding domain. EMBO J. 19(18):4997-5006.


Song YH, Yoo CM, Hong AP, Kim SH, Jeong HJ, Shin SY, Kim HJ, Yun DJ, Lim CO, Bahk JD, Lee SY, Nagao RT, Key JL, Hong JC. (2008) DNA-binding study identifies C-box and hybrid C/G-box or C/A-box motifs as high-affinity binding sites for STF1 and LONG HYPOCOTYL5 proteins. Plant Physiol. 146: 1862-1877.

Sun XD, Ni M. (2011) HYPOSENSITIVE TO LIGHT, an alpha/beta fold protein, acts downstream of ELONGATED HYOCOTYL 5 to regulate seedling de-etiolation. Mol Plant 4: 116-26


