Mechanism of Light Signaling in Controlling Chloroplast Biogenesis

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology in the Graduate School of Duke University

2017
ABSTRACT
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Abstract

Phytochromes are red and far-red light receptors that initiate photomorphogenesis by reprogramming both nuclear and plastidial genomes. Early light signaling events include translocation of photoactivated phytochromes from the cytoplasm to subnuclear photobodies and phytochrome-mediated degradation of a group of transcription factors, PHYTOCHROME INTERACTING FACTORs (PIFs). The degradation of PIFs not only inhibits the elongation of hypocotyl but also promotes chloroplast development by activating photosynthetic genes. However, the mechanisms by which phytochrome signaling initiates chloroplast development remain elusive. The main challenge in determining these mechanisms has been that previous genetic screens have been unable to distinguish mutants involved in plastidial gene regulation from mutants of essential components of chloroplast functionality (Chen et al., 2010; Chen and Chory, 2011). We have previously reported a new phytochrome signaling component, HEMERA (HMR), which is a transcriptional coactivator required for both phytochrome signaling and chloroplast development. The hmr mutant has a combination of long-hypocotyl and albino phenotypes, representing the founding member of a new class of photomorphogenetic mutants that has been overlooked by previous genetic screens (Chen et al., 2010; Chen and Chory, 2011). We hypothesized that these tall-and-albino mutants define uncharacterized components of phytochrome signaling required for chloroplast development. To investigate this hypothesis, we conducted a forward genetic screen for tall-and-albino mutants, which identified a novel phytochrome signaling component named Regulator-for-Chloroplast-Biogenesis-by-Light (RCBL). To determine the
evolutionary history of RCBL, I acquired the homologous sequences of RCBL and its paralog, REGULATOR-FOR-CHLOROPLAST-BIOGENESIS (RCB), from the available genomes and transcriptomes of a wide range of land plants. Phylogenetic analyses of these sequences demonstrate that RCBL and RCB diverged after the emergence of seed plants, but their mutant phenotypes show they are not functionally redundant. Characterization of rcbl mutants show that RCBL is required for both red and far-red light signaling, and it acts genetically downstream of phytochrome A and B. Similar to HMR, RCBL is also essential for photobody assembly, PIF1 and PIF3 degradation, and the expression of PIF-dependent light-responsive genes. Knocking out four PIFs (pifq, pif1/pif3/pif4/pif5) in the rcbl mutant background largely rescued the elongated hypocotyl phenotype of rcbl, indicating that the phytochrome-mediated phenotype of rcbl is dependent on PIFs. In chloroplasts, RCBL is required for transcription of plastid-encoded photosynthesis genes. However, this defective chloroplast phenotype of rcbl cannot be rescued by knocking out PIFs, suggesting RCBL plays a PIF-independent role in chloroplast development.

Since RCBL is involved in both phy signal transduction and chloroplast biogenesis, I examined whether RCBL is dual-localized to the nucleus and chloroplasts. Fluorescently-tagged RCBL shows dual-localization to chloroplasts and nuclei in both tobacco and Arabidopsis. Additionally, RCBL protein can be detected in protein fractions isolated from nuclei and plastids. I therefore conclude that RCBL is a dual-localized protein, which suggests that RCBL might be directly involved in both nuclear and plastidial events of photomorphogenesis.
Further investigation of the structure of RCBL revealed that the C-terminus of RCBL contains a domain similar to *E. coli* thioredoxin but without the canonical catalytic CxxC motif. Biochemical analyses confirmed that RCBL lacks thioredoxin reductase activity. Instead, *in vitro* experiments suggest that RCBL directly interacts with RCB through its C-terminal thioredoxin-like domain.

Taken together, this study revealed a previously uncharacterized early phytochrome signaling component which plays a critical role in chloroplast development, and demonstrated a mechanistic link between the nucleus and plastids during the initiation of photomorphogenesis.
Dedication

To my parents, Eric Chin-Hui Yang and Dany Li-Ju Yeh.
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List of Abbreviations

phy  phytochrome

Re  Red continuous light

FRc  Far-Red continuous light

PAS domain  Per-ARNT-Sim domain

GAF domain  cGMP-specific phosphodiesterases-Adenylyl cyclases-FhIA domain

HKRD  Histidine Kinase-Related Domain

HIR  High-Irradiance Response

VLFR  Very-Low-Fluence Response

LFR  Low-Fluence Response

bHLH  basic Helix-Loop-Helix

PIF  Phytochrome-Interacting Factor

APB  Active PhyB Binding

APA  Active PhyA Binding

NLS  Nuclear Localization Signal

PB  Photobody

Kbs  Kilobases

NEP  Nuclear-Encoded RNA Polymerase

PEP  Plastid-Encoded RNA Polymerase

PAP  PEP-Associated Proteins

GLK1/GLK2  Golden-Like1/2
**PhANGs** Photosynthesis-Associated Nuclear Genes

**GUN1** Genomes Uncoupled 1

**HMR** HEMERA

**PIR** Phy Interacting Domain

**TAD** Transcriptional Activation Domain

**RCB/MRL7/** REGULATOR FOR CHLOROPLAST BIOGENESIS/

**SVR4/ECB1** MesoPHYLL-CELL RNAi LIBRARY LINE

7/SUPPRESSOR OF VARIATION 4/EARLY CHLOROPLAST BIOGENESIS 1

**RCBL/MRL7-L/** REGULATOR FOR CHLOROPLAST BIOGENESIS-

**SVR4-L** FOR-LIGHT-SIGNALING/MESO PHYLL-CELL RNAi LIBRARY LINE 7-LIKE/SUPPRESSOR of VARIATION 4-LIKE

**ENU** N-ethyl-N-nitrosourea

**DAPI** 4’,6- diamidino-2-phenylindole

**trx** Thioredoxin

**RAxML** Randomized Axelerated Maximum Likelihood

**RT-qPCR** Real-Time quantitative Polymerase Chain Reaction

**pifq** pif1, pif3, pif4, pif5

**dCAPs** derived Cleaved Amplified Polymorphic sequences

**WHY1** WHIRLY1

**TnT** Transcription-and-Translation coupled system
GST  Glutathione S-Transferase

NMR  Nuclear Magnetic Resonance spectroscopy
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Chapter 1. Introduction to Phytochrome Signaling and Chloroplast Biogenesis

1.1 Plant Growth and Development is Extremely Plastic in Response to Environmental Light Cues

As a sessile organism, the post-embryonic developmental growth of a plant is extremely flexible to accommodate changing environmental cues, of which light is the most important (Casal et al., 2004). Light governs almost all of the developmental transitions in the plant life cycle (Figure 1), including seed germination (Bentsink and Koornneef, 2008), seedling establishment as an autotroph, floral induction (Alvarez-Buylla et al., 2011) and ultimately senescence (Arsovski et al., 2012; Nemhauser and Chory, 2002; Sullivan and Deng, 2003).

Figure 1. Light regulates most plant developmental stages.

Light governs multiple developmental transitions, including germination, de-etiolation (photomorphogenesis), vegetative growth, and floral transition. Figure adapted from Kami et al., (2010).
The best studied developmental transition in light signaling is seedling establishment. When seedlings first encounter light, they undergo a transition from the heterotrophic dark-grown mode (skotomorphogenesis) to an autotrophic light-grown mode (photomorphogenesis). Skotomorphogenesis features elongated hypocotyls, closed embryonic leaves (cotyledons), and apical hooks (Figure 2, left seedling). These structures help germinated seedlings rapidly seek light as well as protect the fragile shoot apical meristem, containing stem cells, from mechanical damage while the cotyledons emerge from the soil (Gallego Bartolomé et al., 2011). When plants are exposed to light, photomorphogenesis is initiated by global transcriptional reprogramming, which causes the inhibition of hypocotyl elongation as well as the opening and greening of cotyledons (Wu et al., 2010) to transition into a photoautotrophic lifestyle (Figure 2, right seedling) (Kami et al., 2010).

**Figure 2. Light triggers de-etiolation of Arabidopsis seedlings.**

The dark-grown seedling is shown on the left, with apical hook, closed cotyledons (embryonic leaves), and elongated hypocotyl (embryonic stem), whereas the light-grown seedling is shown on the left, with open green cotyledons and a short hypocotyl.

The transition from skotomorphogenesis to photomorphogenesis, named de-etiolation, requires coordination of two genome-containing organelles, the nucleus and
plastids (Dyall et al., 2004). During de-etiolation, about one-third of the nuclear genes are differentially regulated (Hu et al., 2013; Leivar et al., 2012; 2009; Jiao et al., 2007). In plastids, light enhances the DNA binding affinity and transcriptional activity of chloroplast-encoded RNA-polymerases (Oh and Montgomery, 2014; Finster et al., 2013; Liere and Börner, 2007). These processes transform non-photosynthetic etioplasts to photosynthetically-active chloroplasts.

1.2 Phytochromes are Red and Far-red Photoreceptors Which Initiate Photomorphogenesis

To initiate the photomorphogenesis, plants utilize multiple photoreceptors which are collectively able to accurately sense the quality, intensity, directionality, and periodicity of light (Kami et al., 2010; Sullivan and Deng, 2003).

![Figure 3. Multiple photoreceptors perceive different wavelengths of light.](image)
module, including a GAF domain (conjugated with a chromophore, phytochromobilin), and a C-terminal dimerization and PB localization module, including a PRD domain for nuclear localization. (C) The ground (inactive) state of phytochrome, named Pr, is able to absorb red light. After absorbing red light, phytochrome becomes activated (Pfr form), and it can be converted back to inactive form by exposure to far-red light. Figure adapted from www.unil.ch and Van Buskirk et al., (2012).

In *Arabidopsis*, photoreceptors can be categorized into five classes based on the perceived wavelength: UV-B is perceived by UV RESISTANCE LOCUS 8; UV-A/blue light is detected by cryptochromes, phototropins, and zeitlupes (ZEILUPE/FALVIN-BINDING, KELCH, F-BOX1 (FKF1)/LOV KELCH PROTEIN2 (LKP2)); red (R)/far-red (FR) light is sensed by phytochromes (phys) (Figure 3A). Among all photoreceptors, phys are the best-studied for their roles in initiating photomorphogenesis (Chen et al., 2004; Chen and Chory, 2011). Perceiving red light is critical for plant survival because it reflects the availability of photosynthetic energy as well as the potential presence of neighboring competitors (Chen et al., 2004; Franklin, 2005; Chen and Chory, 2011; Casal, 2013).

A phytochrome protein consists of an N-terminal photosensory module with a \_Per-ARNT-Sim (PAS) domain, a cGMP-specific phosphodiesterases-Adenylyl cyclases-FhlA (GAF) domain and a PHY domain, as well as a C-terminal Histidine Kinase-Related Domain (HKRD) (Figure 3B) (Rockwell, 2006). The GAF domain of each phytochrome molecule is covalently linked to a chromophore, phytochromobilin. The configuration of phytochromobilin changes when phys perceive R or FR light. This configuration change leads to a conformational change of the phy holo-protein from the inactive, R-light-absorbing Pr form to the active, FR-light-absorbing Pfr form (Figure 3C) (Butler et al., 1959). The Pfr form can be photoconverted back to Pr form by absorbing
FR light. In addition, the Pfr form is thermodynamically unstable and can spontaneously convert back to Pr form, a process named dark reversion (Rockwell et al., 2006). Because the majority of phy-mediated physiological responses are induced by R light and inhibited by FR light, the Pfr form is considered the biologically active form (Franklin and Quail, 2009; Li et al., 2011).

In *Arabidopsis*, phys are encoded by five genes, which are named *phyA-E*. PhyA and phyB are the most predominant phytochromes with distinct functions. PhyA is photo-labile and perceives continuous FR light and broad spectrum in very low light; PhyB-E are relatively photo-stable and mediate R light responses (Sharrock and Quail, 1989). It has been shown that knocking out all phys causes *Arabidopsis* grown in red light to be nearly transcriptionally blind (Hu et al., 2013; Strasser et al., 2010), indicating that phys are the only photoreceptors perceiving red/far-red light and conducting downstream signaling.

Phy responses have been thoroughly studied and categorized into four modes of action, based on the fluence of light and the proportion of Pfr versus Pr (Fankhauser and Casal, 2004; Casal et al., 1998). Hypocotyl inhibition and cotyledon opening require constant light exposure, which cannot be achieved by an hourly pulse of the same wavelength of light and total fluence. This High-Irradiance Response (HIR) requires constant exposure to high fluence of R and FR light, and is mediated by phyB and phyA respectively. Examples of HIR include inhibition of hypocotyl elongation and opening of the apical hook and cotyledons. On the other hand, some light-mediated responses follow the Bunsen-Roscoe law of reciprocity, which means that they are triggered by total
fluence independent of the fluence rate or irradiation time. These responses can be further categorized into two types: Very-Low-Fluence Response (VLFR) and Low-Fluence Response (LFR). VLFR is non-reversible and extremely sensitive to a broad range of light. PhyA plays a major role in VLFR to induce germination in low light conditions. On the other hand, LFR is reversible by exposure to FR. PhyB is responsible for the LFR, which includes the promotion of seed germination and leaf movement.

1.3 Activated Phytochromes Initiate Transcriptional Reprogramming by Regulating the Stability and Activity of PHYTOCHROME-INTERACTING FACTORS

Phys mediate almost all of the transcriptional reprogramming caused by R (Hu 2013). The central mechanism by which phys reprogram the transcriptome is by regulating a family of basic Helix-Loop-Helix (bHLH) transcriptional factors (TFs) named PHYTOCHROME-INTERACTING FACTORs (PIFs) (Huq et al., 2004; Huq and Quail, 2002; Khanna et al., 2004; Leivar et al., 2008b; Ni et al., 1998; Oh et al., 2004).
The PIF family consists of seven members: PIF1, 3, 4, 5, 6, 7, 8. They all possess a C-terminal bHLH DNA binding and dimerization domain, and an N-terminal Active PhyB Binding (APB) domain. In addition, PIF1 and 3 interact with the Pfr form of phyA through an Active PhyA Binding (APA) domain (Figure 4A). Each PIF plays both distinct and overlapping roles in regulating photomorphogenesis (Figure 4C). For example, PIF1, PIF3, PIF4, PIF5 and PIF7 promote hypocotyl elongation by activating growth genes (Al-Sady et al., 2008; Fujimori et al., 2004; Huq et al., 2004; Leivar et al., 2008a; Lorrain et al., 2009). PIF1 is solely responsible for the inhibition of germination (Oh et al., 2004; 2007), while PIF1, PIF3, and PIF5 inhibit chloroplast development by repressing the expression of chlorophyll biogenesis enzymes (Figure 4C) (Huq et al., 2004; Stephenson et al., 2009). Removal of four PIFs, as in the pifq quadruple mutant (pif1/pif3/pif4/pif5) leads to a constitutive photomorphogenic phenotype of in the dark (Figure 4B), suggesting that PIFs are the master negative TFs of photomorphogenesis (Leivar et al., 2008b; Leivar and Quail, 2011; Shin et al., 2009; Kim et al., 2011).

To initiate photomorphogenesis, activated phys directly interact with PIFs and promote their phosphorylation and subsequent degradation by the ubiquitin-proteasome system. PIF degradation is an extremely quick process, with PIF1 and PIF3 having a documented half-life of only ten to fifteen minutes when exposed to light (Shin et al.,
Light-dependent multisite phosphorylation of PIF3 is required for its degradation (Ni et al., 2013). The responsible kinases have been published recently, named Photoregulatory Protein Kinases 1-4 (PPK1-4) (Ni et al., 2017). Casein kinases 2 (CK2) has been also suggested to phosphorylate PIFs in vitro, however, its activity is not light-regulated (Bu et al., 2011b; 2011a). Taken together, these results show that multiple kinases are involved in the phosphorylation of different PIFs, which needs to be further investigated. PIF3 are ubiquitylated via an E3 ligase named LRB (Ni et al., 2014). However, LRB is not the only E3 ligase mediating light-dependent degradation of PIF3, since the degradation of PIF3 in lrb1,2,3 mutants is delayed, but not completely abolished (Ni et al., 2014; Zhu and Huq, 2014). Therefore, other E3 ligases remain to be identified.

1.4 Photobody-Localization of PhyB Correlates with PIF Degradation

At the cellular level, one of the earliest light responses is the translocalization of photoactivated phyB from the cytoplasm to the nucleus, where they accumulate in subnuclear foci called photobodies (Van Buskirk et al., 2014; Chen et al., 2005; Van Buskirk et al., 2012). Inactive phyB resides in the cytoplasm with the putative Nuclear Localization Signal (NLS) masked by the N-terminal photosensory module (Chen et al., 2005). Once activated, phyB undergoes a conformational change to expose the NLS and is then transported into the nucleus where it is further localized to photobodies (PBs) (Chen et al., 2005; Nagatani, 2004; Sakamoto and Nagatani, 1996; Van Buskirk et al., 2012; Yamaguchi et al., 1999). The size of PBs increases, but the number of which decreases with the quality and intensity of light (Figure 5) (Chen et al., 2003; Van Buskirk et al., 2014).
Figure 5. The morphology of photobodies correlate with red-light intensity and hypocotyl growth

Upper panel: Confocal images of nuclei in 4-d-old Arabidopsis seedlings expressing GFP fused with phyB. Seedlings were grown under different light intensity of Rc (from left to right, 0.5, 1, 2, and 8 µmol m$^{-2}$s$^{-1}$). Lower panel: morphology of seedlings grown in the condition listed above. Figure adapted from Van Buskirk et al., (2012).

PBs are proposed to be sites of phosphorylation, ubiquitination, and degradation of PIF3, as suggested by the result that PIF3 must co-localize with phyB and phyA PBs prior to its degradation (Al-Sady et al., 2006; Bauer et al., 2004). In addition to PB localization, PIF3 phosphorylation and degradation depend on direct interaction with phyB or phyA (Al-Sady et al., 2006). It has been proposed that the physiological function of PBs is to serve as reservoirs of active phys to repress the protein level and activity of PIF3 to ultimately inhibit hypocotyl elongation at night in seedlings grown under diurnal conditions (Van Buskirk et al., 2014). Although it is widely accepted that PBs are protein degradation sites, there are other PB constituents not involved in protein degradation (Van Buskirk et al., 2012; Chen, 2008) such as photostable PIF7 (Kidokoro et al., 2009), suggesting PBs might possess other functions. Another hypothesis is that PBs serve as sites of transcriptional regulation, which is based on the observation that PIFs localize to PBs prior to their degradation (Van Buskirk et al., 2012; Chen, 2008). However, the
precise function of PBs is still not fully understood.

1.5 Photomorphogenesis Requires Coordination Between Nuclear and Plastidial Genomes to Conduct Global Transcriptional Reprogramming

Photomorphogenesis not only requires PIF-mediated transcriptional reprogramming in the nucleus but also the activation of plastid-encoded genes to build the photosynthetic apparatus in chloroplasts. Plastids contain a prokaryotic-like 120-160 kilobase (Kb) circular genome which encodes about 130 genes (Palmer, 1985). These genes are transcribed by two types of RNA-polymerases: two phage-type Nuclear-Encoded RNA polymerases (NEP), and a bacterial-type Plastid-Encoded RNA Polymerase (PEP) (Figure 6) (Liere et al., 2011; Pfannschmidt et al., 2015; Börner et al., 2015).

Although most plastidial genes can be transcribed by both types of RNA-polymerases (Figure 6) (Krause et al., 2000), housekeeping genes are preferentially transcribed by NEP (Figure 6A) (Hajdukiewicz et al., 1997). On the other hand, the majority of photosynthetic genes are transcribed by PEP (Figure 6B) (Hajdukiewicz et al., 1997; Börner et al., 2015; Hübschmann and Börner, 1998; Allison et al., 1996). Once chloroplasts differentiate, the dominance of RNA-polymerase is switched from NEP to PEP, which is possibly mediated by the inhibition of NEP activity by PEP-transcribed tRNA<sup>Glu</sup> (Maliga, 1998; Hanaoka et al., 2005). This switch leads to reprogramming of plastidial transcriptome to increase the production of photosynthesis-related proteins as well as establish chloroplast development (Leon et al., 1998). During de-etiolation, phy signaling in the nucleus also induces plastid-encoded photosynthetic genes by increasing
the activity of PEP complex (Thum et al., 2001; Chun et al., 2001). However, the mechanism by which phy signaling in the nucleus communicates with plastids remains unknown.

![Figure 6](image)

**Figure 6. Plastidial genes are transcribed by two distinct RNA polymerases.**

(A) Schematic illustration of NEP (RPOTmp), a phage-type RNA polymerase in the chloroplasts. Based on current evidence, NEP may act as a single-subunit enzyme and other unknown transcription factors (TF) may support the promoter binding of NEP. Most of NEP promoters consist of a conserved YRTA motif. NEP preferentially transcribes plastidial housekeeping genes. (B) Upper panel: the core subunits of PEP, including α (RpoA), β (RpoB), β’ (RpoC1), β” (RpoC2), and σ (Sigma factor, Sig1-Sig6). Sigma factors recognize conserved elements at positions -10 and -35 to initiate transcription (red arrow). The C-terminal domains of α subunits interact with DNA and other transcription factors, and the N-terminal domain may interact with other core complexes of PEP. Lower panel: PEP associates with a battery of nuclear-encoded PEP-associated proteins (PAPs). These PAPs are required for the transcription of photosynthetic genes in the chloroplasts.

The composition of the PEP complex includes eubacteria-like α, β, β’, and β” core subunits, sigma factors (Figure 6B), and a group of PEP-Associated Proteins (PAPs) identified by proteomic approaches (Pfalz and Pfannschmidt, 2013; Steiner et al., 2011; Pfalz, 2006). Although sigma factors and PAPs are encoded in the nucleus and both are required for PEP-mediated transcription, their functions are different. Sigma factors are
required for promoter specificity of the PEP complex, but PAPs are essential for the activity and assembly of the PEP complex (Melonek et al., 2012; Pfalz, 2006; Pfalz et al., 2015; Steiner et al., 2011; Yagi et al., 2012; Pfannschmidt et al., 2000; Suzuki et al., 2004). Compared to PAPs, the function and regulation of sigma factors are better understood. The expression of nuclear-encoded sigma factors is regulated by environmental stimuli. For example, blue light and stress are able to induce the expression of SIG5 (Tsunoyama et al., 2002; Nagashima et al., 2004). Moreover, phy regulate the expression of SIG2 and SIG6 (Oh and Montgomery, 2014). All sigma factors regulate the binding specificity of PEP (Kanamaru and Tanaka, 2014). For the function of PAPs, several reports demonstrate that they are essential for the activity of the PEP complex, and might be crucial for PEP assembly (Steiner et al., 2011; Pfalz, 2006; Wang et al., 2016; Pfalz et al., 2015; Yu et al., 2013; Gao et al., 2011; 2012; Yagi et al., 2012; Wimmelbacher and Bornke, 2014). However, there is still limited knowledge of the configuration of PAPs within the PEP complex as well as the functionality of individual PAPs.

The evolutionary origin of PAP proteins is still a mystery. While the core PEP subunits and sigma factors are conserved with the cyanobacterial origin of chloroplasts (Hu and Bogorad, 1990; Pfannschmidt et al., 2000; Suzuki et al., 2004), the PAP proteins are plant-specific (Yagi and Shiina, 2014; 2012). This suggests that PAP proteins were possibly acquired after the establishment of endosymbiosis. Plant-specific PAPs offer a light-dependent regulatory machinery to fine tune the DNA binding activity of PEP (Finster et al., 2013) and thus regulate PEP-dependent gene expression in chloroplasts.
When and how these proteins were acquired by chloroplasts is still unclear.

1.6 Anterograde and Retrograde Signaling Pathways Between the Nucleus and Chloroplasts Play a Critical Role in Seedling Establishment

Interorganellar communication involves both anterograde and retrograde signaling pathways, which are important for seedling establishment and stress adaptation. Anterograde signaling pathways from the nucleus to organelles are critical for chloroplast development because over 90% of plastid proteins are encoded in the nucleus (Surpin and Chory, 1997; Leister, 2003; Surpin et al., 2002; Barkan, 2011). Therefore, anterograde signaling pathways play a critical role in maintaining the stoichiometry of nuclear- and chloroplast-encoded photosynthetic proteins (Woodson and Chory, 2008). It has been shown that the expression of GOLDEN-LIKE1 (GLK1) and GOLDEN-LIKE2 (GLK2) are dependent on phys (Oh and Montgomery, 2014; Oh et al., 2013). These two GLK genes are required for the expression of Photosynthesis-Associated Nuclear Genes (PhANGs) (Waters et al., 2009; Oh and Montgomery, 2014).

When plastids are affected by environmental stimuli, they exert signals through retrograde signaling pathways to regulate nuclear gene expression. This type of signaling event was first exemplified by application of a chloroplast-destroying herbicide, which resulted in the repression of hundreds of PhANGs (Woodson et al., 2012; Woodson and Chory, 2008; Martín et al., 2016; Koussevitzky et al., 2007). Genetic screens have identified a number of genomes uncoupled (gun) mutants, in which PhANGs fail to be repressed by plastid retrograde signals. Further characterization of the gun1 mutant demonstrated that GUN1 is involved in multiple stress-induced retrograde signaling
Recent publications have further demonstrated that when plants are exposed to high-intensity light, chloroplasts utilize retrograde signaling pathway to suppress PIF-induced genes (Martín et al., 2016).

Among the players in anterograde and retrograde signaling pathways, SIG2 and SIG6 are involved in both signaling pathways to coordinate light signaling and chloroplast development (Oh and Montgomery, 2014; Woodson et al., 2012). These two are the most important sigma factors for chlorophyll synthesis and chloroplast development (Ishizaki et al., 2005; Kanamaru et al., 2001; Shirano et al., 2000). Oh and Montgomery (2014) have demonstrated that the expression of SIG2 and SIG6 depends on phys, suggesting a light-dependent anterograde signaling mechanism to regulate chloroplast development. On the other hand, Woodson et al. (2012) showed that SIG2 and SIG6 play partially redundant roles in exerting chloroplast signals to control PhANG expression in the nucleus. It is still unknown whether other proteins play similar roles in both antero- and retrograde signaling pathways.
1.7 Identification of HEMERA, a Nucleus and Chloroplast Dual-Localized Phy Signaling Component Required for Chloroplast Biogenesis

Figure 7. HMR is a dual-localized protein, which is required for phytochrome signaling and chloroplast development.

(A) Representative image of 4-d-old Rc grown PBG and hmr-1/PBG seedlings. (B) Confocal microscopy images of PBs in PBG and hmr-1/PBG. PBs are visualized by phyB-GFP fluorescence. (C) Schematic illustration of the HMR domain structure. HMR possesses two active phy interacting domains (PIR1 and PIR2), and a C-terminal 9 amino acid Transcriptional Activation Domain (9a.a.TAD) (Galvão et al., 2012; Qiu et al., 2015). (D) Proposed model of HMR’s nuclear function. HMR binds directly to PIFs at the promoter of Class B genes. HMR’s TAD is required for the activation of these genes, possibly through recruitment of transcriptional machinery or stabilization of the PolII complex. While activating gene expression, HMR also promotes the degradation of PIFs. This degradation-coupled-transcriptional-activation mechanism allows plants to quantitatively control hypocotyl elongation in the light. Figure adapted from Chen et al., (2010); Qiu et al., (2015)

The identification and characterization of hemera (hmr) mutants shed light on the molecular mechanisms of communication between light signaling and chloroplast development. Because HMR has distinct functions in regulating transcription in both the nucleus and chloroplasts, the hmr mutant possesses a unique combination of tall, albino, and seedling lethal phenotypes in Arabidopsis. With this combination of phenotypes, hmr represents the founding member of a novel class of photomorphogenetic mutants (Figure
7A) (Chen et al., 2010; Chen and Chory, 2011). In addition, nuclear and plastidial dual-localization of HMR has been shown by multiple methods, including biochemical fractionation and immunostaining in Arabidopsis and maize (Chen et al., 2010; Pfalz et al., 2015). This further supports the hypothesis that HMR has distinct roles in different compartments to regulate gene expression in photomorphogenesis, which is consistent with the tall-and-albino phenotype of hmr null mutants.

![Figure 8](image)

**Figure 8.** HMR is required for multiple phy signaling events in the nucleus as well as PEP-dependent gene expression in chloroplasts.

(A) Immunoblot detecting phyA proteins in 4-d-old 10 µmol m$^{-2}$ s$^{-1}$ Rc grown Col-0, hmr-2, PBG, hmr-1, and phyB-9 seedlings. Actin was used as a loading control. (B) Immunoblots detecting PIF1 and PIF3 in 4-d-old Rc grown Col-0, hmr-2, PBG, and hmr-1 seedlings. (C) RT-qPCR analyses of selected PIF-induced genes. Class A genes are PIF-induced and HMR-repressed. Class B genes are PIF-induced and HMR-induced. (D) RT-qPCR analyses of PEP- and NEP-dependent genes. (C and D) RNA samples were...
prepared from 4-d-old 10 µmol m⁻²s⁻¹ Rc grown Col-0, *pifq, hmr-5*, and *hmr-5/pifq* seedlings. Error bars represent standard deviation of three replicates. Red or blue arrows show increase or decrease in gene expression, respectively. Figure adapted from Qiu et al., (2015); Chen et al., (2010).

Dissecting the nuclear function of HMR has recently yielded major breakthroughs. As a phy signaling component, HMR is required for multiple early phy signaling events, including light-dependent localization of phyB to PBs (Figure 7B), light-triggered degradation of phyA, PIF1, and PIF3 (Figure 8A and B) (Chen et al., 2010), as well as activation of PIF1 and PIF3 target genes (Figure 8C) (Qiu et al., 2015). Consistent with its role in phy signaling, HMR preferentially interacts with activated phyB and phyA (Galvão et al., 2012), as well as with the master transcription factors PIFs (Qiu et al., 2015). Knocking out four PIFs (PIF1, PIF3, PIF4, and PIF5) rescues the elongated hypocotyl phenotype of *hmr* in the light (Qiu et al., 2015), suggesting that the nuclear function of HMR is dependent on PIFs. Surprisingly, the C-terminal of HMR possesses a nine amino acid Transcriptional Activation Domain (TAD) (Figure 7C). The TAD domain is required for the activation of a subset of PIF-induced, growth-promoting genes, as well as the degradation of PIF1 and PIF3 *in vivo* (Chen et al., 2010; Galvão et al., 2012; Qiu et al., 2015). Although there seems to be a conflict between HMR’s function in PIF degradation and promoting PIF activity, these dual functions may explain the complicated gene expression pattern of PIF-induced genes in *hmr* mutants. Qiu et al. (2015) subcategorized PIF-induced gene into two classes, Class A and B, based on their expression patterns. The Class A genes are HMR-independent and upregulated in *hmr* due to an increase of PIF abundance, whereas the Class B genes require PIFs and HMR’s TAD to activate their transcription. Therefore, the expression of Class B genes is
downregulated in \textit{hmr} mutants regardless of PIF abundance (Figure 8C). Taking all of this evidence together, Qiu et al. proposed a HMR-dependent degradation-coupled transactivation mechanism to express PIF-induced growth-promoting genes, which can tightly control the abundance and activity of transcription factors to fine-tune hypocotyl growth in the light (Figure 7D) (Qiu et al., 2015).

In chloroplasts, HMR/pTAC12/PAP5 is one of the PAPs, which is required for the proper function of PEP (Pfalz, 2006; Qiu et al., 2015). The characterization of maize HMR/pTAC12/PAP5 has demonstrated that HMR is able to bind to single-stranded DNA and RNA and is required for the assembly of the PEP complex (Pfalz et al., 2015). In addition, HMR has been shown to directly interact with pTAC7/PAP12 (Yu et al., 2013) and pTAC14/PAP7 (Gao et al., 2011; 2012). However, the actual molecular function of HMR in regulating the PEP complex is still poorly understood.

As a multiply-targeted protein, an intriguing question is whether HMR plays distinct roles in different compartments or if the pleiotropic phenotypes of \textit{hmr} mutants can be attributed to a specific pool of the protein. For HMR, the quintuple \textit{hmr/pifq} mutant only partially rescues the hypocotyl phenotype, but has no effect on the albino phenotype, indicating that HMR plays distinct roles in the nucleus and chloroplasts: a nuclear, PIF-dependent role in regulating hypocotyl elongation, and a separate PIF-independent plastidial function in promoting chloroplast biogenesis (Qiu et al., 2015).

\subsection*{1.8 Conclusions and outstanding questions}

Upon exposure to light, plants undergo massive transcriptional reprogramming to switch from skotomorphogenesis to photomorphogenesis to adopt a photoautotrophic
lifestyle. During photomorphogenesis, the inhibition of hypocotyl growth and differentiation of chloroplasts are the biggest developmental changes, which requires coordination between the nucleus and chloroplasts. In the past three decades, the signaling mechanism of phy has been extensively studied, from which many important regulators have been identified and characterized. The central mechanism of phy signaling is initiated when plants are exposed to light. Photoactivated phys are translocated into the nucleus and initiate transcriptional reprogramming to regulate seedling establishment. Not surprisingly, most phy signaling components are nuclear-localized. Chloroplast development requires many proteins encoded in both the nuclear and chloroplast genomes. Tight communication between these two compartments is required to initiate chloroplast biogenesis, to maintain the appropriate stoichiometry of components in plastidial protein complexes. However, our understanding of how plants decode light stimuli and coordinate transcription of the nuclear and plastidial genomes to initiate photomorphogenesis is still rudimentary. The complex nature of communication between these two cellular compartments may have hindered scientists from finding the components involved in the multiple pathways that initiate photomorphogenesis.

The identification of HMR indicates a mechanistic link between the nucleus and plastids, suggesting a close relationship between phy signaling and plastid transcription. HMR plays distinct roles in the nucleus and chloroplasts: in the nucleus, it is a transcriptional co-activator that regulates the activity and degradation of PIFs; in chloroplasts, HMR acts as a core component of the PEP complex to regulate the plastidial transcription (Pfalz, 2006; Steiner et al., 2011).
The characterization of HMR reveals a new pathway connecting the nucleus and chloroplasts upon illumination, but it raises another question: are there other components like HMR that have been overlooked by previous genetic screens? To address this question, we conducted a genetic screen for using the tall-and-albino phenotype as the screening criteria to search for novel phy signaling components required for chloroplast biogenesis. This screen has successfully identified several hmr-like mutants, and my Ph.D. project focused on one of the mutants, named regulator-for-chloroplast-biogenesis-in-light-signaing (rcbl). The overall goal of this project was to determine the function of RCBL in phy signaling and chloroplast biogenesis.

Chapter Two describes the identification and characterization of rcbl mutants in phy signaling and chloroplasts biogenesis. My results show that RCBL is required for multiple early events in the phyB and phyA signaling pathways as well as PEP-dependent plastidial gene expression.

RCBL has been shown to be a chloroplast protein (Qiao et al., 2011; Powikrowska et al., 2014a). However, whether the RCBL protein is also localized to the nucleus to regulate phy signaling pathways was previously unknown. In Chapter Three, I determine the subcellular localization and potential partitioning mechanism of RCBL. Indeed, RCBL is dual-targeted to the nucleus and chloroplasts as shown by confocal imaging of fluorescently-tagged RCBL and subcellular fractionation experiments. To my surprise, RCBL proteins from nuclei and plastids show similar molecular weight by SDS-PAGE, suggesting RCBL may be processed in chloroplasts prior to being transported into the nucleus.
To determine the molecular function of RCBL, I examine the possible biochemical function of RCBL in Chapter Four. My studies reveal that RCBL and RCB are structurally similar to thioredoxin (trx), but without any reductase activity. Instead, RCBL and RCB interact directly with each other through their trx-like fold.

Taken together, my dissertation project has discovered a novel dual-targeted phy-signaling component required for chloroplast biogenesis, and has uncovered a potential molecular mechanism for its role in regulating photomorphogenesis. These results have laid the foundation to a better understanding of the crosstalk between nuclear and plastidial transcription during early seedling establishment.
Chapter 2. Identification and Characterization of *Regulator-for-Chloroplast-Biogenesis-in-Light-Signaling* mutants

2.1 Introduction

The identification and characterization of *hmr* mutants suggests the existence of a novel class of photomorphogenic mutants that have been overlooked by previous genetic screens (Chen et al., 2010). Originally, the genetic screen carried out in Chen *et al.* (2010) was designed to identify mutants defective in PBs formation, leading to the discovery of *hmr*. HMR is essential for phy signaling, interacts preferentially with activated phys and the downstream transcription factors, PIFs, and HMR also serves as a transcriptional co-activator to activate a subset of PIF-induced, growth promoted genes (Figure 8C) (Chen *et al.*, 2010; Galvão *et al.*, 2012; Qiu *et al.*, 2015). Surprisingly, HMR carries another function in the chloroplasts as a core subunit of PEP required for PEP-dependent gene expression (Figure 8D) (Pfalz, 2006; Qiu *et al.*, 2015). Given that *hmr* had not been identified in previous screens for phy signaling mutants, other unknown components involved in the coordination between the nucleus and chloroplasts in photomorphogenesis could also have been previously omitted. Therefore, a genetic screening strategy needs to be designed to identify those omitted mutants. However, the confocal-based screening method used to identify *hmr* is time-consuming and labor-intensive. Because *hmr* mutants possess a distinctive tall-and-albino phenotype, we used the appearance of seedlings as the screening criteria. From this genetic screen, we have successfully isolated several mutants. Among these mutants were a pair of paralogous genes, named *regulator-for-chloroplast-biogenesis* (*rcb*) and *regulator-for-chloroplasts-biogenesis-in-light-signaling* (*rcbl*). The characterization of the *rcb* mutant was done by
other members of our laboratory showing that, like HMR, RCB is required for phy signaling and transcription of plastidial PEP-dependent genes. In my dissertation studies, I investigated the function of RCBL in photomorphogenesis by genetic, immunohistochemical, and biochemical approaches.

In this chapter, I performed genetic analyses to dissect the roles of RCBL in de-etiolation, including phy signaling and chloroplast development. First, I describe the screening strategy to identify *hmr*-like mutants. This screening was done by a former lab member, He Wang. Next, through a collaboration with Dr. Weigel’s lab at the Max Planck Institute, the causal gene of *rcbl-1/PBG* was identified using next-generation sequencing and SHOREmap (Schneeberger et al., 2009; Schneeberger and Weigel, 2011). Intriguingly, BLAST analysis shows that *RCBL* is a paralog of *RCB*. To investigate the phylogenetic relationship between RCBL and RCB, I obtained sequences of RCBL and RCB from different species and used a maximum-likelihood method to construct a phylogenetic tree with bootstrapping. To determine the role of RCBL in the phy signaling pathway, I characterized phy responses, light-dependent proteolysis, and the expression of light-responsive genes in *rcbl* null mutants. The results described in this chapter show that *rcbl* is a new member of the *hmr*-class of photomorphogenic mutants, and further determine that *RCBL* acts downstream of *phys* and is involved in the proteolysis-coupled transcriptional activation of a subset of PIF-dependent growth-promoting genes.
2.2 Results

2.2.1 Identification and Verification of Regulator-for-Chloroplast-Biogenesis-in-Light-Signaling (rcbl) Mutants

![Diagram](A) Schematic illustration of tall-and-albino screen for hmr-like mutants (B) Representative images and hypocotyl length quantification of 4-day-old PBG and rcbl-1/PBG seedlings grown under continuous 10 µmol m\(^{-2}\) s\(^{-1}\) red light. Hypocotyl measurements were taken from at least 30 seedlings for each genotype. Hypocotyls of the rcbl-1/PBG seedlings were significantly longer than those of PBG seedlings. Error bars represent standard error. **P<0.001. (C) Representative confocal images showing the subnuclear localization of PHYB-GFP in epidermal cells at the top of the hypocotyl of 4-d-old Rc grown PBG and rcbl-1/PBG. (D) Box-and-whisker plots for the average volumes or diameter of PBs per nucleus in 4-d-old PBG or rcbl-1/PBG grown under 10 µmol m\(^{-2}\) s\(^{-1}\) Rc. The average volume and diameter of photobodies in PBG (0.91 µm\(^3\) and 0.86 µm respectively) are significantly larger than those in rcbl-1/PBG. Centerlines in each box indicate medians; the top and bottom lines of the boxes indicate the 25th and 75th percentiles, and the whiskers extend to the maximum and minimum data points. Quantification of photobodies was done in 44 nuclei of PBG and 42 nuclei of rcbl-1/PBG. (E) Box-and-whisker plots for the number of large (left panel, volumes greater than 0.73 µm\(^3\)) or small (right panel, volume smaller than 0.73 µm\(^3\)) PBs per nucleus in the indicated genotypes. On average, each nucleus of PBG seedlings possesses three large PBs, in contrast to those of rcbl-1/PBG, which have less than one. For small PBs, each nucleus of PBG seedlings has two small PBs on average while rcbl-1/PBG averages 18 small PBs.

The physiological characterization of hmr null mutants enabled us to perform a forward genetic screen to search for other components that, like HMR, act in both phy
signaling pathways and chloroplast development. To perform the hmr-like screen, PBG seeds (PHYB-GFP in Landsberg erecta [Ler] background) were mutagenized with N-ethyl-N-nitrosourea (ENU) and viable plants (M1) were selected. Seeds were collected from individual M1 plants. M2 lines were screened under either continuous R and FR light (8 mmol m\(^{-2}\) s\(^{-1}\)). Mutagenized lines with both elongated hypocotyl and albino phenotypes were preserved for a secondary PB morphology screen (Figure 9A). Lines displaying defective PB formation were saved. To date, more than six additional mutants have been found by phenotyping. The mutant, 24-21, referred to as regulator-for-chloroplast-biogenesis-in-light-signaling (rcbl-1/PBG), is one of the mutants possessing elongated hypocotyl and albino phenotypes (Figure 9B), and it is also defective in PB formation (Figure 9C, D, E).

2.2.2 Using SHORE Map to Identify the Causal Gene of rcbl Mutation and Complementation of rcbl-1/PBG by Ectopically Expressing RCBL cDNA

To clone the gene responsible for rcbl-1/PBG’s phenotype, we generated a mapping population by crossing the heterozygous 24-21 (phyB-GFP in Ler) with the Columbia-0 (Col-0) ecotype. Through our collaboration with the Weigel lab at the Max Planck Institute, the mapping populations of rcbl-1/PBG were subjected to deep sequencing and analyzed by SHOREmap (Schneeberger et al., 2009; Schneeberger and Weigel, 2011). These analyses mapped the rcbl-1/PBG mutation to At2g31840, which was confirmed by direct sequencing. rcbl-1/PBG has a G-to-A mutation at position 786, changing a tryptophan codon to a premature stop (Figure 10C). Prior to further physiological characterization, rcbl-1/PBG was been backcrossed to PBG three times to eliminate extra mutations caused by ENU treatment.
Figure 10. Complementation of rcbl-1/PBG and domain structure of RCBL.

(A) Representative images of 4-day-old PBG, rcbl-1/PBG, and rcbl-1/PBG/RCBL-HA-His seedlings grown under continuous 10 µmol m⁻² s⁻¹ red light. (B) Hypocotyl length quantification of seedlings from (A). Hypocotyl measurements were taken from at least 30 seedlings for each genotype. Expression of RCBL-HA-His is able to rescue the elongated hypocotyl and greening phenotypes of rcbl-1/PBG. Error bars represent standard error. Different letters denote significant difference among means (Tukey-Kramer HSD, P<0.0001) (C) Predicted domain structure of RCBL (AT2G31840) with alleles used in this study. RCBL possesses a predicted chloroplast transit peptide from amino acid 1-48, a predicted bipartite nuclear localization signal (NLS) from amino acid 118-145, and a thioredoxin-like fold from amino acid 212-319. Arrows indicate the mutations in rcbl-1/PBG and rcbl-10.

To further determine whether At2g31840 is the causal gene for the tall-and-albino phenotype of rcbl-1/PBG, I generated a transgenic Arabidopsis line expressing At2g31840 cDNA (pCHF1-UBQ10-RCBL-(PT)4P-3HA-6His) in the rcbl-1/PBG background. Ectopically expressing RCBL cDNA complements the tall-and-albino phenotype in the rcbl-1/PBG background (Figure 10A, B). This confirms that At2g31840 is the causal gene of rcbl-1/PBG. The genotyping primers were designed based on dCAP Finder 2.0 (Neff et al., 2002), and are listed in Table 1.
RCBL has been previously reported as an essential component required for chloroplast biogenesis and PEP-dependent gene expression, under the names SVR4-like and MRL7-like (Yua et al., 2014; Powikrowska et al., 2014a; Qiao et al., 2011). RCBL has been shown to be a chloroplast protein, but unlike RCB, RCBL is not localized to nucleoids (Yua et al., 2014; Powikrowska et al., 2014a; Qiao et al., 2011). Although RCBL is not localized in the nucleoids, nucleoid morphology is altered in the rcbl mutant. Therefore, it has been proposed that RCBL might be involved in nucleoid differentiation or organization (Powikrowska et al., 2014a; 2014b). Unlike RCB, RCBL is highly expressed in the leaf base of monocotyledons, suggesting that its expression is developmentally regulated and it might play a more important role in early chloroplast development (Powikrowska et al., 2014a).

2.2.3 RCBL is Required for Chloroplast Development

The albino phenotype suggests that RCBL is required for chloroplast biogenesis, which is largely dependent on the expression of chloroplast-encoded genes. It has been reported that PEP-dependent genes are downregulated in knock-down MRL7-L-RNAi Arabidopsis seedlings (Qiao et al., 2011), and photosynthetic mRNA and protein levels are also largely reduced in svr4-like (GABI_518H02, same as rcbl-10) (Powikrowska et al., 2014a). To examine whether rcbl is defective in expressing PEP-dependent genes, I tested the expression levels of psbA and psbB, which encode reaction center proteins of photosystem II; psaJ, which encodes subunit J of photosystem I; and rbcL, which encodes the large subunit of RuBisCO. As expected, all four genes are down-regulated in rcbl-1/PBG and rcbl-10 compared to their parental genotypes (PBG and Col-0 respectively) (Figure 11A). This indicates that RCBL is required for the expression of
PEP-dependent genes. For the housekeeping plastidial genes, which are mostly transcribed by NEP, I examined the expression of *rpoB* and *rpoC1*, both of which are subunits of PEP RNA polymerase, as well as *rps15* and *rpl20*, both of which are chloroplast ribosomal proteins. The results show that NEP-dependent genes are up-regulated in *rcbl-10* and *rcbl-1/PBG* compared to their parental genotypes (Figure 11B), indicating that RCBL is not required for the expression of NEP-dependent genes. Taken together these results show that RCBL is required for plastidial PEP-dependent transcription, which is essential for chloroplast biogenesis.

![Graph showing expression levels of various genes](image)

**Figure 11.** RCBL is required for PEP-dependent gene expression in chloroplasts.

(A) RT-qPCR analyses of selected PEP- and NEP-dependent genes in 4-d-old *PBG, rcbl-1/PBG, Col-0,* and *rcbl-10* seedlings grown in 10 µmol m⁻² s⁻¹ Rc light. Transcript levels were normalized to those of *PP2A*. Error bars represent the standard deviation of three replicates. The expression of each gene in *rcbl-1/PBG* or *rcbl-10* was compared with expression levels in *PBG* or *Col-0*, respectively, using Student’s t-test. **P < 0.0001.
2.2.4 RCBL Encodes a Predicted Dual-localized Protein with a C-Terminal Thioredoxin-Like Fold

To determine the function of the RCBL protein, I performed protein structure prediction with multiple online prediction applications. RCBL contains two predicted localization signals: a chloroplast transit peptide (TP) from amino acid 1-48 (ChloroP 1.1, [www.cbs.dtu.dk/services/ChloroP](http://www.cbs.dtu.dk/services/ChloroP)) (Emanuelsson et al., 1999), and a bipartite NLS from amino acid 118-145 predicted by NLS mapper ([nls-mapper.iab.keio.ac.jp](http://nls-mapper.iab.keio.ac.jp)) (Kosugi et al., 2009) (Figure 10C). The C-terminus of RCBL (amino acids 212-319) resembles a thioredoxin-like fold (trx-like) predicted by Phyre2 (Kelley and Sternberg, 2009), InterPro (Mitchell et al., 2015), ProSite (Sigrist et al., 2013), and the NCBI Conserved Domains Database (Marchler-Bauer et al., 2015). In addition, the middle of the RCBL protein (amino acids 80-202) contains a glutamic acid-rich region. It has been suggested that this negatively-charged region may play a role in chloroplast genome differentiation or organization (Powikrowska et al., 2014b).

2.2.5 Phylogenetic Analysis Indicates that RCBL and Its Paralogue, RCB, Diverged After the Emergence of Seed Plants

BLASTp analysis revealed RCBL’s paralogue, RCB, sharing 39% identity and 58% similarity in their amino acid sequences. The *rcb* mutant is also tall-and-albino, showing that it is not functionally redundant with *rcbl*. Qiao *et al.* (2011) investigated the phylogenetic relationship between *RCB/MRL7/SVR4/ECB1* and *RCBL/MRL7-like/SVR4-like*. However, their phylogenetic analysis fails to resolve the divergence of *RCB* and *RCBL*.

To further determine the detailed evolutionary relationship between *RCBL* and
RCB, I obtained nucleotide sequences of RCBL and RCB paralogues from seed plants, ferns, lycophytes, and bryophytes. Genomes of seed plants have been previously published (Goodstein et al., 2012; Sundell et al., 2015), while fern, lycophyte, and bryophyte sequences were obtained from transcriptome data generated by the One Thousand Plants Project (www.onekp.com) (Matasci et al., 2014). Transcriptome mining was conducted via the BlueDevil python pipeline (Li et al., 2014). I also used the NCBI BLAST sequence analysis tool to search for RCBL and RCB sequences from algae genomes including Klebsormidium flaccidum (Hori et al., 2014), Chlamydomonas reinhardtii (Merchant et al., 2007), and Micromonas pusilla CCMP1545 (Worden et al., 2009). However, no RCBL and RCB homologs were found in these algae genomes, suggesting that RCBL and RCB evolved after land plants emerged. This suggests that RCBL and RCB might be important for adapting to dry environments with high light intensity.

With this collection of RCBL and RCB paralogs, I utilized Randomized Axelerated Maximum Likelihood (RAxML) to construct phylogenetic trees with bootstrapping (Stamatakis, 2014). The resulting phylogeny reveals that there is only a single copy of an ancient, pre-duplicated RCBL/RCB in ferns, lycophytes, and bryophytes, while seed plants have distinct RCBL and RCB sequences (Figure 12). This result shows that RCBL and RCB diverged after the emergence of seed plants, suggesting that they might have played a role in the adaptation of seed plants to terrestrial life.
Figure 12. Phylogenetic analysis of RCB and RCBL.

RCBL and RCB diverged after the emergence of seed plants. Homologues of *RCBL* and *RCB* from 7 representative seed plants were obtained by BLAST analysis with sequences obtained from Phytozome (Goodstein et al., 2012), Congenie (congenie.org) (Sundell et al., 2015), and the Amborella Genome Database (www.amborella.org) (Amborella Genome Project, 2013).
2.2.6 Acquisition of Additional *rcbl* Alleles

After identifying the causal gene of the *rcbl-1/PBG* mutation, I requested another T-DNA insertion line disrupting At2g31840 from the *Arabidopsis* T-DNA insertion mutant collection in the *Arabidopsis* Biological Resource Center (www.arabidopsis.org). This *rcbl* allele, *rcbl-10*, has a T-DNA insertion after nucleotide 702, which is within the predicted trx-like fold (Figure 10).

![Diagram](A) Schematic illustration of the RCBL exon-intron structure and corresponding protein structure. The *rcbl-10* and *rbcl-1/PBG* mutations are labeled by a triangle and a line, respectively. Red arrows indicate primers for RT-qPCR analysis. (B) RT-qPCR analysis for the expression of RCBL in *rcbl-1/PBG* and *rcbl-10* compared to their parental genotypes, *PBG* and Col-0 respectively. (C) RT-PCR for full-length transcripts of RCBL in 4-d-old Rc grown *rcbl-10* and Col-0. The *PP2A* transcript served as control.

To determine if *rcbl-1/PBG* and *rcbl-10* are null alleles, I performed RT-qPCR to determine the *RCBL* transcript level in 4-d-old Rc-grown Col-0, PBG, or *rcbl* seedlings. *RCBL* transcript cannot be detected in *rcbl-1/PBG* or *rcbl-10* (Figure 13B). To further examine whether full-length *RCBL* transcripts can be detected in *rcbl-10*, I did RT-PCR
to amplify the *RCBL* full-length transcript in Col-0 and *rcbl-10*. As shown in Figure 13C, *RCBL* full-length transcripts were amplified in light-grown Col-0, but absent in *rcbl-10*. The similar phenotypes between *rcbl-10* and *rcbl-1/PBG* support the idea that At2g31840 is the gene corresponding to the *rcbl-1/PBG* mutation (Figure 14A and C).

To identify other missense *rcbl* alleles, we requested eight more *rcbl* alleles based on the *Arabidopsis* TILLING service (Till et al., 2003b; 2003a). Among these TILLING alleles, *rcbl-11* to *rcbl-13* possess missense mutations in the N-terminal portion of RCBL, and *rcbl-14* to *rcbl-16* have missense mutations within the C-terminal trx-like fold (Figure 10). *rcbl-17* and *rcbl-18* have intronic mutations close to the exon-intron junction, which might affect the splicing of RCBL mRNA. *rcbl-17* is mutated at the fourth nucleotide of intron 3, and *rcbl-18* is mutated at the end of intron 4. Both introns 3 and 4 are within the trx-like fold (Figure 13A). The characterization of these RCBL TILLING lines is described in 2.2.9.

### 2.2.7 Characterization of *rcbl* Null Alleles Indicates That RCBL is Required for phy Signaling.

It has been shown that *hmr* and *rcb* are defective in multiple aspects of phy signaling, including phy-mediated hypocotyl inhibition, light-mediated degradation of phyA, PIF1, and PIF3, as well as activation of a subset of PIF-induced genes (Chen et al., 2010; Qiu et al., 2015; Galvão et al., 2012) (Yoo et al., in prep). To dissect the function of RCBL in the phy signaling pathway, I examined various physiological and molecular phy responses in *rcbl* null mutants. First, to determine whether RCBL is required for phyB and phyA responses in different light intensities, I assayed fluence response curves in Rc- and FRc-grown *rcbl* null mutant lines. Second, to examine whether RCBL is also
required for signal transduction in different light conditions, I measured photo-inhibition responses in continuous blue (Bc) and continuous white (Wc) light. Third, to investigate whether RCBL is required for phy-mediated proteolytic events, I extracted protein from Re-grown seedlings and subjected them to SDS-PAGE followed by immunoblotting to detect phyA, PIF1, and PIF3 protein levels. Fourth, to determine if RCBL is also involved in the transcriptional activation of HMR-dependent PIF-induced genes, I did RT-qPCR analysis to quantify the relative transcript level of target genes in Re-grown rcbl-10 versus Col-0.

2.2.7.1 Fluence Response Curves of rcbl Null Mutants

The tall-and-albino phenotype of rcbl indicates that rcbl is defective in multiple phy-mediated responses. Here I examined two well-known modes of action of phyB and phyA in two rcbl null alleles to determine whether RCBL is required for the phy-mediated responses.

![Figure 14. RCBL is required for phyB- and phyA-mediated high irradiance responses.](image)
phyB is the major photoreceptor for high irradiance responses (HIR) in red light. I examined the HIR-R of phyB by measuring the hypocotyl growth of 4-d-old seedlings under a range of intensities of Rc. The results show that the hypocotyls of rcbl-10 and rcbl-1/PBG are taller than in their parental genotypes under a range of R light intensities (Figure 14A, B), indicating that RCBL is essential for phyB-mediated HIR-R.

For far-red light sensing, phyA is primarily responsible. The best-studied mode of phyA action is HIR in far-red light. Therefore, I tested whether phyA-mediated HIR-FR is defective in rcbl mutants with a fluence response curve experiment. Figure 14C and D show that both rcbl-10 and rcbl-1/PBG mutants have intermediate hypocotyl phenotypes compared to their parental genotypes and phyA-211, implying that RCBL is also required for the phyA HIR-FR response.

2.2.7.2 Responses of rcbl Mutants to Blue and White Light

For blue light sensing, plants utilize other photoreceptors including cry and phots to repress hypocotyl growth (Casal, 2000). To investigate whether RCBL is also involved in light signaling pathways mediated by other photoreceptors, I measured blue light- and white light-mediated photoinhibition of hypocotyl growth in rcbl mutants. My results show that rcbl mutants possess comparable hypocotyl length compared to their parental
genotypes in continuous blue light (Bc) and white light (Wc) (Figure 15). This indicates that RCBL is not required for cryptochrome- and phyA-mediated blue light responses. Moreover, the results indicate that RCBL specifically mediates phy responses, including phyB- and phyA-mediated photoinhibition of hypocotyl growth.

Figure 15. RCBL is not required for blue light sensing.

(A) Upper panel, representative images of 4-d-old 33 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) Wc-grown Col-0, \( rcb1-10 \), \( PBG \), and \( rcb1-1/PBG \) seedlings. Lower panel, box-and-whisker plots for hypocotyl length measurements. (B) Upper panel, representative images of 4-d-old 10 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) Bc-grown Col-0, \( rcb1-10 \), \( cry1-1 \), \( PBG \), and \( rcb1-1/PBG \). Lower panel, box-and-whisker plots for hypocotyl length measurements. Different letters denote significant difference among means (Tukey-Kramer HSD, \( P<0.0001 \)). Centerlines in each box indicate the medians; the top and bottom lines of the boxes indicate the 25th and 75th percentiles, and the whiskers extend to the maximum and minimum data points. More than 30 seedlings were measured for each genotype.

2.2.7.3 Light-Dependent Proteolysis of phyA and PIFs in \( rcb1 \) Mutants

To further dissect which early phy signaling events require RCBL, I first characterized the light-dependent degradation of phyA and PIFs (Al-Sady et al., 2006; Lorrain et al., 2009). Since phyA co-localizes with PBs before its degradation, and HMR and RCB are required for these processes (Seo et al., 2004; Chen et al., 2010) (Yoo et al., in prep), I examined whether phyA proteolysis is abolished in \( rcb1 \) mutants under both Rc
and FRc conditions. The immunoblots in Figure 16A show that in 4-d-old Rc-grown seedlings, phyA accumulates in both *rcbl-1/PBG* and *rcbl-10*. This demonstrates that RCBL is required for the light-dependent proteolysis of phyA.

The central mechanism for initiating photomorphogenesis is the degradation of PIF1 and PIF3, which inhibits hypocotyl elongation and promotes chloroplast differentiation (Park et al., 2004; Al-Sady et al., 2006). Similar to phyA, the degradation of PIFs also requires co-localization to PBs, and HMR and RCB are both involved in the light-dependent degradation of PIFs (Chen et al., 2010; Qiu et al., 2015) (Yoo et al., in prep). To examine whether RCBL is necessary for the degradation of PIF proteins, I examined the PIF1 and PIF3 protein abundance in Rc-grown 4-d-old *rcbl-1/PBG* and *rcbl-10*. PIF1 and PIF3 accumulate in both Rc-grown *rcbl-1/PBG* and *rcbl-10*, but not in their parental genotypes (Figure 16B). These results show that RCBL is required for light-dependent proteolysis of PIF1 and PIF3.

![Figure 16. RCBL is required for the degradation of phyA PIF1, and PIF3.](image)

(A) Western blot showing phyA protein level in 4-d-old D-grown Col-0, Rc-grown Col-0, *rcbl-10*, *PBG*, and *rcbl-1/PBG* seedlings. RPN6 was used as a loading control. (B) Western blot showing PIF1 and PIF3 protein level in 4-d-old Rc-grown Col-0, *rcbl-10*, *hmr-22*, *PBG*, and *rcbl-1/PBG*. RPN6 was used as a loading control.

### 2.2.7.4 Characterization of PIF Transcriptional Activity in *rcbl* Mutants

HMR is a transcriptional co-activator for a subset of growth-related PIF target genes (Qiu et al., 2015). Two classes of PIF target genes have been defined by Qiu *et al.*
Class A (PIF-induced direct target genes) and Class B (PIF-induced and HMR-induced genes). Class A consists of PIF-induced and HMR-repressed genes, including MPL1, MYB27, BGAL7, and SAUR36. Class B consists of PIF-induced and HMR-induced genes, representing PIL1, IAA29, ATHB2, and XTR7. HMR’s C-terminal TAD is necessary for the activation of this subset of genes as well as for the degradation of PIFs (Qiu et al., 2015). To examine whether RCBL is also involved in the activity of PIFs, I examined the expression of PIF target genes in rcbl-10. As shown in the left panel of Figure 17A, Class A PIF-induced direct target genes are up-regulated in Rc grown rcbl-10 compared to Col-0. However, the Class B genes are down-regulated in rcbl-10 compared to Col-0 (Figure 17B). These data indicate that similar to HMR, RCBL is required for both the degradation of PIF1 and PIF3 and the transcriptional activation of Class B genes, suggesting that RCBL may also be involved in the degradation-coupled transcriptional activation mechanism proposed by Qiu et al. (2015).

Figure 17. RCBL is required for expression of PIF- and HMR-induced genes.

(A) RT-qPCR analyses of selected PIF-induced HMR-repressed genes (Class A) and (B) PIF-induced HMR-induced genes (Class B) in 4-d-old Col-0 and rcbl-10 grown under 10 µmol m⁻² s⁻¹ Rc. Transcript
levels from the RT-qPCR experiments were calculated relative to those of PP2A. Error bars represent the standard deviation of three replicates. Red and blue arrows indicate an increase or decrease of gene expression in rcbl-10 compared to Col-0, respectively.

### 2.2.8 Pinpointing the Genetic Position of RCBL in phy Signaling Using Epistasis Analysis

In previous sections, I demonstrated that RCBL is required for phy signaling in Arabidopsis. To further confirm that RCBL genetically acts downstream of phys, I generated double mutants between rcbl-10 and phyB-9 or phyA-211 and examined the genetic epistatic relationship in these double mutants. Although rcbl-10 is taller than Col-0 in Rc, rcbl-10/phyB-9 is not taller than phyB-9 in Rc (Figure 18A, B). The rcbl-10/phyA-211 double mutant was not taller than phyA-211 in FRc (Figure 18 E, F). These results show that RCBL acts downstream of phyB and phyA.

YH is a constitutively active mutant allele of phyB which displays a constitutive photomorphogenic phenotype even when grown in the dark (Su and Lagarias, 2007; Hu et al., 2009). To acquire more evidence to show that RCBL acts downstream of phyB, I generated rcbl-1/YH double mutants and characterized their hypocotyl phenotype. The 4-d-old dark grown rcbl-1/YH seedlings in Figure 18C and D have a tall hypocotyl phenotype compared to YH, indicating that RCBL does act downstream of phyB. Taken together, the double mutant analyses show that phyB and phyA mutations are epistatic to rcbl, indicating that RCBL acts downstream of phyB and phyA.
Figure 18. **RCBL acts downstream of phyB and phyA.**

(A) Representative images of 4-d-old 10 µmol m\(^{-2}\) s\(^{-1}\) Rc-grown Col-0, *rcbl-10*, *phyB-9* and *rcbl-10/phyB-9* seedlings. (B) Box-and-whisker plots for hypocotyl length measurements of seedlings from the genotypes in (A). (C) Representative images of seedlings of 4-d-old dark-grown *YH*, *PBG*, and *rcbl-1/YH*. (D) Box-and-whisker plots for hypocotyl length measurements of seedlings from the genotypes in (E). Different letters denote significant difference among means (Tukey-Kramer HSD, P<0.0001) (E) Representative images of 4-d-old 10 µmol m\(^{-2}\) s\(^{-1}\) FRc-grown Col-0, *rcbl-10*, *phyA-211* and *rcbl-10/phyA-211* seedlings. (F) Box-and-whisker plots for hypocotyl length measurements of seedlings from the genotypes in (E). (B, D, and F) Centerlines in each box indicate the medians; the top and bottom lines of the boxes indicate the 25th and 75th percentiles, and the whiskers extend to the maximum and minimum data points. More than 30 seedlings were measured for each genotype.

Because **RCBL** and **RCB** are paralogous and their mutant lines share phenotypic similarity, I asked whether **RCBL** and **RCB** act in the same genetic pathway to regulate photoinhibition of hypocotyl growth. To examine the epistatic relationship between **RCBL** and **RCB**, I generated a *rcbl-10/rcb-10* double mutant line. Both *rcbl-10* and *rcb-10* possess elongated hypocotyls under Rc, but the double mutant shows no additive
effect (Figure 19A and B), indicating that RCBL and RCB are in the same genetic pathway to regulate phy signaling.

![Figure 19. RCBL and RCB act in the same genetic pathway to regulate hypocotyl elongation.](image)

(A) Representative images of 4-d-old 10 µmol m^-2 s^-1 Rc-grown Col-0, rcbl-10, rcb-10 and rcbl-10/rcb-10 seedlings. (B) Box-and-whisker plots for hypocotyl length measurements of seedlings from the genotypes in (A). Different letters denote significant difference among means (Tukey-Kramer HSD, P<0.0001).

To determine whether the rcbl defect of hypocotyl photoinhibition is dependent on PIFs, I generated rcbl-10/pifq quintuple mutants by crossing rcbl-10 with a pif1, pif3, pif4, pif5 (pifq) quadruple mutant (Leivar et al., 2009). Characterization of rcbl-10/pifq mutants reveals that the elongated hypocotyl phenotype of rcbl-10 under Rc is largely rescued, implying that this phenotype is caused by the accumulation of PIFs (Figure 20A). The slightly taller phenotype of rcbl-10/pifq compared to pifq indicates that RCBL may be involved in the regulation of other PIFs which are not included in the pifq mutant. The phenotype of dark-grown rcbl-10/pifq follows the photomorphogenic short hypocotyl phenotype in pifq, implying that pifq is epistatic to rcbl-10 in hypocotyl elongation. Taking these data together with the data in Figure 18, I conclude that RCBL acts genetically between phyS and PIFs.
Figure 20. The elongated hypocotyl phenotype of *rcbl-10* is dependent on PIFs.

(A) Left, representative images of 4-d-old 10 µmol m⁻² s⁻¹ Re-grown Col-0, *rcbl-10*, *pifq* and *rcbl-10/pifq* seedlings. Right, box-and-whisker plots for hypocotyl length measurements of each genotype. (B) Left, representative images of 4-d-old dark-grown Col-0, *rcbl-10*, *pifq* and *rcbl-10/pifq* seedlings. Right, box-and-whisker plots for hypocotyl length measurements of each genotype. Centerlines in each box indicate the medians, the top and bottom lines of the boxes indicate the 25th and 75th percentiles, and the whiskers extend to the maximum and minimum data points. Different letters denote significant difference among means (Tukey-Kramer HSD, P<0.0001). More than 30 seedlings were measured for each genotype.

Surprisingly, unlike the hypocotyl phenotype, the albino phenotype of *rcbl-10* is not rescued in *rcbl-10/pifq* (Figure 20). I further characterized the expression of plastid-encoded genes in *rcbl-10/pifq*. In Figure 21, *rcbl-10/pifq* is impaired in expressing plastid-encoded genes transcribed by PEP but has increased expression of genes transcribed by NEP, which is a similar phenotype to the *rpoB* mutant which lacks PEP. These results indicate that RCBL has another function in regulating PEP-dependent gene expression, which is not dependent on PIFs. In sum, RCBL mediates photomorphogenesis by two distinct functions: (1) PIF-dependent hypocotyl photoinhibition, and (2) PIF-independent chloroplast development.
Figure 21. In chloroplasts, RCBL possesses a distinct role which is independent of PIFs.

(A) RT-qPCR analyses of selected PEP-dependent genes in 4-d-old Col-0, pifq, rcbI-10, rcbI-10/pifq seedlings grown under 10 µmol m⁻² s⁻¹ Rc. (B) RT-qPCR analyses of selected NEP-dependent genes in 4-d-old Col-0, pifq, rcbI-10, and rcbI-10/pifq seedlings grown under 10 µmol m⁻² s⁻¹ Rc. Transcript levels from RT-qPCR experiments were calculated relative to those of PP2A. Error bars represent the standard deviation of three replicates.

2.2.9 Characterization of rcbI Missense Mutations from TILLING Project

Other rcbI alleles requested from the Arabidopsis TILLING service (Targeting Induced Local Lesions in Genomes, (Till et al., 2003b; 2003a)) are listed in 2.2.6 and Figure 10. Genotyping primers for these lines were designed based on dCAPS (Neff et al., 1998; 2002), and are listed in Table 1. To eliminate extra mutations, most of the rcbI TILLING lines have been backcrossed to Col-0 three times. Preliminary characterization of TILLING lines under Rc showed that rcbI-15 is hypersensitive to Rc. Therefore, I performed a more detailed characterization of rcbI-15.
2.2.9.1 rcbl-15 is Hypersensitive to Red Light

To characterize whether rcbl-15 is hypersensitive to both Rc and FRc, I performed a fluence response curve experiment as described in 2.2.7.1. In Figure 22A and B, the hypocotyl length of rcbl-15 is shorter than the wild-type from 0.15 to 4.7 µmol m$^{-2}$s$^{-1}$, indicating that rcbl-15 is hypersensitive in phyB-mediated Rc HIR. In Figure 22C and D, the hypocotyl length of rcbl-15 mutants is only shorter than wild type at 0.18 µmol m$^{-2}$ s$^{-1}$, indicating rcbl-15 plays a less prominent role in phyA-mediated FRc HIR.

![Figure 22. rcbl-15 is hypersensitive to Rc but not FRc.](image)

(A) Representative images of 4-d-old Col-0, rcbl-10, and rcbl-15 seedlings grown under 10 µmol m$^{-2}$ s$^{-1}$ Rc. (B) Fluence response curves for Rc. Relative hypocotyl length of 4-d-old phyB-9 (solid gray line), rcbl-15 (solid red line), Col-0 (solid black line) seedlings grown in various intensities of Rc light or in the dark. (C) Representative images of 4-d-old Col-0, rcbl-10, and rcbl-15 seedlings grown under 10 µmol m$^{-2}$ s$^{-1}$ FRc. (D) Fluence response curves for FRc. Relative hypocotyl length of 4-d-old phyA-211 (solid gray line), rcbl-15 (solid red line), Col-0 (solid black line) seedlings grown in various intensities of FRc light or in the dark.

2.2.9.2 PIF-Induced Genes Are Down-Regulated in rcbl-15

The results of section 2.2.7.4 demonstrate that RCBL is only required for the expression of Class B genes but not for Class A genes. To determine how Class A and Class B genes are regulated in rcbl-15, I harvested 4-d-old Rc grown rcbl-15 and Col-0
seedlings, and extracted total RNA for performing RT-qPCR. In Figure 23, the upper panel shows that both Class A and Class B genes are down-regulated in rcbl-15 compared to Col-0, implying that the abundance of PIF proteins might be decreased in rcbl-15. Since removal of PIFs decreases hypocotyl elongation, this result may explain the shorter hypocotyl phenotype of rcbl-15 in Rc. However, without knowing the actual protein abundance of PIFs in rcbl-15, it is hard to determine whether the activation of Class B genes is defective in rcbl-15.

2.2.9.3 Both PEP- and NEP-Dependent Genes Are Upregulated in rcbl-15

One interesting phenotype of rcbl-15 mutants is that their cotyledons appear to be greener than in wild-type. Moreover, fluence response experiments showed that rcbl-15 is hypersensitive to Rc. Hence, chloroplast genes in rcbl-15 might also be more responsive to red light than in wild-type. To examine whether the plastid-encoded genes are up-regulated in rcbl-15, I examined the expression of PEP- and NEP-dependent genes as described in 2.2.3 in 4-d-old Rc grown rcbl-15 seedlings. Interestingly, the expression levels of both PEP- and NEP-dependent genes are up-regulated in rcbl-15 (Figure 23C, D). This result indicates that plastidial transcriptional and translational components, as well as the photosynthesis apparatus, are highly expressed in rcbl-15. This might be the cause of the greener phenotype of rcbl-15.

Taken together these results show that rcbl-15 is a gain-of-function mutant for both phy signaling and chloroplast biogenesis. More characterization is required to determine the detailed mechanism of how rcbl-15 regulates genes in both compartments.
Figure 23. *rcbl*-15 is defective in class B gene expression but promotes NEP-dependent gene expression in chloroplasts.

RT-qPCR analyses of selected (A) Class A PIF-induced HMR-repressed genes, (B) Class B PIF-induced HMR-induced genes, (C) PEP-dependent genes, and (D) NEP-dependent genes in 4-d-old Col-0 and *rcbl*-10 seedlings grown under 10 μmol m\(^{-2}\) s\(^{-1}\) Rc. Transcript levels from the RT-qPCR experiments were calculated relative to those of *PP2A*. Error bars represent the standard deviation of three replicates.

2.3 Discussion

Previous studies of HMR have demonstrated that HMR plays distinct roles in the nucleus and chloroplasts. In the nucleus, HMR is a transcriptional co-activator with PIFs
to express growth-promoting genes, which might happen at the PBs, to quantitatively control growth in the light (Chen et al., 2010; Qiu et al., 2015; Galvão et al., 2012). In chloroplasts, HMR is a core subunit of the PEP complex to transcribe photosynthetic-related genes (Qiu et al., 2015; Pfalz et al., 2015; Pfalz, 2006). Because of this dual functionality, hmr mutants possess both tall-hypocotyl and albino-cotyledon phenotypes. The unique phenotype of hmr opens up a possibility for a forward genetic screen to identify more components involved in the coordination of nuclear and plastidial gene expression in photomorphogenesis. Here, I have described the identification and characterization of a hmr-like mutant, rcbl, in phy signaling and chloroplast development. My results have shown that RCBL is also required for multiple events in early phy signaling, including PB formation, phyA and PIF proteolysis, as well as the transcriptional activity of PIFs. In addition, genetic analyses demonstrate that RCBL acts downstream of phy but upstream of PIFs. In chloroplasts, RCBL is necessary for PEP to express photosynthetic genes. Collectively, this characterization shows that our tall-and-albino screen has successfully identified new phy signaling components required for chloroplast biogenesis.

It is quite intriguing that we isolated two paralogous mutants, rcbl and rcb, from the hmr-like screen (Yoo et al., in prep). Although the phylogenetic tree of RCB/MRL7 and RCBL/MRL7-like has been reported in Qiao et al. (2011), my phylogenetic analysis is more in-depth with more homologues from bryophytes, ferns and gymnosperms, providing more detailed information about the evolutionary history of RCBL and RCB in basal plants. My results show that RCBL and RCB diverged after the emergence of seed plants (Figure 12). The phylogenetic relationship between RCBL homologues generally
follows the currently agreed upon green lineage phylogeny, supporting the credibility of my phylogenetic analysis.

Like RCBL, RCB is also required for early phy signaling events and chloroplast biogenesis. This implies that RCBL and RCB are not functionally redundant, because each single mutant shows the same tall-and-albino phenotype. The epistatic analysis of *rcbl* and *rcb* shows no additive effects, indicating that they act in the same genetic pathway to regulate phy-mediated responses. This observation suggests that although RCBL and RCB have diverged evolutionarily, they may act together in a complex involved in phy signaling.

In contrast, RCBL and RCB are likely to play different roles in the chloroplasts. Unlike HMR, neither protein has been found in biochemically purified PEP complexes (Pfalz, 2006; Steiner et al., 2011)). However, previous reports (Yua et al., 2014; Qiao et al., 2011; Powikrowska et al., 2014a; Yu et al., 2011) and our own results demonstrate that both *rcbl* and *rcb* null mutants possess a similar phenotype as PEP-depleted plants, which are defective in expressing PEP-dependent genes, but have elevated expression of NEP-dependent genes (Allison et al., 1996). This indicates that both RCBL and RCB are required for the proper function of the PEP complex. Surprisingly, knocking out four PIFs in *rcbl* and *rcb* reveals dramatically different results: *rcbl/pifq* retains the *rcbl* albino phenotype (Figure 21), while *rcb/pifq* has green cotyledons in Rc (Yoo et al. in prep). This indicates that the removal of PIFs can bypass the RCB function in chloroplasts, but RCBL plays a PIF-independent role in regulating chloroplast biogenesis. PIF1, PIF3, and PIF5 have been shown to repress nuclear-encoded photosynthetic genes (Huq et al., 2004; Stephenson et al., 2009). Our results suggest that an unknown anterograde signal from
PIFs might inhibit PEP activity. The de-repression of PEP by removal of PIFs overcomes the chloroplast defect in *rcb* but not in *rcbl*. However, we still cannot rule out the possibility that the bypass phenotype of *rcb/pifq* could be due to an increase in *RCBL* expression. In addition, the detailed molecular mechanism of how RCBL and RCB affect the function of PEP still need more investigation. For instance, it remains to be determined whether they are required for PEP assembly, or for the promoter recognition ability of the PEP complex.

In 2.2.9, preliminary characterization of TILLING missense alleles revealed a potential gain-of-function allele, *rcbl-15*. Fluence response curves, PIF target gene expression, and plastidial gene expression collectively show that *rcbl-15* is hypersensitive to light and has higher NEP and PEP complex activity. The mutation in *rcbl-15* is L217F, which converts a small amino acid to a bulky amino acid in the trx-like fold. Since the phenotype of *rcbl-15* is subtle, L217F probably does not significantly change the overall structure of RCBL. More biochemical analyses are required to uncover the detailed molecular mechanisms of *rcbl-15* in phy signaling and chloroplast development.

2.4 Materials and Methods

2.4.1 Screening

To perform the *hmr*-like screen, PBG seeds (PHYB-GFP in Landsberg *erecta* (Ler) background) were mutagenized with ENU and viable plants (M1) were selected. The seeds from the M1 plants (M2) were individually collected. M2 lines were screened under continuous R or FR light (8 mmol m⁻² s⁻¹), and lines with both elongated hypocotyl and albino phenotypes were preserved for further characterization. To date, more than 6 additional loci have been found by phenotyping.
2.4.2 Plant Materials and Growth Conditions

The PHYB-GFP (PBG) (Ler) line has been previously described (Yamaguchi et al., 1999). The rcbl-1/PBG mutant line was isolated from a tall-and-albino screen and backcrossed to PBG three times. The rcbl-10 (Col-0) mutant line is originally from GABI-Kat T-DNA insertion line GK-518H02 (Rosso et al., 2003; Li et al., 2003). The accession number of rcbl-10 is CS449718 (Arabidopsis Biological Resource Center, ABRC) and N449718 (Nottingham Arabidopsis Stock Centre, NASC). Genotyping primers for rcbl-1 and rcbl-10 are listed in Table 1. rcbl-11 to rcbl-18 are from the Arabidopsis TILLING project, and were backcrossed to Col-0 at least 3 times before characterization. Table 1 lists the stock center accession numbers of TILLING lines and the genotyping CAPS (Cleaved Amplified Polymorphic Sequences) or dCAPs markers (derived Cleaved Amplified Polymorphic Sequences) (Konieczny and Ausubel, 1993). Col-0 and phyB-9 (Col-0) were used as controls for physiological studies. The pifq mutant was previously characterized by Peter Quail’s laboratory (Leivar et al., 2008b).

Seeds were surface-sterilized and plated on half-strength Murashige and Skoog (MS) growth medium without sucrose as described previously (Chen et al., 2010). After sowing on plates, seeds were stratified in the dark at 4°C for 5 days. Seedlings were grown at 21°C in a LED chamber (Percival Scientific, Perry, IA) under the indicated light conditions. For the “artificial-dark” condition, seeds were stratified in the dark at 4°C for 5 days, and then seeds were treated with 3 h of monochromatic FR light (10 µmol m⁻² s⁻¹) to convert residual active phyB from the Pfr to the Pr form before the dark treatment to induce phyA-dependent germination; the Quail laboratory has demonstrated that the effect of this “artificial-dark” is similar to that of “true-dark” (Oh et al., 2007; Leivar et
al., 2008b). Fluence rates of light were measured with an Apogee PS200 spectroradiometer (Apogee Instruments Inc. Logan, UT).

2.4.3 Measurements of Hypocotyl Length

For the measurement of hypocotyl length, seedlings were scanned using an Epson Perfection V700 photo scanner, and the length of hypocotyls was measured using NIH ImageJ software (imagej.nih.gov) Data were collected from at least 30 seedlings per genotype per treatment.

2.4.4 Confocal Live-Cell Imaging and Quantification of Photobody Morphology

For quantification of PB morphology, seedlings were mounted on Superfrost slides (VWR, Radnor, PA; Cat. #48311-600) by using ddH₂O and 22 x 40 mm coverslips (no. 1.5, VWR, Radnor, PA; Cat. #48393-172). The nuclei of epidermal cells from the upper one-third of the hypocotyl were imaged via a Zeiss LSM 510 inverted confocal microscope (Carl Zeiss, Jena, Germany). GFP was detected by using a 100x Plan-Apochromat oil immersion objective, 488 nm excitation from an argon laser, and the manufacturer’s default “Green only” detection setting (505-550 nm bandpass detector). Images were collected using LSM 510 software version 4.2. Images were processed using Adobe Photoshop CC software (Adobe Systems, Inc., San Jose, CA).

To determine the size and number of PBs, the volume and the diameter of PBs were calculated with Huygens Essential. The detected objects were automatically defined by seed-threshold level, and manually corrected if the detection could not represent the image. The volume and diameter of the PBs were calculated with the Huygens object
analyzer. Microsoft Excel was used to sort the PB information for each nucleus and to generate box-and-whisker plots.

### 2.4.5 Positional Cloning via SHOREmap

rcbl-1/PBG (Ler) was crossed to Col-0 to generate an F2 mapping population. Genomic DNA was extracted from pooled green F2 and albino F2 plants. An Illumina Paired-end library with 300 bp insert size was constructed based on the manufacturer’s instructions. 80 bp paired-end reads were generated on an Illumina Genome Analyzer II. Polymorphisms (SNPs, indels, or large deletions) were identified by SHOREmap (Schneeberger et al., 2009; Schneeberger and Weigel, 2011). The corresponding SNP in rcbl-1/PBG co-segregated with albinism. Within the identified region, the polymorphism was confirmed by PCR and sequencing.

### 2.4.6 Protein Extraction and Western Blot Analysis

Protein was extracted as described in Shen et al. (2008) with some changes. The extraction buffer consisted of 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA (pH 8.0), 5% SDS, 20% glycerol, 20 mM DTT, 40 mM β-mercaptoethanol, 2 mM PMSF, 1 × protease inhibitor cocktail (Roche), 80 μM MG132 (Sigma), 80 μM MG115 (Sigma), 1% phosphatase inhibitor cocktail 3 (Sigma), and 10 mM N-ethylmaleimide. Seedlings were ground directly in extraction buffer in a 1:2 (mg/μL) ratio, boiled for 10 min and then centrifuged at 15,000 x g for 10 min at room temperature. The supernatant was then collected for further analysis.

Protein samples were separated on an SDS-PAGE mini-gel, transferred onto a nitrocellulose membrane, probed with the indicated primary antibodies, and then incubated with secondary goat anti-rabbit goat anti-moantibodies conjugated with
horseradish peroxidase (Bio-Rad). Signals were detected with a chemiluminescence reaction using a SuperSignal® kit (Pierce). Polyclonal anti-HMR antibodies (Chen et al., 2010) were used at a 1:500 dilution. Polyclonal anti-RPN6 antibodies (Enzo Life Sciences) were used at a 1:1000 dilution. Polyclonal anti-PIF1 and anti-PIF3 antibodies (Chen et al., 2010) were used at a 1:500 dilution. Monoclonal anti-phyA antibodies were used at 1:1000 dilution. Polyclonal anti-PIF1 and anti-PIF3 antibodies were used at 1:500 dilutions.
Chapter 3. Determining the Subcellular Localization of RCBL

3.1 Introduction

RCBL has been reported to encode a chloroplast protein, localized to the stroma, as detected by sub-chloroplast fractionation experiments (Qiao et al., 2011; Powikrowska et al., 2014a). But unlike HMR and RCB, RCBL does not localize to plastidial nucleoids. Though RCBL is not localized in the nucleoids, nucleoid morphology is altered in the rcbl mutant. Thus, it has been proposed that RCBL might be involved in nucleoid differentiation or organization (Powikrowska et al., 2014a; 2014b). The albino phenotype in rcbl, displaying low abundance of photosynthetic pigments and chloroplast proteins, as well as abnormal chloroplast ultrastructure, indicate that RCBL is required for the biogenesis of chloroplasts (Qiao et al., 2011; Powikrowska et al., 2014a; Yua et al., 2014). However, these results cannot explain the phy-mediated phenotypes of rcbl mutants shown in Chapter 2. This raised a question: is it possible that RCBL has a dual subcellular localization pattern, and localizes to the nucleus?

HMR has been shown to be dual-localized to both the nucleus and chloroplasts and it carries out distinct functions in both cellular compartments to initiate photomorphogenesis. Because of the similar phenotype between rcbl and hmr mutants, RCBL might also be a dual-localized protein. In addition, NLS Mapper predicts a bipartite NLS (amino acids 118-145) in RCBL. To answer whether RCBL is dual-localized to the nucleus and chloroplasts, I examined the subcellular localization of RCBL by fluorescent-protein tagging and biochemical fractionation. To detect native RCBL protein, I designed an antigen for generating polyclonal RCBL antibodies, which was purified by antigen-conjugated CNBr beads. Unfortunately, the purified RCBL
antibody failed to detect the endogenous RCBL protein in plant extracts. Therefore, to characterize the subcellular localization of RCBL, I turned to fluorescently-tagged RCBL expressed in tobacco and *Arabidopsis*. To ensure the fluorescently-tagged RCBL functions normally, I characterized the complementation phenotype of these transgenic lines. Next, I performed biochemical fractionation to isolate chloroplast and nuclei protein fractions from 2-day-old *Arabidopsis* seedlings expressing HA-tagged RCBL. The protein samples were subjected to SDS-PAGE and detected using HA antibodies with *in vitro* translated full-length or truncated RCBL protein as control.

### 3.2 Results

#### 3.2.1 Generation of Polyclonal RCBL Antibodies

With an antibody against RCBL, we can perform various assays to characterize endogenous RCBL. For instance, we can use immunoblotting to study whether RCBL abundance is regulated in different conditions or genetic backgrounds, immunocytochemistry to visualize the subcellular localization and even the partitioning pattern of RCBL *in situ*, and co-immunoprecipitation to see whether RCBL interacts with other proteins.

To generate RCBL antibodies, I designed a construct for expressing recombinant RCBL antigen with a 6 His-tag for affinity purification. GST-RCBL-antigen (aa 95-232) (Figure 24A) was induced and purified with GST beads and then cleaved by thrombin for GST tag removal. The cleaved RCBL-antigen was subjected to SDS-PAGE for gel cutting and extraction. We sent the purified RCBL-antigen to a commercial company for generating antiserum. To purify the RCBL antibody from the antiserum, GST-fusion RCBL-antigen (Figure 24B) was purified and permanently conjugated with CNBr-
activated Sepharose 4B beads. The antiserum was further purified by affinity chromatography. The efficacy of this purified RCBL antibody was examined by probing Western blots with different concentrations of purified RCBL antigen as well as the plant extract. Unfortunately, the purification of RCBL antibody did not yield a usable antibody to detect endogenous RCBL protein.

Figure 24. Purification of recombinant RCBL antigen and examination of antibody specificity.

(A) Schematic illustration of recombinant proteins used for RCBL antigen expression. (B) Coomassie Blue stained SDS-PAGE gel for examining induction and purification of GST-RCBL-antigen. The GST-RCBL-antigen is labeled by a red arrow. “B,” before induction; “I,” after induction; “L,” lysate; “P,” post-binding; “E1” to “E4,” elution tubes 1-4. (C) Immunoblot showing the sensitivity of antigen detection. His-tagged RCBL antigen was subjected to SDS-PAGE and detected by purified RCBL antibody.

3.2.2 Generation and Characterization of Transgenic Arabidopsis Expressing Tagged RCBL

Because of the failure of RCBL antibody preparation, I used epitope-tagged RCBL protein as an alternative to study the subcellular localization of RCBL. I generated RCBL overexpression lines overexpressing *RCBL-HA-HIS* and *RCBL-HA-YFP* in *rcbl-10*. The plasmid constructs were prepared as described in 3.4.3. Both tagged forms of RCBL
rescued the elongated hypocotyl and greening phenotypes of *rcbl-10* under Rc (Figure 25A, B, Figure 26A, B). Additionally, RT-qPCR analyses of Class A, B, PEP- and NEP-dependent genes in these transgenic lines showed that both HA-His and HA-YFP tagged RCBL can rescue the gene expression defects of *rcbl-10* (Figure 25C, Figure 26C). Both RCBL-HA-His and RCBL-HA-YFP constructs can rescue the phenotype of *rcbl-10*, indicating these two recombinant RCBL proteins are functional *in planta*. Therefore, these two lines were used for further subcellular localization and biochemical studies.
Figure 25. RCBL-HA-His complements *rcbl-10*.

(A) Representative images of 4-d-old Rc-grown Col-0, *rcbl-10*, *rcbl-10/RCBL-HA-His* #2, *rcbl-10/RCBL-HA-His* #12, and *phyB-9* seedlings. (B) Box-and-whisker plots for hypocotyl length measurements of lines shown in (A). Different letters denote significant difference among means (Tukey-Kramer HSD, P<0.0001). (C) RT-qPCR analyses of selected PIF-induced HMR-repressed genes (Class A), PIF-induced HMR-induced genes (Class B), NEP-dependent genes, and PEP dependent genes in Col-0, *rcbl-10*, *rcbl-10/RCBL-HA-His* #2, and *rcbl-10/RCBL-HA-His* #12 grown under 10 µmol m$^{-2}$ s$^{-1}$ Rc. Transcript levels from the RT-qPCR experiments were calculated relative to those of *PP2A*. Error bars represent the standard deviation of three replicates.
Figure 26. RCBL-HA-YFP complements rcbl-10.

(A) Representative images of 4-d-old Rc-grown Col-0, rcbl-10, and rcbl-10/RCBL-HA-YFP seedlings. (B) Box-and-whisker plots for hypocotyl length measurements of lines shown in (A). Different letters denote significant difference among means (Tukey-Kramer HSD, P<0.0001). (C) RT-qPCR analyses of selected PIF-induced HMR-repressed genes (Class A), PIF-induced HMR-induced genes (Class B), NEP-dependent genes, and PEP dependent genes in Col-0, rcbl-10, and rcbl-10/RCBL-HA-YFP grown under 10 µmol m⁻² s⁻¹ Rc. Transcript levels from the RT-qPCR experiments were calculated relative to those of PP2A. Error bars represent the standard deviation of three replicates.

3.2.3 Fluorescently-Tagged RCBL is Dual-Localized to Nucleus and Chloroplasts

To determine the subcellular localization of RCBL, I generated a fusion construct expressing RCBL with C-terminal CFP, RCBL-(PT)4P-CFP-Flag, abbreviated as RCBL-
The fusion protein was transiently expressed under the constitutive CaMV 35S promoter in tobacco leaves. In Figure 27A, confocal microscopy imaging shows that the RCBL-CFP protein is localized to both the nucleus and chloroplasts. To further determine the subcellular localization of RCBL in Arabidopsis and to assess the functionality of the fusion protein, I characterized the RCBL-HA-YFP Arabidopsis transgenic line described in the previous section. Confocal imaging shows that RCBL is localized to both compartments (Figure 27B).

**Figure 27. Fluorescently-tagged RCBL is dual-localized to the nucleus and chloroplasts in tobacco and Arabidopsis.**

(A) Confocal images showing subcellular localization of RCBL in tobacco epidermal leaf cells transiently expressing RCBL-CFP-Flag. RCBL-CFP-Flag (green) is present in both the nucleus and chloroplasts. Nuclei (marked by white arrows) and plastid chromosomes (marked by yellow arrows) were labeled with DAPI (blue). Chloroplasts were shown by chlorophyll autofluorescence (red). Scale bars represent 10 µm. (B) Confocal images showing subcellular localization of RCBL-HA-YFP (green) in seedlings grown under 4-day-old Rc. RCBL-HA-YFP can be seen in both nuclei and plastids. Chloroplasts were shown by chlorophyll autofluorescence (red, marked by yellow arrows). DIC shows nucleus (marked by white arrows) and plastids. Scale bars represent 10 µm.

**3.2.4 Determination of the Size of Mature RCBL Using Biochemical Fractionation**

To examine the nuclear localization of RCBL biochemically, I fractionated the
nuclei and chloroplasts from 2-d-old Rc grown RCBL-HA-YFP or RCBL-HA-His seedlings, and detected RCBL-HA-His in both fractions by Western blotting with an anti-HA antibody. As shown in Figure 28A and B, RCBL-HA-YFP and RCBL-HA-His can be detected in both fractions.

The majority of nuclear-encoded chloroplastic proteins have an N-terminal transit peptide allowing them to be imported by translocons. Once the preprotein is imported into chloroplasts, the transit peptide is cleaved. In contrast, nuclear import is carried out by binding of importin and does not require proteolytic cleavage. For the transportation of dual-localized proteins, there are two possible scenarios. If the dual-localized protein utilizes their signal peptides separately for targeting to the nucleus and chloroplasts, I should detect both an uncleaved nuclear form of the protein and a cleaved plastidial form. Another possibility is a sequential transport mechanism, in which the dual-localized protein needs to be transported into one compartment before the other one. HMR and WHIRLY1 (WHY1) have shown to have only one cleaved mature form in the nucleus and chloroplasts (Nevarez et al., 2017; Isemer et al., 2012; Krause and Krupinska, 2009). Transplastomic WHY1 can be detected in the nuclear fraction, which supports this retrograde protein translocation mechanism. Taking into account the observation that HMR, RCB and RCBL have similar mutant phenotypes and are involved in the same genetic pathways, RCBL is likely to use a similar localization mechanism as HMR and RCB. I hypothesize that RCBL uses this retrograde protein translocation mechanism, so I would observe the same mature form in both nuclear and chloroplastic fractions.

To examine this hypothesis, I generated constructs to express full-length RCBL-HA-His, and a form lacking the transit peptide (based on ChloroP prediction),
RCBLΔ48-HA-His, in an in vitro transcription-and-translation coupled system (TnT). These in vitro produced proteins were used as size controls to compare with the RCBL-HA-His protein from transgenic lines. Surprisingly, both nuclear and chloroplast pools of RCBL-HA-His migrate faster than RCBL-HA-His and slightly faster than RCBLΔ48-HA-His in SDS-PAGE (Figure 28B), indicating that the mature form of RCBL protein is cleaved. Intriguingly, comparing the mobility of the nuclear pool with the plastidial-pool of RCBL, both migrate at the same speed, indicating they are about same size. This result supports my hypothesis, suggesting that RCBL may need to be transported into chloroplasts and then into the nucleus through an unknown mechanism.

![Image](image.png)

Figure 28. RCBL-HA-YFP and RCBL-HA-His can be detected in both plastidial and nuclear fractions.

(A) Immunoblot of RCBL-HA-YFP from nuclear and chloroplast fractions. Protein extracts of the whole plant (T), nuclear (N) and chloroplasts (C) fractions from 2-d-old transgenic Arabidopsis expressing RCBL-HA-YFP were separated by SDS-PAGE. RCBL-HA-YFP protein was detected with anti-HA antibodies. (B) RCBL-HA-His protein is enriched in both nuclear and chloroplast protein fractions. Protein extracts from whole plants (T), and both nuclear (N) and chloroplast (C) fractions from 2-d-old transgenic Arabidopsis expressing RCBL-HA-His were separated by SDS-PAGE. RCBL-HA-His was detected with anti-HA antibodies. TnT-produced RCBL-HA-His and RCBLΔ48-HA-His were used as size references to compare with RCBL-HA-His protein in different fractions. Ferredoxin:Sulfite reductase (SiR) and Histone H3 were used as controls for the total and nuclear fractions, respectively.

3.3 Discussion

Multiple studies have shown that RCBL is localized to the chloroplasts (Qiao et al., 2011; Powikrowska et al., 2014a), and biochemical fractionation experiments
demonstrate the subplastidial localization of RCBL is in the stroma (Powikrowska et al., 2014a). However, in Chapter 2, I showed that RCBL is required for phy signaling and is involved in multiple phy signaling events, which presumably happen inside of the nucleus. In addition, HMR and RCB have been demonstrated to be dual-targeted proteins in the nucleus and chloroplasts (Chen et al., 2010; Nevarez et al., 2017)(Yoo et al. in prep). In this chapter, I have demonstrated that RCBL is a chloroplastic and nuclear dual-targeted protein by characterizing fluorescently-tagged RCBL in planta, and with biochemical fractionation experiments in Arabidopsis.

While RCBL and RCB are both dual-localized to chloroplasts and the nucleus, their localization pattern differs at the suborganellar level. In the nucleus, fluorescently-tagged RCB shows a speckle pattern in tobacco (Yoo et al., in prep), whereas recombinant RCBL protein shows a more even distribution. Immunostaining of endogenous RCB in PBG seedlings shows the co-localization of RCB speckles with PBs (Yoo et al., in prep). However, it still needs to be determined whether RCBL/MRL7-like/SVR4-Like also co-localizes with PBs. In chloroplasts, RCB/MRL7/SVR4 is in the thylakoid fraction (Powikrowska et al., 2014a), with a punctate pattern as shown by confocal microscopy (Qiao et al., 2011; Yua et al., 2014). Our results also support these observations (Figure 27, Yoo et al., in prep). Posikrowska et al. (2014a) has shown that the morphology of nucleoids in the two mutants were altered, but the altered patterns in the two mutants look different from each other. Additionally, our results imply that RCBL and RCB play distinct roles in chloroplasts. Taken together, the difference in chloroplast localization patterns may explain the functional divergence of RCBL and RCB in chloroplasts.
The partitioning mechanism of HMR has been thoroughly characterized by our colleague Dr. Nevarez (Nevarez et al., 2017). He defined the exact transit peptide of HMR, and found that the NLS of HMR is not functional. However, the transit peptide and the NLS of RCBL have not yet been thoroughly characterized. To determine the correct size of the transit peptide, proteomic analyses using mass spectrometry are required. Examination of the NLS can be done by using site-directed mutagenesis to replace the positively charged amino acids in the predicted NLS with alanine and then determine if it still localizes to the nucleus.

When characterizing the mature form of HMR, surprisingly, the nuclear pool of HMR shows the same size as the cleaved, chloroplast pool of HMR in SDS-PAGE (Nevarez et al., 2017). Another example for this group of bizarre dual-targeted proteins is WHY1. It has been shown that transplastomically-expressed WHY1 can still be detected in the nucleus (Chen et al., 2010; Isemer et al., 2012; Krause et al., 2005; Krause and Krupinska, 2009). This implies the existence of an uncharacterized chloroplast-to-nucleus protein translocation mechanism. Both RCBL and RCB mature proteins are similar sizes in both pools (Figure 28) (Yoo et al. in prep), and both are smaller than their respective full-length in vitro translated control proteins. These results indicate that the targeting mechanism of RCBL and RCB may be similar to the one used by HMR and WHY1, which need to be processed in the chloroplasts before being transported into the nucleus. Nevertheless, we still cannot rule out the possibility that a nuclear-localized protease might recognize and cleave the RCBL, RCB, and HMR preproteins for maturation. To rule out this possibility, chloroplast import experiments and characterization of transplastomic Arabidopsis are necessary for RCBL and RCB.
Since I have shown that RCBL is a dual-localized protein, our next question is whether the partitioning of RCBL is regulated by environmental stimuli. Preliminary results from co-expressing fluorescently-tagged RCBL and RCB in tobacco have shown that RCB alters the partitioning pattern of RCBL, leading to co-localization with RCB nuclear speckles. The speckle pattern has never been observed when RCBL is expressed alone. This potential partitioning mechanism of RCBL needs to be further studied for a better understanding of the communication between the nucleus and chloroplasts.

3.4 Materials and Methods

3.4.1 Expression and Purification of RCBL Antigen

Restriction sites, vector information, and primers for generating pET42b-RCBL-antigen and pET15b-TEV-RCBL-antigen are listed in Table 4.

GST-tagged RCBL-antigen (aa 95-232) was expressed in E. coli strain BL21(DE3) (Agilent Technologies) with the pET42b vector (Novagen). Induced cells were harvested by centrifugation at 5000 x g for 10 min at 4°C, and lysed by French press in lysis buffer containing PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.4 mM KH2PO4 [pH 7.5]), 100 mM PMSF, and 1 x protease inhibitor cocktail (Sigma-Aldrich). Lysates were cleared by centrifugation at 20,000 x g for 20 min at 4°C. The cleared lysates were incubated with Glutathione Sepharose 4B (GE; 17-0756-01) equilibrated in PBS buffer at 4°C for 3 hours. Beads were then washed four times with wash buffer (PBS). Finally, GST-tagged proteins were eluted with elution buffer (PBS with Glutathione).

The purification of 6xHis-tagged RCBL-antigen was done similarly as above. The compositions of buffers were changed due to the different affinity tag. The lysis buffer used here contained PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.4 mM
KH2PO4 [pH 7.5]), 10 mM imidazole, 100 mM PMSF, and 1 × protease inhibitor cocktail (Sigma-Aldrich). The protein was bound to Chelating Sepharose Fast Flow (GE Healthcare). Wash buffer consisted of PBS with 20 mM imidazole. His-tagged protein was eluted with elution buffer (PBS with 250 mM imidazole).

### 3.4.2 Purification of RCBL Anti-Serum

Purified GST-tagged RCBL antigen was dialyzed in 4 L of coupling buffer (0.1 M NaHCO3, 0.5 M NaCl, pH 8.3). The serum was also dialyzed in 4 L of 1 × PBS. Both dialyses were performed at 4ºC with gentle magnetic stirring overnight. 330 mg of CNBr Sepharose beads (GE Healthcare) were activated and washed 3 times with 10 mL of 1 mM HCl, and the supernatant was removed by gentle centrifugation (500 x g). The activated CNBr beads were washed twice with 10 mL of coupling buffer. GST-tagged proteins were bound to the CNBr beads in 5 mL of coupling buffer at 4ºC with rotation overnight. The bound beads were transferred to a column and rinsed with 15 mL of coupling buffer. Washed beads were blocked by 10 mL of 0.1 M Tris buffer pH 8.0 with rotation at room temperature for an hour. Blocked beads were transferred back to the column and washed with alternating acidic (0.1 M NaAc, 0.5 M NaCl, pH 4) and basic (0.1 M Tris, 0.5 M NaCl, pH 8) wash solutions three times. The blocked and washed beads were then incubated with dialyzed serum with 10 mL of PBS at 4ºC with rotation overnight.

Antibody-bound beads were applied to a column and washed with 10 mL of 1 × PBS. The antibody was eluted with 1.5 mL of 0.1 M glycine (pH 2.4) with 0.1 M Tris (pH 8.8) and 0.1 M NaCl. The eluted antibody was dialyzed in storage buffer (50% glycerol in 1 × PBS), and was then aliquoted and stored at -80 ºC.
3.4.3 Plasmid Construction and Generation of Transgenic *Arabidopsis* Lines

The constructs used for generating transgenic *Arabidopsis* expressing UBQ10p::RCBL-(PT)_4P-3HA-6His and UBQ10p::RCBL-(PT)_4P-3HA-YFP were made by Gibson Assembly (New England Biolabs). For the *RCBL-HA-His* construct, a DNA fragment encoding (PT)_4P-3HA-6His was subcloned into the PstI and SalI sites of the pCHF1 vector (Fankhauser et al., 1999). Then, pCHF1-(PT)4P-3HA-6His was cut with EcoRI and PstI prior to Gibson Assembly with the UBQ10 promoter and *RCBL* CDS. The UBQ10 promoter (At4g35320) was amplified by PCR using primers #2578 5’-CAGCTATGAC CATGATTACG GTTTTTGTGA TCATTCTTGTA TAC-3’ (forward) and # 2527 5’- GAAGAATCAT GGTATTGTTT TATAGAAGAA GAAGAAG-3’ (reverse). The RCBL CDS was amplified from cDNA using primers #2579 5’- TTCTTCTATA AAACAATACC ATGATTCTTC CATTTTCGAC ACAGTTCACT TGC-3’ (forward) and #2580 5’-GGGGGTGGGG GTGGGGGTGG GGTGGGATT CACACTTACA TCGACTAAAG G-3’ (reverse). For the *RCBL-HA-YFP* construct, the pCHF1 vector was cut by EcoRI and PstI prior to Gibson Assembly with the UBQ10 promoter, *RCBL* CDS, and the (PT)4P-3HA-YFP tag. The UBQ10 promoter and RCBL CDS were amplified using the same PCR primers as above, and (PT)4P-3HA-YFP was amplified by primers #2473 5’-CCCACCCCCA CCCCCACCCC CACCCCCGCG GCCGTTTACC CATAACG-3’ (forward) and #2474 5’-ATACGAACGA AAGCTCTGCA TTACTTGTA CAGCTGTCGA TG-3’ (reverse). *RCBL-HA-His* and *RCBL-HA-YFP* transgenic lines were generated by transforming rcbl-10 heterozygous plants with *Agrobacterium tumefaciens* GV3101 containing pCHF1-UBQ10::RCBL-(PT)4P-3HA-6His or pCHF1-UBQ10::RCBL-(PT)4P-3HA-YFP plasmids. Transgenic
plants were selected on ½ MS plates containing Gentamycin and then homozygous rcbl-10 plants were identified by genotyping.

3.4.4 Confocal Imaging

For quantification of PB morphology, seedlings were mounted on Superfrost slides (VWR, Radnor, PA; Cat. #48311-600) using ddH2O and 22 x 40 mm coverslips (no. 1.5, VWR, Radnor, PA; Cat. #48393-172). The epidermal cells of tobacco leaves and hypocotyl epidermal cells of Arabidopsis were imaged via a Zeiss LSM 510 inverted confocal microscope (Carl Zeiss, Jena, Germany). For observation of the subcellular localization of RCBL fusion proteins, 458 nm excitation (for CFP) or 514 nm excitation (for YFP) from an argon laser were used with the manufacturer’s default CFP-only (475-525 nm) or YFP-only (520-550 nm) detection settings. Images were collected using LSM 510 software version 4.2. Images were processed using Adobe Photoshop CC software (Adobe Systems, Inc., San Jose, CA).

3.4.5 Nuclear and Chloroplast Fractionation

Seedlings from the RCBL-HA-His line grown for 2 days in Rc were frozen and homogenized in liquid nitrogen. The chloroplast fractionation was carried out by a protocol described previously (Chen et al., 2010). For chloroplast fractionation, 1 g of Arabidopsis seedlings were ground in 2 mL of cold grinding buffer (GB) containing 50 mM HEPES-KOH (pH 7.3), 0.33 M sorbitol, 0.1% BSA, 1 mM MnCl2, and 2 mM EDTA with 1 × EDTA-free cOmplete™ protease inhibitor cocktail (PIC, Roche). The plant extract was filtered through a Falcon® 40 μm cell strainer (Corning), and centrifuged at 2600 x g for 2 min to spin down chloroplasts. Chloroplasts were re-suspended in 0.2 mL of GB buffer and applied to a Percoll gradient (80% and 40%), followed by
centrifugation at 2600 x g for 10 min with the minimum deceleration setting. Chloroplasts in the interface between the gradients were collected. For nuclear fractionation, homogenized tissue was mixed with an equal volume of nuclei extraction buffer containing 20 mM PIPES-KOH (pH 7.0), 10 mM MgCl₂, 12% hexylene glycol, 0.25% Triton X-100, 5 mM β-mercaptoethanol, and 1 × EDTA-free cOmplete PIC (Roche). The lysate was then filtered through a 40 µm cell strainer. The filtered lysate was loaded on top of 2 mL of 30% Percoll (Sigma) in 5 mM PIPES-KOH (pH 7.0), 10 mM MgCl₂, 3% hexylene glycol, 0.25% Triton X-100, and 5 mM β-mercaptoethanol, and centrifuged at 700 x g for 5 min at 4°C. The nuclear pellet was dissolved in nuclei extraction buffer. Protein extracts from chloroplasts and nuclear fractions were resolved by SDS-PAGE, and RCBL-HA-His protein was detected by immunoblotting using anti-HA antibodies (12CA5, Roche). The purity of chloroplasts and nuclear fractions were monitored with antibodies against plastidial ferredoxin:sulfite reductase (SiR) and nuclear histone H3 (ab10799, Abcam, Cambridge, MA). The anti-SiR antibody was kindly provided by Dr. Sabine Heinhorst at the University of Southern Mississippi (Chi-Ham et al., 2002).
Chapter 4. Examining the Biochemical Function of RCBL

4.1 Introduction

Chapters two and three have demonstrated that RCBL is a nuclear and plastidial dual-localized protein required for early phy signaling events and chloroplast biogenesis. Yua et al. (2014) showed that RCB/AtECB1/MRL7 has thioredoxin activity in vitro. However, the molecular function of RCBL has not yet been characterized.

To gain insights into the molecular function of RCBL, I performed domain structure analyses of the RCBL protein by using InterPro (www.ebi.ac.uk/interpro) (Finn et al., 2017) and Phyre2 (www.sbg.bio.ic.ac.uk/phyre2) (Kelley et al., 2015). As described in 2.2.4, these analyses suggested that RCBL contains a C-terminal thioredoxin-like fold from amino acid 212-319. To determine whether the C-terminus of RCBL forms a trx-like fold, we produced a recombinant His-tagged C-terminal portion of RCBL for NMR spectroscopy analysis. Dr. Pei Zhou’s laboratory in the Duke University Department of Biochemistry performed structural analysis by NMR spectroscopy. Although the primary sequences of RCBL and RCB are not conserved with Escherichia coli (E. coli) trx1, the secondary and tertiary structures of RCBL and RCB are conserved with E. coli trx. Although RCB/MRL7 has been shown to have thioredoxin activity in vitro (Yua et al., 2014), alignments of E. coli trx with RCBL and RCB/MRL7 show that they do not possess any cysteine residues in the catalytic cleft. This suggests that neither of them should have reductase activity, contradictory to the results from Yua et al. (Yua et al., 2014). To solve this dilemma, I generated constructs for expressing recombinant RCBL and RCB/MRL7 proteins in E. coli, and purified them for the insulin-based
thioredoxin activity assay described in Holmgren, (1979). The *in vitro* thioredoxin assay demonstrates that neither RCBL or RCB has reductase activity.

Because RCBL and RCB have shown no reductase activity, I investigated whether the trx-like fold can perform another molecular function. In addition to acting as reductases, trx-like folds have been shown to have other functions. The *E. coli* trx is also a processivity factor of phage T7 DNA polymerase (Huber et al., 1986; Mark and Richardson, 1976; Huber et al., 1987; Ghosh et al., 2008; Tran et al., 2012b; Etson et al., 2010; Himawan and Richardson, 1992; Tabor et al., 1987), and the active catalytic site is not required for this processivity function (Huber et al., 1986). An alternative hypothesis for the biochemical function of RCBL is that the trx-like fold can serve as a protein-protein interaction domain. Since RCBL and RCB are paralogous, and their null mutant phenotypes in *Arabidopsis* are similar, RCBL and RCB might function in the same protein complex. To test whether RCBL and RCB are in the same protein complex in *Arabidopsis*, I performed co-immunoprecipitation with transgenic *Arabidopsis* expressing RCBL-HA-His and used the anti-RCB antibody to detect the presence of RCB. Next, I examined whether RCBL and RCB interact directly, and which region of RCBL is required and sufficient for interaction. For this, I generated several GST-tagged RCBL proteins covering different regions, and then performed a GST pull-down assay. The results described in this chapter show that RCBL forms a C-terminal trx fold, but without reductase activity. Instead, it utilizes the C-terminal trx fold to interact with RCB. Collectively, these results suggest that RCBL and RCB form a functional complex to perform their function in phy signaling and chloroplast biogenesis.
4.2 Results

4.2.1 The Secondary and Tertiary Structure of C-Terminal RCBL and RCB are Conserved with *Escherichia coli* trxl

![Secondary structure alignment](image)

**Figure 29.** The primary Sequences of RCBL or RCB are not conserved with *E. coli* trxl.

Primary sequence alignment of trx-like fold of RCBL (aa 207-336), RCB (aa 200-314), and *E. coli* trxl(aa1-108). The alignment was done by Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo) (Sievers et al., 2011) and visualized by BoxShade (www.ch.embnet.org/software/BOX_form.html).

To further investigate the biochemical function of RCBL, I focused on characterizing the C-terminal trx-like fold — the only recognizable domain in RCBL. Although the primary protein sequences of RCBL (amino acid, aa 207-336), RCB (aa 200-314), and *E. coli* trxl do not show much similarity (Figure 29), alignment of the secondary structures of the trx-like folds of both RCBL and RCB using PROMALS3D (Pei et al., 2008a; Pei and Grishin, 2014) show that they are conserved with the *E. coli* trxl (Figure 30A). Furthermore, the structure of the trx-like fold in RCBL was successfully solved by nuclear magnetic resonance spectroscopy (NMR) (Figure 30B, left panel). The trx-like fold of RCB was calculated and modeled based on the structure of the trx-like fold of RCBL (Figure 30B, middle panel). I used Chimera (www.egl.ucsf.edu/chimera) (Pettersen et al., 2004) to perform tertiary structure alignment between the trx-like fold of RCBL, RCB, and *E. coli* trxl. The results in Figure 30 support the hypothesis that the tertiary structure of trx-like fold of RCBL and
RCB are conserved with *E. coli* trx1.

However, the alignment also reveals that both RCBL and RCB lack the canonical catalytic motif (CxxC at *E. coli* trx amino acid 32-35, Figure 30), suggesting that the trx-like fold of RCBL and RCB might not have redox activity.

**Figure 30. Secondary and tertiary structures of RCBL or RCB are conserved with *E. coli* trx.**

(A) Secondary structure based amino acid sequence alignment of *E. coli* trx (pdb: 1xob), C-terminal RCBL (aa 208-350), and C-terminal RCB (aa 200-332). The alignment and secondary structure predictions were done by PROMALS3D (Pei et al., 2008a; Pei and Grishin, 2014; Pei et al., 2008b). Red characters represent predicted alpha helices, and blue characters represent beta sheets. The consensus amino acid sequence and structure are labeled at the bottom of the alignment. Pink cylinders represent conserved alpha helix regions, and blue squares represent conserved beta sheet regions. (B) Tertiary structure alignment of C-terminal RCBL (aa 208-350, red, structure solved by NMR spectroscopy), C-terminal RCB (aa 200-332, blue, structure modeled based on RCBL trx), and *E. coli*trx (amino acid 1-108, gray, 1xob, reduced structure solved by NMR spectroscopy). The catalytic site of *E. coli*trx is labeled in magenta. Cysteines in all tertiary structures are shown in ball-and-stick structure.

### 4.2.2 Expression and Purification of RCBL and RCB Recombinant Proteins

To examine whether the trx-like fold of RCBL and RCB have redox activity, I generated and purified His-tagged RCBL and RCB derived proteins *in vitro* (Figure 31A). Recombinant proteins were purified with Ni-columns and subjected to SDS-PAGE and
Coomassie Blue staining. Each of the purified proteins show a single strong band at the predicted molecular weight, which is labeled by red arrows in Figure 31C.

4.2.3 RCBL and RCB Do Not Possess Redox Activity

Next, I performed a trx activity assay using insulin as the substrate to determine the reductase activity of RCBL and RCB (Holmgren, 1979). Reduction of insulin causes increased absorbance at 650 nM. The results show that the trx-like fold of RCBL and RCB cannot reduce insulin (Figure 31B). These data suggest that the trx-like folds of RCBL and RCB have no redox activity. Thus, while the C-terminal trx-like folds of RCBL and RCB are structurally conserved with E. coli trx, they lack catalytic sites and redox activity.

![Figure 31. Recombinant RCBL and RCB proteins do not possess trx activity.](image)

(A) Schematic illustration of recombinant proteins used for trx assay. (B) Absorbance at 650 nm over time for the recombinant proteins in (A). trx reduces insulin and causes an increase in turbidity, which was measured by monitoring absorbance at 650 nm. The slope of each curve shows the initial velocity of the reducing reaction mediated by the recombinant protein added. (C) Recombinant proteins were resolved by SDS-PAGE and stained with Coomassie Blue. Each recombinant protein is denoted with a red arrowhead and labeled on top of the lane.

4.2.4 RCBL and RCB Interact in vivo

trx-like folds have been shown to have functions other than as reductases, including serving as a protein-protein interaction domains (Martin, 1995; Atkinson and Babbitt, 2009). For example, E. coli trx1 is required for the processivity of phage T7 polymerase, and this function is independent of its redox activity (Huber et al., 1986;
1987; Tran et al., 2012a; 2012b; Etson et al., 2010). Another example is the b’ trx-like
fold of protein disulfide isomerase, which is required for substrate binding (Van Dat
Nguyen et al., 2008; Kortemme et al., 1996).

Because RCBL and RCB are paralogous, have non-redundant functions, and are
involved in the same genetic pathway in phy signaling, it is likely that they interact with
each other and may function in the same complex to regulate phy signaling.

To examine whether RCBL is associated with RCB in vivo, I performed co-
immunoprecipitation using 2-d-old Rc grown RCBL-HA-His seedlings. RCB protein was
detected with anti-RCB antibodies. Figure 32 shows that RCB can be co-
immunoprecipitated with RCBL-HA-His, indicating that RCBL and RCB are associated
in vivo.

![Figure 32. RCBL interacts with RCB in vivo.](image)

(A) RCBL-HA-His can pull-down RCB in vivo. Seedlings of the RCBL-HA-His line and Col-0 were grown
in 10 µmol m⁻² s⁻¹ R light for 2 days. Proteins were extracted and subjected to immunoprecipitation using
anti-HA Affinity Matrix (Roche). The input and immunoprecipitated RCBL-HA-His (top panel) and RCB
(bottom panel) were detected by immunoblotting using anti-HA and anti-RCB antibodies, respectively.

4.2.5 The Thioredoxin-Like Folds of RCBL and RCB are Required for their Interaction

To determine whether RCBL interacts with RCB directly, we conducted GST
pull-down assays using recombinant GST-RCBL and truncated proteins to pull down in
vitro-translated HA-RCB (Figure 33).
Figure 33. GST-fusion RCBL-derived proteins and HA-tagged RCB-derived proteins.

Schematic illustration of recombinant proteins used in GST pull-down assays in Figure 34. TP, transit peptide; NLS, nuclear localization signal; trx-like fold, thioredoxin-like fold.

Data from pull-down assays show that only the trx-like domain of RCBL (GST-RCBL-trx, amino acids 202-329) can pull-down HA-RCB protein. The other recombinant RCBL proteins or GST alone were not able to pull down HA-RCB (Figure 34A), indicating that the trx-like domain of RCBL is sufficient and necessary for the interaction. Additionally, the failure of GST-RCBL-C to pull down RCB suggests the existence of an inhibitory domain at the C-terminal end of RCBL (aa 330-350).

Figure 34. The trx-like fold of RCBL is required and sufficient for direct interaction with RCB in vitro.

(A) The trx-like fold of RCBL is required and sufficient to interact with RCB in vitro, while the C-terminal extension of RCBL (330-350) is an inhibitory region for this interaction. GST pull-down assays were performed using E. coli-expressed GST-RCBL, GST-RCBL-N, GST-RCBL-C, GST-RCBL-trx or GST alone to pull down in vitro transcribed and translated HA-RCB proteins. Upper panel, 10% input and bound
HA-RCB were detected by immunoblots using anti-HA antibodies. (B) The trx-like fold of RCB is also sufficient to interact with RCBL in vitro. GST pull-down assays were performed as in (A) but the prey was HA-RCB-trx. Upper panel, 10% input and bound HA-RCB-trx were detected by immunoblotting using anti-HA antibodies. Immobilized GST or GST-RCBL-derived proteins are shown in the corresponding SDS-PAGE gels stained with Coomassie Blue.

Because the RCB protein also possesses a C-terminal trx-like fold, I examined whether the trx-like fold of RCB is sufficient to directly interact with RCBL. I utilized in vitro-translated HA-RCB-trx as prey in GST pull-down assays using the same set of recombinant GST-RCBL truncation proteins as before (Figure 34B). Figure 34B shows that HA-RCB-trx only can be pulled down by GST-RCBL-trx, but not by the other GST-RCBL truncations or GST alone. This result indicates that HA-RCB-trx is sufficient for the interaction between RCBL and RCB. However, HA-RCB can be pulled down by GST-RCBL-trx suggesting RCB does not have a strong inhibitory domain like RCBL. Based on the in vivo and in vitro results shown above, RCBL directly interacts with RCB through its trx-like domain.

4.3 Discussion

Although multiple papers have reported RCB/MRL7/SVR4/ECB1 and RCBL/MRL7-like/SVR4-like to be involved in chloroplast transcription, only one paper has reported a potential biochemical function of RCB, showing that RCB might have a reductase activity in vitro (Yua et al., 2014). To investigate the molecular mechanism of RCBL in phy signaling and chloroplast development, I examined the function of the C-terminal trx-like fold in RCBL. In this chapter, I have shown that RCBL and RCB are structurally conserved with E. coli trx but without any available cysteine residues in the catalytic vicinity. This implies that neither RCB nor RCBL should have reductase activity, which contradicts Yua et al.’s findings (2014). Our trx activity assay supports the
prediction that RCBL and RCB have no reductase activity. Alternatively, RCBL interacts with RCB in vivo, and in vitro pull-down assays demonstrate that the trx-like fold of RCBL is necessary and sufficient for the direct interaction with RCB. Collectively, these results show that the trx-like fold of RCBL may serve as a protein-protein interaction domain instead of a reductase.

The trx-activity assay using insulin as a substrate was developed in 1979 (Holmgren, 1979) and has been widely used. The best advantage of this assay is that the reductase activity can be easily monitored by measuring the increase in absorbance caused by insulin precipitation. However, we still cannot completely rule out the possibility that RCBL or RCB have different substrate specificities, which could make them unable to reduce insulin in this assay.

Multiple trx-like domains have been identified in which catalytic activity is not required for biological function. The best-known example is that E. coli trx is used as a processivity factor for the phage T7 DNA polymerase (Etson et al., 2010; Tran et al., 2012b; Huber et al., 1986; 1987; Himawan and Richardson, 1992; Ghosh et al., 2008; Mark and Richardson, 1976; Tabor et al., 1987; Bedford et al., 1997). It has further been shown that E. coli trx stabilizes, (Huber et al., 1987) increases the accuracy of, (Tran et al., 2012b; Ghosh et al., 2008; Tabor et al., 1987) and reduces microscopic hopping of the T7 polymerase (Etson et al., 2010). Another example is recently discovered TrxZ in Arabidopsis, whose reductase activity is not required for its structural support of the PEP complex (Wimmelbacher and Bornke, 2014; Arsova et al., 2010), and the reductase activity may only be a fine-tuning mechanism to regulate the function of PEP. For the protein disulfide isomerase, which contains four trx-like domains, two of the trx-like
domains have no reductase activity but they instead play roles in substrate recognition and binding (Klappa et al., 1998; Kemmink et al., 1997; van Lith, 2004; Ellgaard and Ruddock, 2005).

Since RCBL is a dual-localized protein, its interacting partners in might differ between subcellular compartments. In chloroplasts, although neither RCB nor RCBL have been identified from biochemical studies of PEP interacting proteins (pTAC or PAP), the PEP-deficient phenotype of *rcbl* and *rcb* mutants still implies that they might be peripheral components involved in regulation of the complex. The altered nucleoid phenotype in both mutants led to one hypothesis that they might interact with histone-like DNA binding proteins in plastids to maintain the structure of nucleoids (Powikrowska et al., 2014b). To identify other interacting proteins within chloroplasts, a chloroplast fractionation combined with co-immunoprecipitation and proteomic analyses will be necessary. In addition, the interacting proteins will need to be validated by more GST pull-down assays.

In the previous chapters, I have demonstrated that RCBL plays a role in phy signaling and acts genetically between *phy* and *PIFs*. HMR has been reported to interact preferentially with activated phyB and phyA, as well as all members of PIF family. Our preliminary results for RCB show that it interacts with phyB and HMR but not PIFs. In contrast, co-immunoprecipitation results using RCBL-HA as bait have not shown interaction with phyB or HMR, though GST pull-down assays do suggest a direct interaction between RCBL and HMR *in vitro*. So far, the only confirmed RCBL interacting protein is RCB, but other phy signaling components may still possibly interact with RCBL. In addition, when co-expressed with RCB, the abundance of nuclear RCBL
is increased and shows a speckle pattern, suggesting that interaction between RCBL and other proteins might affect its subcellular partitioning, suborganellar localization, and possibly function. More characterization is required to identify other nuclear RCBL interaction partners.

4.4 Materials and Methods

4.4.1 Expression and Purification of Recombinant C-terminal

His-tagged C-terminal RCBL (aa 208-350) was expressed in E. coli strain BL21(DE3) (Agilent Technologies) with the pET15b vector (Novagen). Induced cells were harvested by centrifugation at 5000 x g for 10 min at 4ºC, and lysed by French press in lysis buffer containing PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.4 mM KH₂PO₄ [pH 7.5]), 10 mM imidazole, 100 mM PMSF, and 1 × protease inhibitor cocktail (Sigma-Aldrich). The cell extract was cleared by centrifugation at 20,000 x g for 20 min at 4ºC. The cleared cell extract was incubated with Ni-Chelating Sepharose Fast Flow (GE Healthcare) and equilibrated in lysis buffer at 4ºC for 3 hours. The Ni-chelating beads were washed four times with wash buffer (PBS with 20 mM imidazole). His-tagged proteins were eluted with elution buffer (PBS with 250 mM imidazole).

4.4.2 Thioredoxin Activity Assay

Thioredoxin activity assays were performed as described previously (Holmgren, 1979). Reactions were carried out in reaction buffer containing 0.1 M potassium phosphate (pH 7.0), 2 mM EDTA, 0.167 mM insulin as the substrate, and 1 uM of commercially-purified E. coli trx (ab115709, Abcam, Cambridge, MA) or His-tagged C-terminal RCBL. Reactions were started by adding 0.5 mM dithiothreitol DTT. The
reduction of insulin generated turbidity which was detected by measuring the absorbance at 650 nm each min.

4.4.3 Co-Immunoprecipitation

Co-immunoprecipitations were performed as described previously (Chen et al., 2010; Galvão et al., 2012; Qiu et al., 2015) with modifications. Transgenic RCBL-HA-His and Col-0 seedlings were grown in continuous red light (10 µmol m\(^{-2}\) s\(^{-1}\)) for 48 h, and were then incubated with 25 µM MG132 and 25 µM MG115. One gram of treated seedlings were collected and ground in liquid nitrogen. Homogenized samples were resuspended in 1 mL of co-immunoprecipitation buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 2 mM DTT, and 0.1% Nonidet P-40. To inhibit protein degradation, the buffer was supplied with 40 µM MG132, 40 µM MG115, and 1 × EDTA-free protease inhibitor cocktail (Roche). The crude extracts were centrifuged at 20,000 g for 10 min at 4°C, repeated twice. An aliquot (0.9 mL) of the supernatant was mixed with 100 µL of anti-HA Affinity Matrix (Roche) and then incubated at 4°C for 4 h. The RCBL-HA-His bound beads were washed four times with 1 mL co-immunoprecipitation buffer, and the immunoprecipitated proteins were released by boiling the beads in 1 × Laemmli protein sample buffer. Protein samples were loaded on an 8% SDS-PAGE and the input samples, immunoprecipitated RCBL-HA-HIS, and RCB were detected by immunoblotting with mouse anti-HA monoclonal (Invitrogen) or rabbit anti-RCB polyclonal antibodies (Yoo et al. in prep).

4.4.4 GST Pull-Down Assay

GST pull-down assays were performed as described previously (Chen et al., 2010; Galvão et al., 2012; Qiu et al., 2015). Briefly, GST fusion full-length or fragmented
RCBL proteins were expressed in E. coli strain BL21(DE3) (Agilent Technologies) with pET42b derived constructs (Novagen). Induced cells were harvested by centrifugation at 5000 x g for 10 min at 4°C, and lysed by French press in E buffer containing 50 mM Tris-HCl (pH7.5), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% DMSO, 2 mM DTT, and 1 × protease inhibitor cocktail (Sigma-Aldrich). The cell extracts were cleared by centrifugation at 20,000 x g for 20 min at 4 °C, followed by ultra-centrifugation at 50,000 g for 15 min at 4°C. Cleared cell extracts were incubated with glutathione Sepharose beads (GE Healthcare) equilibrated in E buffer at 4°C for 3 h. Beads with immobilized GST fusion proteins were washed four times with E buffer supplemented with 0.1% Nonidet P-40.

Prey proteins with a C-terminal (PT)$_4$P linker and 3xHA tag were synthesized using pCMX-PL2 plasmids (Umesono et al., 1991) and the TnT T7 Coupled Reticulocyte Lysate System (Promega) following the manufacturer’s protocol. The in vitro-translated prey proteins were diluted with E buffer with 0.1% Nonidet P-40 and incubated with the beads with immobilized GST fusion proteins at 4°C for 3 h. The beads were washed four times with E buffer with 0.1% Nonidet P-40. Bound proteins were eluted by boiling in 1 × Laemmli protein sample buffer and separated by 8% SDS-PAGE. Input and affinity-purified proteins were detected by immunoblots using goat anti-HA polyclonal antibodies (GenScript). The amount of GST-fusion proteins bound to the glutathione beads was visualized by staining the SDS-PAGE gel with Coomassie Brilliant Blue. Primers used to clone the constructs for GST pull-down assays are listed in Table 5.
Chapter 5. Summary

Recent work has established a new class of phy signaling components essential for chloroplast biogenesis, which has been overlooked by previous photomorphogenic screens (Chen et al., 2010; Pfalz et al., 2015; Qiu et al., 2015). Characterization of HMR, the first identified member of this group, has established that it has distinct functions in the nucleus and chloroplasts (Chen et al., 2010; Galvão et al., 2012; Qiu et al., 2015; Nevarez et al., 2017; Pfalz, 2006; Steiner et al., 2011; Pfalz et al., 2015). The tall-and-albino phenotype of hmr is an easy readout for identifying more components involved in the coordination of phy signaling and chloroplast development. This screen has yielded several mutants, and I focused on characterizing one of them, regulator-for-chloroplast-biogenesis-in-light-signaling (rcbl), to determine RCBL’s function in photomorphogenesis.

5.1 REGULATOR-FOR-CHLOROPLAST-BIOGENESIS-IN-LIGHT-SIGNALING is a Novel Regulator of Phy Signaling and Chloroplast Biogenesis

To determine the function of RCBL, I characterized the phenotype of rcbl null mutants in early phy signaling events and plastidial transcription. Results in Chapter 1 demonstrate that RCBL is required for the expression of plastidial PEP-dependent genes for chloroplast biogenesis, degradation of phyA, PIF1 and PIF3, and the activation of HMR-dependent PIF-induced genes. In addition, the characterization of rcbl-15 reveals a gain-of-function mutant which shows hypersensitivity to phyB and phyA signaling.
5.2 RCBL is Dual-Localized to Nuclei and Chloroplasts, and the Proteins in Both Pools Share the Same Molecular Weight

Although RCBL was first reported as a plastidial protein (Powikrowska et al., 2014a; Qiao et al., 2011), the phy-defective phenotypes of rcbl mutants suggested that it might also localize to the nucleus. By using fluorescence imaging and biochemical fractionation, I have provided evidence that RCBL is dual-localized not only to chloroplasts, but also the nucleus. Unexpectedly, fractionation results show that the mature forms of RCBL in both compartments have similar molecular weights, and these mature proteins are smaller than full-length in vitro-produced RCBL. Similar cases have been reported, such as WHY1 and HMR (Nevarez et al., 2017; Isemer et al., 2012; Grabowski et al., 2008), and a plastid-to-nucleus translocation mechanism was proposed for this group of proteins. It is likely that the mature form of RCBL also needs to be processed in the chloroplasts before being transported into the nucleus.

5.3 RCBL and RCB Do Not Possess Reductase Activity but Directly Interact With Each Other Through Their trx-Like Folds

To investigate a possible molecular function of RCBL, I examined the biochemical functionality of the C-terminal trx-like fold. A previous report showed that the parologue of RCBL and RCB possess trx activity in vitro (Yua et al., 2014). Our structural studies, on the other hand, show that the trx-like folds in RCBL and RCB are conserved with E. coli trx1, but without any cysteine residues in the catalytic vicinity. Consistently, purified RCBL and RCB recombinant proteins showed no reductase activity in vitro. On the other hand, I have shown that RCBL and RCB directly interact with each other utilizing their trx-like folds. This suggests a molecular mechanism in which RCBL and RCB form a functional complex to exert their roles in phy signaling.
and chloroplast biogenesis, which may explain why RCBL and RCB do not act redundantly.

5.4 Comparison of RCBL and RCB Reveals That They Play Distinct Roles in Regulating the Activity of the PEP Complex

BLAST and phylogenetic analyses reveal the paralogous relationship between RCBL and RCB. Most of their mutant phenotypes showed that they are not redundant, and they are probably involved in similar processes to regulate photomorphogenesis. Surprisingly, when knocking out four PIFs in rcbl or rcb, the albino phenotype is rescued in rcb but not in rcbl. One possibility is that PIF-mediated anterograde signaling is de-repressed in rcb/pifq, which can overcome the PEP defect of the rcb mutant. Although the detailed mechanism of this proposed PIF-mediated anterograde signaling pathway remains elusive, these results clearly show that RCBL and RCB play distinct roles in the chloroplast biogenesis, and RCBL appears to play a more important role in regulating the activity of PEP.

5.5 Conclusions and Proposed Molecular Mechanism of RCBL in Regulating Photomorphogenesis

Collectively, the results in this study indicate that RCBL is a phy signaling component, which is also required for chloroplast biogenesis. Based on multiple lines of evidence, I propose a molecular model in which nuclear RCBL interacts with RCB to regulate phyB localization to PBs and the activation and degradation of PIF1 and PIF3. The plastidial pool of RCBL is essential for the proper function of the PEP complex and it cannot be bypassed by releasing PIF-mediated anterograde inhibition of chloroplast
development. On the other hand, both nuclear and plastidial RCB contribute to the activity of the PEP complex.

![Proposed model of RCBL in phy signaling and chloroplast biogenesis.](image)

**Figure 35. Proposed model of RCBL in phy signaling and chloroplast biogenesis.**

Nuclear RCBL interacts with RCB to regulate the localization of activated phyB to PBs, PIF target gene expression, and PIF degradation. This RCBL-RCB complex may influence the function of the phyB-HMR-PIF complex. In the chloroplast, the activity of the PEP complex is regulated by a group of PAP proteins, including HMR. Additionally, a PIF-mediated anterograde signaling mechanism inhibits PEP activity. The plastidial pool of RCBL is essential for the activity of the PEP complex, which acts downstream of PIFs. On the other hand, removal of PIFs can bypass the effect of RCB on PEP activity.

The identification of *rcbl* is another strong piece of evidence that previous studies have failed to identify a class of photomorphogenic mutants which play vital roles in both the nucleus and chloroplasts. In addition, neither RCBL nor RCB have been identified by biochemical pull-down analyses of the pTAC or PEP complexes, indicating there are likely to be more key players in regulating plastidial transcription that cannot be
identified by this type of approach. Given the complex nature of crosstalk between chloroplasts and the nucleus, my tall-and-albino screening method provides another tool for investigating the communication between two organelles. In addition, finding new components involved in the early phy signaling pathway can also shed light on the assembly and function of PBs. These findings enhance our understanding of how plants decode light stimuli and coordinate nuclear and plastidial transcription to promote photomorphogenesis.
## Appendix A

Table 1. Alleles and genotyping primers used in this study

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutation</th>
<th>Accession</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Enzyme (if needed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rcbl-1</td>
<td></td>
<td></td>
<td>AGAGAAGGGCGATTCAAGTCATAT</td>
<td>CTGGAAGTAATAATGACCAG</td>
<td></td>
</tr>
<tr>
<td>rcbl-10</td>
<td></td>
<td>CS449718</td>
<td>ATATTGACCATCATCATTGC</td>
<td>GCAAGAATAGAGAGGAGACCA</td>
<td>N/A</td>
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<td>rcbl-11a</td>
<td></td>
<td>CS94127</td>
<td>GTTACTTTGAGATATGACTC</td>
<td>GTTACTTTGAGATATGACTC</td>
<td>StyI</td>
</tr>
<tr>
<td>rcbl-12a</td>
<td></td>
<td>CS90549</td>
<td>GACAGTGAAGATGAGGAGATG</td>
<td>CCTCCTCTCTCACCATATC</td>
<td>HinFI</td>
</tr>
<tr>
<td>rcbl-13b</td>
<td></td>
<td>CS93937</td>
<td>GATCCAGTAGAGAGGCAGGACGCTTGA</td>
<td>AGCATCTTCAGGCAATATAG</td>
<td>Bpml</td>
</tr>
<tr>
<td>rcbl-14a</td>
<td></td>
<td>CS92183</td>
<td>GAGAAGGAGTTTGGGCAAG</td>
<td>CAACCAAGTCAGCGATCAG</td>
<td>BglII</td>
</tr>
<tr>
<td>rcbl-15a</td>
<td></td>
<td>CS92919</td>
<td>GAGAAGGAGTTTGGGCAAG</td>
<td>CAACCAAGTCAGCGATCAG</td>
<td>BglII</td>
</tr>
<tr>
<td>rcbl-16a</td>
<td></td>
<td>CS94960</td>
<td>GAGAAGGAGTTTGGGCAAG</td>
<td>CAACCAAGTCAGCGATCAG</td>
<td>MboII</td>
</tr>
<tr>
<td>rcbl-17b</td>
<td>g770r</td>
<td>CS95653</td>
<td>TTGTTCACACCGCTCTCAAGGTTT</td>
<td>GAGACACCTATCTCAGATAC</td>
<td>BstXI</td>
</tr>
<tr>
<td>rcbl-18a</td>
<td>c1213t</td>
<td>CS95650</td>
<td>GCTGTATGGTGGTGTGGTGTT</td>
<td>GGTTAGAGATCGGTCTGTTG</td>
<td>BsrI</td>
</tr>
</tbody>
</table>

*a* Genotyped by CAPS (Cleaved Amplified Polymorphic Sequence) markers.

*b* Genotyped by dCAPS (Derived Cleaved Amplified Polymorphic Sequences) markers.
## Appendix B

Table 2. Primers used in RT-qPCR analyses for detecting plastidial genes

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene</th>
<th>Primer for cDNA synthesis</th>
<th>Forward primer for RT-qPCR</th>
<th>Reverse primer for RT-qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCG00020</td>
<td>psbA</td>
<td>TAGATGGAGCCTCAACAGCAGCTA</td>
<td>ACATTTCTTCTTAGCGGCTT</td>
<td>CGTCCTTGACTATCAACTACTGA</td>
</tr>
<tr>
<td>ATCG00680</td>
<td>psbB</td>
<td>CATCCAAATCTGGATCAATACCAG</td>
<td>GAATTAGATCGTGCGACTTTGA</td>
<td>CTAGCAACCATGCCAAATGTGTC</td>
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<tr>
<td>ATCG00490</td>
<td>rbcL</td>
<td>CTTCACAAGCAGCAGCTAGTTCAGG</td>
<td>GGAGATGATTCTGTACTACAAT</td>
<td>GTCCCTCATTACGAGCTTTGTAC</td>
</tr>
<tr>
<td>ATCG00630</td>
<td>psaJ</td>
<td>GGGAAATGTTAATGCATCTTG</td>
<td>AAACATATCTTTCCGTAGCAC</td>
<td>CTCTATTAATAAATCTGCTAACG</td>
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<tr>
<td>ATCG00190</td>
<td>rpoB</td>
<td>CAATGATAGTGTGTAACCAATCTTC</td>
<td>CTAGTGGACATTATGCACTTTGT</td>
<td>CAGATTTATAAGTAAGCATCTCTTG</td>
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<tr>
<td>ATCG00180</td>
<td>rpoC1</td>
<td>GTATAGCTTCTCGATTTCTCG</td>
<td>GATGCAATTGGAGCTTTATCG</td>
<td>CGATAGGAACCTCTCTTGAAGC</td>
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<td>ATCG00660</td>
<td>rpl20</td>
<td>TGTGCAAGTATTTCCGGATTAAG</td>
<td>GAGCTTTAGGTTCCGCTCATC</td>
<td>ATTACGGCAATTATTCCGAGTG</td>
</tr>
<tr>
<td>ATCG01120</td>
<td>rps15</td>
<td>CTGATTTAATTAATTTATACGTA</td>
<td>GATACGAAGACTTTACTCAG</td>
<td>CAAATAAGCCACAGTGTTGAC</td>
</tr>
</tbody>
</table>
## Appendix C

Table 3. Primers used in RT-qPCR analyses for detecting nuclear genes

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
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<tr>
<td>AT1G69960</td>
<td>PP2A</td>
<td>TATCGGATGACGATTCTTCGTGCAG</td>
<td>GCTTGGTCGACTATCGAATGAGAG</td>
</tr>
<tr>
<td>AT2G31840</td>
<td>RCBL</td>
<td>GAAGCGAGTCTCGATGATCC</td>
<td>GCTTCTGCATCTCTCTCCTC</td>
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<tr>
<td>AT2G46970</td>
<td>PIL1</td>
<td>AAATTGCTTCTACGCCATTACGTGG</td>
<td>TTCTAAGTTTGGAGGCGGACGCAG</td>
</tr>
<tr>
<td>AT4G16780</td>
<td>ATHB2</td>
<td>TCACAGTACTCTCAATCCGAAGC</td>
<td>CCGTAAGAAACTCGAGTCTCTAC</td>
</tr>
<tr>
<td>AT4G32280</td>
<td>AUX/IAA29</td>
<td>CACCATCATTGCCCCGTATCA</td>
<td>CACAGTAGGCCGTTGGGA</td>
</tr>
<tr>
<td>AT4G14130</td>
<td>XTR7</td>
<td>CACCGTCACTGCTTTACTTCTTGG</td>
<td>CATTGTTGTGAAGAACATAAG</td>
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<tr>
<td>AT5G20710</td>
<td>BGAL7</td>
<td>TCTACACGGTTACGTCAATG</td>
<td>AACGCTGAGAAGTGAATGT</td>
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<tr>
<td>AT5G14180</td>
<td>MPL1</td>
<td>TCAGCTACGGGTGTGTTTAGAT</td>
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</tr>
<tr>
<td>AT3G53200</td>
<td>MYB27</td>
<td>CAATAGTTCGCGGTTGGAAG</td>
<td>TGGAGCTGAAAGATGATTCT</td>
</tr>
<tr>
<td>AT2G45210</td>
<td>SAUR36</td>
<td>GTTCAGGATACAAACCGGATA</td>
<td>TTGAGTTTGGCTAATGCTT</td>
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</tbody>
</table>
### Appendix D

Table 4. Primers used in constructs for recombinant protein expression (traditional restriction enzyme and ligase cloning)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Vector</th>
<th>RE used</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET42b-RCBL-antigen</td>
<td>pET42b</td>
<td>SacI/XhoI</td>
<td>GGCGAGCTCTCTCCTCCTCGAAGTAGGAATTAT</td>
<td>GCCCTCGAGTCAAGGACTAATATCTTTGACACC</td>
</tr>
<tr>
<td>pET15b-TEV-RCBL-antigen</td>
<td>pET15b-TEV</td>
<td>NdeI/XhoI</td>
<td>ATAGTACATATGGAGACCTTTTGCCGGAAGAG</td>
<td>CAGAGCTCGAGTCAAGGACTAATATCTTTGACACC</td>
</tr>
</tbody>
</table>
## Appendix E

Table 5. Primers used in construct preparation (Gibson Assembly)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Vector</th>
<th>RE used</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET28a-RCBL</td>
<td>pET28a</td>
<td>BamHI/HindIII</td>
<td>CAGCAAATGGGTCGCGCTATGATTCTTCCATTTTCCAC</td>
<td>CTCGAGTGGCGGCCCCATTATATATACACCTTACA                                      TCGACTAAAG</td>
</tr>
<tr>
<td>pET28a-RCBL-Δ206</td>
<td>pET28a</td>
<td>BamHI/HindIII</td>
<td>CAGCAAATGGGTCGCGCTATGATTCTTCCATTTTCCAC</td>
<td>CTCGAGTGGCGGCCCCATTATATACACCTTACA                                      ACTAAAG</td>
</tr>
<tr>
<td>pET28a-RCB-Δ51</td>
<td>pET28a</td>
<td>BamHI/HindIII</td>
<td>CAGCAAATGGGTCGCGCTATGATTCTTCCATTTTCCAC</td>
<td>CTCGAGTGGCGGCCCCACTTACAGTTACG GGTTAC</td>
</tr>
<tr>
<td>pET28a-RCB-A98</td>
<td>pET28a</td>
<td>BamHI/HindIII</td>
<td>CAGCAAATGGGTCGCGCTATGATTCTTCCATTTTCCAC</td>
<td>CTCGAGTGGCGGCCCCACCTAAAC GTACGGGGTTAC</td>
</tr>
<tr>
<td>pET28a-RCB-Δ197</td>
<td>pET28a</td>
<td>BamHI/HindIII</td>
<td>CAGCAAATGGGTCGCGCTATGATTCTTCCATTTTCCAC</td>
<td>CTCGAGTGGCGGCCCCACCTAAAC GTACGGGGTTAC</td>
</tr>
<tr>
<td>pET42b-RCBL</td>
<td>pET42b</td>
<td>NcoI/PstI</td>
<td>GGTATTGAGGGACGCGGGTCATCCCCTTCGAAGTGTG</td>
<td>GTCGAGCGAGCTCGCCCTGATATTCAATTACACTTAC                                      ATCGACTAAAG</td>
</tr>
<tr>
<td>pET42b-RCBL-N</td>
<td>pET42b</td>
<td>NcoI/PstI</td>
<td>GGTATTGAGGGACGCGGGTCATCCCCTTCGAAGTGTG</td>
<td>GTCGAGCGAGCTCGCCCTGATATTCAATTACACTTAC                                      ATCGACTAAAG</td>
</tr>
<tr>
<td>pET42b-RCBL-C</td>
<td>pET42b</td>
<td>NcoI/PstI</td>
<td>GGTATTGAGGGACGCGGGTCATCCCCTTCGAAGTGTG</td>
<td>GTCGAGCGAGCTCGCCCTGATATTCAATTACACTTAC                                      ATCGACTAAAG</td>
</tr>
<tr>
<td>pET42b-RCBL-trx</td>
<td>pET42b</td>
<td>NcoI/PstI</td>
<td>GGTATTGAGGGACGCGGGTCATCCCCTTCGAAGTGTG</td>
<td>GTCGAGCGAGCTCGCCCTGATATTCAATTACACTTAC                                      ATCGACTAAAG</td>
</tr>
<tr>
<td>pCMX-PL2-HA-RCB</td>
<td>pCMX-PL2-HA</td>
<td>PstI/BamHI</td>
<td>TAGCTCTGCAGATGATGTTTCCTTCGCTGTTGC</td>
<td>GGGGGGATCCTAACCAGTTACGGGGTTACATTAG</td>
</tr>
<tr>
<td>pCMX-PL2-HA-RCB-trx</td>
<td>pCMX-PL2-HA</td>
<td>EcoR/ BamHI</td>
<td>TCTCGAGAAGCTGTGATATCGAAGATGTAATACAGTGTGG</td>
<td>TACTAGCTACTATGGCCGAGTTTAATACGGCAATCATAT                                      GAAACAAATTC</td>
</tr>
<tr>
<td>pCMX-PL2-RCBL(PT)4P-3HA-6His</td>
<td>pCMX-PL2</td>
<td>EcoR/ BamHI</td>
<td>TCTCGAGAAGCTGTGATATCGAAGATGTAATACAGTGTGG</td>
<td>TACTAGCTACTATGGCCGAGTTTAATACGGCAATCATAT                                      GAAACAAATTC</td>
</tr>
<tr>
<td>pCMX-PL2-RCB(PT)4P-3HA-6His</td>
<td>pCMX-PL2</td>
<td>EcoR/ BamHI</td>
<td>TCTCGAGAAGCTGTGATATCGAAGATGTAATACAGTGTGG</td>
<td>TACTAGCTACTATGGCCGAGTTTAATACGGCAATCATAT                                      GAAACAAATTC</td>
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Biography

Emily Jie-Ning Yang was born on June 15th, 1989 in Taipei, Taiwan to Eric Chin-Huei Yang and Dany Li-Ju Yeh. She attended National Taiwan University in Taipei, Taiwan and graduated in 2011 with a Bachelor of Science in Life Science. She will receive her Ph.D. in Biology, with a minor in Cell Biology from Duke University in July 2017. During her graduate studies, she was awarded the JBC/Herb Tabor Young Investigator Award in 2017 for the identification and characterization of the molecular function of RCBL. She was also awarded an International Conference of Arabidopsis Research travel grant in 2017, a Summer Research Fellowship from Duke Graduate School in 2016, Departmental Grant-In-Aid and Departmental Fellowships from the Department of Biology at Duke in 2015, a Conference Travel Award from the Duke Graduate School in 2015, and served as a mentor for the Howard Hughes Medical Institute Vertically Integrated Partners Program at Duke University in 2013 and 2014. During her undergraduate studies, she received the Best Poster Award from the Department of Life Science, National Taiwan University in 2011, an Undergraduate Research Fellowship from Republic of China National Science Council (Grant # 99-28150C-001-004-B) in 2010, and a Summer Undergraduate Internship from the Institute of Plant and Microbial Biology in Academia Sinica, Taiwan in 2009.

Publications:
