The role of cryptochromes in the sexual life cycle of *Chlamydomonas reinhardtii*

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Dissertation

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1. Introduction

Light is an important environmental stimulus influencing development, morphogenesis and physiology of most living organisms on Earth. Photosynthetic organisms that include higher plants, algae and cyanobacteria survive by converting light into an energy reservoir which is stored as carbohydrates by photosynthesis. Moreover, plant and algal movements (e.g. phototropism and phototaxis), and their developmental cycle are influenced by light (Kami et al., 2010). In addition, the entrainment of circadian biological clocks is also induced by light/dark cycle (Johnson et al., 1991; Kondo et al., 1991; Niwa et al., 2013).

Photosynthetic organisms accurately perceive spectral composition of light over the wavelength range from UV-B to far-red via employing multiple photoreceptors which initiate the corresponding signaling pathways to optimize the photosynthesis efficiency or avoid photo-damage (Galvao and Fankhauser, 2015). These light responsive photoreceptors are typically equipped with a chromophore to absorb and respond to specific wavelengths of light. To date, diverse types of sensory photoreceptors, such as phytochromes (PHYs), UV-B resistance 8 (UVR8), rhodopsins, cryptochromes (CRYs), phototropins (PHOTs), Aureochromes and members belonging to the family of ZTL/FKF1/LKP2 are found in nature to regulate light dependent developmental processes (e.g. germination and flowering), photosynthesis, photo-orientation, and control of the circadian clock (Hegemann, 2008; Masuda, 2013; Christie et al., 2015; Galvao and Fankhauser, 2015). Among these, CRYs are flavoproteins that serve as the sensory UV-A/blue light photoreceptor in insects, plants, fungi, and bacteria, while in mammals, CRYs have lost their light-sensing ability and play an important role in the central oscillator of the circadian clock (Cashmore, 2003; Lin and Shalitin, 2003; Lin and Todo, 2005; Chaves et al., 2011). CRYs share high sequence and structure similarity with DNA photolyases, which use light to repair UV-damaged DNA (Sancar, 2003). CRYs and photolyases originate from a common evolutionary ancestor and constitute the CRYs/photolyases superfamily.

1. Introduction

1.1 The research on CRYs/photolyases family

During evolution, most CRYs have lost the ability to repair damaged DNA and have obtained other biochemical functions, e.g. de-etiolation, photoperiodic flowering and circadian clock (Lin et al., 1995; Malhotra et al., 1995). The CRYs/photolyases family members contain the N-terminal highly conserved photolyase homologous region (PHR) domains that bind light-absorbing chromophores, such as methenyltetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deazariboflavin (8-HDF) and flavin adenine dinucleotide (FAD). In addition, each CRY also has a CRY C-terminal Extension (CCE) signaling domain which is not found in photolyases. Interestingly, the CCE domains in CRYs are of variable lengths and poorly conserved (Chaves et al., 2011).

The CRYs/photolyases superfamily is divided into three different categories: i) cyclobutane pyrimidine dimer (CPD) photolyases, ii) (6-4) photolyases and iii) CRYs based on their functions. According to amino acid sequence similarities and phylogenetic analyses, the family members of CPD photolyases are further clustered into class I, II and III CPD photolyases; (6-4) photolyases are divided into eukaryotic (6-4) photolyases and prokaryotic (6-4) photolyases; CRYs are divided into plant CRYs, CRY-DASHs (CRY-*Drosophila*, *Arabidopsis*, *Synechocystis* and *Homo*s), as wells as animal or animal-like CRYs (Chaves et al., 2011; Oberpichler et al., 2011; Fortunato et al., 2015; Mei and Dvornyk, 2015). Recently, a new photolyase subfamily, named PHR2 (photolyase/blue receptor 2), was reported to be present exclusively in the green plant lineage (Mei and Dvornyk, 2015). PHR2 subfamily are another class of CPD photolyases since they have the ability to catalyze the photo-repair of CPD. Notably, the PHR2 subfamily members form a sister group of CRY-DASHs (Mei and Dvornyk, 2015); plant CRYs are closely related to class III CPD photolyases, while animal CRYs cluster together with eukaryotic (6-4) photolyases (Öztürk et al., 2008; Beel et al., 2012).

Crystal structures of all classes of members from CRYs/photolyases family have been determined (Müller and Carell, 2009; Zhang et al., 2013; Scheerer et al., 2015).

The structures of the members from this family are highly conserved; however, they have variations in structural details such as, protein surface charges, modes of interaction with DNA and electron transfer pathways with different key amino acids, length and conformation of the C-terminal domain, and chromophore binding in the inter-domain loops (summarized in Scheerer et al., 2015).

1.1.1 Photolyases

Photolyases are enzymes that use 350-500 nm light to repair DNA lesions caused by UV light (200-300 nm). Upon exposure to harmful UV radiation, two major lesions are formed in the DNA of organisms, i) CPD (occupying 80-90% of the photo-lesions) and ii) (6-4) pyrimidine-pyrimidone adducts (occupying 10-20%) in single or double strands DNA. These lesions are repaired by CPD photolyases and (6-4) photolyases, respectively (Sancar, 2003; Chaves et al., 2011). However, CPD photolyases and (6-4) photolyases share high sequence similarities, as well as similar structures and reaction mechanisms.

As mentioned above, the four classes (class I, II, III and PHR2) of CPD photolyases are distributed in different kinds of organisms to perform the similar catalytic reaction. The class I CPD photolyases are found in various microbial organisms, including bacteria, euglenozoa, alveolata and fungi, but not in land plants and animals, while class II CPD photolyases have been identified in all classes of organisms (Lucas-Lledo and Lynch, 2009; Mei and Dvornyk, 2015). Class III CPD photolyases are bacteria-specific photolyases that stay close to plant CRYs (Öztürk et al., 2008). PHR2 members are green plants-specific CPD photolyases. However, it seems that their functions have been diversified. For example, PHR2 has been shown to repair CPD formed in both chloroplast and nuclear DNA in *Chlamydomonas reinhardtii* (Chlamydomonas thereafter), while it only performs nuclear DNA repair activity in *Arabidopsis thaliana* (Chen et al., 1996; Petersen and Small, 2001).

1.1.2 CRYs

1.1.2.1 Plant CRYs

Plant CRYs are mainly found in green plants. They have been extensively studied in *Arabidopsis thaliana*. Two plant CRYs in *A. thaliana* (AtCRY1 and AtCRY2) are mainly involved in the process of de-etiolation, photoperiodic control of flowering, guard cell development, stomata opening, leaf senescence, pathogenic responses (summarized in Liu et al., 2016). Among them, de-etiolation and photoperiodic flowering are two major blue light-induced morphogenetic steps regulated by AtCRY1 and AtCRY2, respectively (Guo et al., 1998; El-Din El-Assal et al., 2001; Yu et al., 2010). However, AtCRY2 also controls the hypocotyl response under dim blue light and AtCRY1 participates in the regulation of flowering time in some conditions (Lin et al., 1998; Blazquez et al., 2003; Mockler et al., 2003), suggesting that the functions of these two AtCRYs are distinct and only partially overlapping. Actually, these two CRYs have many different characteristics. For example, AtCRY2 mediates blue light-induced responses mainly in the nucleus, while AtCRY1 localizes not only in nucleus, but also in the cytosol to some extent to promote cotyledon expansion and root elongation (Wu and Spalding, 2007; Liu et al., 2016). AtCRY1 is stably expressed, while AtCRY2 is ubiquitinated and degraded by the proteasome pathway in photobodies of the nucleus after light illumination (Ahmad et al., 1998; Lin et al., 1998; Shalitin et al., 2002; Yu et al., 2007; Yu et al., 2009; Weidler et al., 2012; Zuo et al., 2012).

Both AtCRY1 and AtCRY2 form homodimers through interaction of their PHR domain (Sang et al., 2005; Rosenfeldt et al., 2008). Although light does not appear to affect the dimerization of AtCRYs, blue light illumination leads to the conformation changes of PHR domains via altering the state of the combined FAD, and subsequently activates the CCE domains to pass on the signals (Yang et al., 2000; Wang et al., 2001; Yang et al., 2001; Yu et al., 2007). *In vitro* spectroscopic analyses show that purified AtCRY1 and AtCRY2 contain the oxidized form of FAD as the ground state. Upon blue light illumination, FAD in AtCRYs incorporates an electron

and changes to a semi-reduced neutral FAD radical (FADH·), which is capable of absorbing a broader spectrum of light, e.g. green light (Zeugner et al., 2005; Banerjee et al., 2007; Bouly et al., 2007). Blue light illumination finally induces the phosphorylation of AtCRYs at the CCE domains. AtCRY1 and AtCRY2 seem to be phosphorylated via different pathways. *In vitro* analysis showed that purified AtCRY1 could be phosphorylated by the red/far-red photoreceptor PHYA, which has been tested with kinase activity (Ahmad et al., 1998; Ahmad et al., 1998; Yeh and Lagarias, 1998). Interestingly, AtCRY1 could also be phosphorylated in the *phyA* mutant strain and *in vitro* without addition of any kinase after blue light exposure (Bouly et al., 2003; Shalitin et al., 2003; Özgür and Sancar, 2006), suggesting that AtCRY1 has the ability of autophosphorylation, although no significant kinase related domain has been found in AtCRY1. In contrast, autophosphorylation is not detected in AtCRY2 (Özgür and Sancar, 2006). Instead, the phosphorylation of CCE of AtCRY2 is performed by Casein kinase 1 in a blue light dependent manner (Tan et al., 2013).

1.1.2.2 Animal CRYs

There are also CRYs from the animal lineages. Animal CRYs are classified into two distinct groups according to their functions within the circadian clock. The circadian clock, an endogenous timekeeper, is enhanced by environmental cues like light/dark cycle and temperature. As a result, it generates rhythms in metabolic processes in eukaryotic and some prokaryotic organisms with a periodicity of about 24 hours under a free running conditions (Bass, 2012). Circadian clocks, in a simplified scheme, consist of three major parts: one central oscillator, the input pathways (including photoreceptors-induced pathways) to the central oscillator, and the output pathways connected to distinct phases of the oscillator that regulate rhythms in behavior, physiology, and metabolism in an organism (Hurley et al., 2016). The central oscillator usually includes transcriptional activators and inhibitors acting in transcription/translation feedback loops. In *Drosophila*, there is a blue light-responsive animal CRY that acts in the circadian input pathway. It is referred to as type I animal CRY. In contrast, light-irresponsive animal CRYs such as in mice, humans, and other vertebrates act as transcriptional repressors within the central oscillator. They have

been designated as type II animal CRYs. However, some animals (e.g., zebrafish and monarch butterfly) have both types of animal CRYs. Interestingly, both type I and II animal CRYs have retained the two chromophore-binding sites in the conserved PHR domain, even though type II animal CRYs have evolved to be light-insensitive proteins, which indicates the different roles of the combined chromophores in these two types animal CRYs (Chaves et al., 2011; Xing et al., 2013).

Type I animal CRY is well characterized in *Drosophila* where one CRY (dCRY) is encoded. dCRY has no DNA repair activity, and serves as a circadian blue light photoreceptor involved in light-mediated entrainment of the circadian clock (Chaves et al., 2011). In the transcriptional/translational feedback loops of its circadian clock, the transcriptional activators CLOCK and CYCLE promote the transcription of the clock genes *Period* and *Timeless*, as well as of *dCRY*. The encoded PER and TIM work as a complex and translocate into the nucleus to inhibit their own transcription by binding to the CLOCK/CYCLE complex. dCRY interacts with TIM in a light-dependent manner and induces the degradation of TIM by ubiquitin-proteasome pathway via E3 ligase Jetlag (Ceriani et al., 1999; Rosato et al., 2001; Stanewsky, 2002; Peschel et al., 2009). Interestingly, dCRY is also degraded by interaction with Jetlag after light illumination (Peschel et al., 2009). Moreover, dCRY is also ubiquitylated by a Cullin-RING E3 ubiquitin ligase (CRL4) which employs Brwd3 (Bromodomain and WD repeat domain containing 3) as substrate receptor (Ozturk et al., 2013). In a dCRY mutant lacking the last 19 aa of the CCE, the truncated dCRY binds TIM in the dark as well as after a light pulse and TIM is no longer degraded in a light-dependent manner, suggesting the involvement of CCE of dCRY in the interaction with TIM and conformational changes after light pulses in *Drosophila* (Busza et al., 2004). Besides its function as a circadian photoreceptor, dCRY is required for maintaining rhythmicity of the circadian clock of peripheral tissues and sensing magnetic fields (Krishnan et al., 2001; Gegear et al., 2010; Qin et al., 2016).

For type II animal CRY, most research was done in mice. Two copies of type II animal CRY (mCRY1 and mCRY2) were identified in mice to be involved in the oscillator of the circadian clock. The circadian clock of *mCRY1* knockout strain

exhibits a shortened free-running period while the free-running period of *mCRY2* knockout strain is prolonged; a strain lacking both *mCRY* genes gets arrhythmic (Okamura et al., 1999; van der Horst et al., 1999; Vitaterna et al., 1999). Light induction of *Period* gene expression could also be observed in strain lacking both *mCRY* genes (Okamura et al., 1999). These results lead to the conclusion that type II CRYs of mammals are a central part of the circadian oscillator, instead of serving as circadian photoreceptors. Similar to *Drosophila*, the mammalian circadian oscillator also consists of interconnected transcriptional/translational feedback loops. The transcription heterodimer, CLOCK/BMAL1 activates the transcription of *mCRY1, mCRY2*, three *Period* genes, and clock-controlled genes (summarized in Chaves et al., 2011). mCRY1 has the capability to bind to CLOCK/BMAL1 complex alone at the promoter to inhibit CLOCK/BMAL1-induced transcription without releasing the complex from the promoter. Besides, mCRY1 can also form a complex with PER2 (Schmalen et al., 2014) to repress transcription by dissociating CLOCK/BMAL1 from the promoter (Ye et al., 2014). Besides interacting with PERs, mCRYs also undergo post-translational modification like ubiquitylation and phosphorylation to be involved in the mammalian circadian clock (Yagita et al., 2002; Harada et al., 2005; Hirano et al., 2014). For example, the phosphorylation of mCRY2 at Ser-557 by the priming kinase DYRK1A (dual-specificity tyrosine phosphorylation-regulated kinase 1A) (Kurabayashi et al., 2010) occurs in the nucleus. This phosphorylation promotes the subsequent phosphorylation at Ser-553 by GSK-3β (glycogen synthase kinase 3β), which leads to a more efficient degradation of mCRY2 by proteasome pathway (Harada et al., 2005). The crucial role of phosphorylation at Ser-557 of mCRY2 to maintain the proper clock oscillation was also confirmed in the *mCRY2* (S557A) mutant mice (Hirano et al., 2014). In contrast, the phosphorylation of mCRY1 on Ser-588 increases its protein stability by inhibiting FBXL3-mediated degradation, and prolongs the circadian period (Gao et al., 2013).

1.1.2.3 CRY-DASHs

CRY-DASHs are considered as a new subclade of the CRYs/photolyases family because of its close relationship with animal CRYs (Brudler et al., 2003). Up to now,

CRY-DASH homologs have been found in cyanobacteria, other eubacteria, archaea, fungi, plants, eumetazoa, and most vertebrates, but not in mammals (Chaves et al., 2011; Mei and Dvornyk, 2015). In *Arabidopsis*, CRY-DASH is a dual-localized protein in chloroplasts and mitochondria (Kleine et al., 2003). However, reports about the function of CRY-DASH are still rare. Recently, comprehensive phylogenetic analysis demonstrates that CRY-DASHs cluster close with PHR2 proteins (Mei and Dvornyk, 2015), a new group of photolyases which has been shown to have the DNA repair activity of CPD (Petersen and Small, 2001), raising the possibility of DNA repair activity of CRY-DASHs. Indeed, in contrast to CPD photolyases with repair activity of CPD in single strand or double strands DNA, CRY-DASHs from bacterial, plant, and animal sources were found to repair CPD specifically in single strand DNA (Selby and Sancar, 2006), in agreement with the indication from their crystal structure (Huang et al., 2006). However, in the green alga *Ostreococcus tauri*, the CRY-DASH homolog, CPF2 (CRY photolyase family 2) exhibits only the CPD-damaged double strands DNA repair activity (Heijde et al., 2010). Another research in mucoromycotina fungi showed that CRY-DASH has nearly all the characteristics of photolyases, such as fully complementing the *E. coli* photolyase mutant and repairing *in vitro* CPD lesions in single strand or double strands DNA (Tagua et al., 2015). In addition, sequence-independent binding of CRY-DASH to undamaged DNA in *Synechocystis* and *Arabidopsis* was also reported, suggesting the involvement of CRY-DASH in signaling (Brudler et al., 2003; Kleine et al., 2003). Indeed, *Synechocystis* CRY-DASH was found to be a transcriptional repressor of at least eight genes (Brudler et al., 2003). Still, more efforts are necessary to clarify the role of CRY-DASH in signaling pathways.

1.1.2.4 Research of some special CRYs

Except the three clear-divided clades of CRYs, there are still some CRYs which stay at the base of the phylogenetic tree with ambiguous evolutionary trajectory. For example, a CRYp in diatoms exhibits weak homology with plant CRYs and class III CPD photolyases. However, it shows spectroscopic features of a chromophore composition like a CRY-DASH. It alters the protein expression level of light protection

proteins and light-harvesting proteins (Juhas et al., 2014). CPF1 proteins in diatoms and green algae *Ostreococcus* not only display efficient (6-4) photolyase activity, but also show a role in the circadian clock of a heterologous mammalian cell system (Coesel et al., 2009; Heijde et al., 2010). Phylogenetically, they are situated at the basis of the eukaryotic (6-4) photolyases and animal CRYs (Beel et al., 2012). So it seems that the ancestor of eukaryotic (6-4) photolyases and animal CRYs has dual or even more functions. Another case is CryB from the purple photosynthetic bacterium *Rhodobacter sphaeroides*. CryB lacks the typical CCE domain of CRYs, and belongs to the FeS-BCP family, a new identified subgroup employing 6,7-dimethyl-8-ribityllumazine as an antenna chromophore and binding a [4Fe-4S] cluster in the catalytic domain (Oberpichler et al., 2011; Geisselbrecht et al., 2012). Although CryB is capable of close binding of single strand DNA and lower binding of double strands DNA and single strand RNA, it shows no repair activity for CPD in single-stranded DNA *in vitro*. Interestingly, it exhibits the CRY ability to regulate the gene expression of pigment-binding proteins and the amount of photosynthetic complexes (Hendrischk et al., 2009). So it is described as a CRY but represents a bacterial type without CCE domain.

In summary, former research about members from CRYs/photolyases family has paved the way for studies of other members in this family. The green alga Chlamydomonas was selected as model organism to study the function of the members of this family in this work. It encodes all three types CRYs, one plant CRY (pCRY), one animal-like CRY (aCRY), and two CRY-DASHs.

1.2 The model organism Chlamydomonas

Chlamydomonas is a unicellular green alga with several mitochondria, two anterior flagella for motility and mating and a large basal U-shaped chloroplast for photosynthesis (Harris, 2009). It contains a primitive visual system, eyespot, which is situated at the edge of the chloroplast and involved in phototaxis (Schmidt et al., 2006; Kreimer, 2009). Chlamydomonas is a haploid organism and reproduces by binary fission in optimal growth conditions. Sexual growth is often induced by adverse conditions (e.g. N-starvation) for genetic crosses and tetrad analysis in the laboratory.

Chlamydomonas grows not only photoautotrophically, but also heterotrophically with acetate as its sole carbon source. This ability allows the research of mutants in photosynthetic apparatus; otherwise mutants would not survive if obligatory photoautotroph (Harris, 2001).

Various molecular and genetic tools and methods have been established for this alga (summarized in Jinkerson and Jonikas, 2015). For example, artificial miRNA technology is widely used to knock down the expression of nuclear genes of interest. Many mutants have been generated by insertional mutagenesis with antibiotics selection marker (Gonzalez-Ballester et al., 2011; Li et al., 2016). Targeted modification of the nuclear genome is also available, by approaches such as zinc-finger nucleases (Sizova et al., 2013) and homologous recombination (Sodeinde and Kindle, 1993; Gumpel et al., 1994), as well as the CRISPR/Cas9 system (Jiang et al., 2014). Transgenes encoding transformation markers, reporter genes, fluorescent proteins, epitope tagged proteins, and therapeutic proteins are successfully expressed in this alga (Jinkerson and Jonikas, 2015). Furthermore, the genome of the nucleus, chloroplast and mitochondria are available and all three genetic compartments can be transformed (Boynton et al., 1988; Kindle et al., 1989; Randolph-Anderson et al., 1993). A comprehensive online database (https://phytozome.jgi.doe.gov/pz/portal.html#! info?alias=Org_Creinhardtii) including latest version of genome sequences (Merchant et al., 2007) along with a large number of expressed sequence tags (ESTs) exists that has paved the way to perform proteomics research and basic molecular studies. The protein compositions of several sub-proteomes for flagella (Pazour et al., 2005), for the eyespot (Schmidt et al., 2006; Eitzinger et al., 2015) or for the chloroplast (Terashima et al., 2011) are also open to public, which offer the basic localization information about proteins in Chlamydomonas. Furthermore, resources of wild-type, mutant strains, and plasmid constructs for transformation are also available to the research community. These merits have conferred Chlamydomonas as an excellent model organism in the research fields of flagellar, basal bodies (centrioles), chloroplast biogenesis, photosynthesis, light perception, cell-cell recognition, circadian clock and cell cycle control (summarized in Harris, 2001 and Harris, 2009). For light perception, this alga

is equipped with multiple photoreceptors which absorb a wide range of wavelengths of the visible spectrum.

1.2.1 The photoreceptors found in Chlamydomonas and their role

So far, several types of photoreceptors (rhodopsins, PHOT, UVR8 and CRYs) have been identified in Chlamydomonas and were partially characterized. At least seven rhodopsin-related proteins are present in the genome of Chlamydomonas (Hegemann et al., 2001). Among them, two kinds of rhodopsins, channelrhodopsins (ChRs) and histidine kinase rhodopsins (HKRs) have been characterized (summarized in Hegemann, 2008; Luck et al., 2012). Two bacterial-type ChRs (ChR1 and 2) are present in the eyespot to perceive mainly green and blue light. They are mainly involved in phototactic and photophobic responses in Chlamydomonas (Nagel et al., 2002; Sineshchekov et al., 2002; Nagel et al., 2003; Berthold et al., 2008). When light is perceived by the retinal chromophore of the ChRs, the conformation of the ChRs are changed to allow H^+, Na^+, K^+, and Ca^{2+} to transmit from one side to the other side of the membrane and induce the photocurrent to promote a following signaling cascade (Berthold et al., 2008). Recently, it was found that trafficking of these rhodopsins into the eyespot is even connected with the intra-flagellar transport (Awasthi et al., 2016). Less is known about the HKRs, which generally contain domains of rhodopsin, a histidine kinase, a response regulator, and in some cases an effector domain like an adenylyl or guanylyl cyclase. Studies about HKR1 in Chlamydomonas showed that HKR1 is localized in eyespot and is bimodally switched by absorbing UV-A and blue light *in vitro* (Luck et al., 2012).

Chlamydomonas PHOT is a well-studied blue light sensory photoreceptor. It is a membrane-associated protein in Chlamydomonas (Huang et al., 2002), and is distributed in the eyespot, flagellar and the cell body (Huang et al., 2004). It is involved in the development of the eyespot size, the regulation of expression level of ChR1 and phototactic behavior in gametes (Trippens et al., 2012). Moreover, PHOT regulates the light-induced expression level of genes related to chlorophyll and carotenoid biosynthesis and light-harvesting complexes in vegetative cells, both

under blue and red light (Im et al., 2006). Furthermore, it is involved in the switch-off of chemotaxis towards nitrite in pregametes (Ermilova et al., 2004). In addition, it participates in the regulation of light-dependent steps in the sexual life cycle as outlined below (Huang and Beck, 2003). Recently, it was shown that PHOT acts also as the photoreceptor that mediates feedback regulation of photosynthesis of high light acclimation (Petroutsos et al., 2016).

In addition to the above mentioned photoreceptors, a UVR8 homolog was found in Chlamydomonas to induce UV-B acclimation by initiating the signaling pathway via interacting with COP1 in a UV-B dependent manner (Tilbrook et al., 2016). UVR8 in Chlamydomonas shares the sequence and structure similarity of UVR8 in *Arabidopsis*. It is a functional photoreceptor since it can complement the *Arabidopsis uvr8* mutant. UVR8 in Chlamydomonas regulates the expressions of multiple genes related to photosynthesis and stress in a UV-B dependent manner to induce UV-B acclimation and protection (Tilbrook et al., 2016).

Moreover, four CRYs, an aCRY, a pCRY and two CRY-DASH proteins are also encoded in *C. reinhardtii* (Beel et al., 2012). The pCRY (former known as Chlamydomonas Photolyase Homologue1, CPH1) is closely clustered with AtCRY1 and AtCRY2 from *Arabidopsis* and considered as a possible evolutionary precursor of all plant CRYs (Lariguet and Dunand, 2005). *In vitro* analyses of the conserved PHR domain of pCRY in Chlamydomonas were performed (Immeln et al., 2007; Langenbacher et al., 2009; Immeln et al., 2010). Data from UV-visible spectroscopy, fluorescence spectroscopy, and thin layer chromatography analysis demonstrate that oxidized FAD is the exclusive flavin chromophore of pCRY-PHR and absorbs the blue light region with maxima at 368 and 449 nm (Immeln et al., 2007). Furthermore, the blue light absorption induces the radical formation of FAD, PHR domain autophosphorylation and secondary structure alteration (Immeln et al., 2007; Immeln et al., 2010). Interestingly, ATP binding of the PHR domain stabilizes its radical state after illumination, suggesting the significance of ATP binding in pCRY signaling (Immeln et al., 2007). *In vivo* analysis shows that pCRY in Chlamydomonas is rapidly degraded by the proteasome pathway in a blue and red light-dependent manner

(Reisdorph and Small, 2004). pCRY in Chlamydomonas may also act as a negative regulator of circadian phototaxis of vegetative cells (Forbes-Stovall et al., 2014). In addition, studies of pCRY in Chlamydomonas showed that it controls key features of the circadian clock including period and phase, and the developmental processes in sexual life cycle like gametogenesis and zygote germination (Müller et al., in preparation for resubmission). The two CRY-DASH proteins from Chlamydomonas are still under investigation.

Recently, aCRY was functionally characterized in Chlamydomonas using an insertional *acry* mutant with a strongly reduced level (down to approximately 20%) compared to wild type. In response to blue and red light, the transcript levels of several genes of chlorophyll and carotenoid biosynthesis, light harvesting complexes, nitrogen metabolism, the cell cycle and the circadian clock are altered in the *acry* mutant when comparing with wild type (Beel et al., 2012; Beel et al., 2013). Moreover, yellow but not far-red light causes comparable changes on the transcript level of selected genes between *acry* mutant and wild type. These data are in agreement with *in vitro* data showing that blue, yellow, and red light is absorbed by the neutral radical state of flavin in aCRY, but not far-red light. It is thus assumed that the neutral radical of the flavin chromophore acts as a dark form of the sensor that absorbs in almost the entire visible spectrum (below 680 nm). A conversion by red light of the neutral radical state to the anionic fully reduced state was found by spectroscopic analyses to go hand in hand with conformational changes of aCRY (Spexard et al., 2014). Moreover, an essential role of an unusually long-lived tyrosyl radical at position 373 of aCRY was found recently to be involved in the red light response (Oldemeyer et al., 2016). Heterologously expressed and purified aCRY forms a dimer in the dark, while partial oligomerization was observed upon illumination via disulfide bridge formation at cysteine 482 (Oldemeyer et al., 2016). Phylogenetically, aCRY in Chlamydomonas stays at the base of a subgroup consisting of animal CRYs and eukaryotic (6-4) photolyases; moreover, it is closely related to CPFs from *O. tauri* and *Phaeodactylum tricornutum*, which are shown to have dual or triple functions (Coesel et al., 2009; Heijde et al., 2010), raising the possibility of multi-functionalities of aCRY in Chlamydomonas (Beel et al., 2013). Despite these advances on the molecular

spectroscopic and protein chemical level of aCRY, its *in vivo* functions are still elusive to a large extent (as detailed in Zou et al., submitted).

1.2.2 The involvement of light in the sexual life cycle of Chlamydomonas

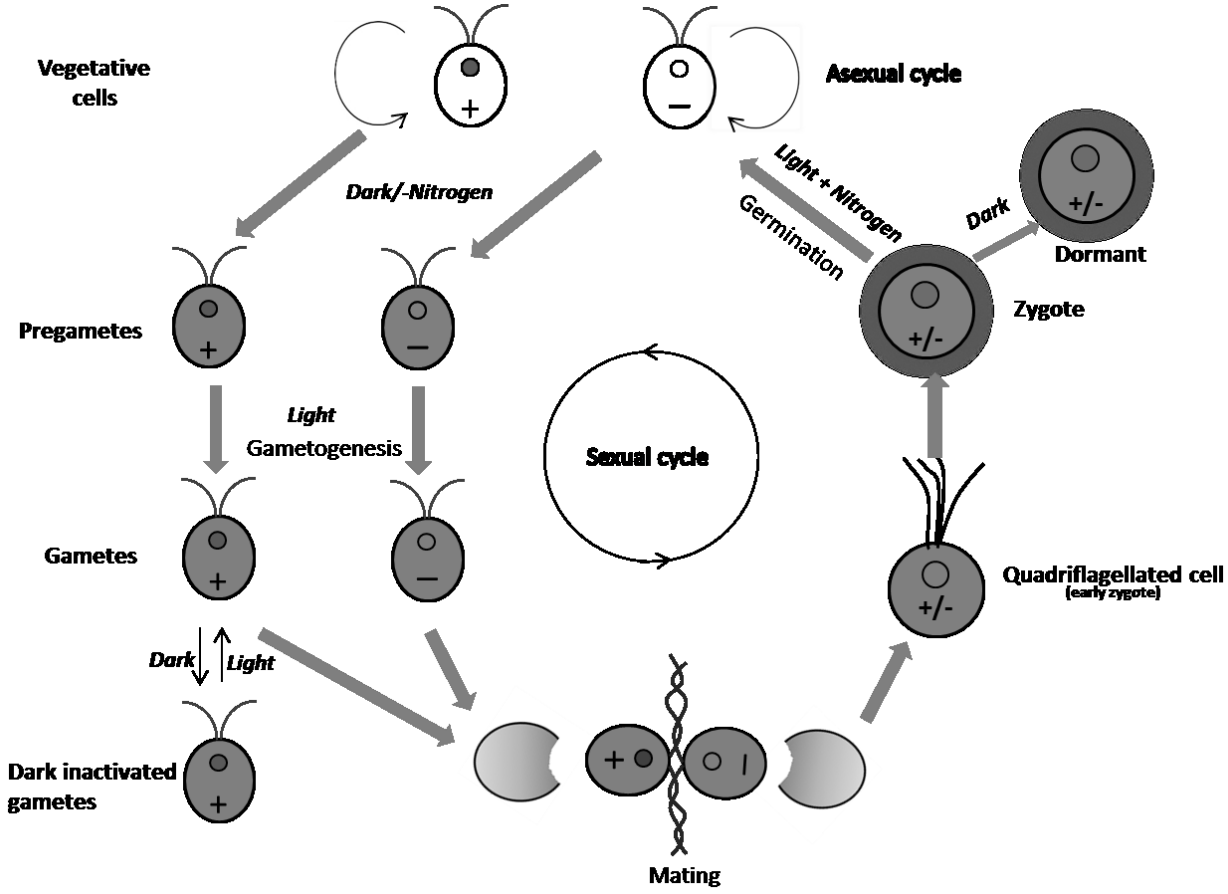

Figure 1. Life cycle of Chlamydomonas (Zou et al., submitted).
The life cycle of Chlamydomonas (modified from Huang and Beck, 2003). Haploid vegetative cells perform asexual reproduction under optimal growth conditions. They turn into pregametes under nitrogen deprivation conditions in darkness. Light induces the formation of gametes. Gametes may lose their mating ability and turn to dark inactivated gametes upon dark treatment. For simplicity, only the conversion of the plus strain is shown. When gametes of two different mating types are mixed, they will mate and fuse to a quadriflagellated cell that is called early zygote. The early zygotes convert to mature zygotes after exposure to 15-18 hours light followed by 5 days in the dark (Jiang and Stern, 2009). In the absence of any light, zygotes will stay as dormant cells; when light and nitrogen are available, they will undergo meiosis and germinate to release four vegetative cells, two plus and two minus.

Chlamydomonas undergoes both asexual and sexual life cycles (Fig. 1). Under optimal growth conditions, Chlamydomonas reproduces asexually by binary fission, which doubles the population quickly (~8 hours). In contrast, Chlamydomonas will

turn to the sexual life cycle when the growth condition is non-optimal, e.g. nitrogen depletion. It is known that light influences the sexual life cycle of Chlamydomonas as well as growth conditions. Nitrogen depletion leads to the conversion of haploid vegetative cells to pregametes (Huang and Beck, 2003; Goodenough et al., 2007). Illumination provokes the transition from pregametes to gametes, which achieve full mating ability (Beck and Acker, 1992). While some Chlamydomonas strains do not differentiate at all into gametes in the dark but absolutely require light for this process, others can differentiate to gametes in the dark to a small extent, but need light for higher conversion rates (Saito et al., 1998). Gamete maintenance is also dependent on light. Consequent dark treatment of mature gametes leads to the loss of mating ability producing so called dark-inactivated gametes. Further light exposure recovers the mating ability. The conversion from pregametes to gametes and from dark-inactivated gametes to reactivated gametes, also known as gametogenesis and restoration, are mainly influenced by blue light, but to some extent also by red light (Weissig and Beck, 1991; Pan et al., 1997), indicating the participation of blue and/or red light photoreceptors in these two processes (as summarized in Zou et al., submitted). When mature gametes of the plus and minus mating types are mixed, they get close with the help of the agglutinin secreted at the top of the flagellar, and finally fuse into one cell with four flagellar (also called quadriflagellated cell, QFC) after releasing the cell wall. The maintenance of the quadriflagellated form for several hours offers a good opportunity for mating ability-related research, such as mating ability and mating maintenance ability test. The quadriflagellated cells finally lose the flagellar and become mature diploid zygotes. The germination process of the zygote into four haploid vegetative cells requires again light as well as nitrogen-containing media. In darkness, zygotes will become dormant cells (Gloeckner and Beck, 1995). Occasionally, 1-5% young zygotes fail to express the zygotic-specific genes and resume the vegetative growth as a stable mt^+/mt^- diploid in the presence of enough nitrogen. When these cells are kept under nitrogen-free condition again, they develop to be minus gametes and can mate with the plus haploid gametes, suggesting plus is recessive to minus (Ebersold, 1967; Galloway and Goodenough, 1985; Goodenough et al., 2007).

1.3 Main aims of the PhD work

Previous research in Chlamydomonas demonstrated that PHOT influences the processes of mating ability, the restoration of mating ability of dark-inactivated gametes, and zygote germination (Huang and Beck, 2003). Interestingly, it has been revealed that red light is also involved in the sexual processes of gametogenesis to some extent, although blue light has a major effect (Weissig and Beck, 1991; Pan et al., 1997). So far, there is no evidence for a red light absorbing photoreceptor that is involved in the sexual life cycle of Chlamydomonas. Because no phytochrome is encoded in the Chlamydomonas genome (Mittag et al., 2005; Merchant et al., 2007), aCRY is a candidate that might play a role in the process of gametogenesis and/or zygote germination in addition to PHOT. But also pCRY may act in concert with other blue light photoreceptors in these processes.

Based on the selected mutant strains of aCRY and pCRY in Chlamydomonas (Beel et al., 2012; Müller et al., in preparation for resubmission), the expression level of aCRY at different stages of the Chlamydomonas sexual cycle was characterized. Moreover, the involvement of aCRY in the light regulated steps of the sexual cycle and the participation of pCRY in zygote germination were investigated. Furthermore, the connection between aCRY and other photoreceptors (PHOT and ChR1) during pregamete to gamete transition, as well as the role of aCRY in the process of phototaxis of gametes were characterized.

2. Results

2.1 aCRY in vegetative cell is primarily present as a soluble protein, but is also associated with membranes to some extent (Zou et al., submitted)

The soluble aCRY protein of vegetative cells accumulates at its highest level at the beginning of the day and is present at its lowest level at the beginning of the night (Beel et al., 2012). To investigate if aCRY may be also associated with membranes such as the Chlamydomonas blue light receptor PHOT, subfractions of vegetative cells harvested during the day (LD4) were prepared. For this purpose, total proteins were separated into fractions containing soluble, peripheral membrane, and transmembrane proteins (s. 6.2.4.4) and analyzed in immunoblots along with anti-aCRY antibodies. Anti-LOV1 (PHOT) and anti-CYTf antibodies were used as controls for a membrane-associated (PHOT) and a transmembrane (CYTf) protein, respectively. As shown before (Huang et al., 2002), PHOT was strongly enriched in the peripheral membrane fraction, weakly present in the soluble fraction and missing in the transmembrane fraction (Fig. 2). CYTf, that is known to be located in thylakoid membranes (Willey et al., 1984), appeared predominantly in the transmembrane fraction (Fig. 2). aCRY was mainly present in the soluble fraction, but also detectable in the membrane-associated fraction to a smaller extent (Fig. 2). aCRY did not accumulate in any significant amount in the transmembrane protein fraction, consistent with the theoretical prediction by TMHMM Server (Sonnhammer et al., 1998) that it lacks transmembrane domains (Fig. 3). As a result, aCRY is predominantly present as a soluble protein but also has the ability to associate with membranes to some part in vegetative cells.

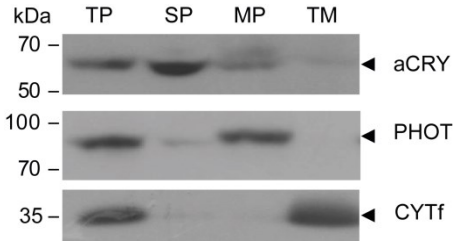

Figure 2. The distribution of aCRY in different subfractions (Zou et al., submitted).

Soluble proteins (SP), membrane-associated proteins (MP), and transmembrane proteins (TM) of total proteins (TP) were prepared from vegetative cells of the mt^+ strain. 30 µg proteins of each fraction were separated by 9% SDS–PAGE and immunoblotted with anti-aCRY, anti-LOV1 (PHOT) and anti-CYTf antibodies, respectively.

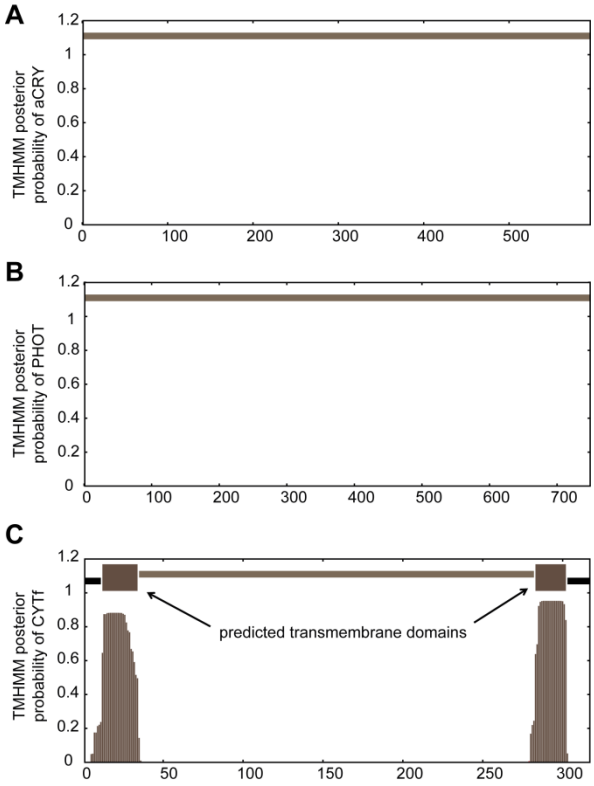

Figure 3. The transmembrane domain prediction of aCRY, PHOT and CYTf.
The transmembrane domain prediction of aCRY (Cre06.g278251.t1.1 in Phytozome), PHOT (Cre03.g199000.t1.2 in Phytozome) and CYTf (BAA00844.1, Genbank) were performed on the TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM/). The results show that aCRY (A) and PHOT (B) have no transmembrane domain, while CYTf (C) has two transmembrane domains.

2.2 Soluble and membrane-associated aCRY accumulates differentially during the sexual cycle (Zou et al., submitted)

Light is involved in the process of gametogenesis, where primarily blue and to some extent red light are effective (Weissig and Beck, 1991, Fig. 1). To examine if aCRY with its ability to absorb in the blue and red region of the visible spectrum (Beel et al., 2012) may have a function in the sexual life cycle, the expression status of aCRY at different stages of the Chlamydomonas life cycle was analyzed. Soluble proteins from vegetative cells, pregametes, gametes, early zygotes and late zygotes were prepared

and analyzed by immunoblots with anti-aCRY antibodies. As shown before (Beel et al., 2012), soluble aCRY is well expressed in vegetative cells (LD0, Fig. 4A). In contrast, aCRY accumulation is strongly reduced in pregametes and gametes, both in cells of mating type plus and minus. The strong reduction was also found in early zygotes (Fig. 4A). In contrast to these results, soluble aCRY was visible under all tested conditions (under dark, and after 6, 12 and 24 h illumination) in late zygotes, albeit it was more abundant after illumination (Fig. 4B). Furthermore, a modified form of aCRY was observed to a significant extent, whose nature was unclear (Fig. 4B). One possibility is that aCRY gets phosphorylated during this stage of the sexual cycle. To check for this, proteins of a crude extract were treated with λ protein phosphatase, but the signal of the potentially modified aCRY was not changed (Fig. 4C), excluding phosphorylation. Thus, the nature of the modification in late zygotes is still unknown. These data show that soluble aCRY is differentially expressed at different life stages of Chlamydomonas.

Since aCRY is also present in the fraction of membrane-associated proteins in vegetative cells to a certain extent, it was also checked whether the observed changes of soluble aCRY during the life cycle can be found with membrane-associated aCRY. Surprisingly, membrane-associated aCRY accumulates in pregametes and gametes of both mating types (Fig. 5A). Only in early zygotes its level is strongly reduced. Membrane-associated aCRY was also found in the membrane fraction of late zygotes, but no modified form was detected there (Fig. 5B). In contrast to soluble aCRY in late zygotes, membrane-associated aCRY is most abundant in the dark. These data suggest that the soluble forms and membrane-associated forms of aCRY are subject to different control mechanisms during the life cycle despite in early zygotes where they are both present in small amounts.

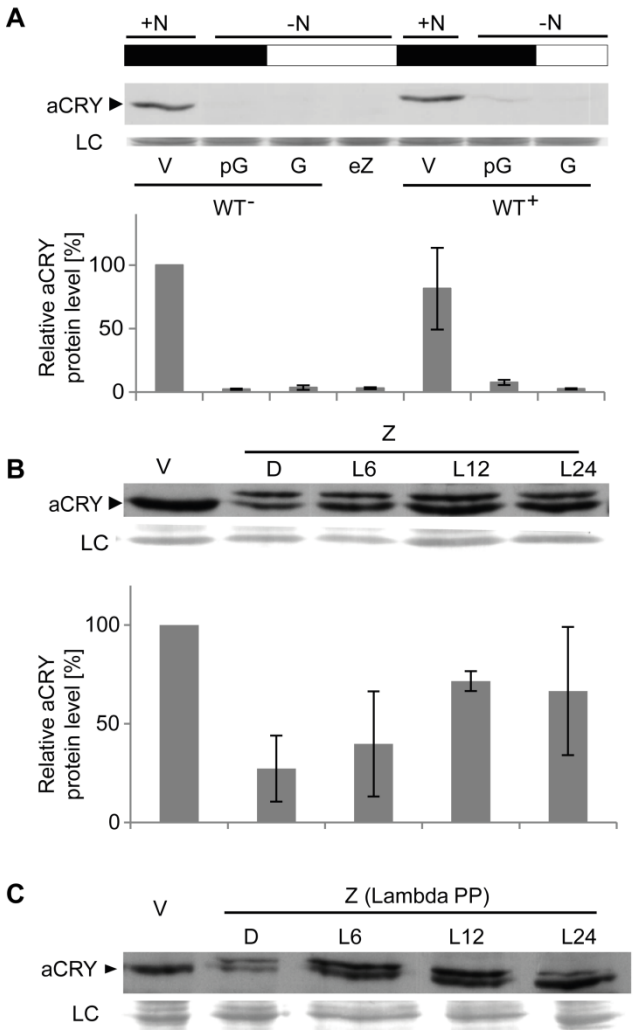

Figure 4. The expression profile of soluble aCRY in vegetative cells (V), pregametes (pG), gametes (G), early zygotes (eZ) and late zygotes (Z) (Zou et al., submitted).

A, The expression of soluble aCRY in V, pG, G of mating type plus and minus (mt^+ and mt^-) cells and eZ. V, pG, G and eZ were prepared as described in Methods. +N or –N represents the presence or absence of a nitrogen source in the media. Black bars on the top indicate darkness and white bars indicate light. 75 µg proteins of each sample were separated by 9% SDS-PAGE and immunoblotted with anti-aCRY antibodies. Unspecified protein bands from the PVDF membrane stained with Coomassie Brilliant Blue R250 after immunochemical detection were used as loading control (LC). Quantified aCRY protein levels of three biological replicates relative to the protein level of V cells of the mt^- strain are shown in the diagram with standard deviation (SD). B, The expression of aCRY in the soluble protein fraction of late zygotes under darkness (D), and after 6 h (L6), 12 h (L12), and 24 h (L24) illumination, respectively. The expression of aCRY in vegetative cells (V) was used as a control; the levels of aCRY (only the lower bands in late zygotes) were quantified based on three biological replicates; error bars show SD. The preparation of samples was described in Methods. C, The expression of aCRY in late zygotes after protein phosphatase treatment. To check whether aCRY is modified by phosphorylation in late zygotes, crude extracts of native soluble proteins were treated with Lambda Protein Phosphatase (Lambda PP) for 30 min at 30°C according to the manufacturer's instructions. 75 µg proteins of each sample were separated by 9% SDS–PAGE and immunoblotted with anti-aCRY

antibodies. Proteins of vegetative cells were used as a control. Unspecified protein bands from the PVDF membrane were selected as loading control (LC).

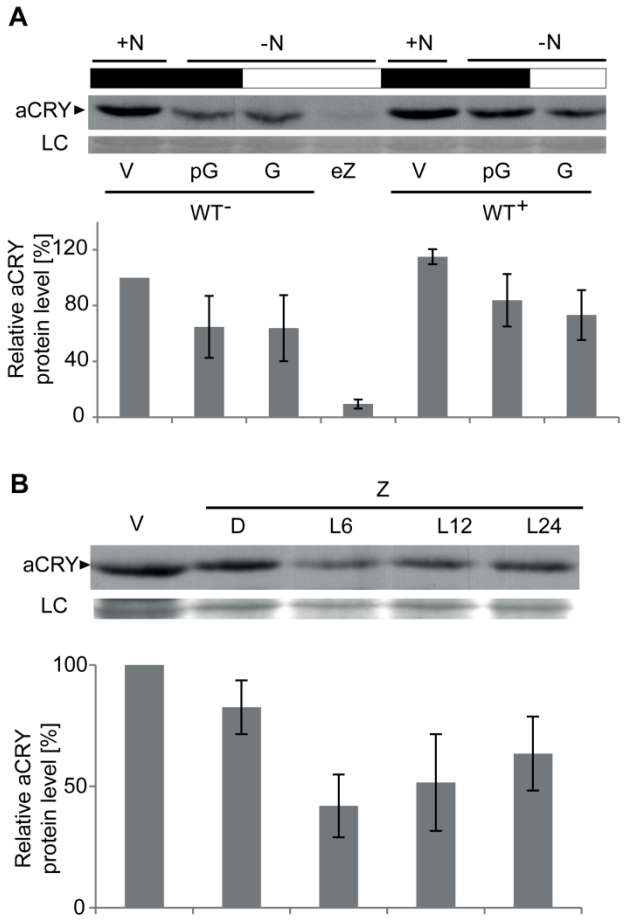

Figure 5. The expression of aCRY in the membrane fractions of vegetative cells, pregametes and gametes of mt^+ and mt^- wild type strains, as well as of early zygotes and late zygotes (Zou et al., submitted).
A, The expression level of aCRY in the membrane fraction of vegetative cells (V), pregametes (pG) and gametes (G) of mt^+ and mt^- strains and early zygotes (eZ). V, pG, G, and eZ were prepared and labeled as described in Fig. 4. Three biological replicates were performed. Error bars indicate the standard deviation (SD). B, The expression of aCRY in the membrane fraction of late zygotes (Z) under dark (D) as well as after 6 h (L6), 12 h (L12), and 24 h (L24) illumination. The expression of aCRY in membrane fraction of V was used as a reference. The aCRY levels in the membrane fraction of later zygotes were quantified based on three biological replicates; error bars indicate SD.

2.3 The degradation of aCRY in pre-gametes, gametes and early zygotes

2.3.1 Soluble aCRY is degraded by the proteasome pathway (Zou et al., submitted)

To check whether soluble aCRY may be degraded by the proteasome pathway, the cell-permeable proteasome inhibitor (MG 132) was added prior to harvesting. In this case, soluble aCRY accumulates in pregametes, gametes and early zygotes (Fig. 6), suggesting that aCRY is indeed degraded via the proteasome pathway. Even in vegetative cells, soluble aCRY accumulates at a higher rate in the presence of the proteasome inhibitor, suggesting that soluble aCRY undergoes degradation via the proteasome pathway even in these cells to a certain extent.

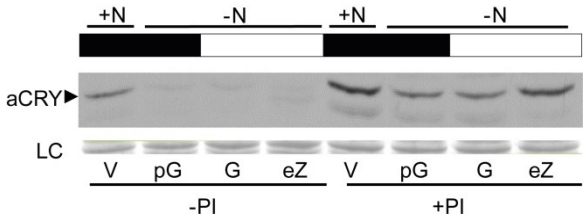

Figure 6. Degradation of soluble aCRY via the proteasome pathway in pregametes (pG), gametes (G), and early zygotes (eZ) (Zou et al., submitted).
Samples were prepared and the figure was labeled as described in Fig. 4. The samples were either treated (+PI) or not treated (-PI) with proteasome inhibitor MG-132 (carbobenzoxy-leucyl-leucyl-leucinal) 1h before harvesting with a final concentration of 10 mM.

2.3.2 Search for possible proteasome-derived interaction partners of aCRY

Protein degradation via the proteasome pathway involves tagging of the substrate protein with several ubiquitin molecules by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s), as well as the following degradation of the tagged protein by the 26S proteasome. E3 interacts with substrate proteins and also with an E2 to form an E2-E3-substrate complex. The E3 ligases are represented by a small amount of HECT (homologous to the E6-associated-protein carboxyl terminus) domain family members and the vast majority of RING (really interesting new gene) and RING-related E3s (Metzger et al., 2012). Since several interaction partners of CRYs which also serve as E3 ligase have been revealed in other organisms (Table 1), a BLAST searches against the

Chlamydomonas proteome with these candidates as queries were performed. The closest homologs of each protein are listed in Table 1. Kelch_F-box and ROC114 (rhythm of chloroplast 114) were selected to perform more detailed experiments (e.g. pull-down assay) to analyze a potential interaction with aCRY.

Table 1. List of E3 ligases that interact with CRYs in other organisms and their homologs in Chlamydomonas.

Name	Homolog in Chlamydomonas	Reference
COP1 (*Arabidopsis*)	Cre02.g085050.t1.2	(Yang et al., 2001; Shalitin et al., 2002; Cashmore, 2003)
CDT2 (mouse)	No	(Tong et al., 2015)
FBXL3 (mouse)	No	(Busino et al., 2007)
Brwd3 (*Drosophila)*	Cre02.g078150.t1.1	(Ozturk et al., 2013)
Jetlag	No	(Peschel et al., 2009)
Kelch_F-box	Cre01.g013050.t1.1	(Schumann et al., 2011)
ROC114	Cre02.g095900.t1.2	(Niwa et al., 2013)

2.3.2.1 Kelch_F-box and ROC114 in Chlamydomonas

Domain analysis shows that Kelch_F-box contains one F-Box domain and one Kelch domain, which are assumed to interact with E2 and the substrate, respectively. ROC114 is an F-box containing protein, which is related to the light induced degradation of ROC15, a clock component in Chlamydomonas involved in phase-resetting of the circadian clock by light (Niwa et al., 2013).

2.3.2.1.1 Pull-down tests of Kelch_F-box and ROC114 with aCRY

The plasmids for overexpressing Kelch_F-box and ROC114 that contain codon-adapted sequences of Kelch_F-box (pKelch_F-box, s. Appendix) and ROC114 (pROC114, s. Appendix) were synthesized from the Thermo Fisher Scientific Company. At the 5' and 3' end of the Kelch_F-box or ROC114 fragment, restriction sites for *Nco*I and *Xho*I were designed, respectively. The Kelch_F-box and ROC114 were ligated into the expression vector pET28a after digestion by the restriction enzymes *Nco*I and *Xho*I. Then the plasmids were transformed into the *E. coli* strain XL-blue separately for plasmid replication. Plasmids were isolated, digested by *Xho*I

and *Nco*I and further checked by agarose gel electrophoresis. pYZ3, which contains the full length of Kelch_F-box, was digested by *Xho*I and *Nco*I and the expected bands of 2423 bp and 5231 bp were detected (Fig. 7A). pYZ2, which contains the full length of ROC114 was digested by *Xho*I and *Nco*I and the expected bands of 4034 bp and 5231 bp were detected (Fig. 7B). pYZ3 and pYZ2 were then transformed to the *E. coli* strain BL21 separately for protein overexpression. The IPTG concentration and the induction time duration were optimized using the small amount protein expression protocol (s. 6.2.2.4). For a bigger protein demand, proteins were overexpressed using the large amount protocol (s. 6.2.2.5).

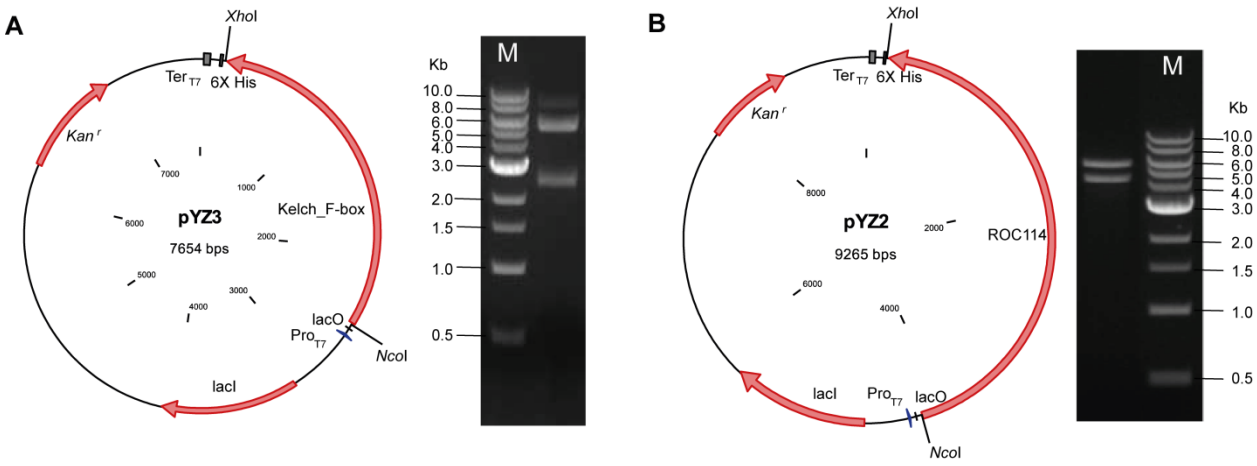

Figure 7. Plasmids used for the overexpression of Kelch_F-box (A) and ROC114 (B).
The codon-adapted sequences of Kelch_F-box and ROC114 were introduced to pET28a, respectively. Pro_{T7}: T7 promoter; lacO: lac operator DNA sequence; Ter_{T7}: T7 terminator; *Kan*^{r}: Kanamycin resistance; M: marker, Kb: kilobase. The constructed plasmids were tested by gel electrophoresis after digestion with *Xho*I and *Nco*I. The predicted fragment sizes after digestion of pYZ3 are 2423 bp and 5231 bp, while the predicted fragment sizes after digestion of pYZ2 are 4034 bp and 5231 bp.

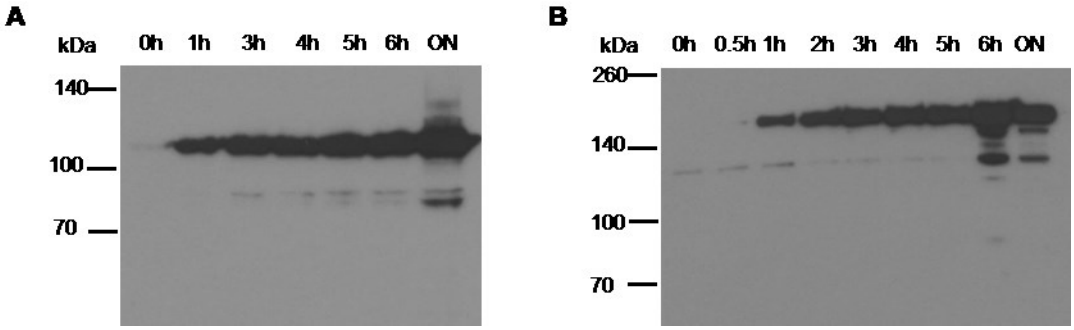

Figure 8. Time course of overexpression of Kelch_F-box (A) and ROC114 (B).

The labels on the top of the figures represent the time duration after adding IPTG. ON means overnight (~16 h). For Kelch_F-box, the induction was done at 16°C with 0.2 mM IPTG, while the induction of ROC114 was performed at 30°C with 1 mM IPTG. The 6 × His-tagged Kelch_F-box in the immunoblot is higher than its predicted size (86 kDa). The 6 × His-tagged ROC114 in the immunoblot is also higher than its predicted size (138 kDa).

From the optimization test, it was found that the expression of Kelch_F-box reached its highest level after an overnight induction with 0.2 mM IPTG (Fig. 8A). However, at this time point, other unspecific proteins may be also expressed. Therefore, the time point of 6 h IPTG induction was chosen for enriching a large amount of proteins. The amount of overexpressed ROC114 reached its highest level at 6 h after induction with 1 mM IPTG (Fig. 8B). Other unspecific proteins were also observed at this time point. Therefore, the cells were harvested at 5 h after IPTG induction.

The overexpressed Kelch_F-box and ROC114 were purified (s. 6.2.2.6) before they were applied to the Dynabeads (Life Technologies) for a pull-down assay (s. 6.2.4.9). Purified 6 × His tagged proteins were first added to the beads for binding and the excess proteins were washed away. Soluble proteins of the crude extract from vegetative cells and pregametes of Chlamydomonas were applied to react with the column-binding proteins and the excess proteins were washed away. Finally, all proteins bound to the column were eluted with imidazole containing buffer and analyzed by immunoblot using anti-aCRY antibodies.

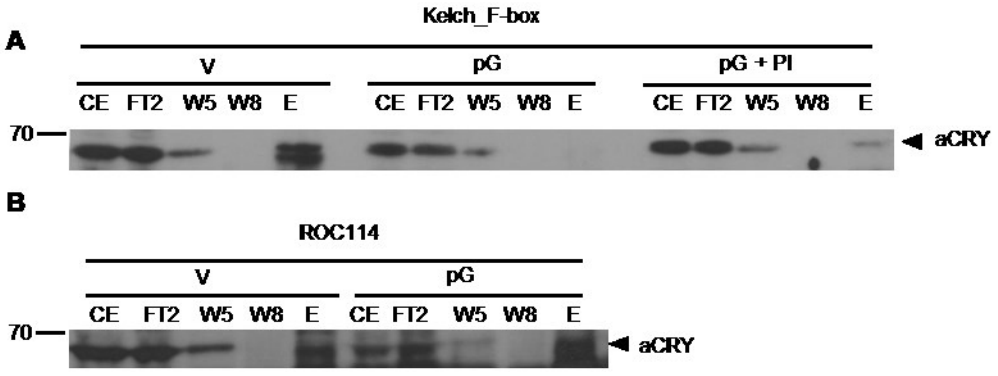

Figure 9. Pull-down assay of Kelch_F-box (A) and ROC114 (B) with aCRY.
After the soluble protein of purified Kelch_F-box was attached to the Dynabeads, the soluble crude protein extracts from vegetative cells (V), pregametes (pG), or pregametes with

proteasome inhibitor treatment (pG+PI) were added for binding. Similarly, the soluble proteins of purified ROC114 were first attached to the Dynabeads and then soluble crude proteins from V or pG were added for binding. CE, crude extract from Chlamydomonas; FT2, flow through 2; W5, wash step 5; W8, wash step 8; E, elution.

The results from the pull-down assay using purified Kelch_F-box and soluble proteins from vegetative cells showed that most of the aCRY cannot bind to the Dynabeads with purified Kelch_F-box since the amount of aCRY in crude extracts is nearly the same as that in the Flow Though 2 (Fig. 9A). However, in the final elution step, aCRY is visible after long time exposure (1 h), suggesting that aCRY interacts with Kelch_F-box weakly. When proteins from crude extracts of pregametes, which contain low amount of soluble aCRY (Fig. 4A), were used to test the interaction with Kelch_F-box, no aCRY was detected in the elution (Fig. 9A), suggesting that soluble aCRY in pregametes cannot interact with Kelch_F-box or the interaction between these two proteins are too weak to be detected. Interestingly, when soluble protein extracts from pregametes were treated with proteasome inhibitor to prevent the degradation of aCRY, a weak band of aCRY was observed (Fig. 9A), suggesting that the interaction between aCRY and Kelch_F-box occurs. Thus, it seems that aCRY is degraded by the proteasome pathway with Kelch_F-box as the substrate acceptor. When purified ROC114 proteins were used to perform the pull-down assay, soluble aCRY was present in the elution, although most of soluble aCRY were washed away in Flow Through 2 (Fig. 9B) after applying soluble crude extracts from vegetative cells or pregametes. This suggests that ROC114 could interact with aCRY in both vegetative cells and pregametes. To verify the interactions, yeast two hybrid tests were also performed.

2.3.2.1.2 Yeast Two Hybrid experiments of Kelch_F-box and ROC114 with aCRY

Yeast Two Hybrid analysis is an *in vivo* method to detect protein-protein interactions (Uetz et al., 2000). Two protein domains, a DNA binding domain (BD) and an activation domain (AD) are required in the Yeast Two Hybrid assay. Fused proteins are constructed by connecting each protein of interest with either the BD or the AD of the transcription factor. The protein fused to the BD, which binds the upstream activator sequence of the promoter, is called ‘bait’, whereas the protein fused to the

AD is referred as ‘prey’. If interaction occurs between the bait and the prey, the BD and AD are brought in close proximity and thus reconstitutes a functional transcription factor, resulting in transcription of a reporter gene (Bruckner et al., 2009).

To check the potential interaction between aCRY and Kelch_F-box/ROC114 using the Yeast Two Hybrid assay, plasmids with fused proteins of AD-Kelch_F-box, BD-Kelch_F-box, AD-ROC114 and BD-ROC114 (named pYZ5, pYZ6, pYZ7 and pYZ8, respectively, Fig. 10 and 11) were constructed. Furthermore, the constructed plasmids were also sequenced at the connection region (s. Appendix). The plasmids expressing fused protein of BD-aCRY and AD-aCRY were constructed by Wenshuang Li.

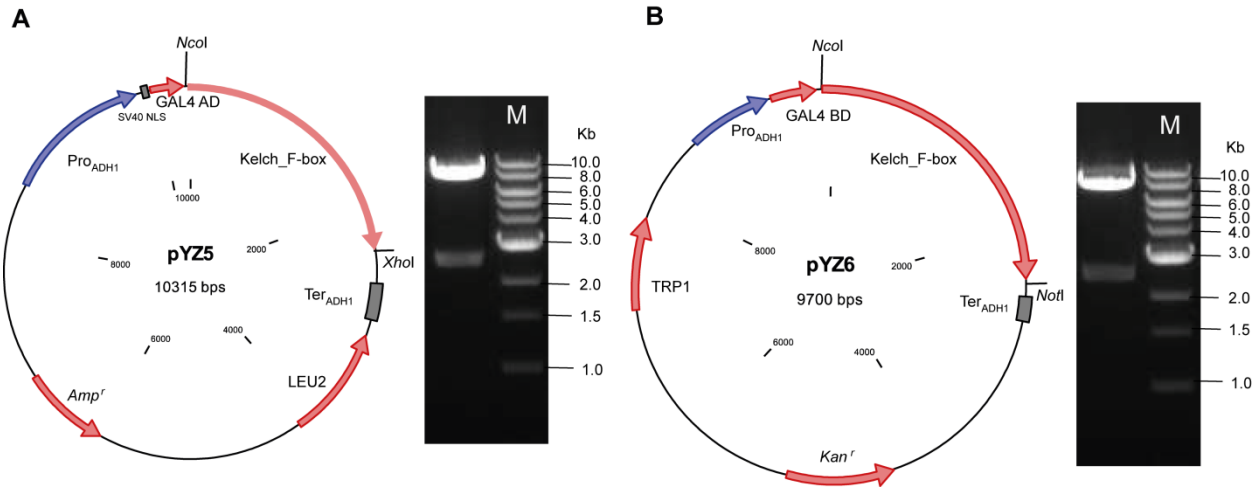

Figure 10. Plasmid construction of Kelch_F-box fused to GAL4-AD and GAL4-BD.
The codon-adapted sequence of Kelch_F-box was introduced to pGADT7 and pGBKT7, which are plasmids that express the GAL4 activation domain (AD) and DNA binding domain (BD), respectively. Pro_{ADH1}: ADH1 promoter; SV40 NLS: SV40 nuclear localization sequence; GAL4-AD: GAL4 activating domain; Ter_{ADH1}: ADH1 terminator; *Amp*^{r}: Ampicillin resistance; LEU2: leucine nutritional marker; *Kan*^{r}: Kanamycin resistance; TRP1: tryptophan nutritional marker; M: marker, Kb: kilobase pair. The constructed plasmids pYZ5 and pYZ6 were tested by gel electrophoresis after digestion with the restriction enzymes indicated in the plasmid maps. The predicted sizes after digestion of pYZ5 are 2423 bp and 7892 bp, while the predicted sizes after digestion of pYZ6 are 7625 bp and 2435 bp.

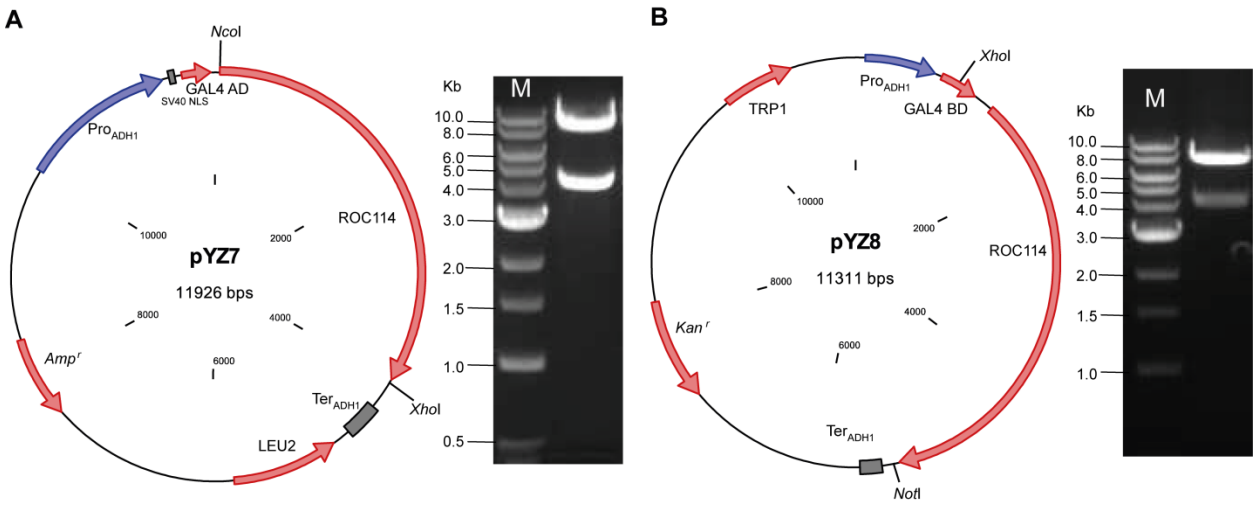

Figure 11. Plasmid construction of ROC114 fused to GAL4-AD and GAL4-BD.
The codon-adapted sequence of ROC114 was introduced to pGADT7 and pGBKT7, respectively. The abbreviations are shown as in Fig. 10. The constructed plasmids pYZ7 and pYZ8 were tested by gel electrophoresis after digestion with the restriction enzymes indicated in the plasmid maps. The predicted sizes after digestion of pYZ7 are 4034 bp and 7892 bp, while the predicted sizes after digestion of pYZ6 are 6956 bp and 4355 bp.

Plasmids with BD fusing proteins were transformed into the Y2HGold strain, while plasmids with AD fusing proteins were transformed into the Y187 strain (s. Table 15). Auto-activation tests of BD fusing aCRY, Kelch_F-box and ROC114 were first carried out. In the positive interaction control of SV40 large T-antigen and Murine p53, all the mating strains grew on minimal synthetic dropout media (SD) selection plates lacking Leu and Trp (SD-Leu/-Trp), which confirm the presence of both GAL4-AD and GAL4-BD plasmids. Media lacking Leu, Trp and His (SD-His/-Leu/-Trp) were used to test for protein interactions. In the negative control of SV40 large T-antigen and Lamin, the mating strains grew only on SD-Leu/-Trp media, but not grew on SD-His/–Leu/-Trp media (Fig. 12). In the self-activation test of BD fusing protein of aCRY or ROC114 with the AD protein alone, all the mating strains showed the same grown conditions as the negative control (Fig. 12), suggesting that there is no self-activation for BD fusing aCRY and ROC114. In the self-activation test of BD fusing protein of Kelch_F-box with only AD protein (Fig. 12), the mating strains weakly grew on SD-His/-Leu/-Trp media but not on SD-His/-Leu/-Trp media with 5 mM 3-AT, a competitive inhibitor of the His3 reporter for suppressing false positives, suggesting that a very weak auto-phosphorylation occurs in the BD fusing Kelch_F-box protein.

So the self-activation test shows that all these three fusing proteins are in principle suitable for the Yeast Two Hybrid assay.

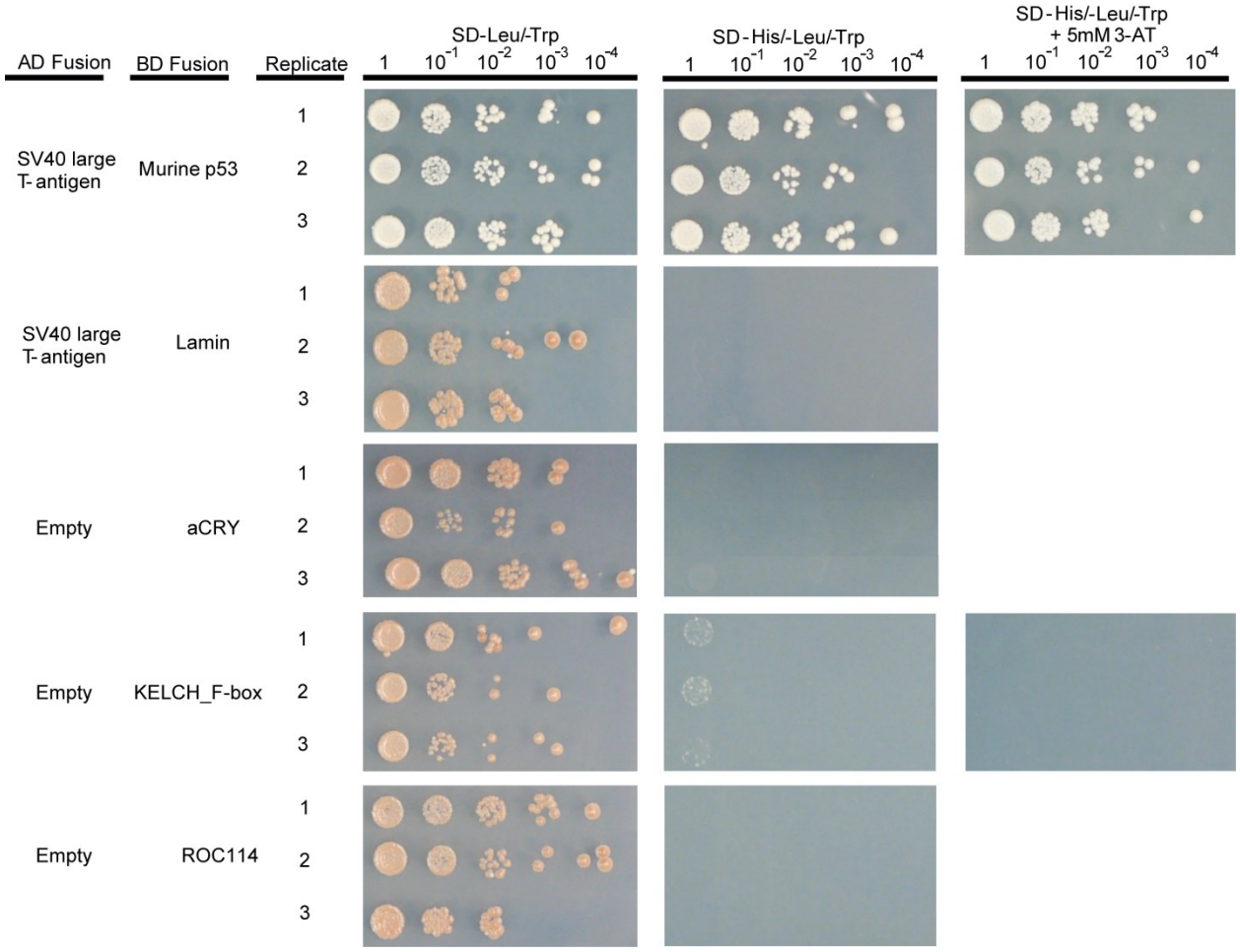

Figure 12. Self-activation tests of aCRY, Kelch_F-box and ROC114 fused to BD in Yeast Two Hybrid assays.

The cDNAs encoding aCRY, Kelch_F-box and ROC114 were fused to the GAL4-BD. Empty fusion vectors containing only GAL4-AD served as mating partners. The cDNAs encoding BD fusing murine p53 and Lamin, interacting with AD fusing SV40 large T-antigen, were used as positive and negative controls, respectively. Cells with OD600 of 1.0 were serially diluted 10-fold for four times and spotted onto minimal synthetic dropout media (SD) selection plates as indicated. Yeast strains containing both GAL4-AD and GAL4-BD plasmid constructs carrying the Leu and Trp reporters, respectively, were grown on selective media lacking Leu and Trp (SD-Leu/-Trp) to confirm the presence of both GAL4-AD and GAL4-BD plasmids. Media lacking Leu, Trp and His (SD-His/-Leu/-Trp) were used to test for protein interactions. 3-AT served as a competitive inhibitor of the His3 reporter for suppressing false positives. Each set of tests was performed for three biological replicates.

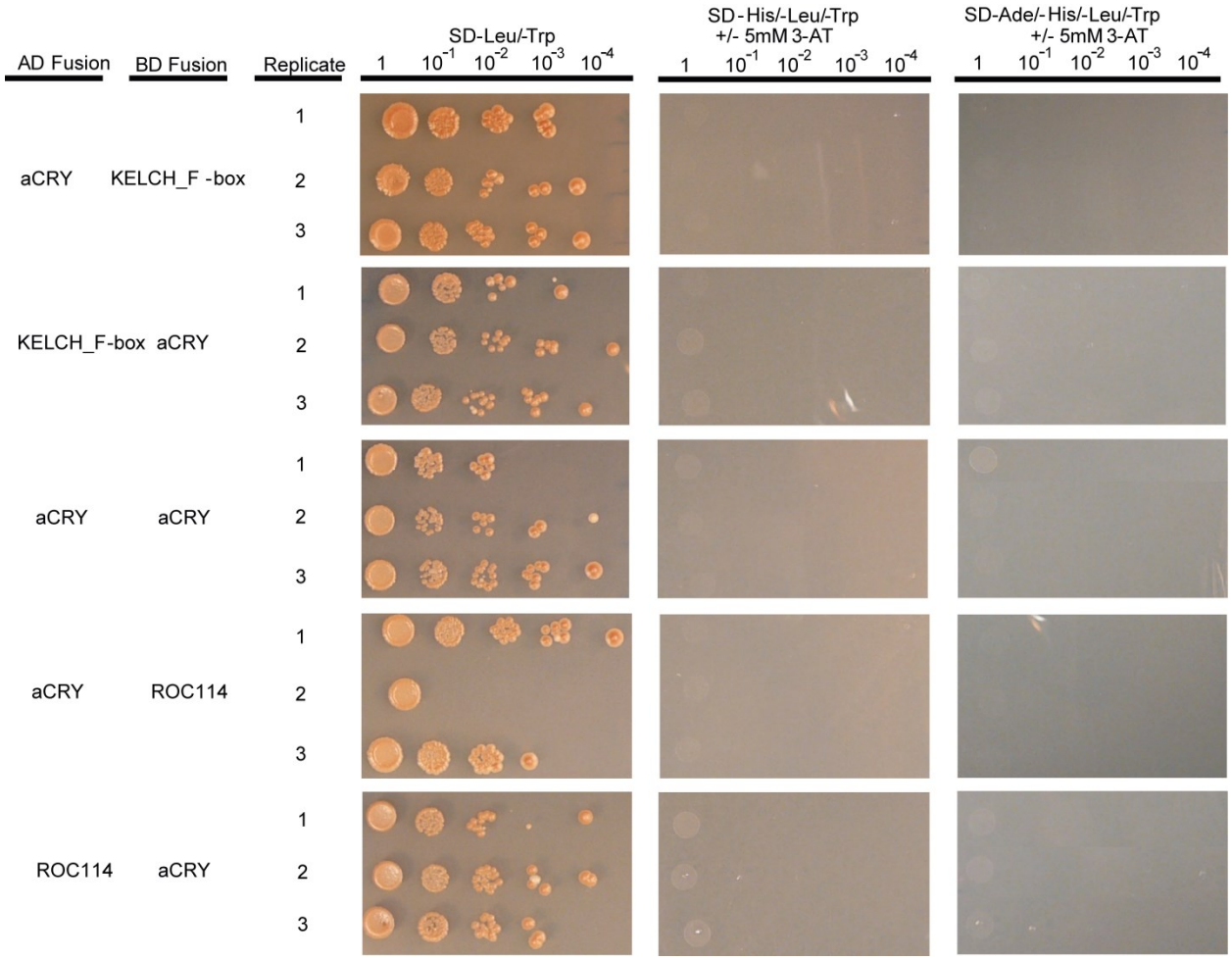

Figure 13. Yeast Two Hybrid assays between aCRY and Kelch_F-box or ROC114.
5mM 3-AT was only applied to the media for the interaction test of AD fused aCRY and BD fused Kelch_F-box. The labeling is labeled as in Fig. 12 and the more restrictive media lacking Leu, Trp, His and Ade (SD-Ade/-His/-Leu/-Trp) were used to test for very strong protein interactions.

For the interaction test between BD-Kelch_F-box and AD-aCRY, 5mM 3-AT was added to the media to remove the weak auto-activation. All the mating strains grew on SD-Leu/-Trp media, suggesting that the mating process goes well. However, no clear colony was visible in tests of AD-aCRY and BD-Kelch_F-box, as well as in BD-aCRY and AD-Kelch_F-box (Fig. 13), suggesting that no interaction occurs in yeast. Similarly, there was no visible colony growing in the mating strains containing fused protein of AD-aCRY and BD-ROC114, as well as BD-aCRY and AD-ROC114 (Fig. 13). Furthermore, the interaction of aCRY and aCRY was also tested, since aCRY seems to form a dimer in the dark using the C-terminal extension (Oldemeyer et al.,

2016). Unfortunately, no yeast colony containing fused protein AD-aCRY and BD-aCRY was observable, suggesting that aCRY cannot form a dimer in yeast cells. All these tests were done both under darkness and under constant light of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 23°C and 30°C. All the results were similar and there were no growing colonies on the selective media. This suggests that light and temperature are not involved in the interaction between aCRY and Kelch_F-box or ROC114 in yeast.

2.4 The role of aCRY in gametogenesis and phototactic behavior (Zou et al., submitted)

2.4.1 aCRY is a negative regulator in mating

In vitro spectral analysis and *in vivo* physiological analysis revealed that aCRY has the ability to perceive blue and red light to mediate physiological responses (Beel et al., 2012). In the step of light-dependent gametogenesis, blue and red light are also two major light qualities that affect the gametes formation process (Weissig and Beck, 1991). Because CC-124 and CC-125 are routinely used for sexual cycle-related experiments (Suzuki and Johnson, 2002; Huang and Beck, 2003), an *acry* mutant strain generated by insertional mutagenesis (CRMS101, Beel et al., 2012) was selected as the parental strain to be crossed with CC-124. One progeny of mt^- with paromomycin resistance was chosen to cross with CC-125 (detailed crossing procedure is depicted in Fig. 14). Finally, one mt^+ progeny with low aCRY expression level (~16.7%, named $acry_{mut}$, Fig. 15) was selected for the mating ability test. To generate a complemented strain, $acry_{mut}$ was transformed with pKP39, a vector containing the full length of aCRY and conferring the host with hygromycin resistance (Beel et al., 2012) and screened for the expression level of aCRY with the wild type (CC-125) as a control. Finally, two complemented strains, designated $acry_{compl}$ #15 and $acry_{compl}$ #26, were chosen since they produced nearly the same amount of aCRY as the wild type (Fig. 15).

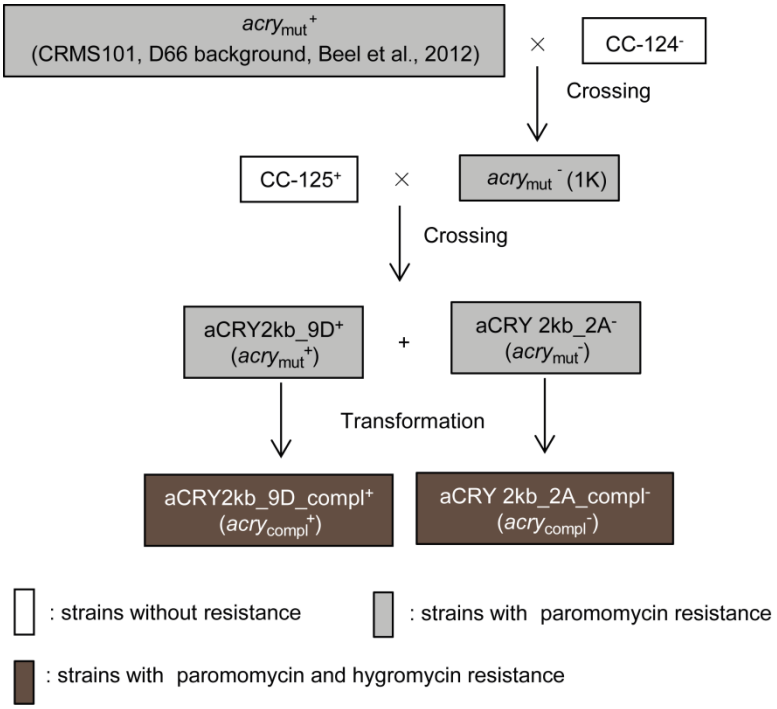

Figure 14. The genetic background of the *acry*$_{mut}$ and *acry*$_{compl}$ strains used in this work.
CRMS101 is generated by insertional mutagenesis with paromomycin resistance. *acry*$_{mut}$ (1K) is the resulting strain after the first crossing step.

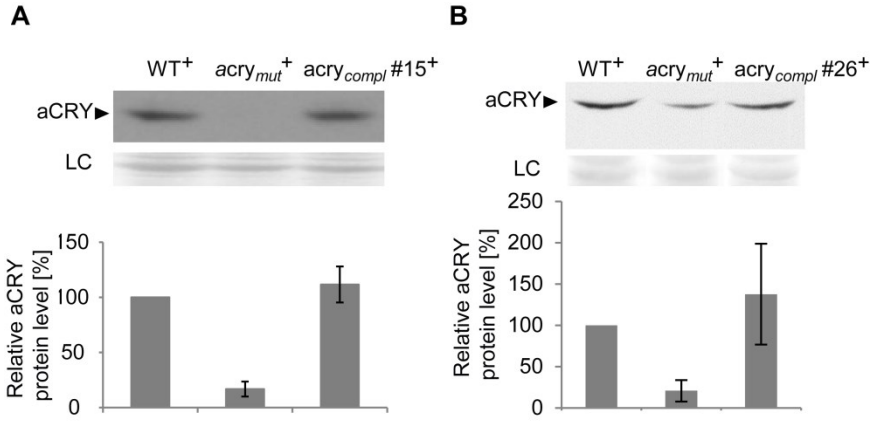

Figure 15. Quantification of the protein level of aCRY in mutant and complemented strains used for the mating ability test.
The expression level of soluble aCRY in CC-125 (WT), *acry*$_{mut}$ and *acry*$_{compl}$ strains (#15 and #26) were determined. Unspecified protein bands from the PVDF membrane were used as loading controls (LC). The quantification of aCRY in these strains is based on three independent biological replicates and shown with standard deviations.

In a next step, the mating ability of wild type, *acry*$_{mut}$, and the two complemented strains *acry*$_{compl}$ #15 and *acry*$_{compl}$ #26, was examined by mating with the wild type

strain of opposite mating type (CC-124). In the presented results, the gametes formation rate of 1h-illuminated pregametes of wild type (G1) was set to 100%. It turns out that the pregametes of wild type exhibited a relative low mating ability rate (29.0%, Fig. 16A). In wild-type strains, as shown before (Beck and Acker, 1992), 1 h light exposure strongly increases the mating ability, suggesting the involvement of photoreceptors. In *acry*$_{mut}$, the mating ability was significantly higher than that of wild type both in pre-gametes and G1 gametes (Fig. 16A). Furthermore, the mating ability of the complemented strains displayed a lower value than *acry*$_{mut}$ both in pregametes and gametes (Fig. 16A), similar to wild type. These data suggest that aCRY is involved in the mating ability and it seems to exert a negative regulation in the mating pathway. Upon its reduction, the mating ability increases. However, after illumination, there is no significant difference in the increase of mating ability from pregametes to gametes for wild type, *acry*$_{mut}$ and *acry*$_{compl}$ (Fig. 16B), suggesting that the increase of mating ability after light exposure seems not to be regulated by aCRY.

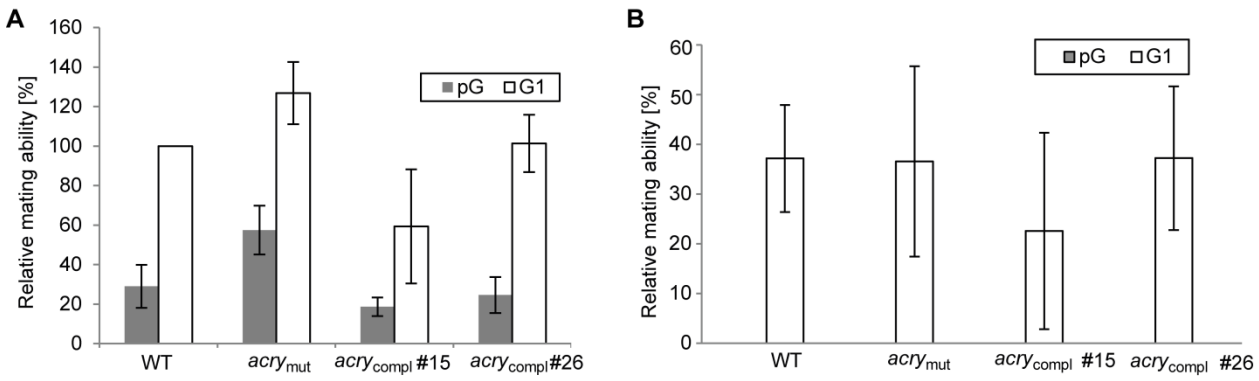

Figure 16. The mating ability assay of wild type, *acry*$_{mut}$ and two *acry*$_{compl}$ strains (Zou et al., submitted).
For the mating ability test, pregametes (pG) and pregametes illuminated for 1 h with light (G1) were used. A. The mating ability of G1 gametes of wild type (WT) was set to 100%. At least three biological replicates were performed and the diagram was labeled with error bars of standard deviation. B. The increase of the mating ability from pregametes to gametes after illumination in WT, *acry*$_{mut}$ and two *acry*$_{compl}$ strains. The mating ability of pregametes of each strain was set to zero. The relative mating ability of G1 of each strain was calculated using the mating ability of G1 subtracting that of pregametes.

2.4.2 aCRY promotes the loss of mating ability in darkness

Incubation of mature gametes in the dark leads to a loss of their mating ability (Beck and Acker, 1992; Huang and Beck, 2003). To test the possible role of aCRY in the

mating maintenance ability, gametes of wild type, *acry*$_{mut}$ and the complemented strains were kept in the dark for 1 h. The dark treatment resulted in a significant loss of mating ability in the wild-type strain (Fig. 17), in agreement with former studies (Beck and Acker, 1992; Pan et al., 1997; Huang and Beck, 2003). Interestingly, dark treatment only provoked a minor loss of mating ability in *acry*$_{mut}$, indicating that aCRY appears to be an indispensable component for the loss of the mating ability pathway. The *acry*$_{compl}$ strains displayed a similar mating ability reduction after dark treatment as wild type, which further confirms the negative role of aCRY in the regulation of mating maintenance.

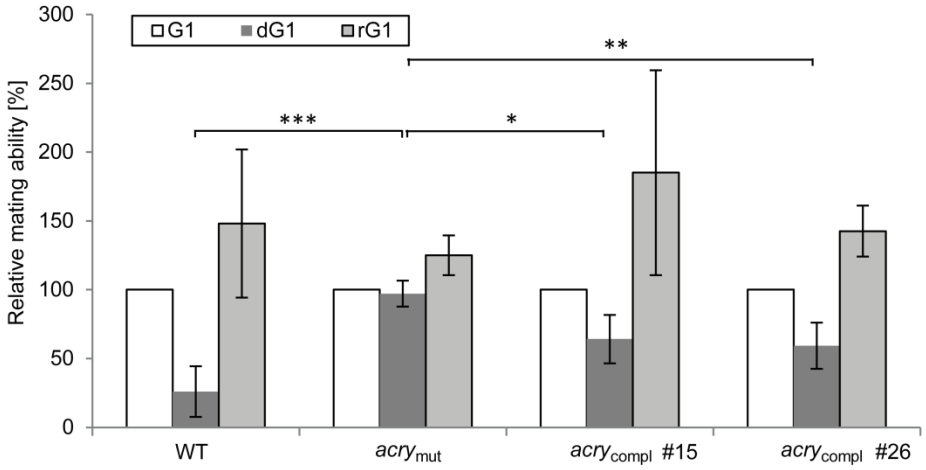

Figure 17. Mating maintenance ability assay and mating ability restoration tests of dark inactivated gametes in wild type (WT), *acry*$_{mut}$ and two *acry*$_{compl}$ strains (Zou et al., submitted). Gametes with 1 h illumination (G1) were put in darkness for 1 h to deactivate the mating ability and named dG1 (dark-inactivated G1 cells). The dG1 cells were then illuminated for 1 h and turned to rG1 (reactivated G1 cells). The mating ability of gametes was set to 100% separately according to each strain. The y-axis represents the relative mating ability. Mean values and standard deviation of three biological replicates were shown. Student's t-test was performed (***, $p<0.001$; **, $p<0.01$; *, $p<0.05$).

Light exposure of dark-inactivated gametes can restore their mating ability. Blue light, as well as red light to some extent, is able to recover the mating ability of dark-inactivated gametes (Pan et al., 1997). As a result, the dark-inactivated gametes were treated with 1 h light and their mating ability was also checked. In wild type and complemented strains, light largely recovers the mating ability of dark-inactivated gametes, while the mating ability is not greatly improved in *acry*$_{mut}$ (Fig. 17), suggesting that the mating ability has reached a plateau level. However, there is no

significant difference existing in the mating ability of reactivated gametes of wild type, *acry*$_{mut}$ and complemented strains.

2.4.3 aCRY affects the phototactic behavior of gametes

The effect of aCRY on the phototactic behavior of Chlamydomonas G1 gametes was analyzed. For that purpose, equal amounts of pregametes were evenly distributed in a flat culture flask with shaking under light for 1 h. The swimming behavior of the cells was compared immediately after shaking (control, 0 min) and after shaking and being kept in a light gradient for 20 min (s. 6.2.4.6). Cells from wild type and *acry*$_{compl}$ accumulated to a large extent towards the highest light intensity (Fig. 18). Accumulation towards the highest light intensity also occurred in *acry*$_{mut}$, however at a lower accumulation rate. Many cells were still widely distributed over the Falcon tube and present at the regions with low light intensities. These data suggest that aCRY contributes to the regulation of phototactic behavior of gametes.

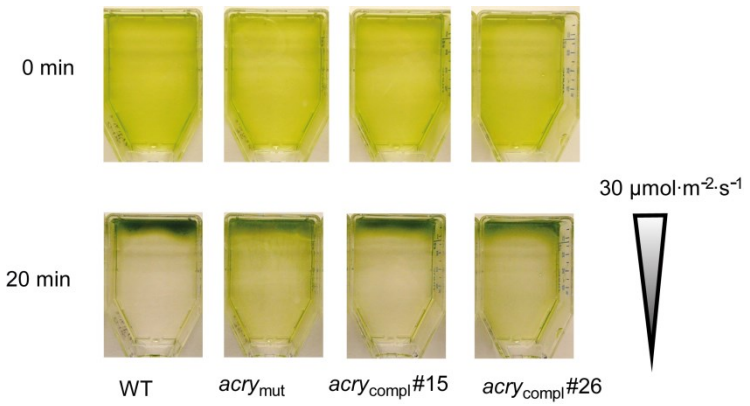

Figure 18. Phototactic behavior of gametes of wild type (WT), *acry*$_{mut}$ and its complemented strains (Zou et al., submitted).
Pregametes of WT, *acry*$_{mut}$, *acry*$_{compl}$ #15 and *acry*$_{compl}$ #26 were distributed equally to culture flasks, illuminated for 1h and exposed to a dim light gradient ranging from 30 µmol·m^{-2}·s^{-1} to not measurable low light intensities (as indicated by triangular bars). Flasks were left under these dim light conditions for 20 min without shaking and documented with a camera.

2.5 Both aCRY and pCRY are involved in zygote germination

2.5.1 Zygote germination rate is influenced by the light quality (Zou et al., submitted)

In the sexual life cycle of Chlamydomonas, both gametogenesis and zygote germination are light-regulated steps (Treier et al., 1989; Weissig and Beck, 1991). Both blue light and to a small extent red light are involved in the gametogenesis step. The role of different light spectrums on germination was not known so far. Therefore, it was tested if blue and red light that are mainly absorbed by the neutral radical form of aCRY also influence germination. The used wavelength of blue and red light was 465 nm and 635 nm, respectively, according to the absorption peak of aCRY (Beel et al., 2012). Blue light exhibited high ability to induce zygote germination (Fig. 19), similar to white light. But also red light had a profound effect on germination (32.0%, Fig. 19). As it is known that zygotes cannot germinate in the dark (Gloeckner and Beck, 1995), these results indicate that germination is not only affected by blue, but also partially by red light. Thus, in addition to the photoreceptor PHOT, which controls germination, a red light receptor or a photoreceptor network could also be involved.

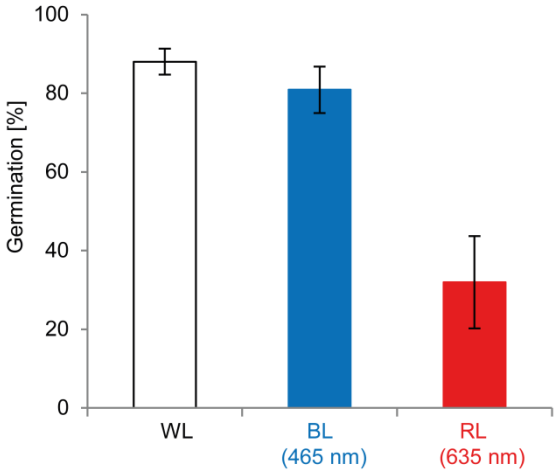

Figure 19. The influence of blue and red light in germination (Zou et al., submitted).
Blue (465 nm), red (635 nm) and white light (s. 6.2.4.7) were used to illuminate wild type zygotes during the germination process. About 50 zygotes were counted for each condition and each replicate (n=3). Mean values and standard deviations are shown.

2.5.2 aCRY influences zygote germination (Zou et al., submitted)

In wild-type strains of Chlamydomonas, diploid zygotes can undergo meiosis and germinate into haploid vegetative cells only upon illumination and the presence of a nitrogen source. In order to find out a potential role of aCRY in this process, the *acry*$_{mut}$ used for the mating ability test along with another mt$^-$ progeny with strongly reduced aCRY levels (~13% compared to wild type mt$^-$, Fig. 20) were selected to perform the germination assay. In the germination test, crosses between CC-125 and CC-124 were used as wild type control. Homozygotes of wild type (WT $^+$ x WT$^-$) showed a germination value of about 90% (Fig. 21). Interestingly, both heterozygotes of wild type and *acry*$_{mut}$ (WT$^+$ × *acry*$_{mut}^-$, *acry*$_{mut}^+$ × WT$^-$) exhibited a significant lower germination rate (approx. 70%, Fig. 21). Furthermore, the germination of the homozygote of *acry*$_{mut}$ reached a germination level of only 40% (Fig. 21), which is significantly different to the germination level in wild-type homozygotes and heterozygotes of wild type and *acry*$_{mut}$. These data suggest that aCRY plays an important role in the germination process. To further verify the role of aCRY in germination, the above mentioned complemented mt$^+$ strain, along with another aCRY complemented mt$^-$ strain (83% aCRY expression when using CC-124 as wild type control, Fig. 20) was used. In the homozygotes of *acry*$_{compl}$, the germination rate (82%) was significantly higher than that in homozygotes of *acry*$_{mut}$ (Fig. 21). All these data demonstrate that aCRY indeed influences the zygote germination step.

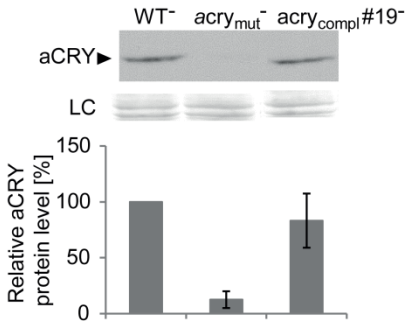

Figure 20. Quantification of the aCRY complemented strain of mating type minus used in zygote germination.
The expression level of soluble aCRY in wild type (WT, CC-124) was used as the control and was set to 100%. The expression level of aCRY in *acry*$_{mut}$ strain and the corresponding complemented strain (*acry*$_{compl}$ #19) is demonstrated. Unspecific protein bands from the PVDF membrane were

selected as loading control (LC). The quantification of aCRY in these strains is based on three independent biological replicates and shown with standard deviations.

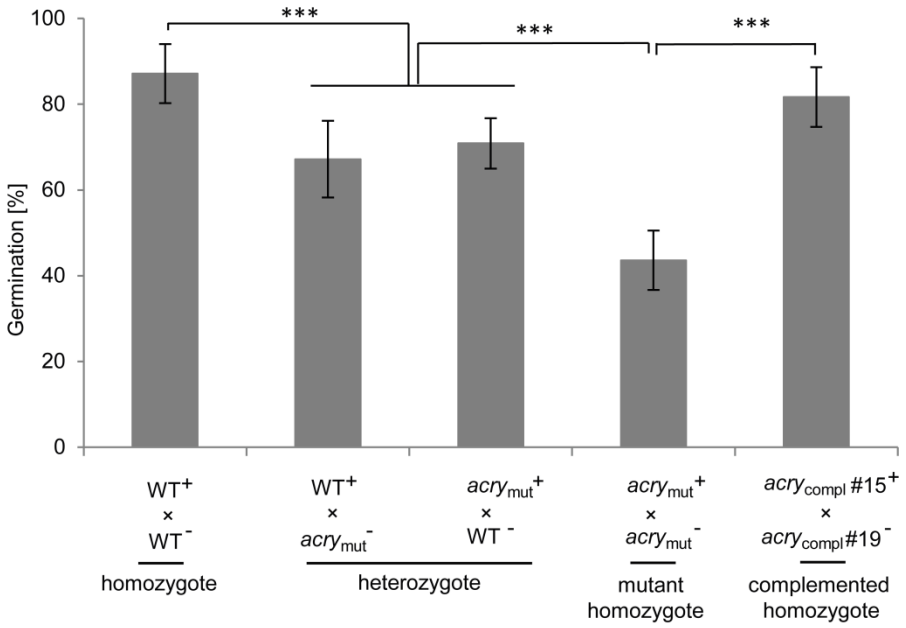

Figure 21. Germination assay of wild type, aCRY mutant and complemented strains (Zou et al., submitted).
The germination profile of crossings between wild type (WT), *acry*$_{mut}$ and the *acry*$_{compl}$ strains. Germination of the crossing between the wild-type strains CC-124/mt$^-$ (WT$^-$) and CC-125/mt$^+$ (WT$^+$) is shown as homozygote (WT$^+$ x WT$^-$), as well as the crossing between WT and *acry*$_{mut}$ as heterozygote (WT$^+$ x *acry*$_{mut}^-$ or *acry*$_{mut}^+$ x WT$^-$) and the crossing between two *acry*$_{mut}$ strains as mutant homozygote (*acry*$_{mut}^+$ x *acry*$_{mut}^-$). In addition, the complemented homozygote (*acry*$_{compl}$ #15$^+$ x *acry*$_{compl}$ #19$^-$) is shown. At least three biological replicates were performed and the diagram was labeled with error bars of standard deviations. Student's t-tests were performed (***, $p<0.001$).

2.5.3 pCRY influences zygote germination

The influence of pCRY on zygote germination was also investigated. The *pcry*$_{mut}$ generated via insertional mutagenesis with D66 background (CRMS102) were crossed with CC-124 (WT$^-$) and subsequent with CC-125 (WT$^+$). Progenies of both mating types carrying the *pcry*$_{mut}$ allele were generated (detailed information of crossing is shown in Fig. 22). While approximately 90% of the zygotes germinated in the WT$^-$ × WT$^+$ cross, only 60% of the zygotes germinated in the WT$^+$ × *pcry*$_{mut}$ and

pcry$_{mut}$ × WT^{-} crosses and the germination rate was only 25% for the *pcry*$_{mut}$ homozygous zygotes (Fig. 23). The difference between the germination of wild type homozygotes, *pcry*$_{mut}$ heterozygotes and *pcry*$_{mut}$ homozygotes was also compared and it is always significant between each set. These results demonstrate that pCRY has a strong influence on germination, which is further confirmed by the analyses of a complemented strain in the background of CC-124 (*pcry*$_{compl}$), in which the pCRY levels were in the range of 93% (in collaboration with Nico Müller). The heterozygous zygotes of the cross between this complemented strain and wild type exhibit a germination rate of approximately 80% (Fig. 23) and the difference between *pcry*$_{mut}$ homozygotes and complemented heterozygotes is very high, suggesting also the participation of pCRY in germination.

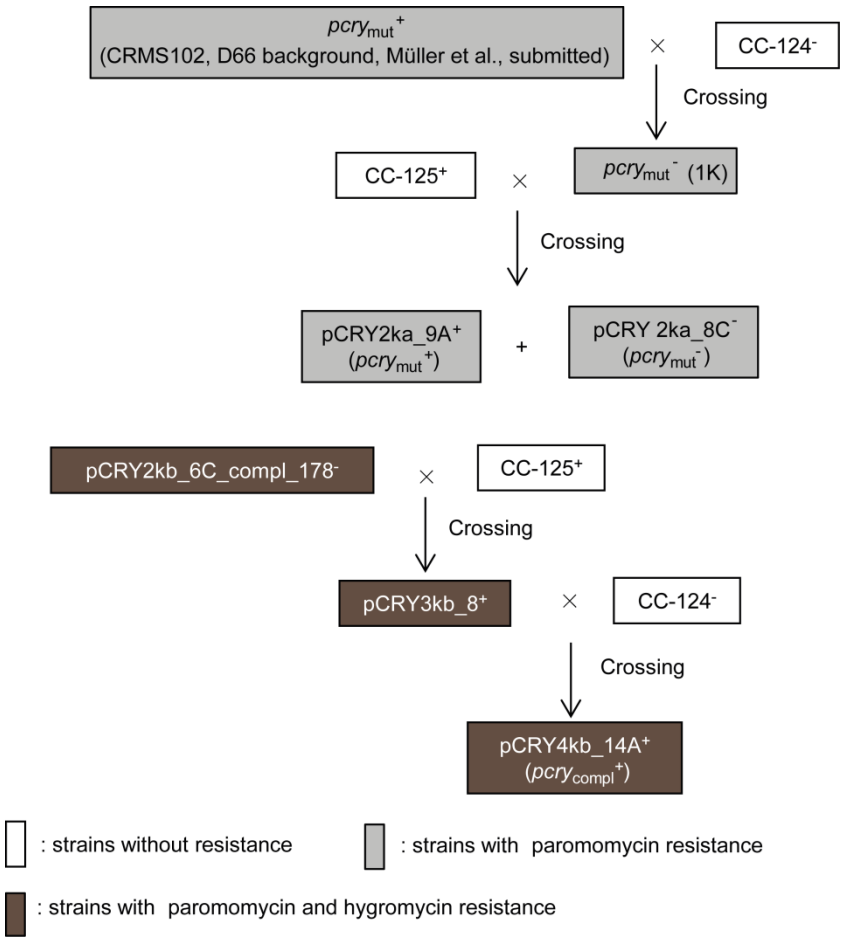

Figure 22. The genetic background of the *pcry*$_{mut}$ and *pcry*$_{compl}$ strains used in this work.

CRMS102 is generated by insertional mutagenesis with a cassette containing a paromomycin resistance gene. *pcry*$_{mut}$ (1K) is the result of the first crossing step. pCRY2kb_6C_178 is a complemented strain with SAG73.72 background from Nico Müller.

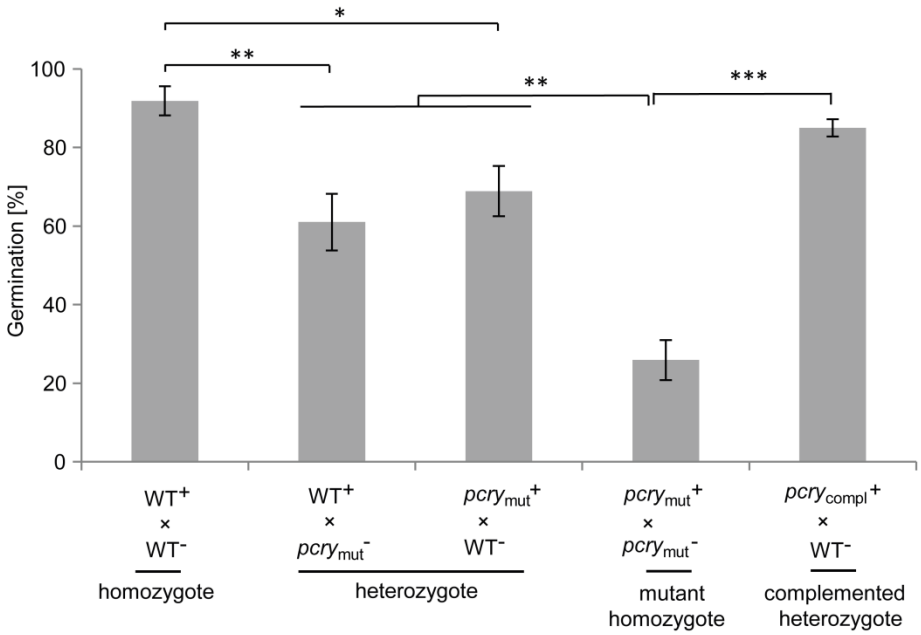

Figure 23. pCRY influences the germination efficiency (Müller et al., in preparation for resubmission).

Crossings of mating type plus (mt^+) and minus (mt^-) of different combinations are shown: WT, wild type strain; *pcry*$_{mut}$, *pcry* mutant strain; *pcry*$_{compl}$, complemented strain of the *pcry* mutant. Germination of the crossing between wild-type strains CC-124/mt^- (WT^-) and CC-125/mt^+ (WT^+) is shown as homozygote ($WT^+ \times WT^-$), as well as the crossing between WT and *pcry*$_{mut}$ as pCRY heterozygotes ($WT^+ \times$ *pcry*$_{mut}^-$ or *pcry*$_{mut}^+ \times WT^-$) and, the crossing of two *pcry* mutant strains as pCRY homozygotes (*pcry*$_{mut}^+ \times$ *pcry*$_{mut}^-$). In addition, the heterozygote of a complemented strain/mt^+ (*pcry*$_{compl}^+$) and a WT CC-124/mt^- (*pcry*$_{compl}^+ \times WT^-$) is shown. The asterisks indicate significant differences estimated by the Student's t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). At least three biological replicates were performed and the diagram is labeled with error bars of standard deviations.

2.6 The relationship between aCRY, PHOT and ChR1 in Chlamydomonas (Zou et al., submitted)

aCRY and PHOT are two important photoreceptors involved in two light-dependent steps in the sexual life cycle, gametogenesis and zygote formation. PHOT has been well studied in Chlamydomonas with respect to the role as a positive regulator in the mating ability, the restoration of mating after dark treatment, and the zygote germination (Huang and Beck, 2003). This work demonstrates that aCRY is a negative regulator in mating ability and the maintenance of mating ability, but a

positive regulator in zygote germination. Furthermore, aCRY and PHOT seem to work jointly to regulate some light-dependent genes, e.g. LHCBM6, GSA and PDS under blue and red light (Im et al., 2006; Beel et al., 2012). The expression of these genes is also changed in the PHOT knockdown strains under red light, even though PHOT shows no absorption in the red light region of the spectrum. These data suggest an interconnection between these two photoreceptors.

2.6.1 aCRY regulates the expression level of PHOT

It is interesting but unknown if the expression level of PHOT after the switch from pregametes to fully differentiated gametes would change in *acry*$_{mut}$ compared to wild type, indicating that both photoreceptors may be part of a network. For this purpose, proteins from membrane fractions, including the membrane-associated as well as transmembrane proteins, of pregametes and gametes exposed for 4h to light of wild type, *acry*$_{mut}$ and *acry*$_{compl}$ were isolated and checked at first in immunoblots with anti-aCRY antibodies. As expected (s. Fig. 5), membrane-associated aCRY accumulates at a similar rate in pregametes and gametes and has reduced levels in the *acry* mutant and high levels in a complemented strain. For comparing PHOT levels during gamete induction, the level of PHOT in the membrane fraction of pregametes of each strain was set to 100% (wild type, *acry* mutant and complemented strain, respectively). Notably, PHOT accumulates at a significant higher level in gametes of the *acry* mutant compared to wild type (Fig. 24). This effect is changed in the complemented strain, where PHOT levels in gametes compared to pregametes are similar to wild type. Thus, the increased level of PHOT in the *acry* mutant is in concert with the higher mating ability in the *acry* mutant (Fig. 16A) and the positive role of PHOT in mating ability (Huang and Beck, 2003). Moreover, these data suggest that the expression of aCRY and PHOT are interconnected in gametes.

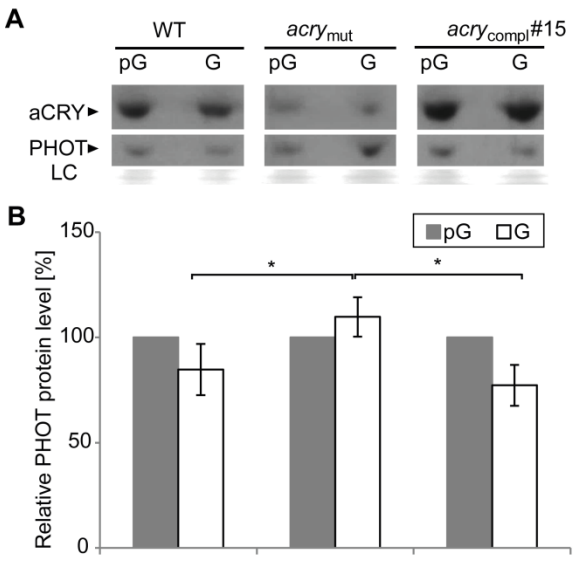

Figure 24. The expression level of PHOT in the membrane fraction of pregametes (pG) and gametes (G) of wild type (WT), *acry*$_{mut}$ and *acry*$_{compl}$ #15 (Zou et al., submitted).
A. Immunoblot analysis of aCRY and PHOT. 75 µg proteins were separated by SDS-PAGE and immunoblotted with anti-LOV1 antibodies for detecting PHOT. B. Quantification of PHOT in relative expression level. It was based on at least three independent biological replicates. The level of PHOT in pG was set to 100%. Error bars of standard deviations are shown. Student's t-test of relative expression level between different sets were performed (*, $p<0.05$).

2.6.2 aCRY affects the expression of ChR1

It has been shown that PHOT affects the expression of ChR1 in vegetative cells and regulates the phototactic behavior in gametes (Trippens et al., 2012). Since aCRY affects the expression of PHOT and the phototactic behavior in G1 gametes, the expression level of ChR1 in pregametes and G1 gametes was also analyzed (in collaboration with Sandra Wenzel). Two important results were observed: (i) the expression level of ChR1 is strongly reduced in gametes compared to pregametes in wild type as well as in the complemented strain of the *acry* mutant. In the *acry* mutant, it is also reduced but to a lesser extent; (ii) the relative expression level of ChR1 in gametes is altered in *acry*$_{mut}$ where it is increased compared to wild type and the complemented strain (Fig. 25). These results clearly suggest that the expression of ChR1 in gametes is also influenced by aCRY.

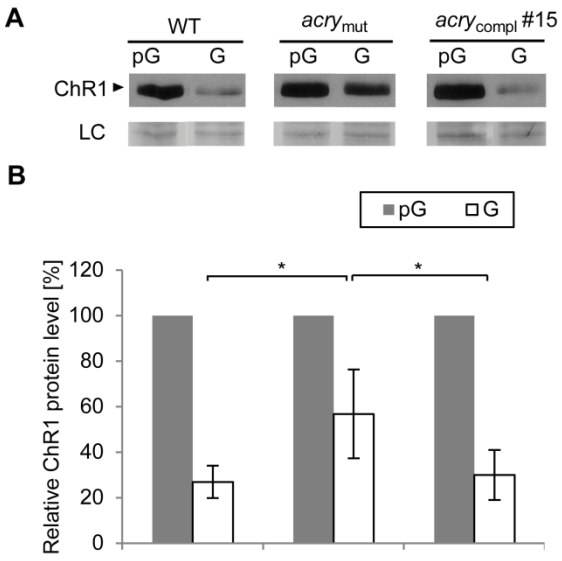

Figure 25. ChR1 expression level of gametes in wild type (WT), *acry*$_{mut}$ and the *acry*$_{compl}$ #15 strain (Zou et al., submitted).
A. Immunoblot analysis of ChR1. 25 µg proteins were used for immunoblot with anti-ChR1 antibodies. B. Quantification of ChR1 in relative expression level. It was based on at least three independent biological replicates. The level of ChR1 in pG was set to 100%. Error bars of standard deviations are shown. Student's t-test of relative expression level between different sets were performed (*, $p<0.05$).

3. Discussion

Light plays a crucial role in the life cycle of the green alga Chlamydomonas. It serves not only as the energy source for photosynthesis, but also works as a stimulus for this alga to entrain light-induced processes by employing multiple photoreceptors (Kianianmomeni and Hallmann, 2014). Blue light and its absorbing receptors are known to control the cell and life cycles of some algae, including Chlamydomonas or the marine diatom *P. tricornutum* (Huang and Beck, 2003; Oldenhof et al., 2004; Huysman et al., 2013). The LOV (light, oxygen, or voltage) domain-containing receptors PHOT as well as aureochrome are involved in these processes. Here, the spectrum of involved photoreceptors with aCRY for life cycle control in Chlamydomonas has been extended. It was shown before that blue and to a smaller extent red light are involved in the process of gametogenesis (Weissig and Beck, 1991; Pan et al., 1997) of Chlamydomonas. In fact, blue and red light are also involved in the process of germination (Zou et al., submitted). Thus, aCRY with its ability to absorb not only blue light but also red light based on its flavin neutral radical (Beel et al., 2012; Spexard et al., 2014; Oldemeyer et al., 2016) represented a premium candidate for controlling these processes. Indeed, it is found that aCRY plays a key role in the steps of gametogenesis and zygote germination. Using *acry* mutant strains and their corresponding complemented strains, aCRY was found to be involved in three steps of the sexual cycle (i) pregamete to gamete transitions, known as gametogenesis, (ii) the maintenance of mating ability under dark and (iii) zygote germination.

3.1 The expression profile and the role of aCRY in vegetative cells

The differential expression pattern of aCRY in vegetative cells, pregametes, gametes and early as well as late zygotes is also of interest along with its differences in the appearance of soluble and membrane-associated aCRY in those cell types. In land plants and animals, CRYs are often present as soluble proteins and perform roles in the nucleus and/or cytosol (Chaves et al., 2011; Juergens et al., 2015; Liu et al., 2016). In Chlamydomonas, two forms of aCRY, soluble and membrane-associated

are present (Zou et al., submitted). In vegetative cells, both soluble aCRY and membrane-associated aCRY exist, although the expression level of soluble aCRY varies at different time point of the day-night cycle (Beel et al., 2012). Soluble aCRY seems to form a homodimer and even an oligomer during the day when performing its asexual functions (Wenzel and Mittag, personal communication, Zou et al., submitted), consistent with *in vitro* data showing that heterologously expressed full-length aCRY is present as a dimer in the dark and partially oligomerizes after light exposure (Oldemeyer et al., 2016). *In vivo* studies in vegetative cells showed that aCRY is capable to influence the light-dependent expression of multiple genes related to chlorophyll and carotenoid biosynthesis, light-harvesting complexes, nitrogen metabolism, cell cycle control, and the circadian clock under blue, yellow and red light (Beel et al., 2012). However, it is still unclear which state of aCRY (soluble aCRY and/or membrane-associate aCRY) has been employed to perform these functions in vegetative cells.

3.2 The expression profile and the role of aCRY in pregametes and gametes

In pregametes and gametes, soluble aCRY is strongly degraded, apparently by the proteasome pathway as shown with inhibitor studies. In contrast, membrane-associated aCRY is rather stable in pregametes and gametes, indicating that individual functions of these forms may exist and leaving the possibility that the membrane-associated form of aCRY in pregametes and gametes may have other interaction partners. In the gametogenesis step, aCRY is shown to be a negative regulator in the processes of mating and mating maintenance (Zou et al., submitted). As soluble aCRY is degraded in pregametes and gametes, it could be assumed that the membrane-associated aCRY plays a role during mating. It's worthwhile to find out the membrane interaction partner(s), which may be important for the initiation step of the signaling pathway to regulate the mating.

The conversion from pregametes to gametes, also known as gametogenesis, is light regulated (Treier et al., 1989). Light was found to facilitate gametogenesis by acceleration of nitrogen depletion through photosynthesis and work as an essential

factor to the conversion from pregametes to gametes (summarized in Harris, 2009). On the molecular level, nitrogen deprivation and light also induces the alteration in the expression of certain genes, also including mating-unrelated ones. Among them, gametes specific genes such as *GAS3*, *GAS28* and *GAS96* and mating type specific genes such as *FUS1* (mating type plus) and *MID* (mating type minus), as well as the gene encoding agglutinin are expressed in the process of gametogenesis (von Gromoff and Beck, 1993; Ferris et al., 1996; Lin and Goodenough, 2007). To check whether aCRY influences the expression of mating specific and mating type specific genes after light illumination, the expression levels of *GAS28*, *FUS1* and *SAG1* (encoding the agglutinin in mating type plus) of pregametes and gametes after 5 hours illumination were checked in the mating type plus of wild type, *acry* mutant strain and the complemented strain under blue and red light. The results showed no significant difference between wild type, *acry* mutant strain and the complemented strain (s. Appendix), suggesting that aCRY seems not to affect these genes after light exposure.

In the gametogenesis step, it is revealed that blue and red light provoke the conversion from pregametes to gametes (Weissig and Beck, 1991). Although it is predicted that another red light photoreceptor is present in Chlamydomonas (Beel et al., 2013), aCRY seems to be the only described photoreceptor that can perceive red light so far (Beel et al., 2012). It is reported that gametogenesis is also controlled by PHOT that represents a membrane-associated protein (Huang et al., 2002; Huang and Beck, 2003). However, the role of aCRY and PHOT are contrary in the gametogenesis step. In *phot* knock-down strains, the conversion of pregametes to mature gametes is reduced (Huang and Beck, 2003) suggesting a positive regulation for PHOT, while in the *acry* mutant, the mating ability of the partially mating-competent pregametes of strain CC-125 (Saito et al., 1998) as well as of gametes is significantly increased. However, the increase of mating ability from pregamete to gametes after light illumination is nearly the same in wild type, *acry* mutant and complemented strains, which suggests that aCRY may be just a component of the mating ability pathway, rather than being the photoreceptor that directly perceives the light and regulates the gametes formation step. However, it cannot be ruled out that

aCRY could also participate in the gametogenesis after light illumination. Because PHOT, the photoreceptor involved in the gametogenesis step, is normally expressed in the *acry* mutant strain, it seems possible that the weak role of aCRY in this step is covered by PHOT. So a double mutant of aCRY and PHOT is desirable to clearly show the role of aCRY in gametogenesis. Moreover, there is a significant loss of mating ability of dark-exposed gametes in *phot* knockdown strains similar to wild type whereas PHOT is involved in the restoration of the mating ability of dark-inactivated gametes (Huang and Beck, 2003). In contrast, in the *acry* mutant, high mating ability is maintained upon dark exposure of mature gametes (Zou et al., submitted), opposite to wild type, suggesting that aCRY integrates into the signaling pathway that leads to the loss of mating ability in the dark. Although light was shown to be involved in the restoration of the mating ability of dark-inactivated gametes (Pan et al., 1997), the clear influence of aCRY in this step was not found.

The role of aCRY in the sexual steps of mating ability and mating maintenance seems, however, counterintuitive for a photoreceptor, since aCRY appears to act as a negative regulator in the dark in both functions and cannot improve the mating performance after illumination. To our knowledge, photoreceptors ChR1 and PHOT are active in promoting responses in the alga only in the illuminated forms, while in the dark, the activity of the ion channel ChR1 and the LOV/kinase domains of PHOT, respectively, are strongly reduced (Hegemann, 2008). In contrast, phytochrome is active in both functional states, P_r and P_{fr}, to regulate different physiological pathways in plants, while in the dark the P_r form is accumulated only slowly (Rockwell et al., 2006). Till now, no evidence shows that aCRY exhibits any enzymatic function in the dark. Therefore, a possible explanation for the negative regulation by aCRY in the dark is that dissociation of aCRY from an enzyme such as a phosphatase thereby releases an inhibitory interaction for the mating process. Clearly, it is worthwhile to further investigate such bimodal activities of aCRY in the dark as well as in the light.

3.3 The expression profile and the role of aCRY in early and late zygotes

In early zygotes, both soluble and membrane-associated aCRY are absent from the cell. One possible explanation for this is that aCRY completes its role in the process

of mating and is degraded to be out of function. In mature zygotes, both soluble and membrane-associated aCRY are present again to various extents, providing the possibility that aCRY regulates the light-involved germination.

Blue and red light can promote the germination step, although blue light has a higher impact on this process (Zou et al., submitted). It has been demonstrated that aCRY is capable to absorb blue, yellow and red light by conversion from the neutral to reduced/oxidized state of its chromophore FAD (Beel et al., 2012). Studies about PHOT demonstrated that PHOT is a blue light photoreceptor to mediate zygote germination process. However, the germination rates of the homozygotes of wild type and phot knock-down strains don't show a high difference (Huang and Beck, 2003), even the expression level of PHOT in the mutants are less than 10%, suggesting the involvement of other photoreceptor(s) in zygote germination. Since no other red light photoreceptor, such as phytochromes, is encoded in the Chlamydomonas genome, aCRY is the only one photoreceptor sensitive to red light till now. As a result, it is conceivable that aCRY is involved in this light-involved process. Indeed, aCRY plays a role as a positive regulator for the light-dependent germination step (Zou et al., submitted) during which the zygote undergoes meiosis, resulting in four vegetative cells. Thus, both aCRY and pCRY exert the same role as PHOT in this process (Huang and Beck, 2003) and are acting complementary with PHOT. Furthermore, the blue light photoreceptor pCRY is also shown as a positive regulator in germination (Müller et al., in preparation for resubmission). As a result, it seems that the light-regulated germination is a controlled by various photoreceptors to ensure the proceeding of this crucial step in the Chlamydomonas life cycle.

3.4 The photoreceptor network between aCRY, PHOT and ChR1

The role of both aCRY and PHOT in the same physiological processes, such as regulation of expression of light induced genes in vegetative cells, mating ability and zygote germination, unavoidable raises the possibility of a close relationship between these two photoreceptors. Interestingly, the expression of PHOT is influenced in the

transition state from pregametes to gametes by aCRY. In the *acry* mutant, more PHOT is expressed during the pregamete-gamete switch, which is in agreement with the positive role of PHOT in mating ability (Huang and Beck, 2003) and the higher amount of mating ability found in the *acry* mutant. Data from this work clearly suggest that aCRY is interconnected with PHOT within a functional network (Zou et al., submitted). Former data showed an influence of a red light receptor, most probably aCRY, on the expression of certain genes associated with photosynthetic function that require normal PHOT expression level of wild type (Im et al., 2006). Moreover, it is intriguing that both aCRY and PHOT exist (also) as membrane-associated proteins, especially in pregametes, gametes and late zygotes in case of aCRY. This offers the possibility of a signal network in the vicinity of the plasma membrane. In the moss *Physcomitrella patens*, an interaction between PHOT and the red light photoreceptor phytochrome at the plasma membrane was found. In this case, phytochrome interacts with PHOT in its photo-activated state exclusively at the plasma membrane and activates a direct cytoplasmic signal to guide directional responses to light (Jaedicke et al., 2012). Because no phytochrome is encoded in the genome of *Chlamydomonas* (Mittag et al., 2005; Merchant et al., 2007), it is conceivable that membrane-associated aCRY may act in Chlamydomonas together with PHOT to guide the same kind of response, for example phototaxis. Indeed, aCRY is really involved in the process of phototactic behavior in gametes (Zou et al., submitted). It has been shown that PHOT is capable to regulate the expression level of ChR1 (Trippens et al., 2012), the dominant primary eyespot-located photoreceptor responsible for phototaxis. Similarly, the expression level of ChR1 is also altered in *acry* mutant strain (Zou et al., submitted). Moreover, in several photo-responses of *Arabidopsis*, CRYs and PHOTs are found reciprocally modulate blue light-induced responses, such as phototropic curvature, hypocotyl elongation and anthocyanin accumulation through direct or indirect interactions (Kang et al., 2008). Although it has been shown that aCRY seems to interconnect with PHOT to regulate the conversion from pregametes to gametes in Chlamydomonas, no direct interaction of aCRY and PHOT has been revealed till now (yeast two hybrid analysis shown no direct interaction, Li and Mittag, personal communication). As a result, aCRY, PHOT

and ChR1 seems to form a photoreceptor network (Fig. 26) that could regulate several crucial steps (e.g. mating, zygote germination and phototactic behavior) in Chlamydomonas. Clearly, the detail information of this photoreceptor network is needed to be further clarified in future.

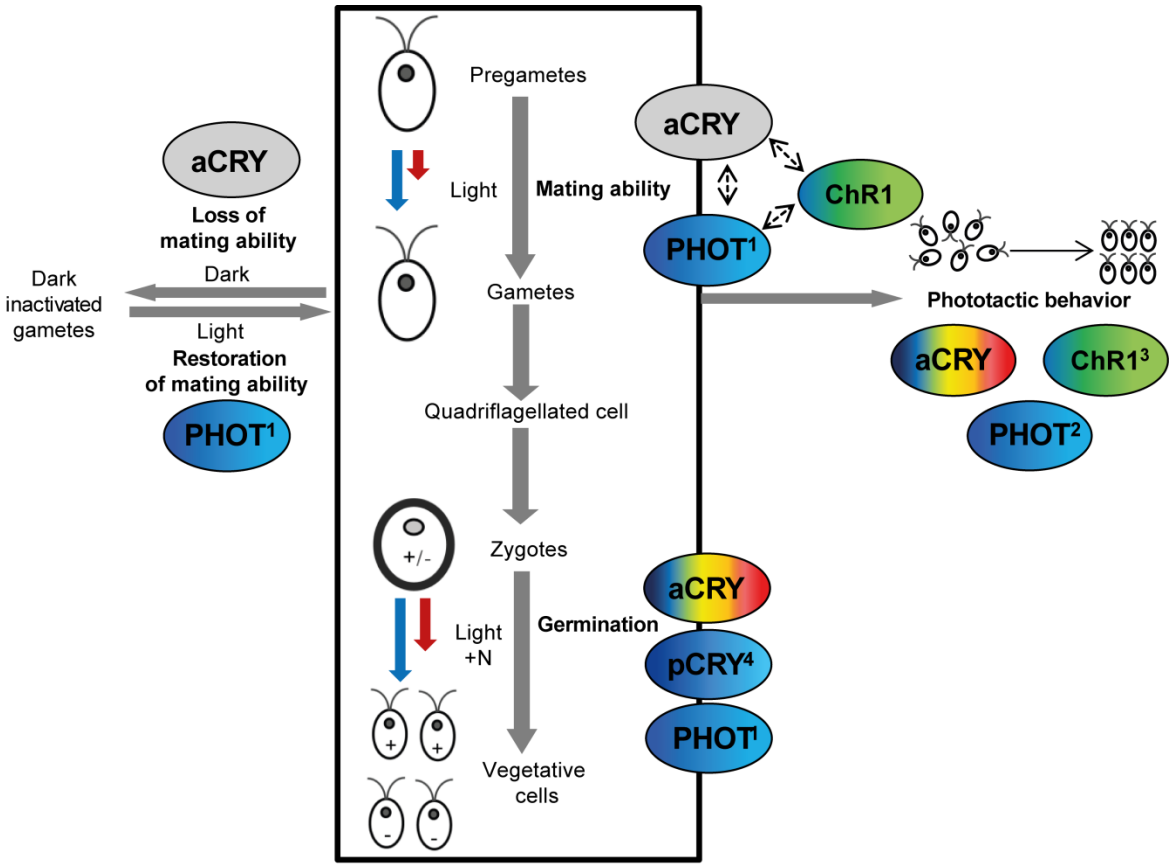

Figure 26. Overview of the role of photoreceptors in the Chlamydomonas sexual life cycle and the photoreceptor network (in collaboration with Sandra Wenzel, Zou et al., submitted).
Ellipses with different colors represent the aCRY, PHOT and ChR1 photoreceptors with their absorbing light spectrum indicated by the color. The negative roles of aCRY in gametogenesis seem not based on light and are shown with gray background. During the transition from pregametes to gametes, the interconnection between aCRY and PHOT as well as ChR1 are indicated with dotted double-end arrows. Superscript numbers on the photoreceptors represent references (1, Huang and Beck, 2003; 2, Trippens et al., 2012; 3, Berthold et al., 2008; 4, Müller et al., in preparation for resubmission). For the two light dependent steps in the sexual life cycle, gametogenesis and germination, blue light and red light arrows indicate light qualities, affecting these pathways to various extents (indicated by the length of the arrow).

3.5 The degradation of aCRY in Chlamydomonas

It is demonstrated that soluble aCRY proteins in pregametes, gametes and early zygotes are degraded by the proteasome pathway. Degradation of CRYs is commonly found in various organisms. For example, pCRY2 in *Arabidopsis* is phosphorylated in a blue-light-dependent manner and the phosphorylation of pCRY2 results in photomorphogenic responses and eventually degradation (Shalitin et al., 2002). Although COP1 has been revealed to be involved in pCRY2 degradation, the degradation is only reduced but not abolished in *cop1* mutants. In addition, the stability of pCRY2 is controlled by SPA and PHYA (Weidler et al., 2012), both of which don't show E3 ligase activity. So it is still unclear which E3 ligase, besides COP1, also interacts with pCRY2 and induces the proteasomal degradation. In *Drosophila*, CRY is degraded in a blue-light-dependent manner, via CUL4-ROC1-DDB1-Brwd3 enzyme complexes with Brwd3 as the substrate receptor (Ozturk et al., 2013), while in mouse, mCRY1 is degraded via CUL4A-DDB1-CDT2 enzyme complexes with CDT2 as the substrate receptor. mCRY1 and mCRY2 are also degraded by the Skp1-Cul1-FBXL3 ubiquitin ligase complex (Busino et al., 2007) and the AMP-activated protein kinase-mediated phosphorylation of mCRY1 promotes the interaction of mCRY1 with FBXL3 (Lamia et al., 2009). Interestingly, another FBXL protein, FBXL21, could also interact with mCRYs but stabilizes them by ubiquitination (Hirano et al., 2013).

Protein degradation by the proteasome pathway involves the interaction between substrate and the E3 ligase of the proteasome complex. Skp-Cullin-F-box (SCF) protein complexes are the most prevalent E3 ubiquitin ligases in green plants. The F-box protein specially interacts with the target proteins via a protein-protein interaction domain, such as Trp-Asp (WD) repeats and leucine-rich repeats (LRR), Kelch repeats, or F-box associated domains (Gagne et al., 2002; Xu et al., 2009). So one F-box protein containing Kelch repeats (Kelch_F-box) and ROC114 were selected as two candidates since they probably have E3 ligase activity (Schumann et al., 2011; Niwa et al., 2013).

For the interaction test between aCRY and Kelch_F-box, it is suggested that there is an interaction between soluble aCRY and Kelch_F-box in vegetative cells and proteasome inhibitor-treated pregametes, but not in pregametes. Since most soluble aCRY is degraded in pregametes, it is reasonable that no interaction signal between aCRY and Kelch_F-box could be detected. This is the only F-box protein that also contains a Kelch domain found in Chlamydomonas, while 103 F-box proteins containing a Kelch domain are found in *A. thaliana* (Schumann et al., 2011). In land plants, besides protein recognition for inducing proteasomal degradation, these proteins evolve to have diverse functions including regulation of phytochrome A-mediated light signaling, the involvement of photomorphogenesis and regulation of the circadian clock (Harmon and Kay, 2003; Suetsugu and Wada, 2013).

However, no evidence from the Yeast Two Hybrid assays shows that aCRY interacts with Kelch_F-box. Additionally, the interaction between soluble aCRY in vegetative cells and pregametes and ROC114 could be detected in the pull-down assay, but not in Yeast Two Hybrid analysis. One explanation for this is the different second or third structure formation of aCRY, Kelch_F-box or ROC114 in yeast compared to that in Chlamydomonas. Another possibility is that the interactions between aCRY and Kelch_F-box or ROC114 in the pull-down assay are not direct interactions, so they cannot be detected in the Yeast Two Hybrid assay. It also cannot be ruled out the possibility that the Yeast Two Hybrid assay is not suitable for the interaction test of aCRY since the dimerization of aCRY *in vivo* and *in vitro* (Oldemeyer et al., 2016; Zou et al., submitted; Wenzel and Mittag, personal communication) can also not be detected. So other methods, like co-immunoprecipitation should be employed to test the interaction between aCRY and Kelch_F-box or ROC114 and much work is still needed to characterize the E3 ligase that serves as the aCRY receptor to induce its degradation.

4. Summary

Cryptochromes are blue light sensory flavoproteins in plants, insects, fungi, and bacteria and are part of the circadian oscillator in mammals. An animal-like cryptochrome (aCRY) from Chlamydomonas was recently characterized as a blue and red light photoreceptor that plays a crucial role in the regulation of light-dependent genes involved in chlorophyll and carotenoid biosynthesis, light-harvesting complex assembly, nitrogen metabolism, cell cycle control and the circadian clock. In this work, the role of aCRY and a Chlamydomonas plant cryptochrome were investigated with regard to the life cycle of this alga.

aCRY was found to be mainly a soluble protein, which is also attached to membranes to some extent. Soluble aCRY accumulates in vegetative cells and mature zygotes, but not in pregametes, gametes and early zygotes. The degradation of soluble aCRY in pregametes, gametes and early zygotes is processed by the proteasome pathway. E3 ligases candidates, such as Kelch_F-box and ROC114 were primarily found to interact with aCRY in a pull-down assay but not in a yeast two hybrid approach, which needs to be further clarified in future. In contrast to soluble aCRY, membrane-associated aCRY accumulates in vegetative cells, pregametes, gametes, and late zygotes, but not in early zygotes. The role of the different distributions of aCRY in soluble or membrane-associated proteins in Chlamydomonas is still unknown.

Based on an available *acry* mutant, aCRY mutant strains of mating type plus and minus with similar genomic background as wild-type strains were generated by backcrossing. Also complemented strains of these mutants were generated. Based on these strains, aCRY was found to act as a negative regulator in mating ability and in maintenance of mating ability, in contrast to the blue light photoreceptor phototropin that acts positively in mating ability and in the restoration of the mating ability. During gametogenesis, aCRY is interconnected with phototropin and channalrhodopsin1, forming a photoreceptor network. aCRY was also found to

participate in the regulation of phototactic behavior in gametes, possibly via adjusting the expression level of channalrhodopsin1, the direct photoreceptor related to phototaxis.

My work also revealed that mainly blue, as well as red light are involved in zygote germination, during which the zygote undergoes meiosis. In addition to phototropin, both aCRY and plant cryptochrome positively regulate zygote germination. These results indicate that not only phototropin but also aCRY and plant cryptochrome are key regulators of the sexual cycle of Chlamydomonas.

5. Zusammenfassung

Cryptochrome sind blaulichtwahrnehmende Flavoproteine in Pflanzen, Insekten, Pilzen sowie in Bakterien, wogegen sie in Säugetieren Bestandteil des circadianen Oszillators sind. Ein tier ähnliches Cryptochrom (aCRY) aus Chlamydomonas wurde kürzlich als Blau- und Rotlicht-Photorezeptor charakterisiert, der eine wichtige Rolle bei der Regulation von lichtabhängigen Genen spielt, welche in die Chlorophyll- und Carotinoid-Biosynthese, in die Assemblierung von Lichtsammelkomplexen, den Stickstoffmetabolismus, die Kontrolle des Zellzyklus und der circadianen Uhr involviert sind. In dieser Arbeit wurden die Funktionen von aCRY und einem pflanzlichen Cryptochrom aus Chlamydomonas hinsichtlich des Lebenszyklus dieser Alge untersucht.

aCRY wurde als ein primär lösliches Protein charakterisiert, welches aber auch zu einem gewissen Teil an Membranen gebunden ist. Das lösliche aCRY akkumuliert in vegetativen Zellen und vollentwickelten Zygoten, nicht jedoch in Pregameten, Gameten und Zygoten aus einem frühen Entwicklungsstadium. Der Abbau des löslichen aCRY in Pregameten, Gameten und Zygoten des frühen Entwicklungsstadiums findet über den Proteasom-Weg statt. E3-Ligase-Kandidaten wie etwa Kelch_F-box und ROC114 wurden als Interaktionspartner von aCRY in einem „pull-down-assay“ identifiziert, nicht jedoch in einem Hefe-Zwei-Hybrid-System. Diese Diskrepanz muss in Zukunft noch geklärt werden. Im Gegensatz zum löslichen aCRY akkumuliert das membranassoziierte aCRY in vegetativen Zellen, Pregameten, Gameten und vollentwickelten Zygoten, nicht jedoch in Zygoten des frühen Entwicklungsstadiums. Die Aufgaben dieser unterschiedlichen Verteilung von aCRY in der löslichen und der membrangebundenen Proteinfraktion von Chlamydomonas sind noch unbekannt.

Basierend auf einer verfügbaren *acry*-Mutante wurden über Rückkreuzungen aCRY-Mutantenstämme der Paarungstypen Plus und Minus mit einem ähnlichen genetischen Hintergrund wie der Wildtyp erzeugt. Außerdem wurden auch komplementierte Stämme dieser Mutanten generiert.

5. Zusammenfassung

Basierend auf der Untersuchung dieser Stämme konnte aCRY als ein negativer Regulator der Paarungsfähigkeit und des Erhalts der Paarungsfähigkeit identifiziert werden, während im Gegensatz dazu der Blaulicht-Photorezeptor Phototropin positiv auf die Paarungsfähigkeit und die Wiederherstellung der Paarungsfähigkeit wirkt. Während der Gametogenese ist aCRY mit Phototropin und Kanalrhodopsin 1 verbunden und formt damit ein Photorezeptor-Netzwerk. aCRY ist außerdem an der Regulation des phototaktischen Verhaltens von Gameten beteiligt, möglicherweise über die Regulation des Expressionsniveaus von Kanalrhodopsin 1, dem direkt an der Phototaxis beteiligten Photorezeptor.

Meine Arbeiten haben außerdem gezeigt, dass hauptsächlich Blaulicht, aber auch Rotlicht, an der Keimung der Zygoten beteiligt sind, während derer die Zygote eine Meiose durchläuft. Zusätzlich zu Phototropin zeigen aCRY und das pflanzliche Cryptochrom eine positive Regulation der Zygotenkeimung. Diese Resultate legen nahe, dass nicht nur Phototropin, sondern auch aCRY und das pflanzliche Cryptochrom Schlüsselkomponenten der Regulation des sexuellen Lebenszyklus von Chlamydomonas sind.

6. Materials and Methods

6.1 Materials

6.1.1 Laboratory equipment

Table 2. Lists of used devices

Equipment	Description	Manufacturer
Autoclaves	①Varioklav® ②HMC HV-85L	①H+P Labortechnik, München ②Hirayama Manufacturing Corporation, Jena
Blot apparatuses	①PerfectBlueTM Semi-Dry-Elektroblotter SEDEC M ②PerfectBlueTM Tank-Elektroblotter Web M	①/②PEQLAB Biotechnologie GmbH, Erlangen
Centrifuges	①Eppendorf 5415 D with rotor F45-24-1 ②Eppendorf 5415 R with rotor FA-45-24-11 ③Hermle Z323K with following rotors: 220.72V04, 220.87V01, 221.16 ④Ultracentrifuge Avanti™ J-30 I with Rotor JA-25.50, JA-10, and JS24.38 and corresponding tubes	①/②Eppendorf AG, Hamburg ③Hermle, Wehingen ④Beckmann Instruments Inc., Palo Alto, CA USA
Clean bench	Holten LaminAir Model 0.9	Reinraum, Klima- and Labortechnik Maahl, Kaarst
Electrophoresis chambers	①Hoefer™ HE 33, mini horizontal submarine unit ②BlueMarine™ 200	①Amersham Pharmacia Biotech Europe GmbH, Freiburg ②SERVA Electrophoresis GmbH, Heidelberg

Continued

Electrophoresis power supply	EPS 301	Amersham Pharmacia Biotech Europe GmbH, Freiburg
Freezer	NEB Cool	New England Biolabs GmbH, Frankfurt
Magnetic stirrer	IKAMAG® REO, IKAMAG® RCT	IKA® -Werke GmbH & Co. KG, Staufen
Microscopes	①Zeiss Axiophot with AxioCam Icc1 (with phase contrast and fluorescence) ②Axiostar plus ③Zeiss Stemi 2000	①/②/③ Carl Zeiss Microscopy GmbH, Göttingen
PCR thermal cycler	peqSTAR 96× Universal Gradient	PEQLAB Biotechnologie GmbH, Erlangen
pH-meter	Mettler Toledo InLab® Routine Pro pH	Mettler-Toledo GmbH, Giessen
Photometer	Photometer Helios beta	Thermo Elektron Corporation
Pipettes	Pipetman P2, P20, P200, P1000 and P5000	Abimed GmbH, Langenfeld
Rotating shaker	Roto-Shake-GenieTM	Scientific Industries, Inc., USA
Scales	MC1 Analytic AC 120 S, MC1 Laboratory LC 620 S	Sartorius AG, Göttingen
Spectrophoto−meter	NanoDrop 2000	Thermo Fisher Scientific, USA
Thermo shaker	TS1 ThermoShaker	Biometra GmbH, Göttingen
Vacuum concentrator	Vacuum concentrator Savant Speed vac® Plus SC 110	Savant, Holbrook, NY
HPLC (Mass pectrometer)	UltiMate 3000	Dionex, Sunnyvale, USA
Vortex mixer	Vortex-Genie® 2	Scintific Industries, New York, USA
FPLC (Fast protein liquid chromatography)	ÄKTA Explorer FPLC System	Amersham Pharmacia Biotech
Counting chamber	Thoma chamber	Fein Optic, Jena

6.1.2 Consumable supplies

Table 3. List of consumable supplies

Products	Manufacturer
Cuvettes, polystyrene, single-use	Carl Roth GmbH + Co. KG, Karlsruhe
Eppendorf tubes (1.5 ml and 2 ml)	Carl Roth GmbH + Co. KG, Karlsruhe
Falcon tubes, polypropylene (15 ml and 50 ml)	Carl Roth GmbH + Co. KG, Karlsruhe
Nitrocellulose membrane (for Neuhoff protein determination)	Sartorius Stedim Biotech GmbH, Göttingen
Nitrocellulose membrane (for immunoblot)	Bio-Rad, München
Parafilm® "M"	Carl Roth GmbH + Co. KG, Karlsruhe
PCR tubes (0.2 ml and 0.5 ml)	AHN Biotechnologie GmbH, Nordhausen
PVDF membrane	Bio-Rad Laboratories GmbH, München
Rotilabo®-Spritzenfilter steri (0.22 and 0.45 µm)	Carl Roth GmbH + Co. KG, Karlsruhe
Rotiprotect®-latex/ nitril	Carl Roth GmbH + Co. KG, Karlsruhe
Standard microscope slides and coverslips	Carl Roth GmbH + Co. KG, Karlsruhe
Standard 96-well PCR plates	Eppendorf AG, Hamburg
Tips for Pipetman pipettes	Carl Roth GmbH + Co. KG, Karlsruhe
Wooden toothpicks	Carl Roth GmbH + Co. KG, Karlsruhe
Centrifugal filters	Merck Millipore, Darmstadt

6.1.3 Chemicals, buffers and solutions

All chemicals used in the experiments had p.A. (pro Analysis) quality and were purchased from the following companies: Roth (Karlsruhe), Sigma (Steinheim), Merck (Darmstadt), Sigma-Aldrich (München) and Takara Bio Europe SAS (France), Becton, Dickinson and Company (USA) and AppliChem (Darmstadt). All buffers and solutions were prepared with double stilled water (ddH_2O).

Table 4. Chemicals, buffers and solutions for media

Buffers and solutions	Recipe
Beijerinck's solution	0.3 M NH_4Cl 13.6 mM $CaCl_2 \cdot 2H_2O$ 4 mM $MgSO_4 \cdot 7H_2O$
Beijerinck's solution -NH_4Cl	13.6 mM $CaCl_2 \cdot 2H_2O$ 4 mM $MgSO_4 \cdot 7H_2O$
(K)(PO_4)-buffer (1M)	1M KH_2PO_4 was added to 1M K_2HPO_4 till pH is 7
Trace (Hutner et al., 1950)	2.2 g $ZnSO_4 \cdot 7H_2O$ 1.14 g H_3BO_3 0.506 g $MnCl_2 \cdot 4H_2O$ 0.499 g $FeSO_4 \cdot 7H_2O$ 0.161 g $CoCl_2 \cdot 6H_2O$ 0.157 g $CuSO_4 \cdot 5H_2O$ 0.110 g $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$ Above chemicals were dissolved separately in ddH_2O and then mixed; 5 g Na_2EDTA was added and boiled; after cooling down, pH was adjusted to 6.5-6.8 with 10% (w/v) KOH; ddH_2O was added to 100 ml and the solution was filtrated (the color should be green and turn to violet after several days)
Salts 1 for N10 medium	0.28 M NH_4Cl 0.136 M $CaCl_2 \cdot 2H_2O$
Salts 2 for N10 medium	0.162 M $MgSO_4 \cdot 7H_2O$
1M Potassium phosphate buffer for N10 medium (pH7.0)	28.8 g K_2HPO_4 14.4 g KH_2PO_4 100 ml ddH_2O

Table 5. Chemicals, buffers and solutions for protein biochemical experiments

Buffers and solutions	Recipe
0.5 M EDTA (pH = 8.0)	9.306 g of Na_2EDTA 30 ml ddH_2O. pH was adjusted to 8.0 with 1 M NaOH. ddH_2O was added to 50 ml.
Crude extraction buffer for Chlamydomonas	10 mM Tris pH 7.5 80 mM NaCl 10 ml 0.5 M EDTA pH 8.0 1% glycerol Solution was autoclaved and stored at 4°C.

Continued

Crude extraction buffer with PIC and DTT for Chlamydomonas	900 µl crude extraction buffer for Chlamydomonas 2 µl 0.1M DTT 100 µl 10 × PIC (1 pill solved in 10 ml extraction buffer)
Destaining solution for Amidoblack	90% (v/v) methanol, 10% (v/v) acetic acid
Amidoblack solution	0.5% (w/v) amidoblack in destaining solution as stock solution The stock solution was diluted with destaining solution till OD_{630}=10.
4 × SDS sample buffer	2.42 g Tris, solved in ddH_2O, pH was adjusted to 6.8 with HCl 8g SDS 40 g glycerol ddH_2O was added till 84 ml; small amount of bromophenol blue was added as indicator; solution was separated into aliquots of 840 µl and stored in -20°C. For the 4 × SDS sample buffer used in the experiment, 160 µl 2.5M DTT was added into 840 µl aliquot.
9% resolving gel	10.75 ml ddH_2O 7.5 ml 30% acrylamide mix 6.3 ml 1.5 M Tris/HCl (pH 8.8) 0.25 ml 10% SDS 16 µl TEMED 0.2 ml 10% (w/v) ammonium persulfate The solution was overlaid with isopropanol; the gel polymerized for at least 1h.
Stacking gel	6.8 ml ddH_2O 1.7 ml 30% acrylamide mix 1.25 ml 1.0 M Tris/HCl (pH 6.8) 0.1 ml 10% SDS 16 µl TEMED 0.1 ml 10% (w/v) ammonium persulfate (mixed gently before addition of ammonium persulfate) Suitable comb was inserted and the gel polymerized for at least 30 min.
5 × SDS PAGE running buffer	125 mM Tris 1.02 M glycerol

Continued

	0.5% (w/v) SDS
"Semi-dry" Transfer buffer	25 mM Tris 156 mM glycerol 20% (v/v) methanol 0.375% (w/v) SDS
Coomassie solution	0.25% (w/v) Brilliant Blue R-250 45% (v/v) methanol 45% (v/v) ddH_2O 10% (v/v) acetic acid The solution was well mixed and filtered through Whatman Grade 1 Qualitative Filter Paper to remove unsolved staff
Destaining solution for Coomassie	10% (v/v) acetic acid 45% (v/v) methanol 45% ddH_2O

Table 6. Chemicals, buffers and solutions for immuno-chemical experiments

Buffers and solutions	Recipe
10 × TBS	0.2 M Tris 1.5 M NaCl pH value was adjusted to 7.5 with HCl
1 × TBS-Tween	100 ml 10 × TBS 0.5 ml Tween 20 ddH_2O was added to 1L
Blocking solution	5% (w/v) milk powder 1 × TBS-Tween
Luminol-solution 1	9.68 ml Tris/HCl pH 8.5 0.1 ml luminol stock solution 0.22 ml p-coumaric acid stock solution (stock solution: 0.22g luminol solved in 5 ml DMSO, 0.15g p-coumaric acid solved in 10 ml DMSO, stored at -20°C, protected from light)
Luminol-solution 2	10ml Tris pH 8.5 5.7 µl H_2O_2

Table 7. Other used chemicals, buffers and solutions

Buffers and solutions	Recipe
Mating buffer	0.6 mM $MgCl_2$ 1.2 mM HEPES pH was adjusted to 6.8 with 1 M KOH
1 × PBS	137 mM NaCl 2.7 mM KCl 10 mM $Na_2HPO_4 \cdot 2\ H_2O$ 1.8 mM KH_2PO_4 The final pH was about 7.4
2 × *E. coli* lysis buffer	84 mM $Na_2HPO_4 \cdot 2H_2O$ 16 mM $NaH_2PO_4 \cdot H_2O$ 600 mM NaCl 20% (v/v) Glycerin 1% (v/v) Triton X-100 20 mM Imidazol Solution was filtered with a 0.2 µm filter and stored at 4°C.
1 × *E. coli* lysis buffer mixture	900 µl 2 × *E. coli* Lysis Buffer 1.4 µl 14.5 M ß-mercaptoethanol (final concentration: 20 mM) 200 µl 10mg/ml lysozyme (final concentration: 1 mg/ml) 400 µl 1 × PIC (1 pill in 50 ml ddH_2O) 500 µl ddH_2O
TAE buffer (Tris-acetate-EDTA) (50 ×)	2 M Tris 5.71% (v/v) glacial acetic acid 0.05 M EDTA pH8.0
Electrophoresis agarose gel 1% (for separating 0.4 - 10.0 kb DNA fragment)	0.6 g Agarose in 60 ml 1 × TAE buffer The mixture was boiled till all agarose was solved; ethidium bromide was added with the final concentration of 0.5 µg/ml when the temperature was cooled down to 50°C, the gel was poured to the chamber with proper comb.
2 M LiAc with PIC	2.04 g in 10 ml ddH_2O 20 µl 2 × PIC was added to 1 ml
0.4 M NaOH with PIC	4 ml 1M NaOH in 10 ml 20 µl 2 × PIC was added to 1 ml
PEG MW 3350 (50% w/v)	25 g PEG 3350 15 ml ddH_2O Solution was stirred until dissolved and ddH_2O was

Continued

	added to exactly 50 ml; the final solution was sterilized by filtration
10/1 TE Buffer	10 ml 1 M Tris/HCl, pH = 8.0 2 ml 0.5 M EDTA, pH = 8.0 ddH_2O was added till 1L
Single-stranded carrier DNA (2.0 mg/ml)	100 mg salmon sperm DNA 50 ml sterile 10/1 TE-Buffer Solution was stirred overnight at 4°C
1 M 3-AT	4.204 g 3-Amino-1,2,4-Triazol ddH_2O was added till 50 ml Solution was sterilized by filtration. If added to plates, the medium should be cooled down to 50°C.
Purification buffer A	84 mM $Na_2HPO_4 \cdot 2H_2O$ 16 mM $NaH_2PO_4 \cdot H_2O$ 200 mM NaCl 10% (v/v) Glycerin Solution was filtered through 0.2 µm to remove gas and stored at 4°C; 140 µl ß-mercaptoethanol was added prior to use.
Purification buffer B	84 mM $Na_2HPO_4 \cdot 2H_2O$ 16 mM $NaH_2PO_4 \cdot H_2O$ 200 mM NaCl 10% (v/v) Glycerin 500 mM Imidazol Filter through 0.2 µm to remove gas and store at 4°C; add 140 µl ß-mercaptoethanol prior to use.
Pull-down buffer	3.25 mM phosphate buffer pH 7.4 70 mM NaCl 5% (v/v) Glycerol 1 pill PIC EDTA-free (Roche) was added per 10ml
Binding/wash buffer	50 mM phosphate buffer pH 8.0 300 mM NaCl 0.01% Tween-20 1 pill PIC EDTA-free (Roche) was added per 10ml
Wash buffer for pull-down assay	50 mM phosphate buffer pH 8.0 70 mM NaCl 5% (v/v) Glycerol 1 pill PIC EDTA-free (Roche) was added per 10ml
Elution buffer	300 mM Imidazol; 50 mM phosphate buffer pH 8.0; 300 mM NaCl; 0.01% (v/v) Tween-20

6.1.4 Enzymes, inhibitors and inducers, antibiotics, markers, and kits

Table 8. List of used enzymes, inhibitors, antibiotics and markers

Enzymes	Manufacturer
Q5® High-Fidelity DNA Polymerase	New England Biolabs GmbH, Frankfurt
Restriction endonucleases	New England Biolabs GmbH, Frankfurt
T4 DNA Ligase, 5 U/µl	Roche GmbH, Mannheim
10 × T4 DNA Ligase buffer	Roche GmbH, Mannheim
T4 Polynucleotide Kinase, 10 U/µl	New England Biolabs GmbH, Frankfurt
Antartic Phosphatase, 5 U/µl	New England Biolabs GmbH, Frankfurt
Lambda Protein Phosphatase, 400 U/µl	New England Biolabs GmbH, Frankfurt

Table 9. List of used inhibitors and inducers

Inhibitors	Manufacturer
3-Amino-1,2,4-Triazol	Sigma-Aldrich Chemie GmbH, München
MG132 (proteasome inhibitor)	Peptide institute, Inc., Japan
IPTG	PEQLAB Biotechnologie GmbH, Erlangen

Table 10. List of used antibiotics

Antibiotics	Manufacturer
Ampicillin	Carl Roth GmbH + Co. KG, Karlsruhe
Hygromycin B	Carl Roth GmbH + Co. KG, Karlsruhe
Kanamycin	Carl Roth GmbH + Co. KG, Karlsruhe
Paromomycin sulfate salt	Sigma-Aldrich Chemie GmbH, München

Table 11. List of used markers

Markers	Manufacturer
100 bp DNA ladder, effective size range:100 bp to 1517 bp	New England Biolabs GmbH, Frankfurt
1 kb DNA ladder, effective size range: 500 bp to 10002 bp	New England Biolabs GmbH, Frankfurt
Gel loading dye, purple (6 ×)	New England Biolabs GmbH, Frankfurt
Spectra™ multicolor broad range Protein ladder (10-260kDa)	Life Technologies GmbH, Darmstadt

Table 12. List of used kits

Kits	Manufacturer
GeneJET gel extraction kit	Fisher Scientific GmbH, Schwerte
High pure plasmid isolation kit	Roche GmbH, Mannheim
Matchmaker® gold yeast two-hybrid system	Takara Bio Europe SAS, France
QIAquick PCR Purification Kit	Qiagen GmbH, Hilden
RNeasy Plant Mini kit	Qiagen GmbH, Hilden
QuantiTect SYBR Green RT-qPCR	Qiagen GmbH, Hilden
Dynabeads ® His-Tag Isolation and Pulldown	Fisher Scientific GmbH, Schwerte

6.1.5 Antibodies

Table 13. List of used antibodies

Antibodies	Name	Working dilution	Manufacturer
First antibodies	anti-aCRY	1:5000	Pineda Antikörper Service, Berlin Germany
	anti-LOV1 (for detecting PHOT)	1:2000	Donated by Peter Hegemann
	anti-Cytochrome f	1:15,000	Purchased from Agrisera
	anti-ChR1	1:6000	Donated by Peter Hegemann
Second antibody	monoclonal anti-rabbit IgG (conjugated to a horseradish peroxidase)	1:6666	Sigma-Aldrich, Steinheim Germany

6.1.6 Used Strains

Table 14. List of used and generated strains of Chlamydomonas

Strains	General discription
SAG73.72	mt^+, ordered from Sammlung von Algenkulturen der Universität Göttingen, also known as CC-3348 (http://chlamycollection.org/strain/cc-3348-wild-type-mt-sag-73-72-c8/). It can grow on nitrate.
D66	mt^+, a cell wall-deficient strain, also known as CC-4425 *cw15 nit2*-203. It is a *cw15 nit1* strain suitable for transformation.
CC-125	mt^+, one basic “137c” wild type strain; carrying the *nit1* and *nit2* mutations and cannot grow on nitrate as its sole N source. CC-125 carries the AGG1 (agg1+) allele for

Continued

	phototactic aggregation.
CC-124	mt$^-$, one basic "137c" wild type strain; carrying the nit1 and nit2 mutations, and cannot grow on nitrate as its sole N source. CC-124 carries the *agg1* (agg1-) allele for phototactic aggregation.
CRMS101 (Beel et al., 2012)	mt$^+$, the original *acry* mutant strain with D66 background. The difference with D66 is the insertion of an *APHVIII* cassette which confers the strain with paromomycin resistance.
CRMS102	mt$^+$, the original *pcry* mutant strain with D66 background. The difference with D66 is the insertion of an *APHVIII* cassette which confers the strain with paromomycin resistance.
acry*$_{mut}$ (1K)	mt$^-$, a progeny of crossing between CC-124 and CRMS101; with paromomycin resistance.
aCRY2kb_2A*	mt$^-$, a progeny of crossing between aCRY1kb and CC-125; with paromomycin resistance.
aCRY2kb_9D*	mt$^+$, a progeny of crossing between aCRY1kb and CC-125; with paromomycin resistance.
aCRY2kb_2A_compl. #19*	mt$^-$, a transformant of aCRY2kb_2A with pKP39**; with paromomycin and hygromycin B resistance.
aCRY2kb_9D_compl. #15/#26*	mt$^+$, transformants of aCRY2kb_9D with pKP39**; with paromomycin and hygromycin B resistance.
***pcry*$_{mut}$ (1K)**	mt$^-$, progenies of crossing between CC-124 and CRMS102; with paromomycin resistance.
pCRY2kb_6C*	mt$^-$, progenies of crossing between pCRY1kb and SAG73.72; with paromomycin resistance.
pCRY2ka_8C*	mt$^-$, a progeny of crossing between pCRY1ka and CC-125; with paromomycin resistance.
pCRY2ka_9A*	mt$^+$, a progeny of crossing between pCRY1ka and CC-125; with paromomycin resistance.
pCRY2kb_6C_compl. #178	mt$^-$, a transformant of aCRY2kb_2A with pNM003***; with paromomycin and hygromycin B resistance.
pCRY3kb_8*	mt$^+$, a progeny of crossing between pCRY2kb_6C_compl. #178 and CC-125; with paromomycin and hygromycin B resistance.
pCRY4kb_14A*	mt$^+$, a progeny of crossing between pCRY3kb_8 and CC-124; with paromomycin and hygromycin B resistance.

*: generated strains

**: pKP39 is a vector that contains promoter sequences and the aCRY gene from Chlamydomonas, as well as the hygromycin B resistance region (Beel et al., 2012).

***: pNM003 is a vector that contains promoter sequences and the pCRY gene from Chlamydomonas, as well as the hygromycin B resistance region (Müller et al., in preparation for resubmission).

Table 15. List used strains of other species

Strains	General description
***E. coli* XL1-Blue**	*sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A46, *thi*, *rel*A1, Tn10 (tet^r), *lac*$^-$, F' [*pro*AB$^+$, *lac*^{q}, *lac*ZΔM15] (Bullock, 1987)
***E. coli* BL21 (DE3)**	*E. coli* str. B F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *dcm*$^+$ tet^r *gal* λ(DE3) *endA* Hte [*argU proL*Camr] [*argU ileY leuW* Strep/Specr] (Studier and Moffatt, 1986)
***Saccharomyces cerevisiae* Y2HGold**	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1*$_{UAS}$*–Gal1*$_{TATA}$*–His3, GAL2*$_{UAS}$*–Gal2*$_{TATA}$*–Ade2 URA3 : : MEL1*$_{UAS}$*–Mel1*$_{TATA}$ *AUR1-C MEL1* (Nguyen, unpublished)
***S. cerevisiae* Y187**	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met–, URA3 : : GAL1*$_{UAS}$*–Gal1*$_{TATA}$*–LacZ, MEL1* (Harper et al., 1993)

6.1.7 Media

Media have been prepared with distilled water (ddH_2O) and autoclaved for 30 min at 121°C if not otherwise specified.

Table 16. Media for culturing *E. coli*

Medium	Composition
LB liquid medium or agar plates	25 g LB powder (ready to use product, from Roth); dissolved in 1 L ddH_2O with 1.5% (w/v) agar if making agar plates
LB liquid medium or agar plates with Ampicillin	LB Medium with 100 µg/ml Ampicillin
LB liquid medium or agar plates with Kanamycin	LB Medium with 50 µg/ml Kanamycin
SOC (Super Optimal broth with Catabolite repression)	100 ml autoclaved LB medium 1 ml filter sterilized 2 M glucose store with 1ml aliquot at -20°C

Table 17. Media for culturing *S. cerevisiae*

Media for yeast were autoclaved for 15 min at 121°C.

Medium	Composition
SD-Ade/-His/-Leu/-Trp agar plates	26.7 g Minimal SD base 600 mg DO-Supplement (-Ade/-His/-Leu/-Trp) 900 ml ddH_2O pH was adjusted to 5.8 with 1 M NaOH ddH_2O was added till 1L 18 g Agar-Agar
SD-His/-Leu/-Trp agar plates	26.7 g Minimal SD base 600 mg DO-Supplement (-Ade/-His/-Leu/-Trp) 100 mg Adenine-hemisulfate-salt 900 ml ddH_2O pH was adjusted to 5.8 with 1 M NaOH ddH_2O was added till 1L 18 g Agar-Agar
SD-Leu liquid medium or agar	26.7 g Minimal SD base 640 mg DO-Supplement (-Leu/-Trp) 100 mg L-Trp 900 ml ddH_2O pH was adjusted to 5.8 with 1 M NaOH ddH_2O was added till 1L with 1.8% (w/v) agar if making agar plates
SD-Trp liquid medium or agar plates	26.7 g Minimal SD base 640 mg DO-Supplement (-Leu/-Trp) 100 mg L-Leu 900 ml ddH_2O pH was adjusted to 5.8 with 1 M NaOH ddH_2O was added till 1L with 1.8% (w/v) agar if making agar plates
SD-Leu/-Trp agar plates	26.7 g Minimal SD base 640 mg DO-Supplement (-Leu/-Trp) 900 ml ddH_2O pH was adjusted to 5.8 with 1 M NaOH ddH_2O was added till 1L 18 g Agar-Agar
YPD liquid medium or agar plates	20 g Peptone 10 g Yeast extract 900 ml ddH_2O

Continued

	pH was adjusted to 5.8 with 1 M NaOH ddH_2O was added till 1L 50 ml 40% (w/v) sterilized glucose was added to autoclaved medium when it has been cooled to ~55°C; with 1.8% (w/v) agar if making agar plates
YPDA liquid medium or agar plates	20 g Peptone 10 g Yeast extract 100 mg Adenine-hemisulfate-salt 900 ml ddH_2O pH was adjusted to 5.8 with 1 M NaOH ddH_2O was added till 1L 50 ml 40% (w/v) sterilized glucose was added to autoclaved medium when cooled to ~55°C; with 1.8% (w/v) agar if making agar plates

Table 18. Media for culturing Chlamydomonas

Medium	Composition
TAP liquid or agar plates (Harris, 1989)	2.42 g Tris base 25 ml Beijerinck's solution (s. 6.1.3) 1 ml (K)(PO_4)-buffer (s. 6.1.3) 1 ml trace (s. 6.1.3) 900 ml ddH_2O was added and mixed well, pH was adjusted with acetic acid to 7.0, ddH_2O was added till 1L with 2% (w/v) agar if making agar plates
TAP-N	2.42 g Tris base 25 ml Beijerinck's solution-NH_4Cl (s. 6.1.3) 1 ml (K)(PO_4)-buffer (s. 6.1.3) 1 ml trace (s. 6.1.3) 900 ml ddH_2O was added and mixed well, pH was adjusted with acetic acid to 7.0, ddH_2O was added till 1L
N10 plates	1.2 g Sodium acetate, water free 5 ml Potassium phosphate buffer (s. 6.1.3) 2.5 ml Salts 1 (s. 6.1.3) 2.5 ml Salts 2 (s. 6.1.3) 1 ml trace metals (s. 6.1.3) 15 g agar ddH_2O was added to 1L.
TAP plates with 3% Difco agar	1LTAP media with 3 g Difco agar

Continued

TAP plates with 1.5% Difco agar	1LTAP media with 1.5 g Difco agar
TAP agar plates with paromomycin	TAP medium with 2% (w/v) agar and 50 µg/ml paromomycin
TAP agar plates with hygromycin	TAP medium with 2% (w/v) agar and 10 µg/ml hygromycin

6.2 Methods

6.2.1 Molecular biological methods

Molecular methods were performed based on Molecular Cloning: A Laboratory Manual (Sambrook and Russell, 2001) with some modifications.

6.2.1.1 Plasmid isolation using high pure plasmid isolation kit

A picked colony was incubated in 2.5 ml LB with corresponding antibiotics at 37°C for 12-16 hours. The bacterial cells from the culture (cell density of 1.5-5.0 A_{600} units per ml) were harvested and the plasmids were isolated according to the instruction of the High Pure Plasmid Isolation Kit (s. 6.1.4, Table 12).

6.2.1.2 Plasmid digestion, agarose gel electrophoresis and DNA fragments purification from gel

The concentration of the plasmid was determined via NanoDrop2000. The digestion of plasmid was performed according to the recommendation of the restriction endonucleases description from NEB (http://www.neb-online.de/en/). Heat inactivation of the enzyme was performed if indicated. DNA samples which were mixed with the loading dye first, as well as the appropriate DNA ladder, were loaded on the agarose gel in 1 × TAE running buffer (s. 6.1.3, Table 7) and run at 100-110 V (10 V/cm_2) for 40-60 min. The DNA bands on the agarose gel were visualized under UV light and recorded by a camera. If needed, DNA fragments were purified from the gel by using the UV light with higher wavelength (366nm) to attenuate the mutation. After cutting the gel piece with a scalpel, the gel block with the desired DNA fragment was purified according to the instruction of the gel extraction kit (s. 6.1.4, Table 12).

6.2.1.3 Plasmid ligation with T4 DNA Ligase

Fifty nanogram digested vectors were used for ligation. Based on a 1:3 molar ratio (vector : insert) of sticky ends, the amount of insert DNA fragment was calculated and added to the T4 DNA Ligase reaction according to the protocol of the manufacture. T4 DNA Ligase was always added at last. The reaction was incubated at 16°C

overnight. Before transformation, the reaction was heat-inactivated at 65°C for 10 min. 5 µl from the 20 µl reaction solution were transformed into 50 µl competent cells (s. 6.2.2.3).

6.2.1.4 Protein concentration determination using Neuhoff method (Neuhoff et al., 1979)

A calibration curve was established using BSA (Bovine serum albumin, MP Biomedicals, France) as protein standard. The following concentrations of BSA were used: 0, 1, 2, 3, 4 and 5 µg/µl. Samples were denatured in 1 × SDS sample buffer (s. 6.1.3, Table 5) at 96°C for 3 min. 2 µl of the denatured sample was loaded onto the center of 1×1 cm acetate membrane squares (triplicates were used) and dried. Then, the membrane with protein samples were stained with an Amidoblack solution (s. 6.1.3, Table 5) in reaction vessels for 2 min, destained with destaining solution (s. 6.1.3, Table 5) for 3 times and dried. The dried membrane was cut along the pre-recorded squares and each sample was dissolved in 1 ml H_2O-free DMSO in a 2 ml Eppendorf tube by strong mechanical shaking. The absorbance at OD_{630} was recorded for the establishment of a calibration curve or determination of the protein concentration according to the calibration curve.

6.2.1.5 Western blot

6.2.1.5.1 SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

25 ml 9% resolving gel (s. 6.1.3, Table 5) was prepared first and poured into a fixed gel caster and overlaid with 1-2 mm 2-propanol for at least 1 h. The 2-propanol was discarded and the resolving gel was cleaned with distilled water before 10 ml stacking gel (s. 6.1.3, Table 5) was poured above it. The stacking gel was inserted with a proper comb and polymerized for at least 30 min. Each gel was run at 40 mA was applied for about 2.5 h to allow the migration of bromphenol blue not to exceed over 9 cm in the resolving gel.

6.2.1.5.2 Immunoblot

A blotting chamber was used for one or two gels for the transfer from gel to activated PVDF. Before the transfer, the filter paper (Whatman paper, 6 pieces per gel with sizes of 9×17 cm) should be prepared; the PVDF membrane (size 9×17 cm) was incubated in methanol for a few seconds for activating. The gel (max 9×16 cm) was cut and incubated in transfer buffer for 15 min under 2D-shaking. The blotting chamber was arranged with the order of minus stainless steel electrode, 3 pieces of filter paper, gel, membrane, 3 pieces of filter paper, and plus platinum electrode. For the gel size of 9×17 cm, blotting was done for 90 min at 225 mA for one gel (the electronic current is calculated according to the following formula: mA = 1.5 (thickness of the gel) × gel amount × wide of one gel (cm) × length of one gel (cm) (e.g. 1.5×1×9×17 = 229.5 mA)).

PVDF-membranes were transferred directly into the hybridization tube. The membrane with attached proteins was incubated in 25 ml of blocking buffer in a hybridization tube overnight at 4°C or room temperature for 2h with rotating on a RotiShaker. After incubation, 25 ml blocking buffer with the appropriate amount of 1^{st} antibody (the final concentration of used 1^{st} antibody in blocking buffer is listed in 6.1.5) was added into the hybridization tube to replace the blocking buffer. The membrane was incubated with 1^{st} antibody for 1.5 h at room temperature or overnight at 4°C (indicated for each antibody in 6.1.5, Table 13). Each membrane was washed with 25 ml 1 × TBS-Tween for 8 min for three times and incubated in appropriate amount 2^{nd} antibody for 1 h at room temperature. Then the membrane was washed with 25 ml 1 × TBS-Tween for 15 min for three times, and 25 ml 1 × TBS without Tween for 5 min for one time. Finally, the membrane was ready for detection.

Before the detection, Luminol 1 und 2 were prepared freshly. The membrane was incubated with Luminol 1 and 2 under dim red light for 3 min. Thereafter, the membrane was arranged in the photo box and exposed to x-ray films for suitable time. The exposed x-ray films were incubated for 1.5-3 min in developer solution, washed in water, incubated in fixation solution for 1-2 min and afterwards washed in water.

Then the x-ray films were dried and the marker bands were labeled according to the fixed membrane. The PVDF-membrane was stained with Coomassie Brilliant Blue solution for 1 min and washed with destaining buffer for 15 min for 3 times until the protein bands were significantly distinguishable.

6.2.2 Methods related to *E. coli*

6.2.2.1 Cell cultivation of *E. coli*

E. coli cells were incubated overnight at 37°C in LB liquid media or on LB agar plates with appropriate antibiotics according to the plasmid selection marker. Frequently used strains were stored at 4°C. Otherwise, isolated plasmids from *E.coli* (s. 6.2.1.1) were stored in -20°C and transformation (s. 6.2.2.3) was performed when needed.

6.2.2.2 Preparation of *E. coli* competent cells

5 µl *E. coli* competent cells were pre-cultured in 2.5 ml LB media at 37°C with shaking overnight. 0.5 ml pre-culture were transferred into 100 ml LB main culture to enlarge the population. When the OD_{600} value of the culture reached 0.4-0.6, indicating that the cells were in the middle log phase, the cells were spin down under 4,000 g for 5 min at 4°C. The pellet was resuspended in 10 ml pre-cooled 0.1 M $CaCl_2$ solution and incubated on ice for 30 min. Then, the cells were centrifuged and the pellet was gently suspended in 2 ml pre-cooled 0.1 M $CaCl_2$/10% glycerol solution. The cell mixture was distributed in 100 µl aliquot in 1.5 ml Eppendorf tubes in liquid nitrogen and stored at -80°C.

6.2.2.3 Transformation in *E. coli*

Pre-prepared competent *E. coli* cells were thawed on ice for ~30 min before the transformation. Then the ligation mixture (50 ng)/plasmid (10-50 ng) was added to thawed cells, gently mixed, and incubated on ice for ~45 min. Eppendorf tubes with cells and plasmids mixture were moved from ice immediately to a 42°C water bath for 90 s, returned to ice immediately and then incubated on ice for ~5 min. Then 1 ml of SOC-media (s. 6.1.7, Table 16) was added to the cells, and incubated at 37°C for

one hour with shaking at 300 rpm. Cells were centrifuged at 600 g for 1 min to remove 800 µl of the supernatant. The pellet was resuspended in the rest culture (~300 µl) and spread on LB plates containing corresponding antibiotics. The resulting colonies were picked after incubation overnight at 37°C.

6.2.2.4 Protein overexpression in *E. coli*: optimizing conditions

The selected colony was firstly pre-cultured in 2.5 ml LB media with applicable antibiotics overnight at 37°C. 1 ml pre-culture was inoculated to 100 ml LB main culture and cultured at 30°C with shaking at 200 rpm. The OD_{600} value was checked every hour till it reached 0.6-0.7. Then IPTG (final concentrations: 0.2 mM or 1 mM) were used to induce the protein overexpression and 2 ml cells were harvested at different time points (e.g. 0h, 1h, 2h, 4h, 6h and 24h) with 4,000 g, 4°C for 5 min. The OD_{600} value was recorded at each point for further calculation of the volume of 1 × SDS buffer that was needed to dissolve the pellet (see below). Cell pellets were frozen in liquid nitrogen and stored at -80°C.

To check the protein expression status of the overexpressed protein in *E. coli*, the cell pellets were thawed for 10 min on ice and suspended with 1 × SDS sample buffer (s. 6.1.3, Table 5) depending on the OD_{600} value. The desired volume of 1 × SDS sample buffer was calculated with the following formula: 20 µl×OD_{600}/0.15. The samples were well mixed and boiled at 80°C for 5 min to denature all proteins. The cell debris was removed by centrifugation at 16,000 g for 10 min and the supernatant was transferred to a new tube and used for further Western blot analysis (s. 6.2.1.5).

6.2.2.5 Large scale protein overexpression in *E. coli*

The selected colony was pre-cultured in 50 ml LB media with applicable antibiotics overnight at 37°C. 30 ml pre-culture was inoculated in 750 ml LB media with shaking of 200 rpm at 30°C. The OD_{600} value was checked every hour till the OD_{600} reached 0.6-0.7. Then IPTG with appropriate concentration (determined in 6.2.2.4) was used to induce the protein overexpression and all cells were harvested at appropriate time point (determined in 6.2.2.4) at 4,000 g, 4°C for 5 min. After centrifugation, the cell

pellets were resuspended in 10 ml 1 × PBS (s. 6.1.3, Table 7) and were transferred to a clean 50 ml Beckman centrifuge tube for another centrifugation at 4,000 g and 4°C for 5 min. The weight of the pellet was recorded after discarding the supernatant. Cell pellets in 50ml tube were frozen in liquid nitrogen and stored at -80°C.

6.2.2.6 Protein extraction in *E. coli* and purification by FPLC

The frozen cell pellet was thawed on ice for 1h and resuspended in 1 ml 1 × *E. coli* lysis buffer mixture (s. 6.1.3, Table 7) per gram pellet and incubated for 30 min at a shaking rate of 50-100 rpm. Then the cells were disrupted by ultrasound on ice with 6 s pulses and 6 s pauses in between for about 8 min. The insoluble material and the cell debris were removed by centrifugation at 16,000 g at 4°C for 20 min. The supernatant with overexpressed soluble protein was transferred to a new tube for further purification. 150 µl supernatant was taken out and mixed with 50 µl 4 × SDS sample buffer (s. 6.1.3, Table 5). The mixture was boiled at 96°C for 3 min and detected by Western blot (s. 6.2.1.5).

For proteins that need to be purified, the overexpressed soluble crude extract was filtered by 0.2 µm filter and then purified by FPLC system using HiTrap^{TM} Talon crude (GE Healthcare Life Sciences) according to the user instruction. The final purification buffer B containing high imidazole (s. 6.1.3, Table 7) was changed to the purification buffer A with low amount of imidazole (s. 6.1.3, Table 7) using centrifugal filters (Merck Millipore). A small portion of the purified protein was denatured for protein concentration determination and the rest was kept at -80°C.

6.2.3 Methods related to *S. cerevisiae*

6.2.3.1 Cell cultivation and strain conservation of *S. cerevisiae*

The cultivation and storage of *S. cerevisiae* cells was performed according to *Yeast Protocols Handbook* (*www.clontech.com*). To rejuvenate of frozen strains (Y2HGold and Y187), a small portion of the frozen stock with glycerol was spread on YPD agar plate (s. 6.1.7, Table 17) and incubated at 30°C for 3-5 days until colonies were about 2 mm in diameter. One single colony was chosen with a sterilized toothpick and evenly mixed in 200 µl ddH_2O. Then the mixture was spread onto a fresh YPDA plate and incubated at 30°C overnight and stored as working stock at 4°C for two months at most. For storage of more than one year, a single colony was transferred into 20 ml YPDA and incubated overnight at 30°C on the shaker at 150 rpm. Then, the yeast cells were spun down at 1,000 g for 3 min and mixed with 50% of YPDA and 50% glycerol (generally 0.5-3 ml). Aliquots were stored at -80°C.

6.2.3.2 Plasmid transformation in *S. cerevisiae*

Plasmid transformation in *S. cerevisiae* was performed based on *Quick and Easy* TRAFO Protocol in yeast (Gietz and Woods, 2002). A yeast strain colony from the stock plate was inoculated into 10 ml of YPDA media and incubated for 16 h at 30°C with shaking at 150 rpm to become vigorous enough for transformation. 2 ml active cells were spun down at 1,000 g for 1 min and the supernatant was removed as much as possible. Generally, the tube was inverted on a dry paper towel for about 1 min after discard the supernatant. 360 µl transformation mixture (including 240 µl 50% (w/v) PEG 3350, 36 µl 1.0 M LiAc, 50 µl 2 mg/ml well mixed boiled single-stranded - carrier DNA (s. 6.1.3, Table 7) and 34 µl water containing 0.1-1 µg plasmid DNA) were added into the yeast pellet and mixed carefully but thoroughly. Then the final mixture was incubated in a water bath at 42°C for 60 min. Cells were separated with transformation solution by centrifuging at 1,000 g for 1 min. The pellet was resuspended with 1.0 ml ddH_2O, 10 µl and 100 µl of which were transferred onto plates of appropriate SD selection media (s. 6.1.7, Table 17). Transformants with plasmids were visible after 3-4 days incubation at 30°C.

6.2.3.3 Protein extraction from yeast (Zhang et al., 2011)

A yeast strain colony was incubated in 2 ml of appropriate SD selection media (s. 6.1.7, Table 17) in test tubes with shaking at 30°C overnight. Cells were harvested prior to stationary phase (OD_{600} = 1.0) by centrifugation at full speed for 3 min at 4°C. Cells were pre-treated with 150 µl 2.0 M LiAc (s. 6.1.3, Table 7) and then 150 µl 0.4 M NaOH for 5 min on ice. Each time cells were centrifuged at full speed of 4°C for 3 min before changing solutions. Cells were resuspended in 100 µl 1 × SDS sample buffer (s. 6.1.3, Table 5) and boiled for 5 min at 95°C. Then, the cell lysate was centrifuged to remove cellular debris. The supernatant containing the whole protein extract of yeast was transferred to a new fresh tube and stored at -80°C. The concentration of the sample was determined by the Neuhoff method (s. 6.2.1.4). The expression level of targeted protein was tested by Western blots (s. 6.2.1.5).

6.2.3.4 Yeast Two Hybrid assay

6.2.3.4.1 Construction and confirmation of fusion genes

Fusion genes were generated easily since same or compatible restriction sites were present at the end of the synthetic genes and the used vectors. The fusion constructs were sequenced (s. Appendix) with T7 sequencing primers (*Matchmaker® Gold Yeast Two-Hybrid System User Manual, www.clontech.com*) to confirm that all fused genes were expressed in-frame consistent with GAL4-AD or GAL4-BD.

6.2.3.4.2 Transformation of vectors

pGADT7 and pGADT7-derived plasmids (AD vectors) were transformed into Y187 strain separately; pGBKT7 and pGBKT7-derived plasmids (BD vectors) were transformed into Y2HGold strain respectively (s. 6.2.3.2). SD-Leu agar plates were used to screen the transformants with pGADT7 and pGADT7-derived vectors, while SD-Trp agar plates were used for screening transformants with pGBKT7 and pGBKT7-derived vectors,. Afterwards, these plates were incubated in a chamber at 30°C for 3-5 days until colonies appeared.

For each transformation, three individual colonies (for three biological replicates) were transferred onto new SD selection agar plates (s. 6.1.7, Table 17) and incubated at 30°C for one day till enough cells were grown for mating. The positive transformants were stored for long-term use (s. 6.2.3.1).

6.2.3.4.3 Mating

The mating step of prey strain (the transformed Y187 strain with different AD vectors) and bait strain (the transformed Y2HGold strain with different BD vectors) allows the mixture of plasmids which confer the diploid yeast strain with the ability to survive on SD-Leu/-Trp media. Proper amount of cells from prey strains and bait strain were transferred with a sterile toothpick into 200 µl YPDA liquid media (s. 6.1.7, Table 17). Then, 5 µl of the prey yeast strain suspension was pipetted onto a fresh YPDA plate and dried under sterile atmosphere. Thereafter, 5 µl of the bait yeast strain suspension was dropped on top of the dried prey strain spot to allow mating. The mating plates were covered with Parafilm and incubated at 30°C for 2-4 days.

6.2.3.4.4 Auto-activation test of bait

The pretest of auto-activation of bait was performed to ensure that the bait (GAL4-BD fusion protein) could not autonomously activate the reporter genes in Y2HGold with no fusing protein in GAL4-AD. If the fused bait protein had auto-activation activity, 3-AT was added to the media lacking histidine to serve as a competitive inhibitor of the HIS3 reporter to reduce the possibility of false positive interaction.

The proper concentration of the 3-AT was optimized by testing the growth of the mating mixture under different concentrations ranging from 1 mM to 15 mM. In general, the final concentration of 3-AT in the media was 1 mM, 3 mM, 5 mM, 7 mM, 9 mM or 15 mM. The suitable concentration of 3-AT in the media was determined after performing the protein-protein interaction test assay (s. 6.2.3.4.5) of BD-fusing proteins and AD empty protein.

6.2.3.4.5 Protein-protein interaction assay

A small fraction of the cell mixture from the YPDA mating plate was transferred onto SD-Leu/-Trp plate and incubated at 30°C for 3-5 days to remove the unmated haploid cells.

For each diploid strain that was used for protein-protein interaction test, it was incubated on SD-Leu/-Trp plates (s. 6.1.7, Table17) for normal growth control, and on plate of SD-His/-Leu/-Trp for normal interaction test and on plate of SD-Ade/-His/-Leu/-Trp for strong interaction test. When there was an auto-activation, 3-AT was added to the test plates.

Firstly, the diploid colonies grown on SD-Leu/-Trp were resuspended in 500 µl 10/1 TE buffer (s. 6.1.3, Table 7) and served as stock solution. The stock solutions were diluted ten times with 10/1 TE buffer and measured at OD_{600}. The OD_{600} of each stock solution was diluted to OD_{600} value of 1.0 with 10/1 TE buffer to 100 µl. Then, the 100 µl mixture was diluted using standard 96-well PCR plate at the ratio of 1:10, 1:100, 1:1,000 and 1:10,000. The cells were pipetted up and down to ensure that they were well mixed for each dilution step. Then, 5 µl of each dilution were dropped onto each of the three plates mentioned above and dried under sterile bench. Cells were incubated at 23°C and 30°C, under light or darkness to explore whether temperature or light had some influence on the interaction. The growth conditiond of the yeast diploid cells were recorded with camera after 9 days.

6.2.4 Methods related to Chlamydomonas

6.2.4.1 Cell cultivation of Chlamydomonas

Strains were pre-cultured and cultured under 12 h light/12 h dark (LD12/12) cycle with a light intensity of 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in TAP media (s. 6.1.7, Table 18), unless otherwise indicated. Cells were harvested at the indicated light/dark time points, where LD0 represents the beginning of the day and LD12 represents the beginning of the night.

6.2.4.2 Counting cells of Chlamydomonas

To know the cell density of Chlamydomonas in the culture, cell counting was performed with a Thoma chamber. In general, a cell mixture of 180 µl cell culture plus 20 µl Lugol's solution (Merck) was prepared. 10 µl of the mixture were dropped into the Thoma chamber with cover slide. The cells of five unconnected medium squares were counted. The cell concentration of the culture was calculated based on the following formula:

$$\text{Cells/ml} = \frac{\text{Cell number in 5 squares}}{5 \times 0.2 \times 0.2 \times 0.1 \times 10^{-3}\ \text{ml}} \times 10/9 = \text{cell number in 5 squares} \times 5/9 \times 10^5$$

6.2.4.3 Transformation of Chlamydomonas with glass beads

50 ml Chlamydomonas cells prior to log phase with a concertation of about 1×10^6 cells/ml were harvested at LD2 with the moderate speed (1,400 g for 3 min). The pellet was resuspended with 10 ml freshly melted autolysin. Then cells were transferred into a Nunc-Flask and shaken under light for 20 min. The suitability for the transformation was checked by mixing 10 µl cells and 10 µl 1% Triton-X100 completely on the coverslip under the microscope. If 80% cells have been lysed after 5 min, the autolysin-treated cells were spun down at 1,400 g for 3 min and re-suspended in 2 ml fresh TAP.

The sterilized glass beads (dimeter of 0.45-0.5 mm) were washed with H_2SO_4, and then with distilled water till the pH value is 7. The washed glass beads were dried

and autoclaved. 300 mg glass beads were filled into the 0.5 ml PCR tubes and autoclaved again before use. The autoclaved glass beads were washed twice with fresh TAP. The transformation mixtures, including 300 µl cells and 0 µg (control), 1 µg or 5 µg DNA, were added to the prepared PCR tube with glass beads, and then vortexed for 15-30 s at top speed on a Fisher Vortex Genie 2 mixer. The supernatant with transformants were transferred into a 50 ml Falcon Tube with 10 ml fresh TAP and shaken for ~18 h under constant light. Then cells were spun down at 1,400 g for 3 min and resuspended gently in 0.5 ml fresh TAP. 250 µl cells were pipetted to selective TAP-agar plates and spread evenly. The plates with transformants were incubated under constant light conditions for 7-10 days till colonies were visible.

6.2.4.4 Cell harvest and crude protein extraction in Chlamydomonas

Vegetative cells were incubated in the pre-cultures for 3 days. Subsequently, a main culture with 1 ml pre-culture was incubated in main culture for another 3 days before harvest. Cells were spun down at 4,000 g for 3 min at 4°C in a 50 ml Falcon tube and transferred to 1.5 ml Eppendorf tube. Then the cells were harvested by centrifugation at 4,000 g for 3 min at 4°C and frozen immediately in liquid nitrogen. The sample could be stored in -80°C for less than two weeks or used for crude protein extraction immediately.

Total protein extraction (comprising soluble, membrane-associated and transmembrane proteins) using boiling procedure was performed according to Schulze et al. (2013). In detail, cells were suspended with 400 µl 2 × SDS sample buffer (s. 6.1.3, Table 5) per 100 mg cell dry weight and incubated at 80°C for 5 min. Then, the cell suspension was vortexed at the highest speed, frozen rapidly in liquid nitrogen, and thawed at 80°C for 5 min. After repeating the mixing and freezing step once again, the total protein extracts were incubated for another 5 min in an 80°C water bath, and the remaining cell debris was spun down at 16,100 g for 20 min.

The crude soluble protein extraction using glass beads procedure was performed according to Zhao et al., (2004). In detail, glass beads (dimeter at 0.25-0.3 mm) were filled to 2/3 in 1.5 ml Eppendorf tubes with security caps and washed with extraction

buffer (without DTT and PIC, s. 6.1.3, Table 5) two times. The frozen cell pellet, as well as 150 to 200 µl (depending on the pellet size) of extraction buffer with DTT and PIC (s. 6.1.3, Table 5) was transferred to the washed glass beads on ice. The glass beads with cells-buffer mixture were strongly vortexed for 1 min for five times with 1 m interval on ice. The mixture with broken cells was separated from the glass beads by centrifugation at a speed of 1,000 g for 10 s and then transferred to a new fresh tube for further centrifugation at 16,100 g for 15 min.

The glass beads method is suitable for breaking the cell wall of vegetative cells, pregametes, gametes and early zygotes but not for late zygotes. In order to break the hard cell wall of the late zygotes for protein extraction, samples were grounded in liquid nitrogen using a mortar and pestle (Aoyama et al., 2014). The cell power was resolved in extraction buffer with DTT and PIC (s. 6.1.3, Table 5) and then centrifuged at 16,100 g for 15 min. Supernatant with soluble protein crude extract (light green-yellow) was transferred into a new fresh 1.5 ml Eppendorf tube and dissolved in 1 × SDS sample buffer (s. 6.1.3, Table 5). The protein crude extract was denatured and negative charged after heat at 96°C for 3 min.

After obtaining the soluble protein, the pellets (containing membrane-associated proteins and transmembrane proteins) were either directly resolved with 2 × SDS-sample buffer or used for subfractions separation. To separate peripheral membrane proteins from transmembrane proteins, the pellets were washed two times with ice-cold 1 × PBS (s. 6.1.3, Table 7). Then the pellet was incubated with 30 ml 0.1 M sodium carbonate buffer (pH 11.8) at 4°C with gentle agitation for 1 h (Fujiki et al., 1982). After ultra-centrifugation at 93,000 g, the remaining pellet containing the transmembrane protein fraction was washed with 1 ml 0.1 M sodium carbonate buffer for 3 times and resolved in 2 × SDS-sample buffer. 30 ml supernatant fraction containing the membrane-associated proteins was precipitated with 7.5 ml 100% (w/v) TCA overnight at 4°C and spin down at 5,000 g for 10 min. The pellets were transferred to 1.5 ml Eppendorf tube and washed three times with 1 ml ice-cold acetone, then dried and resolved directly in 2 × SDS-sample buffer (Schulze et al., 2013).

Protein concentrations were determined by the Neuhoff method (s. 6.2.1.4). The immunoblots (s. 6.2.1.5) for the detection of aCRY, PHOT and Cytochrome f were performed with the same procedure as Beel et al. (2012), except that the dilution for the anti-LOV1 antibodies (for PHOT detection) was 1:2,000 (kindly provided by Peter Hegemann group, Berlin, Germany) and for the anti-Cytochrome f antibodies 1:15,000 (purchased from Agrisera). The protein levels were quantified using ImageJ 1.46r (Wayne Rasband, National Institutes of Health, USA).

6.2.4.5 Generation of pregametes, gametes, early zygotes and late zygotes (Zou et al., submitted)

Vegetative cells were transferred to TAP without NH_4^+ (TAP-N) at the end of the light period (LD12) and kept for 14 h in darkness to induce pregametes. Afterwards the pregametes were exposed to white light for 6 h for the transition to gametes. Then mature gametes of opposite mating type were mixed to allow mating for 4 h without stirring and zygote pellicles (early zygotes) were formed in the media and transferred to the plates under a flow of sterile air. In order to obtain mature zygotes, early zygotes were incubated for 16 to 18 h under light and then put in the dark for another 5 days. The harvest of zygotes samples were performed based on Wegener et al. (1989) with little modification. The mature zygotes on the plates were exposed to chloroform vapors for 90 s to kill unmated cells. The zygote pellicles were scraped off the plates with a razor blade and suspended in 4 mL TAP. To further destroy unmated cells, two times of 30 s continuous sonication were performed at a power of 25 to 30 W using a Sonicator (Bioblock Scientific-Vibra Cell^{TM} 72405) equipped with a microtip. After sonication, the volume was adjusted to 20 mL with TAP media and centrifuge at 1,500 g for 5 min. The zygote pellet was resuspended in 20 mL TAP media and this washing procedure was repeated two times (Wegener et al., 1989). Then the pure zygotes were harvested immediately, after 6 h, 12h and 24h white light illumination.

6.2.4.6 Mating ability, mating maintenance ability and phototactic behavior test (Zou et al., submitted)

Liquid cultures of vegetative cells (cell density 0.5-1×10^7 cells/ml) were centrifuged (2,000 g for 3 min) and resuspended in nitrogen-free TAP media at LD12. Cells of the tester strain mt^+ and the mating partner strain mt^- were treated differentially. Vegetative cells of the tester strain were incubated in culture flasks (Nunc®) with shaking at 70 rpm/min for 15 h in the dark to generate pregametes, while the vegetative cells of the mt^- partner strain were incubated for 12 h in darkness, followed by a 3 h light treatment (60 µmol·m^{-2}·s^{-1}) to induce the formation of gametes. Pregametes of the tester strains were put under white light with a fluence of 60 µmol·m^{-2}·s^{-1} for 1 h (called G1 cells) to induce the mating ability. The mating ability was tested by mixing the tester strain with the mating partner, which was added in approximately 2-fold excess to the tester strain. Then the mixture was treated under dark for 1 h to allow mating (Beck and Acker, 1992). The mating efficiency was determined by recording the quadriflagellated and biflagellated cells under the microscope with phase-contrast after fixation with 0.2% glutaraldehyde and calculating according to Beck and Acker (1992).

To test the mating maintenance of the strains, G1 cells were put into a dark box for hour to deactivate their mating ability. The mating ability dark-inactivated gametes was also examined as described.

G1 cells were also used for phototactic behavior tests. For this purpose, the gametes were put into a dark place. Light was introduced from one side with an intensity of 30 µmol·m^{-2}·s^{-1} and pictures were taken from above.

6.2.4.7 Germination assay (Zou et al., submitted)

For the germination assay, vegetative cells of each mating type were separately cultivated on solid TAP media for three days under LD12/12 and subsequently transferred to solid N10 media (Jiang and Stern, 2009) having only 1/10 of the normal amount of nitrogen of TAP. They were kept on N10 for additional three days under

LD12/12 to induce gametogenesis. Then, cells were suspended in mating buffer (0.6 mM $MgCl_2$, 1.2 mM HEPES, pH 6.8) with a cell density of approx. 1×10^7 cells/ml and shaken at 70 rpm/min for three hours in the light to obtain motile individual gametes (Suzuki and Johnson, 2002). Cells of two different mating types were mixed and then incubated for one hour in the light to allow mating. The mating mixture was applied to 3% Difco agar TAP plates and dried under a sterile bench. The dried plates were incubated under LD12/12 for three days. To analyze the germination rate, the unmated vegetative cells were scratched from the top of the plates with a razor blade, and then the agar blocks with the attached three-day-old zygotes were cut and transferred to 1.5% Difco agar TAP plates. Zygotes were distributed separately with a glass needle under a binocular microscope and treated for 30s with chloroform to kill the unmated gametes surrounding the zygotes. Then plates with zygotes were returned to LD12/12 conditions. Germinated zygotes were counted as positive depending on whether one or more spores from a tetrad could form colonies. The germination rate reached a plateau level usually until the tenth day after mating. Used light intensities were 30 $\mu mol\cdot m^{-2}\cdot s^{-1}$ for white light, 30 $\mu mol\cdot m^{-2}\cdot s^{-1}$ for blue light (SuperFlux LED from Lumitronix LED-Technik, peak at 465 nm, FWHM of 18.5 nm) and 41 $\mu mol\cdot m^{-2}\cdot s^{-1}$ for red light (SuperFlux LED from Lumitronix LED-Technik, peak at 635 nm, FWHM of 17 nm).

6.2.4.8 Mating and tetrad separation of Chlamydomonas

The desired mating type plus and minus strains were firstly pre-cultured on fresh TAP plates for 3 days at 23°C under constant light or LD12/12 for the population enlargement. To induce gametes, vegetative cells of both mating types were transferred to N10-plate for 3 days at 23°C under LD12/12.

Proper amount of cells from the N10 plates were suspended in 3 ml mating buffer (s. 6.1.3, Table 7) with cell density of about $1\text{-}5\times10^6$ cells/ml in Nunc flasks without clumps. If the cell density is too high, the formed zygotes would be strongly connected and not easy to be separated into individuals. The cells were kept on a shaker with 70 rpm directly under light for 3 hours. Pre-test of mating were performed

by mixing 10 µl mt^- cells and 10 µl mt^+ cells on a slide and the aggregation was checked under the microscope. If no aggregation was observable, the cells of both mating types could be kept on the shake for longer time before mating. If there were enough cells that aggregated to form a clump under the microscope, mating was performed by mixing the two opposite mating type strains and allowed to proceed for 1 h under light without shaking. 300 µl of mating mixture was pipetted onto a 3%-Difco-Agar-plate (3 or 4 drops per plate) and dried under sterile laminar box. The dried plates were sealed with parafilm and put under light for 18 h to mature the zygotes. Then, the plates were wrapped separately with foil and stored for 5-7 days in darkness.

A raster on the 1.5% Difco-Agar-plate was prepared for the distribution of zygotes and formed tetrads. Unmated vegetative cells were scraped from the top of the cell spot with a curved cutting edged scalpel, since zygospores stick close to 3% Difco-Agar while vegetative cells do not. Several pyramidal agar blocks with zygotes were cut out using the scalpel with flat cutting edge and transferred to the 1.5% Difco-Agar-plate invertedly. Under the microscope, zygospores are large yellow cells with a black cell wall. The zygotes were distributed on the crossing points of the raster with the glass needle and exposed to a glass dish containing a thin (1-2 mm) layer of chloroform to kill the surrounding vegetative cells that have been accidentally transferred from the 3% Difco-Agar-plate. The plates with well-arranged zygotes were kept for 16-20 hours under very low light for germination. The germinated zygote could be divided into four or eight tetrads by touching the cell wall of the zygote using a glass needle. The tetrads were distributed along the designed lines and grown under constant light conditions until visible colonies can be picked for subsequent analysis. The left plates were monitored daily for contamination.

6.2.4.9 Pull-down experiments using purified overexpressed protein.

The soluble crude protein extract from vegetative cells or pregametes were prepared using glass beads method (Zhao et al., 2004). 300 ml frozen cells harvested in log phase were broken using glass beads method with cold pull-down buffer with PIC (s.

6.1.3, Table 7). The insoluble fraction was removed by centrifugation for 30 min at 4°C with a speed of 16,000 g. The supernatant was collected for pull-down assay.

For pull-down assay, 50 µl magnetic Dynabeads solution (s. 6.1.4, Table 12) was used for one test. Firstly, the Dynabeads was washed with 300 µl Binding/wash buffer (s. 6.1.3, Table 7) for three times. According to manufacturer's instructions, 50 µl magnetic Dynabeads has the ability to combine 80 µg histidine-tagged overexpressed protein of 28 kDa. As a result, the amount of the purified proteins that were suitable for 50 µl magnetic Dynabeads was calculated using the following formula according to user's instruction:

Purified proteins (µg) = (the molecular mass of the purified protein × 80)/28

The volume of the purified proteins was adjusted to 700 µl with Binding/wash buffer when the purified proteins were applied to Dynabeads. Afterwards, the purified proteins and Dynabeads were rotated for 10 min at 4°C to allow binding. The unbound proteins in the supernatant were named FT1 (flow though 1). The Dynabeads were washed two times with 300 µl binding/wash buffer and the supernatant after washing was named W1 and W2. The Dynabeads were then washed two times with 300 µl Wash buffer (s. 6.1.3, Table 7) and the supernatant after washing was named W3 and W4. 700 µl crude proteins extract from Chlamydomonas was added to the Dynabeads and rotated for 1h at 4°C to allow the possible interaction to occur. The fraction of proteins from Chlamydomonas that were not attached to the beads were named FT2. The Dynabeads were then washed four times with 300 µl Wash buffer and the supernatant after washing was named W5, W6, W7 and W8. To finally elute all the proteins attached to the beads, 120 µl Elution buffer (s. 6.1.3, Table 7) was applied to the Dynabeads and they were rotated for 10 min at 4°C. The Dynabeads were attracted to one side and the supernatant was collected, denatured with SDS and checked by immunoblot (s. 6.2.1.5).

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Appendix

1. Sequence of vector pKelch_F-box; the synthetic codon adapted coding sequence for Kelch_F-box is underlined with grey background, while the start codon is in bold.

CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAA
AATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGGCCGCTACAGGGCGCTCCCATTCGCCATTCAGGCTGCGCAACT
GTTGGGAAGGGCGTTTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGG
TAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGACGTAATACGACTCACTATAGGGCGAATTGGC
GGAAGGCCGTCAAGGCCGCATCC**ATG**GCCAGCGATAATGCACCGGCAGGTCCGCTGCTGAGCGAAGAAAGCAGCGCATGTCTGCT
GGATCGTCTGGATAGCAATATTCTGGTTAGCATTCTGAGCTTTCTGACACCGCGTGAAGTTGTTACCGCAAGCCTGACCTGTAAACA
GCTGGCAGCAGCAGCCGAAGCACCGGTTCTGTGGCGTCAGCTGTATGATAGCGCACTGTGTCCGCGTGTTAAAGAACGTCATCTGC
CGCATCCGAGCCAGATTAGCGATTGGCGTGGTGTTGTTGTTCGTGGTCTGCTGCTGCGTGAACTGCCGCATGGTGCATGGGAACGT
CTGCAGCCGGAAGGTAATCGTCAGCTGCGTCCGTGTCCTCGTGAAGGTCATGCAGCAAGCAGCTGGGGTCGTGATAGCATGATTCT
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AGCAGCGGCAGCCGCAGCTGAACCGCCTAGTGCACGTCCGGCAGGCCGTGATGAAGAACGTCGTGCAGGTCCGTATCATCATCAC
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TGTTCGTTATGGTCATAGCGCAACCCGTTGTGGTCCGGATGGTGGCTGGCTGGCAGTTTATGGTGGTATGCAGGCAGGCGGTTATG
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TCTGTTTGTTTTTGGTGGCATTGCACGTCGTGGTAGCACCACCCGTCTGAGCGTTATTGATCTGCGTACCCTGACCGTTGATCGTCC
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CCGGCAAATGAACCGCATGATCGTAATGCAATGGGTCGTTGTCATGCAAGCGTTCTGCTGGGTAGCAAACTGCTGATGTTCGGTGG
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CGGGTTCAGGTGGTAGTGCAGAAGGCGAAGAGGCAGCAGATGAACTCGAGCTGGGCCTCATGGGCCTTCCGCTCACTGCCCGCTT
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2. Sequence of vector pROC114; the synthetic codon adapted coding sequence for ROC114 is underlined with grey background, while the start codon is in bold.

CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAA
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GGAAGGCCGTCAAGGCCTAGGCGCGCCATGAGCTCCC**ATG**GGCAGCACCCGTGGTAGCAAACGTGGTGCAGCAAGCGCACAGCA
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CGTGACCATGCTGAGCAGCCCAGCAGCGGGTGTTCGTCCGTGTTGGGCAATGCTGAATGTTATTCTGGAAGGTCTGCTGGCAGCC
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TACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAAC
AAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC

3. The sequences alignment of plasmids and its sequenced parts. The restriction enzyme cutting sites are indicated.

pYZ5:

```
pYZ5-sequencing      ----------NNNNNNNNNNNNNNNNNNNNGAGATACCCCACCAAACCCAAAAAAAGAG
pYZ5                 GATGATGTATATAACTATCTATTCGATGATGAAGATACCCCACCAAACCCAAAAAAAGAG
                                                   ****************************
                                                    NcoI
pYZ5-sequencing      ATCTTTAATACGACTCACTATAGGGCGAGCGCCGCCATGGCCAGCGATAATGCACCGGCA
pYZ5                 ATCTTTAATACGACTCACTATAGGGCGAGCGCCGCCATGGCCAGCGATAATGCACCGGCA
                     ************************************************************

pYZ5-sequencing      GGTCCGCTGCTGAGCGAAGAAAGCAGCGCATGTCTGCTGGATCGTCTGGATAGCAATATT
pYZ5                 GGTCCGCTGCTGAGCGAAGAAAGCAGCGCATGTCTGCTGGATCGTCTGGATAGCAATATT
                     ************************************************************

pYZ5-sequencing      CTGGTTAGCATTCTGAGCTTTCTGACACCGCGTGAAGTTGTTACCGCAAGCCTGACCTGT
pYZ5                 CTGGTTAGCATTCTGAGCTTTCTGACACCGCGTGAAGTTGTTACCGCAAGCCTGACCTGT
                     ************************************************************

pYZ5-sequencing      AAACAGCTGGCAGCAGCAGCCGAAGCACCGGTTCTGTGGCGTCAGCTGTATGATAGCGCA
pYZ5                 AAACAGCTGGCAGCAGCAGCCGAAGCACCGGTTCTGTGGCGTCAGCTGTATGATAGCGCA
                     ************************************************************

pYZ5-sequencing      CTGTGTCCGCGTGTTAAAGAACGTCATCTGCCGCATCCGAGCCAGATTAGCGATTGGCGT
pYZ5                 CTGTGTCCGCGTGTTAAAGAACGTCATCTGCCGCATCCGAGCCAGATTAGCGATTGGCGT
                     ************************************************************

pYZ5-sequencing      GGTGTTGTTGTTCGTGGTCTGCTGCTGCGTGAACTGCCGCATGGTGCATGGGAACGTCTG
pYZ5                 GGTGTTGTTGTTCGTGGTCTGCTGCTGCGTGAACTGCCGCATGGTGCATGGGAACGTCTG
                     ************************************************************

pYZ5-sequencing      CAGCCGGAAGGTAATCGTCAGCTGCGTCCGTGTCCTCGTGAAGGTCATGCAGCAAGCAGC
pYZ5                 CAGCCGGAAGGTAATCGTCAGCTGCGTCCGTGTCCTCGTGAAGGTCATGCAGCAAGCAGC
                     ************************************************************
```

pYZ6:

```
pYZ6-sequencing      NNNNNNNNNNNGNNTGTATCGCCGGA-TTTGNNN-ACGACTCACTATAGGGCGAGCCGCC
pYZ6                 CAAAGACAGTTGACTGTATCGCCGGAATTTGTAATACGACTCACTATAGGGCGAGCCGCC
                               *  ************ ****    **************************
                                                                    NcoI
pYZ6-sequencing      ATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGCATATGGCCATGGCCAGCGAT
pYZ6                 ATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGCATATGGCCATGGCCAGCGAT
                     ************************************************************

pYZ6-sequencing      AATGCACCGGCAGGTCCGCTGCTGAGCGAAGAAAGCAGCGCATGTCTGCTGGATCGTCTG
pYZ6                 AATGCACCGGCAGGTCCGCTGCTGAGCGAAGAAAGCAGCGCATGTCTGCTGGATCGTCTG
                     ************************************************************

pYZ6-sequencing      GATAGCAATATTCTGGTTAGCATTCTGAGCTTTCTGACACCGCGTGAAGTTGTTACCGCA
pYZ6                 GATAGCAATATTCTGGTTAGCATTCTGAGCTTTCTGACACCGCGTGAAGTTGTTACCGCA
                     ************************************************************

pYZ6-sequencing      AGCCTGACCTGTAAACAGCTGGCAGCAGCAGCCGAAGCACCGGTTCTGTGGCGTCAGCTG
pYZ6                 AGCCTGACCTGTAAACAGCTGGCAGCAGCAGCCGAAGCACCGGTTCTGTGGCGTCAGCTG
                     ************************************************************

pYZ6-sequencing      TATGATAGCGCACTGTGTCCGCGTGTTAAAGAACGTCATCTGCCGCATCCGAGCCAGATT
pYZ6                 TATGATAGCGCACTGTGTCCGCGTGTTAAAGAACGTCATCTGCCGCATCCGAGCCAGATT
                     ************************************************************
```

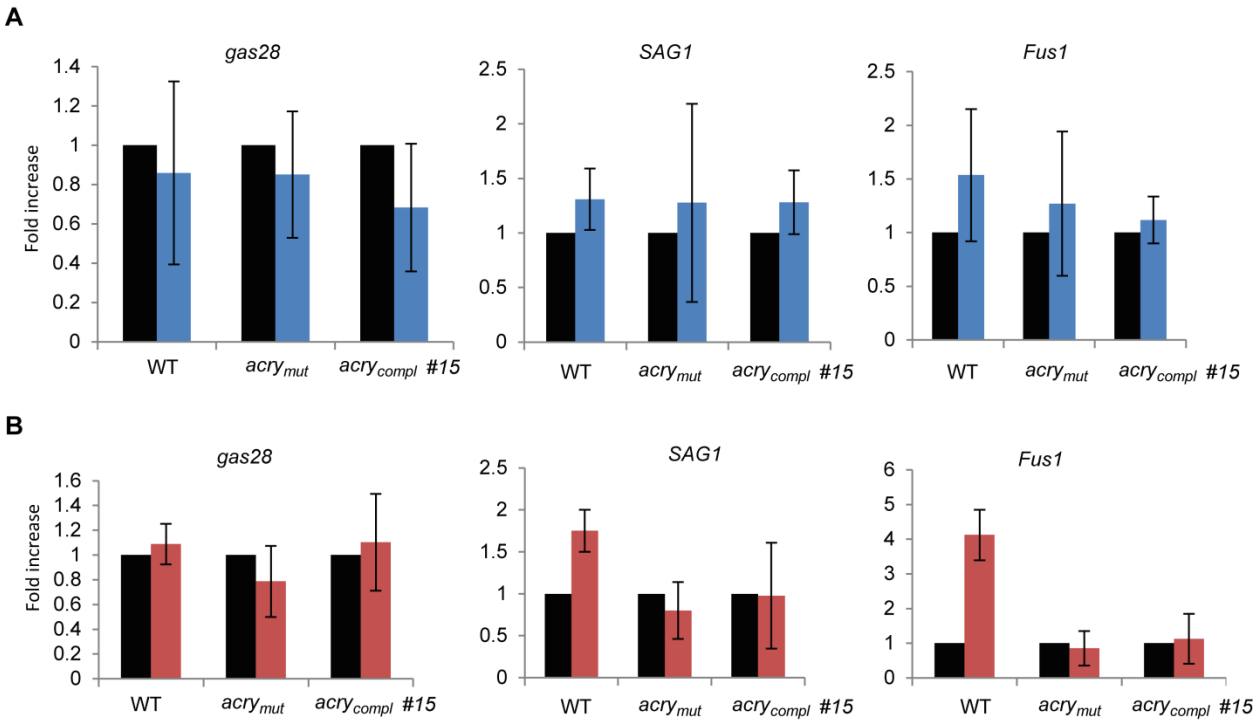
pYZ7:

```
pYZ7-sequencing      NNNNNNNNNGNNGANACCCCNCCAAACCCAAAAAAAGAGATCTTTAATACGACTCACTAT
pYZ7                 TTCGATGATGAAGATACCCCACCAAACCCAAAAAAAGAGATCTTTAATACGACTCACTAT
                           *  ** ***** ****************************************
                                 NcoI
pYZ7-sequencing      AGGGCGAGCGCCGCCATGGGCAGCACCCGTGGTAGCAAACGTGGTGCAGCAAGCGCACAG
pYZ7                 AGGGCGAGCGCCGCCATGGGCAGCACCCGTGGTAGCAAACGTGGTGCAGCAAGCGCACAG
                     ************************************************************

pYZ7-sequencing      CAGCAGCAACCGAGCAAACGTCTGCGTACCGCACCGCATGAAGATGTTAGCAGCAGCGGT
pYZ7                 CAGCAGCAACCGAGCAAACGTCTGCGTACCGCACCGCATGAAGATGTTAGCAGCAGCGGT
                     ************************************************************

pYZ7-sequencing      GATACCGATCTGCTGCAGATGCTGCCTCCGGAACTGCTGCTGGTTGTTGTTGGTAATCTG
pYZ7                 GATACCGATCTGCTGCAGATGCTGCCTCCGGAACTGCTGCTGGTTGTTGTTGGTAATCTG
                     ************************************************************

pYZ7-sequencing      ACACCGCGTGAATGTCAGCCGCTGCGTCTGACCTGTAAAGGTCTGCGTGATGTTGTTGAT
pYZ7                 ACACCGCGTGAATGTCAGCCGCTGCGTCTGACCTGTAAAGGTCTGCGTGATGTTGTTGAT
                     ************************************************************

pYZ7-sequencing      AGCGCAGTTGCAGATACCCTGACCGTTACCAATGATATTGCACAGTATCTGGTTCTGGAA
pYZ7                 AGCGCAGTTGCAGATACCCTGACCGTTACCAATGATATTGCACAGTATCTGGTTCTGGAA
                     ************************************************************
```

pYZ8:


pYZ8-sequencing NNNNNNNNNNNNNANNNATCGCCGGA-TTTGTAATACGACTCACTATAGGGCGAGCCGCC
pYZ8 CAAAGACAGTTGACTGTATCGCCGGAATTTGTAATACGACTCACTATAGGGCGAGCCGCC
NcoI
pYZ8-sequencing ATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGCATATGGCCATGGGCAGCACC
pYZ8 ATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGCATATGGCCATGGGCAGCACC
pYZ8-sequencing CGTGGTAGCAAACGTGGTGCAGCAAGCGCACAGCAGCAGCAACCGAGCAAACGTCTGCGT
pYZ8 CGTGGTAGCAAACGTGGTGCAGCAAGCGCACAGCAGCAGCAACCGAGCAAACGTCTGCGT
pYZ8-sequencing ACCGCACCGCATGAAGATGTTAGCAGCAGCGGTGATACCGATCTGCTGCAGATGCTGCCT
pYZ8 ACCGCACCGCATGAAGATGTTAGCAGCAGCGGTGATACCGATCTGCTGCAGATGCTGCCT
pYZ8-sequencing CCGGAACTGCTGCTGGTTGTTGTTGGTAATCTGACACCGCGTGAATGTCAGCCGCTGCGT
pYZ8 CCGGAACTGCTGCTGGTTGTTGTTGGTAATCTGACACCGCGTGAATGTCAGCCGCTGCGT
pYZ8-sequencing CTGACCTGTAAAGGTCTGCGTGATGTTGTTGATAGCGCAGTTGCAGATACCCTGACCGTT
pYZ8 CTGACCTGTAAAGGTCTGCGTGATGTTGTTGATAGCGCAGTTGCAGATACCCTGACCGTT
pYZ8-sequencing ACCAATGATATTGCACAGTATCTGGTTCTGGAACAGAGCAAACAGTTTGAACAGGGTGGT
pYZ8 ACCAATGATATTGCACAGTATCTGGTTCTGGAACAGAGCAAACAGTTTGAACAGGGTGGT
pYZ8-sequencing GCAAGCGTTAGCAATGGTTGGGTTGCACGTTTTCTGGCAAAATTTCCGCGTATCAAAAAA
pYZ8 GCAAGCGTTAGCAATGGTTGGGTTGCACGTTTTCTGGCAAAATTTCCGCGTATCAAAAAA
pYZ8-sequencing CTGGATGTTAAATGTAGCCAGAGCGTTCTGACCCTGTTTGCACGTCATATGGTTCGTGAT
pYZ8 CTGGATGTTAAATGTAGCCAGAGCGTTCTGACCCTGTTTGCACGTCATATGGTTCGTGAT

4. qPCR analysis of mating specific genes and mating type specific genes in pregametes and gametes after 6 h illumination.



The black bar represents the expression level of each gene in pregametes, while the blue or red bar represents the expression level under blue or red light. The expression level of each gene in pregametes was set as 1; the relative expression level in gametes was calculated based on pregametes.

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Abbreviations

8-HDF	8-hydroxy-7, 8-didemethyl-5-deazariboflavin
aa	amino acid
aCRY	animal-like cryptochrome
$acry_{compl}$	*acry* complemented strain
$acry_{mut}$	*acry* mutant strain
AD	activating domain
AtCRY	CRY in *A. thaliana*
ATP	adenosine triphosphate
BD	binding domain
BLAST	basic local alignment search tool
bp	base pair
Brwd3	bromodomain and Trp-Asp repeat-containing protein 3
cAMP	cyclic adenosine monophosphate
CCE	cryptochrome C-terminal extension
CDT2	cell division cycle protein
ChR	channelrhodopsin
COP1	constitutive photomorphogenic 1
CPD	cyclobutane pyrimidine dimer
CPF1	cryptochrome/photolyase family 1
CPH1	Chlamydomonas photolyase homologue 1
CRL4	cullin-RING E3 ubiquitin ligase
CRY	cryptochrome
CryB	one cryptochrome in bacterium *Rhodobacter sphaeroides*
CRYp	a plant like cryptochrome in diatom *Phaeodactylum tricornutum*
CUL1	cullin 1

Abbreviations

Da	dalton
DASH	*Drosophila*, *Arabidopsis*, *Synechocystis* and *Homo*
dCRY	*Drosophila* CRY
ddH_2O	double distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DYRK	dual-specificity tyrosine phosphorylation-regulated kinase
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tags
FAD	flavin adenin dinucleotide
FBXL	F-box and leucine rich repeat protein
FeS-BCPs	Fe-S bacterial cryptocchromes and photolyases
FKF	flavin-binding Kelch repeat F-box protein
FPLC	fast protein liquid chromatography
g	gravity
GAS	gametes specific gene
GSA	glutamate-1-semialdehyde aminotransferase
GSK-3β	glycogen synthase kinase 3β
HECT	homologous to the E6-associated-protein carboxyl terminus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HKR	histidine kinase rhodopsin
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	lysogeny broth
Lhc	light-harvesting protein
LKP	light, oxygen and voltage Kelch protein
LOV	light, oxygen and voltage

mCRY	mouse cryptochrome
MTHF	methenyltetrahydrofolate
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pCRY	plant cryptochrome
$pcry_{compl}$	*pcry* complemented strain
$pcry_{mut}$	*pcry* mutant strain
PDS	phytoene desaturase
PEG	polyethylene glycol
PER	period
PHOT	phototropin
PHR	photolyase homologous region
PHR2	photolyase/blue receptor 2
PHY	phytochrome
PIC	protease inhibitor cocktails
PPase	λ protein phosphatase
P_r and P_{fr}	phytochrome red and far-red state
PVDF	polyvinylidene fluoride
RING	really interesting new gene
RNA	ribonucleic acid
ROC	rhythm of chloroplast
rpm	revolutions per min
RT-qPCR	quantitative reverse transcription PCR
SCF	Skp-Cullin-F-box

SD	synthetic defined medium
SDS	sodium dodecyl sulfate
SPA	suppressor of phytochrome A-105
TAE	tris-acetate-EDTA
TAP	tris-acetate-phosphate
TBS	tris-buffered saline
TEMED	tetramethylethylenediamine
TIM	timeless
UV	ultraviolet
UV-A	ultraviolet A
UV-B	ultraviolet B
UVR8	UV resistance locus 8
V	voltage
v/v	volume/volume
w/v	weight/volume
YPD	yeast extract peptone dextrose
YPDA	yeast extract peptone dextrose with adenine-hemisulfate-salt
ZTL	zeitlupe

Curriculum Vitae

PERSONAL DATA

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EDUCATION

2012-2016 **PhD candidate, Friedrich-Schiller-University Jena, Germany**

I pursue my doctor degree in the lab of Prof. Maria Mittag group. We characterize the role of cryptochromes in the sexual life cycle of *Chlamydomonas* based on the mutants generated via insertional mutagenesis.

2009-2012 **Master Degree of Cell Biology in Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China** (Final score: 83.73/100)

I spend three years in evolutionary research of green algae and the characterization of role of FBPase II in *Chlamydomonas*. During this time period, I also learnt Perl programming.

2005-2009 **Bachelor Degree of Biotechnology in Shandong Normal University, Jinan, China** (Final score: 81.39/100)

I completed 4 semesters of general biology and 4 semesters of biotechnology.

PUBLICATIONS

Ting Ni, Jipei Yue, Guiling Sun, **Yong Zou**, Jianfan Wen, Jinling Huang, 2012. Ancient Gene Transfer from Algae to Animals: Mechanisms and Evolutionary Significance. BMC Evol. Biol. 2012, 12:83

Nico Müller, Sandra Wenzel, **Yong Zou**, Sandra Künzel, Severin Sasso, Daniel Weiß, Katja Prager, Arthur Grossman, Tilman Kottke, and Maria Mittag, 2016. Plant Cryptochrome Controls Key Features of the Circadian Clock and the Developmental Cycle in *Chlamydomonas reinhardtii*, in preparation for resubmission.

Yong Zou, Sandra Wenzel, Nico Müller, Katja Prager, Tilman Kottke and Maria Mittag, 2016. The Animal-like Cryptochrome of *Chlamydomonas* Influences Its Sexual Cycle and is Part of a Photoreceptor Network, submitted.

POSTER PRESENTATION

Mar. 31-Apr. 03, 2014	MiCom 2014 (title: The Role of Photoreceptors in Photoperiod Control in *Chlamydomonas reinhardtii*)
Mar. 25-28, 2015	Meeting DFG research group FG 1261 on "Specific Light-Driven Reactions in Unicellular Model Algae" (title: The involvement of an animal-like and a plant cryptochrome in the life cycle of *Chlamydomonas reinhardtii*, with Nico Müller and Sandra Wenzel)

ORAL PRESENTATION

Oct. 15-16, 2013	JSMC symposium (title: The Role of Photoreceptors in Photoperiod Control in *Chlamydomonas reinhardtii*)
Sep. 02-03, 2014	JSMC symposium (title: The Role of Animal-like Cryptochrome in Sexual Life Cycle in *Chlamydomonas reinhardtii*)
Oct. 16-18, 2014	Meeting DFG research group FG 1261 on "Specific Light-Driven Reactions in Unicellular Model Algae" (title: The Role of Animal-like Cryptochrome in Sexual Life Cycle in *Chlamydomonas reinhardtii*)
Feb. 20-21, 2015	Süd-ostdeutsches Pflanzenphysiologentreffen (title: The involvement of aCRY and pCRY in the life cycle of *Chlamydomonas reinhardtii*, with Nico Müller)
Apr. 13-16, 2015	MiCom 2015 (title: The role of an animal-like cryptochrome in the sexual life cycle of the unicellular green alga *Chlamydomonas reinhardtii*)
Aug. 23-29, 2015	6th European Phycological Congress (EPC6) in London (title: The role of an animal-like cryptochrome in the life cycle of the unicellular green alga *Chlamydomonas reinhardtii*)

Eigenständigkeitserklärung

Ich erkläre hiermit, dass die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena mir bekannt ist. Diese vorliegende Dissertation habe ich selbst angefertigt und keine anderen als die angegebenen Hilfsmittel, Mitteilungen oder Quellen benutzt.

Bei der Auswahl und Auswertung des Materials haben mich die in der Danksagung meiner Dissertation genannten Personen unterstützt. Personen, die bei der Anfertigung wissenschaftlicher Daten beteiligt waren, sind auch in der Arbeit angegeben.

Ich erkläre, dass ich keine Hilfe eines Promotionsberaters in Anspruch genommen habe und dass Dritte weder unmittelbar, noch mittelbar geldwerte Leistungen von mir für Arbeiten genommen haben, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

Weiterhin erkläre ich, dass ich die Dissertation nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe. Ferner habe ich nicht versucht, diese Doktorarbeit oder eine andere Abhandlung dieser Arbeit bei einer anderen Hochschule als Dissertation einzureichen.

Jena, den

Yong Zou

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