What is LOV?

An Analysis of LOV Domains and Their Potential Role in Plant Oxygen Sensing

MASTERTHESIS

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Abstract
In order to survive, grow and reproduce, it is crucial for organisms to sense and react to their environments. Light-Oxygen-Voltage (LOV) domains are well-known environmental sensors that are widespread in bacteria, archaea, plants and other eukaryotes. While plant LOV proteins have yet only been found to have light-sensing roles, the conservation and divergent roles of LOV domains in other kingdoms of the tree of life suggests a possible additional role for plant LOV domains in oxygen sensing. Here, we describe the role of plant LOV domains in light perception and we investigate whether plant LOV proteins could indeed function in oxygen sensing by analyzing the roles and mechanisms of LOV domains in light sensing proteins of *Arabidopsis thaliana* as well as oxygen sensing roles of LOVs in other species. Both commonalities and differences are seen for light-sensing proteins regarding the roles of their LOV domains, and their functioning appears to be flexible. Looking at the oxygen sensor proteins, it is noticeable that all described oxygen-sensing proteins function via changes in redox status of the FMN/FAD cofactors, and that it was often seen to go together with light sensing functions. Known plant LOV proteins could thus possibly have an extended redox-based oxygen sensing role. Lastly, as some highly similar PAS domains are found to be involved in Light-Oxygen-Voltage, we propose a new protein family LOV related (LOVr).

Plain language summary
Sensing and responding to the environment is of crucial importance for organisms to ensure their survival, growth and reproduction. This is especially important for plants, which are very susceptible to changes in environmental conditions as they are restricted to living in one place. Some of the most well-known environmental sensors are Light-Oxygen-Voltage (LOV) domains, which are parts of proteins that sense and pass on a signal to activate specific responses to the environment. Proteins that contain these domains are found to be widespread in different species, including bacteria, fungi and plants. While these LOV proteins in plants have only been found to play a role in light-sensing so far, the different roles of LOV proteins in other species could mean that plant LOV proteins might also play a role in the sensing of oxygen. To see if this is indeed the case, here we describe the roles and mechanisms of light-sensing LOV domains in the plant model organism *Arabidopsis thaliana,* and look at the oxygen sensing roles of LOV domains in other species. There seem to be differences in how LOV domains function, and the roles that they play in light-sensing proteins. LOV proteins in other species such as bacteria and fungi are able to sense oxygen via changes that occur in a small molecule bound to the LOV domain. These changes are dependent on oxygen concentration, and in turn pass on the oxygen signal to other parts of the protein. Interestingly, light and oxygen sensing in these proteins often goes hand-in-hand. The light-sensing plant LOV proteins bind to the same small molecule, and perhaps these known LOVs indeed are able to sense oxygen as well. Lastly, the definition of LOV domains seems to have changed over time, and some highly similar proteins are found to also function in Light-Oxygen-Voltage sensing. We thus propose a new protein family related to LOV proteins, termed LOV related (LOVr).

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List of abbreviations

ASK = Arabidopsis SKP1-like

CDFs = CYCLING DOF FACTORs

CNBHD = Cyclic Nucleotide-Binding Homology Domain

ENV1 = ENVOY

ERFVII = Group VII ETHYLENE RESPONSE FACTOR

ETC = Electron Transport Chain

FAD = Flavin Adenine Dinucleotide

FKF1 = FLAVIN-BINDING KELCH REPEAT F-BOX 1

FMN = Flavin Mononucleotide

GGP = GDP-L-galactose phosphorylase

GI = GIGANTEA

hERG = human Ether á Go-go Related

HIF = HYPOXIA-INDUCIBLE FACTOR

LKP2 = LOV KELCH PROTEIN 2

LOV = Light-Oxygen-Voltage

PAS / Per Arnt Sim = Period-ARNT-Singleminded

PCO = PLANT CYSTEINE OXIDASE

PHOT = Phototropin

PRT6 = PROTEOLYSIS6

PRR5 = PSEUDORESPONSE REGULATOR 5

ROS = Reactive Oxygen Species

SCF = SKP-Cullin-Rbx-F-box

sLOV = short Light-Oxygen-Voltage

STAS = Sulfate Transport Anti-Sigma antagonist

STK = Serine/Threonine kinase

TOC1 = TIMING OF CAB EXPRESSION 1

UPS = Ubiquitin Proteasome System

ZTL = ZEITLUPE

Introduction

Survival, growth and reproduction of organisms is highly dependent on their ability to sense and respond to their ever-changing environment. Organisms are subjected to changes in multiple environmental factors such as temperature, light, carbon dioxide and oxygen levels, water availability and nutrients. Thus they have evolved to live and thrive within an optimal range of these factors. Fluctuations of environmental signals beyond the organisms optimal ranges often lead to stressful situations. It is thus important for organisms to sense and react to their environments in a manner that allows optimal growth. Plants, being sessile organisms, are often the most susceptible to changes in environmental conditions and hence it is of crucial importance to be able to rapidly sense and adjust to their often fast changing conditions (Lamers et al., 2020). Some of the most well-known environmental sensors are the Light-Oxygen-Voltage (LOV) domains, initially identified as blue-light sensing modules in plant phototropin photoreceptors (Huala et al., 1997; Salomon et al., 2000). In contrast to the other major photoreceptors phytochromes (red/far-red light sensing) and cryptochromes (UV-A/blue light sensing), which regulate multiple developmental processes, phototropins are mainly involved in regulating directed movement (Christie, 2007; Ito et al., 2012; Kagawa, 2003; Kami et al., 2010; Kinoshita et al., 2001; Sakai et al., 2001). This includes chloroplast movement, stomatal opening, cotyledon and leaf expansion, leaf flattening and phototropism. PHOT1 and PHOT2, the two phototropins in *Arabidopsis thaliana*, each possess two LOV domains termed LOV1 and LOV2 (Christie et al., 2002).

LOV domains are relatively small modules of around 110 amino acids, that consist of a characteristic pattern of α-helices and β-strands, making them part of the larger Period-ARNT-Singleminded (PAS) protein family (Harper et al., 2003; Herrou & Crosson, 2011; Taylor & Zhulin, 1999). PAS/LOV domains have been shown to follow the secondary structure Aβ-Bβ-Cα-Dα-Eα-Fα-Gβ-Hβ-Iβ, where a five-stranded antiparallel β-sheet is connected by four α-helices (Figure 1A) (Losi, 2007). LOV domains are often characterized by their binding to a flavin chromophore cofactor in the binding pocket that is formed by this PAS fold (Christie et al., 1999; Crosson et al., 2003a). LOV domain containing proteins are thus generally considered flavoproteins, being bound to either a flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). The conserved **GXNCRFLQ** motif located on the Eα-helix plays an important role, with the cysteine residue functioning in the formation of a flavin-cysteinyl adduct (Briggs, 2007; Glantz et al., 2016; Salomon et al., 2000). Multiple sequence alignment of phototropin LOV domains show the presence of this highly conserved motif and cysteine residue in almost all classes of plants (Figure 1B).

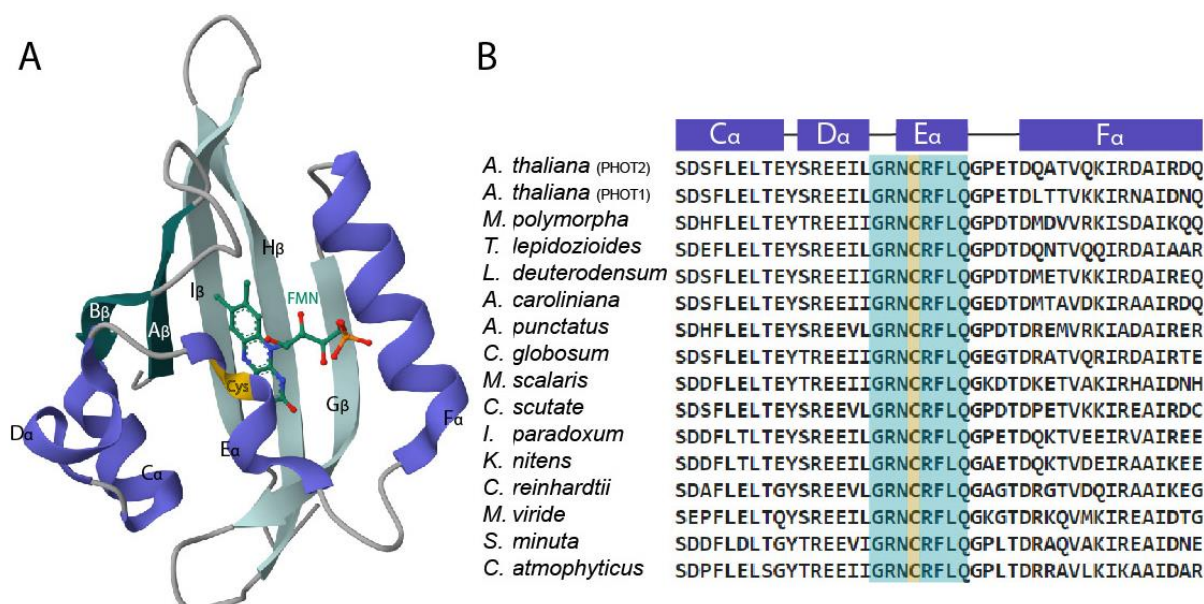

Figure 1. Structure and multiple sequence alignment of the phototropin LOV2 domain. (**A**) Shown is the cartoon crystal structure from the LOV1 domain of phototropin2 from *Arabidopsis thaliana*. Adapted from RCSB Protein Data Bank (Nakasako et al., 2007). (**B**) Shown are the sequences of PHOT LOV2 domains in flowering plants (*Arabidopsis thaliana*), ferns (*Azolla caroliniana*), lycophytes (*Lycopodium deuterodensum*), mosses (*Takakia lepidozioides*), liverworths (*Marchantia polymorpha*), hornworths (*Anthoceros puctatus*), Zygnematophyceae (*Mougetotia scalaris*), Coleochaetophyceae (*Coleochaete scutate, Chaetosphaeridium globosum*), Klebsormidiophyceae (*Interfilum paradoxum, Klebsormidium nitens*), Chlorokybophyceae (*Chlorokybus atmophyticus*), Mesostigmatophyceae (*Mesostigma viride, Spirotaenia minuta*) and Chlorophytes (*Chlamydomonas reinhardtii*). The conserved **GXNCRFLQ** motif is colored. The corresponding secondary Cα-Dα-Eα-Fα structures are shown above the alignment. Sequences were obtained from UniProt (The UniProt Consortium, 2023), aligned using EMBL-EBI MAFFT (Katoh & Standley, 2013) and viewed using MView (Brown et al., 1998).

Furthermore, putative LOV domains are found to be widespread in bacteria, archaea and other eukaryotes as well (Crosson et al., 2003b; Krauss et al., 2009). While short-LOV (sLOV) proteins consisting of just the LOV domain do exist, often they are coupled to and regulate a variety of other domains such as kinases, F-boxes, phosphodiesterases, Sulfate Transport Anti-Sigma antagonist (STAS) domains and zinc fingers (Crosson et al., 2003b; Glantz et al., 2016; Rani et al., 2013). Activation of these partner domains is needed for signaling output and thus protein functioning. Bacterial proteins with similar LOV domains have for example been found to sense oxygen and link this to aerotaxis (movement towards oxygen) and eukaryotic proteins with similar motifs function as voltage-gated potassium channels (Dixon, 1998; Edwards et al., 2006; Huala et al., 1997; Krauss et al., 2009). However, some differences do exist in the definition of LOV domains. While initially termed after the light, oxygen and voltage sensing proteins in which these similar motifs occur (Huala et al., 1997), they are now generally defined as PAS domains that function as the light, oxygen or voltage sensors of proteins, via a flavin chromophore cofactor that is bound in their binding pocket (Christie et al., 1999; Herrou & Crosson, 2011; Salomon et al., 2000).

Although plant LOV domains have so far only been described to function in light sensing, plants are known to sense and respond to differences in oxygen levels as well. In flowering plants (angiosperms), the PLANT CYSTEINE OXIDASE (PCO) branch of the PROTEOLYSIS6 (PRT6) N-degron pathway is the only known mechanisms of O_2 sensing, resulting in degradation of substrates through the ubiquitin proteasome system (UPS) (Gibbs et al., 2011; Licausi et al., 2011). These pathway substrates, the Group VII ETHYLENE RESPONSE FACTOR (ERFVII) transcription factors, regulate anaerobic gene expression under low oxygen conditions. Interestingly, a similar yet mechanistically different mechanism for

oxygen sensing exists in mammals, through which HYPOXIA-INDUCIBLE FACTORs (HIFs) is degraded in the presence of oxygen (Erbel et al., 2003; Ratcliffe et al., 1998). The HIF system, whose discovery led to a Nobel Prize in physiology in 2019, consists of a heterodimer formed by two PAS proteins containing two PAS domains each.

Recent work concerning oxygen sensing in plants has shown how plants utilise oxygen to adapt to altitudes. In particular chlorophyll biosynthesis and expression of hypoxia-related genes have been shown to be dependent on altitude via sensing of atmospheric oxygen (Abbas et al., 2022). While some plants are adapted to high altitudes and are even found above 6000 meters (Angel et al., 2016; Dentant, 2018), most plants will experience stress at high altitudes due to the low temperature, low precipitation, high radiation and lower partial pressure of oxygen (pO_2) (Körner, 2003). Yet, climate change is predicted to not only influence the growth range of plants on latitudes, with species moving towards the polar margins, but also on altitudes (Kullman, 2002; Lenoir et al., 2008; Peñuelas & Boada, 2003; Walther et al., 2005). As the temperatures are rising, plant species have been shown to be displaced towards higher elevations. Still, plants will have to face the lower oxygen levels that accompany this shift in growth range.

Despite our increasing knowledge in oxygen biology, little is known about exactly how plants sense and respond to oxygen changes. Conservation of LOV domains throughout the tree of life could suggest a possible extended role for plant LOV domains in oxygen sensing. However, while the role of LOV domains as photosensory modules is well described, their possible role in oxygen sensing remains unknown, mainly due to the fact that all experiments related to LOV domain containing proteins are performed at ambient oxygen levels (21% O_2). To understand whether plant LOV proteins could indeed have a role in oxygen sensing, first the functioning of LOV domains in light sensing in *Arabidopsis thaliana* will be discussed. This thesis will then look into oxygen sensing LOV proteins in other species, and the involvement of a flavin cofactor. We show that the mechanisms of light-sensing LOV domains are flexible and that all described oxygen-sensing proteins function via changes in redox status of the flavin cofactors. Furthermore, oxygen sensing was often accompanied by light sensing functions, pointing to a possible extended redox-based oxygen sensing role of known plant LOV proteins. Lastly, as some highly similar PAS domains are found to be involved in Light-Oxygen-Voltage, we propose a new protein family LOV related (LOVr).

Light sensing

Not only is light important for plants in the process of photosynthesis, but it is also used as a signaling cue (Christie et al., 2015; Kami et al., 2010). Light intensity, quality, direction and duration all influence plant development and physiology. As previously mentioned, plants possess different types of photoreceptors, each one specialized in sensing light of a specific wavelength and regulating its own specific responses.

Multiple different blue-light sensing proteins with LOV domains are known to exist in *Arabidopsis thaliana*. Apart from the two LOV domains in PHOT1 and PHOT2 (Figure 2A), LOV domains are found in the FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1)/ LOV KELCH PROTEIN 2 (LKP2) / ZEITLUPE (ZTL) family (Figure 2B) and the PAS/LOV protein (PLP) family (Figure 2C). Here, the mechanism of light sensing and signal transduction for each family is described.

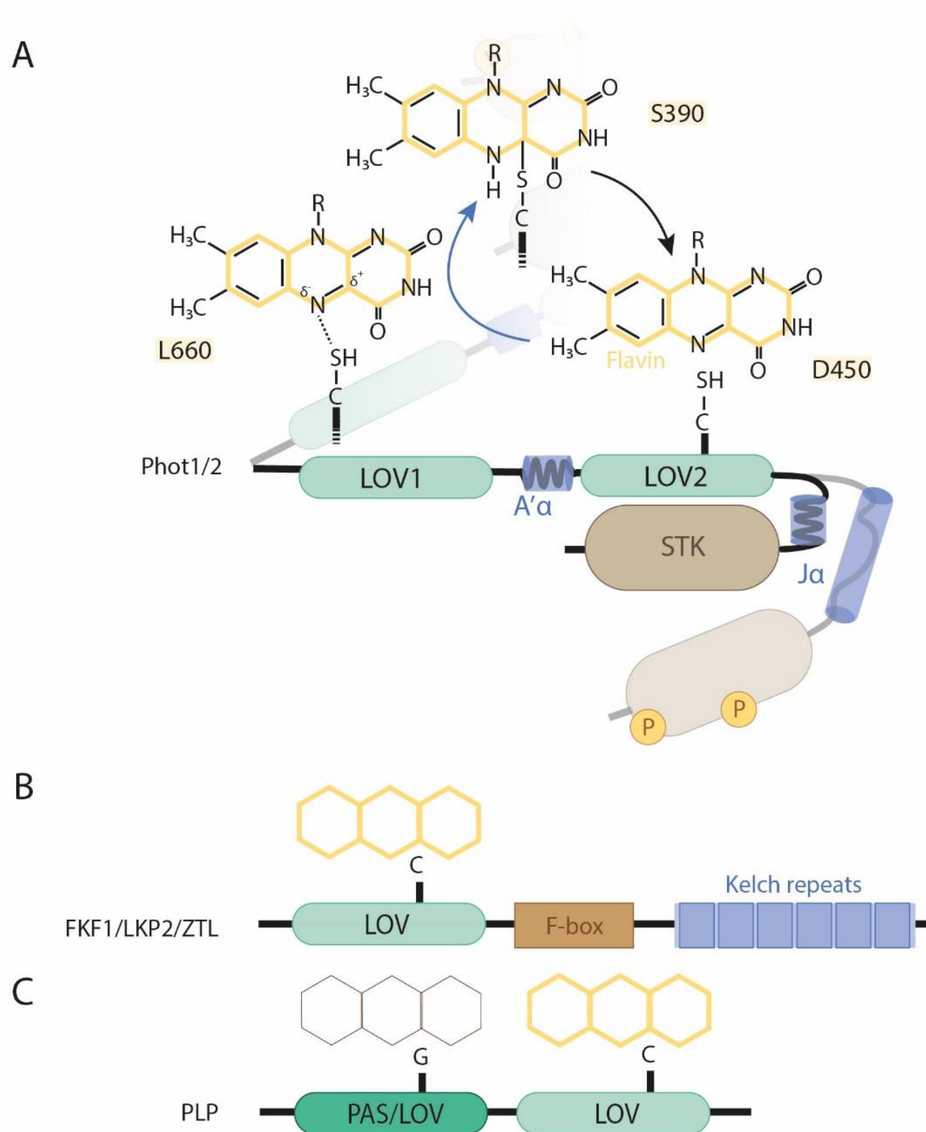

Figure 2. The three light sensing LOV protein families in *Arabidopsis thaliana*. Shown are the domains of phototropins (**A**), FKF1/LKP2/ZTL (**B**) and PLP (**C**). (**A**) The phototropin LOV1 domain is used in dimerization, while an FMN cofactor bound to LOV2 undergoes a photochemical cycle upon blue light sensing, leading to conformational changes in the phototropin protein through A'α and Jα helices that cause autophosphorylation and activation of the STK domain. (**B**) FKF1/LKP2/ZTL proteins consist of an N-terminal FMN binding LOV domain, F-box and 6 Kelch repeats. (**C**) PLP proteins consist of an N-terminal glycine containing PAS/LOV domain, that is thought to bind FMN, and an C-terminal FMN binding LOV domain. Figures were created using Adobe Illustrator.

Phototropins

As previously mentioned, phototropins are essential for directed movements, such as stomatal opening, chloroplast relocation, leaf and cotyledon expansion, flattening of leaves, and phototropism (Christie, 2007; Ito et al., 2012; Kagawa, 2003; Kami et al., 2010; Kinoshita et al., 2001; Sakai et al., 2001). These blue-light activated responses together allow plants to maximize their photosynthetic efficiency. Phototropins function through two tandemly aligned N-terminal LOV domains (LOV1 and LOV2) connected to a C-terminal Serine/Threonine Kinase (STK) domain (Christie et al., 1999). While LOV1 and LOV2 are structurally almost identical and have both been found to bind to a FMN cofactor, they appear to have distinct functions (Christie et al., 2002). LOV2, the LOV domain most closely located to the STK domain, seems to be the predominant light sensing LOV of both PHOT1 and PHOT2. Mutant

studies where the flavin-cysteinyl adduct forming cysteine has been replaced with an alanine residue show that functional LOV2 alone is enough for phototropin function, while LOV1 is not.

The phototropin LOV domains undergo a photochemical reaction cycle (Figure 2A). This cycle starts under dark conditions with the FMN cofactors in the ground state, which form a non-covalent bond with the LOV domains (Eitoku et al., 2005; Ito et al., 2012; Jones & Christie, 2008; Kennis et al., 2003; Pfeifer et al., 2010). The ground state is referred to as D_{447} or D_{450}, after the wavelength of light that it absorbs. Within microseconds of blue-light (390-500 nm) illumination, a flavin-cysteinyl adduct is formed in two steps. First, an intermediary reactive triplet state (L_{660}) is created. This leads to the formation of a bond between the sulfur atom on the sidechain of the conserved cysteine residue in the Eα-helix, and the C4a atom of the isoalloxazine ring of FMN. This state, called S_{390}, in which the photoadduct is formed, leads to local changes of the phototropin protein. A Glutamine (Q) residue on the Iβ strand of LOV2 forms a hydrogen bond with the FMN cofactor, which changes upon light activation (Jones & Christie, 2008; Nash et al., 2008; Nozaki et al., 2004). The oxygen in the glutamine side chain rotates, in turn triggering structural changes. Through the Iβ strand, a neighboring α-helix (Jα) then undergoes a two-step conformational change (Nakasone et al., 2007). In an initial reaction, the linker dissociates from the LOV2 domain, after which the helix unfolds. Furthermore, an additional alfa-helix on the N-terminal side of LOV2 (A’α) was found to act together with Jα, as deletion of this helix eliminated unfolding of Jα (Zayner et al., 2019). Through unfolding of the Jα-helix , LOV2 which normally acts as an inhibitor of the STK domain, dissociates from the kinase domain and consequently autophosphorylation of two serine residues in the STK domain can occur (Inoue et al., 2011). With activation of the kinase, phototropin targets can be phosphorylated, which is needed for the next steps in blue-light signaling. Binding of the flavin cofactor to the LOV domains is dark reversible (Kasahara et al., 2002). For phototropin LOV domains, the S_{390} state falls back to the ground state within several seconds to minutes, after which the cycle can be repeated.

LOV1 instead has been suggested to be involved in dimerization of phototropins (Nakasako et al., 2008; Nakasone et al., 2014). While LOV2 either remains as a monomer in solution (PHOT2) or dimerizes depending on concentration (PHOT1), the LOV1 domains of both PHOT1 and PHOT2 are shown to dimerize in an anti-parallel orientation (Nakasako et al., 2008). Different types of interactions are seen for LOV1 dimers of PHOT1 and PHOT2 respectively. Whereas PHOT1-LOV1 dimerizes through a disulfide bridge, PHOT2-LOV1 dimers are mainly formed through hydrogen bonds between the subunits. Dimerization of LOV1 is light independent. Even though conformational changes may occur by binding to FMN, similarly to LOV2, the disulfide bridge and other bonds are enough to keep the subunits from dissociating. Dimerization of phototropins likely leads to signal amplification, thus fine-tuning the responses (Łabuz et al., 2022). Furthermore, LOV1 has also been linked to regulation of kinase photosensitivity (Oide et al., 2018; Okajima et al., 2014). Compared to the full protein, the LOV2 domains of shortened phototropins without LOV1 have been shown to revert back to the ground state faster, consequently only activating STK for shorter periods of time. Structural changes at the N- and C-termini of LOV1 could be involved in this regulation, in a similar way to how structural changes of A’α and J α of LOV2 help regulate the STK domain. Still, the precise role of LOV1 remains unknown.

FKF1/LKP2/ZTL

Blue-light sensing and signaling in LOV domains of FKF1/LKP2/ZTL proteins occurs in a similar fashion to that of phototropins, through a photochemical cycle with a D_{450} ground state and a S_{390} activated state (Imaizumi et al., 2003). However, unlike phototropins, blue-light sensing does not lead to activation of a kinase (Han et al., 2004; Ito et al., 2012; Zoltowski & Imaizumi, 2014). The ZTL/FKF1/LKP2 family is involved in the regulation of the circadian clock and photoperiodism of flowering by targeted ubiquitination and protein degradation. The C-terminal side of ZTL/FKF1/LKP2 proteins consists of an

F-box and 6 Kelch repeats (Figure 2B). While targets are ubiquitinated and thus marked for degradation by SKP-Cullin-Rbx-F-box (SCF) E3 ligases that are formed by the F-box by interacting with Arabidopsis SKP1-like (ASK) proteins, specificity is mediated by interactions of these protein targets with the Kelch repeats. However, the Kelch domain is only open to bind targets when these proteins are in their monomeric form. In contrast to phototropins, proteins of the FKF1/LKP2/ZTL family have a single LOV domain that functions in both dimerization and signal transduction. ZTL/FKF1/LKP2 members form homo or heterodimers via interactions between the Gβ/Hβ/Iβ-sheets, in an anti-parallel configuration that is also seen for phototropin LOV1 (Pudasaini et al., 2017).

Apart from their role in dimerization, FKF1/LKP2/ZTL LOV domains are also known to directly interact with other proteins (Kim et al., 2007; Kwon et al., 2022). An example is the interaction between ZTL and GIGANTEA (GI), which functions in the stabilization of ZTL (Kim et al., 2007; Zoltowski & Imaizumi, 2014). As GI transcription is controlled by the circadian clock, with its highest protein abundance in the late afternoon, the ZTL-GI interaction is dependent on the time of the day, this way maintaining circadian oscillations. Due to LOV functioning, the ZTL-GI interaction is enhanced under blue light. The same cysteine in the conserved **GXNCRFLQ** motif that is used in FMN binding in phototropins is needed for this enhanced interaction (Kim et al., 2007). While FMN was still bound after mutation of this cysteine to an alanine, the ZTL-GI interaction was strongly reduced and consequently less ZTL was found in immunoprecipitation experiments. As binding of GI interrupts the dimerization via LOVs, FKF1/LKP2/ZTL proteins can only bind with GI as monomers. Interactions of target proteins with Kelch repeats thus only occur upon binding to GI. Protein levels of CYCLING DOF FACTOR1 (CDF1) for example, which binds to the Kelch repeats of FKF1, quickly go down with high levels of GI and FKF1 (Kwon et al., 2022; Sawa et al., 2007). Homologs TIMING OF CAB EXPRESSION 1 (TOC1) and PSEUDORESPONSE REGULATOR 5 (PRR5), have also been shown to directly bind to the LOV domain of ZTL, leading to their degradation (Kwon et al., 2022). In this case, other interactions mediated by the LOV domain such as dimerization and GI binding, thus seem to inhibit degradation of TOC1 and PRR5.

Although the photochemical cycle of this family of F-box proteins is thus similar to that of the earlier discussed phototropins, the conformational changes following blue-light sensing appear to be different for ZTL specifically (Pudasaini et al., 2017; Trozzi et al., 2021). Whereas simple switching of a glutamine residue undergoing a hydrogen bond with FMN leads to structural changes in PHOT1/2 and FKF1/LKP2, the same glutamine residue is thought to have a more heterogeneous conformation in ZTL. Here, this glutamine is found to be more dynamic, undergoing multiple different conformations that in turn relay conformational changes to the C- and N-terminal domains. This might allow for the dual functioning of ZTL both in light (e.g. GI binding, targeted degradation) and dark (e.g. TOC1/PRR5 degradation) conditions. Additionally, it is shown that signaling is even possible in absence of the glutamine via movement of a coupled phenylalanine residue towards the flavin cofactor (Trozzi et al., 2021). Moreover, FKF1/LKP2/ZTL proteins have very different dark-reversion rates (Ito et al., 2012; Zoltowski & Imaizumi, 2014). While dark-reversion of ZTL already takes ~1.6 hours in contrast to the seconds to minutes that are seen for phototropins, dark-reversion for LKP2 and FKF1 has been shown to have a half time of 62.5 or even more than 100 hours (Pudasaini et al., 2015; Zikihara et al., 2006). These much higher rates are likely explained by an extra stretch of residues between the Eα and Fα helices, known as the E-F loop, as deletion of this loop lead to a much faster reversion rate (Zoltowski & Imaizumi, 2014). The photochemical cycles of LOV domains thus have some slight variations between proteins and show flexibility in their possible signaling mechanisms.

PAS/LOV

Relatively little is known about the roles and functioning of PAS/LOV proteins. The PLP gene encodes three different proteins dependent on splicing variation, referred to as PLPA, PLPB and PLPC (Ogura et

al., 2008). The proteins consist of a PAS domain on the N-terminal side and a LOV domain on the C-terminal side, with a truncated LOV domain in PLPC (Figure 2C). The N-terminal PAS domain and surrounding sites shows more similarity to phototropin LOV domains than PAS domains, which is why these proteins have been proposed to be LOV/LOV proteins (LLP) instead (Kasahara et al., 2010). However, instead of the conserved cysteine residue characteristic for LOV domains, the *A. thaliana* PLP PAS domain contains a glycine residue (Gly^{66}). FMN is still found to bind to this PAS/LOV domain, but no functional photochemical cycle is seen in reaction to light unless the glycine is replaced by cysteine. Instead, flavin binding to this N-terminal LOV/PAS domain is thought to be needed to maintain the right structure for dimerization to occur, similarly to the light-independent dimerization of phototropin LOV1 domains.

The full-length C-terminal LOV domains of PLPA and PLPB on the other hand have been shown to bind a flavin cofactor with the conserved cysteine, suggesting PLP proteins to act as blue light receptors (Kasahara et al., 2010; Ogura et al., 2008). Recent research shows that PLP functions as a negative regulator of GDP-L-galactose phosphorylase (GGP), a precursor in the L-galactose pathway of ascorbate synthesis (Bournonville et al., 2023). Light induction counteracted this inhibition, in turn promoting synthesis of the antioxidant ascorbate.

Oxygen and voltage sensing

The original name for LOV domains came from their appearance in light, oxygen or voltage sensing proteins, but many of those proteins that were originally described to be putative oxygen and voltage sensing LOV proteins by Huala et al. (1997), don't appear to bind a flavin cofactor. While still being called Light-Oxygen-Voltage domains, these flavin-binding domains thus mostly seem to be involved in light sensing and less is known about their roles in the sensing of oxygen and voltage. Especially for *A. thaliana*, all previously described LOV domains are known to function in light sensing, but no other functions have been reported so far. Still, some oxygen sensing LOV proteins have been found in bacteria and fungi, and will be described here.

Indirect oxygen or redox sensing?

A known LOV domain containing oxygen sensor is the nitrogen fixation protein NifL in *Azotobacter vinelandii* (Dixon, 1998). NifL is considered to be a LOV domain because of its oxygen sensing role and bond to the flavin cofactor FAD (Figure 3A). Although this protein does not have the full **GXNCRFLQ** motif with a cysteine residue, it does contain some other previously identified to be conserved amino acids, such as the phenylalanine, threonine-glycine-tyrosine and glutamic acid upstream of the motif (Figure 3E) (Glantz et al., 2016). NifL functions together with NifA, in a complex that is specific to the γ-subdivision of Proteobacteria (Dixon, 1998; Martinez-Argudo et al., 2004). NifA regulates synthesis of nitrogenase, an enzyme that is needed for these bacteria to fixate nitrogen. While oxygen is crucial for the aerobic *A. vinelandii* to provide the large amounts of ATP that are used by the nitrogenase, oxygen also inactivates nitrogenase. NifL is thought to act as an indirect oxygen sensor, by using signaling molecules such as FAD and $FADH_2$ (Zhang et al., 2023). Low levels of O_2 increase concentrations of NADH and $FADH_2$, as the electron transport chain (ETC) produces less ATP (Purcell et al., 2010). FAD/$FADH_2$ levels thus reflect the intracellular redox status. Only the oxidized form of NifL is able to inhibit NifA activity by sequestering NifA in the NifL-NifA complex (Dixon, 1998; Zhang et al., 2023). Under low oxygen levels, high levels of $FADH_2$ thus reduce NifL, this way eliciting redox-induced conformational changes that affect binding to NifA. O_2 sensing through NifL is thus a method to control synthesis of nitrogenase, so that it is only inhibited when it likely will be deactivated by oxidative conditions and

synthesis would be wasteful. Indeed, addition of O_2 to the reduced form of NifL quickly led to oxidation to its active NifA inhibiting form, and this redox response is no longer seen in NifL proteins where the C-terminal LOV domain is removed (Dixon, 1998).

Another case in which redox status of a FAD cofactor is indirectly affected by oxygen concentrations via changes in the ETC, is for the *Escherichia coli* aerotaxis protein Aer, which helps *E. coli* seek favorable oxygen environments (Figure 3B) (Edwards et al., 2006). While the exact mechanisms remain unknown, Aer's FAD cofactor could for example be reduced by NADH, or directly by a dehydrogenase component of the ETC. The photosensory function of LovK, a LOV-histidine kinase in *Caulobacter crescentus*, has been shown to be dependent on redox changes reflecting the oxygen environment as well (Purcell et al., 2010). Reduction of its FMN cofactor led to weakening of its light-dependent ATPase activity. Due to the oxygen sensing function of LovK, it can thus work as a conditional photosensor. Similarly, redox affects functioning of the sLOV protein ENVOY (ENV1) in the fungus *Trichoderma reesei* (Lokhandwala et al., 2015). This protein needs both blue-light and oxygen for dimerization to occur. ENV1 even remains dimeric when switched back to dark conditions, and was only found to return to its monomeric state under reducing conditions.

Looking at the oxygen sensing mechanisms of these proteins, the importance of redox status of the flavin cofactor is clear. Oxygen sensing relies on changes in redox status of their flavin cofactors leading to conformational changes in the proteins. One could thus argue that instead of being (in)direct) oxygen sensors, these LOV proteins function as redox sensors.

Oxygen and voltage sensing PAS proteins

Up to date, no voltage sensing flavin-binding LOV domains have been identified. But while the originally described putative LOV proteins from Huala et al. (1997) might no longer be considered LOV proteins, they are extremely similar to "real" LOVs. The N-terminal PAS domain of voltage sensing potassium channels of the KCNH family (ERG, EAG, ELK) such as human ether a go go (hERG) follows the Aβ-Bβ-Cα-Dα-Eα-Fα-Gβ-Hβ-Iβ secondary structure, similarly to known LOV domains (Figure 3A, B, D) (Codding et al., 2020; Dai & Zagotta, 2017). Their PAS domains have been shown to be critical for voltage dependent activation of the channel, and interactions between these domains with the C-terminal cyclic nucleotide-binding homology domains (CNBHD) seem to regulate shifts in voltage sensitivity (voltage dependent potentiation). Although a heme-binding domain has recently been identified in human ERG3, no bound cofactors have been found yet for other members of the KCNH family (Burton et al., 2020).

Of course, LOVs are a type of PAS domain, and consequently many things such as protein fold and sensing-function are shared between the two. Another (direct) oxygen sensing PAS protein is for example the FixL from *Bradyrhizobium japonicum* (Figure 3C) (Wright et al., 2018). Like LOV protein NifL, FixL functions in a two-component system with FixJ so that nitrogenase genes are only expressed under low oxygen concentrations. Here as well, the oxygen sensing role of FixL comes from a bound heme group instead of a flavin cofactor such as FMN or FAD, thus excluding FixL from the LOV protein family. However, unlike hERG, FixL does not show high sequence similarity to "true" LOV domains. Multiple sequence alignment of the human ether á go-go related (hERG) and FiXL proteins to *A. thaliana* proteins shows high similarities of hERG to phototropins, PLP and LKP2/FKF1 proteins, with the cysteine residue even being conserved, while this is not seen for FixL (Figure 3E). PAS proteins that are more similar to known LOV domains than to other PAS domains thus exist, having sensing functions other than light. The role of the hERG protein in light, oxygen or voltage sensing, combined with its conserved folds <u>and</u> sequences, thus suggests that although it doesn't bind a flavin cofactor, its sensing and signaling mechanisms are regulated in a very similar way.

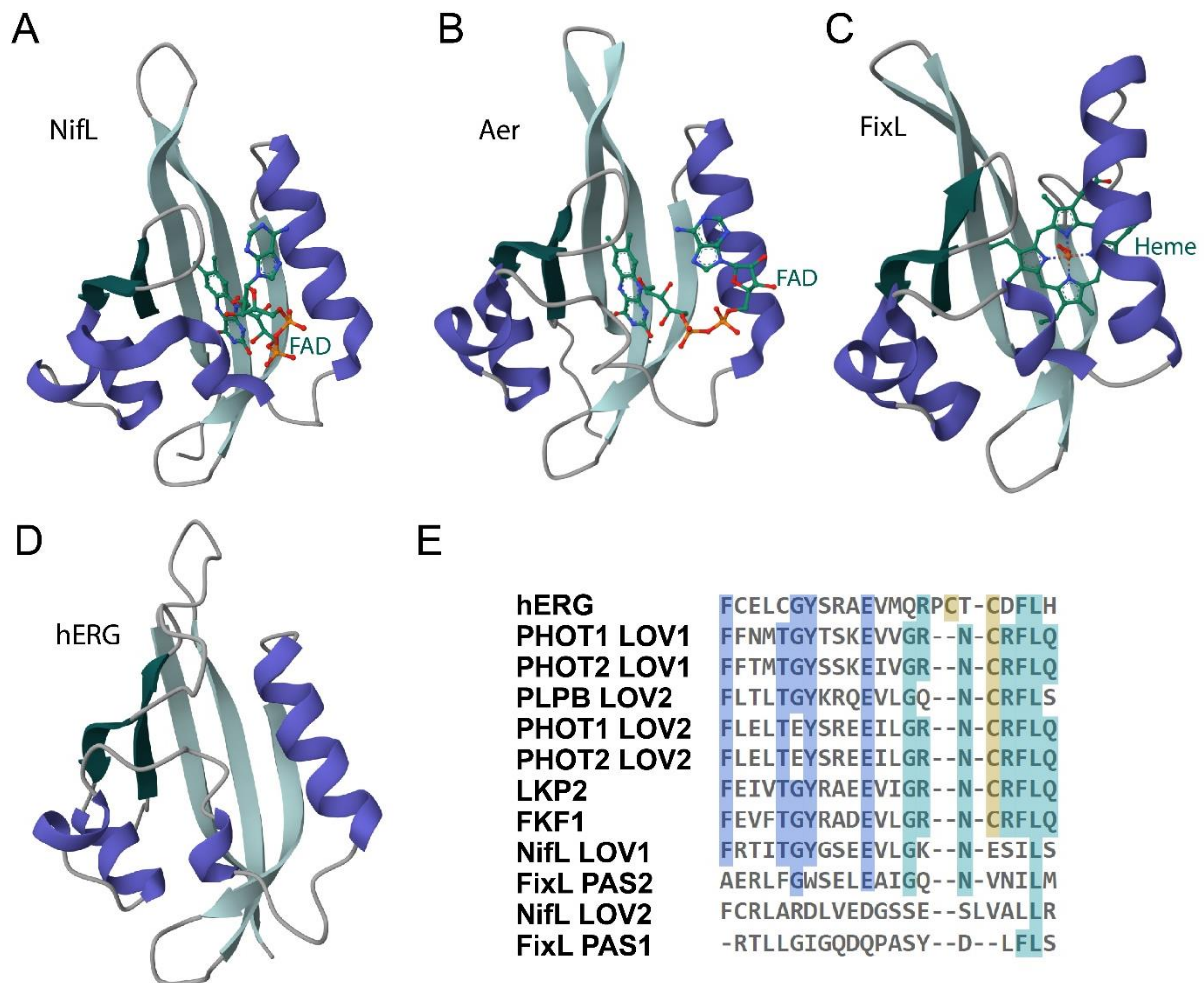

Figure 3. Secondary structures and multiple sequence alignment of "true" LOV domains and similar PAS domains. Shown are cartoon crystal structures (**A**) NifL in *Azotobacter vinelandii* and (**B**) Aer in *Escherichia coli*, and those of the PAS domains (**C**) FixL in *Bradyrhizobium japonicum* and (**D**) hERG in humans. Structures are adapted from the RCSB Protein Data Bank (Cabral et al., 1998; Gong et al., 1999; Key et al., 2006; Maschmann et al., 2022). (**E**) Conserved amino acids of the GXNCRFLQ motif are shown in green and yellow. Other previously found highly conserved amino acids are shown in blue. Sequences were obtained from UniProt (The UniProt Consortium, 2023), aligned using EMBL-EBI MAFFT (Katoh & Standley, 2013) and viewed using MView (Brown et al., 1998).

Discussion

As previously mentioned, plant LOV proteins have yet only been found to have light-sensing roles, but the conservation of LOV domains in other kingdoms of the tree of life could suggest a possible additional role for plant LOV domains in oxygen sensing. To understand whether plant LOV proteins could indeed function in oxygen sensing, first the roles and mechanisms of LOV domains in light sensing proteins of *Arabidopsis thaliana* was discussed. Secondly, the oxygen sensing roles of LOVs in other species was described, as well as similarly functioning oxygen and voltage sensing PAS proteins.

Differential roles and mechanisms of LOV domains

Looking at the light sensing PHOT1, PHOT2, ZTL, FKF1, LKP2 and PLP proteins, both commonalities and differences are seen for the roles of their LOV domains and in their photochemical cycles of FMN binding. Dimerization occurs for many LOV domains, as a way to regulate and finetune the blue-light

response, like in the case of phototropin LOV1 (Łabuz et al., 2022; Oide et al., 2018), but also for regulation of protein function. LOV domains are namely found to interact with other proteins as well, to regulate stability and function of the LOV protein itself (e.g. ZTL-GI), or to regulate levels of target proteins (e.g. ZTL-TOC1/PRR5) (Kwon et al., 2022). LOV domains are linked to other domains such as kinases or F-boxes and Kelch repeats, which are activated via signal transduction, but the previously thought to be conserved mechanisms of LOV signaling to these partner domains appear to be flexible. In the case of the ZTL for instance, signal transduction does not depend on simple switching of the glutamine residue upon light activation, like is seen in other LOV proteins, but is found to undergo multiple different more dynamic conformations (Trozzi et al., 2021). Additionally, signaling appears to function without this glutamine as well, due to movement of other linked residues. Furthermore, some LOV domains even seem to function independent of a photochemical cycle, as for example seen for the dimerization of phototropin LOV1 and the proposed PAS/LOV1 domain of PLP (Nakasako et al., 2008; Ogura et al., 2008). Although an FMN cofactor is bound in these cases, light-sensing is thus not crucial for functioning of these domains.

LOV proteins as redox sensors

Looking at the oxygen sensor proteins, oxygen levels regulate their function for instance by only forming dimers (ENV1) or functioning as an inhibiter (NifL) under the presence of O_2 (Lokhandwala et al., 2015; Zhang et al., 2023). It is noticeable that all described oxygen-sensing proteins function via changes in redox status of the FMN/FAD cofactors, which reflect oxygen concentrations (Purcell et al., 2010). It might thus be proposed that instead of being Light-Oxygen-Voltage sensors, LOV domains are simply redox sensors.

In line with this, Yee et al. (2015) show that multiple LOV proteins can react to light even without the cysteine residue, as both flavin-cysteinyl adduct formation and the 1-electron reduced (Semiquinone) form of flavin depend on flavin protonation and subsequent conformational changes. They suggest that LOV photoreceptors may have arisen from redox sensing flavoproteins. In that case, it could be possible that phototropins, FKF1/LKP2/ZTL and PLP proteins do not only have a light-sensing function, but also respond to FMN redox changes induced by oxygen or voltage. Indeed, oxygen sensing in both fungi (e.g. ENV1) and bacteria (e.g. LovK) was seen to go together with light sensing functions (Lokhandwala et al., 2015; Purcell et al., 2010).

PAS vs LOV

LOV domains are diverse, differing both in mechanism (e.g. FMN vs FAD binding, glutamine switching) and function (e.g. dimerization, sensing, interacting with other proteins, inhibiting partner domains) between and within proteins. Despite their diverse roles and functions, these domains are defined as LOV domains because 1) they are PAS domains following the Aβ-Bβ-Cα-Dα-Eα-Fα-Gβ-Hβ-Iβ secondary fold, 2) often having the conserved **<u>GXNCRFLQ</u>** motif, and 3) they are found in proteins involved in the sensing of light, oxygen or voltage 4) via a flavin chromophore that is bound in their binding pockets (Crosson et al., 2003b; Losi, 2007; Salomon et al., 2000; Taylor & Zhulin, 1999). However, the definition of LOV domains seems to have changed over time, after many were found to be flavin-binding domains. Originally seen as a domain that is found in light, oxygen or voltage sensing proteins (Huala et al., 1997), LOV domains now generally seem to be identified as flavin-binding PAS domains. Yet, with this new definition, many of those original proteins described to have putative LOV domains (after which LOVs are named), do not seem to be LOV proteins after all. Especially those proteins involved in oxygen and voltage sensing do not all bind to a flavin cofactor.

Still, some PAS proteins are extremely similar to known LOVs, not only sharing similarities in sensing-function and secondary structure, but also in their sequence. A new protein family could thus be proposed, LOV related (LOVr), where LOVr domains are PAS fold domains involved in the sensing of

light, oxygen and/or voltage, with higher similarity to LOV domains (and their **GXNCRFLQ** motif) than to other PAS domains, but that do not necessarily bind a flavin cofactor. The PAS domains of KCNH proteins such as hERG would then thus be considered LOVr proteins.

Future perspectives

More experiments should be done on known LOV, LOVr and other PAS proteins to see if they play an extended role in oxygen sensing in plants. Experiments related to LOV proteins are normally performed at ambient oxygen levels, focusing on their light-sensing roles. Future research could thus analyze possible changes of redox status in these proteins under different oxygen conditions, including hypoxic environments. Furthermore, it could be investigated whether plants with the cysteine mutations that prevent light sensing of these proteins, still respond to changes in oxygen levels. Further studying of LOV domains will help to better understand the overall plant-environment sensing mechanisms and functions of the PAS superfamily, and additionally might lead to a better understanding of plant oxygen biology.

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