Photochemical Mechanisms in Brassica Rapa Photoreceptors

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PHOTOCHEMICAL MECHANISMS IN *BRASSICA RAPA*
PHOTORECEPTORS

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PHOTOCHEMICAL MECHANISMS IN *BRASSICA RAPA* PHOTORECEPTORS

A Thesis Presented to the Graduate Faculty of

Dedman College

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with a

Major in Chemistry

by

Shital Kale
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ABSTRACT

Light plays a significant role in plant growth and development. Plants utilize light as an information source to synchronize physiological processes with daily environmental changes. *Arabidopsis thaliana* is a useful genetic model to better understand plant circadian clocks and seasonal (photoperiodic) flowering. In *Arabidopsis*, plant cells contain several photoreceptors that can absorb different wavelengths of light. These photoreceptors including the ZTL LOV domain family, crytochromes, phytochromes and phototropins regulate many aspects of physiology in plants. These plant photoreceptors function as important cues for regulation of the plant circadian clock and photoperiodic flowering. The circadian clock and flowering time contribute to agricultural productivity. Given that global climate changes have a negative impact on agricultural crop production, and negatively impact circadian and photoperiodic processes, it is imperative that we understand the chemical basis for these pathways. Further, abiotic stresses cause molecular, physiological and biochemical changes that affect plant growth, development, and ultimately yield. Plants are sessile organisms that cannot escape from a persistently changing environment. *Brassica* species, which are closely related to *Arabidopsis thaliana*, are major world agricultural crop plants. To understand the biological functions of LOV photoreceptors in integrating environmental change into plant physiology, we focused on performing photochemical characterization of the BrLKP2 family in *Brassica* to identify possible functional differences between agricultural crops and the genetic model *A. thaliana*. 
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<tr>
<td>ARNT</td>
<td>Aryl Hydrocarbon Receptor Nuclear Translocators</td>
</tr>
<tr>
<td>AsLOV2</td>
<td><em>Avena sativa</em> LOV2</td>
</tr>
<tr>
<td>BMAL1</td>
<td>Brain and Muscle ARNT-Like 1</td>
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<tr>
<td>BrLKp2</td>
<td><em>Brassica rapa</em> LOV KELCH PROTEIN 2</td>
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<tr>
<td>CCA1</td>
<td>CIRCADIAN CLOCK ASSOCIATED 1</td>
</tr>
<tr>
<td>CCT</td>
<td>Cry C-terminal</td>
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<tr>
<td>CDF1</td>
<td>Cyclic DOF Factor 1</td>
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<tr>
<td>CLCOCK</td>
<td>Circadian Locomotor Output Cycles Kaput</td>
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<tr>
<td>CO</td>
<td>CONSTONS</td>
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<tr>
<td>CRY</td>
<td>Cryptochrome</td>
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<tr>
<td>DASH</td>
<td><em>Drosophila, Arabidopsis, Synechocystis, Human</em></td>
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<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FKF1</td>
<td>FLAVIN-BINDING, KELCH REPEAT, F-BOX1</td>
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<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
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<tr>
<td>FT</td>
<td>FLOWERING LOCUS</td>
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<tr>
<td>GI</td>
<td>GIGANTEA</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-galactopyranoside</td>
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<tr>
<td>LHY</td>
<td>LATE ELONGATED HYPOCOTYL</td>
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<tr>
<td>LKP2</td>
<td>LOV KELCH PROTEIN 2</td>
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<tr>
<td>LOV</td>
<td>Light-oxygen-voltage</td>
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<tr>
<td>LovK</td>
<td>LOV-histididine kinase</td>
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<td>PAS</td>
<td>Per-Arnt-Sim</td>
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<td>Pfam</td>
<td>Protein families</td>
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<td>PYP</td>
<td>Photoactive Yellow Protein</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>S-phase kinase-associated protein 1</td>
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<td>SIM</td>
<td>Single-minded</td>
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<td>Ultraviolet-visible</td>
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CHAPTER 1: INTRODUCTION

Plants as sessile organisms must develop extensive signal transduction pathways to adapt to a constantly changing environment. Climate changes and global warming are considered as severe environmental problems with adverse impact on almost all aspects of plant development, growth, reproduction and ultimately yield (1). Plants constantly monitor the surrounding environment through a sensor signaling mechanism. Light is one of the most important environmental factors, that plays a major role in plant signaling. Plants utilize light not only for photosynthesis but also as an information source to adjust their physiological processes (2). Light regulates a variety of responses including phototropism, stomatal opening, circadian clock, light inhibition of hypocotyl elongation, and the expression of specific genes (2,3). Control of these responses in plants is located within specific wavelengths in different regions of visible spectrum that are detected by different photoreceptors. These photoreceptors accurately survey ambient light conditions and regulate many aspects of physiology in plants to adjust to surrounding environmental changes. Plants contain various classes of photoreceptor molecules: red/far-red light sensing photoreceptors called phytochromes, blue light sensing cryptochromes, phototropins and members of the ZTL family (2, 3, 4).

The circadian clock and photoperiodic flowering contribute to agricultural productivity. *Arabidopsis thaliana* is a useful genetic model to understand the circadian clock mechanism and
biological function of other crop species like *Brassica* (1, 6). The diploid *Brassica* species are important sources of vegetables in our daily diet. In particular, *B. rapa* genomes are widely used vegetable crops in northeast Asia. To understand the biological function of *Brassica* species, it is important to know the photochemical activation of their photosensory proteins, which are responsible for cell signaling (1, 6, 7).

1.1 PAS domains: Sensor signaling modules

Survival of single-cell and multicellular systems requires accurate sensitivity to, and integration of, signals to sense and respond to their intracellular and extracellular environment. At the molecular level, signal receptor proteins that are of modular composition, detect a wide range of chemical and physical stimuli and, in response, regulate the biological activity of functionally diverse effector domains. Unlike other sensor modules, PAS domains are important sensory modules that are capable of monitoring changes in light, oxygen, redox potential, and small ligands. PAS domains are found in proteins from all kingdoms of life and regulate activities of effector domains including histidine and serine/threonine kinases, circadian clock proteins, voltage-activated ion channels, nucleotide phosphodiesterases and transcription factors (8, 9). PAS domains serve as signaling modules in multiple domain containing proteins. As a result, cellular response to changes in environmental conditions are controlled through PAS-containing transducers, regulators and receptors.

PAS stands for Per-ARNT-Sim domains from the name of the proteins in which imperfect repeat sequences were first identified. These include *Drosophila* period (PER), vertebrate aryl hydrocarbon receptor nuclear transporter (ARNT) and single-minded proteins (SIM) (8, 9, 10). PAS domains regulate various functions within signal transduction proteins and serve as an important mediator of protein-protein interaction. For example, the circadian
clock of mammals is generated by transcription factors that feed back and repress their own transcription. PAS domain containing BMAL1 and CLOCK transcription activators heterodimerize and activate transcription factors by binding to an E-Box gene (8).

PAS domains often bind covalently or non-covalently to a cofactor, including flavin nucleotide, p-coumaric acid, heme, carboxylic acid and divalent metal cations. The phototropic response in plants, fungi and bacteria is regulated by LOV-domain family of blue light photoreceptor, a subclass of PAS domain that binds covalently to flavin cofactor in response to blue light absorption (8, 10).

1.1.1 Structure and mechanism of PAS domains

PAS domains were first identified by homologous regions of 50 amino acids in proteins PER, ARNT and SIM. To date, the Pfam database includes 21,000 entries of PAS domain containing proteins. These are found in all kingdoms of life where 81%, 13% and 6% derived from prokaryotes, eukaryotes and archaeal proteins respectively (9). PAS domains were initially described as a PAS/PAC motif or S1/S2 box to form a single globular protein which contains 100 amino acids, these motifs were consolidated to a single domain upon determination of the first PAS structures. The first three-dimensional structure of PAS domains containing photoactive yellow protein (PYP) from Halorhodospira halopila, showed that the PAS fold is composed of a single anti-parallel, five-stranded β-sheet and the several α-helices of PAS fold create a pocket adjacent to the β-sheet. More than a dozen PAS domain structures have been solved and deposited in the Protein Data Bank. Crystal structures of PAS domains show the same overall protein folding as the PAS domain of PYP (8, 10).

The PAS domain refers to a single protein region, composed of several intervening α-helices Ca, Da, Ea and Fa and central antiparallel five standard β-sheet Aβ, Bβ, Gβ, Hβ, and Iβ
in the topological order B-A-I-H-G that is, 2-1-5-4-3 (8). The conserved PAS core senses and responds to signals and generates structural and dynamic changes within the core, from which the PAS core propagates a signal via α-helical and coiled-coil linkers at the N- or C- terminal to the covalently attached effector domain. Within one protein, multiple PAS domains are named as PAS A and PAS B (12).

Several PAS domains bind to cofactors to mediate signal transduction and initiate protein-protein interactions. The LOV subclass of photosensory PAS domains bind to flavin cofactors to induces conformational changes in response to blue-light. For example, the photosensory PAS domain of *N. crassa* Vivid was reported to undergoes structural and dynamic changes of the region of β-sheet upon illumination with light (11).

### 1.2 Flavin based blue light photoreceptor

Light is a ubiquitous signal for biological function in terrestrial and aquatic ecosystems, and many organisms across multiple kingdoms have the capability to respond to this environmental cue. Several blue light photoreceptors dynamically control a wide range of biological and physiological processes in response to light via binding to flavin cofactors. Blue light sensing photoreceptors regulate physiological responses, including signal transduction in plants, circadian clock responses, DNA-repair enzymes, cyclase activity and stress responses (12). Most blue light photoreceptor families, including BLUF, cryptochrome and LOV, utilize blue-light absorbing flavin chromophores in the form of either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). BLUF, LOV and CRY photoreceptors containing the flavin chromophore demonstrate different photochemistry, signaling transduction mechanisms and biological function upon absorption of blue light (13, 14).
1.2.1 Cryptochrome

Cryptochromes (CRYs) are identified in a wide array of organisms including plants and animals, where they function as a blue light sensing photoreceptors. Cryptochromes are homologous to DNA photolyases, but lack DNA repair activity (3,13). CRYs can be divided in three families based on their sequence similarity i) plant CRYs ii) animal CRYs and iii) CRY-DASH proteins (13). Plant CRYs were first discovered in Arabidopsis thaliana, where they are responsible for regulation of seed germination, elongation and phototropism (15). In A. thaliana, cryptochrome 1 and 2 (Atcry1 and Atcry2) are localized mainly in the nucleus. Atcry1 has roles in regulating seedling de-etiolation and Atcry2 takes part in photoperiodic flowering and regulates low-light dependent ubiquitination and degradation in the nucleus. Arabidopsis Atcry1 and Atcry2 are composed of N-terminal photolyase homology region (PHR) and C-terminal extension (CCT). The PHR domain regulates the photosensing and CCT domain, which functions as a protein-protein interaction module to regulate effector proteins such as the ubiquitin ligase COP1 (15, 24). The N-terminal or C-terminal carry short sequence motifs, known as the DAS motif, which contains acidic and serine-rich sequence (14). Animal CRYs are found in insects, mammals and fish, where they act as either light-dependent or light independent regulators of the circadian clock. Recent studies indicate that cryptochromes are important for birds in magnetic orientation during migration and also act as magnetoreceptor of butterflies to sense magnetic field (13, 14).

1.2.2 BLUF

BLUF stands for blue light sensing using FAD. Upon absorption of blue light, BLUF domains covalently attached effector domains such as adenyl cyclase, EAL domains and GGDEF domains. Protein containing BLUF domains include adenylate cyclase in Euglena
gracilis, YcgF in Escherichia coli, and Slr1694 in Synechocystis sp. PCC6803 (16). BLUF domains are composed of a ferredoxin-like fold with two α helices flanking the isalloxazine ring of bound FAD molecule, which is perpendicular to a five-stranded β-sheet. AppA functions as a transcriptional regulator of photosynthetic gene expression. AppA contains two domains, an N-terminal BLUF domain that non-covalently binds FAD cofactor, and at the C-terminal a cysteine-rich domain is bound to photosynthetic transcriptional genes repressor PpsR. Upon absorption of blue light, AppA induces a conformational change that prevents AppA from interacting with PpsR, which inhibits photosynthetic gene expression (16). BLUF domains are more unique than other flavin containing blue light photoreceptors. Upon blue-light excitation, BLUF domain leads to 10nm red-shifted signaling state from dark state. This conformation is due to conserved glutamine and tyrosine residues nearby to active site FAD cofactor (13, 15)

1.2.3 LOV domain family

A specific subset of the PAS domain superfamily, the LOV domains function as blue light sensors to control cellular responses to light via flavin cofactor. Flavin-binding LOV domains are protein photosensors known to be conserved across a diverse range of proteins in prokaryotes, eukaryotes and archaea. These photosensory LOV domains are couple to a wide array of multi-domain proteins, including kinases, DNA-binding domains (Zinc finger, leucine zipper), phosphodiesterase, F-box domains, STAS domains that control a blue light dependent physiology (11).

In A. thaliana, LOV domain containing proteins ZTL, LKP2 and FKF1 take part in regulation of circadian timing and photoperiodic flowering. The fungus Neurospora crassa contains two LOV domain photoreceptors, White Collar-1 and Vivid, that regulate blue light responses and reset the circadian clock. (17, 18, 19). In recent studies, LOV proteins ENVOY, a
The homolog of *N. crassa* VIVID, regulates cellulase expression, metabolic function and stress responses in the fungus *T. reesei*, which functions as a model organism for plant cell wall degradation (21). LOV domains containing proteins are also abundant in bacteria. Flavin containing LOV-STAS proteins, YtvA functions as a blue light photoreceptor and regulator of stress factor in *Bacillus subtilis*. On blue light absorption, histidine autophosphorylation is regulated in *B. abortus* by a LOV domain coupled histidine kinase (11).

### 1.3 Light sensing photoreceptors in plants

Plants constantly monitor various changes in the environment through a sensor signaling mechanism. Environmental factors have wide regulatory influence on growth and development in plants. Light is one of the most important environmental clues for plants. Plants utilize light not only for photosynthesis but also information source to adjust their physiological responses and biological events. Plant cells contain several photoreceptors that can absorb different wavelength ranges of light. These photoreceptors include phytochromes, blue light sensing cryptochromes and phototropins (2,3). These photoreceptors sense changes in the ambient light condition and adjust their development, morphology and metabolic path to optimize their fitness to the surrounding environment.

Phytochromes and cryptochromes regulate various developments such as seed germination and hypocotyl elongation. In plants, to date, three LOV domains containing blue light photoreceptor families have been identified. The first one is phototropins, which are responsible for stomatal opening, phototropism, chloroplast and leaf movement. The second is the ZTL/FKF1/LKP2 family and third is PAS-LOV protein (PLP), which signaling mechanisms are not determined yet (3, 15).
1.3.1 ZTL/FKF1/LKP2 family

The ZEITLUPE (ZTL), FLAVIN BINDING, KELCH REPEAT, F-BOX (FKF1) and LOV KELCH PROTEIN2 (LKP2) group are LOV domain based blue-light photoreceptors that are known to be involved in protein degradation and protein-protein interaction (3,19). All three members of this family share a conserved structure containing a LOV domain at the N-terminus coupled to an F-box domain involved in SCF complex formation and protein degradation. Function is facilitated by kelch repeats at the C-terminal domain that take part in protein-protein interaction (Fig 1).

![Figure 1: A. Thaliana ZTL LOV domain family](image)

The three members of ZTL LOV domain family are composed of a five-standard antiparallel β-sheet flanked on one side by series of α-helix consistent with other LOV/PAS domain proteins. The sequence alignments of ZTL group proteins more closely resemble LOV1 domains compared to the LOV2 domain of plant phototropins. The blue light photoreceptors ZTL, FKF1 and LKP2 contain bound flavin cofactor. Upon illumination of blue light, a
conserved cysteine residue forms a C4a adduct with flavin. The adduct spontaneously breaks in darkness. In studies, it was confirmed that adduct scission occurs with time constants for FKF1 and LKP2 of more than 65 hours but for ZTL only 2.6 hours (2, 19, 20). Combined biochemical, genetic and the structural studies of ZTL provided new mechanisms, which indicate how an internal biological clock is regulated by light (20). These include important roles of Gigantea (GI) that takes part in the stability and abundance of ZTL proteins. GI proteins control ZTL protein degradation through directly binding to the LOV domain of ZTL. This GI-ZTL complex is enhanced in response to blue light. Similarly, FKF1 regulates photoperiodic flowering by activation of CO transcriptions. Upon absorption of blue light LOV domain of FKF1 bind GI and this complex degrades CDF1 proteins and regulates CO gene transcription (2).

1.3.2 PAS/LOV proteins

The *A. thaliana* PAS/LOV PROTEIN (PLP) gene is encoded into three PLP splice variants, which are named as PLPA, PLPB and PLPC. PLP contains one PAS domain at the N-terminus and LOV domain at the C-terminus. PLP containing PAS domains are different from other LOV domains of PAS family because they lack the conserved cysteine residue for photochemical activation. PLP interacts with other proteins, but protein:protein interaction is diminished upon illumination of blue light. PLP contains a LOV domain so it is known as blue light photoreceptor but physiological function and biochemical activity have not been proven (3, 23).

1.3.3 Phototropins

Phototropins are blue light photoreceptors that play fundamental roles in regulation of plant growth and development. Phototropins are plasma membrane bound photoreceptors that that have a significant role in the regulation of phototropism, chloroplast movement and stomatal
opening in seed plants. Since the first identification of *Arabidopsis* phototropin in 1997, phototropins have been identified in fern *Adiantum capillus-veneris*, moss *Physcomitrella patens* and green alga *C. reinhardtii*. Plant phototropin structures are divided into two segments: photosensory domain LOV1 and LOV2 located at N terminus and serine/threonine kinase domain at C terminus. These are connected by a short linker termed the Jα helix. LOV2 domains are known for regulation of phototropin signaling, whereas function of LOV1 remains unclear but it takes part in phototropin dimerization. In the dark state, phototropins are phosphorylated and the C-terminal containing kinase domain is inactivated. Upon absorption of blue light the photoreceptor is autophosphorylated on multiple site and the kinase domain is activated with light-stimulated unfolding of the Jα helix. The LOV1 and LOV2 domain show similar photochemistry like LOV domain of PAS superfamily (15).

### 1.4 Homologs of *Arabidopsis* photoreceptors genes in *B. rapa*

Economically, *Brassica* species are used as source of dietary vegetables and plant oils. The genus *Brassica* includes a number of important crops such as *B. napus*, *B. rapa*, *B. carinata* and *B. juncea*, which provide about 12% of the worldwide vegetable oil production. The three-diploid species *B. rapa* (A genome), *B. nigra* (B. genome) and *B. oleracea* (C genome) have undergone genome triplication that have involved considerable gene loss since their divergence from *Arabidopsis*, leading to questions about how well conserved the circadian and photoperiodic pathways are in agricultural crops (6, 26).

Plants growth is sensitive to the environment. Global climate changes have a negative impact on the agricultural crop production. Plants as sessile organisms cannot escape from persistently changing environment. Abiotic stresses cause molecular, physiological and biochemical changes that affect plant growth, development, and ultimately yield. *B. rapa* crop
plants are adversely affected by drought, cold and heat that induce stress related symptoms (37). The plant circadian clock and photoperiodic flowering coordinate their physiology and metabolism to daily environmental changes. *Arabidopsis thaliana* acts as a model for other crop plants to better understand circadian clocks and photoperiodic flowering. The circadian clock comprises regulatory transcription and translation feedback loops to modulate circadian rhythm and protein function. In *Arabidopsis*, photoreceptors play an important role to control the circadian clock and photoperiod dependent flowering (1, 5,7).

The circadian clock consists of a series of transcriptional and post transcriptional feedback loops to create oscillations in gene expression. The clock genes are described on the base of their timing of expression pattern (1, 5). In *Arabidopsis*, the Myb-type-2 transcription factors LHY and CCA1 are expressed at dawn, and directly activate expression of daytime PRR gene family members PRR9 and PRR7. CCA1 and LHY act as negative elements to suppress several evening genes, including, GI, TOC1, PRR5, LUX, ELF3, and ELF4. The PRR genes, including PRR9, PRR7 and PRR5 inhibit expression of CCA1, LHY and RVE8. RVE8 genes induce the expression of evening genes. The F-box containing ZTL family regulates protein levels of TOC1 and PRR5. The F-box of ZTL family members interact with SKP1, CULLIN, and RBX1 to form SCF complex that targets TOC1 and PRR5 genes for ubiquitination and subsequent degradation (1, 5, 6). The photoperiodic flowering in *Arabidopsis* is promoted through function of FT and CO. FT gene expression is induced by the transcriptional activator CO protein. CDF1 and other CDFs are the transcription factors that repress CO expression during the morning. In the afternoon, CDFs are degraded by a complex of FKF1 and GI. The timing of flowering largely depends upon daily expression of CO. FKF1, GI and CDF proteins play major roles in accelerating the expression of CO (2).
Flowering is a highly important trait for the cultivation of crop plants. Variations in circadian rhythms and photoperiodic flowering affect growth and yield of plants. The *Brassica* species are the closest crop families to the genetic model *A. thaliana* (1, 26). Homologs of photoreceptor genes of *Arabidopsis* are present in *B. rapa* (Fig.2 B). Despite considerable gene loss after genome triplication of *B. rapa*, three copies of LKP2 (termed a, b and c) are present and involved in regulation of plant growth and development (Fig. 2 A). In order to understand the biological functions of *Brassica* crop plants, it is vital to understand the photochemical mechanism of these photoreceptors.

**Figure 2: Functional domains and sequence alignments of photoreceptors:** A) Functional domains of BrLKP2 photoreceptor B) Sequence alignments of blue light photoreceptors

The light induced activation is dependent on flavin C4a adduct formation. The photoadduct formation leads to conformational changes that activate biological responses. In term of signaling, adduct formation in the phototropin LOV2 domain results in dissociation of the Jα helix that is located at C-terminal of LOV domain. The signal propagation in phototropin LOV2 is initiated by N5 protonation, that results in rotation of a conserved glutamine side chain
to alter H-bonding to N5 position. The active-site glutamine residue is believed to be essential for function in all LOV proteins (28, 29). Surprisingly, in *Brassica* and AtLKP2 photoreceptors a leucine is contained at the position equivalent to the conserved glutamine found in all other LOV proteins. Notably, Gln to Leu substitutions abolished light induced activity in all other LOV proteins that have been reported. Recent work demonstrated that the Gln-flip mechanism is not necessitated for signal transduction in AtZTL. Since, G80 residue of AtZTL affect allostery and tune signal duration (20). To understand signal transduction of blue light photoreceptors of *Brassica rapa*, we used biochemical techniques to distinguish how active-site substitution and conserved residues affect photochemistry.
2.1 Photochemical characterization of *B. rapa* photoreceptors

Photochemistry of blue-light photoreceptor is characterized by light induced formation of a covalent adduct between cysteine residue and bound flavin cofactor. Herein, we studied photochemistry of BrLKP2 photoreceptors through photochemical characterization.

2.1.1 BrLKP2 photochemistry

Modular LOV domain photoreceptors are present in plants, bacterial species, and fungi. The flavin-binding LOV domains act as reversible photoswitches to regulate a diverse array of blue-light signal transduction pathway (3, 13, 27). The photocycle of LOV domain photoreceptors are regulated by photochemical properties of the bound flavin cofactor and surrounding environment. Upon illumination of light, LOV photoreceptors undergo a cyclic photoreaction similar to that of the photocycle of phototropin. LOV domains show light induced absorbance changes upon transfer from the dark state to the light state. The dark-state spectra is characterized by an oxidized flavin chromophore that absorbs maximally around 450 nm with vibronic structure near 425 and 475 nm. Upon blue light irradiation, the chromophore bleaches in the 450 nm region with formation of new absorption peak at 390 nm (13, 39).
The photoreceptor undergoes photochemical activation in which photoexcited flavin cofactor and conserved cysteine residue form a covalent adduct (Fig.3 A). The stability of the photoadduct is variable among members of the LOV domain family. The photoadduct thermally decays back to the non-covalent dark state within several seconds to many hours (27, 28). Despite the same photochemical reaction, the LOV domain photoreceptors are separated into two groups based on their photoadduct decay kinetics. The LOV2 domains of phototropins are fast cyclers with short photocycle lifetimes. Some phototropins with LOV1 domains and fungal and bacterial LOV domain photoreceptors are slow cyclers with long photocycle lifetimes. Sequence variations of active site residues within photoreceptor show changes in the active site environment may responsible for tuning photocycle kinetics. The photoadduct decay rates of AsLOV2 \((t_{1/2}\sim80\text{s})\) and EL222 \((t_{1/2}\sim30\text{s})\) are shorter, whereas YtVA \((t_{1/2}\sim3000\text{s})\), LovK\((t_{1/2}\sim6000\text{s})\) and VVD \((t_{1/2}\sim18000\text{s})\) are placed into slow cycling class with longer photocycle kinetics (27, 28).

BrLKP2c undergoes a photocycle consistent with other LOV proteins (Fig.3 B). UV-Vis spectrophotometry indicates the presence of an oxidized flavin in the dark. The ground state of the flavin cofactor shows absorption spectra of protein with maximum absorption at around 450 nm. Upon illumination of blue light, the 450 nm absorption band is bleached, leading to formation of a 380 nm absorbing species. Of particular interest to the current study is the rate of spontaneous adduct decay upon return to darkness.
According to resent studies, plant LOV domain photoreceptors show various photoadduct decay rates, which are important to dictating sensitivity to the environmental light. Photoadduct can decay to the ground state via either light stimulated adduct decay or a thermal decay. The following kinetic model represent explanation of photodynamic equilibrium (19). The transformation from light state to dark state is considered as general reversible reaction.

\[ \text{light}(L) \rightleftharpoons \text{dark}(D) \]

The cysteiny1 C4a adduct returns back to the dark state through either a thermal process or photochemical process.
Rate of adduct decay: thermal

\[
\frac{d(L)}{dt} = -k_T (L)
\]  

(1)

Rate of adduct decay: photochemical

\[
\frac{d(L)}{dt} = -k_R (L)
\]  

(2)

Rate of adduct formation: photochemical

\[
\frac{d(L)}{dt} = -k_F (D)
\]  

(3)

Therefore, the overall rate can be defined as

\[
\frac{d(L)}{dt} = -k_F (D) - (k_R + k_T)(L)
\]  

(4)

By using steady state equilibrium approximation, we obtained new equation,

\[
(k_R + k_T)(L) = k_F (D)
\]  

(5)

The following equation can be arranged to define (L): (D) ratio as

\[
\frac{(L)}{(D)} = \frac{k_F}{(k_T + k_R)}
\]  

(6)

Here the kinetic constants, \(k_F\) and \(k_R\) can be defined in the term of fluence rate \(F(\lambda)\), absorption cross section \(\sigma(\lambda)\), quantum yields of adduct formation \(\phi_F\) and adduct decay \(\phi_R\).

Thus, the rate constant \(k_F\) and \(k_R\) are defined by following expression,
By combining the equations 6, 7 and 8, we obtained following equation,

\[ k_R = \phi_R \int_{360 \text{nm}}^{500 \text{nm}} F(\lambda) \sigma_R(\lambda) \, d\lambda \]  

(7)

\[ k_F = \phi_F \int_{360 \text{nm}}^{500 \text{nm}} F(\lambda) \sigma_F(\lambda) \, d\lambda \]  

(8)

By combining the equations 6, 7 and 8, we obtained following equation,

\[ \frac{(L)}{(D)} = \frac{\phi_F \int_{360 \text{nm}}^{500 \text{nm}} F(\lambda) \sigma_F(\lambda) \, d\lambda}{k_T + \phi_R \int_{360 \text{nm}}^{500 \text{nm}} F(\lambda) \sigma_R(\lambda) \, d\lambda} \]  

(9)

The final equation (9) is used to evaluate the ratio of light and dark of LOV domain proteins under a certain range of fluence rate. Here we used quantum yield \( \phi_F = 0.5 \) and \( \phi_R = 0.30 \), which are derived from other systems. We use 360 nm- 500 nm spectral window because that range is relevant for LOV domain signaling in plants. By using the equation (9), we can predict the relative populations of light to dark states of LOV domains photoreceptors.

### 2.1.2 Recovery rate of kinetics

Upon blue-light excitation, the oxidized flavin cofactor absorbs a photon, leading to formation of the singlet excited state and then interconversion to a triplet excited state. From the triplet state, the sulfur atom of the conserved cysteine residue forms a covalent adduct with isoalloxazine ring (27, 28). BrLKP2c proteins were photoexcited then allowed to decay spontaneously to the ground state to obtain a recovery rate for the photocycle. UV-absorption spectrophotometry was performed to collect spectra at defined intervals for several hours. The photoadduct decay rates were calculated by fitting the light to dark state recovery data into a
mono-exponential function. The recovery rate of BrLKP2c and b is long compared the LOV domains of ZTL family (Table 1) (Fig-4).

The previous study shows that a proton-transfer reaction from N (5) is rate-limiting step for the thermal reversion. The recovery rate of kinetics not only depend upon photochemical properties but also active site residues within the protein (32,33). The longer photokinetics can be affected by various factors including pH, solvent accessibility, hydrogen bonding, and the flavin electronic environment (27, 28). The BrLKP2c photoreceptor shows a longer lifetime (t1/2~32 to 35 hours) as compared to other flavin binding LOV domain photoreceptors including AsLOV2 (t1/2~80s), VVD (t1/2~18000s) and EL222 (t1/2~30s). The adduct decay rate is different compared to its homologs within the ZTL family. FKF1 and LKP2 LOV domains show longer kinetics (τ> 65 hours) as compared to ZTL, which converts from light state to dark state within few hours (τ=2.7 hours) (2,19). Substitutions of active site residues within LOV domains have revealed mechanisms of adduct decay formation. According to previous studies, mutagenesis at conserved residues affect photocycle lifetime through indirect interactions such as increased solvent accessibility to the active site (31). For example, a Ile74Val variant accelerates the rate of photoadduct decay in VVD-36. Further, a Gln153Leu mutation strongly affects photocycle dynamics in AsLOV2 (13, 27, 28). Given that BrLKP2 contains a Leu at this equivalent position, we created a Leu149Gln substitution to determine if it was contributing to the long photocycle kinetics in BrLKP2. Indeed, substitution at Leu149 had a significant effect on adduct decay kinetics in BrLKP2c (Table 1).
**Figure 4: Photocycle kinetics:** Mono-exponential fitting of BrLKP2c dark state recovery kinetics trances at 450 nm and 478 nm wavelength

<table>
<thead>
<tr>
<th>Variant</th>
<th>Amino acid residue</th>
<th>Time constant (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrLKP2c</td>
<td>29-165</td>
<td>&gt;115200</td>
</tr>
<tr>
<td>BrLKP2b*</td>
<td>29-165</td>
<td>144000</td>
</tr>
<tr>
<td>AtZTL</td>
<td>29-165</td>
<td>6200</td>
</tr>
<tr>
<td>AtLKP2**</td>
<td>16-165</td>
<td>491014</td>
</tr>
<tr>
<td>BrLKP2c L149Q</td>
<td>29-165</td>
<td>18000</td>
</tr>
</tbody>
</table>

**Table 1: Recovery rate kinetics of photoreceptors:** The kinetic measurement of BrLKP2c and variants. * and ** indicate data are provided by and Shannon Swisher and Jameela Lokhandwala respectively. (Zoltowski lab. SMU)
2.1.3 Eyring and Arrhenius kinetics of adduct decay

The wild type BrLKP2c protein shows a typical LOV domain photocycle. Upon illumination of light, the photoadduct spontaneously decays back to dark state with a time constant of approximately 35 hours at room temperature. The temperature dependence of photokinetics can provide insight to the mechanism of adduct decay and facilitate understanding of functional differences between photoreceptors. Towards this end, we performed Eyring and Arrhenius analysis temperature dependence study of the dark state recovery kinetics of BrLKP2c and L 149 Q mutant (Fig.6, table 2). Before characterize the protein at high temperature, the thermostability of protein was determined by thermofluor assay. We observed that proteins are stable at above 323K temperature (Fig. 5).

![Graph showing temperature dependence of protein stability](image)

**Figure 5: Thermostability of photoreceptor**

Eyring plot analysis reveals that proteins are less temperature dependence because of low enthalpy of activation and entropy of activation is less favorable at high temperature. Arrhenius
data displays that energy of activation is increased at high temperature. As per study, conserved active site residues within LOV domains play a key role to accelerate the recovery rate of kinetics (28).

Figure 6: Eyring and Arrhenius kinetics of adduct decay: A) Arrhenius analysis of BrLKP2c B) Eyring analysis of BrLKP2c

Table 2: Thermodynamic parameters for dark state reversion: The thermodynamic parameters $E_a$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are determined by fits to different temperature points.

<table>
<thead>
<tr>
<th>Variant</th>
<th>$E_a$ (kJmol$^{-1}$)</th>
<th>$\Delta H^\ddagger$ (kJmol$^{-1}$)</th>
<th>$\Delta S^\ddagger$ (JK$^{-1}$mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>85.80</td>
<td>83.30</td>
<td>-61.60</td>
</tr>
<tr>
<td>Mutant</td>
<td>55.40</td>
<td>52.40</td>
<td>-144.80</td>
</tr>
</tbody>
</table>
2.1.4 Solvent effect

According to study, N (5) deprotonation and steric stabilization are the primary mediators of photoadduct decay kinetics (34). Several studies have led to general mechanism of adduct decay. First, solvent-isotope effects show abstraction of proton is rate limiting step. Second, adduct decay is catalyzed by small molecule bases. Third, residue substitution acts as an intrinsic base that regulate solvent access.

![Image of graph showing effect of solvent on photoadduct decay kinetics of BrLKP2c](image.png)

**Figure 7: Effect of solvent:** Photoadduct decay kinetics of BrLKP2c

Early studies shown that the base act as an effective enhancer of the dark recovery rates of AsLOV2. According to base catalysis mechanism, LovK constructs show different recovery
rates at various imidazole concentration (28, 40). Here we found that the dark state recovery rates of BrLKP2c was enhanced by increasing concentration of extrinsic base (Fig. 7).

2.3 Analysis of dimerization

Previous studies demonstrated that Cys residue near the protein active site can be involved in a conformation switch that modulates dimerization and protein function (17, 35). To examine whether similar processes could be happening in BrLKP2c, we studied dimerization under reducing and oxidizing conditions. The result from SDS-PAGE and SEC suggest that the BrLKP2c proteins show dimeric association of (Fig.8).

In recent studies, it is revealed that ZTL domain form anti-parallel dimer through extensive contacts along β-scaffold (20). The interaction between helical E-F interface and associated form α helical dimer. The β-sheet anti-parallel dimer interfaces are stabilized by interaction of C45, F47 and I153 residue within core. The R95 residue, which is located within loop of Eα-Fα helices, forms salt bridge with the phosphate moiety of chromophore of VVD for stabilization of dimer interface (17, 18). The present study demonstrates that BrLKP2c exists as a dimer that may be similar to ZTL. Notably, in the absence of reducing agents BrLKP2 proteins formed dimers under denaturing conditions, but dimerization was abolished in presence of reducing condition. Therefore, disulfide linked dimerization is likely involved in BrLKP2 function.
Figure 8: **Analysis of dimerization:** A) SDS-PAGE analysis-Lane 12 shows monomer (+BME) and Lane-13 shows disulfide linked dimer in absence of reducing condition (-BME). B) SEC demonstrates dimerization peaks at high and low concentration of protein.
CHAPTER 3: SUMMARY

*Brassica* species, which are closely related to *Arabidopsis*, are an internationally important crop plant. *Brassica* oilseeds, are known as canola oil, which provide edible vegetable oil to the world. *Arabidopsis thaliana* assisted as a genetic model plant to understand clock mechanism and function. The blue light photoreceptor LKP2 not only enhance cell elongation but also increase cell number and ploidy in hypocotyl in *Arabidopsis*. The triplicated genome of LKP2 family of *Brassica rapa* could take part in plant growth and development. Here, we studied photochemistry of BrLKP2 photoreceptors to understand their biological function in *Brassica rapa*. Previous studies showed that, active site Gln residues take part in regulation of LOV domain signaling. Despite Leu to Gln mutation, the photocycle lifetime of BrLKP2c is longer as compared to *Arabidopsis* ZTL. This likely due to AtZTL contains a Gly residue which lies in a motif known to alter photocycle kinetics (20). According to our studies, the biochemical function of BrLKP2 photoreceptor remains illusive.
CHAPTER 4: MATERIALS AND METHODS

4.1 Cloning, protein expression and purification

BrLKP2c constructs were cloned using the nucleotides encoding full-length *Brassica Rapa* of LKP2. PCR products were verified by agarose gel electrophoresis. PCR products of BrLKP2c and the GST parallel vector were digested with EcoRI and NotI restriction sites. BrLKP2c constructs 29-165, 33-169 were cloned for expression in *E. coli*. Site-specific mutation was carried out according to the quick-change protocol. All protein constructs and mutants were verified through Genwiz Sequencing Service.

All GST constructs were expressed in JM109 *E. coli* strains. Cells were grown at 37 °C until reaching an OD$_{600}$ of 0.5-0.6. At this point, temperature was lowered to 18 °C for 40 min and then induced by addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were grown at 18 °C for about 18-20 hrs at 220 rpm. The cell pellets were harvested by centrifugation and stored in 100 mM NaCl, 50 mM HEPES (pH8) and 10% glycerol (stabilizing buffer) at -80 °C.

For protein extraction, the frozen cell pellets were thawed at room temperature and lysed using sonication. The cell lysate was clarified with centrifugation at 18,000 rpm at 4 °C for 1-hour. For first stage of protein purification, the soluble fraction was loaded onto glutathione
affinity columns (Qiagen). After binding, GST-tagged proteins were treated with 6-His-TEVprotease overnight at 4 °C to remove GST affinity tag. An additional round of Ni-NTA chromatography was conducted to eliminate 6His TEV protease from cleaved GST proteins prior to final purification with a Superdex S200 size exclusion chromatography.

4.2 Size Exclusion Chromatography

To verify protein purity, the final eluted protein from affinity chromatography was loaded onto a Hiload Superdex 200 16/60 gel filtration column (GE Lifesciences) pre-equilibrated with stabilizing buffer. The eluted protein fractions were collected and verified through electrophoresis and UV-Vis spectroscopy method, and then proteins were concentrated for photochemical characterization experiments.

4.3 SDS-PAGE

The purity of purified protein was verified through SDS PAGE. Protein samples were mixed with 1x SDS loading buffer and denatured by heating at 70 °C for five min prior to loading onto a 12% Tris-Glycine Gel. After completion of electrophoresis, the gel was removed and submerged overnight in Coomassie solution for staining. The stained gel was soaked in destain solution until protein bands became visible. The gel was then washed several times in dH2O before drying.

4.4 Determination of protein concentration

LOV-domain protein contains a flavin chromophore, which absorbs light in the UV region of the spectrum. The concentration of flavin bound protein constructs were determined by using the Lamber-Beer equation.

\[ A = \varepsilon b c \]
where $c$ is the concentration (M), $A$ is the absorbance; $\varepsilon$ is the extinction coefficient in M$^{-1}$ cm$^{-1}$, $b$ is the pathlength in cm. Using the known value of the molar extinction coefficient at 450 nm from previous studies ($\varepsilon_{450\text{nm}} = 1310.0$ M$^{-1}$ cm$^{-1}$), the concentration of protein was measured at 450 nm (36).

**4.5 UV-Vis absorbance spectroscopy and kinetics**

UV-Vis absorbance measurement for all constructs were performed using a temperature-controlled Agilent 8453 spectrophotometer. Purified proteins were concentrated to 50 $\mu$M for UV-Vis absorbance measurement and kinetics. Spectra of protein in dark and light states were recorded using a clean quartz cuvette with a pathlength of 1.0 cm. Light-adapted protein samples were prepared by photobleaching on ice via irradiation with a broad-spectrum white flood light source (150 W) or blue light laser. Kinetics of photoadduct decay was analyzed by measuring the absorption at 450 nm and 478 nm as a function of time. All values for 450 and 478 nm were corrected by subtracting the absorbance at 600 nm, which was used as a baseline.

**4.6 Thermofluor assay**

The thermofluor assay was conducted using a qPCR instrument to check the thermal stability of protein at high temperature. The temperature and fluorescence were monitored by running the protein sample including buffer and protein thermal shift dye in triplicate. The protein thermal shift dye fluoresces in hydrophobic environments. The fluorophore dye allows to differentiate between folded and unfolded states of protein. At low temperature, fluorescence is not observed because protein is folded and hydrophobic surfaces are not exposed. Upon an
increase the temperature, protein starts to unfold, hydrophobic areas become exposed. When hydrophobic surfaces of protein bind to dye, resulting in an increase in fluorescence emission. To determine the meting point of protein, the fluorescence intensity is plotted as a function of temperature.

### 4.7 Eyring and Arrhenius analyses

To study temperature dependent kinetics of photoadduct decay, all protein constructs were characterized at different temperatures. The resultant data at various temperatures were fit using a monoexponential equation. The Arrhenius analysis was performed to obtain the activation energy. The enthalpy and entropy of activation for adduct formation was calculated by using Eyring equation.

The following equation of Eyring analysis,

$$
\ln \left(\frac{k_T}{T}\right) = -\frac{\Delta H^\ddagger}{RT} \times \frac{1}{T} + \ln \left(\frac{k_B}{h}\right) + \frac{\Delta S^\ddagger}{R}
$$

Where, $k_T$, $T$, $R$, $k_B$ and $h$ are rate constant, temperature in K, molar gas constant ($8.31 \text{ J mol}^{-1} \text{ K}^{-1}$) and Boltzmann’s constant ($1.38 \times 10^{-23} \text{ JK}^{-1}$) and Planck’s constant’s ($6.626 \times 10^{-34} \text{ J s}$) respectively. The enthalpy of activation ($\Delta H^\ddagger$) and entropy of activation ($\Delta S^\ddagger$) were determined by measuring rate constant values at different temperature. Plotting $\ln \left(\frac{k_T}{T}\right)$ against $\frac{1}{T}$ gives a straight line with slope component and intercept component representing the enthalpy and entropies of activation respectively.

### 4.8 Base catalysis

It is known that small biological bases such as imidazole enhance the rate of thermal decay from cysteiny1-c4a adduct state back to the non-bonded dark state. Solvent accessibility
and base catalysis in proteins were assayed by the ability of imidazole to access the active site and catalyze adduct reversion. A stock solution containing 1.0 M imidazole, 100 mM NaCl, 50 mM Hepes (pH 8.0) and 10% glycerol were prepared. Protein samples were diluted to a concentration of 50 µM and keep constant at various concentration of imidazole ranging from 0-500 mM imidazole. Kinetic parameters of imidazole catalysis were calculated by measuring the absorbance at 450 nm and 478 nm as a function of time.
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