THE IDENTIFICATION AND CHARACTERIZATION OF *SEEDLINGS HYPER-RESPONSIVE TO LIGHT* 2 (*SHL2*), A GENE IMPLICATED IN DEVELOPMENTAL RESPONSES TO LIGHT

A Dissertation

by

MI-SEON SEONG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Botany

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ABSTRACT

The Identification and Characterization of *Seedlings Hyper-responsive to Light* 2 (*SHL2*), a Gene Implicated in Developmental Responses to Light. (December 2006)

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Mutants showing developmental hyper-responsiveness to limited light were screened and designated as *seedlings hyper-responsive to light* (*shl*). These mutants showed an etiolated phenotype similar to wild type in the dark, yet had shorter hypocotyls, larger cotyledons, and more advanced development of true leaves than wild type in low light. The *SHL* genes act (genetically) as light-dependent negative regulators of photomorphogenesis, possibly in a downstream signaling or developmental pathway that is shared by the major photoreceptor genes (*CRY1*, *PHYA,* and *PHYB*) and other photoreceptors (*CRY2*, *PHYC*, *PHYD*, and *PHYE*). *shl1* and *shl2* were shown to be partially dependent on *HY5* activity for their light-hyperresponsive phenotypes.

shl1-1 showed a defect in responding to auxin in its root development in both white and yellow light conditions, and showed a defect in responding to auxin in hypocotyl elongation in yellow light. Compared to wild type, both *shl1-1* and *shl2-2* showed increased hypocotyl length in response to cytokinin in white light. Gibberellin (GA) partially recovered *shl1-1* mutant phenotype in yellow light, whereas showed no effect on hypocotyl elongation of *shl2-2* in this light condition. These altered responses of *shl1-1* and *shl2-2* to multiple phytohormones in different light regimes suggests that cross-talks among light and hormones regulate *SHL1* and *SHL2*.

One of the *SHL* genes, *SHL2* was cloned by map-based positional cloning and shown to be allelic to the previously identified locus designated *murus3(mur3)* and *katamari1(kam1)*. *MUR3/KAM1* encodes a XyG galactosyltransferase. Sequence analysis demonstrated that our original EMS generated reference allele *shl2-2* is probably not a null mutant, therefore the phenotypes of T-DNA insertion null mutant in *SHL2,* SALK_074435 were studied in different light conditions. Unlike *shl2-2*, SALK_074435 had a slightly short hypocotyl phenotype in the dark (though not to the extent of the *det/cop/fus* mutants). A consideration of the phenotypes and molecular lesions of *shl2-2* and *mur3* alleles, along with the phenotypes of null alleles *kam1* and SALK_74435, suggests that SHL2/MUR3/KAM1 may be involved in hypocotyl elongation in low light through the modification of xyloglucan in the plant cell wall, and may play a role in hypocotyl elongation in the dark through proper organization of the endomembrane.

DEDICATION

I dedicate this dissertation to my God who allowed me to study his wonderful creation, my husband David who has supported and encouraged me, my two precious children Daniel and Grace who are God's gift and give me joy every day, my parents who have supported me throughout my life, my father-in-law who inspired me on research, and my mother-in-law who supported me.

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CHAPTER I
INTRODUCTION

Light signal transduction and photomorphogenesis

Light is one of the most important environmental factors in plant biology. Light acts as a developmental cue and energy source for plants. As a developmental cue, light regulates leaf development, germination, and flowering time. Since plants are sessile organisms, the strategies used to sense light are critical to their survival. There are three classes of response to light: Low fluence responses (LFRs), the very-low-fluence responses (VLFRs), and high-irradiance responses (HIRs). In LFRs, plants respond between 1 and 1000 μmol $m^{-2}s^{-1}$ and sense red light (R) to far-red light (FR) ratios (R/FR) in their environment. LFRs are R/FR reversible and two subsets of LFRs are known. The first, shade avoidance, is the ability of plants to elongate their stems in response to a low ratio of red/far-red light under a leaf-canopy, or from nearby leaves (Kendrick and Kronenberg, 1994; Smith and Whitelam, 1990). The second, the end-of-day far-red response is the ability of plants to react to change in light quality at dusk (McNellis et al., 1994a). In a VLFR, between 0.1 and 1 μmol $m^{-2}s^{-1}$ of light can be sensed, and its responses, such as seed germination mediated by PHYA (Shinomura et al., 1996), are not reversible. In the HIRs, plants respond to ≥1000 μmol $m^{-2}s^{-1}$ of light (Neff et al., 2000). PHYA is involved in the VLFR and the FR-HIR, and PHYB is a major pothotoreceptor involved in the LFRs and R-HIR during photomorphogenesis (Nagy and Schafer, 2002; Quail, 2002).

Two distinct developmental programs control seedling development in a manner that is dependent on light conditions. In the dark, plants develop elongated hypocotyls with small pale cotyledons and closed apical hooks (skotomorphogenesis). When they are

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This dissertation follows the style and format of Plant Physiology.

exposed to light, hypocotyl elongation is inhibited, apical hooks open, cotyledons unfold, and chloroplasts develop (photomorphogenesis).

Genetic analysis has been a very useful method to identify the genes involved in photomorphogenesis (Chory et al., 1996). In this method, a mutant population with all possible phenotypes is created and a mutant with desirable phenotypes is screened followed by the identification of the gene that is responsible for the phenotypes (Jander et al., 2002). The pioneering genetic screening for photomorphogenic mutants were performed in two different environments. In the light environment, the mutants that displayed the dark-grown phenotypes were screened, and in the dark, the mutants that showed light-grown phenotypes were screened. From the genetic screens for mutants with dark-grown phenotypes in the light, *long hypocotyl* or *hy* mutants were identified (Koornneef et al. 1980). Except *hy5,* most of them were photoreceptor mutations. Mutants with defect in chromophore biosynthesis or attachment are *hy1*, *hy2*, and *hy6* (Parks and Quail, 1991; Chory 1992). Mutants with defect in apoproteins of phytochromes are *hy3, hy4,* and *hy8* whose genes encode the apoproteins of PHYB, CRY1, and PHYA respectively (Nagatini et al., 1991a; Parks and Quail, 1993; Ahmad and Cashmore, 1993).

To sense light conditions, plants employ multiple photoreceptors. In Arabidopsis, five phytochrome apoproteins encoded by *PHYA* to *PHYE* genes (Sharrock and Quail, 1989), three cryptochromes, and two phototropins (Liscum and Briggs, 1995; Briggs et al., 2001; Chen et al., 2004) are identified. Phytochromes are divided into two groups based on their stability in the light. Type I phytochromes are found at very high levels in etiolated seedlings but are degraded rapidily upon transfer to light (photo-labile), whereas type II phytochromes are relatively stable even though they are present at lower levels. In Arabidopsis, only PHYA belongs to type I phytochrome and PHYB-E belongs to type II phytochromes (Quail, 1997; Sharrock and Clack, 2002). PhytochromeA (PHYA) is responsible for de-etiolation in far-red light, whereas phytochromeB (PHYB)

plays the major role in perceiving red light (Pepper et al., 1994; Yamamoto et al., 1998; Neff et al., 2000). The mutations in *PHYB* (*hy3*) showed elongated hypocotyls, petioles, stems, and root hairs, and accumulated less light-inducible proteins such as chlorophyll a/b-binding (CAB) in the light. As adults, *phyB* mutants flower early and showed increased apical dominance (Reed et al., 1993). The role of PHYA has been studied through the *phyA* mutant that showed etiolated pattern under continuous far-red light (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993) and through overexpression of *PHYA* (Nagatani et al., 1991b). Whereas *PHYB* overexpression increased the sensitivity of transgenic seedlings specifically to red light, *PHYA* overexpression caused tobacco transgenic seedlings to be more sensitive to both far-red light and white light (Cherry et al., 1991; Nagatani et al., 1991b).

phyD and *phyE* single mutants showed no discernable phenotyles. However *phyD* and *phyE* mutants in a *phyB* mutant background showed subtle phenotypes indicating *phyD* and *phyE* have a degree of redundant function with *phyB* (Aukerman et al., 1997; Devlin et al., 1998, 1999). *PHYC* overexpression studies suggest its role in primary leaf expansion (Halliday et al., 1997; Qin et al., 1997). It is unlikely that phytochromeC (PHYC), phytochromeD (PHYD), and phytochromeE (PHYE) play as important role in plant development as PHYA and PHYB.

Cryptochromes and phototropins are blue/ultraviolet A (UV-A) light receptors. Cryptochromes are involved in regulating photomorphogenesis together with phytochromes (Briggs and Huala, 1999; Cashmore et al., 1999; Lin, 2000), whereas phototropins play a major role in phototropism (Briggs and Huala, 1999) and organelle movements such those observed in chloroplasts (Jarillo et al., 2001; Kagawa et al., 2001; Ohgishi et al., 2004; Lin, 2002). The *cry1* (*hy4*) mutant was identified through genetic screens for long hypocotyls in white light (along with *phyB/hy3*) (Koorneef et al., 1980). It showed greatly reduced inhibition of hypocotyl elongation as well as reduced blue-light-induced expression several genes in blue light (Ahmad and Cashmore, 1993).

Cryptochrome 2, CRY2 is involved in de-etiolation responses to low blue- light fluence rates, whereas CRY1 is the major photoreceptor under high blue light fluence rates (Ahmad and Cashmore, 1993; Lin et al., 1998). Phototropin was first identified as a plasma membrane protein that showed blue light-dependent phosphorylation in pea, Arabidopsis, and other plants (Gallagher et al., 1988; Short and Briggs, 1994; Christie and Briggs, 2001; Lin, 2002), and its gene was cloned from Arabidopsis phototropic deficient mutant *nph1* (non phototropic hypocotyl). Another phototropin involved in phototropism was identified and named as PHOT2 (originally NPL1 for *NPH1*-like). PHOT1 alone is involved under low blue light, but PHOT1 and PHOT2 have redundant functions under higher light fluence rates (Liscum and Briggs, 1995; Sakai et al., 2001).

Partial purification of phytochrome (Butler et al., 1959; Butler et al., 1964) and later complete purification of full-length PHYA holoprotein from multiple plant species allowed the study of its biochemical properities. Phytochromes have been found as soluble homodimers with each ~125kD polypeptide subunit consisting of an apoprotein covalently bound to a linear tetrapyrrole chromophore (Jones and Quail, 1986). However, Sharrock and Clack (2004) recently showed cross interactions between typeII (PHYB-PHYE) phytochromes. The apoprotein is divided into two major domains: an amino-terminal domain (signal input domain) and a carboxy-terminal domain (signal output domain) (Quail, 1997). An amino-terminal domain has the binding site for the chromophore and a carboxy-terminal domain is needed for dimerization and is involved in the transmission of light signals (Pepper, 1998). The N-terminal domain has four subdomains: P1, P2, P3, and P4. C-terminal domain contains two subdomains: a PER-ARNT-SIM (PAS)-related domain (PRD) and a histidine kinase-related domain (HKRD). A PRD has PAS-A and PAS-B domains (Bolle et al., 2000) found in many organisms playing important signaling roles in protein-protein interactions in response to oxygen, redox potential, and light (Taylor and Zhulin, 1999).

Phytochromes have two conformers that show reversibility by red (R) and far-red (FR) light (Butler et al., 1959) in LFRs: an R-absorbing Pr form and a FR-absorbing Pfr form that is considered to be biologically active (Quail, 1997). Structural rearrangements occur during this phototransformation of phytochromes (Park et al., 2000). Higher plant phytochromes are light and chromophore-regulated serine/threonine kinases, unlike phytochromes in cyanobacteria that autophosphorylate on histidine/aspartate (Yeh and Lagarias, 1998). Substrates of phytochromes include PKS1 protein kinase substrate 1), CRY1, CRY2, and Aux/IAA (Ahmad et al., 1998).

Subcellular localization of phytochromes is regulated by light. They are present in the cytoplasm in the dark but translocated to the nucleus upon exposure to light (Kircher et al., 2002; Sakamoto and Nagatani, 1996; Yamaguchi et al., 1999). Nuclear translocation of phytochromes requires a Pr to Pfr conformational change (Murphy and Lagarias, 1997). PHYA translocate to nucleus in FR, indicating that Pr configuration of phyA can be translocated to nucleus if it has been previously conformed to Pfr (Kircher et al., 1999).

Plant cryptochromes are related to DNA photolyases, UV-A/blue light–induced flavoproteins, that repair UV-B and UV-C-induced damage on DNA, but they possess no DNA repair activity (Sancar, 2003). N-terminal of cryptochromes contains photolyase homology region (PHR) with a primary catalytic chromophore flavin adenine dinucleotide (FAD) and a second light-harvesting chromophore, a pterin or deazaflavin, noncovalently bound (Lin et al., 1995; Sancar, 2003). C-terminal domains of cryptochromes are variable, but very important for their function on Arabidopsis CRY1 and CRY2. Even though there is little similarity on C-terminal domains among crytochromes, most of plant cryptochromes examined showed three motifs, known as DAS (DQXVP-acidic-STAES) domains (Lin and Shalitin, 2003). CRY3 is significantly different from CRY1 and CRY2 and closely related to the cryptochrome identified from cyanobacterium *Synechocystis*. It has no C-terminal extension, but has an N-terminal

transient peptide sequence mediating its import into mitochondria and chloroplast (Kleine et al., 2003). CRY2 is located in the nucleus and associated with chromosomes (Guo et al., 1999; Cutler et al., 2000). CRY1 is enriched in the nucleus in the dark but mainly in the cytoplasm in the light (Yang et al., 2000). The phototropin contains a photosensory N-terminal domain and a serine/threonine kinase C-terminal domain. N-terminal domain is composed of two LOV (light, oxygen, voltage) domains, a subset of PAS domains. These two LOV domains are called LOV1 and LOV2, and FMN (flavin mononucleotide) molecule is bound to these domains. Blue light perceived by flavins activates a serine/threoinin kinase in C-terminal domain and phosphorylates itself (Briggs and Christie, 2002). LOV2 domain is critical for phototropism (Cristie et al., 2002).

Early intermediates of phytochrome signaling

Through the pioneering genetic screens for mutants in Arabidopsis that showed the characteristics of light-grown seedlings in the dark, the constitutive photomorphogenic (*COP*) and de-etiolated (*DET*) loci were identified (Chory et al., 1989; Deng et al., 1991; Hou et al., 1993; von Arnim and Deng, 1996). Similar mutants with anthocyanic cotyledons have been designated *fusca* (*fus*) (Kwok et al., 1996). *COP/DET/FUS* genes showed overlapping, but largely similar pleiotropic photomorphogenic phenotypes including chloroplast development, inhibition of hypocotyl elongation, and activation of light-inducible genes in the dark (Kwok et al., 1996; Yamamoto et al., 1998). That all the *cop/det/fus* mutations are recessive implies that their gene products act as negative regulators of photomorphogenesis in the dark (Pepper et al., 1994; Kwok et al., 1996). Epistasis analyses by double mutation with each photoreceptor showed that these genes act downstream of multiple photoreceptors. They are considered to be late signaling components since different light qualities perceived by different photoreceptors cause the same developmental responses (Neff et al., 2000).

DET1 is localized in the nucleus but does not bind DNA, but instead acts via protein-protein interaction (Pepper et al., 1994; Pepper and Chory, 1997). The expression of *DET1* is not regulated by light indicating that the posttranscriptional modifications or protein-protein interaction maybe involved in its function, and that it may play a role as a signal transduction element, not a downstream effector molecule (Pepper and Chory, 1997). DET1 binds to Histone 2B in a nucleosome context and regulates gene expression by remodeling chromatin (Benvenuto et al., 2002). DET1 physically interacts with COP10 and UV-damaged DNA-binding protein 1a (DDB1a) and forms a CDD (COP10, DDB1, and DET1) complex (Yanagawa et al., 2004). The CDD complex physically interacts with COP9 signalosome (CSN), COP1, and proteasome subunits and enhances the ubiqutin-conjugating enzyme (E2) activity (Yanagawa et al., 2004). CSN increases the stability of the CDD complex (Suzuki et al., 2002). DET1 stabilizes the CDD complex and is probably necessary for nuclear localization of the complex (Yanagawa et al., 2004). DET1, together with COP1, is part of an E3 complex in mammals (Wertz et al., 2004). DDB1, DET1, and COP1 together regulate c-Jun ubiquitination in mammals (Wertz et al., 2004). However the role of DDB1 in CDD complex is not yet specified. COP10, an E2 ubiquitin-conjugating enzyme variant (Suzuki et al., 2002), alone has an ability to enhance the ubiquitin E2 activity (Yanagawa et al., 2004).

COP9 signalosome (CSN) was first identified in Arabidopsis and later discovered in other eukaryotes (reviewed in Harari-Steinberg and Chamovitz, 2004). CSN is localized to the nucleus (Chamovitz et al., 1996) and composed of eight protein subunits (CSN1-CSN8), which are encoded by the pleiotropic *cop/det/fus* loci (von Arnim, 2003). CSN shares similarity to the lid sub-complex of the 26S proteasome (von Arnim, 2003) and the eukaryotic translation initiation factor eIF3 to a lesser extent (reviewed in Harari-Steinberg and Chamovitz, 2004). CSN is involved in many developmental pathways such as photomorphogenesis, environmental stress, disease resistance, and hormone signaling, perhaps by regulating the ubiquitin-proteasome system (von Arnim, 2003). CSN is involved in ubiquitin-proteasome-mediated protein degradation in various ways.

First, a metalloprotease subunit of CSN regulates SCF (Skp1/Cullin/F-box protein)-type ubiquitin E3 ligase activities by removing an ubiquitin-like protein Nedd8/Rub1 from cullin (Lyapina et al., 2001; Zhou et al., 2001; Hochstrasser, 2002). Second, CSN associates with kinases such as inositol 1, 3, 4-trisphosphate 5/6 (5/6-kinase) and their substrates, suggesting a role as a master docking station for a SCF complex, a kinase, and its substrate (Harari-Steinberg and Chamovitz, 2004). Third, CSN is also involved in deubiquitination in mammals (Groisman et al., 2000) and in fission yeast (Zhou et al., 2003). The relation of CSN and COP1 varies in different organisms. In Arabidopsis, CSN is suggested to contribute to the nuclear localization of COP1 or to the COP1 stability in nucleus (Wang and Deng, 2004). In murine embryonic fibroblasts, the suppression of COP1 by subunit 3 of the COP9 signalosome (CSN3) is required for the tumor suppressor p53-dependent cell cycle arrest induced by Myeloid leukemia factor 1 (MLF1) (Yoneda-Kato et al., 2005).

COP1 contains a ring-finger-type zinc binding motif, a coiled coil domain (COIL), and WD-40 repeats, indicating that COP1 may bind to nucleic acids and to other proteins (Yamamoto et al., 1998). It has been suggested that DET1 acts upstream of COP1 in the cases of dark adaptation and seed germination (Ang and Deng, 1994). COP1 is localized in the cytoplasm in the light and transferred to the nucleus in the dark. Functioning as an E3 ubiquitin ligase, COP1 is involved in the protein degradation of positive light signaling regulators such as CRY2, HY5, HYH, LAF1, HFR1 and PHYA (Holm et al., 2001; Ang et al., 1998; Seo et al., 2003; Seo et al., 2004; Jang et al., 2005; Sharrock and Clark, 2002), thereby repressing photomorphogenesis. Photoactivated CRY1 and CRY2 physically interact with COP1 and repress its activity under the blue light (Wang et al., 2001). COP1 interacting proteins called CIP1 (Matsui et al., 1995), CIP4 (Yamamoto et al., 2001), CIP7 (Yamamoto, 1998), and CIP8 (Torii et al., 1999) have been identified.

Through further pioneering genetic screens for mutants that show the characteristics of dark-grown seedlings in the light, mutations defective in photoreceptors and HY5 have

been identified (Ahamad and Cashmore, 1993; Reed et al., 1993; Somers et al., 1993; Oyama et al., 1997). HY5, a positive regulator of photomorphogenesis and bZIP-type transcription factor (Ang et al., 1998; Chattopadhyay et al., 1998), binds to the G-box, one of the light-responsive cis-acting elements (LREs) found in light-regulated promoters. HY5 has shown to interact genetically with DET1 (Pepper and Chory, 1997) and physically with COP1 and SPA1 (Ang et al., 1998, Saijo et al., 2003). In the dark, HY5 interacts with nuclear localized COP1 through COP1 WD repeat domain and is targeted for protein degradation by the 26S proeasome (Holm et al., 2001).

To identify the early signaling components for photoreceptors, extensive genetic screens isolating photomorphogenic mutants in different light conditions, global gene expression studies, and the yeast two-hybrid system using the C-terminal domain of phytochromes have been performed. These studies have identified several overlapping or photoreceptor specific signaling components (Neff et al., 2000). Overlapping signaling components of PHYA and PHYB have been identified through both the yeast two-hybrid system and genetic screening. PIF3 (PHYTOCHROME INTERACTING FACTOR 3), NDPK2 (NUCLEOTIDE DIPHOSPHATE KINASE 2), and PKS1 (PHYTOCHROME KINASE SUBSTRATE 1) were identified through yeast two-hybrid system using the C-terminal domain of phytochromes, whereas *COG1*, *PFT1* (PHYTOCHROME AND FLOWERING TIME1), *PRR7*, *PDF1*, and *PSI2* genes have been identified through genetic approaches.

A nuclear localized bHLH protein, PIF3 is a negative regulator of phyB-mediated hypocotyls elongation and cotyledon opening, but functions as a positive regulator of CHS induction by both phyA and phyB (Kim et al., 2003). PIF3 binds to phyA to a lesser extent (Ni et al., 1998; Ni et al., 1999). It binds to G-box *cis*-element found in light-regulated genes and this complex interacts with light activated form (Pfr) of phyB (Martinez-Garcia et al., 2000). However, this interaction may be transient since PIF3 protein is accumulated in the nucleus in the dark requiring constitutive

photomorphogenesis 1 (COP1), but degraded rapidly in the COP1-independent way upon the exposure of red (R) and far-red light (FR) (Bauer et al., 2004). PKS1 and NDPK2 are localized in the cytoplasm. PKS1 interacts with the C-terminal domain of phyA and phyB and revealed phosphorylation by oat phyA serine/threonine kinase *in vitro* (Fankhauser et al., 1999). PKS1 is a negative regulator of phy B signaling and a positive regulator of VLFR responses mediated by phyA (Lariguet et al., 2003). NDPK2 is localized in both the cytoplasm and nucleus. It is homologous to animal tumor suppressors and is suggested to positively regulate phyA and phyB signaling (Choi et al., 1999).

A Dof transcription factor COG1 and PSI2 were suggested to be negative components of phyA and phyB signaling (Park et al., 2003; Genoud et al., 1998), whereas PEF1 and PRR7 are positive signaling components (Ahmad and Cashmore, 1996; Kaczorowski and Quail, 2003). *PRR7 (PSEUDO-RESPONSE REGULATOR7)* is involved in both seedling de-etiolation and circadian clock (Kaczorowski and Quail, 2003). *pft1* loss of function mutant showed hyposensitve to far-red and hypersensitive to red light, suggesting that PFT1 may function in phytochrome signaling at a node where phyA and phyB signaling converge. PFT1, a nuclear protein, showed an ability to activate transcription in yeast, suggesting that it may function as a transcription co- activator (Cerdan and Chory, 2003).

SUB1 and HFR1 are identified as overlapping signaling components of both phyA and cryptochrome (Guo et al., 2001; Fairchild et al., 2000). SUB1 is a calcium binding protein localized in the cytoplasm and may regulate HY5 negatively and regulate photomorphogenesis positively (Guo et al., 2001). HFR1 is a bHLH transcription factor involved in both PHYA and CRY1-medated responses. It forms homodimers or heterodimers eith PIF3 (Fairchild et al., 2000; Duek and Fankhauser, 2003). COP1 E3 ligase is involved in degradation of HFR1 transcription activators by ubiquitinating them as targets of protein degradation. This represses photomorphogenesis (Jang et al., 2005).

HRB1 (hypersensitive to red and blue 1) and PIF4(SRL2) are involved in both red and blue light signaling with *pif4 (srl2)* epistatic to *hrb1* in light-regulated gene expression responses (Huq and Quail, 2002; Kang et al., 2005). Both of them are localized in the nucleus and are negative regulators on de-etiolation (Kang et al., 2005, Huq and Quail, 2002).

PHYA specific signaling components that have been identified are FHY1, FHY3 (far-red elongated hypocotyls) (Whitelam et al., 1993), FIN2 (Soh et al., 1998), FIN5 (Cho et al., 2003), LAF1 (long after far-red light 1) (Ballesteros et al., 2001), LAF3 (Hare et al., 2003), LAF6, SPA1 (Hoecker et al., 1998), FHL, EID1 (Emplfindlicher Im Dunkelroten licht 1), HFR1/RSF1/REP1 (Farchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000), and FAR1 (far-red-impaired response) (Hudson et al., 1999), FIN219 (Hsieh et al., 2000), and PAT1 (Bolle et al., 2000). All of these proteins except SPA1 and EID1 are positive elements in the phyA pathway (Whitelam et al., 1993; Hoecker et al., 1998; Soh et al., 1998; Hudson et al., 1999) and most of those signaling components are located in the nucleus except FIN219 (Hsieh et al., 2000), PAT1 (Bolle et al., 2000), and FHY1. FHY1 is localized in both the cytoplasm and the nucleus (Desnos et al., 2001), whereas FIN219 and PAT1 exit in the cytoplasm. LAF1, HFR1 and HY5 are transcription factors (Ballesteros et al., 2001; Chattopadhyay et al., 1998; Duek and Fankhauser, 2003), whereas FAR1 and FHY3 are transposase-related proteins (Hudson et al., 2003). COP1 E3 ligase is involved in degradation of LAF1 and HY5 transcription activators. This represses photomorphogenesis (Jang et al., 2005). SPA1 (SUPPRESSOR OF PHYTOCHROME A), a coiled-coil protein, interacts with COP1 directly and promotes the ubiquitination of LAF1 only at low COP1 concentration, perhaps by stimulating COP1 activity (Seo et al., 2003). Also, its interaction with COP1 in dark reduces the degradation of HY5 (Saijo et al., 2003).

Genes involved in PHYB signalling such as *GI*, *ELF3*, *ELF4*, *SRR1* (sensitivity to red light reduced), *PEF2*, *PEF3*, *SRL1*, and *SRL2 (PIF4), RED1* have been identified (Reed et al., 1993; Ahmad and Cashmore, 1996; Wagner et al., 1997, Huq et al, 2000; Liu et al., 2001, Staiger et al, 2003; Doyle et al., 2002; Khanna et al., 2003). Most of them act positively in the phyB signaling pathway except *SRL1* and *SRL2* (Huq et al., 2000). *SRL2* encodes a nuclear localized PIF3-related protein (PIF4) that can bind to light regulated promoters like PIF3 (Huq and Quail, 2002). *SRR1* is localized in both nucleus and the cytoplasm (Staiger et al., 2003). *RED1* encodes a cytochrome P450 and CYP83B1, which is involved in catalyzing the N-hydroxylation of indole-3-acetaldoxime (IAOx) to synthesize indole glucosnolates. IAOx is also a precursor for the biosynthesis of IAA. Therefore in *red1* mutants, synthesis of indole glucosinolates is blocked, resulting in hyperaccumulation of auxin. This hyperaccumulation of auxin reduced responsiveness to red light, indicating auxin level is important for seedling de-etiolation under red light (Hoecker et al., 2004).

A novel Ser/Thr protein phosphatase (AtPP7) has been identified as a positive regulator of cryptochrome signaling component in Arabidopsis. *PP7* shows high sequence similarity to the *Drosophila* retinal degeneration C protein phosphatase that acts as a blue light signaling component (Møller et al., 2003).

Microinjection experiments, in which signal molecules are injected into plant tissue directly, suggest that Ca^{2+}/calmodulin, trimeric G proteins, and cGMP are involved in PHYA signaling pathway (Neuhaus et al., 1993; Bowler and Chua, 1994; Bowler et al., 1994).

Changes in the quality of the light reflected from neighboring vegetation can be sensed by plants and triggers the alteration of the plant architecture and dramatically accelerate flowering known as the shade avoidance response (Smith and Whitelam, 1997). PHYB is known as the major photoreceptor for this response, with minor roles of PHYD and

PHYE, whereas PHYA moderates this response (Devlin et al., 2003). The photoreceptors involved in shade avoidance have been widely studied, but the downstream components have not been well characterized. HAT4 (ATHB2), a homeodomain-Leu zipper gene, has been the only gene that shows transcriptional change in response to the reduced R: FR ratio (Carabelli et al., 1993) and its overexpression resulted in a constitutive shade-avoidance response in Arabidopsis (Schena et al., 1993). But recent DNA microarray study of genes expressed differently in response to stimulated shade has identified many potential shade-specific signaling components (Devlin et al, 2003).

In addition to the seedling developmental pathways described above, another developmental pathway controlling hypocotyl elongation in dim light has been described (Desnos et al., 1996; Sidler et al., 1998). Desnos et al. (1996) showed that the hypocotyls length of *prc1-1* was promoted in dim light while inhibited in the dark and in high fluence rate light. This study indicates the presence of PROCUSTE-independent pathway in dim light. PRC1-1 was identified as one of cellulose synthases, cesA6 (Refrégier et al., 2004). Together with other cell wall related genes that are regulated by light, PRC1-1 suggests that light regulates the expression of cell wall related genes to change the architectures of plants. The overexpression of another gene, designated *AtPGP1*, which belongs to the family of ATP binding cassette-containing (ABC) transporters, caused maximum hypocotyl elongation under dim light at ~20 µmol $m^{-2}s^{-1}$ (Sidler et al., 1998). These reports indicate that plants in dim light use different mechanism from plants in the dark to elongate their hypocotyl length to respond to low light condition. In support of a low light specific signaling pathway, we have identified mutants with hyper-responsiveness to light through the genetic screening for low light specific mutants and designated them as seedlings hyperresponsive to light (*shl*s). Whereas these seedlings showed an etiolated phenotype identical to wild type in the dark, they have shorter hypocotyls, larger cotyledons, and develop leaves earlier than wild type in low light (Pepper et al., 2001).

The involvement of auxin and other phytohormones in light signaling has been reported. Recently, together with another *ABC* (ATP-binding cassette) transporter gene *AtMDR1*, *AtPGP1* showed its involvement in basipetal auxin transport by participating in auxin efflux (Murphy et al., 2002; Lin and Wang, 2005). These data indicate that auxin transport is involved in hypocotyl elongation in low light. The genetic screens for mutants defective in responding to light or mutants with light grown phenotype in the dark also have led to the identification of many genes involved in both light and phytohormone signaling pathways. Many of these studies showed that hormones mediate photomorphogenesis. Light controls the production of many hormones such as ethylene, GA, and brassinosteroid and also changes the sensitivity to hormones. However their interaction is different depending on plant species, tissue types, and developmental stages (reviewed in Halliday and Fankhauser, 2003).

Photomorphogenesis, growth and regulation of the cell wall

Hypocotyl elongation under the control of light regulation is accomplished by cell elongation (Desnos et al., 1996). Plant cell elongation is driven by internal turgor pressure and limited by the cell wall extensibility (Taiz, 1984). Cell wall extension depends on the cell wall composition and the modification of exiting cell wall structures (Darley et al., 2001). Persson et al. (2005) analyzed publicly available microarray data to identify the genes that are corregulated with CESA genes and showed that the gene expression of COP1-interacting protein (CIP7), a positive regulator of light regulated genes was coregulated with CESA1, 3, and 6, suggesting its role as a bridge between light signaling and cell wall modification. Another example of interaction between light and cell wall modification was identified through the genomic analysis of the shade avoidance response using DNA microarrays (Devlin et al., 2003). In their study (Devlin et al., 2003), xyloglucan endotransglycosylase (*At-XTH24*) was up-regulated by the far-red light indicating that xyloglucan is involved in shade-avoidance response

The primary cell wall controls the plant cell shape and morphology. The primary cell walls of flowering plants consist of cellulose-xyloglucans network embedded in a pectins and structural proteins (Carpita and Gibeaut, 1993). Cellulose forms cellulose microfibrils, semicrystalline aggregates of linear polymers of β-1, 4-linked glucose residues (Emons and Mulder, 2000) and gives considerable mechanical strength to plant cells. Cellulose microfibril pattern determines wall properties and is regulated by cell type and developmental stage (Emons and Mulder, 2000). Cellulose is synthesized by plasma membrane-bound cellulose synthase complexes (Fagard et al., 2000). Plant cellulose synthase (CesA) proteins consist of six particles arranged in a ring, called particle rosette. In *Arabidopsis thaliana,* at least ten cellulose synthase (CesA) genes were found (Emons and Mulder, 2000; Fagard et al., 2000). The expression of CesA proteins in each species is different based on tissue types and cell wall types. One of the cellulose synthase mutants, *prc1-1* was identified in Arabidopsis by decreased hypocotyl cell elongation and increased radial expansion in hypocotyls in the dark, where it showed normal hypocotyl elongation in white light (Desnos et al., 1996). *PROCUTE1 (PRC1)* encodes one of Arabidopsis cellulose synthase, AtCesA6, also called IXR2 (Desnos et al., 1996; Fagard et al., 2000; Richmond, 2000).

Xyloglucans are the principal hemicellulosic components in flowering plants and form tight non-covalent association via hydrogen-bond with cellulose microfibrils (Valent and Albersheim, 1974; Hayashi et al., 1987, 1994a, b; Hayashi, 1989), and probably tether adjacent microfibrils (Fry, 1989). Xyloglucans possess a 1, 4-β-glucan backbone with 1, 6-α-D-xylosyl residues on three consecutive glucose residues, called XXXG (Fry et al., 1993). Each 'XXXG' building block of xyloglucans may carry D-galactose in β-1, 2-linkage at the second and/or third xylose residue resulting in XLXG, XXLG, and XLLG. Subsequent addition of L-fucose in α-1, 2-linkage at a specific galactosyl unit gives XXFG and XLFG (Madson et al., 2003). The final forms of xyloglucans are synthesized by glycosyltransferases that act on backbone structures in the Golgi apparatus, and transported to the cell surface by vesicles (Reiter, 2002). Several Arabidopsis

glycosyltransferases have been found. A xylosyltransferase in XyG biosynthesis is presumed to be encoded by Arabidposis AtXT gene (Faik et al., 2002). *AtFUT1* encodes fucosyltransferases (Perrin et al., 1999). *MUR3/KAM1* encodes XyG galactosyltransferase which is specific for the third Xyl residue within the XXXG building block (Madson et al., 2003).

Xyloglucan metabolism is important in cell expansion because the cellulose xyloglucan network is considered the major constraint to turgor-driven cell expansion (Chen et al., 2002). Several enzymes are suggested to be involved in xyloglucan metabolism: endo-ß-1,4-glucanases(EGases), xyloglucan endotransglucosylase/hydrolase (XTHs) and exo-glycosylhydrolases (glycosidases) (Rose and Bennett, 1999). EGases hydrolyze β-1, 4-linkages on the reducing-end side of unsubstituted glucose residues (Fanutti et al., 1993) and play a role in xyloglucan turnover during fruit ripening and organ abscission (Mølhøj et al., 2002). The involvement of EGases in auxin induced cell elongation was reported, but the effect of EGases in cell growth is poorly understood (Mølhøj et al., 2002). Four glycanases (α-fucosidase, α-xylosidase, β-galactosidase and β-glucosidase) are able to modify xyloglucan oligosaccharides and/or xyloglucan (Fry, 1995). Their involvement in cell expansion is not obvious. However their activity may indirectly affect on cell elongation. For example, they might alter xyloglucan-cellulose framework or increase the activity of endo-acting enxymes by removing the side chains from xyloglucan (Rose and Bennett, 1999). Arabidopsis has 33 XTH genes across all five chromosomes (Yokoyama and Nishitani 2001b; Rose et al., 2002). Expression analysis of the Arabidopsis XTH gene family showed that they exhibit different organ- or tissue-specific profiles (Yokoyama and Nishitani, 2001b). They also show different responses to plant hormones (Yokoyama and Nishitani, 2001b) and different responses to environmental stimuli (Xu et al., 1996). Peña et al (2004) showed that galactosylation of XyGs enhance the activity of Arabidopsis hypocotyl *XTH*s, which cleave XyGs and rejoin the cut ends with new partners resulting in cell elongation (Takeda et al., 2002). Light regulation of *XTH* genes has been reported. Some *XTH* genes such as *At-XTH 4,*

At-XTH15, At-XTH22, At-XTH24, and *At-XTH30* are upregulated by darkness (Xu et al., 1996). *At-XTH15* is down-regulated by PHYA (Kuno et al., 2000, Rose et al., 2002) and *At-XTH 24* is up-regulated by PHYA (Devlin et al., 2003). It has been reported that the expression of XTR7 is downregulated by red, far-red and blue light (Kuno et al., 2000; Kang et al., 2005). Phytohormones such as auxin and GA regulate the expression of xyloglucan endotransglycosylases (XTHs) (Catalá et al., 2001), suggesting that phytohormones play a role as an intimediator of light regulated cell elongation by modifying the cell wall components.

Over the past decades, the understanding of light signaling mechanisms in plants has been progressed significantly. However, photomorphogenesis is not a simple process. It is a coordinated development of the complex network including multiple hormone signaling pathways and cell wall synthesis. Intensive studies of hormone and light crosstalks have been conducted. However, the mechanism of how plants modulate their cell wall components and how they rearrange them to elongate cells in different light conditions requires further understanding.

CHAPTER II
IDENTIFICATION OF *shl* MUTANTS*

Introduction

Light quality, intensity, duration, and direction affect many aspects of plant development. The ability to monitor such signals is critical for their survival. To understand the mechanisms of how plants respond to their different light conditions, genetic screens have been used.

First genetic screens targeted the long *hypocotyls (hy)* mutants in white light and for light-grown or 'de-etiolated' phenotype in darkness. Genetic screens for the long *hypocotyls (hy)* mutants in white light resulted in the identification of photoreceptors and HY5, a transcriptional regulator (Ahmad and Cashmore, 1993; Reed et al, 1993; Somers et al., 1993; Oyama et al., 1997). And finally, genetic screens for light-grown or "de-etiolated" phenotype in darkness (Chory et al., 1989; Deng et al., 1991) have identified negative regulators of photomorphogenesis (Deng et al., 1992; Pepper et al., 1994; Wei et al., 1994a). Second genetic screens have been more specific and physiology-based, such as searching for phyA-specific and phyB-specific signaling components. Recently, physiological, pharmacological, and molecular approaches using two-hybrid screens in yeast have led to the identification of many important photo regulatory genes (Neff et al., 2000).

The ability of plants to sense light quality has been examined by studying shade avoidance responses in the photoreceptor mutants and found to be accomplished mainly

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*Reprinted with permission from "*shl*, a new set of Arabidopsis mutants with exaggerated developmental responses to available red, far-red, and blue light" by Pepper AE, Seong-Kim M, Hebst SM, Ivey KN, Kwak S, Broyles DE, 2001. Plant Physiology, 127, 295-304. 2001 by the American Society of Plant Biologists.

by phyB with minor roles for phyA, phyD, and phyE (Devlin et al, 2003). Decreased light intensity can also be sensed by plants and its effects on plant development have been studied. Decreased light intensity increases ethylene production in Arabidopsis rosettes and upregulates auxin-inducible genes resulting in changes in plant architecture (Vandenbussche et al., 2003b). Different light quality and light quantity regulate leaf elevation angles separately (Hangarter, 1997; Vandenbussche et al., 2003b). In pigweed, both light quantity (photosynthetic photon flux density, PPFD) and quality (red:far-red ratio, R:FR) were involved in plant height, whereas light quantity (PPFD) only regulated total dry matter accumulation and partitioning but not the dry matter accumulation to the stem (Rajcan et al., 2002). This study also showed that the occurrence of floral primordia, flowering and initiation of seed set was delayed in low PPFD.

To identify the genes involved in low light quantity (PPFD), we screened for mutants with phenotypic effects in low light-a threshold condition in which the normal photoperception pathways are only partially active, leading to limited deetiolation responses in wild-type(WT) seedlings. Using screens performed in low light, we obtained two classes of mutants: 1) those which had completely etiolated phenotypes, and 2) those which had completely de-etiolated phenotypes. Whereas some of the mutations in the former class mapped to known genetic loci (*PHYB, CRY1*), others appeared to be novel genetic loci (characterization of these will be presented elsewhere). Here, we present our initial analysis of several mutants with exaggerated developmental responses to available light.

Results

Mutant Screen

To identify novel regulatory components at the interface of light signaling and development especially responding to light quantity, we screened M_2 seed pools from

±28,000 individual ethyl methane sulfonate mutagenized M_1 plants. Aliquots from 16,420 seed pools were divided and screened simultaneously in low-intensity white light (4 µmol $m^{-2}s^{-1}$) and in darkness. An additional 9,540 seed pools were screened in darkness and under a yellow-green filter (24 µmol $m^{-2}s^{-1}$) that depleted much of the photomorphogenetically active B, R, and FR regions of the spectrum (the yellow-green filter was technically advantageous in that WT seedlings showed less phenotypic variance than that observed in low white light). Under each of these conditions, WT seedlings displayed a long hypocotyls and unfolded but poorly developed cotyledons.

We identified 380 M_2 families that segregated multiple individuals with short hypocotyls and expanded cotyledons in low light. In darkness, 202 of the 380 M_2 families segregated individuals with de-etiolated phenotypes, and an additional 99 families segregated individuals with severe developmental abnormalities (e.g. no root, fused cotyledons, and fascinated). The remaining 79 M_2 families had normal etiolated phenotypes in darkness. In the M_3 generation, 15 of these families (±19%) exhibited heritable light-hyperresponsive phenotypes. The candidate mutants obtained from these families were designated *shl* for seedlings hyperresponsive to light.

Genetic Characterization of *shl* Mutants

All 15 *shl* mutants were recessive in back-crosses to WT Columbia ecotype (Col-0). Mutant lines were assigned to complementation groups by F_1 complementation analysis. Three complementation groups, designated *shl1, shl2*, and *shl5* contained multiple alleles (with five, four, and two alleles, respectively). Various alleles of *shl1* and *shl2* were obtained from both the yellow-green light and the low-intensity white light conditions, indicating that the two light regimes were effectively similar. The remaining four mutant lines fell into mono-allelic complementation groups, indicating that our screens were far from “saturating”.

Phenotypic analysis of the F_2 progeny from back crosses to Columbia (Col-0 or Col-0 seeds carrying the glabrous mutation (Col-*gl1*) indicated that in 14 of 15 mutant lines, the light-hypersensitive trait was conditioned by a single gene (a subset of these data is presented in Table I). In the remaining line, mutant progeny were observed segregating in a ratio near 1:15 ($P > 0.70$), suggesting that the mutant phenotype in this line was due to recessive alleles at two unlinked loci. F_3 seeds were obtained by selfing of 20 of these F_2 progeny. Ten of the F_3 families segregated *shl* mutant individuals. This result closely fits ($P > 0.4$) the expectation for an F_2 population segregating two unlinked recessive loci, in which 7/16 of the individuals with WT phenotypes would be expected to carry at

Table I. *Segregation analysis of shl mutants.*

Mutants were back-crossed to WT Col-0 ecotype, and F_2 progeny were scored in low light for WT or light hyperresponsive (shl¯) phenotypes. Chi-squared (χ^2) analysis was applied using the null hypotheses (n.h.). Hypotheses indicated by (r) were rejected.

Cross	WT	shl¯	Ratio	n.h.	χ^2	*P*
shl1-1 × WT Col-0	624	200	3.12:1	3:1	0.235	>0.70
shl2-2 × WT Col-0	471	163	2.89:1	3:1	0.161	>0.70
shl3 shl4 × WT Col-0	521	32	16.28:1	3:1	108.9	<0.01 (r)
				15:1	0.211	>0.70
shl5-1 × WT Col-0	425	123	3.46:1	3:1	1.9	>0.15

least one mutant allele at both loci. Furthermore, mutant to WT ratios near 1:15 were consistently obtained in subsequent back-crosses to Col-0 and in out-crosses to Landsberg erecta. The putative double mutant line complemented all other lines, and the loci were tentatively designated *shl3* and *shl4*. Neither *shl3* nor *shl4* had an obvious morphological phenotype in the single-mutant homozygous state, although one of these loci had a subtle quantitative effect on hypocotyls length in high-irradiance FR light.

After two back-crosses to Col-0, representative alleles of the *shl1, shl2, shl5* complementation groups, as well as *sh3 shl4* double mutant, were out-crossed to

Landsberg *erecta* to create F_2 mapping populations. Molecular genotyping of 94 mutant F_2 individuals using PCR-based markers localized *shl1* to the top of chromosome 1, showing complete cosegregation with single sequence length polymorphism (SSLP) marker nga59. A mapping population of 94 mutant F_2 individuals was used to map *shl2* to a location on chromosome 2, ± 7.0cM telomeric to PHYB. Genetic mapping of *shl3* and shl4 were limited by the relatively small number of mutant individuals in the F_2 generation. However, we found convincing linkage of one of these loci to chromosome 1, between SSLP marker nga63 (11.48 cM) and cleaved amplified polymorphic sequence (CAPS) marker CAT3 (29.91cM). A smaller mapping population (38 mutant individuals) was used to locate *shl5* to chromosome 5, in close proximity to SSLP marker nga225 (± 1.3 cM).

shl Mutant Phenotypes

After 7d in low white light, *sh1, shl2, shl5* and the *sh3 shl4* double mutant had comparatively short hypocotyls and expanded cotyledons relative to WT (Fig. 1). Precocious development of the first set of true leaves was readily apparent in *sh2* and *shl3 shl4* and was also evident in *shl1* and *shl5*-particularly after 8 to 9 d in low light. All

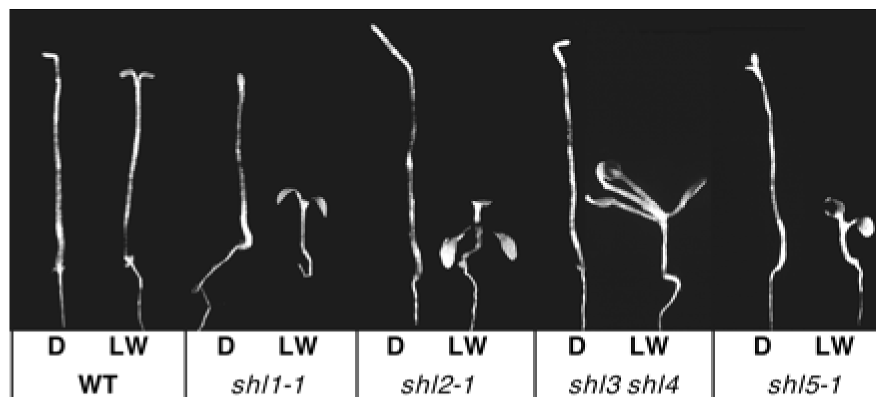

Figure 1. Morphologies of WT and *shl* mutant seedlings. Seedlings were grown for 7d on Murashige and Skoog/phytagar/2 % (w/v) Suc media in darkness (D), in low white (LW) at a fluence of 4 µmol $m^{-2}s^{-1}$.

four mutant lines had a normal etiolated morphology in darkness (Fig.1). A minority of *shl5-1* seedlings had partially open, but not expanded, cotyledons (as shown). The frequency of such seedlings was not reproducible from experiment to experiment. Hypocotyl length was used as simple quantitative measure of seedling developmental sensitivity to light (Fig. 2). In darkness, the strongest allele of *shl1* had slightly shorter hypocotyls than WT. The *shl3 shl4* double mutant and the strongest alleles of the *shl2* and *shl5* complementation groups had dark-grown hypocotyls lengths that were indistinguishable from WT. However, each of the mutants showed enhanced sensitivity to white light over a wide range of white light fluence conditions. For example, *shl1-1* showed 26% inhibition of hypocotyls growth at 0.37 µmol $m^{-2}s^{-1}$– a condition that had no effect on WT hypocotyl length. All of the *shl* mutants showed significantly enhanced

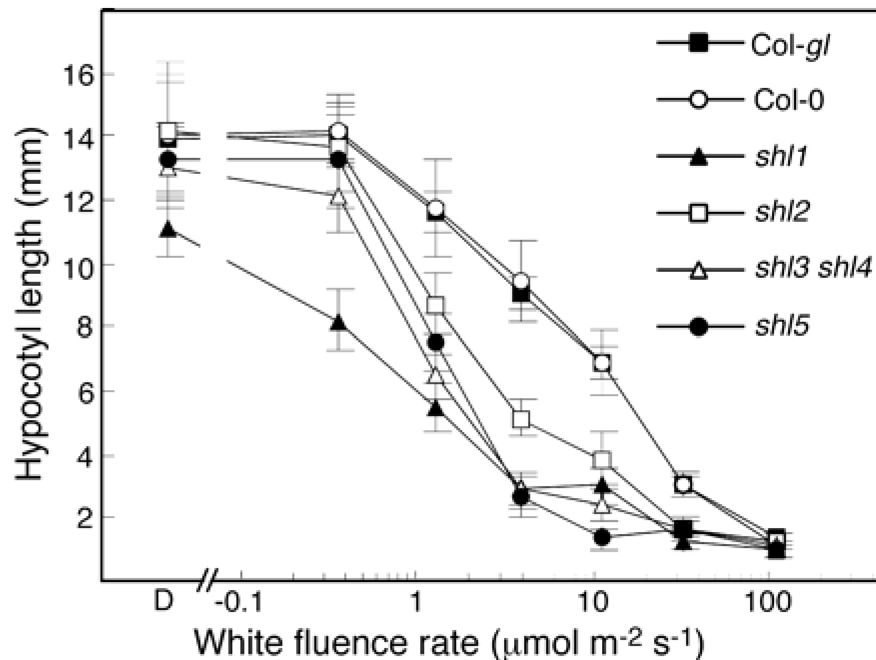

Figure 2. Hypocotyl length responses to white light of varying intensity in WT and *shl* mutant seedlings. Hypocotyls were measured in seedlings grown for 6 d. Error bars = SD.

inhibition of hypocotyls growth in th range of 1 to 30 µmol $m^{-2}s^{-1}$. At an intensity of 110 µmol $m^{-2}s^{-1}$, growth of WT and *shl* mutant hypocotyls was similarly inhibited.To determine the spectral dependence of expression of the *shl* phenotypes, *shl1-1, shl2-1*, *shl3 shl4*, and *shl5-1* were examined in narrow-spectrum R, FR, B, and green (G) light (Fig. 3). Each mutant displayed enhanced responsiveness to light of each of these spectral conditions. The *shl5* mutant showed comparatively less responsiveness to FR and (to a lesser extent) B than the other *shl* mutants, which showed similar patterns of responsiveness in the light conditions tested. Additional alleles of *shl1* (*shl1-2*) and *shl2* (*shl2-2, shl2-3, shl2-4*) showed qualitatively similar responses to those of those of the reference alleles shown in figure 3. In all cases, the shorter hypocotyls length of the *shl* mutant was accompanied by increased expansion of the cotyledons relative to the WT controls.

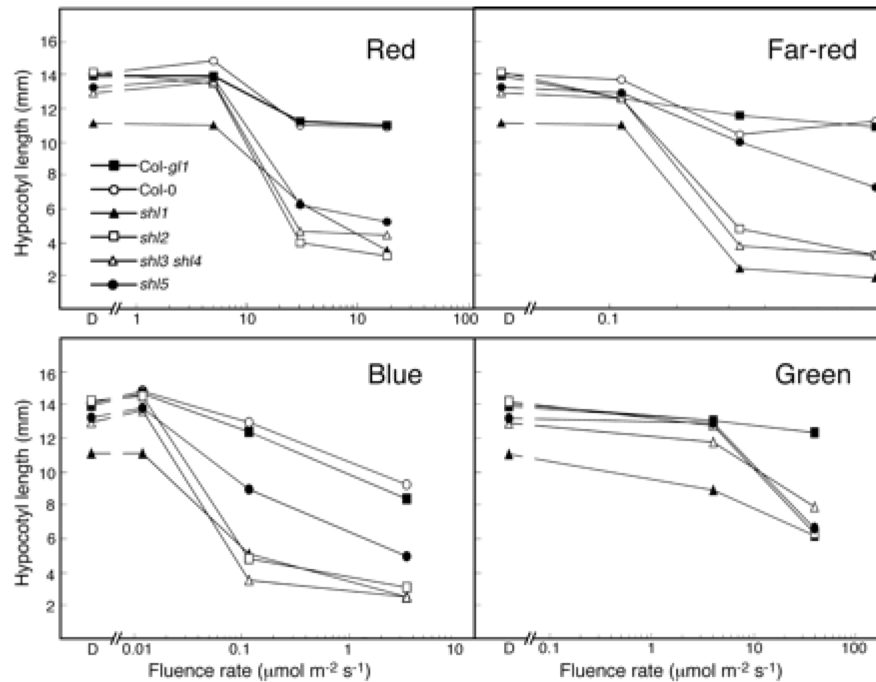

Figure 3. Hypocotyl length responses to various spectral conditions in WT and *shl* mutant seedlings. Hypocotyls were measured in seedlings grown for 6 d in R, FR, B, and G narrow-spectrum light sources at the range of fluences indicated. D, Dark condition.

Phenotypes of *shl* mutants were also examined in mature plants. All of the *shl* mutants displayed shorter petioles and a more compact rosette than WT. Plants carrying the most severe mutant allele of *shl1* showed a dramatic reduction in fertility and a moderate decrease in apical dominance (Table II). Whereas the *shl2-1* mutation and the *shl3shl4* double mutation resulted in modest increases (± 2-fold) in the accumulation of anthocyanin, the *shl5* mutation resulted in more dramatic increases (±10-fold). Finally, severe *shl2* alleles showed a moderate late-flowering phenotype.

Table II. *Phenotypic analysis of shl mutant plants.*
Plants were examined at the flowering stage, after ±35 d growth in long-day (16-h) conditions. A minimum of eight plants was examined for each determination. Anthocyanin content was measured as a ratio of ($A_{530} - A_{657}$)/g fresh wt. The number of elongated inflorescence axes was used as an indicator of apical dominance. Total leaf number was used as a measure of flowering time.

Genotype	Anthocyanin	Inflorescence Axes	Leaf No.
Col-0	1.20 ± 0.47	1.0 ± 0.00	8.38 ± 0.52
Col-*gl1*	1.18 ± 0.34	1.25 ± 0.46	8.50 ± 0.76
shl1-1	1.06 ± 0.31	2.86 ± 1.67	10.86 ± 2.27
shl2-1	2.46 ± 0.34	1.42 ± 0.49	14.10 ± 1.55
shl3 shl4	3.06 ± 0.45	1.34 ± 0.66	9.49 ± 0.96
shl5-1	13.67 ± 3.29	1.0 ± 0.00	8.42 ± 0.53

*Anthocyanin content was calculated as A530-A637/gram fresh weight

Discussion

To identify mutants in genes acting at the interface of light perception and developmental pathways-“downstream” from the photoreceptors and photoreceptor-specific signaling elements-we employed broad-spectrum white light to cast a “wide net” for mutants that were light hyposensitive or hypersensitive to a wide range of spectral conditions. At the onset, mutant seed pools were “counter-screened” in darkness to eliminate mutants in the *det/cop/fus* class and those with severe pleiotropic developmental defects. In pilot experiments, we found that under low-light conditions, even unmutagenized WT seed stocks gave rise to abnormal seedlings with a relatively

short hypocotyls and well-developed cotyledons at a low, but potentially problematic, frequency. This frequency appeared to increase with the age of the seeds, and with the length of time that the seeds are stored in an imbibed state. We concluded that a typical en masse screen of M_2 seedlings for mutants with exaggerated de-etiolation responses would yield an overwhelming number of seedlings with phenotypes that were not due to heritable mutation. To avoid this source of false mutants, we screened M_2 families derived from single M_1 plants and identified pools that segregated multiple individuals with light-hyperresponsive phenotypes. By this strategy, we isolated recessive light-hyperresponsive mutants in eight genetic loci.

On the basis of their recessive nature, we expect that the *SHL* genes act as negative regulators of photomorphogenesis. However, they are functionally distinct from mutants in *det/cop/fus* class in that they give rise to phenotypes that are hyperresponsive to available light, rather than light independent.

There is a formal possibility that *shl* mutants are extremely weak alleles of mutants in *det/cop/fus* class that express overt phenotypes only in the light. However over, the overwhelming majority of mutants in the *det/cop/fus* class have been mapped (Chory et al., 1989; Chory et al., 1991; Deng et al., 1991; Wei and Deng, 1992; Miserá et al., 1994; Wei et al., 1994b; Franzmann et al., 1995). *shl1* and *shl5* do not appear to be closely linked to any of these mapped loci.

The *shl1, shl2, shl5* mutants and the postulated *shl3 shl4* double mutant are phenotypically distinct from other identified light-hypersensitive mutants. The *spa1* (Hoecker et al., 1998) and *eid1* (Buche et al., 2000) mutants appear to be FR-specific in their phenotypic expression. *psi2* (Genoud et al., 1998) displays hypersensitivity to both R and FR light, but is dependent on *PHYB* and *PHYA*, respectively, for these effects and did not show a significant phenotype when tested in a range of B light intensities. Mutations in *SUB1*, a Ca^{2+} binding protein, show enhanced responsiveness to B and FR,

but not to R (Guo et al., 2001). Finally *shy1* (Kim et al., 1996) and *srl1* (Huq et al., 2000) have R-light-dependent phenotypes. *srl1* was located on chromosome 2 near the mapped location of *shl2*, but its phenotypic expression is strictly dependent on PHYB. In contrast, *shl2* was not strictly dependent on *PHYB* even for its R-light hypersensitivity. Furthermore, all four alleles of *shl2* showed clear hyperresponsive phenotypes in R, FR, B, and G light. Finally, although one of the *shl3* or *shl4* loci had a subtle hyperresponsive phenotype in FR (as a single mutant), we did not detect any linkage of either loci to nga 168, which is linked to *SPA1* on chromosome 2 (Hoecker et al., 1998), or to nga8, which is linked to *EID1* on chromosome 4 (Buche et al., 2000).

shl1, *shl2*, *shl3 shl4* double mutant, and (to a lesser extent) *shl5* exhibit hyperresponsive phenotypes in FR, R, B, and G. One interpretation of this finding is that the *SHL* genes are acting in a downstream signaling pathway that is shared by *CRY1*, *PHYA*, and *PHYB* and possibly other photoreceptors (*CRY2, PHYC, PHYD,* and *PHYE*). This downstream placement of the *SHL* genes would place them at or near the interface where light signal transduction elements are interacting with developmental regulators. The phenotypes of the *shl* mutants may be due to mutations in signaling molecules or other regulators that result in an increase in the sensitivity of a particular signaling process or amplify the developmental responses. In this respect, *SHL3* and *SHL4* appear to have at least partially overlapping functions. Several of the mutants also had light-related phenotypes as adult plants, displaying short petioles, elevated anthocyanin (*shl2, shl3 shl4*, and *shl5*), and in the case of *shl2*, a moderate late-flowering phenotype similar to that seen in plants overexpressing *CRY1* (Lin et al., 1996).

Materials and methods

Plant Materials, Growth Conditions, and Mutant Isolation

Arabidopsis ecotype Col-*gl1* seeds were obtained from Lehle Seeds (Round Rock, TX).

Col-*gl1* seeds were mutagenized by imbibation in 0.3% (v/v) ethyl methane sulfonate for 12h, followed by extensive washing with H_2O. M_1 seed were sown on soil to achieve a final density of 0.25 plants cm^{-2}, grown under an 8-h day-length regime for 40d, then transferred to a 16-h day length in order to stimulate flowering. This protocol produced mature plants with a stout, erect inflorescence, thus preventing entanglement and greatly facilitating the harvest of independent M_2 seed pools from individual M_1 plants. Aliquots of ±80 seeds from each M_2 seed pool were surface sterilized (Chory et al., 1989), resuspended in sterile 0.1% (w/v) phytagar, then cold treated at 4 ^oC for 40h. Seed pools were then dispersed onto duplicate plates containing Murashige and Skoog/phytagar/ 2% (w/v) Suc media. Seeds were illuminated for 4h with white light (100 µmol $m^{-2}s^{-1}$) to ensure optimal germination, then screened simultaneously in darkness and in either low-intensity white light (4 µmol $m^{-2}s^{-1}$) or under a yellow-green acrylic filter (24 µmol $m^{-2}s^{-1}$). Mutants were identified after 7 to 8 d. Unless stated otherwise, experiments were performed at 23 ^oC± 0.5 ^oC under a 16-h day-length regime.

Genetic Analysis

The genetic methods employed have been described previously (Chory et al., 1989; Pepper and Chory, 1997). Routine phenotyping for complementation, segregation, and mapping experiments was performed under low white light or under a yellow-green acrylic filter. Genomic DNAs were isolated using the micropreparation method described by Pepper and Chory (1997). Mapping of *shl* mutants was performed using PCR-based CAPS (Konieczny and Ausubel, 1993) and SSLP (Bell and Ecker, 1994; Lukowitz et al., 2000) markers. Mutants were back-crossed to WT Col-0 or Col-*gl1* at least three times prior to comprehensive phenotypic analysis.

Analytical Methods

Occasional seedlings with obvious severe developmental defects were omitted from any

phenotypic analyses. For measurements of hypocotyls length, 30 seeds of each genotype were evenly dispersed onto Murashige and Skoog/phytagar/ 2% (w/v) Suc media in a 7-mm grid pattern. All seeds were subjected to 4h of white light (100 µmol $m^{-2}s^{-1}$) prior to placement in the dark or in various light regimes for 6 d. Hypocotyls were straightened using forceps if necessary, and then measured under a stereo dissecting microscope using a 0.5-mm ruler. Hypocotyls of seedlings growing appressed to the agar media were not measured. Analyses of anthocyanin content (by an acid-methanol extraction), flowering time, and apical dominance were performed as described in Pepper and Chory (1997).

Light Sources

Narrow-spectrum R and FR light were supplied by light-emitting diode arrays (models SL515-670 [670-nm maximum] and SL515-735 [735-nm maximum], respectively; Quantum Devices, Inc., Barneveld, WI). Narrow-spectrum B light (420-nm maximum) was supplied by Coralife Actinic 03 fluorescent aquarium bulbs (Energy Savers Unlimited, Inc., Carson, CA) filtered through a Kopp 5-57 blue glass filter (Kopp Glass, Inc., Swissvale, PA). White light was supplied by an equal mixture of cool-white and Grow-lux wide-spectrum fluorescent bulbs (Sylvania, Danvers, MA). A 2472 yellow-green acrylic filter (Polycast Technology, Stamford, CT) with a transmission maximum of ±550 produced light that was partially depleted in the photomorphogenetically active UV, B, R, and FR regions of the spectrum. Narrow-spectrum G light (±520-nm maximum) was produced by a 2092 green acrylic filter (Polycast Technology), as described previously (Ahmad and Cashmore, 1993; Lin et al., 1996). Dark experiments were performed in a passively ventilated dark box. Fluence rates of white, R, B, yellow, and G light were measured with a quantum photometer (model LI-189, LI-COR, Lincoln, NE). Fluence rates of FR light were measured using a radiometer (model IL1400, International Light, Newburyport, MA) with FR probe (model SEL033, International Light).

CHAPTER III
GENETIC INTERACTIONS WITH PHOTORECEPTORS AND HY5*

Introduction

Different light quality is perceived by three different groups of photoreceptors, phytochromes, cryptochromes, and phototropins (Quail, 2002a). In Arabidopsis, there are 5 phytochromes encoded by *PHYA* to *PHYE* genes (Sharrock and Quail, 1989), 2 cryptochromes, and 2 phototropins (Liscum and Briggs, 1995; Briggs et al., 2001; Chen et al., 2004). PHYA is responsible for de-etiolation in far-red light, whereas PHYB plays the major role in perceiving red light (Neff et al., 2000). *phyD* and *phyE* mutants showed subtle phenotypes only in a *phyB* mutant background indicating *phyD* and *phyE* have a degree of redundant function with *phyB* (Aukerman et al., 1997; Devlin et al., 1998, 1999). Overexpression studies of *PHYC* suggest its role in primary leaf expansion (Halliday et al., 1997; Qin et al., 1997). Cryptochromes and phototropins are both blue light receptors. Cryptochromes are involved in regulating photomorphogenesis together with phytochromes, whereas phototropins play a major role in phototropism and chloroplast movement (Ohgishi et al., 2004; Lin, 2002).

Downstream of phytochrome and cryptochrome signaling components are studied through physiological, pharmacological, molecular, and genetic approaches (Neff et al., 2000). One of shared signaling components of photoreceptors is the positive regulator HY5. HY5 is located in nucleus and activates light-regulated genes through interaction with the G-box on their promoters (Chattopadhy et al., 1998).

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*Reprinted with permission from "*shl*, a new set of Arabidopsis mutants with exaggerated developmental responses to available red, far-red, and blue light" by Pepper AE, Seong-Kim M, Hebst SM, Ivey KN, Kwak S, Broyles DE, 2001. Plant Physiology, 127, 295-304. 2001 by the American Society of Plant Biologists.

To test for functional dependence of the *shl*s on photoreceptors and HY5, we have made double mutant combinations of each *shl* mutant with phyA*, phyB, cry1,* or *hy5*. Here we present that the light-hyperresponsive phenotype of the *shl1* mutant was only partially dependent on *PHYB* and *PHYA* in R and FR respectively, but probably strictly dependent on *CRY1* in B light. The light-hyperresponsive phenotype of the *shl2* mutant was only partially dependent on *PHYB, PHYA*, and *CRY1* in R, FR, and B, respectively. Both *shl* mutants showed a partial dependence on HY5 activity for expression of their light-hyperresponsive phenotypes, indicating there is a separate pathway from HY5 on hypocotyl elongation in low light.

Results

Genetic Interactions with Photoreceptors PHYA, PHYB, and CRY1

The photoreceptors PHYA, PHYB, and CRY1 play predominant-but not exclusive-roles in seedling photomorphogenetic responses to FR, R, and B, respectively (Whitelam et al., 1993; Reed et al., 1994; Ahmad and Cashmore, 1997; Neff and Chory, 1998; Casal and Mazzella, 1998). To test for functional dependence of the *shl* phenotypes on each of these photoreceptors, *shl1-1* and *shl2-2* were placed in double-mutant combinations with *phyA-211, phyB-9,* and *cry1-B36* (in the Col-0 genetic background). Hypocotyl phenotypes of *shl phyA*, *shl phyB*, and *shl cry1* double mutants were determined in FR, R, and B, respectively.

As shown in Figure 4, the *shl1* mutant retained significant phenotypic effects on hypocotyls length in both the *phyA* mutant and *phyB* mutant backgrounds. In the cross of *shl1* to *cry1*, five homozygous *cry1* mutant individuals were identified in the F2 generation by PCR; all had a long hypocotyl phenotype in B, similar to the *cry1* control. However, in the F3 progeny from these five F_2 individuals, no novel phenotypes were observed. Thus, we could not definitively identify a phenotype for the *shl1 cry1* double

mutant. Given that *shl1* is not linked to *cry1*, we would have expected that two-thirds of the five F_2 individuals would have been heterozygous for *shl1*. The probability that at least one of the five F_2 individuals was heterozygous for *shl1* is approximately 99.6%. Thus, there is a strong possibility that the phenotype of *shl1* in B light is strictly dependent on *CRY1* activity. This hypothesis is supported by the fact that there were no homozygous *cry1* individuals with a hypocotyl phenotype that was shorter that the *cry1* mutant control. However, it remains remotely possible that *shl1* does indeed exert an effect in the *cry1* mutant background and that none of the homozygous *cry1* mutant

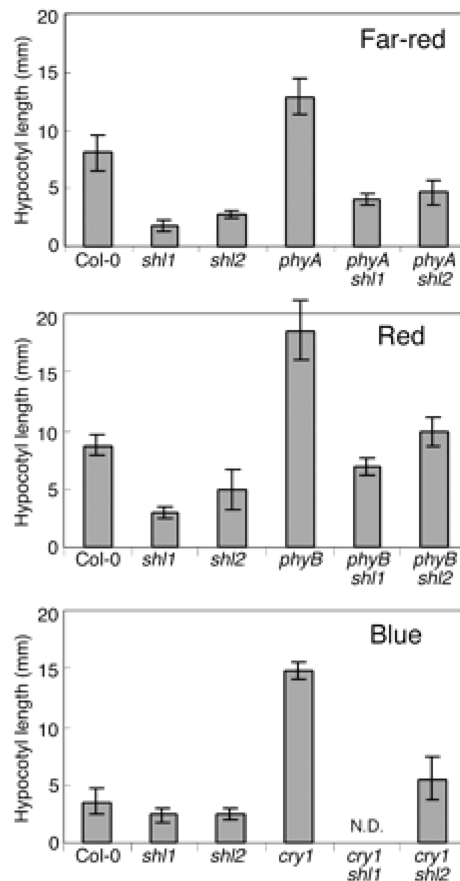

Figure 4. Genetic interactions between *shl1-1* and *shl2-2,* and the photoreceptor mutants *phyA-211*, *phyB-9*, and *cry1-B36*. Hypocotyls were measured in seedlings grown for 7 d in R (64.4 µmol m^{-2} s^{-1}), FR (7.8 µmol m^{-2} s^{-1}), and B (2.78 µmol m^{-2} s^{-1}). These intensities were selected to provide effective phenotypic discrimination between WT and photoreceptor mutants. N.D., Not determined. Error bars = SD.

background and that none of the homozygous *cry1* F_2 individuals were heterozygous or homozygous for *shl1*. The light-hyperresponsive phenotype of the *shl2* mutant was only partially dependent on *PHYB, PHYA*, and *CRY1* in R, FR, and B, respectively (Fig. 4). For example, although the *shl2 phyB* had slightly longer hypocotyls than WT, it was still significantly inhibited compared to the *phyB* single mutant.

Genetic Interactions with HY5

HY5 is a basic-Leu zipper transcription factor that positively regulates seedling de-etiolation and in the process actively promotes the inhibition of hypocotyls elongation (Koorneef et al., 1980; Oyama et al., 1997). The hypocotyl phenotypes of *shl1 hy5* and *shl2 hy5* double mutants were examined in moderate white light (Fig. 5). The phenotypes of the double mutants were additive, with both *shl* mutants showing a partial dependence on HY5 activity for expression of their light-hyperresponsive phenotypes.

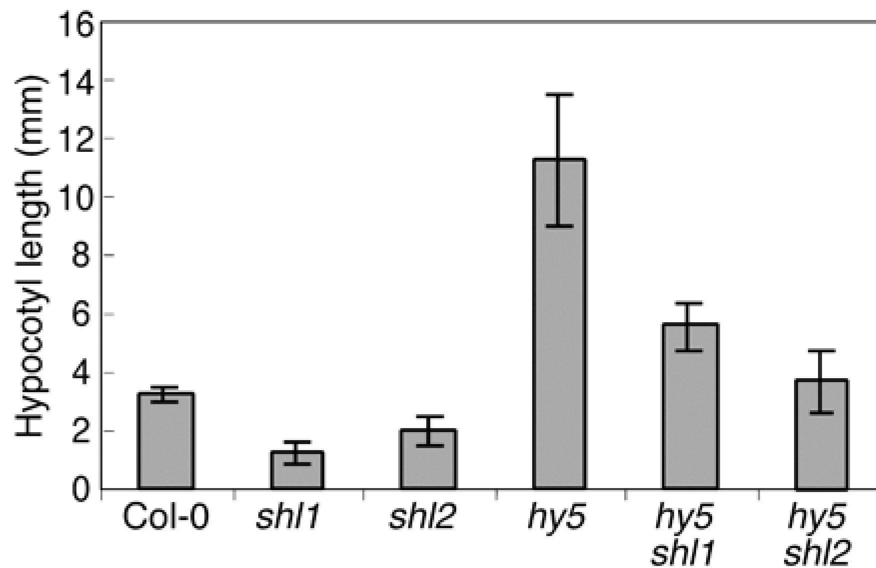

Figure 5. Genetic interactions between *shl1* and *shl2-2* and the *hy5-5C* mutant. Hypocotyls were measured in seedlings grown for 7d in white light at an intensity of 45µmolm^{-2}s^{-1}. Error bars = SD.

Interestingly, *shl1*, which as a single mutant showed the greater inhibition of hypocotyls length in this light condition, also showed the greater degree of dependence on *HY5* activity.

Discussion

The photoreceptors PHYA, PHYB, and CRY1 play the dominant roles in seedling photomorphogenetic responses to FR, R, and B, respectively (Reed et al., 1994; Ahmad and Cashmore, 1997; Casal and Mazzella, 1998; Neff and Chory, 1998). It is interesting to note that the FR, R, and B phenotypes of *shl1* and *shl2* were only partially dependent on PHYA, PHYB, and CRY1, respectively. However, the roles played by these major photoreceptors are not exclusive. For example, both Pr and Pfr absorb in the B region of the spectrum (Smith, 1986). PHYA plays a subsidiary role in B inhibition of hypocotyl elongation (Whitelam et al., 1993; Ahmad and Cashmore, 1997; Casal and Mazzella, 1998; Neff and Chory, 1998). CRY2 plays a significant role in B-dependent inhibition of hypocotyl elongation at low fluence levels (<10 μmol $m^{-2}s^{-1}$), similar to those used in our phenotypic analyses. Finally, PHYB plays a minor role in FR-stimulated opening of the apical hook (Neff and Chory, 1998), and other phytochromes (PHYC, PHYD, and PHYE) are either known to, or presumed to, play subsidiary roles in various photomorphogenetic responses to R and FR (Aukerman et al., 1997; Poppe and Schäfer, 1997; Devlin et al., 1999). Thus, the phenotypes of *shl* mutants in R, FR, and B may be dependent on signals generated by a larger set of photoreceptors with partially overlapping, and often synergistic, activities that may include PHYA through PHYE and both CRY1 and CRY2.

Although PHYA and PHYB are required for full activity or CRY1 (Ahmad and Cashmore, 1997; Casal and Mazzella, 1998; Neff and Chory, 1998), CRY1 can also act independently of PHYA and PHYB (Casal and Mazzella, 1998; Neff and Chory, 1998). All of the *shl* mutants showed substantial hyperresponsiveness to G light. Both Pr and

Pfr have absorption minima in the green region of the spectrum (Smith, 1986), and hypersensitivity to G light has previously only been observed in transgenic plants overexpressing *CRY1* (Lin et al., 1996). This result strongly indicates that the *shl* mutations affect pathways that are downstream from cryptochrome(s), as well as the phytochromes. In this respect, it is interesting to note the possible dependence of *shl1* on *CRY1* for expression of its B-hyperresponsive phenotype. This finding would suggest a direct interaction between *SHL1* and *CRY1* in B signaling.

Unlike *sub1*, which is entirely dependent on the activity of *HY5* for the expression of its B and FR hyper-responsive phenotype (Guo et al., 2001), both the *shl1* and *shl2* mutant phenotypes were only partially independent of *HY5*. *shl1-1* was more dependent on *HY5* for its phenotypic effect than was *shl2-2*, suggesting that a significant portion of the photomorphogenetic signaling generated in the *shl1* mutant exerts its effect through *HY5* and that *SHL1* may act in a pathway that is upstream from *HY5* and other regulators. Since we do not know for certain that *shl1-1* is a null allele, all we can conclude is that signals generated by these mutations do act through *HY5*, but also act through alternate pathways. Recently sequence analysis of *shl2-2* demonstrated that *shl2-2* may not be a null mutant. Therefore genetic interaction analysis with photoreceptors and HY5 using a *SHL2* null mutant needs to be studied.

Material and methods

Plant Materials, Growth Conditions, and Double Mutant Isolation

Arabidopsis ecotype Col-*gl1* seeds were obtained from Lehle Seeds (Round Rock, TX). Null mutants *phyA211*, *phyB*-9, and *cry1-B36* (in the Col-0 ecotype background) were obtained from Jason Reed (Reed et al., 1994). The *hy5-5C* null allele was isolated in the Col-0 background as a suppressor of *det1-1* (Pepper and Chory, 1997). This *hy5* allele was back-crossed twice to WT Col-0 and was homozygous for the WT *DET1* allele.

Seeds were surface sterilized (Chory et al., 1989), resuspended in sterile 0.1% (w/v) phytagar, then cold treated at 4 ^{o}C for 40h. Seeds were then dispersed onto duplicate plates containing Murashige and Skoog/phytagar/ 2% (w/v) Suc media. Seeds were illuminated for 4h with white light (100 µmol $m^{-2}s^{-1}$) to ensure optimal germination before transferring to different light conditions.

Our strategy for the identification of *shl phyA, shl phyB, shl cry1*, and *shl hy5* double mutants was partially dependent on an assumption that the *shl* mutations acted in a fully recessive manner. *shl1-1* and *shl2-2* alleles were crossed with *phyA-211*, *phyB-9*, *cry1-B36*, and *hy5-5C*. We phenotyped the F_2 generation under conditions that gave excellent discrimination between the WT and *phyA* (7.8 µmol $m^{-2}s^{-1}$ FR), *phyB* (64.4 µmol $m^{-2}s^{-1}$ R), *cry1* (2.78 µmol $m^{-2}s^{-1}$ B), and *hy5* (45 µmol $m^{-2}s^{-1}$ white light) and identified individuals with phenotypes that were similar to *phyA-211*, *phyB-9*, *cry1-B36*, and *hy5-5C* controls. These F_2 individuals, assumed to be homozygous for their respective photoperception-deficient alleles, were then examined in the F_3 generation for the appearance of distinct short hypocotyls progeny at a frequency consistent with the segregation of the recessive *shl* mutant (1, short; 3, long). In the absence of such progeny, the phenotype of the double mutant could not be conclusively determined. F_2 and F_3 individuals homozygous for the *cry1-B36* mutant allele were identified by a PCR-based assay: oligonucleotide primers CRY1-F2 (5´-GATCAAACAGGTCGCGTGG-3´) and CRY1-R2 (5´TTTCATGCCACTTGGTTAGACC-3´) failed to produce an amplication product in the homozygous *cry1-B36* mutant.

Light Sources

Narrow-spectrum R and FR light were supplied by light emitting diode arrays (models SL515-670 [670nm maxium] and SL515-735[735-nm maxium], respectively; Quantum Devices, Inc., Barneveld, WI). Narrow-sepctrum B light (420-nm maximum) was supplied by Coralife Actinic 03 fluorescent aquarium bulbs (Energy Savers Unlimited,

Inc., Carson, CA) filtered through a Kopp 5-57 blue glass filter (Kopp Glass, Inc., Swissvale, PA). White light was supplied by an equal mixture of cool-white and Grow-lux wide-spectrum fluorescent bulbs (Sylvania, Danvers, MA). A 2472 yellow-green acrylic filter (Polycast Technology, Stamford, CT) with a transmission maximum of ± 550 produced light that was partially depleted in the photomorphogenetically active UV, B, R, and FR regions of the spectrum. Narrow-spectrum G light (±520-nm maximum) was produced by a 2092 green acrylic filter (Polycast Technology), as described previously (Ahmad and Cashmore, 1993; Lin et al., 1996). Dark experiments were performed in a passively ventilated dark box. Fluence rates of white, R, B, yellow, and G light were measured with a quantum photometer (model LI-189, LI-COR, Lincoln, NE). Fluence rates of FR light were measured using a radiometer (model IL 1400, International Light, Newburyport, MA) with FR probe (model SEL033, International light).

CHAPTER IV
ANALYSIS OF HORMONE RESPONSES ON *shl*s

Introduction

Both light and phytohormones regulate plant growth and development. Hypocotyl elongation, which has been a useful quantitative trait for analysis of the regulation of plant growth (Gray et al., 1998) provides an example of this interaction. Light, cytokinin, and abscisic acid (ABA) reduce hypocotyl elongation, whereas brassinosteroids, auxin, and gibberellins (GAs) increase hypocotyl elongation (Gray et al., 1998, and refs. therein). Ethylene increases hypocotyl elongation in high light (75 µ mol $m^{-2}s^{-1}$) (Smalle et al., 1997), but reduces it in darkness (Crocker et al., 1913) and in low light (40 µmol $m^{-2}s^{-1}$) (Collett et al., 2000).

Light and hormone signals interact with each other. Phytochromes alter gibberellin levels in different ways based on their developmental stages and their tissue types (Halliday and Fankhauser, 2003). For example, phytochromes enhance gibberellin (GA) biosynthesis during seed germination (Kamiya and Garcia-Martinez, 1999), but reduce GA biosynthesis at the seedling stage (Halliday and Fankhauser, 2003). Light intensity regulates GA metabolism (Hedden and Kamiya, 1997). Pea seedlings grown in low light (40 µmol $m^{-2}s^{-1}$) accumulated GA_{20} sevenfold more than the seedling grown in a high light condition (386 µmol $m^{-2}s^{-1}$), whereas seedling grown in the dark produced 25% of that in high light condition (Gawronska et al., 1995). Plants respond differently to exogenous ethylene depending on light intensity. At the light intensity 75 µmol$m^{-2}s^{-1}$, ethylene increased the hypocotyl elongation (Smalle et al., 1997), whereas it inhibited hypocotyl elongation in low light (40 µmol $m^{-2}s^{-1}$) (Collett et al., 2000). Dim-red light increases auxin transport resulting in hypocotyl elongation (Shinkle et al., 1998). Hypocotyl elongation in shade and in dim light is mediated by auxin transport (Jensen et al., 1998). Light represses the expression of a negative regulator in auxin signaling

SHY2/IAA3 in the presence of sucrose, but red light increases the expression of *SHY2/IAA3* (Tian and Reed, 1999, Tian et al., 2002). Expression of *FIN219*, a phytochromeA signaling component, was induced by auxin, suggesting that phytochrome systems interact with auxin regulation (Hsieh et al., 2000). The addition of high levels of cytokinin in growth media produced dark grown morphologies similar to the phenotypes of *det1* and *cop1* such as short hypocotyl, expanded cotyledon, and chloroplast development (Chory et al., 1994). However, the mode of interaction of these hormones with light is poorly understood. Hormones might act as 'second signals' in response to light, or they may use different pathways from light that converge to regulate the same target genes.

shl1 showed reduced apical dominance (Table II), suggesting that auxin signaling might have been disrupted. To understand the possible defects or altered responses of *shl*s in responses to phytohormones, *shl1* and *shl2* mutants were grown with exogenous phytohormones such as GA, auxin, cytokinin, and ethylene at various concentrations and in various light fluences. Analysis of the *shl* mutants indicates that phytohormones interact differently with light based on light quantity and quality.

Results

The Effect of Auxin on Root Growth and on Hypocotyl Elongation

30nM of the synthetic auxin 2, 4-D was added to Murashige-Skoog (MS) media to test possible defects of *shl1-1* and *shl2-2* in auxin responses by the method of Beemster and Baskin (2000). The plates were placed in white (100 µmol $m^{-2}s^{-1}$) and yellow light (30 µmol $m^{-2}s^{-1}$) for 8 days and their root and hypocotyl length were measured. The yellow light condition was used instead of low white light to get better screening of *shl* mutants from wild type. Wild type sometimes showed false *shl* like phenotype in low white light condition. In all genotypes (WT, *shl1-1*, *shl2-2*) grown in the media without auxin showed reduced root growth in yellow light compared to the root growth in white light

(Figs. 6, 7). This may be caused by the reduced auxin transport to root in yellow light. In the presence of auxin, wild-type and *shl2-2* seedlings showed reduced root elongation and formed lateral roots (data not shown) in response to exogenous auxin in both white and yellow light, whereas the root elongation of *shl1-1* was not reduced in white light but significantly promoted in yellow light (Fig. 6, 7). *shl1-1* also showed inhibited lateral root formation (data not shown) in both yellow and white light, indicating that *shl1-1* is hyposensitive to auxin. This result is consistent with the reduced apical dominance of

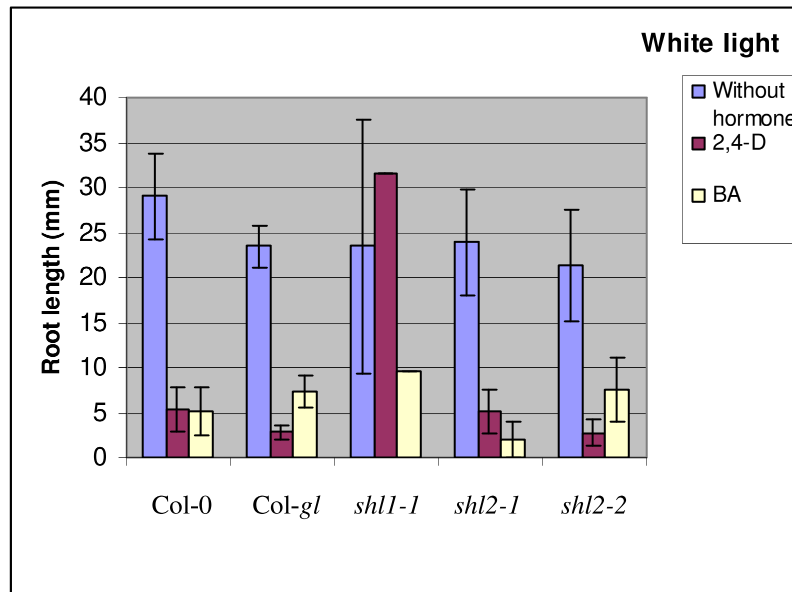

Figure 6. Effects of auxin and cytokinin on the root elongation of wild type, *shl1-1, shl2-1*, and *shl2-2*. Plants were grown for 8 days in white light (100µ molm^{-2}s^{-1}). Data are means ± SD (*n* = 3-27except *shl1-1* in 2, 4-D and BA which had one sample).

shl1-1 (Table II, on pp 25) that indicated a possible defect of *shl1-1* in auxin response.

Compared to WT, *shl1-1* and *shl2-2* hypocotyls showed no difference in responding to 2, 4-D at the concentration of auxin that we used in white light. In yellow light, WT and *shl2-2* showed shorter hypocotyls than their no hormone controls (Figs. 8, 9), whereas

the hypocotyl elongation of *shl1-1* was not decreased significantly compared to WT and *shl2-2*, indicating that *shl1-1* is hyposensitive to auxin, consistent with the root growth result.

The Effect of GA on Hypocotyl Elongation

To study the possible involvement of GA in the functions of *SHLs*, Col-*gl*, *shl1-1*, and *shl2-2* were treated with 10µM GA and their hypocotyl lengths were measured. In white light, Col-*gl*, *shl1-1*, and *shl2-2* showed longer hypocotyls than their no hormone controls with no difference in responding to GA between WT and *shl* mutants(Fig. 8). However, in yellow light, *shl1-1* and *shl2-2* showed altered responses to GA (Fig. 9): *shl1-1* was significantly more sensitive to GA than wild-type, whereas *shl2-2* showed hyposensitivity to GA (Fig. 9). Considering *shl1-1* is hyposensitive to auxin, this data

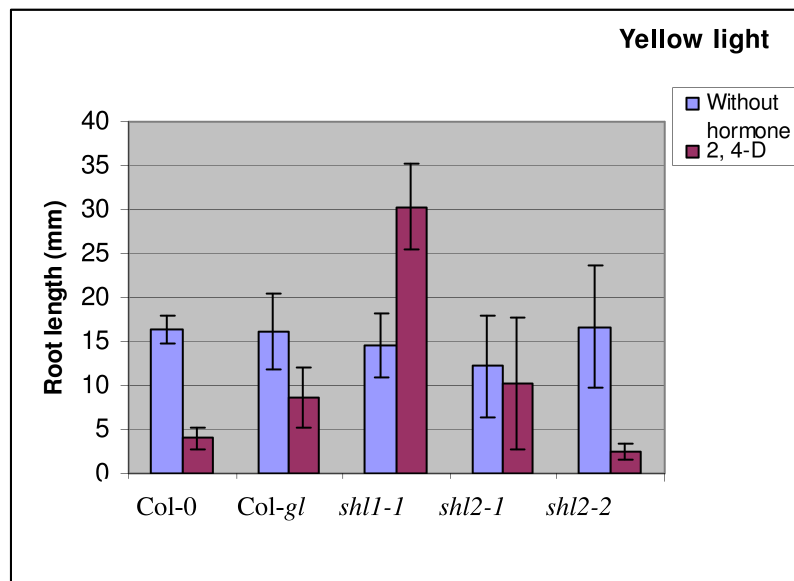

Figure 7. Effects of 2, 4-D on the root elongation of wild type, *shl1-1, shl2-1*, and *shl2-2*. Plants were grown for 8 days in white light (30 µmol $m^{-2}s^{-1}$). Data are means ± SD (*n* = 2-27).

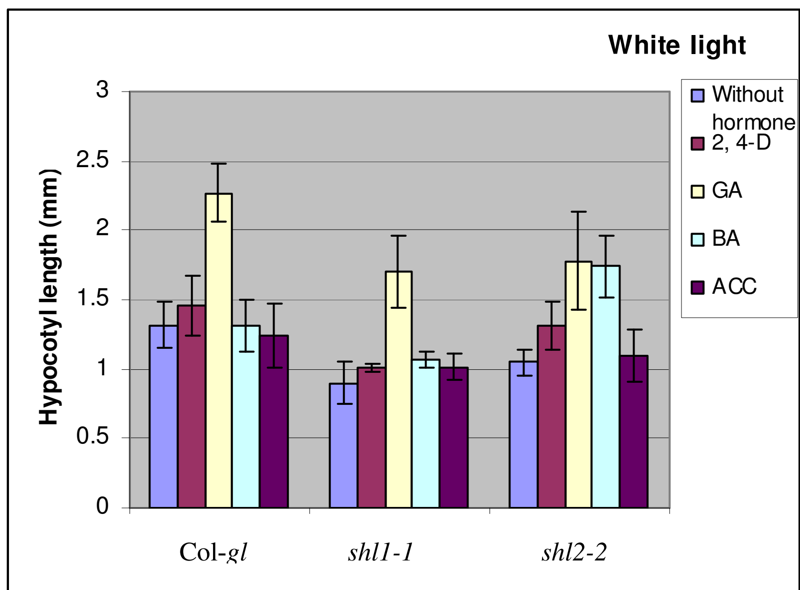

Figure 8. The effect of exogenous 2, 4-D, GA, BA, and ACC on hypocotyl elongation of wild type, *shl1-1,* and *shl2-2*. Plants were grown for 8 days in white light (100 µmol $m^{-2}s^{-1}$). Data are means ± SD (n = 4-16).

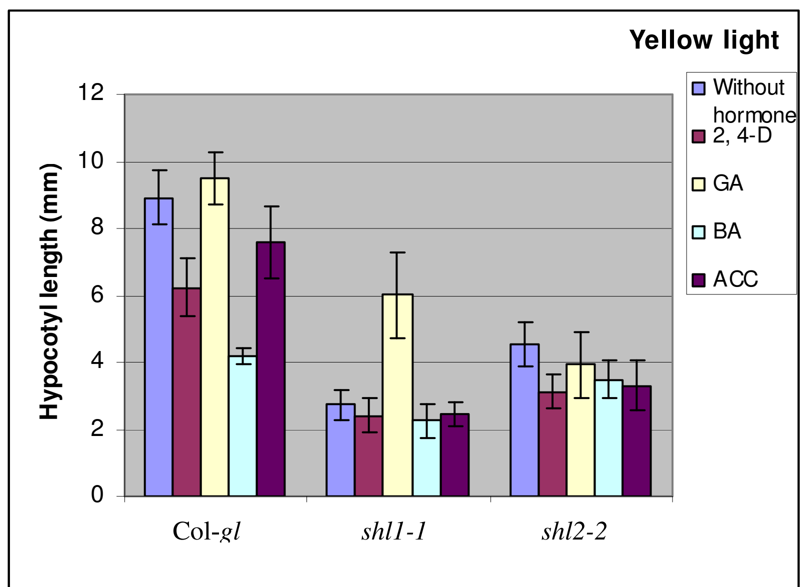

Figure 9. The effect of exogenous 2, 4-D, GA, BA, and ACC on hypocotyl elongation of wild type, *shl1-1,* and *shl2-2*. Plants were grown for 8 days in yellow light (30 µmol $m^{-2}s^{-1}$). Data are means ± SD (n = 4-16).

suggests that exogenous GA may partially rescue auxin signaling defect in *shl1-1*. The hyposensitity of *shl2-2* to GA in yellow light suggests that GA is involved in hypocotyl elongation in yellow light condition and that *shl2-2* has a defect in that GA involvement.

The Effect of Cytokinin on Root Growth and Hypocotyl Elongation

To test for possible defects in response to cytokinin, *shl1-1*, and *shl2-2* were grown on media with 5 µM of 6-benzyladenine (BA). The root elongation in white light and the hypocotyl growth in both white and yellow light were measured. Like the wild-type, *shl1-1* and *shl2-2* showed reduced root length (Fig. 6), suggesting *shl* mutants are not defective in response to cytokinin in root elongation in white light.

This concentration of BA did inhibit the hypocotyl elongation of wild-type in white light (Fig. 8), however, it significantly reduced the hypocotyl elongation of WT in yellow light (Fig. 9), indicating that the effect of cytokinin on the hypocotyl elongation is light dependent. *shl* mutants showed altered responses to cytokinin in their hypocotyl elongation from wild type (Figs. 8, 9). Cytokinin significantly promoted hypocotyl elongation of *shl2-2* in white light ($P < 0.001$) compared to WT and *shl1-1*. However *shl2-2* showed slight difference from WT in yellow light. BA slightly increased the hypocotyl elongation of *shl1-1* in white light, but the difference was not statistically significant.

The Effect of Ethylene

To test their possible defects or altered responses to ethylene, wild type, *shl1-1*, and *shl2-2* were subjected to 1 µM ACC in white (100 µmol $m^{-2}s^{-1}$) and yellow light (24 µmol $m^{-2}s^{-1}$). In white light, ACC did not affect hypocotyl elongation of wild-type or *shl2-2*, but did promote the growth of *shl1-1*. However, this was not significant (Fig. 8), suggesting that *shl1-1* maybe slightly more sensitive to ethylene than wild type. At 24

μmol $m^{-2}s^{-1}$, ethylene reduced hypocotyl elongation of wild type, *shl1-1*, and *shl2-2* in yellow light, but the reduction of *shl1-1* was diminished (Fig. 9). This altered response of *shl1-1* in white and yellow light suggests that ethylene production in *shl1-1* may be slightly reduced both in white and yellow light (or ethylene response by light regulation is slightly perturbed in *shl1-1*). Considering the concentration and light condition used, this data is consistent with previous studies (Smalle et al., 1997; Collett et al., 2000).

Discussion

Exogenous auxin reduces root elongation and promotes lateral root formation (Beemster and Baskin, 2000; Wightman and Thimann, 1980). Roots are more sensitive than hypocotyls to auxin; therefore even the concentration of auxin that promotes hypocotyl elongation can be inhibitory for root growth. However at the low concentration, auxin increases root elongation (Evans et al., 1994). Seedlings grown in yellow light showed reduced root elongation compared to the seedlings grown in white light. Inhibited root growth was also observed in the shade (a far-red rich light regime) (Morelli and Ruberti, 2000). This may be caused by the lack of auxin for root growth in the root because of the lateral auxin redistribution of the hypocotyl causing low endogenous concentration of auxin in the root (Morelli and Ruberti, 2000). Exogenous auxin increased root elongation of *shl1-1* in yellow light (Fig. 7) suggesting that auxin transport or auxin sensitivity in *shl1-1* might have been impaired resulting in lack of endogenous auxin level in the root. Therefore exogenous auxin might have created stimulatory concentration of auxin for root growth in *shl1-1* root, whereas the root elongation of WT and *shl2-2* were reduced possibly because of the inhibitory concentration of auxin.

In addition to the tissue types, plants show different responses to auxin depending on environmental conditions. For example, Arabidopsis seedlings grown in Low Nutrient-deficient growth Media (LNM) showed longer hypocotyls in response to exogenous auxin than seedling grown in 0.5 X MS medium (Smalle et al., 1997). High temperature

also increased auxin-mediated hypocotyl elongation in Arabidopsis (Gray et al., 1998). Auxin promoted hypocotyl elongation of the superroot mutant, *sur1* in the light, but inhibited hypocotyl elongation in the dark (Boerjan et al., 1995). Plants also respond differently to exogenous auxin depending on their intrinsic auxin level and/or sensitivity. Wild-type Arabidopsis seedlings grown on media containing a range of auxin concentrations showed reduced hypocotyl elongation in light intensity 40µmolm^{-2}s^{-1}, whereas *axr1-12* and 35S-*iaaL* (reduced auxin response and level mutants) showed increased hypocotyl elongation in the light intensity 40 µmol m^{-2}s^{-1} (Collett et al., 2000). Consistent with those data, auxin increased hypocotyl elongation of wild type, *sh11-1*, and *shl2-2* in white light, but decreased hypocotyl elongation in yellow light 30µmol m^{-2}s^{-1} (Figs. 8, 9).

Auxin transport might be involved in hypocotyl elongation in low light as it is in the FR-rich light. Hypocotyl elongation in the FR-rich light occurs through the lateral auxin redistribution to epidermal and cortical cells of the hypocotyl (Morelli and Ruberti, 2000). Jensen et al. (1998) showed that auxin transport inhibitor 1-naphthylphthalamic acid (NPA), reduced the hypocotyl elongation in light intensity between 0.1 and 100 µmolm^{-2}s^{-1} and was greatest when the light intensity was around 50 µmol m^{-2}s^{-1}. NPA also reduced hypocotyl elongation in blue, red, and far-red light, but was less effective under red light. Recent studies about ATP-binding cassette (ABC) transporter proteins, AtMDR1 and AtPGP1 also support that the auxin transport is involved in hypocotyl elongation in dim light (Lin and Wang, 2005). Downregulation of *AtPGP1* reduced hypocotyl elongation under low light (Sidler et al., 1998). Functional analyses showed that AtPGP1 and AtMDR1 are involved in polar auxin transport and auxin-mediated development (Noh et al., 2001) by mediating auxin efflux for basipetal auxin transport (Lin and Wang, 2005). In white light, hypocotyls have low concentration of auxin, resulting from transport to the root and/or maybe from less production of auxin (Vandenbussche et al., 2003). Thus exogenous auxin promotes the hypocotyl elongation. In decreased light intensity, several auxin inducible genes were up-regulated, indicating

more auxin production in low light condition (30 µmol $m^{-2}s^{-1}$) (Vandenbussche et al., 2003b). Therefore inhibiton of hypocotyl elongation of wild type, *sh11-1*, and *shl2-2* in response to exogenous auxin in yellow light (Fig.9) might result from the inhibitory high concentration of auxin by adding exogenous auxin to the hypocotyl that has enough stimulatory concentration of auxin for growth.

Relative to WT, both *shl1-1* and *shl2-2* showed altered responses to GA in low light (yellow light). In yellow light, *shl1-1* was hypersensitive to GA, whereas *shl2-2* showed hyposensitivity to GA (Fig. 9). Considering *shl1-1* showed a defect in responding to auxin in yellow light, this data suggests that exogenous GA can partially rescue the defect.

Both auxin and gibberellin are required for stem elongation, but use different mechanisms for regulating stem elongation: auxin promotes cell elongation, whereas GA stimulates cell division (Yang et al., 1996). However they interact each other. Auxin promotes GA biosynthesis in pea stems (*Pisum sativum*) (Ross et al., 2000) and in barley stems (Wolbang et al., 2004). A GA biosysnthesis inhibitor, paclobutrazol (PAC) abolished stimulation of hypocotyl elongation by auxin in light, indicating that auxin promotes hypocotyl elongation through GA signaling in light intensity 75 µmol $m^{-2}s^{-1}$ (Saibo et al., 2003). However, auxin and GA showed independent effects on hypocotyl elongation in the light intensity 40 µmol $m^{-2}s^{-1}$ (Collett, 2000). Similar to Collett (2000), our data shows that auxin and GA are involved in hypocotyl elongation partly independently in low light. Insensitivity of *shl2-2* to GA in yellow light indicates that *SHL2* might be controlled by GA.

Cytokinin is involved in shoot initiation to control meristem (Schmulling, 2002) and it inhibits root elongation (Baskin et al., 1995). Wild-type, *shl1*, and *shl2-2* all showed reduced root length in white light (Fig. 6), indicating there is no defect or altered root response to cytokinin in white light.

Both cytokinin and light inhibit hypocotyl elongation independently and additively, and the morphogenic responses can be saturated by either light or cytokinin (Su and Howell, 1995). Therefore cytokinin dose not have a relevant effect on hypocotyl elongation in normal light condition (Su and Howell, 1995). In our study, *shl2-2* showed increased hypocotyl elongation in response to exogenous cytokinin (BA) in white light (Fig. 8). As we described earlier, *shl2-2* also showed altered response to GA, indicating that there may be cross talk between GA and cytokinin in the regulation of *SHL2*.

Ethylene increases the hypocotyl length in the light (75 µmol $m^{-2}s^{-1}$) when the concentration is higher than 1µM ACC (Smalle et al., 1997). In white light (100 µmol $m^{-2}s^{-1}$), ACC (1 µM) has not shown any effect on hypocotyl elongation of wild-type and *shl2-2*, consistent with the results of Smalle et al. (1997). Ethylene inhibits hypocotyl elongation in darkness (Kieber et al., 1993; Crocker et al., 1913) and ACC inhibits hypocotyl elongation in the light intensity 40 µmol $m^{-2}s^{-1}$ (Collett et al., 2000). Consistent with previous studies, ethylene reduced hypocotyl elongation of wild type, *shl1*, and *shl2-2* in yellow light, but ethylene inhibited the hypocotyl elongation of *shl1* less than WT and *shl2-2* (Fig. 9). This data confirms that plants respond differently to exogenous ethylene depending on light intensity.

Ethylene synthesis is inhibited by light which reduces the activity of ACC oxidase (ACO) (Kao and Yang, 1982; Finlayson et al., 1998). Ethylene production was increased in low R: FR as a shade avoidance response, whereas it was decreased in red light (Pierik et al., 2004). However, in Arabidopsis rosettes, a low R: FR condition, without changing the light intensity did not increase ethylene production, whereas the decreased light intensity (30 µmol $m^{-2}s^{-1}$) increased ethylene production in Arabidopsis rosettes. This suggests that the increase of ethylene production in low R: FR was from low light intensity and that light intensity and the light quality signaling can be separated (Vandenbussche et al., 2003). Consistent with the increased ethylene production, several genes involved in ethylene biosynthesis were up-regulated in low light intensity.

In low R: FR (0.32, 180 µmol $m^{-2}s^{-1}$), exogenous ethylene gas increased stem elongation at the low concentration, but the high concentration of 0.20 µLL^{-1} was inhibitory (Pierik et al., 2004). Taken together, exogenous ACC might inhibit the hypocotyl elongation in low light because of inhibitory concentration. However there is still a possibility that ethylene sensitivity in low light is higher than in white light resulting in reduced hypocotyl elongation with exogenous ACC.

ACC (1 µM) slightly promoted the growth of *shl1*, whereas it did not change the hypocotyl elongation of WT and *shl2-2*. In yellow light, the hypocotyl elongation of *shl1* was not inhibited as much as WT and *shl2-2*. This indicates that ethylene production in *shl1* is reduced both in white and yellow light (or ethylene response by light regulation is slightly disrupted in *shl1*).

Plants respond differently to exogenous hormones in low light conditions, perhaps because of different endogenous level of hormones in dim light or because sensitivity to hormones is dependent on light conditions. Our data show that there is cross-talk among hormones regulating hypocotyl elongation through SHLs and that their interactions are dependent on light intensity. Cytokinin and ethylene might interact to regulate hypocotyl elongation through *SHL1* in white light, and auxin, ethylene, and GA might be involved in regulating *SHL1* in low light. *SHL*2 may be regulated by cytokinin in white light and by GA in low light.

Materials and methods

Plant Materials and Growth Conditions

Arabidopsis *shl1-1* and *shl2-2* mutants were previously isolated by their short hypocotyls in yellow light (24 µmol $m^{-2}s^{-1}$) (Pepper et al., 2001). These mutations were isolated in the Col-*gl* ecotype background. As a control, Col-0 and Col-*gl1* were used.

Seeds were surface sterilized (Chory et al, 1989), resuspended in sterile 0.1% (w/v) phytagar, then stored overnight at 4 °C. Seeds were then plated on Murashige-Skoog plates (1x Murashige-Skooge salts, 0.8% phytagar, 1XGamborg's B5 vitamin mixture, 2% (w/v) sucrose) with or without hormones. Square tissue culture plates (90- x 90 mm) were used for root growth test and round plates for hypocotyl elongation test. These plates were subjected to at least 4h of white light (100 µmol $m^{-2}s^{-1}$) and placed in white light (100 µmol $m^{-2}s^{-1}$) or in yellow light (30 µmol $m^{-2}s^{-1}$) for 8 days. 90- x 90-mm square tissue culture plates were vertically oriented.

2, 4-D, GA, ACC, and BA were dissolved in 100% ethanol, 90% ethanol, water, and KOH, respectively. The stock solutions were filter-sterilized and added to media after autoclaving to give final concentrations of 30 nM 2, 4-D, 10 µM GA, 1µ M ACC, and 5 µM BA.

Analytical Methods

Hypocotyls were measured under a stereo dissecting microscope using a 0.5-mm ruler and forceps for straightening hypocotyls. To measure root elongation, 90- x 90-mm square tissue culture plates were scanned using a scanner and the root growth were measured on screen using image J (version 1.32j) software (NIH, Bethesda, MD).

CHAPTER V
IDENTIFICATION OF *SHL2*

Introduction

Plants respond to different light conditions perceived by different photoreceptors: phytochromes, cryptochromes, and phototropins (Sharrock and Quail, 1989). Upon perceiving the different qualities and intensities of light, they use specific or overlapping signal transduction pathways to accomplish cellular responses. Developmental responses of plants to light, called 'photomorphogenesis', requires a complicated network of genes. To unravel this network, genetic approaches based on de-etiolation responses have been used to identify the genes involved in skotomorphogenesis and photomorphogenesis (Neff et al., 2000). In genetic approaches, genes are randomly mutated by insertional mutagenesis using T-DNA or transposons, or by chemical mutagenesis, such as with ethyl methane sulfonate (EMS). Mutants with the desired phenotypes are identified through screens, and the genes responsible for the mutant phenotype are identified (Jander et al., 2002). Even though identifying the gene of interest may be more rapid in the case of insertional mutagenesis, chemical mutagenesis requiring map-based cloning has many advantages. It can produce mis-sense mutations in the coding region or promoter mutations without knocking out a protein function (Jander et al., 2002). Mis-sense mutations in the different domains of a gene can lead to the identification of the functions of each domain. In addition, complete sequencing of the Columbia (Col-0) genome and the increased number of available genetic markers can shorten the time required for map-based cloning.

As described earlier (in CHAPTER II; Pepper et al., 2001), to identify the genes involved in the developmental responses of plants to low light, we used chemical mutagenesis and identified the mutants – designated as *shl*s – involved in low photomorphogenic light quantity by screening mutants with exaggerated developmental

responses to available light (Pepper et al., 2001). In this study, we identified *SHL2* by a map-based cloning approach that made extensive use of of T-DNA insertion mutants from the collection developed by Salk Institute Genome Analysis Laboratory (La Jolla, CA).

Most of the light perception genes identified through genetic approaches are upstream components of the light signal transduction except *CESA6* which encodes a cellulose synthase (Fagard et al., 2000). Its mutant $cesA6^{prc1\text{-}1}$ was identified by its phenotype of having short hypocotyls in the dark, probably because of a defect in cell elongation in this mutant (Desnos et al., 1996; Refrégier et al., 2004). Here we report that *SHL2* encodes a possible downstream target of light signaling, a XyG galactosyltransferase I, previously identified as *MURUS3* (Li *et al.,* 2004) */ KATAMARI1* (Tamura *et al.,* 2005).

Results

Map-based Cloning of *SHL2*

shl2 was previously shown to have exaggerated developmental responses to low light including red, far-red, and blue light (Pepper et al., 2001). *shl2-2* was back-crossed twice to WT Col-0 and then out-crossed to Arabidopsis Landsberg *erecta* to create F_2 mapping populations. Initially a population of 94 mutant F_2 progeny was used to map *shl2-2* to a location on chromosome 2, ±7.0 cM telomeric to *PHYB* (Pepper et al., 2001). We used cleaved amplified polymorphic sequence (CAPS, Konieczny and Ausubel, 1993) markers and simple sequence length polymorphism (SSLP, Bell and Ecker, 1994) markers for mapping (Fig. 10). We narrowed the map position to an interval between CAPS markers derived from BACs T24K242 and T9I22. To narrow the map position, we developed simple sequence length polymorphism markers using the Arabidopsis genomic sequence information (http://www.arabidopsis.org/) (Fig. 11, Table III) and we also generated a larger mapping population of 106.

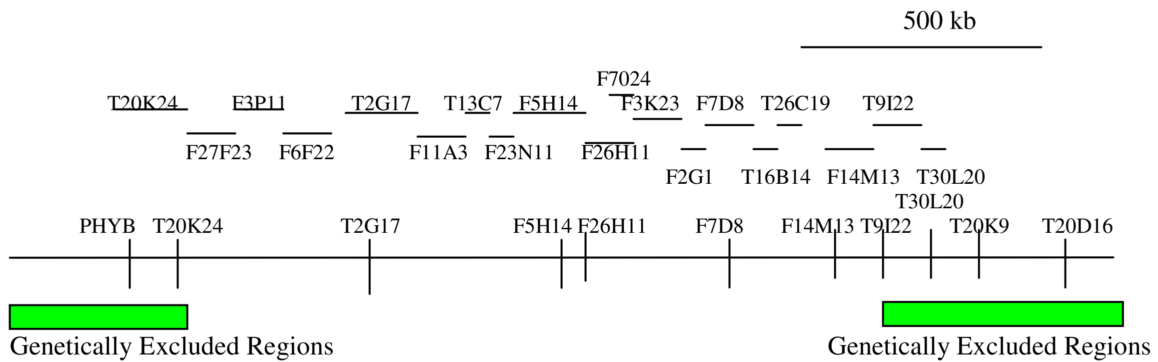

Figure 10. BACs between T24K242 and T9I22. We narrowed the map position to an interval between T24K242 and T9I22. 16 BACs covering this interval were used for transformation of *shl2-2* in an attempt to identify the gene by complementation of the mutant phenotype.

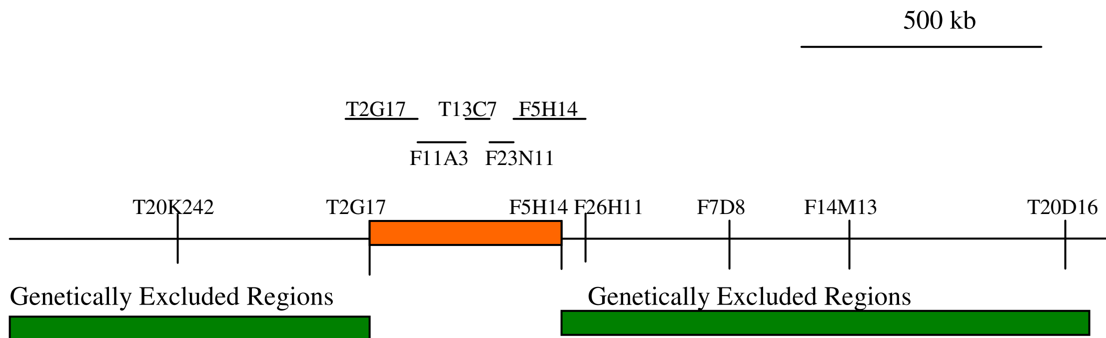

Figure 11. Physical map of the chromosomal region encompassing *SHL2*. The positions of molecular markers are indicated. Green boxes indicate the regions excluded by genetic mapping. The orange box indicates the interval where *SHL2* is located.

Screening of Salk T-DNA Insertion Mutants

Through fine-mapping using the expanded population we narrowed the map position of the *shl2-2* mutation to a region of 315 kb (315,458 bp) (Fig. 11) flanked by markers in T2G17 and F5H14, we used two approaches to identify *SHL2*: sequencing candidate genes in the interval and screening collections of T-DNA insertion mutants from the Salk Institute Genome Analysis Laboratory (La Jolla, CA). In both approaches, we selected the candid genes that were presumably involved in signal transduction or in

protein degradation based on the observation that protein degradation modulates light signaling (Ni, 2005) as well as transcription factors and potential targets of light signaling pathways. We sequenced those genes using templates obtained using *shl2-2* genomic DNA but did not identify any mutation in them (data not shown).

Table III. *Selected Salk T-DNA insertion lines for complementation test.*

T-DNA insertion mutants	Disturbed Gene AGI ID	Gene Alias	Putative Product
SALK_142063	AT2G20780		Putative mannitol transporter
SALK_064179	AT2G20520	*FLA6*	Fasciclin-like arabinogalactan-protein 6 (FLA6)
SALK_057718	AT2G19760	*PRF1/PFN1*	Profilin 1 (PRF1)
SALK_016631	AT2G19230		Putative leucine-rich repeat protein kinase
SALK_073980	AT2G20410		Activating signal cointegrater (ASC-1)-related
SALK_074435	AT2G20370	*MUR3/KAM1*	Xyloglucan galactosyltransferase
SALK_099232	AT2G20380		Kelch repeat-containing F-box family protein
SALK_099579	AT2G20210		Leucine-rich repeat family protein

We screened 61 Salk T-DNA insertion mutant lines in yellow light. Eight lines out of the 48 lines that survived showed at least one seedling with an *shl2-2*-like phenotype like in yellow light (Table IV). To test whether they complement *shl2-2*, the eight T-DNA insertion mutant lines were crossed to *shl2-2* individually and the next generations were screened in yellow light. Seven lines complemented *shl2-2,* indicating that the genes knocked out by T-DNA in those lines were not *SHL2*. However, SALK_074435 showed no complementation when crossed with *shl2-2*. The At2g20370 gene was disrupted by T-DNA in SALK_074435. To confirm that At2g20370 was the gene mutated in *shl2-2,* we sequenced the At2g20370 gene of *shl2-2* mutants and identified a missense mutation (GGA->GAA), changing glycine to glutamic acid on the 246th amino acid.

Identification of *SHL2*

Mutation *shl2* and the gene disrupted in the SALK_074435 T-DNA line is allelic to the previously identified locus designated *MURUS3(MUR3)/KATAMARI1(KAM1)* (Madson *et al.,* 2003; Tamura et al., 2005). It encodes a XyG (xyloglucan) galactosyltransferase I, which is a dual-function protein: it galactosylates the third Xyl residue within XXXG, converting XXXG to XXLG (Madson *et al.,* 2003), and is involved in actin organization and endomembrane organization (Tamura et al., 2005).

The SHL2/MUR3/KAM1 protein also contains the glucuronosyltransferase domain of exostosins (Li et al., 2004) (Figs. 12, 13). Exostosins catalyze the formation of an extracellular glycosaminoglycan heparin sulfate (Esko and Selleck, 2002). Mutations in this class of animal enzymes cause an autosomal dominant disorder, called hereditary multiple exostoses (EXT), which shows multiple outgrowths of the long bones (exostoses) at their epiphyses (Saito et al., 1998). The SHL2/ MUR3/KAM1 protein is a type II membrane protein located in the membrane of Golgi stacks. It consists of a short N-terminal domain in the cytosol side that is responsible for Golgi targeting, a transmembrane domain, and a C-terminal domain located inside the lumen (Tamura et al., 2005).

The missense mutation identified in *shl2-2* is located within the exostosin domain, just like *mur3-1* and *mur 3-2* missense mutations (Madson et al., 2003), whereas *kam1-1* is a nonsense mutation caused by a change of glutamine-62 to a stop codon (Tamura et al., 2005) (Figs. 12, 13).

Discussion

Map-based cloning in Arabidopsis has become considerably easier since the entire Columbia (Col-0) genome was sequenced (The Arabidopsis Genome Initiative, 2000)

and many genetic markers have been developed. Using these resources and the markers we developed, we narrowed the map position of the *shl2-2* mutation to a region of 315 kb flanking T2G17 and F5H14 (Fig. 11). Once we had narrowed the map position of the

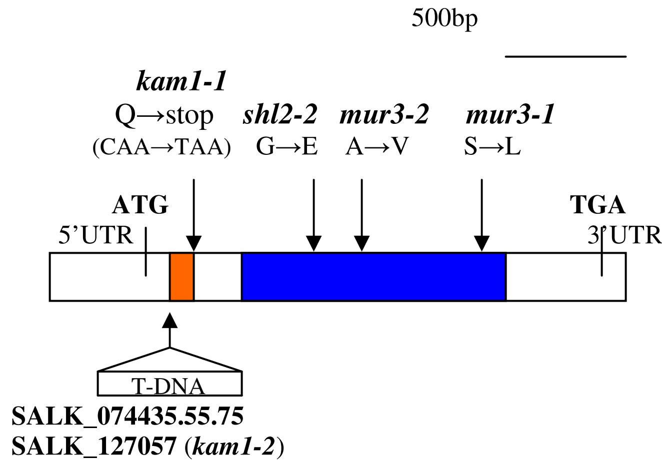

Figure 12. Structure of the *SHL2/MUR3/KAM1* gene. The orange box indicates the predicted transmembrane domain and the blue box indicates the exostosin domain coding region. The site of each mutation of *kam1-1, shl2-2, mur3-2,* and *mur3-1* and the insertion sites of SALK T-DNA lines are indicated. The *shl2-2, mur3-2,* and *mur3-1* mutations are located within the exostosin domain.

```
1   mfprvsmrrr saevsptepm ekgngknqtn ricllvalsl ffwalllyfh fvvlgtsnid
61  kqlqlqpsya qsqpssvslr vdkfpiepha apskpppkep lvtidkpilp papvanssst
kam1-1:q→stop
121 fkpprivesg kkqefsfira lktvdnksdp cggkyiyvhn lpskfnedml rdckklslwt
181 nmckfttnag lgpplenveg vfsdegwyat nqfavdvifs nrmkqykclt ndsslaaaif
241 vpfyagfdia rylwgynisr rdaaslelvd wlmkrpewdi mrgkdhflva gritwdfrrl
  shl2-2:g→e                                          mur3-2:a→v
301 seeetdwgnk llflpaaknm smlvvesspw nandfgipyp tyfhpakdse vfewqdrmrn
361 lerkwlfsfa gaprpdnpks irgqiidqcr nsnvgkllec dfgeskchap ssimqmfqss
421 lfclqpqgds ytrrsafdsm lagcipvffh pgsaytqytw hlpknyttys vfipeddvrk
                                                      mur3-1:s→l
481 rnisieerll qipakqvkim renvinlipr liyadprsel etqkdafdvs vqavidkvtr
541 lrknmiegrt eydyfveens wkyalleegq reagghvwdp ffskpkpged gssdgnggtt
601 isadaaknsw kseqrdktq
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Figure 13. Derived amino acid sequence of the MUR3 protein. The shaded area close to the N terminus represents the predicted transmembrane domain, and the exostosin domain is underlined. Amino acid substitutions caused by *shl2-2, kam1-1* and two *mur3* mutant alleles are indicated (Madson et al., 2003; Tamura et al., 2005).

shl2-2 mutation to an interval of 315 kb flanking T2G17 and F5H14 (Fig. 11). We used two different approaches in an attempt to reduce the time to identify *SHL2*: (1) sequencing candidate genes and (2) complementation tests with sequence-indexed T-DNA insertion mutants. *SHL2* was identified by complementation test with SALK_074435 T-DNA insertion mutant. Our work demonstrated that map-based cloning incorporating many available resources, such as SALK T-DNA insertion mutant lines and gene model information, can shorten the time to identify chemically mutated genes.

SHL2 was allelic to the previously identified mutants *murus3(mur3)/katamari1(kam1)*. The *mur3* mutant was identified based on the altered cell wall monosaccharide composition (Reiter et al., 1997). It failed on galactosylation on "the third xylosyl residue unit within the XXXG core structure", whereas it increased galactosylation of the second xylosyl residue (Madson et al., 2003). The *kam1* mutant was identified by its "defect in the organization of endomembranes and actin filaments" (Tamura et al., 2005). Based on those studies, *SHL2/MUR3/KAM1* which encodes a XyG (xyloglucan) galactosyltransferase I, is suggested to have dual-functions: galactosylation of the third Xyl residue within XXXG (Madson et al., 2003) and an actin organization that is responsible for endomembrane organization (Tamura *et al*., 2005). Comparisons of different mutations in *SHL2/MUR3/KAM1* such as missense mutations (*shl2-2, mur3-1* and *mur 3-2*) identified within the exostosin domain (Madson et al., 2003) and a null mutation (*kam1-1*) might reveal the mechanisms of how SHL2/MUR3/KAM1 work in different light conditions.

Materials and methods

Plant Materials and Growth Conditions

Arabidopsis thaliana shl1 and *shl2* mutants were previously isolated by their short

hypocotyls in yellow light (24 μmol $m^{-2}s^{-1}$) (Pepper et al., 2001). These mutations were isolated in the Col-*gl* ecotype background. Arabidopsis ecotype Col-*gl1* seeds were obtained from Lehle Seeds (Round Rock, TX). SALK T-DNA insertion mutants were obtained from the Salk Institute Genome Analysis Laboratory (La Jolla, CA).

Seeds were surface sterilized (Chory et al., 1989), resuspended in sterile 0.1% (w/v) phytagar, and then stored overnight at 4°C. Seeds were then plated on Murashige-Skoog plates (1 x Murashige-Skoog salts, 0.8 % phytagar, 1 x Gamborg's B5 vitamin mixture, 2% (w/v) sucrose). The plates were subjected to at least 4 h of white light (100 μmol $m^{-2}s^{-1}$) and then placed in white light (100 μmol $m^{-2}s^{-1}$) or in yellow light (30 μmol $m^{-2}s^{-1}$) for 8 days.

Genetic Analysis

The genetic methods employed have been described previously (Chory et al., 1989; Pepper and Chory, 1997). Routine phenotyping for complementation, segregation, and mapping experiments was performed under low white light or under a yellow-green acrylic filter. Genomic DNAs were isolated using the micropreparation method described by Pepper and Chory (1997). Mapping of *shl* mutants was performed using the PCR-based CAPS (Konieczny and Ausubel, 1993) and SSLP (Bell and Ecker, 1994; Lukowitz et al., 2000) markers. Mutants were back-crossed to WT Col-0 or Col-*gl1* at least three times before comprehensive phenotypic analysis.

Sequence Analysis

DNA sequence information of all candidate genes and *SHL2/MUR3/KAM1* was obtained from "The Arabidopsis information Resource (TAIR)" website (http://www.arabidopsis.org/). A dye-based cycle sequencing kit (Applied Biosystems, Foster City, CA) was used for DNA sequencing.

CHAPTER VI
CHARACTERIZATION OF *SHL2*

Introduction

Morphological changes in plants triggered by changes in the environment such as light intensity require modifications in the plant cell wall. Plant cell elongation in the dark depends on the modification of existing cell wall structures and changes in cell wall composition (Darley et al., 2001). For example, the synthesis and normal deposition of cellulose are required for cell elongation (Fagard et al., 2000; Pagant et al., 2002; Refrégier et al., 2004). The integration of whole xyloglucan suppresses cell elongation, whereas xyloglucan oligosaccharide increases the cell elongation in pea stem segments (Takeda et al., 2002). Therefore, the inhibition of cell elongation by light can be accomplished by blocking the pathways involved in cell wall loosening and degradation (Ma et al., 2001).

The regulation of cell wall-modifying enzymes by different environmental cues has been studied (Xu et al, 1996). Cellulose synthase mutant *cesA6*$^{prc1\text{-}1}$ grew short hypocotyls in the dark, probably because of a defect in cell elongation in this mutant (Desnos et al., 1996; Refrégier, et al., 2004). The expression of *TCH* genes are upregulated by touch, wind, rain, and darkness (Braam, 1992; Braam and Davis, 1990; Sistrunk et al., 1994). *TCH4* (renamed as *At-XTH22*) which encodes an Arabidopsis xyloglucan endotransglycosylase is strongly upregulated in expression by touch, heat shock, cold shock, and darkness (Xu et al., 1996). *Meri-5* (renamed as At-*XTH24*) is strongly expressed in the dark, whereas other *At-XTR* genes such as *EXT* (renamed as *At-XTH4*), *XTR4* (renamed as *At-XTH30*), and *XTR7* (renamed as *At-XTH15*) are slightly increased in their expression. Not all *XTR* genes are upregulated by darkness. For example, expression of *XTR2* (renamed as *At-XTH28*) and *XTR3* (renamed as *At-XTH4*) has not shown any change in response to darkness (Xu et al., 1996).

PHYA down-regulated *XTR7* (renamed as *At-XTH15*) (Kuno et al., 2000; Rose et al., 2002) and up-regulated *At-XTH 24* (At4g30270)(Devlin et al., 2003). As described above, cell wall-modifying enzymes involved in hypocotyl elongation in the dark and in shade have been identified. However, cell wall-modifying enzymes involved in hypocotyl elongation in low light have not been identified.

We showed earlier that *SHL2* encoding XyG galactosyltransferase I was involved in hypocotyl elongation, specifically in low light (Pepper et al., 2001). The two other missense mutants *mur3-1* and *mur3-2* in the exostosin domain of *SHL2/MUR3/KAM1,* did not show any significant hypocotyl elongation change in the dark and in high light as seen in *shl2-2*. In contrast, *kam1* mutant, a null mutation of *SHL2/MUR3/KAM1* identified by a "defect in the organization of endomembranes and actin filaments" showed short hypocotyl elongation in the dark (Tamura et al., 2005).

Here we present that the dual function of *SHL2/MUR3/KAM1* might imply two different roles of *SHL2/MUR3/KAM1* depending on light conditions. Firstly, the exostosin domain of *SHL2/MUR3/KAM1* may be responsible for hypocotyl elongation under low light conditions probably through glycosyltransferase activity. Secondly, *SHL2/MUR3/KAM1* might be involved in hypocotyl elongation in the dark by organizing endomembranes and actin filaments to facilitate cellulose synthesis.

Results

Phenotypes of T-DNA Insertion Mutant in *SHL2*, SALK_074435

Sequence analysis demonstrated that our original reference allele *shl2-2* is probably not a null mutant. Therefore the phenotypes of T-DNA insertion mutant in *SHL2,* SALK _074435 were studied in different light conditions to understand the complete function of *SHL2/KAM1/MUR3*. After 7ds in the dark, in yellow, and white light, the hypocotyl

length of SALK_074435 were measured. SALK_074435 exhibited shorter hypocotyls than Col-0 in both low light and in the dark (though not to the *det/cop/fus* mutants). However, hypocotyls of SALK_074435 were shorter in low light than in the dark (Fig. 14).

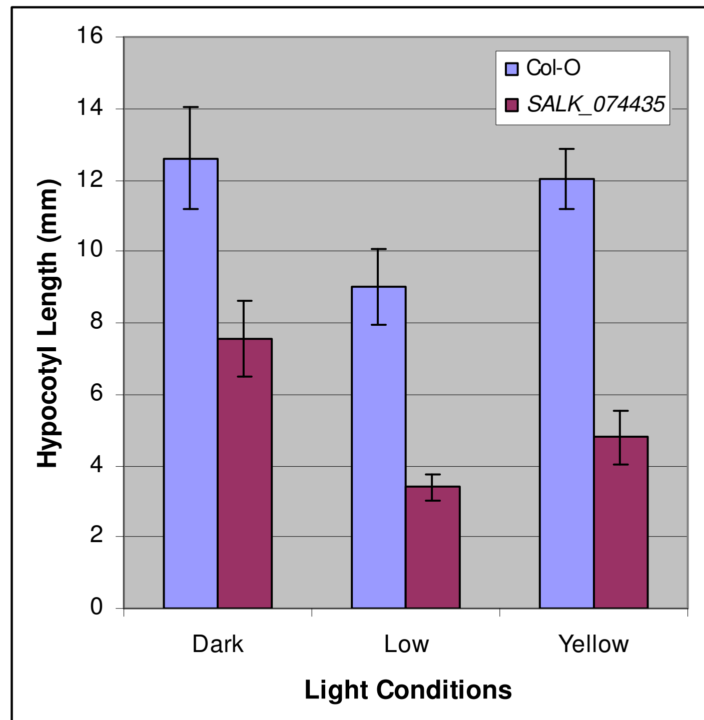


Figure 14. The hypocotyl length of Col-0 and SALK_074435 in the dark, in low light (17 μmol $m^{-2}s^{-1}$), and in yellow light (81 μmol $m^{-2}s^{-1}$). The hypocotyls of SALK _0744355 T-DNA insertion mutants were shorter in all the light conditions compared to Col-0. Data are means (n= 10-21) ± SD.

We also measured the cotyledon sizes of SALK_074435 after 7ds in dark, yellow, and white light. Whereas *shl2-2* showed no significant cotyledon size difference in the dark (data not shown), the cotyledons of SALK_074435 were larger than wild type Col-0 in the dark, in low light, and in yellow light (Fig. 15). However the difference in the dark was small. The cotyledons of both Col-0 and SALK_074435 were not open in the dark, unlike light-grown seedlings.

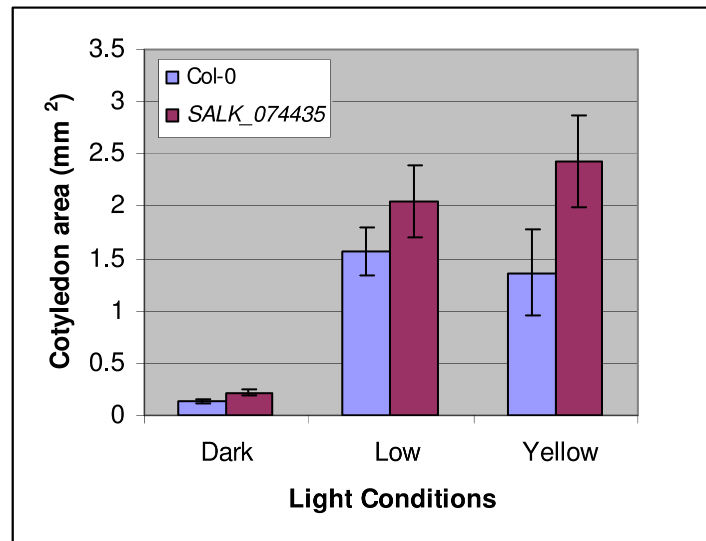


Figure 15. The cotyledon sizes of Col-0 and SALK_074435 in the dark, in low light (17 µmol $m^{-2}s^{-1}$), and in yellow light (81 µmol $m^{-2}s^{-1}$). SALK _074435 T-DNA insertion mutant showed larger cotyledons in the dark, low light and yellow light when compared with Col-0. Data are means (n= 6-10) ± SD.

SALK_074435 Has a Defect in Hypocotyl Cell Elongation

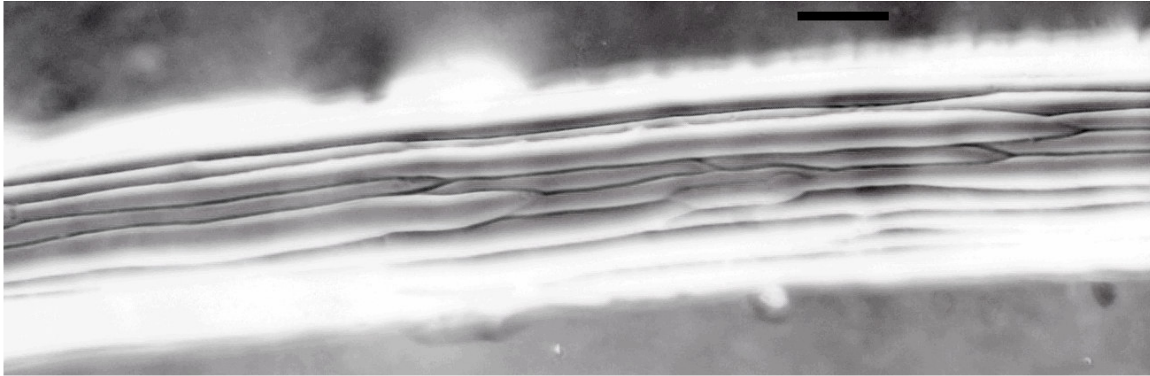
Our previous study showed that *shl2-2* hypocotyls were shorter than Col-0 under low light conditions (Pepper et al., 2001). To understand whether the short hypocotyls of *shl2-2* resulted from a defect in cell elongation, hypocotyl cells of *SHL2/MUR3/KAM1* T-DNA insertion mutant SALK_074435 and wild type (Col-0) grown under low light and yellow light were imprinted to compare the cell sizes. SALK_074435 hypocotyls had wider and shorter cells than Col-0 hypocotyl cells (Fig. 16), indicating that *SHL2/MUR3/KAM1* is involved in cell elongation in these light conditions.

SHL2/MUR3 Gene Expression Patterns Depending on the Light Condition

Since *shl2-2* mutant phenotypes were dependent on the light conditions, *SHL2/MUR3/KAM1* gene expression was studied using quantitative PCR. Given that galactosylation of XyGs increases the activities of Arabidopsis hypocotyl XETs (Peña et

al, 2004), we decided to compare the gene expression pattern of *SHL2/MUR2/KAM1* with XETs. We chose *At-XTH 24* (At4g30270) because its up-regulation by PHYA has been reported (Devlin et al., 2003).

A



B

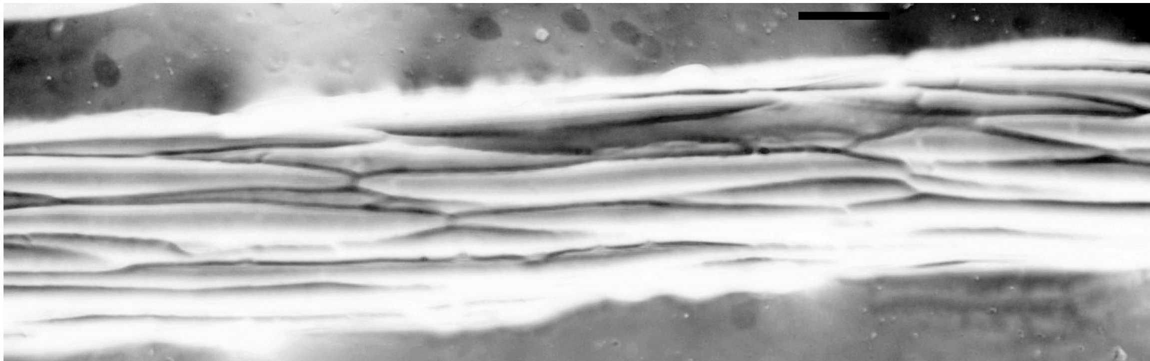


Figure 16. Hypocotyl epidermal cell elongation of Col-0 and SALK_074435 in low light (17 µmol $m^{-2}s^{-1}$). A. Col-0 in low light (17 µmol $m^{-2}s^{-1}$). B. SALK_074435 in low light (17 µmol $m^{-2}s^{-1}$). Epidermal cells of the hypocotyls of Col-0 and SALK_074435 were imprinted. The cells of SALK_074435 are shorter and wider than Col-0. Bar =100µm.

Gene expression levels in other light conditions were compared to gene expression in white light making gene expression level in white light 1 fold. From previous work on the phenotypes of *shl2-2*, a high level of *SHL2/MUR3/KAM1* gene expression was expected in the dark and low light (Pepper et al., 2001). However, gene expression levels

of *SHL2/MUR3/KAM1* did not show any significant difference depending on light conditions, except in far-red light (Fig. 17). In far-red light, *SHL2/MUR3/KAM1* showed 0.59 fold decrease in expression compared to white light (Fig. 17). *At-XTH 24* (At4g30270) was 105.9 times upregulated in the dark and 6 times upregulated in far-red light, consistent with the data of Devlin et al. (2003). *At-XTH 24* (At4g30270) was expressed more in all low light conditions compared to that of the white light (Fig. 17).

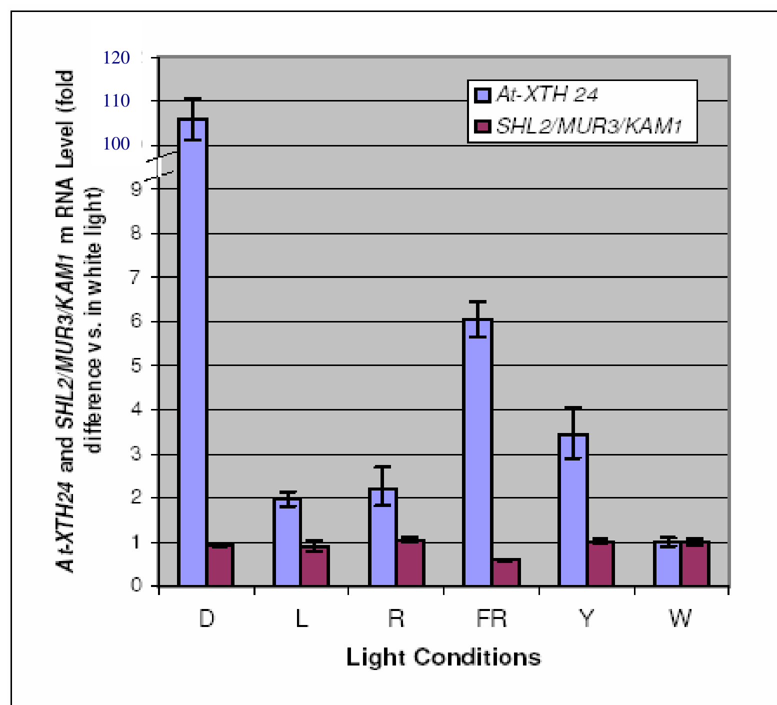


Figure 17. Gene expression of *SHL2/MUR3/KAM1* and *At-XTH 24* (At4g30270) in different light conditions. W (White 104 µmol $m^{-2}s^{-1}$), Y (Yellow 81 µmol $m^{-2}s^{-1}$), L (low 17 µmol $m^{-2}s^{-1}$), FR (Far-red 0.8 µmol $m^{-2}s^{-1}$), R (red 20µmol $m^{-2}s^{-1}$). *At-XTH 24* (At4g30270) was 105.9 fold, 2 fold, 2.2 fold, 6 fold, 3.4 fold more expressed in the dark, low light, red light, far-red light, and yellow light respectively than in the white light. The expression of *SHL2/MUR3/KAM1* have not shown any difference in all light conditions except in the far-red light where *SHL2/MUR3/KAM1* showed 0.6 fold expression compared to in the white light. Bars are SD.

Overexpression of *SHL2MUR3/KAM1*

To confirm that *SHL2/MUR3/KAM1* gene is responsible for *shl2-2* mutant phenotypes, *SHL2/MUR3/KAM1* was expressed in *shl2-2* constitutively under the control of the cauliflower mosaic virus (CaMV) 35S promoter. As controls, a vector itself was also introduced into Col-0 and *shl2-2* by *Agrobacterium*-mediated transformation, referred to as D1-3 and F1-1 respectively. *SHL2/MUR3/KAM1* overexpression in *shl2-2* recovered the hypocotyl elongation of *shl2-2*, but not completely in A6-2 line (Fig. 18). A1 line showed several seedlings with WT phenotype, indicating overexpression of *SHL2/MUR3/KAM1* rescued their mutant phenotype (Data not shown). However, we failed to get their next homozygous generation.

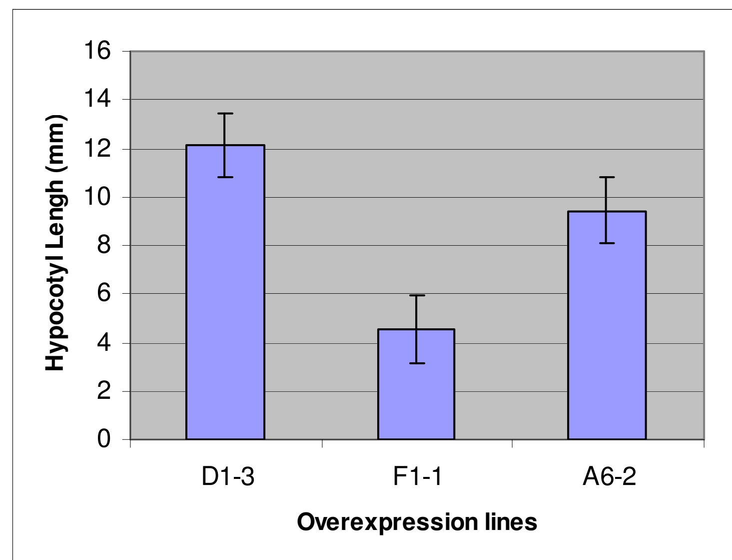


Figure 18. Overexpression of *SHL2/MUR3/KAM1* in *shl2-2*. D1-3: Col-0 with a vector overexpressed , F1-1: *shl2-2* with a vector overexpressed, A6-2: *shl2-2* with *SHL2/MUR3/KAM1* overexpressed. *SHL2/MUR3/KAM1* overexpression recovered the hypocotyl elongation of *shl2-2*, but not completely. Data are means (n= 9-23) ± SD.

To identify the function of *SHL2/MUR3/KAM1* directly, *SHL2/MUR3/KAM1* was expressed in Col-0 constitutively under the control of the cauliflower mosaic virus

(CaMV) 35S promoter, referred to as E1-1~E6-7. As a control, a vector was overexpressed in Col-0, referred to as D1-3. *SHL2/MUR3/KAM1* overexpression lines showed no difference in hypocotyl elongation from a vector overexpressed lines (Fig. 19).

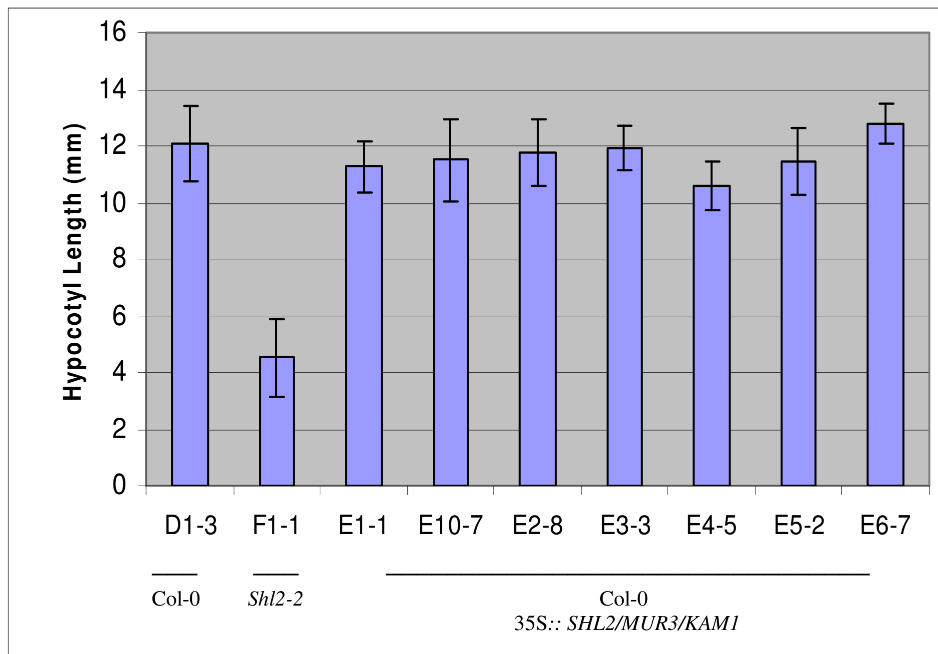


Figure 19. Overexpression of *SHL2/MUR3/KAM1* in Col-0. D1-3: a vector overexpressed in Col-0, E1-1~E6-7: *SHL2/MUR3/KAM1* was overexpressed in Col-0. *SHL2/MUR3/KAM1* overexpression lines show no difference from a vector overexpressed lines. Data are means (n= 22-27, n= 9 for F1-1) ± SD.

Discussion

Sequence analysis demonstrated that our original reference allele *shl2-2* is probably not a null mutant, therefore the phenotypes of T-DNA insertion mutant in *SHL2,* SALK _074435 were studied in different light conditions. SALK_074435 showed shorter hypocotyls than Col-0 when grown in both low light and in the dark, whereas *shl2-2* showed decreased hypocotyl length only in low light (Pepper et al., 2001). This data

indicates that intact proteins are required for hypocotyl elongation both in low light and in the dark. However, hypocotyl elongation of SALK_074435 was decreased more when grown in low light than in the dark, distinguishing this mutant from *cesA6*$^{prc1\text{-}1}$, which showed longer hypocotyls in low light than in the dark (Desnos et al., 1996). This suggests that *SHLl2* is more involved in hypocotyl elongation in low light than in the dark, whereas *cesA6*$^{prc1\text{-}1}$ is more responsible for dark-related hypocotyl elongation than low light-related hycpocotyl elongation. However, another *SHL2* null mutant *kam1-1* showed only one third of wild type hypocotyl elongation, whereas SALK_074435 showed about two thirds. SALK _127057, renamed as *kam 1-2* also appeared to be similar to the *kam1-1* phenotype (Tamura et al., 2005). Considering that SALK_074435 and SALK _127057 have the same T-DNA insertions (TAIR.org), the difference between our data and that of Tamura et al. (2005) may be from different experimental conditions. For instance, Tamura et al. (2005) measured the hypocotyl length when the seedlings were 5-d-old, whereas we sampled seedlings after 7 days of growth.

We cannot determine how much *SHL2* is involved in hypocotyl elongation in the dark, but our data supports that there is a separate regulation for modifying the cell wall for cell extension in hypocotyls in low light (Desnos et al., 1996). We suggest that *SHL2* is involved in the regulation of cell wall extension through glycosyltransferase activity. Considering *mur3* mutants showed normal endomembrane organization (Tamura et al., 2005), and that *shl2-2, mur3-1* and *mur 3-2* all had missense mutations in the exostosin domain and showed normal hypocotyl elongation in the dark, we suggest that the glycosyltransferase activity of the exostosin domain is responsible for hypocotyl elongation in low light. The involvement of *SHL2/MUR3/KAM1* in hypocotyl elongation in the dark may be accomplished through organizing endomembranes and actin filaments.

Cotyledons of SALK_074435 T-DNA insertion mutant were larger than wild type Col-0 when grown in the dark, in low light and yellow light (Fig. 15). However the increase of

cotyledon size in the dark was very slight and the cotyledons were not open (data not shown). Even though the cotyledons of *kam1-1* were slightly open, the cotyledons of *kam1-1* were not like light grown seedlings (Tamura et al., 2005). Taken together with our data, this indicates modification of cell wall components is not enough to produce the light grown phenotype in the dark as seen in upstream light signaling component mutants.

SALK_074435 hypocotyl cells are wider and shorter than Col-0 hypocotyl cells when grown in low and yellow light, indicating that *SHL2/MUR3/KAM1* is involved in cell elongation in these light conditions. *mur3* mutant did not show any significant difference in hypocotyl length and cell elongation compared with the wild type grown in the dark and in high light conditions (60 µmol $m^{-2}s^{-1}$) (Peña et al, 2004), but the phenotypes of *mur3* in low light are not known. In contrast, a *SHL2/MUR3/KAM1* null mutant *kam1-1*, showed significant reduction in cell elongation in the dark (Tamura et al., 2005). All missense mutants in the exostosin domain of *SHL2/MUR3/KAM1* (*shl2-2, mur3-1* and *mur 3-2*) did not show any defects in hypocotyl elongation in the dark and in high light condition, and *mur3* mutants showed normal endomembrane organization in the dark. Taken together, the exostosin domain of *SHL2/MUR3/KAM1* might be responsible for the hypocotyl elongation in low light by elongating the cells, and *SHL2/MUR3/KAM1* might be involved in hypocotyl elongation in the dark using a different mechanism than in low light. This mechanism may involve organizing endomembranes and actin filaments.

SHL2/MUR3/KAM1 might also be involved in hypocotyl elongation in the dark by facilitating the proper export of cell wall components such as glucose for cellulose synthesis or xyloglucan synthesized in the Golgi complex. The actin cytoskeleton is suggested to be involved in endomembrane orgazination and vesicle trafficking (Zheng et al., 2004; Wasteneys and Galway, 2003; Boevink et al., 1998; Brandizzi et al., 2002) and also in cell morphogenesis and elongation (Baluska et al., 2001; Li et al., 2003).

Proper organization of actin filaments is also important for cell wall synthesis by facilitating the secretion of enzymes and polysaccharides involved in cell wall biosynthesis (Blancaflor, 2002). An actin filament depolymerizer Latrunculin B (Lat B) inhibited the Golgi complex to plasmamembrane trafficking of H+-ATPase:GFP, although the endoplasmic reticulum (ER) to Golgi trafficking of sialytransferase was not affected (Kim et al., 2005). *SHL2/MUR3/KAM1* is located in Golgi membrane and has been suggested to interact with actin and regulate its proper distribution (Tamura, 2005). The cellulose synthase mutant *cesA6$^{prc1\text{-}1}$* showed shorter hypocotyls when grown in the dark than in low light, indicating that regulating cellulose synthesis is more important for hypocotyl cell elongation under dark growth condition than in low light (Desnos et al., 1996, Refrégier, et al., 2004). At1G27440 gene, which encodes another exostosin family protein, was coregulated with *CESA4, 7,* and 8 (Persson et al., 2005). Even though its functions are not known, the involvement of exostosin family protein on cellulose synthesis is implied. Therefore, *SHL2/MUR3/KAM1* genes may be involved in hypocotyl cell elongation perhaps by organizing endomembranes and actin filaments to facilitate cellulose synthesis. However, we can not eliminate the possibility that *SHL2/MUR3/KAM1* genes may be involved in xyloglucan transport to the cell wall from Golgi complex in the dark, resulting in elongating hypocotyl cells.

Peña et al (2004) showed that galactosylation of XyGs enhance the activity of Arabidopsis hypocotyl XTHs. XTHs cleave XyGs and rejoin the cut ends with new partners resulting in cell elongation (Takeda et al., 2002). We chose *At-XTH 24* (At4g30270) to compare to *SHL2/MUR3/KAM1* gene expression, because it was reported that *At-XTH 24* (At4g30270) expression is up-regulated by PhyA (Devlin et al., 2003). Considering the phenotypes of *shl2-2* mutants, a high level of *MUR3/SHL2/KAM1* gene expression was expected in the dark and low light (Pepper et al., 2001). However *SHL2/MUR3/KAM1* gene expression levels did not show significant differences depending on the light condition except when grown in far-red light. Unlike

SHL2/MUR3/KAM1, *At-XTH 24* (At4g30270) was expressed more in all low light conditions than in the white light (Fig.17) and most expressed in the dark.

Light regulation of *XTH* genes has been reported. Some *XTH* genes such as At-XTH 4, At-XTH15, At-XTH22, At-XTH24, and At-XTH30 are upregulated by darkness (Xu et al., 1996). *At-XTH15* is down-regulated by PhyA (Kuno et al., 2000, Rose et al., 2002). Therefore the lack of galactosylation of XyGs may give a different effect depending on *XTH* expression regulated by light resulting in *shl2* phenotype. There is also a possibility that *SHL2MUR3/KAM1* is posttranscriptionally regulated by light or hormones.

During cell assembly in living hypocotyls, the physical interaction of XyG and cellulose did not change in the absence of the Gal residues on XyGs in *mur3*, but the tensile strength of cellulose was greatly reduced and the hypocotyls showed abnormal cell swelling. Galactosylation of XyGs increases the activities of Arabidopsis hypocotyl XETs which cleave XyGs and rejoin the cut ends with new partners (Peña et al., 2004). Previous evidence implicated that XET activity is directly involved in growth (Takeda et al., 2002). However because *mur3* mutants have not shown any significant differences in cell elongation compared with wild type grown in the dark and under high light conditions (60 µmol $m^{-2}s^{-1}$), Peña et al. (2004) suggested that decreased XET activity in *mur3* results from a failure to religate XyGs after cell expansion, but has no effect on cell growth (Peña et al, 2004). However, our data suggest that lack of galactosylation of XyGs results in decreased cell elongation in low light probably because of decreased XET activity. Therefore, SHL2/MUR3/KAM1 may be involved in hypocotyl cell elongation by increasing the activity of XETs in low light condition.

SHL2/MUR3/KAM1 was expressed constitutively under the control of the cauliflower mosaic virus (CaMV) 35S promoter. *SHL2/MUR3/KAM1* overexpression in *shl2-2* rescued most of hypocotyls elongation, even though it did not completely rescue the hypocotyls elongation defect (Fig. 18). This data confirms that *SHL2/MUR3/KAM1* is

responsible for *shl2-2* mutant phenotype, consistent with our sequence and complementation test with SALK_074435.

Overexpression of *SHL2/MUR3/KAM1* in Col-0 showed no difference from a vector overexpressed lines (Fig. 19). Together with the analysis of *SHL2* transcript levels, this data suggest that SHL2/MUR3/KAM1 action is unlikely to be regulated at the transcriptional level in low light condition.

Materials and methods

Plant Materials and Growth Conditions

Arabidopsis thaliana shl2-2 mutants were previously isolated by their short hypocotyls when grown in yellow light (24 µmol $m^{-2}s^{-1}$) (Pepper et al., 2001). These mutations were isolated in the Col-gl ecotype background. As a control, Col-0 and Col-*gl1* were used. Arabidopsis ecotype Col-*gl*1 seeds were obtained from Lehle Seeds (Round Rock, TX). SALK_074435 T-DNA insertion mutant was obtained from the Salk Institute Genome Analysis Laboratory (La Jolla, CA).

Seeds were surface sterilized (Chory et al, 1989), resuspended in sterile 0.1% (w/v) phytagar, and then stored overnight at 4 °C. Seeds were then plated on Murashige-Skoog plates (1x Murashige-Skooge salts, 0.8% phytagar, 1x Gamborg's B5 vitamin mixture, 2% (w/v) sucrose) (Murashige and Skoog, 1962). For the hypocotyls elongation test, these plates were subjected to a minimum of 4h of white light (100 µ mol $m^{-2}s^{-1}$) and then placed in the dark, in low-intensity white light (17 µmol $m^{-2}s^{-1}$), under a yellow-green acrylic filter (81 µmol $m^{-2}s^{-1}$), or in white light (100 µmol $m^{-2}s^{-1}$) for 8 days. For the *SHL2* gene expression test, plates were placed in the different light conditions: in the dark, in low-intensity white light (17 µmol $m^{-2}s^{-1}$), under a yellow-green acrylic filter

(81 µmol $m^{-2}s^{-1}$), in far-red light (0.8 µmol $m^{-2}s^{-1}$), in red light (20 µmol $m^{-2}s^{-1}$), or in white light (104 µmol $m^{-2}s^{-1}$) for 8 days.

Analytical Methods

For measurements of hypocotyl length, 30 seeds of each genotype were evenly dispersed onto Murashige and Skoog/phytagar/ 2% (w/v) Suc media in a 7-mm grid pattern. Hypocotyls were straightened using forceps if necessary, and then measured under a stereo dissecting microscope using a 0.5-mm ruler.
For measurements of cotyledon size, cotyledons were cut, put on the transparent film, and scanned. Cotyledon sizes were measured using Image J (version 1.32j) software (NIH, Bethesda, MD, USA).

Imprinting of Hypocotyl Epidermal Cells

Hypocotyls of Arabidopsis Col-0 ecotype and SALK_074435 T-DNA insertion mutant seedlings were used for epidermal imprints using agarose as described in Mathur and Koncz (1996).

Gene Expression Analysis

Arabidopsis Col-0 ecotype seedlings were used for total RNA isolation using a Qiagen RNA isolation kit (Qiagen, Valencia, CA, USA). Total RNA was treated with DNase I to remove genomic DNA contamination and then reverse-transcribed using reverse transcriptase and random decamers (80 µM) to synthesize first-strand cDNAs. Gene-specific primer pairs for *SHL2/MUR3/KAM1* (5'-CTCGGTACAGGCTGTGATAGAC-3' and 5'-CGCATACTTCCAGCTGTTCTCCT-3') and for *At-XTH 24* (At4g30270) (5'-CATAGGAGGTTTCCACAGGG-3' and 5'-CTC GGA CAT AAT AGA CAA GCT AG-3') were used to amplify the cDNA using a PE 3700HT real time machine (Applied

Biosystems). Each reaction was done in triplicate. Eukaryotic translational elongation factor (EF930) was used as an endogenous control. Data was analyzed by comparative C_T method ($\Delta\Delta$ C_T method) according to the Applied Biosystems website manual. Best representing two data for each reaction have been used for data analysis.

Overexpression of *SHL2MUR3/KAM1*

Wild-type *SHL2/MUR3/KAM1* gene was PCR-amplified with gene specific primers and cloned into the binary vector pCBK05 (Riha et al., 2002). The construct was introduced into *shl2-2* and Col-0 by *Agrobacterium*-mediated transformation (Bechtold and Bouchez, 1994). As controls, a vector itself was also introduced into *shl2-2* and Col-0 by *Agrobacterium*-mediated transformation. Transgenic plants were selected on Murashige-Skoog plates containing 50µg/ml kanamycin. Plates were placed in a yellow-green acrylic filter (81 µmol $m^{-2}s^{-1}$) and hypocotyl length was measured.

CHAPTER VII
CONCLUSIONS

Plant development is dependent on many environmental factors, and light is without question a major factor. Developmental processes depending on light called 'photomorphogenesis' have been intensively studied through genetic approaches, molecular approaches such as the yeast two-hybrid system using the C-terminal domain of phytochromes, pharmacological approaches, and physiological approaches (Neff et al., 2000). More recently, global gene expression studies performed using the different light conditions revealed many genes regulated by light. Numerous genes involved in triggering photomorphogenesis when plants are exposed to light, or in inhibiting photomorphogenesis in the dark have been identified by mutational and other approaches. However, the number of genes directly involved in regulating plant development in various light conditions is low. Most of these genes identified are upstream components of light signaling.

To identify the genes involved in responses to limited light, the mutants that showed developmental hyper-responsiveness to limited light were screened. The mutants were designated as seedlings hyperresponsive to light (*shl*). They showed shorter hypocotyls, larger cotyledons, and developed true leaves earlier than wild type in low light. In the dark, they showed an etiolated phenotype typical of the wild type in the dark. *shl1, shl2, shl5,* and *shl3 shl4* (double mutant) seedlings showed limited or no phenotypic effects in darkness, but showed significantly enhanced inhibition of hypocotyl elongation in low-white, red, far-red, blue, and green light across a range of fluences. These results reflect developmental hyper-responsiveness to signals generated by both phytochrome and cryptochrome photoreceptors.

The *shl1* mutant retained significant phenotypic effects on hypocotyl length in both the *phyA* mutant and *phyB* mutant backgrounds but may be dependent on *CRY1* for phenotypic expression in blue light. The *shl2* phenotype was partially dependent on

PHYB, *PHYA*, and *CRY1* in red, far-red, and blue light, respectively. *shl2* and, in particular, *shl1* were partially dependent on *HY5* activity for their light-hyperresponsive phenotypes. The *SHL* genes act (genetically) as light-dependent negative regulators of photomorphogenesis, possibly in a downstream signaling or developmental pathway that is shared by *CRY1*, *PHYA,* and *PHYB* and other photoreceptors (*CRY2*, *PHYC*, *PHYD*, and *PHYE*).

Two of the *shls, shl1-1* and *shl2-2* were studied intensively to understand their possible defects in responses or altered responses to the exogenous phytohormones. Wild type Col-*gl* showed different responses in hypocotyl elongation to phytohormones except GA in white and yellow light. These different responses may be caused by the altered levels of endogenous phytohormones or altered sensitivity to phytohormones in different light intensities. Compared to wild-type, *shl1-1* showed a defect in responding to auxin in its root development both in white and yellow (low) light conditions, whereas it showed a slight defect in responding to auxin in hypocotyl elongation in yellow light. *shl1-1* showed altered response to ethylene in white and yellow light in such a way that the reduced production of ethylene in *shl1* is suggested. Both *shl1* and *shl2-2* showed increased hypocotyls in response to cytokinin in white, whereas WT hypocotyls were not increased. *shl1-1* showed hypersensitivity to GA, whereas *shl2-2* showed hyposensitivity to GA in yellow light. These altered responses of *shl1* and *shl2-2* to multiple phytohormones in different light intensities indicate cross-talk among those hormones that regulate *SHL1* and *SHL2*.

BR may be involved in the hycpocotyl elongation in low light condition. AtPGP involved in the hypocotyl elongation in low light formed a protein-protein interaction with TWD1 whose role in brassinosteroid signal transduction or reception was suggested (Geiser et al., 2003). BR may also be in the downstream of the ethylene signal in the light (Grauwe et al., 2005). Therefore possible defects of *shls* in response to BR will give us more information of the functions of BR in low light.

One of *SHLs*, *SHL2* was shown to be allelic to the previously identified mutants *MUR3/KAM1*. This gene encodes a type II membrane protein XyG galactosyltransferase localized in Golgi membrane that transfers galactose to the third Xyl residue within XXXG (Madson et al., 2003) and organizes actin that is responsible for endomembrane organization (Tamura et al., 2005). Whereas the original *shl2-2* allele showed long hypocotyls like WT in the dark, T-DNA insertion mutant in *SHL2*, SALK _074435 showed slightly shorter hypocotyls in the dark (though they were much longer than those of *cop/det/fus* mutants).

shl2-2, mur3-1, and *mur3-2* alleles each had an amino-acid substitution mutation in the exostosin domain, yet showed normal hypocotyl elongation in the dark, whereas a null mutant *kam1* and T-DNA insertion mutant SALK _074435 showed shorter hypocotyls in the dark, indicating that exostosin domain is required for normal hypocotyl elongation in low light, maybe through galactosylation activity resulting in the increased XET activities (Peña et al., 2004). This suggests that the modification of xyloglucan in the plant cell wall is important in hypocotyl elongation in low light. However specific XTH(s) involved in this process need(s) to be identified.

Mutant phenotypes of T-DNA insertion mutant SALK _074435 and the null mutant *kam1* indicate that the second proposed function of *SHL2/MUR3/KAM1* (organizing actin filament, resulting in proper endomembrane organization), may be important in hypocotyl elongation in the dark. Proper organization of actin filaments facilitates the secretion of enzymes and polysaccharides involved in cell wall biosynthesis (Blancaflor, 2002), perhaps by ensuring the proper endoplasmic reticulum (ER) to Golgi trafficking (Kim et al., 2005). Therefore *SHL2/MUR3/KAM1* might facilitate the proper delivery of the cell wall components such as glucose for cellulose synthesis or xyloglucan synthesized in Golgi resulting in hypocotyl elongation in the dark. We cannot determine how much *SHL2* is involved in hypocotyl elongation in the dark, but our data confirms

that there is a separate regulation on modification of the cell wall resulting in cell extension in hypocotyls in low light as suggested by Desnos et al. (1996).

SHL2/MUR3/KAM1 was expressed equally in different light conditions except in far-red light where it was expressed 0.5 times lower compared to the expression in white light. Alternatively, *SHL2/MUR3/KAM1* activity may be regulated post-transcriptionally, or its gene product may be important for the functions of other genes, such as XTHs, that are regulated by directly or indirectly by light. Identifying specific XTH(s) whose activity is affected by SHL2/MUR3/KAM1 activity using an individual recombinant XTH instead of total XTHs extracted from plant tissue described in Peña et al. (2004) and studying their regulation by light or phytohormones will give us more insight in understanding how the balance of cell wall modyfing enzymes is regulated to control the rate of hypocotyl elongation in low light. We can also screen mutants in *XTH*s, which show hyper-responsive to light.

Our study suggests that plants respond to different light intensities by regulating cell wall modification activities, resulting in changes in cell elongation. Studying the changes of the cell wall components depending on the light environment will give us clearer information about how plants respond to different light conditions at the level of cell wall modification. Gas-liquid chromatography of alditol acetates (Reiter et al., 1993) can be used for determining the monosaccharide composition of cell wall polysaccharides and high-performance anion-exchange chromatography (HPAEC)-pulsed-amperometric detection (Vanzin et al., 2002) can be used for detecting altered XyG structural change. Further, it is likely that the molecular identification of additional *SHL* genes will give us more information on light signaling in low light conditions.

Considering that *shl2-2* was not a null mutant, analysis of hormone responses on *SHL2* null mutant will give us more information on SHL2 and hormone interactions, especially with GA. Hyposensitivity of *shl2-2* to GA indicates that SHL2 is regulated by GA

directly or indirectly. The expression pattern of *SHL2* responding to GA might show us whether or not GA regulates *SHL2* directly.

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APPENDIX

A-1. *Markers derived from the BACs close to the interval between T2G17 and F5H14.*

Marker Name	Primer Sequence (5'--->3')
T2G17-F	CATAGAAAACATCCGAACGATAG
T2G17-R	GCCATGGAGCCTTAGTCAACTC
T2G17B-F	GAGAAAGAGATCGAGAGCTTCTG
T2G17B-R	GACAAAAACTAGACCTCGTGGC
F11A3-F	AAATCAAGCCCAGCCCATTTG
F11A3-R	TTTAGCCTACTAAACGGAATCG
T13C7-F	TACAACGTCGTCACCACCAC
T13C7-R	CCATCATCATCACCGCCA
F23N11-F	GACCCTTTTAAATCGGAACC
F23N11-R	CAAATGTTGGCGTTAGAAGC
F23N11B-F	GACCCTTTTAAATCGGAACC
F23N11B-R	CAAATGTTGGCGTTAGAAGC
F5H14-F	GTGGGATGTGTGATATCTGA
F5H14-R	CGTTTCTCTGGTTGTAGGTG
F26H11B-F	CAGAGAGCCACTTTGCGTGA
F26H11B-R	GCTTTCAACATGAACCGTATGGC
F7D8-F	GCTTGCGCATATTTTGG
F7D8-R	GCATGATCATGGGAATAAGG

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