Modes of action of cryptochrome 2 from *Arabidopsis thaliana*

Zur
Erlangung des Doktorgrades
Der Naturwissenschaften
(Dr. rer. Nat.)

dem Fachbereich Biologie
der Philipps-Universität Marburg
vorgelegt von

Rafael Muñoz Viana
aus Oviedo/Spanien

Marburg/Lahn im Juni 2007

Die Untersuchungen zur vorliegenden Arbeit wurden von Januar 2004 bis januar 2007 am pflanzenphysiologie unter der Leitung von Herrn PD Dr. Alfred Batschauer durchgeführt.

Von Fachbereich Biologie der Philipps-Universitäat Marburg als Dissertation angenommen am:

Erstgutachter: PD Dr. Alfred Batschauer
Zweitgutachter: Prof Dr. Paul Galland

Tag der mündlichen Prüfung am:

Erklärung

Ich versichere, dass ich meine Dissertation

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Selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

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(Rafael Muñoz Viana)

CURRICULUM VITAE

PERSONAL DETAILS:

Name: Rafael Muñoz Viana
Place of birth: Las Palmas de Gran Canaria, Spain
Date of birth: March 31st, 1977

ACADEMIC DATA:

1992-1995:	Bachiller at Auseva High school, Oviedo
1995/2000:	First cycle of chemistry at the University of Oviedo.
2000/2002:	Second cycle of biochemistry and molecular biology at the University of Oviedo.
2002:	Diploma thesis at the department of biomedical sciences, University of Antwerp (Belgium) as an Erasmus student (project title: "Effect of brefeldin A on the cholera toxin induced cAMP accumulation in Caco2 cells").
2003-2004:	PhD at Helle. D. Ulrich laboratory, Max Plank Institute for Terrestrial Microbiology Marburg, Germany (thesis title: "Role of PCNA in the postreplicative DNA repair".)
2004-2006:	PhD at Alfred Batschauer laboratory, department of plant physiology Philipps university Marburg, Germany (tesis title: "Modes of action of cryptochrome 2 from *Arabidopsis thaliana*").

LANGUAGES:

Spanish: Mother tongue, English: Advanced, German: Basic.

PUBLICATIONS:

Banerjee R., Schleicher E., Meier S., Viana R.M., Pokorny R., Ahmad M., Bittl R., Batschauer A. (2007) The signaling state of Arabidopsis cryptochrome 2 contains flavin semiquinone. Journal of Biological Chemistry 282:14916-14922.

Rosenfeldt G., Viana R.M., Mootz H.D., von Arnim A.G., Batschauer A. (2007) Chemically induced and light-independent cryptochrome photoreceptor activation. Molecular Plant (Accepted).

CONGRESSES:

Annual conference of EU RTN group "checkpoint and cancer" Erasmus medical center. Rotterdam, September 15 2003

Ein Teil der während der Promotion erzielten Ergebnisse wurde in folgender Originalpublikation veröffentlicht:

Banerjee R., Schleicher E., Meier S., Viana R.M., Pokorny R., Ahmad M., Bittl R., Batschauer A. (2007) The signaling state of Arabidopsis cryptochrome 2 contains flavin semiquinone. Journal of Biological Chemistry 282:14916-14922.

Rosenfeldt G., Viana R.M., Mootz H.D., von Arnim A.G., Batschauer A. (2007) Chemically induced and light-independent cryptochrome photoreceptor activation. Molecular Plant (Accepted).

ACKNOWLEDGEMENTS

First and most of all I would like to express my gratitude to PD Dr. Alfred Batschauer for trusting and allowing me to work under his supervision, for supporting my research, for his availability for discussion, for advising my work, and for his never-ending patience during these past years.

Hence, I would like to thank Oxana Panajotow and Agnes Debelius for their professionalism and skill-full technical assistance.

Furthermore, I would like to thank each member of the Alfred Batchauer working group for constructive help, stimulating discussions and good working atmosphere. Special thanks go to Dr. Richard Pokorny for the great time working together and for his friendship. I would like also to thank Gebhard A. Kaiser for his technical support.

I am also very grateful to Dr. Israel Fernández López, Carola Rößer, Julia Sommer, Evelyn Vollmeister Dr. Kenneth Berendzen and Dr. Miroslav Vranes for the help and advice correcting some chapters of this thesis.

Finally I would also like to thank my family for their constant and no matter-what support during all these years abroad.

Dedicated to my family

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

8-HDF	8-Hydroxy-5-deazaflavin
Amp	Ampicillin
B	Blue
bp	Base pair
Car	Carbenicillin
CCT1	Cryptochrome 1 C-terminus
CCT2	Cryptochrome 2 C-terminus
cDNA	Coding DNA
CHS	Chalcone synthase
CNT1	Cryptochrome 1 N-terminus
CNT2	Cryptochrome 2 N-terminus
CO	Constans
COP1	Constitutively photomorphogenic 1
CPD	Cyclobutane--Pyrimidin-Dimer
Cry	Cryptochrome
DNA	Desoxyribonucleic acid
dNTP	2´-desoxynucleotid-5´-triphosphate
DTT	Dithiothreithol
E .coli	*Escherichia coli*
EDTA	Ethylendiamintetraaceticacid
FAD	Flavine adenine dinucleotide
FKF1	Flavin-binding Kelch repeat F-box
FMN	Flavin mononucleotide
FR	Far-red
FRET	Fluorescence resonance energy transfer
FT	Flowering locus T
Gen	Gentamicin
GFP	Green fluorescent protein
GUS	Beta-glucuronidase
HA	Hemagluttinin
kDa	Kilo Dalton
Ip	Isoelectric point

Ig G	Immuno globulin G
Kan	Kanamicin
LD	Long day
LKP2	LOV domain kelch protein 2
LOV	Light, oxygen, voltage
MTHF	N^5N^{10}-Methenyl-5,6,7,8-tetrahydrofolate
mRNA	Messenger RNA
NLS	Nuclear localization signal
nm	Nanometer
OD	Optical density
PAS	PER, ARNT, SIM
PCR	Polymerase chain reaction
PEG	Polyethylen glycol
Phot	Phototropin
PHR	Photolyase
Phy	Phytochrome
R	Red
Rif	Rifampicin
RNA	Ribonucleic acid
rpm	Rounds per minute
SCF	Skp/Cullin/F-box
SD	Short day
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Tet	Tetracycline
TBE	Tris borate EDTA
TCA	Tri chloro acetic acid
TEMED	N,N,N,N-Tetramethylendiamine
U	Unit
UV	Ultra violet
vol	Volume
v/v	Volume / volume
w/v	Weight / volume
ZTL	Zeitlupe

SUMMARY

Cryptochromes are photolyase-like blue/UV-A light receptors that regulate various light developmental responses in plants. *Arabidopsis thaliana* cryptochrome 2 (Atcry2) is the major photoreceptor mediating blue light regulation of flowering induction. Although the biological role of crys in plants is well-known, the initial photochemistry underlying cryptochrome activation and regulation remain poorly understood. In the present work we addressed several aspects in the early activation events of cry 2.

Many members of the photoreceptor families and components of the light signalling transduction pathway dimerize. Therefore, we studied wheter Atcry2 dimerizes, too. Immunoprecipitation studies of transgenic *Arabidopsis* extracts expressing both, cry2-GFP and cry2 revealed that full-lenght Atcry2 is a homodimer i*n vivo* in a light-independent fashion. The identity of the domains involved in cry2 homodimerization were investigated, and both CNT2 and CCT2 were found as monomers.

Because of the sequence similarity of cry2 with cry1, heterodimerization between cry1 and cry2 was also studied, but no cry1-cry2 heterodimers were found in our experiments.

The *in vivo* effect of dimerization was investigated using *Arabidopsis* transgenic lines expression either CCT2-GFP or cry2-GFP in addition of the endogenous cry2. Cry2-GFP dimers showed phosphorylation and degradation under blue light in the same way as the endogenous cry2, whereas under the same conditions the CCT2-GFP monomers remained stable and unphosphorylated. Moreover, cry2-GFP was able to promote early flowering in plants kept under short day conditions. Whereas under the same conditions CCT2-GFP expressing transgenic *Arabidopsis* flowered as late as wild-type.

Crys purified from *Escherichia coli* contain two chromophores, which were identified as flavin adenine dinucleotide (FAD) and methenyltetrahydrofolate (MTHF), whereas the presence of MTHF was not found for Atcrys purified from an eukaryotic source as insect cells. The detailed knowledge of which chromophore(s) are attached under natural conditions is important for the interpretation of spectroscopic data of these receptors. Therefore, we overexpressed epitope-tagged cry2 *in planta*. Specific immuno-precipitation of the tagged cry2 protein allowed purification of sufficient

amounts of the photoreceptor to identify its chromophores. Based on fluorescence emission data we found that cry2 binds indeed FAD and MTHF *in planta*. In addition, energy transfer from MTHF to FAD was observed.

Because of their similarity in aminoacid sequence and structure, photolyases have been taken as a model for crys photocycle. However, crys were shown to undergo a photocycle in which semireduced flavin ($FADH^{o}$) accumulates upon blue light irradiation in contrast to photolyase that accumulates fully reduced $FADH^{-}$. Green light irradiation of cry2 causes a change in the equilibrium of flavin oxidation states, and attenuates cry2-controlled responses such as flowering. Here, we provided *in vivo* evidence for semireduced flavin ($FADH^{o}$) being the active FAD redox state in Atcry2 by analysis of the expression of flowering genes, linking *in vitro* with physiological studies.

In order to address further insight into the role of phosphorylation on Atcry2 activity, cry2 protein purification from the plant source following mass spectroscopy was performed. Pitifully, the obtained amounts were too small to allow clear results.
Genes that are specifically blue-light induced in cell cultures were identified by PCR and qRT-PCR that can be used in future studies as reporters for transient studies monitoring cry activity.

Zusammenfassung

Cryptochrome sind mit DNA-Photolyasen eng verwandte UV-A/Blaulicht-Photorezeptoren, die zahlreiche Entwicklungsprozesse in Pflanzen steuern. *Arabidopsis thaliana* Cryptochrom 2 (cry2) spielt hierbei eine zentrale Rolle bei der photoperiodischen Regulation der Blühinduktion. Obwohl die biologischen Funktionen von Cryptochromen in Pflanzen gut untersucht sind, sind deren photochemischen Prozesse, die zur Aktivierung führen, wenig verstanden. In der vorliegenden Arbeit wurden zahlreiche Aspekte der Aktivierung von cry2 untersucht.

Zahlreiche pflanzlichen Photorezeptoren und Komponenten der Lichtsignaltransduktion dimerisieren. Entsprechend wurde hier untersucht, ob dies auch für cry2 der Fall ist. Immunpräzipitationsstudien an Proteinextrakten transgener *Arabidopsis* Pflanzen, die cry2-GFP exprimieren, zeigten, dass cry2 *in vivo* als Dimer vorliegt. Licht scheint keinen Einfluss auf die Dimerisierung zu haben. Bei der Analyse, welche Domänen von cry2 für Dimerisierung notwendig sind, konnten keine eindeutigen Befunde erzielt werden, da sowohl die N-terminale Domäne (CNT2) als auch die C-terminale Domäne (CCT2) als Monomer gefunden wurden.

Aufgrund der hohen Sequenzähnlichkeit von cry2 mit cry1 wurde untersucht, ob diese Heterodimere bilden. Die vorliegenden Befunde gaben darauf keinen Hinweis.

Um die Rolle der Dimerisierung von cry2 auf dessen biologische Funktion zu untersuchen, wurden transgene Linien untersucht, die neben dem endogenen cry2 zusätzlich CCT2-GFP oder cry2-GFP exprimieren. Cry2-GFP Dimere wurden lichtabhängig phosphoryliert und abgebaut, ähnlich dem endogenen cry2. Im Gegensatz hierzu, blieben die CCT2-GFP Monomere unphosphoryliert und stabil. Weiterhin führte die Expression von cry2-GFP zu einer Beschleunigung des Blühens unter Kurztag-Bedingungen im Vergleich zum Wildtyp, nicht aber die Expression von CCT2-GFP.

Pflanzliche Cryptochrome, die in *E. coli* exprimiert wurden, tragen zwei Chromophore (FAD und MTHF). Nach Expression in eukaryontischen Systemen wie Insektenzellen konnte bislang allerdings keine Bindung von MTHF an cry nachgewiesen werden. Kenntnisse darüber, welche Chromophore an cry *in planta* gebunden sind, sind für die Interpretation der spektroskopischen Daten dieser Photorezeptoren bedeutsam. Deshalb wurde in der vorliegenden Arbeit Epitop-markiertes cry2 in Pflanzen exprimiert und

spezifisch über Immunpräzipitation in Mengen aufgereinigt, die eine Identifizierung der gebundenen Chromophore ermöglichte. Die Ergebnisse von Fluoreszenz-Emissionsspektroskopie zeigten, dass cry2 *in planta* sehr wahrscheinlich beide Cofaktoren, FAD und MTHF, bindet. Zusätzlich ergaben diese Untersuchungen Hinweis auf Energietransfer von MTHF auf FAD.

Augrund der Ähnlichkeit von Photolyasen und Cryptochromem in ihrer Aminosäuresequenz and Struktur wurde die Photochemie von Photolyasen als Modell für den Photozyklus von Cryptochromen benutzt. Allerdings zeigten *in vitro* Untersuchungen, dass Cryptochrome nach Anregung mit Blaulicht semireduziertes Flavin ($FADH^{o}$) akkumulieren, im Gegensatz zu Photolyasen, die vollständig reduziertes Flavin ($FADH^{-}$) bilden. Zu Blaulicht zusätzlich gegebenes Grünlicht verschiebt das Gleichgewicht der Oxidationszustände von Flavin hin zu oxidiertem FAD und reprimiert cry2-kontrollierte Prozesse wie die Induktion der Blütenbildung. Hier durchgeführte Untersuchungen über die Wirkung von Grünlicht auf die Expression von Blühgenen lieferten zusätzliche Hinweise darauf, dass cry2 mit semireduziertem Flavin den aktiven Zustand dieses Photorezeptors repräsentiert.

Zum weiteren Verständnis der Phosphorylierung von cry2 wurde versucht, das Protein aus Pflanzen in Mengen zu isolieren, die für nachfolgende massenspektrometrische Analysen ausreichend sind. Leider wurde dieses Ziel nicht erreicht.

Weiterhin wurden die Expression zahlreicher Gene in *Arabidopsis* Zellkulturen durch *PCR*- und quantitative *real-time PCR*-Analysen daraufhin untersucht, ob sie spezifisch durch Blaulicht reguliert werden. Einige Gene konnten hierbei identifiziert werden, die in zukünftigen Untersuchungen als Reporter genutzt werden können, um die Aktivität von Wildtyp und Mutanten Cryptochromen zu untersuchen.

1 INTRODUCTION

Due to their photoautotrophic nature, plants depend upon light energy for their survival. Being sessile organisms that cannot choose their habitat, plants are usually limited in their resources and must adapt their growth and development to their environment. In addition to supply energy for their photosynthetic needs, light gives valuable information about the surrounding environment, and monitoring changes in the quantity, quality and direction of light allows plants to determine their position on time and space.

Light quality represent its energy, and it is determined by its wavelength (λ) given in nanometers (nm). The human eye can see only the region of the light spectrum called visible, ranging from 400 to 700 nm (Fig. 1.1). Plant light perception is expanded beyond the human limit, being able to see near ultraviolet (UV-A) and far-red light (FR) (Batschauer A. 1998).

Quantity of light is referred as fluence rate: the amount of photons by area and time (μmol $m^{-2}s^{-1}$).

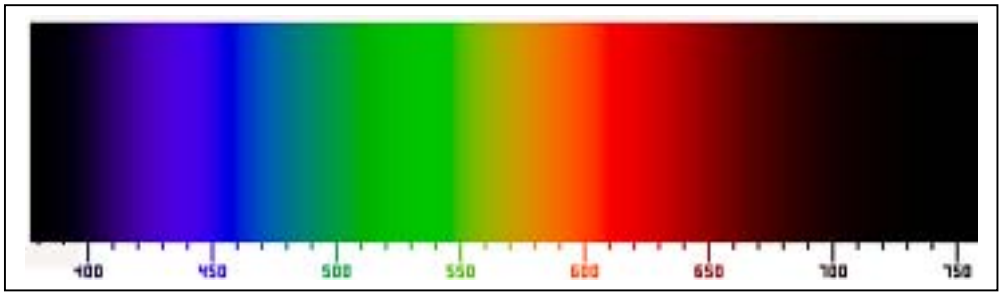


Fig. 1.1 Light quality of the visible region of the spectrum. Numbers indicate the wavelength in nanometers.

Key processes in plant life, like germination (the transition from seed to non-photosynthetic seedling) and de-etiolation (the transition from seedling to photosynthetic plant) are regulated by the environmental light signals. In addition, together with the circadian clock, light enables plants to monitor the photoperiod (the number of hours of light per 24-hour period) allowing them to adapt their timing for photosynthesis and transition to flowering and reproduction.

All this developmental plasticity in response to light signals is conferred by specialized proteins called photoreceptors. The presence of several photoreceptor families allows the correct interpretation of different light cues. In higher plants, four major families of photoreceptors have been identified and characterized in molecular terms (Banerjee R. & Batschauer A. 2005; Gyula P. *et al* 2003). These are the phytochromes (phys), the cryptochromes (crys), the phototropins (phots), and zeitlupe (ZTL) photoreceptor families.

1.1 Light sensors: photoreceptors

The different photoreceptor families detect different light qualities, usually in the visible region of the spectrum. All photoreceptors bind a molecule called chromophore that allows the light intake. Without chromophores photoreceptors are not functional. The nature of the chromophore varies with each photoreceptor family (Fig. 1.2).

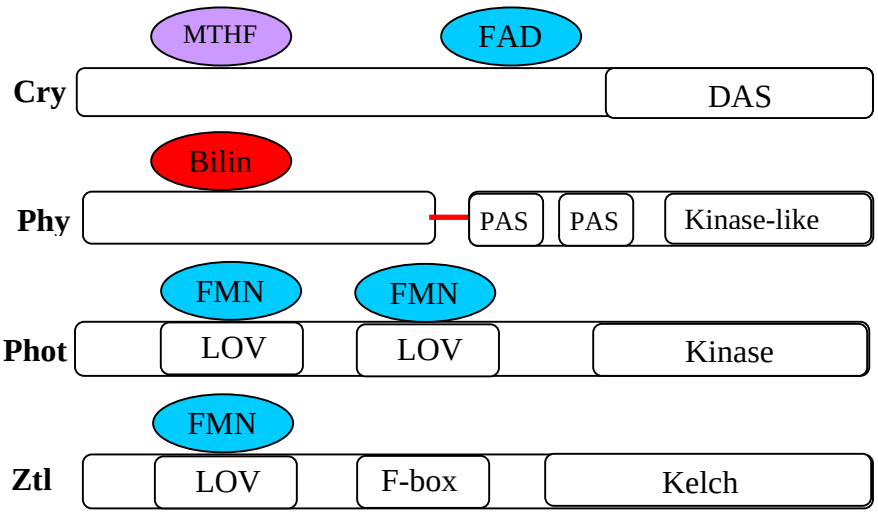


Fig. 1.2 Domain distribution and chromophore composition of photoreceptor families. Phytochrome (Phy), phototropin (Phot), ZTL (zeitlupe), and cryptochrome (Cry). Colours indicate the waveband absorbed by the chromophore, PAS: PER/ARNT/SIM domain, LOV: light, oxygen, voltage domain, FMN: flavin mononucleotide, FAD: flavin adenine dinucleotide, MTHF: N^5N^{10}-Methenyl-5,6,7,8-tetrahydrofolate.

1.1.1 Phytochromes (Phys)

Arabidopsis contains 5 phytochromes named phyA to phyE. They can be separated into three groups according to their sequence homology. PhyA and C conforms each one group, and phyB, D and E the third one (Clack T. *et al* 1994). Studies using *Arabidopsis* phytochrome mutants revealed residual responsivity of *phyAphyB* double mutant seeds to R and FR pulses, suggesting some overlapping function of other phytochromes with phyA and phyB (Poppe C. & Schäfer E. 1997).

Phys can exist in two forms, an inactive red absorbing form (Pr) with maximum absorption at 667 nm, and an active far-red absorbing form (Pfr) with maximum absorption at 730 nm. These two forms are found in different proportions depending on the light conditions, and conformational changes of the protein moiety are associated to the photo-conversion between the Pr and Pfr forms (Quail P.H. 1991). The Pr form is localized on the cytoplasm but the photoactivated Pfr is translocated to the nucleus upon photoconversion (Yamaguchi R *et al* 1999;

Kircher S. *et al* 1999; Nagy F. & Schäfer E. 2000). Curiously, phyA is activated under continuous FR, and blue light. To explain this fact it has been proposed that the Pfr or Pr forms of phyA are not really the active state, but the switch between these forms is the signalling state (Shinomura T. *et al* 2000). Moreover, phyA has also the particularity of being the only labile phy in its Pfr form, being ubiquitinated and degraded by the proteasome after activation (Seo H.S. *et al* 2004).

Phy protein can be divided in N-terminal and C-terminal regions separated by a small hinge region (Fig. 1.2). The C-term has a histidine kinase related domain (HKRD) and a protein-protein interaction zone containing PAS motifs. These motifs are responsible for homodimerization (Jones A.M. & Quail P.H. 1986), and interaction with many partners, including the transcription factor PIF3 (Phytochrome Interaction Factor 3; Ni M. *et al* 1998), the E3 ligase COP1 (Constitutively Photomorphogenic 1; Seo H.S. *et al* 2004), and photoreceptors like cry1 (Ahmad M. *et al.* 1998a) and ZTL (Jarillo J.A. *et al.* 2001b). The N-term contains the chromophore, the linear tetrapyrrole phytochromobilin (Lagarias J.C. & Lagarias D.M.1989; Lagarias J.C. & Rapoport H. 1980).
The C-term seems to be a regulatory, but dispensable part of the phy. On the other hand the N-term seems to carry the signalling domains (Cherry J.R. *et al* 1992, Jordan E.T. *et al* 1997), being active as long as dimeric and localized to the nucleus (Oka Y. *et al* 2004, Matsushita T. *et al* 2003). Phys are phosphorylated at several serines by a light dependent mechanism. The N-terminal serines are phosphorylated in both, Pr and Pfr forms, but phosphorylation of the hinge region happens only when phy is in the Pfr form (Lapko V.N. *et al* 1999), and regulates the interaction of phy with its partners (Oh E. *et al* 2004), pointing to a conformational change due to phosphorylation. In addition, phytochromes are Ser/Thr protein kinases (Yeh K.C. & Lagarias J.C. 1998), being able to phosphorylate many substrates, including cry1 (Ahmad M. *et al* 1998a) amongst them.

The biological role of phys is wide, as they participate in almost all light-triggered processes, like seed germination, de-etiolation, which includes hypocotyl elongation inhibition, cotyledon opening and greening, and light regulated gene expression, shade avoidance, entrainment of the circadian clock, and photoperiodic regulation of flowering (Chory J. *et al* 1996).

1.1.2 Phototropins (Phots)

Arabidopsis contains 2 phototropins (phots), phot1 (Huala E. *et al* 1997) and phot2 (Kagawa T. *et al* 2001; Jarillo J.A. *et al* 2001a). These proteins have three recognizable domains: a Ser/Thr protein kinase domain responsible of blue-light-dependent autophosphorylation (Christie J.M. *et al* 1998), and 2 amino-terminal PAS (PER, ARNT, SIM) domains of the LOV (light, oxygen, voltage) subtype, each binding one FMN (flavin mononucleotide) molecule as chromophore (Christie J.M. *et al* 1999).

It was shown that phot1 is a dimer (Salomon M. *et al* 2004) that undergoes autophosphorylation at several known ser residues in a fluence dependent way (Salomon M. *et al* 2003). Although the subcellular location of phot2 is not yet defined, phot1 is known to be associated with the plasma membrane (Sakamoto K. & Briggs W.R. 2002).

Phototropism is the process, which allows plants to bend towards or away from a light source to allow light capture for photosynthesis or to avoid light damage. In *Arabidopsis*, phototropism is controlled by phots. Phot1 mediates phototropism under low intensity blue light (Liscum E., & Briggs W.R. 1995). While, phot2 responds to high irradiance blue light (Kagawa T. *et al* 2001). The *phot1phot2* double mutant displays impaired phototropic responses at all irradiances (Sakai T. *et al* 2001). No impairment of phototropism is found for *cry1cry2* double mutants, confirming a unique role for phototropins in mediating this blue light response (Lascève G. *et al*. 1999). Phots also participate in other processes, like the inhibition of hypocotyl growth during the very first minutes of blue light irradiation (Folta K.M. & Spalding E.P. 2001), the establishment of light-regulated ion fluxes (Harada A. *et al* 2003), chloroplast movement (Kagawa T. *et al* 2001; Jarillo J.A. *et al* 2001a) and stomata opening (Kinoshita T. *et al* 2001). However, other light regulated processes like transition to flowering (Imaizumi T. *et al* 2003), light regulated gene expression (Ohgishi M. *et al* 2004), or entrainment of the circadian clock (Devlin P.F. & Kay S.A. 2000) do not require the phototropins presence.

1.1.3 ZTL / LPK2 / FKF1

This family comprises 3 proteins in *Arabidopsis*: Zeitlupe (ZTL/LKP1/ADO1; Somers D.E. *et al* 2000), LOV domain kelch protein 2 (LKP2/ADO2; Schultz T.F. *et al* 2001) and Flavin-binding Kelch repeat F-box (FKF1/ADO3; Nelson D.C. *et al* 2000).

ZTL and LKP2 are very similar in their amino acid sequence. Whereas, FKF1 seems to be more divergent (Nelson D.C. *et al* 2000), all zeitlupe members share 3 common domains, a PAS domain, a F-box and a group of 6 Kelch repeats.

The PAS domain is from the LOV subtype, like the one found in phots, and binds FMN (flavin mononucleotide). Upon light excitation light induced absorbance changes are observed similar to phot, suggesting that ZTL/FKF1/LKP2 may also be photoreceptors. However, in contrast to the LOV domain from phot the dark reversion of the FMN in these proteins is very slow (Imaizumi T. *et al* 2003). So far, data supporting a photoreceptor role for these proteins has been obtained only for FKF1 (Imaizumi T. *et al* 2003; Zikihara K. *et al* 2006).

F-box proteins are components of the Skp/Cullin/F-box (SCF) complex (Xiao W. & Jang C.J. 2000), an ubiquitin ligase complex that recruits specific substrates, and usually also itself, for ubiquitination and subsequent proteolysis by the 26S proteasome (Vierstra R.D. 2003). It is clear for ZTL to form SCF complexes in vivo (Han L. *et al* 2004), LKP2 can interact with many ASKs in vitro (Yasuhara M. *et al* 2004), and targets for FKF1 (Imaizumi T. *et al* 2005) and ZTL (Más P. *et al* 2003) mediated degradation have been found, indicating functional ubiquitin ligase activity for the members of this family.

The kelch repeats have β-propeller structure and are protein-protein interaction domains (Adams J. *et al* 2000).

The main function of ZTL and LKP2 is the control of light input to the circadian clock (Schultz T.F. *et al* 2001; Más P. *et al* 2003). Therefore, components of the clock and processes controlled by it are also affected when the dosage of these genes is altered (Somers D.E. *et al* 2004). On the other hand, FKF1 has little effect on circadian clock, although its mRNA level is controlled by it (Nelson D.C. *et al* 2000). FKF1 main function is the control of flowering in a direct way, targeting a repressor of Constants, a crucial gene from the long day pathway, for degradation (Imaizumi T. *et al* 2005).

1.1.4 Cryptochromes (Crys)

Crys are UV-A/blue light photoreceptors which are supposed to have evolved from photolyase (PHR), a DNA repair enzyme (Kanai S. *et al* 1997: Sancar A. 2003). Both, crys and PHRs, contain FAD (flavine adenine dinucleotide) in an uncommon U-shaped conformation as chromophore (Dym O. & Eisenberg D. 2001).

Crys presence is no limited to plants (Ahmad M. & Cashmore A.R. 1993) or photosynthetic organisms (Hitomi K. *et al* 2000), but are also found in bacteria like the enteropathogenic *Vibrio cholerae* (Worthington E.N. *et al* 2003) and animals ranging from human (Hsu D.S. *et al* 1996), to mouse (Kobayashi K. *et al* 1998), chicken (Yamamoto K. *et al* 2001), Zebrafish (Kobayashi Y. *et al* 2000), fruit fly (Emery P. *et al* 1998), or bullfrog (Zhu H. *et al* 2001).
Placental mammals lack PHR, but have crys. Meanwhile it was shown that marsupials have both of them (Kato T. Jr. *et al* 1994). Plants, like marsupials, also contain both, cryptochromes (Hoffman P.D. *et al* 1996; Ahmad M. & Cashmore A.R. 1993) and PHR (Batschauer A. 1993; Ahmad M. *et al* 1997a; Sakamoto A. *et al* 1998).

Two PHR types exist, each of them can repair one kind of DNA damage produced by UV-B. The cyclobutane pyrimidine dimer Pyr<>Pyr is the most common damage, counting for something like 80% of the total damage. The pyrimidine-pyrimidone (6-4) photoproduct Pyr [6-4] accounts for the remaining 20%. PHRs are named like the damage they repair as cyclobutane pyrimidine dimer (CPD) photolyase and (6-4) photolyase.
Plant and animal crys evolved from different PHR ancestors. Both plant crys and 6-4 photolyase, evolved from a CPD photolyase ancestor (Kanai S. *et al* 1997), and later on, the 6-4 photolyase evolved to animal crys (Todo T. 1999).

Arabidopsis contains 3 crys. Cry1 and cry2 are nuclear proteins and share many characteristics such as their specific CCT domain. cry3 is from the cryDASH type (Daiyasu H. *et al* 2004), lacks the CCT of other crys, is targeted to organelles, binds DNA (Kleine T. *et al* 2003), and retains photolyase activity specific for pyrimidine dimmers in single stranded DNA (Selby C.P. & Sancar A. 2006).
Although crys from all kingdoms share many common structural features their functions are different. Nuclear crys of plants do not have photolyase activity (Malhorta K. *et al* 1995; Lin C. *et al* 1995a; Hoffman P.D. *et al* 1996). They control de-etiolation (Ahmad M. & Cashmore A.R. 1993), timing of flowering (Guo H. *et al* 1998) and entrainment of the circadian clock (Somers D.E. *et al* 1998). Obviously, mammals lack de-etiolation and flowering responses, but the role of crys on the circadian clock is more important in mammals than in plants, as crys do not merely set the circadian clock, but are core components of it (Kume K. *et al* 1999; van der Horst G.T. *et al* 1999).
Because the object of study in this thesis are plant nuclear crys their structure and function are described in the following chapters.

1.2 Cryptochromes biological role

1.2.1 Photomorphogenesis: de-etiolation and gene expression

Photomorphogenesis is the process happening after germination when, upon exposure to light, the seedling undergoes de-etiolation. This developmental process sets the seedling for efficient photosynthesis, and is characterized by inhibition of the hypocotyl growth, apical hook opening, cotyledon expansion, chloroplast development, and induction of a gene expression program different from the one in darkness.

Expression of light-regulated genes is mediated by binding of transcription factors to specific promoter sequences in particular the G-boxes (Giuliano G. *et al* 1988). Chalcone synthase (*CHS*, Batschauer A. *et al* 1991) is a well known light-regulated gene participating in the flavonoid synthesis pathway (Winkel-Shirley B. 2001). It has G-boxes in its promoter region (Kaiser T. & Batschauer A. 1995), where transcription factors like HY5 bind (Chattopadhyay S. *et al* 1998) to promote gene expression. Amongst the transcription factors mediating photomorphogenesis we can find HY5 (Oyama T. *et al* 1997), HYH (Holm M. *et al* 2002), LAF1 (Long After Far-red light1, Seo H.S. *et al* 2003) and HFR1 (long Hypocotyl in Far-Red 1; Kim Y.M. *et al* 2002). The activity of all of them depends on COP1 action. COP1 (Constitive Photomorphogenic 1; Deng X.W. *et al* 1991) is an E3 ubiquitin ligase with three recognizable domains (Deng X.W. *et al* 1992). An N-terminal RING-finger that mediates ubiquitinating target proteins and itself (Seo H.S. *et al* 2003), a coiled coil domain responsible of COP1 dimerization (Torii K.U. *et al* 1998), and C-terminal WD-40 repeats protein-protein interaction motifs with propeller structure similar to the kelch repeats of the ZTL family (Holm M. *et al* 2001). COP1 has both nuclear and cytoplasmatic localization signals (Stacey M.G. *et al* 1999), being nuclear in darknesss and relocated to the cytoplasm upon light exposure (von Arnim A.G. & Deng X.W. 1994).

COP1 action is modulated by the SPA (Suppressor of PhyA) family (Yang J. & Wang H. 2006) that has four members in *Arabidopsis*, SPA1 to SPA4, and which are needed by COP1 in different developmental stages (Fittinghoff K. *et al* 2006). SPA1 is the best characterized member of this family. Its domain composition resembles COP1, as it has WD-40 repeats in its C-terminal domain with high homology to the ones of COP1, following a protein-protein interaction coiled-coil domain, and finally in the N-terminal part a kinase-like domain (Hoecker U. *et al* 1999).

SPA1 interacts with both COP1 (Hoecker U. *et al* 2001) and transcription factors like HY5 (Saijo Y. *et al* 2003), HFR1 (Yang J. *et al* 2005b), or CO (Laubinger S. *et al* 2006), forming a bridge between

COP1 and targets for ubiquitination. The ubiquitination of LAF1 (Seo H. *et al* 2003), HFR1 (Jang I.C. *et al* 2005), HYH (Holm M. *et al* 2002) and HY5 (Ang L.H. *et al* 1998) for proteosomal degradation (Fig. 1.3) is proven.

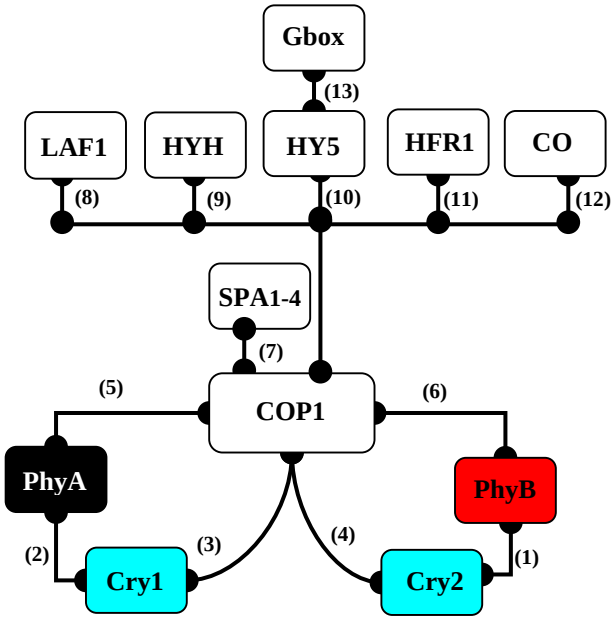


Fig. 1.3 Photoreceptors induce de-etiolation and gene expression by de-activating COP1 and stabilization of multiple transcription factors. All lines represent physical interactions, colour represent photoreceptor blue, red or far-red activation.

(1) Más P. *et al* (2000)
(2) Ahmad M. *et al* (1998)
(3) Yang H.Q. *et al* (2001), Wang H. *et al* (2001)
(4) Wang H. *et al* (2001)
(5) Seo H.S. *et al* (2004)
(6) Yang H.Q. *et al* (2001)
(7) Hoecker U. *et al* (2001), Laubinger S. *et al* (2003)
(8) Seo H. *et al* (2003)
(9) Holm M. (2002)
(10) Ang L.H. *et al* (1998)
(11) Jang I.C. *et al* (2005), Yang J. *et al* (2005).
(12) Laubinger S. *et al* (2006)
(13) Chattopadhyay S. *et al* (1998)

Additionally, COP1 is also able to interact physically with crys (Yang H.Q. *et al* 2001; Wang H. *et al* 2001) and phys (Seo H.S. *et al* 2004). Upon light exposure photoreceptors exclude COP1 from the nucleus (Osterlund M.T. & Deng X.W. 1998), being this exclusion, although slow (von Arnim A.G. *et al* 1997), important to abolish COP1 activity (Subramanian C. *et al* 2004).
Array studies with *cop1*, *hy5* (Ma L. *et al* 2002) and *cry1cry2* (JiaoY. *et al* 2003) mutants further supported the role of all these proteins in gene induction associated to photomorphogenesis.

Studies with photoreceptor mutants revealed a main role for cry1 in photomorphogenesis. The hypocotyl growth inhibition (Ahmad M. & Cashmore A.R. 1993; Ahmad M. *et al* 1995) and the expression of *CHS* or anthocyanin accumulation (Ahmad M. *et al* 1995), a red-coloured product from the phenyl propanoid pathway (Winkel-Shirley B. 2001), are the common outputs in analyses of photomorphogenesis. Cry1 overexpressing *Arabidopsis* plants were hypersensitive to all blue light fluences when the above mentioned outputs were measured (Lin C. *et al* 1996). However, the role of cry2 in *CHS* gene induction is minor. Cry1 is greatly needed for *CHS* expression under blue light, and cry2 compensates weakly the *cry1* mutation (Wade H.K. *et al* 2001). Cry2 has also a minor role in hypocotyl growth inhibition (Lin C. *et al* 1998; Mockler T.C. *et al* 1999), being active under medium-low fluences of blue light (Lin C. *et al* 1998).

1.2.2 Entrainment of the circadian clock

The Circadian clock (circadian: "approximate one day") is an endogenous autoregulatory mechanism that allows living organisms to anticipate rhythmic changes in the environment, like the day-night cycle or seasons over the year, giving adaptive advantage and enhancing plant fitness (Dodd A.N. *et al* 2005, Green R.M. *et al* 2002). Amongst the activities controlled by the circadian clock in plants are the regulation of transcription, leaf movement, growth and photosynthesis, (Barak S. *et al* 2000).

The clock is classically divided in inputs, central oscillator, and outputs. Input pathways receive environmental cues that entrain the central oscillator, which is composed by a negative feedback loop. In turn, the central oscilator controls output components, creating rhythms.

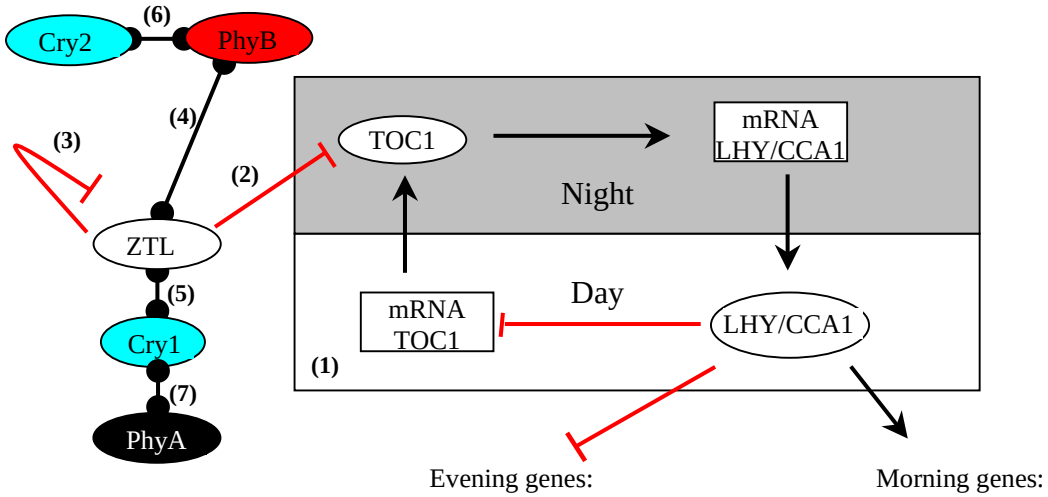


Fig. 1.4 Circadian clock and light input model: Photoreceptors are both inputs and outputs of the clock (Toth R. *et al* 2001). Lines with balls mean protein-protein interaction, black arrows are for positive induction, red arrows are for repression.

(1) Alabadí D. *et al.* (2001), Alabadí D. *et al.* (2002)
(2) Más P. *et al.* (2003)
(3) Kim W.Y. *et al.* (2003), Han L. M. *et al.* (2004)
(4) Jarillo J.A. *et al.* (2001)
(5) Jarillo J.A. *et al.* (2001)
(6) Más P. *et al.* (2000)
(7) Ahmad M. *et al.* (1998)

The *Arabidopsis* clock model is far from be completed. Nevertheless, the known central oscillator is a negative feedback loop composed by *TOC1* (Timing Of CAB expression 1; Strayer C. *et al* 2000), *LHY* (Late Elongated Hypocotyl; Schaffer R. *et al* 1998) and *CCA1* (Circadian Clock Associated 1; Wang Z.Y. & Tobin E.M. 1998). TOC1 promotes *CCA1/LHY* transcription during the night. In the early morning CCA1/LHY proteins induce the transcription of the morning genes, and, at the same time, repress *TOC1* expression by binding to the Evening Elements (EE) of its promoter region (Alabadí D. *et al* 2001 & 2002). EE have been found in the promoter region of many other circadian controlled genes which peak at the end of the

subjective day (evening genes). Therefore, it is very probable that LHY/CCA1 also inhibit their transcription in the same way as they do with *TOC1* (Harmer S.L. *et al* 2000). As the day advances LHY/CCA1 protein level decline, allowing the expression of the evening genes and *TOC1*, which in turn will activate again *LHY/CCA1* transcription during the night starting another loop (Fig. 1.4).

To achieve proper function, the circadian oscillator needs to be entrained. Its phase must be synchronized with the suitable photoperiod running in nature at that time. Light is often the predominant entraining stimulus in plants, although the clock can work under constant dark or light conditions. In plants kept under constant light, the period length is dependent on the fluence rate. This is known as the "Aschoff rule", the clock runs slower under low light intensities (Aschoff J. 1979). Moreover, the spectral distribution of light affects clock entrainment, suggesting an active role of photoreceptors in setting the pace of the oscillator. Indeed, A*rabidopsis* single and double photoreceptors mutants under constant light (Somers D.E. *et al* 1998; Devlin P.F. & Kay S.A. 2000) showed period alteration with special sensitivity for determined fluences and wavelengths. Cry1 and cry2 act redundantly in the blue-light input pathway. Cry1 senses blue light at all fluences and cry2 has a minor redundant role with cry1 under low and medium fluences of blue light.

Additional experiments performed with single, double and triple photoreceptor mutants (Yanovsky M.J. *et al.* 2001) revealed that blue is the most prominent light quality to entrain the clock. Moreover, amongst all photoreceptor mutants tested *cry1cry2* double mutant showed the biggest period lengthening under blue light, and little increase in darkness. Nevertheless, despite all the effect of crys in entrainment and setting the period length, *cry1cry2* double (Devlin P.F. & Kay S.A. 2000) and *phyAphyBcry1cry2* quadruple (Yanovsky M. *et al* 2000) mutants retain rhythmicity. This fact indicates that crys do not play an oscillator role in plants as they do in mammals (van der Horst G.T. *et al* 1999), and that other photoreceptors can provide input to the clock.

The F-box protein and putative blue-light photoreceptor ZTL regulates both TOC1 (Más P. *et al.* 2003) and its own protein level (Kim W.Y. *et al* 2003; Han L. M. *et al* 2004) in a circadian pattern. In addition it interacts with photoreceptors (Jarillo J.A. *et al.* 2001b) giving a physical scaffold for signal transduction (Fig. 1.4). ZTL mutants have longer periods (Somers D.E. *et al* 2000), and overexpresion of ZTL leads to arrhythmicity under continous light or darkness(Somers D.E. *et al* 2004), pointing to an important role for this gene on light input to the clock.

1.2.3 Control of flowering time

Flowering, controlled by environmental conditions and developmental regulation, is the switch from the vegetative to the reproductive state. Since flowering is the key of the species survival, it must be tuned to happen in the most favourable period of the year. This fact is even more important in annual plants, which have only one chance to propagate their seeds.

Diverse pathways converge on the activation of the same flowering time genes (Mouradov A. *et al* 2002). One of these pathways, the photoperiodic regulation of flowering time, is given by the external coincidence of perception of the day length by photoreceptors and the circadian clock expression of flowering-inducing genes. However, the response to period length is not the same amongst different plant species. Depending on their photoperiod dependence we find plants which flower under long day (LD), short day (SD), or are period insensitive.

Most of the current knowledge of the photoperiod pathway comes from the long day plant *Arabidopsis*, where both, FR and B light, promote flowering (Brown, J.A., & Klein W.H. 1971). Daylength is perceived by leaves, but flowering happens in the shoot apical meristem (SAM) (Blázquez M.A. *et al* 2006). In order to explain this fact, it was postulated that a floral signal called florigen is produced in the leave and transported to the SAM. There, it triggers the activation of the floral identity genes *LFY* (Leafy) and *AP1* (Apetala 1) (Simon R. *et al* 1996). Nowadays FT (flowering locus T) mRNA and protein are consider as florigens (Huang T. *et al.* 2005; Corbesier L. *et al* 2007). *FT* is expressed in leaves depending on the simultaneous presence of the CO protein (Constans; Putterill J. *et al* 1995) and light (Suárez-López P. *et al* 2001; Yanovsky M. J. *et al* 2002). CO is a nuclear transcription factor whose mRNA and protein levels are tightly regulated by the circadian clock and photoreceptors (Figs. 1.5, 1.6).

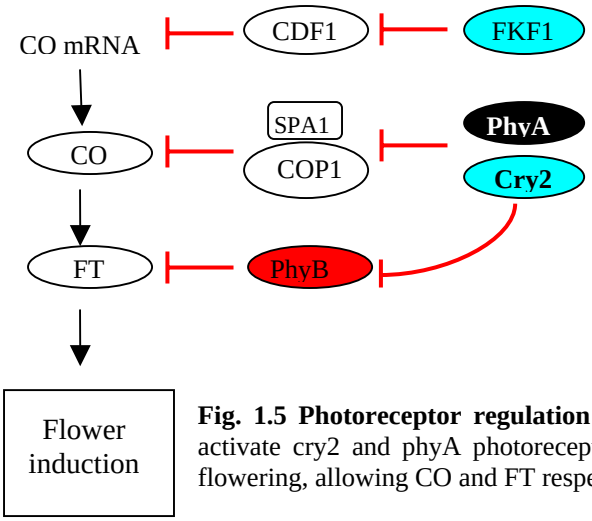


Fig. 1.5 Photoreceptor regulation of flowering time. Blue and far-red light activate cry2 and phyA photoreceptors, which in turn deactivate inhibitors of flowering, allowing CO and FT respectively expression.

CO mRNA levels are under the control of *CDF1* (Cycling Dof Factor1; Imaizumi T. *et al* 2005). *CDF1* is expressed on the very early morning and binds to the *CO* promoter inhibiting its expression during the day. In the late afternoon, *FKF1* is expressed (Nelson D.C. *et al* 2000) and targets CDF1 for degradation *via* its F-box domain, allowing expression of *CO* (Imaizumi T. *et al* 2003; Imaizumi T. *et al* 2005). Although *FKF1* is essential for *CO* expression, lack of either *crys* or *phyA* have no effect on *CO* normal expression (Imaizumi T. *et al* 2003)

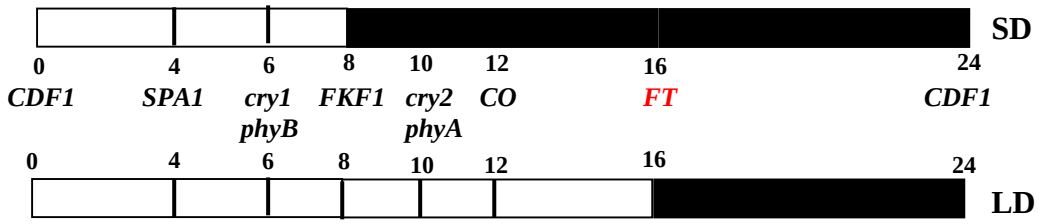


Fig. 1.6 Circadian clock regulated mRNA expression of proteins participating in the photoperiod pathway of flowering induction. Numbers represent the zeitgeber (ZT, time since lights are on) for maximum mRNA expression. Meanwhile the expression of the diverse components of the photoperiod pathway of flowering is the same under both, long day (LD) or short day (SD), FT is expressed only under LD photoperiod, typically 16 hours light: 8 hours darkness.

The CO protein is present in the nucleus at the evening. In short days this time corresponds to darkness and CO is rapidly ubiquitinated by the COP1-SPA complex (Valverde F. *et al* 2004; Laubinger S. *et al* 2006). It is interesting to note that *cop1* mutants can flower even in darkness (Nakagawa M. & Komeda Y. 2004). Only in summer the length of the day is long enough to provide light at the time when CO levels are high. When this happens, activated cry2 and phyA photoreceptors are able to abolish COP1 action thus stabilizing CO protein (Valverde F. *et al* 2004). However, there is another mechanism that represses FT expression. PhyB is a constitutive inhibitor of FT, being the *phyB* mutant able to flower earlier than the wt under long days (El-Assal S.E.D. *et al* 2003; Endo M. *et al* 2005). Genetic studies showed that cry2, cry1 and phyA are able to promote flowering. However, cry2 and phyA have an additional effect in deactivating the inhibitory action of phyB, where cry2 plays a main role in flowering regulation under blue light more than cry1 and phyA (Guo H. *et al* 1998; El-Assal S.E.D. *et al* 2003; Mockler T *et al* 2003).

Temporal gene expression is not the only important factor in photoperiodic regulation of flowering. Spatial distribution of CO (An H. *et al* 2004), FT (Takada S. & Goto K. 2003) and CDF1 (Imaizumi T. *et al* 2005) is specific for the vascular leaf tissue. In addition, FKF1 is also strongly expressed in leaf (Nelson D.C. *et al* 2000).

1.2.4 Biological role of crys in other plants

Data obtained with *cry* mutants in several plant species are in accordance with the previous experiments performed in *Arabidopsis*.

As seen in *Arabidopsis*, cry1 is implied in de-etiolation in pea (*Pisum sativum*, Pscry, Platten J.D. *et al* 2005) tomato (*Lycopenum esculentum*, Lecry; Ninu L. *et al* 1999) and rice (*Oryza sativa*, Oscry; Hirose F. *et al* 2006). In addition, cry1 fluence rate response in pea (Platten J.D. *et al* 2005a) and tomato (Weller J.L. *et al* 2001) corresponds with the one of *Arabidopsis* (Lin C. *et al* 1996). Moreover, At*cry1* mutants can be rescued by Os*cry1*, and mixed constructs of rice and *Arabidopsis* crys1. Cry1 seems to interact with COP1 in both rice and *Arabidopsis* revealing a common mechanism for all plant crys (Zhang Y.C. *et al* 2006).

Manipulation of cry2 results in flowering time alteration independently of the wild type plant response to photoperiod. In addition to the well known role of cry2 in flowering induction in the long day plant *Arabidopsis*, which flowers late when cry2 is lacking, and flowers early when it is overexpressed it (Guo H. *et al* 1998), tomato, a day length insensitive plant, overexpressing Le*cry2* flowers late under both SD and LD (Giliberto L. *et al* 2005). In addition, flowering in rice is induced under SD, but Os*cry2* mutants flower late under both SD and LD (Hirose F. *et al* 2006).

1.3 Cryptochromes structure

Crys are usually divided in two domains, the N-terminal domain (CNT) of 500 amino acids with high homology to photolyases (PHR), and a highly specific C-terminal (CCT) domain, which is found only in crys and has no homology to other known protein domain.

1.3.1 The cryptochrome N terminus (CNT)

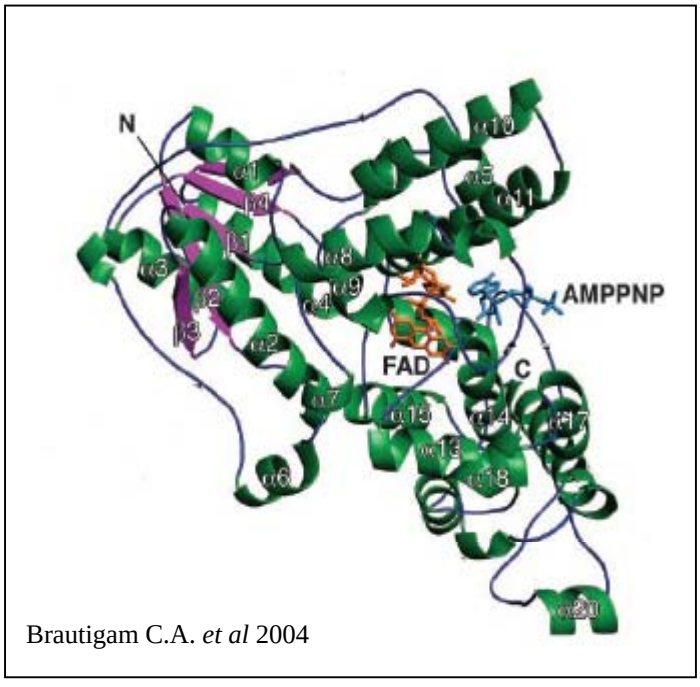
CNT has a similar size as PHR and their sequence identity is around 30%. The CNTs of cry1 and cry2 have a sequence identity of almost 60%. This homology amongst crys is high when different plant species are compared, but it drops lower when the comparison is between animal and plant CNT as they seem to have evolved form different PHR ancestors.

Comparison of available photolyases crystal structures from *E. coli* (Park H.W. *et al* 1995), the cyanobacterium *Synechocyctis* (Tamada T. *et al* 1997), and for the CNT of cry1 (CNT1)

(Brautigam C.A. *et al* 2004) has revealed that all of them have similar structural folds. The carbon backbone of these proteins is super imposable, although the surface charge is different on crys and PHRs. Moreover, despite the high homology between PHR and CNT, no photolyase activity has been found for either cry1 (Lin C. *et al* 1995a) or cry2 (Malhotra K. *et al* 1995; Hoffman P.D. *et al* 1996).

The main chromophore, FAD, is found deeply and non-covalently bound, in the CNT structure. The maximum homology region amongst CNT of different species and kingdoms is always in this FAD binding pocket, being especially important for cry function, as many mutants were found altered in this region or close to it (Ahmad M. *et al.* 1995). Moreover, both crys and PHRs are not active without FAD (Bouly J.P. *et al* 2003, Kleiner O. *et al* 1999a).

A second chromophore acting as a light harvesting antenna is found in PHR. This second cofactor is 5, 10-methenyltetrahydrofolic acid (MTHF) in case of *E. coli* (Johnson J.L. *et al* 1988), or 8-hydroxy-5-deazaflavin (8-HDF) present in *Anacystis nidulans* PHR (Eker A.P.M. *et al* 1990). The presence of the second chromophore in PHR is dispensable for function. In *Arabidopsis* crys, the presence of a second chromophore is not so clear. The cry amino acid sequence of the binding pocket for the second chromophore resembles more the one of 8-HDF. However, MTHF has been found to be bound to full length crys when expressed in *E .coli* (Malhotra K. *et al* 1995). Nevertheless, it should be noted that *E .coli* is not able to produce 8-HDF. When cry is obtained from the eukaryotic insect cell expression system no second chromophore can be found (Lin C. *et al* 1995).



Brautigam C.A. *et al* 2004

Fig. 1.7 CNT1 crystal structure with FAD and the ATP analog AMPPNP bound

The reason for this is unclear and could be caused by weak and non covalent non-covalent binding resulting in its loss during the purification process, as previously reported for the PHR from *Thermus thermophilus* (Ueda T. *et al* 2005), and more recently for cry from *Drosophila* (Berndt A. *et al* 2007). Moreover, no second chromophore was found in the CNT1 structure. Chromophore reconstitution is possible to be performed in *E. coli* PHR (Johnson J.L. *et al* 1988), but attempts to reconstitute CNT1 expressed in *E. coli* (Brautigam C.A. *et al* 2004) or full-length cry1 expressed in insect cells (Lin C. *et al* 1995) with external chromophore have been unsuccessful

Another feature of CNT1 crystal is the capacity to bind ATPPNP, an ATP analog, close to the FAD binding pocket (Brautigam C.A. *et al* 2004, Fig. 1.7), supporting the previous report of cry1 binding ATP (Bouly J.P. *et al* 2003).

1.3.2 The cryptochrome C terminus (CCT)

Depending in the nature of the C-terminal (CCT) extension crys are defined in 2 types, being classified as cry1 or cry2 by the length and sequence of the CCT. AtCCT1 has 182 amino acids and AtCCT2 112, CCTs from animals are mostly smaller.

The CCT is the functional part of the cry protein in regard to signal transduction, as GUS-CCT were functional and able to complement cry mutants (Yang H.Q. *et al* 2000), but CNT did not (Ahmad M. *et al* 1998b). Domain swapping between Atcrys rendered chimeric constructs formed by CNT2+CCT1 and CNT1+CCT2 which gave cry1 and cry2 responses, respectively (Ahmad M. *et al* 1998b). Therefore, CCT gives specific fluence response. CCT2 responds to blue low-fluence rates, and CCT1 to medium/high blue light fluence rates.

CCT1 and CCT2 in *Arabidopsis* have divergent sequence, although common motifs can be found on both of them. They are named D for the common DQXVP residues, A for an Acid stretch of 4 amino acids, and S for the common STAESS residues. The function of these conserved motifs is still unknown. When looking at the distribution of these common DAS motifs one can realize that distances from D to A and from S to the end are similar in both crys, but greater distances are found in cry1 between the beginning of the C-term to D and between A to S (Fig.1.8).

In addition to these common motifs, CCT2, but not CCT1, has a functional bipartite nuclear localization signal (NLS) surrounding the A motif, also present in mouse CCT2 (Kobayashi K.

et al 1998). For *Arabidopsis* cry2 it was shown that this NLS is sufficient for its nuclear localization (Kleiner O. *et al* 1999).

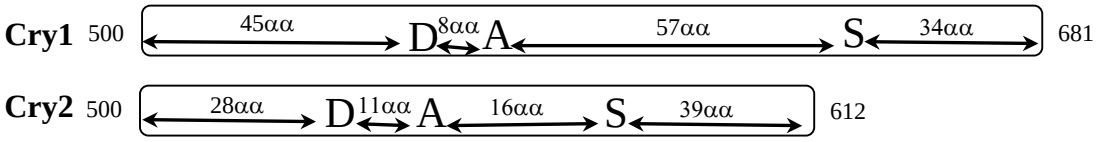


Fig. 1.8 Distribution of common motifs in AtCCT: D (DQXVP), **A** (Acidic), **S** (STAESS)

The discovery and molecular cloning of crys from plant species different from *Arabidopsis*, like tomato (Perrotta G. *et al* 2000), rice (Hirose F. *et al* 2006), and pea (Platten J.D. *et al* 2005b) has allowed a deeper comparison amongst these photoreceptors, making possible the identification of new specific domains for each CCT1 and CCT2 (Fig. 1.9).

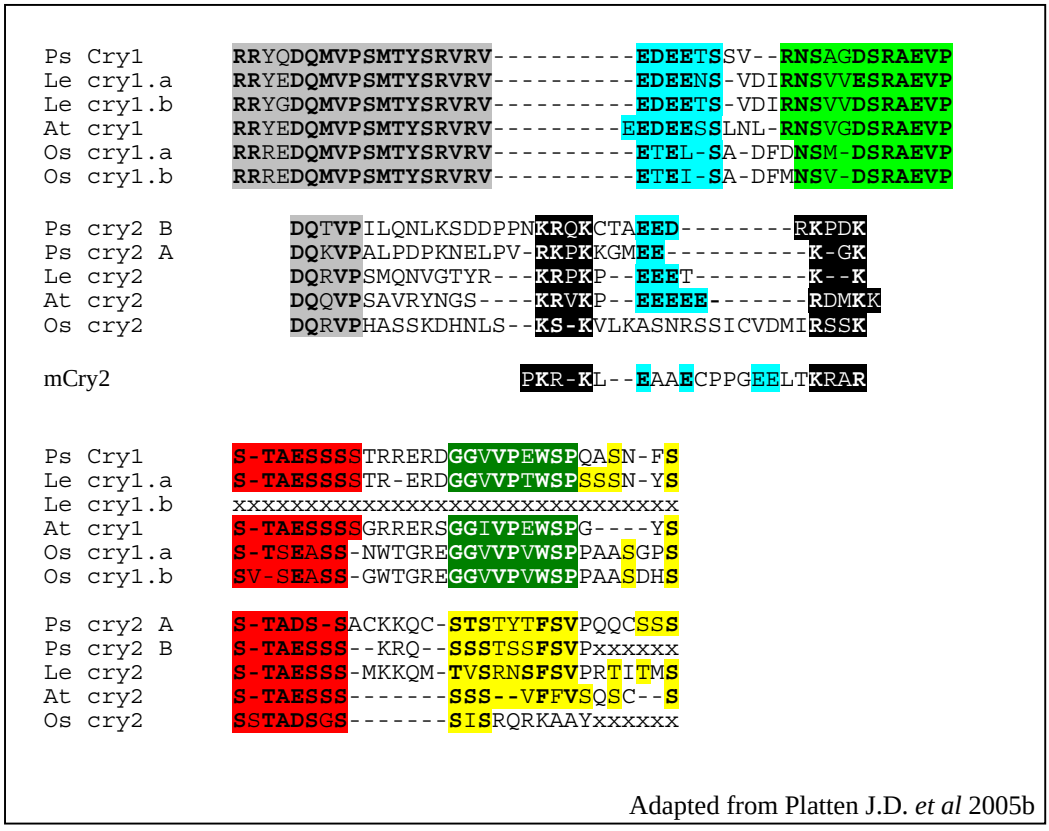


Fig. 1.9 Cryptochrome C-terminal comparison amongst plant species:
Ps: pea (*Pisum sativum*); Le: tomato (*Lycopenum esculentum*); At: thale cress (*Arabidopsis thaliana*); Os: rice (*Oryza sativa*); m: mouse (*mus musculus*). Conserved motifs are highlighted in coloured boxes, bold letters are used for consensus conserved residues. Grey: D motif, Black: bipartite NLS, blue: A (Acid) motif, light green: M (middle), dark green: G, Red: S_1 (STAESSS), yellow: S_2 (Ser rich motif). As C-terminals have different length gaps are labelled as x.

Combining the information of Fig.s 1.8 and 1.9 a detailed map of AtCCT domains composition and distribution can be drawn (Fig. 1.10). Cry1 contains extra domains initial (I), middle (M) and final (F) not present in cry2. Moreover, the D domain of CCT1 is larger than

in CCT2, the shared D consensus DQXVP represents just the minimum conserved sequence. The S domain can be renamed as S_1, and it is followed in CCT2 but not in CCT1 by another Ser rich domain, named S_2. Although this domain is not present in CCT1, another domain, named G for GGVVPxWSP, is found in every plant CCT1 at the same position as S_2 in CCT2.

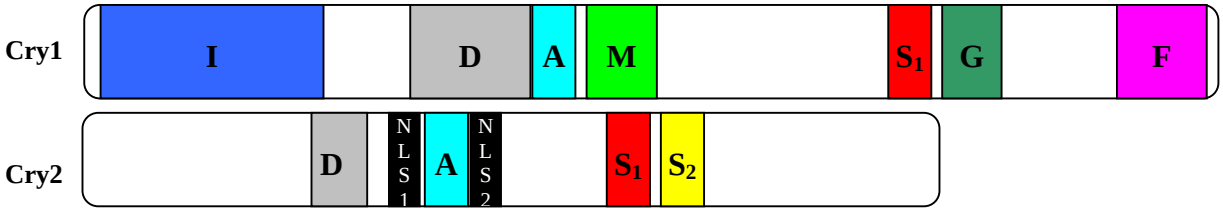


Fig. 1.10 Domain distribution of *Arabidopsis* cryptochromes. Grey: D motif, black: bipartite NLS, blue: A (Acid) motif, light green: M (middle), dark green: G, red: S_1, yellow: S_2 (Ser rich motif).

1.4 Crys activation mechanism

1.4.1 Cryptochrome Photocycle

Because of their huge homology and their evolutionary relationship, the photocycle of *E. coli* CPD PHR has been taken traditionally as a model for cry activation.

PHR binds FAD, and this chromophore can be found in three redox states, oxidized (FADox), one-electron-reduced (semi-reduced, radical form FADH°), and two-electron-reduced (fully reduced $FADH^-$) forms. The flavin cofactor of all photolyases characterized to date becomes oxidized to the FADH° blue-neutral radical, or to FADox during purification under aerobic conditions (Jorns M.S. *et al* 1984). Moreover, a similar oxidation has been seen in FAD from purified cry1 yielding FADox. (Lin C. *et al* 1995a).

The reaction mechanism of CPD photolyase (Sancar G.B. *et al* 1987a & 1987b; Jorns M.S. *et al* 1987) consists of light-driven electron transfer from the fully reduced flavin ($FADH^-$) to the CPD damage in the DNA, creating an instable CPD radical anion and a neutral flavin radical ($FADH^o$). The CPD radical causes a spontaneous cleavage of the carbon bonds within the cyclobutane ring, and transfers the electron back to $FADH^o$ (Fig. 1.11).

In PHR a blue light photon (350-450 nm) can be absorbed by MTHF. The 5, 10-methenyl bridge of the MTHF is responsible for the near-UV absorption at 360 nm. However, once bound to PHR, MTHF absorption undergoes a red shift, absorbing at higher wavelengths (Sancar A. 2003). The absorbed energy is transferred from MTHF to the flavin, having been this

step also documented for cry1 from *Vibrio cholerae* (Saxena C. *et al* 2005) and cry3 from *Arabidopsis* (Song S.H. *et al* 2006; Klar T. *et al* 2007).

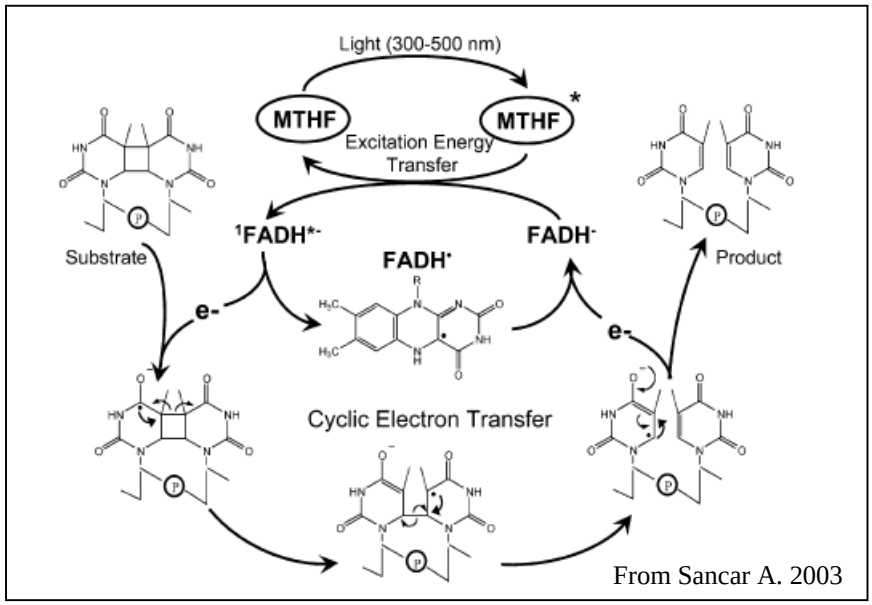


Fig. 1.11 Photocycle of CPD photolyase

If the flavin in PHR is not in the fully reduced state it can be reduced upon excitation, a process called photoreduction. An electron is transferred from the environment to FADox or FADH° to produce the active $FADH^-$ form (Payne G. *et al* 1987). In cry1 photoreduction also occurs, going from FADox to a pretty stable FADH° redox state (Lin C. *et al* 1995a, Kottke T. *et al* 2006). For the photoreduction a group of 3 tryptophans (W) have been found to participate in the transport of the electrons from the medium to the FAD. In *E. coli* PHR W_{382}, W_{359}, and W_{306} are necessary *in vitro* for photoactivation (Aubert C. *et al* 2000), but not *in vivo*, where only the external W306 is needed (Li Y.F. *et al* 1991). In cry1, the electron transfer to the FAD has been also recorded (Giovani B. *et al* 2003). Moreover, the triple W chain is conserved. $W_{400,}$ $W_{377,}$ W_{324} are the residues involved in cry1 electron transfer, and needed for its function *in vivo* (Zeugner A. *et al* 2005).

Very recent work has provided further insight about the *Arabidopsis* cry pohotocycle (Banerjee R. *et al* 2007 Bouly J.P. *et al* 2007). These works correlated the semi reduced FAD redox state of cry with the protein activity, showing a difference in activation between cry and PHR. Because some of the results of this thesis were included in those investigations, they will be further described in the result and discussion sections.

1.4.2 Early events in cry activation

1.4.2.1 Phosphorylation

Phosphorylation is tightly associated with cry activity. It depends not only on blue light, but also on the presence of the FAD cofactor. However, the second chromophore is not necessary for phosphorylation *in vitro*. (Bouly J.P. *et al* 2003 ; Özgür S. & Sancar A. 2006). Moreover, phosphorylation in darkness can be archived by manipulation of the redox state of FAD. *In vitro* chemical reduction of FAD leads to phosphorylation of cry1 (Bouly J.P. *et al* 2003). In addition, cry1 can be autophosphorylated *in vitro* (Bouly J.P. *et al* 2003; Shalitin D. *et al* 2003; Özgür S. & Sancar A. 2006), and the target residues for phosphorylation were identified as serines (Bouly J.P. *et al* 2003). Consistent with autophosphorylation activity, is the fact that, both, cry1 and cry2, bind ATP (Bouly J.P. *et al* 2003; Özgür S. & Sancar A. 2006), being the affinity of cry2 higher than the one of cry1. In addition, the crystal of CNT1 soaked with an ATP analog showed thismolecule in the structure (Brautigam C.A. *et al* 2004). However, GUS-CCT expressed in plant lacks the ATP binding CNT domain, but is constitutively phosphorylated (Shalitin D. *et al* 2002).

Crys can be phosphorylated *in vitro* in the S_1 motif of the CCT by phyA under R, FR or blue light (Ahmad M. *et al* 1998a). Phosphorylation *in vivo* occurs in both crys with different kinetics, and blue light fluence dependency (Shalitin D. *et al* 2002 & 2003). Cry1 gets phosphorylated when exposed to a minimum of 10 μmol m^2 s^{-1} during 15 minutes. This reaction is fluence rate dependent, and higher phosphorylation is obtained with higher light intensities. However, it is important to note that independently of the fluence used always a fraction of cry1 remained unphosphorylated. Cry2 minimum conditions for phosphorylation are 5 μmol m^2s^{-1} during 10 minutes and maximum phosphorylation was reported under 20 μmol m^2s^{-1} of blue light during 15 minutes. Moreover, cry1 can be phosphorylated *in vivo* in *phyA, phyB, phyAB, phyABD, phyBDE* and *cry2* mutants, whereas non functional mutants of *cry1* are not phosphorylated. However, phosphorylation by phyA, of these same *cry1* mutants *in vitro* could be successfully conducted (Ahmad M. *et al* 1998a). In line with the *in vivo* results for cry1 is the finding that cry2 can be phosphorylated *in vivo* in *phyA, phyB, phyD, phyE, phyAB, phyABD, phyBDE,* and *cry1* mutant background. However, in both cases phosphorylation *in vivo* happened under blue light, but never under red light. It is also worth to repeat that CCT2 as a GUS fusion is constitutively phosphorylated *in vivo* (Shalitin D. *et al* 2002) independently of the light conditions. In summary, the current data have shown that plant cryptochromes are rapidly

phosphorylated in blue light, that phosphorylation correlates with biological activity, and that phytochromes are not essentially required for this process. In addition, it has been demonstrated that phosphorylation of cry2 occurs in the absence of cry1 and *vice versa* (Shalitin D. *et al* 2003), indicating that each cry regulates its own phosphorylation. Nevertheless, the sites within cry1 and cry2 that are phosphorylated *in vivo* and *in vitro* have to be determined in order to address the molecular mechanism of cry phosphorylation, and the specific role of each phosphorylation site in their biological function.

1.4.2.2 Conformational change

Because CNT alone is not functional (Ahmad M. *et al* 1998b), but CCT is (Yang H.Q. *et al* 2000), and constructs of any CNT1 or CNT2 with CCT are equally functional, it was postulated that CNT inhibits CCT and that this inhibition is released by a conformational change upon light excitation. In this model CNT would inhibit CCT action and only once activated by light CCT will be released and functional (Fig. 1.12).

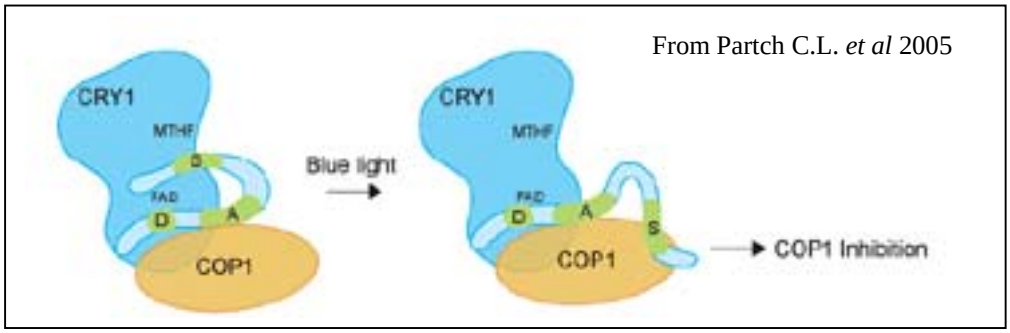


Fig. 1.12 Cryptochrome conformational change model

In vitro interaction between CCT and CNT has been shown, and cry1 exposed to blue light seems to be more sensitive to proteolysis in accordance with a flexible CCT (Partch C.L. *et al* 2005). Moreover, spectral changes by Fourier transform infrared (FTIR) experiments also revealed small changes of the blue light-irradiated CNT1 structure (Kottke T. *et al* 2006).
In any case, it is difficult to predict the event when this conformational change would occur, before or after phosphorylation, and further research is needed to unravel its mechanism.

1.4.2.3 Formation of nuclear speckles

Once activated, Cry1 (Wang H. *et al* 2001) cry2, phyB (Más P. *et al* 2000) and PhyA (Kircher S. *et al* 1999) have been found to form nuclear speckles. These bodies are believed to be situated in places of transcription regulation, where multiple factors interact. In accordance with this hypothesis, COP1 (Wang H. *et al* 2001) and HY5 (Ang L.H. *et al* 1998) have been also found to form nuclear speckles. So far, the identity of some speckles components has been addressed by GFP and related fluorescent proteins fusions. Purification and identification of all the proteins present in the speckles would be expected to be very informative about photoreceptor activity regulation and its signalling.

1.4.2.4 Degradation and signal turnover

Cry2 is regulated at the protein level by light-induced turnover. Both, cry2 protein stability and phosphorylation have been shown to be correlated and dependent on similar fluence rates (Shalitin D. *et al* 2002). High fluences of blue light promote cry2 phosphorylation, becoming unstable and rapidly degraded (Lin C. *et al* 1998; Guo H. *et al* 1999; Shalitin D. *et al* 2002). Under low fluence rates of blue light cry2 is stable and active over long time (Lin C. *et al* 1998). The exact mechanism of cry2 degradation has not been elucidated yet. However, evidence points to ubiquitin labelling by COP1 and proteosomal degradation. Weak mutant alleles of *cop1* showed enhanced stability of phosphorylated cry2. On the other hand, fusion of cry2 with the very stable GUS protein at its N-term enhanced its stability (Guo H. *et al* 1999) under blue light leaving the possibility of involvement of the N-end rule degradation pathway (Varshavsky A. 1996). For animal crys it is known that they are ubiquitinated by an F-box protein (Busino L. *et al* 2007; Siepka S.M. *et al* 2007). Nevertheless, a direct evidence for Atcry2 ubiquitination and the lysines involved in it is still lacking.

Phosphorylation can occur in CCT of both crys but only cry2 is degraded, and CCT1 lacks lysines (K), the amino acid which is targeted for bearing the ubiquitin chains. Therefore, one could speculate that the CCT is the region responsible for degradation. Surprisingly the constitutively active and phosphorylated GUS-CCT2 is highly stable (Yang H.Q. *et al* 2000). Moreover, cry chimeras consisting in domain swapped CNT1+CCT2 and CNT2+CCT1

showed degradation (Ahmad M. *et al* 1998b) giving to both CCT2 and CNT2 a role in its own degradation.

Cry1 is not degraded, but excluded form the nucleus, very probably together with COP1 (Osterlund M.T. & Deng X.W. 1998; von Arnim A.G. & Deng X.W. 1994; von Arnim A.G. *et al* 1997, Wang H. *et al* 2001). COP1 affects photoreceptor localization and stability (Fig. 1.13).

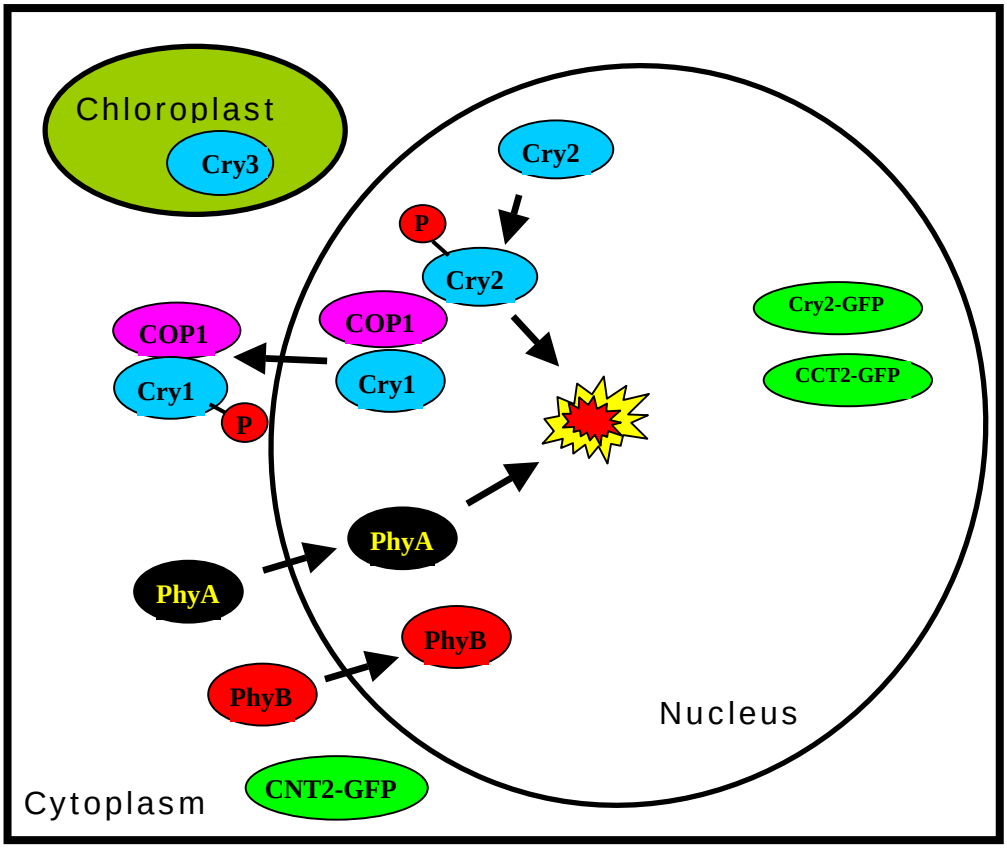


Fig. 1.13 Light dependent subcellular localization of crys and partners

Cry1 seems to join COP1 in its cellular localization (Wang H. *et al* 2001), because, like COP1, it is found in the nucleus in darkness (Cashmore A.R. *et al* 1999), but in the cytoplasm when exposed to light (Yang H.Q. *et al* 2000).

The mentioned above NLS present in Atcry2 is conserved in mouse cry2, and it has been proven to promote its active localization to the nucleus, helping thereby other proteins to enter the nucleus (Sakakida Y. *et al* 2005).

The cellular localization of phys, *bona fide* protein interactors of crys, is also altered upon light irradiation. Phys are cytoplasmatic proteins in darkness. However, once activated by light they are translocated to the nucleus, where they are active (Nagy F. & Schäfer E. 2000). As mentioned above, after activation phyA is unstable being targeted by COP1 for degradation (Seo H.S. *et al* 2004). Whereas, the other phys remain stable and nuclear.

2 Materials & Methods

2.1 Cloning and Plasmids

2.1.1 General cloning procedure

All restriction enzymes were supplied from Fermentas. Restriction cuts of plasmids were performed overnight using 0.5 units of enzyme and the suitable enzyme buffer provided by the manufacturer. Generation of blunt ends was performed with Klenow fragment (Fermentas #EP0051), or T4 DNA polymerase (Fermentas #EP0061) as indicated in each case.
Isolation of DNA fragments was performed with QIAex II gel extraction kit (Quiagen) after running the DNA fragment in an agarose gel and cutting the desired bands following the manufacturer instructions. DNA ligations using T4 DNA ligase (Fermentas #EL0334) were performed overnight.

2.1.2 Plasmids

Complete plasmids maps for pMENCHU can be found in Ferrando A. *et al* 2000. pGIGI is described in Ferrando A. *et al* 2001. Information about pPCV812 is available in Koncz C. *et al* 1989. The pFGC5941 plasmid is shown in Fig. 2.1

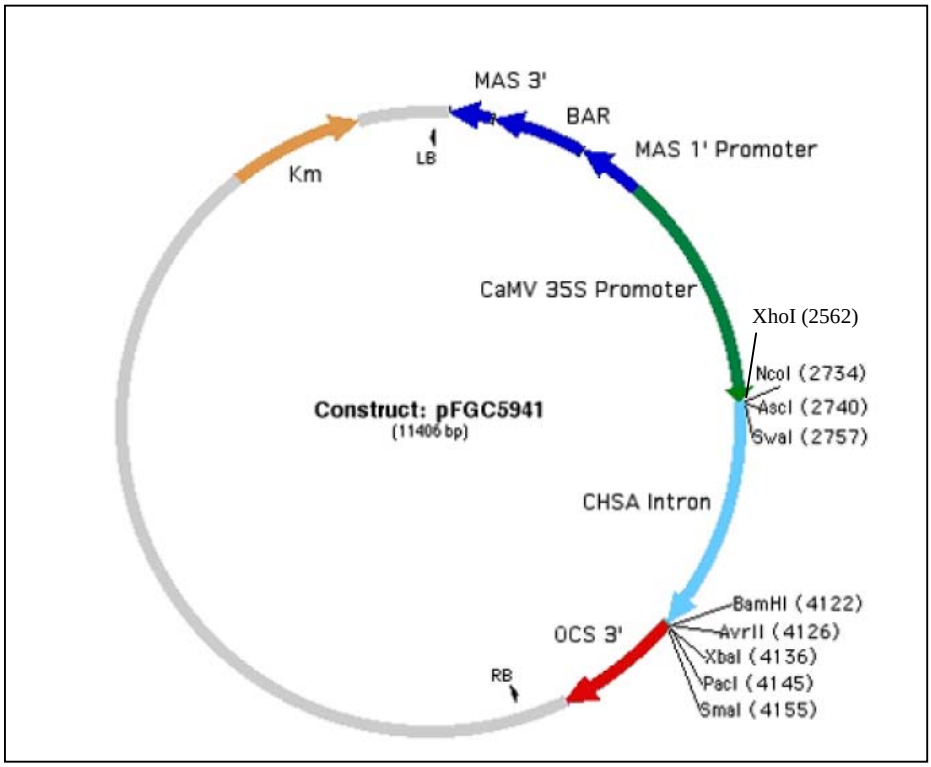


Fig. 2.1 pFGC5941

2.1.3 Cloning Cry2 c-myc and HA tagged versions

Cry2 contains the cutting sites for the following common restriction enzymes: BglII, PstI, EcoRI, HindIII, NcoI. Therefore, they can not be used for clonning purposes. *Cry2* was isolated from a pre-existent pGEM-*cry2* plasmid from our lab. It was cut in XbaI and NcoI sites to generate cohesive ends. The isoschizomer BsmBI enzyme was used to avoid cutting cry2. The fragment was ligated in pGIGI and pMENCHU using NcoI and XbaI sites. Ligation of the BsmBI and NcoI cohesive ends destructed both restriction sites.

pGIGI and pMENCHU plasmids contain a tag, *c-myc* in case of pGIGI and *HA* for pMENCHU, divided with a plant intron (giving c-mycIT and HiA tags) to avoid its expression in bacteria. The cloning placed the tag in the C-terminus of our *cry2* gene. Insert containing twice the overexpressor promoter CaMV35S + cry2-*HiA* + NOS terminator was cut from pMENCHU with SacI, made blunt and released by cutting with XhoI. This fragment with the *HA* epitope was ligated on the pFGC5941 binary vector.

pFGC5941 was cut with BamHI, filled with T4 DNA polymerase (Fermentas #EP0061) to give a blunt end, and cut again with XhoI releasing the *CHSA* intron. Insert was ligated rendering a pFGC5941 3xCAMV35S::CRY-2-HiA NOS construct (Fig. 2.2)

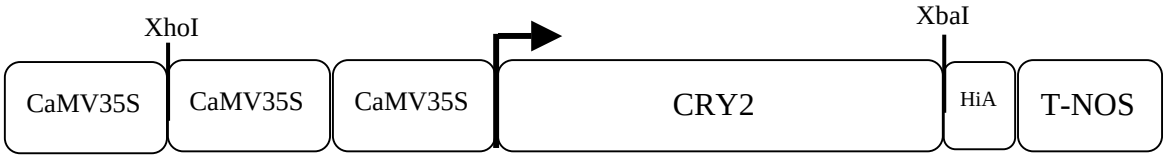


Fig. 2.2 Clonning of cry2-HiA on pFGC5941

Insert containing twice the overexpressor promoter CAMV35S + cry2-cmycIT + NOS terminator was cut from pGIGI with SacI and XhoI. This fragment with the c-myc epitope was ligated on the pPCV812 binary vector. pPCV812 was cut with SacI and XhoI. Insert was ligated overnight using its XhoI and SacI sites rendering a 2xCAMV35S::*cry2-HiA* NOS in pPCV812 (Fig. 2.3).

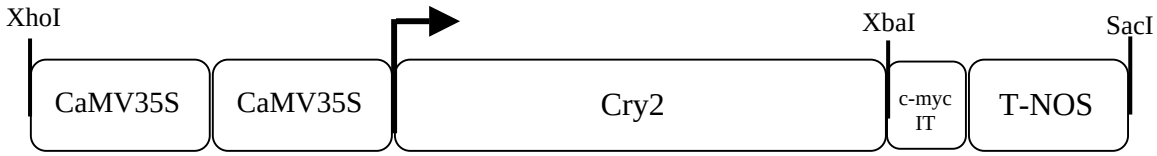


Fig. 2.3 Clonning of cry2-cmycIT into pPCV812

Cry2-cmycIT was also cloned on pFGC5941 following the same strategy as *cry2-HiA*. pGIGI was cut with Sac I, made blunt and released by cutting with XhoI. This fragment with the *c-myc* epitope was ligated on the pFGC5941 binary vector.

pFGC5941 was cut with BamHI, made blunt with T4 DNA polymerase, and cut again with XhoI releasing the CHSA intron. Insert was ligated using its XhoI and blunt end sites rendering a 3xCAMV35S::cry2-myc NOS terminator in pFGC5941 as illustrated in Fig. 2.4

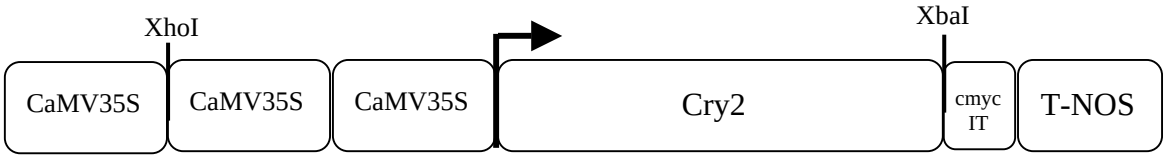


Fig. 2.4 Clonning of *cry2-cmycIT* into pFGC5941

2.1.4 Cloning Cry2-GFP, CNT2-GFP and CCT2-GFP into PCV812 vector

Cry2-GFP, CNT2-GFP and CCT2-GFP were cut from pMAV4 described in (Kleiner O. *et al* 1999) with BamHI and Sac I to release a fragment containing cry2 full length, CNT or CCT fused to GFP. pPCV812-GUS was cut on the BamHI and SacI sites, releasing the GUS fragment. The different fragments and the pPCV812 plasmid were ligated yielding a pPCV812 CAMV35S::cry2 (or truncated version)-GFP NOS terminator as illustrated in Fig. 2.5

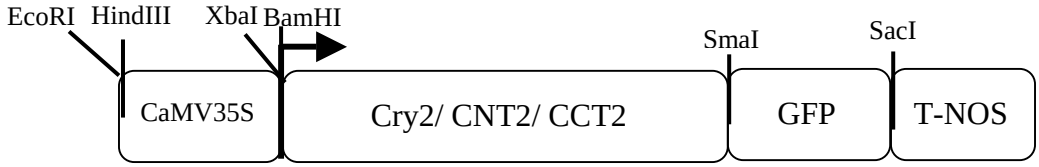


Fig. 2.5 Clonning of cry2-GFP on pPCV812

2.2 Plant material, growth conditions, light treatments and bacterial strains

2.2.1 Plant material

Arabidopsis thaliana wild types used were from *Lansberg erecta* (Ler) and *Columbia* (Col) ecotypes. *Cape verde* island (Cvi) ecotype described in (El-Assal S.E.D. *et al* 2001) was on Ler background. *Arabidopsis* CCT2-GFP, CNT2-GFP and cry2-GFP transgenic plants were generated on Ler background. Cryptochrome single mutants *cry1* (hy4-B104 *Bruggemann E.P.* et al *1998)*, *cry2* (fha-1*Guo H.* et al 1998) were on Ler background as well as *cry1cry2* double mutant (Mockler T. et al 1999). Tobacco was *Nicotiana benthamiana*.

Arabidopsis transgenic lines expressing full length and truncated cry2-GFP versions were already available in our lab. They were generated in Ler by the floral dip method (Clough S.J. &

Bent A.F. 1998) with a GV3101 *Agrobacterium* strain (Koncz C. *et al* 1986) harbouring the pMP90 helper plasmid and carriying the pPCV812 (carbecillin and hygromicin resistant) plasmid. The plasmid containined a full lenght cry2 (amino acids from 1-612) or truncated versions of the protein, CCT2 (amino acids ranging 501-612) or CNT (amino acids ranging 1-500) all of them fused to the green fluorescent protein (*GFP*) (Fig.2.8). The expected size of the protein constructs are: 98 kDa for CRY2 (1-612)-GFP, 89 kDa for CNT2 (1-500) -GFP, 38 kDa for CCT2 (501-612)-GFP. After hygromicin selection, the F_2 offspring was checked for expression of the transgenic protein by western blot (see results section).

2.2.2 Plant growth conditions

2.2.2.1 Growth on soil

Arabidopsis seeds were sown on soil containing pots, and kept for stratification on darkness at 4°C for 3 days, after that, they were moved to the suitable growth chamber with the desired light conditions.

2.2.2.2 Growth on filter paper

5 layers of normal paper and one layer of strong paper, measures 8x8 cm, were pre-soaked twice on normal tap water for 10 minutes, and a final time on distilled water for 10 minutes more. Wet (but not dripping) paper was placed on a box with the strong paper facing up. Seeds were sown in the paper, the boxes were closed and put inside a black box. Stratification was conducted for 3 days on a cold room at 4°C then a light treatment was done for about 6 hours and the boxes were kept in darkness and at 22°C for 5 days. The etiolated seedlings were then ready for harvesting (Dark samples were harvested under dim green light) or light treatment.

2.2.2.3 *Arabidopsis* cell culture

The *Arabidopsis* green and white cell cultures were a gift from Koncz C. (MPI for plant breeding, Köln).Culture was kept at temperature of 22-24°C, and continuous shaking at 100 rpm. Green culture was kept under long day photoperiod (16:8), white in continuous darkness. The cell culture must be subcultured each 7 days in sterile conditions. To subculture 10 ml old culture were added to 40 ml of cell culture medium in a 250 ml Erlenmeyer

Cell culture medium	stock	final	1L	Company (Cat n°)
MS-vitamins	Powder	x1	4.3 g	Duchefa (M0221.0001)
Sacharose	Powder	88 mM	30 g	Roth (4621.1)
Gamborg + B5	x40	x1	2.5 ml	Duchefa (G0210)
NAA	1 mg/ml	0.1 mg/L	100 µl	Sigma (N1641)
Kinetin	1mg/ml	0.5 mg/L	500 µl	Sigma (K3253)

After setting the pH to 5.8 with KOH, the medium must be autoclaved

2.2.3 Light treatments/light conditions

Fluence rates (µmol m^{-2} s^{-1}) of blue light were measured at 440 nm with a light analyser from Gigahertz optik (model BN-9201.2-TF). White light was measured with a Li-190 light sensor (Licor).

2.2.3.1 Light conditions in the growth chambers

Long day (LD) consisted in 16 h light using 160 µmol m^{-2} s^{-1} fluence and 8 h dark (16:8), at 22°C. Short day (SD) consisted in 9 h light and15 h dark (9:15), at 25°C light fluences varies with the experiment from 100 to 160 µmol m^{-2} s^{-1}

2.2.3.2 Light treatments for cry phosphorylation

5 days old seedlings grown in darkness on paper were exposed to blue light, fluence rates modified by removing the lid of the seedling growth box or by applying layers of paper on top of the boxes. Fluence rate is indicated in the text on every case

2.2.3.3 Light treatments for *CO* and *FT* gene expression

Plants were grown on soil under SD (8 h light/16 h darkness) for 7 days before the treatments with monochromatic light. Monochromatic light treatments were for 72 h for gene expression All mono and bichromatic light treatments (blue: 470 +/- 10 nm; green: 563 +/-12 nm) were performed using a fluence rate of 2 µmol m^{-2} s^{-1}, applied through double interference filters (Schott, Germany).

2.2.3.4 Light treatments for blue light dependent gene expression

3 days old green or white cell cultures were kept on darkness overnight. Unless other conditions are specified on the Fig. legend, cultures were kept shaking under 33 µmol $m^{-2}s^{-1}$ blue light for 5 h, controls were kept in darkness for the same time.

2.2.4 *Agrobacterium* Strains

2.2.4.1 *Agrobacterium* strains and plasmids used

The GV3101 strain was used on all cases (Koncz C. & Schell J 1986), except for the p19 helper plasmid which was in the pCH32 (Voinnet O. *et al* 2003)..

GV3101 (pMP90) has a Rif resistance gene included in its genome, the helper plasmid gives Kan resistance and harbors the vir operon required for Ti plasmid replication. Different plasmids carrying the proteins of interest were inserted in the *Agrobacterium* strain by the conjugation method using *E.coli* S-17 as described in Koncz C & Schell J. 1986. yielding the strain collection described below.

Construct	**strain**	**helper**	**plasmid**	**resistance**	**Source**
P19	C58C1	pCH32		Rif/Kan/Tet	Voinnet O. *et al* 2003
Cry2-HiA	GV3101	pMP90	pFGC5941	Rif/Kan/Gen	This work
Cry2-mycIT	GV3101	pMP90	pFGC5941	Rif/Kan/Gen	This work
Cry2-mycIT	GV3101	pMP90	pPCV812	Rif/Kan/Cb	This work
Cry2-GFP	GV3101	pMP90	pPCV812	Rif/Kan/Cb	This work
CHS::GUS	GV3101	pMP90	pBI101	Rif/Kan/Gen	Kaiser T. *et al* 1995
GUS-HiA	GV3101	pMP90	pPCV812	Rif/Kan/Cb	Ferrando A. *et al* 2000
GFPuv	GV3101	pMP90		Rif/Kan/	G.Freymark gift

Antibiotic	**stock (mg/ml)**	**final (µg/ml)**	**Company (Cat N°)**
Rifampicin (Rif)	25	50	Duchefa, Netherlands (R0146)
Tetracycline HCl (Tet)	12,5	10	Duchefa, Netherlands (T0150)
Kanamycin (Kan)	50	25	Roth (T832.2)
Gentamicin sulphate (Gen)	10	15	Duchefa, Netherlands (G0124)
Carbenicillin disodium (Cb)	50	50	Duchefa, Netherlands (C0109)

2.2.4.2 *Agrobacterium* growth conditions

Agrobacteria were grown using YEB medium with suitable antibiotics for 2 days at 200 rpm and 28°C. The second day OD_{600} was checked and set to OD_{600}=0.2. *Agrobacteria* were grown again overnight at the same conditions as before until they reach the maximum of the logarithmic growth phase $OD_{600=}$ 0.8-1.

YEB medium	stock	final	Company (Cat N°)
Beef extract	---	5 g/L	DIFCO labs (0126-17-0)
BactoYeast	---	1 g/L	DB (212720)
Bacto Pepton	---	5 g/L	DIFCO labs (0118-17-0)
Saccharose	---	5 g/L	Roth, (4621.1)
Bacto-Agar	---	15 g/L	DB (214010)
$MgCl_2$	1 M	1.4 mM	Roth (2189.1)
CaC l_2	0.1 M	44 µM	AppliChem (A1873.1000)

2.2.4.3 *Agrobacterium* mediated tobacco infiltration:

This method is described in (Voinnet O. *et al* 2003). It allows the fast overproduction of protein in tobacco leaves by infiltrating *Agrobacterium tumefaciens* through open stomata.
Agrobacteria were incubated in MAM (MES, Acetosyringone, $MgCl_2$) medium at concentration of OD_{600}=1 for 4-5 h before infiltration. The *Agrobacteria* were infiltrated in 1 month old tobacco leaves with a syringe and plants were left 5 days on a LD chamber before harvest.

MAM solution	stock	final	Company (Cat N°)
MES/KOH pH 5.6	1 M	10 mM	Roth (4256.2)
Acetosyringone	150 mM	150 µM	Aldrich (D13,440-6)
$MgCl_2$	1 M	10 mM	Roth (2189.1)

2.3 Protein Methods

2.3.1 Protein extract preparation

Dark grown or irradiated plant samples were prepared under red safe light. Frozen plant material was grinded in liquid nitrogen with a bead mill (Retsch MM200) in presence of 1 volume (1ml of buffer per gram of plant material) of native extraction buffer. The resulting frozen powder was taken to Eppendorf tubes and placed on ice, thawed for 30 min, and centrifuged at 20,000 g for 15 min at 4°C. The supernatant was collected and centrifuged again in the same conditions to obtain clear extracts. Protein amount was measured by the amido-black method.

Native buffer	stock	final	10 ml	Company (Cat N°)
NaCl	5 M	400 mM	800 µl	Roth (3957.1)
Tris pH 7.5	1 M	100 mM	1 ml	Roth (4855.2)
Glycerol	50%	10%,	2 ml	Roth (3783.2)
Dithiothreitol(DTT)	0.5 M	1 mM	20 µl	Applichem (A1101,0025)
EDTA	0.5 M	1 mM	50 µl	Roth (8043.2)
Igepal CA-630	10%	1%	100 µl	Sigma (I-3021)
Complete (EDTA-free)	x10	x1	1 pill	Roche (11836-170-001)
dH_2O	----	---	5.75 ml	

A commercial mix was purchased, PPase inhibitor cocktail setII Calbiochem (Cat N° 524625): Imidazole 2 mM, NaF 1 mM, molybdate 1.15 mM, VO_4^{-3} 1 mM, tartrate 4 mM and used as indicated by the supplier when indicated in the text

2.3.2 Protein measurement by amido black

The amido black determination of protein amount is described in Popov N. *et al* 1975. 5 µL of protein extract was diluted on 195 µl dH_2O, 800 µl amidoblack solution was added and mixed by inverting 4 times, samples were centrifuged for 12-15 min at 20,000 g, supernatants were discarded and pellets were washed with 1 ml washing solution following centrifugation for 12-15 min at 20,000 g. Supernatans were discarded again and pellets were air dried for 30 min at room temperature. Pellets were resuspended in 1 ml 0.2 M NaOH and absoption at 615 nm was measured in a spectrometer (taking 0.2 M NaOH as reference). Results were compared with a BSA standard calibration curve of known concentrations.

Solution	final	0,5L	Company (Cat N°)
Dye			
Methanol	90%	450 ml	Roth (4627.2)
Acetic acid	10%	50 ml	Roth (3738.5)
Amido Black B10	OD10	*	Roth (95901)
Wash			
Methanol	90%	450 ml	Roth (4627.2)
Acetic acid	10%	50 ml	Roth (3738.5)
Measurement			
NaOH (0,2 g/pellet)	0.2 M	4 g	Roth (9598.1)

* Little amounts must be added, mixed and measured at Abs 615 nm until the desired OD10 is reached (dilution of the dye 1:10 is needed before the measurement).

2.3.3 Dephosphorylation by lambda phosphatase (λPPase) treatment

The enzyme from New England biolabs (#P0753S) was used; all reagents were included on the kit. Reaction was kept at 30°C for 30 min, and immediately denatured by boiling at 94°C in presence of SDS denaturing buffer. Controls were made incubating the same amount of sample on the same conditions and buffers but without λPPase

Reagent	stock	final	amount
Sample	any	any	40 µl
Buffer	x10	x1	5 µl
MnCl	20 mM	2 mM	5 µl
λPPase	400 U/µl	400 U	1 µl

2.3.4 Chemical Crosslinking

Protein extract of tobacco leaves expressing Cry2-HA was used for chemically crosslinking. Different amounts of native protein extracts were kept with 10 µl of crosslinker (bufferF), native buffer was added to reach the same final volume of 20 µl in every case, giving protein final concentrations of 0.5 0.75 and 1 µg/µl. These mixtures were incubated at 10°C under red light for 20 min.

BufferF	stock	final (x2)	100 ml (x2)	Company (cat N°)
KH_2PO_4	0.5 M	1,76 mM	352 µl	Roth (3904.1)
Na_2HPO_4	0.5 M	10 mM	2 ml	Roth (4984.1)
NaCl	5 M	136 mM	2.72 ml	Roth (3957.1)
KCl	0,5 M	2,6 mM	0.52 ml	Applichem (A2939,1000)
$MgCl_2$	1 M	5 mM	500 µl	Roth (2189.1)
Glycerol	100%	10%	10 ml	Roth (3783.2)
Formaldehyde	37%	1%	2.7 ml	Roth (4979

2.3.5 Immuno precipitation (Pull-down)

1-2 mg of total protein extracts were incubated with anti-GFP antibody (goat) 1:200 dilution 2 hours in a rotatory wheel at 4°C. Sequently 100 µL of Protein-G agarose, (Roche, Cat n° 1 243 233) diluted in native buffer (1:2) were added to each tube and spun in a rotatory wheel 1 h at 4°C in darkness. Beads were washed 3 times with 1 mL native buffer, and as the next step 3 different methods were used for elution.

For samples that were going to be used right away for electrophoresis 100 ml of denaturing SDS buffer were added to and boiled for 5 min at 95ºC. For the chromophore composition experiments of cry2, two different kinds of elutions were performed.

A mild elution was done by using a glycine buffer at pH 3 and gentle shaking for 3 h at 4°C. Under these conditions the protein is released from the protein-G agarose beads. A stronger elution was done by incubating the beads gently shaking for 2 h at 4°C in the presence of 90 µl of native buffer supplemented with 10 µl of 72% TCA yielding a final TCA concentration of 7%. Under these conditions the protein was denatured and precipitated, releasing the chromophores to the medium. In both cases, the sample was centrifuged at 20,000 g and the chromophore containing supernatant was measured by fluorescence spectroscopy.

2.3.6 Protein electrophoresis

Prior to electrophoresis samples were denatured by boiling at 94°C for 5 min in presence of SDS denaturing buffer. 15 µg of each measured sample or 25 µL of eluate, when the protein amount was unknown, were run on 10% SDS-PAGE mini gel (Biorad) at room temperature with 120 V for 2 h. 20 µg of each measured sample or 100 µl of eluate, when the protein amount was unknown, were loaded on a 10% SDS-PAGE big gels and run at 4°C 40 mA for 6-9 h.

SDS denaturating buffer	**stock/Mw**	**final (x5)**	**100ml (x5)**	**Company (cat N°)**
Tris/HCl pH 6.8	1 M	225 mM	22.5 ml	Roth (4855.2)
Glycerol	liquid	50% (v/v)	50 ml	Roth (3783.2)
SDS	powder	5% (w/v)	5 g	Roth (2326.2)
Blue Bromo-Phenol	powder	0.05%	50 mg	Roth (A512.1)
Dithiothreitol (DTT)	powder	250 mM	3.86 g	Applichem (A1101,0025)
dH_2O	----	----	27.5 ml	

2.3.6.1 SDS PAGE Lämmli

Gels were casted the previous day to use, to allow complete polymerization and enhance the protein separation. Prestained SDS molecular weight markers 7b marker versions I or II (Sigma SDS-7B), were always used to check protein mobility.

Acrylamix	**stock**	**final**	**100ml**	**Company (cat N°)**
Acrylamide	30%	20%	66.6 ml	Roth (3037.1)
Bisacrylamide	2%	0.53%	26.53 ml	Roth (3039.2)
dH_2O	---	---	7.5 ml	------

The Acrylamix must be self prepared

TEMED	---	Roth (2357.3)
Ammonium persulfate (APS)10%		Serva (13375)

Lämmli buffer	**Stock/Mw**	**final**	**1Lx10**	**Company(cat N°)**
Tris/HCl pH 8.3	1 M	25 mM	250 ml	Roth (4855.2)
Glycin	75.7 g/mol	192 mM	145.3 g	Roth (39083)
SDS	10%	0.1%	100 ml	Roth (2326.2)

Two different gel sizes were used the small is referred as mini-gel and the other as big gel.

Mini-gels gel dimensions are: Stacking: 8.3 cm wide x 2 cm high and 0.75 mm of thickness. Separating gels are 8.3 cm wide x 5 cm high and 0.75 mm of thickness

Separating gel (10ml)				
Reagent	Final conc	15%	12%	10%
Acrylamix(20% A + 0,53BisA)	GEL	7,5	6 ml	5 ml
Tris/HCl 1,5 M pH 8,8	375 mM	2,5 ml	2,5 ml	2,5 ml
dH_2O	---	---	1,5 ml	2,5 ml
SDS 10%	0,1%/0,2%	100 µl	100 µl	100 µl
APS 10%	0,04%/0,07%	40 µl	40 µl	40 µl
TEMED 100%	0,05%	6 µl	6 µl	6 µl

Stacking gel (5ml)		
Reagent	Final conc	5%
Acrylamix(20%A + 0,53BisA)	GEL	1,25
Tris/HCl 0,5M pH 6,8	125mM	1,25
dH_2O	---	2,45
SDS 10%	0,2%	100µl
APS 10%	0,07%	38µl
TEMED 100%	0,05%	3µl

Big gels dimensions are: Stacking 18.4 cm wide x 5 cm high and 1.5 mm of thickness. Separating gels are 18.4 cm wide x 13 cm high and 1.5 mm of thickness

Separating gel (40mL)			
Reagent	Final conc	10% gel	12% gel
Acrylamix(20%A + 0,53BisA)	10%	20 ml	24 ml
Tris/HCl 1,5 M pH 8,8	375 mM	10 ml	10 ml
dH_2O	---	10 ml	6 ml
SDS 10%	0,1%	400 µl	400 µl
APS 10%	0,04%	160 µl	160 µl
TEMED 100%	0,05%	24 µl	24 µl

Stacking gel (15ml)		
Reagent	Final conc	5%gel
Acrylamix(20%A + 0,53BisA)	5%	3,75 ml
Tris/HCl 0,5 M pH 6,8	125 mM	3,75 ml
dH_2O	---	7,08 ml
SDS 10%	0,2%	300 µl
APS 10%	0,07%	105 µl
TEMED 100%	0,05%	7,5 µl

The gel cassette was assembled putting 2 big glass plates separated with 3 plastic spacers, 2 in the sides and 1 at the bottom, some hollow space was left on the sides to be filled with agar (1% Agar) to avoid leaking on the joints of the spacers. A solid base was made with a fast polymerization gel 1ml separating gel + 2.4 µl TEMED, The mixture is added to the cassette covering the whole floor of the gel cassette, when the bottom gel was solid the rest of the separating gel can be added, after adding the separating gel isopropanol in water (1:2) was added to make an even level and remove bubbles. When polymerized the isopropanol was removed, washed with dH_2O and air dried. After that the stacking gel was added and the gel is kept at 4°C overnight for next day use.

After run, proteins were detected by western blot analysis or coomassie staining

Coomassie	**final**	**1L**	**Company (cat N°)**
Isopropanol	25%	250 ml	Prolabo (20842,323)
Acetic acid	5%	5 ml	Roth (3738.5)
Coomassie R250	0.1%	1 g	Roth (3862.2)

The gel was destained with water or 5% acetic acid as many times as necessary.

2.3.6.2 2D electrophoresis

2.3.6.2.1 Protein precipitation

Samples were incubated in acetone/10%TCA at –20°C for 1 h, centrifuged at 20,000 g for 10 min at 4°C. Supernatant was removed and pellet was washed twice with cold acetone. After being dried, pellets were resuspended overnight at 4°C in 500 µl 2D buffer (8 M Urea, 2% CHAPS, 0.5% Ampholyte (Pharmalyte), Bluebromophenol, 10 mM DTT).

2.3.6.2.2 First dimension

Proteins were separated according to their isolectric (Íp) point, 18 cm non linear pH: pH 3-11 and pH 4-7 inmobiline drystipes (Amersham, 17-6003-76) were loaded overnight with sample. First dimension was performed overnight following these programs.

pH 4-7 stripes: Gradient 300 V 40 min, step and hold 300 V 40 min, gradient 500 V 1 h, step and hold 500 V 1 h, gradient 1000 V 1 h, step and hold 1000 V 1 h, gradient 8000 V 2 h, step and hold 8000 V 8 h.

pH 3-11 stripes: Gradient 500 V 1 h, step and hold 500 V 1 h, Gradient 1000 V 1 h, step and hold 1000 V 1 h, Gradient 8000 V 2 h, Step and hold 8000 V 2 h.

2.3.6.2.3 Second dimension

Prior to the second dimension run, stripes were equilibrated on 1% DTT containing SDS buffer (50 mM TrisHCl pH 8,8; Urea 6 M Glycerin 30% (v/v); SDS 2% (w/v) 0,002 Blue bromophenol) 15 min and further equilibrated in the previous SDS buffer containing 2.5% Iodoacetamid (Merck). In the second dimension protein was separated according to its molecular weight. Stripes were subjected to a normal 12% SDS-PAGE gel and run overnight. Protein detection was done by western blot or colloidal coomassie and further Mass Spectroscopy analysis.

2.3.6.2.4 Coomassie staining:

Both Maldi and Colloidal coomassie are stainings compatible with MS

Maldi Coomassie for MS	**final**	**1L**	**Company (cat N°)**
Coomassie Brilliant Blue (CBB) R250	0.2%	2 g	Roth (3862.2)
Ethanol	40%	400 ml	
Acetic acid	10%	100 ml	Roth (3738.5)

Colloidal Coomassie for MS	**final**	**Company(cat N°)**
Coomassie Brilliant blue G250	0.08%	Roth (9598.1)
Ortho-phosphoric acid (85%)	2%	Merck (100573)
Ammonium sulfate $(NH_4)_2SO_4$	10%	Roth (9212.1)
Methanol	20%	Roth (4627.2)

On both cases destain washing with MilliQ water performed as many times as necessary

2.3.7 Western-blot

2.3.7.1 Blot transfer

Gels were briefly washed with dH_2O to eliminate the rest of electrophoresis buffer, and incubated 30 minutes in transfer buffer. PVDF membrane (Macherey-Nagel, porablot PVDF) was activated in MeOH and then equilibrated in transfer buffer. Nitrocelulose membranes (Macherey-Nagel, porablot NCP) were only soaked in transfer buffer. 12 Whatman 3MM paper pieces (Macherey-Nagel, Blotting-paper MN 218B) fitting the size of the gel were cut and soaked in transfer buffer previous to assemble the sandwich. In the transfer sandwich the membrane is facing the anode and the gel the cathode. Transfer was performed at 30 mV for 40-60 min,

Transfer Buffer pH=9,2	**Stock**	**final**	**1L**	**Company(cat N°)**
Tris/HCl	powder	48 mM	5.82 g	Roth (4855.2)
Glycin	powder	39 mM	2.93 g	Roth (39083)
SDS	10%	0.1%	10 ml	Roth (2326.2)
Methanol	100%	20%	200 ml	Roth (4627.2)
Set pH to 9.2 with HCl				

TBS-T	**Mw (g/mol)**	**final**	**1Lx10**	**Company(Cat N°)**
Tris/HCl pH 7.5	121.14	20 mM	24.2g	Roth (4855.2)
NaCl	58.44	150 mM	87.7g	Roth (3957.1)
Tween-20	Liquid	0.1%	-----	Roth (9127.1)

2.3.7.2 Antibody incubation

Membranes were blocked with 7% milk- TBST 60 min at room temperature and gentle shaking or overnight at 4°C. Membranes were washed 3 times with TBS-T for 10 min at room temperature with shaking, followed incubation with 1st antibody for 60 min at room temperature with shaking in case of polyclonal antibodies, or overnight at 4°C in case of monoclonal antibodies, washed 3 times with TBS-T for 10 min at room temperature. Then membranes were incubated with the 2nd antibody for 60 min at room temperature. They were washed again 3 times with TBS-T for 10 min at room temperature. One final wash was performed before development using TBS at room temperature for 5 min.

To prepare antibodies dilute them in TBS-T, NaN_3 (Sigma, S-8032) to a final concentration of 15mM was added to increase the antibody solution lifespan being possible to reuse these antibody solutions up to five times. Horseradish peroxidase (HRP) conjugated antibodies

were diluted (1:10.000) in TBS-T + milk 5% and used only once for developing membranes by the ECL method.

Antibody	**working concentration**	**raised in**	**source (cat N°)**
1st			
Anti-CCT2	WB (1:5000); PD(1:200)	Rabbit	M. Ahmad
Anti-Cry2	WB (1:2000)	Rabbit	M. Korneff
Anti-CCT1	WB (1:500)	Rabbit	M. Ahmad
Anti-HA(clone12CA5)	WB(1µg/µL); PD(10µg)	Mouse	Roche (1-583-816)
Anti-HA(clone HA.11)	PD(50µg)	Mouse	Covance (MMS-101R)
Anti-myc(clone9E10)	WB(1µg/µL); PD(12µg)	Mouse	Covance (MMS-150P)
Anti-GFP	WB(1:4000); PD(1:200)	Goat	Rockland (600-101-215)
Anti-GFP(cloneGFP20)	WB(1:2000)	Mouse	Sigma (G6539)
2nd			
Anti-Rabbit IgG-HRP	WB(1:10.000)	Goat	Sigma (A6154)
Anti-Mouse IgG-HRP	WB(1:10.000)	Goat	Sigma (A4416)
Anti-Goat IgG-HRP	WB(1:10.000)	Rabbit	Sigma (A5420)

WB indicates used for Western-blot; PD indicates used for pull-down. Rabbit and goat antibodies are polyclonal, mouse are monoclonal.

2.3.7.3 Enhanced chemo-luminiscence development (ECL)

Solutions A and B were prepared and mixed just prior to membrane development. Mixed solutions were poured over the membrane and exposed to white light for 1 min. The membrane was wrapped in a transparent plastic bag and pasted to the Hypercassette (Amersham, Buckimhamshire UK). Working under red light, Hyperfilm ECL (Amersham, Buckimhamshire UK, Cat N° 108535D) was exposed for the desired time, usually 1 or 5 min. Final development and fixing was conducted under red light using Kodak reagents (developer + fixer Sigma Z354147-1SET). The film was taken to developing solution until bands appeared, washed with water/acetic acid Roth (3738.5) and given to fixer solution until it become transparent. Eventually it was washed with abundant water and dried on air.

Reagent	**Stock**	**final**	**1cap**	**Company (Cat N°)**
Luminol	powder	250 mM	50 mg in 1129 µl DMSO	Fluka (09253)
p-coumaric A	powder	90 mM	20 mg in 1354 µl DMSO	Fluka (28200)
H_2O_2	30%	-------	----------	Merk

Solution A: 1ml Tris/HCl pH 8.5 + 300 µl Luminol + 133.5 µl p-coumaric Acid + dH_2O to 15 ml. the final solution is filtered (0.45 µm) to get rid of undisolved luminol.

Solution B: 9,15 µl H_2O_2 in 15 ml dH_2O

2.3.7.4 Stripping and reprobing membranes

Membranes were incubated in stripping buffer on a water bath at 60°C for 30 min. After that they were washed 3 times with TBS-T. After that membranes were ready to be probed with antibodies again. The stripping procedure can not be performed more than twice without compromising membrane stability.

Stripping buffer	**stock**	**final**	**1 L**	**Company (Cat N°)**
Tris/HCl pH 6.7	1 M	62.5 mM	62.5 mL	Roth (4855.2)
SDS	10%	2%	200 mL	Roth (2326.2)
ß-mercaptoEthanol	14.2 M	100 Mm	------	Roth (4227.1)
dH_2O	------	------	737.5 ml	

The buffer can be stored at room temperature without ß-Me. and 704 µl ß-Me must be added to 100 mL of preheated buffer prior to use.

2.3.7.5 Ponceau Red staining of membranes

Once developed, as control for correct protein transfer membranes were stained with the low sensitive dye red ponceau, only the most abundant proteins can be seen. Membrane was soaked with the stain buffer for 5 min and washed until only the bands were visible. Membrane was air dried.

Stain buffer	**Stock/Mw**	**final**	**200 mL**	**Company (Cat N°)**
Red Ponceau	Powder	0.2%	400 mg	
TCA (tricloro acetic acid)	35%	3%	17 mL	Roth (7437.1)

Wash buffer contains 1% of Acetic acid Roth (3738.5)

2.4 Fluorescence spectroscopy

Fluorometry was performed on a Shimadzu RF-5301 PC equipped with a cuvet cooling device. Hellma precision Quartz cells type 105.250-QS light path 10 mm Cebte 15 mm were used cooled at 10°C.

2.5 Gene expression studies

To check gene expression total RNA was extracted from the aerial part of *Arabidopsis* seedlings or filter-dried cell *Arabidopsis* cell culture using RNeasy kit (Qiagen plant RNAeasy kit, cat N° 74904) according to the manufacturer's instructions. cDNA synthesis was performed with superscript RNase H-reverse transcriptase and oligo dT according to the manufacturer's instructions. Real time PCR was performed using SYBR green I, Platinum Taq DNA polymerase on an I-cycler machine (Biorad), following the instructions of the manufacturer. Data treatment was performed by the Ct method (Pfaffl M.W. 2001). Gene expression data were represented relative to the maximum value among all data sets after normalisation to the *UBQ10* control.

2.5.1 Isolation of mRNA

100 mg of frozen plant material (filtered cell culture or seedlings) were grinded in a bead mill homogenizer (Retsch, MM200) using the Qiagen plant RNAeasy kit as specified by the manufacturer. RNA quality was measured by spectrometry diluting 1:20 to get values in a linear range, 3 µl RNA + 57 µl RNAse free water measured in the UV compatible plastic cuvettes (Uvette Eppendorf cat N° 95201005-1). Absorption at 260 nm was measured being 1 Absorbance unit equal to 40 ng/µl of RNA

2.5.2 cDNA synthesis

Digestion of the genomic DNA (gDNA) of RNA samples was performed only just prior synthesis of cDNA. RNA stocks were kept at –80°C without treatment. gDNA from 1µg of RNA was digested using 1unit Deoxyribonuclease I Amplification grade (DNAseI, Invitrogen Cat N° 18068-015) in a final volume of 10µl in a PCR tube using a PCR Eppendorf, Mastercycler Gradient machine as incubator at 24°C for 15 min. DNAse I was inactivated by the addition of 1 µl of 25 mM EDTA to the reaction mixture and further inactivated by heating at 65°C for 15 min. The cDNA synthesis was performed on the same tube as the digestion. Oligo dT and dNTPs were added to the digested sample and heated at 65°C for 5 min, tubes were cooled on ice and the master mix was added to a final reaction volume of 20 µl.

Reagent	Stock	final	vol(x1)	company(Cat N°)
Oligo(dT)$_{12-18}$ primer	0.5 µg/µl	0.25 µg	0.5 µl	Invitrogen (18418-012)
dNTPs	10 mM	0.5 mM	1 µl	Fermentas (#R0181)
RT-PCR-buffer	x5	x1	4 µl	Invitrogen (18053-017)
DTT	100 mM	10 mM	2 µl	Invitrogen (18053-017)
RNAse inhibitor	40 U/µl	20 U	0.5 µl	Fermentas (#E00311)
SSpol RNAH$^-$	200 U/µl	200 U	1 µl	Invitrogen (18053-017)

The whole mixture was incubated for 2 min at 37°C before superscript polymerase (SSpol) was added. The reaction was incubated at 37°C 50 min and stopped by heating at 70°C for 15 min.

2.5.3 Polymerase Chain Reaction (PCR)

PCR mix was prepared as indicated below multiplying the amounts as many times as desired reactions. The final reaction volume was 20 µl.

Reagent	Stock	final	Vol (µl x1)	Company (Cat N°)
dH_2O	---	---	5	----
PCR buffer	x10	x1	2	Eppendorf (0032007.724)
$MgCl_2$	25 mM	2.5 mM	2	Roth (2189.1)
Oligo up	10 µM	0.5 µM	1	VWR (see table)
Oligo down	10 µM	0.5 µM	1	VWR (see table)
DNTPs	10 mM	0.25 mM	0.5	Fermentas (#R0181)
Taq pol	1 U/µl	2.5 U	2.5	Eppendorf (0032007.724)
cDNA	---	---	2	experiment

PCR program consisted in a initial step of 95°C for 5 min following 35 cycles consistent in a denaturation step at 95°C for 20 sec, an annealing step using a specific temperature for each set of primers (see the result section) for 40 sec and a final elongation step at72°C for 30 sec. After that, a final elongation step was carried out at 72°C for 10 min

2.5.3.1 Agarose electrophoresis

Samples were loaded with Xylen Cyanol (XCL) loading buffer and run in 2% agarose gel (QA agarose molecular biology grade, Qbiogene Cat N° AGAH0500) prepared in TBE (Tris/borate 100 mM, EDTA 1 mM) buffer containing a final concentration of 0.01% of Ethidium bromide (Roth Cat N° 2218.2) at 120 V for 40 min. Results were recorded with a UV transilluminator (gel doc 1000 Biorad)

Reagent	Stock	final	10 ml	Company (Cat N°)
XCL dye	powder	0.1% (w/v)	10 mg	Roth (A513.1)
EDTA	0.5 M	100 mM	2 ml	Roth (8043.2)
Glycerol	liquid	50%	5 ml	Roth (3783.2)
SDS	10%	1%	1 ml	Roth (2326.2)
dH_2O	---	---	2 ml	-----

Quantitative real time PCR (qrtPCR)

PCR master mix was prepared as indicated below multiplying the amounts as many times as desired reactions. Reactions were performed used in a final volume of 20 µl.

Reagent	stock	final	Vol (x1)	company (Cat N°)
dH_2O	---	---	4 µl	---
10x buffer	x10	x1	2 µl	Invitrogen (10966-048)
$MgCl_2$	25 mM	4 mM	3.2 µl	Roth (2189.1)
Oligo up	10 µM	0.5 µM	1 µl	VWR (see table)
Oligo down	10 µM	0.5 µM	1 µl	VWR (see table)
dNTPs	10 mM	0.25 mM	0.5 µl	Fermentas (#R0181)
cDNA	---	---	2 µl	own made
platinum Taq	5 U/µl	1.5 U	0.3 µl	Invitrogen (10966-048)
SYBR GreenI	x10	x1	2 µl	Roche (1 988431)
Fluorescein	0,1 µM	20 nM	4 µl	Biorad (170-8780)

2.5.5 PCR and qrtPCR Primers

The following table contains information for the PCR and qPCR primers used for studies. The gene name, locus, known function and putative cellular localization of the gene product are indicated. Primers are described by their content in CG. Their numbers in our stock list (#N), the size in base (bp) of the product and the product with intron, between brackets, are also listed. For quantitative real time PCR (qrtPCR) primers, were designed avoiding spontaneous primer dimmer (P.D.) formation amongst them. Therefore, they were always designed with a theoretical dimerization free energy (ΔG P.D.) higher than –1, which leads to non-spontaneous dimerization.

Name: catalase2 (*CAT2*)		**locus:** at4g35090	
Process: detoxification		**targeted:** NO	
PCR primers size (+ intron): 297 (373)			
	Sec (5´---->3´)	**%GC**	**bp** **#N**
Primer up:	CGGGACTTCCACGCTCATGGATAACGG	59	27 5
Primer down:	CCCTTCTTCACCACCAACTCTGGTGC	58	26 6
RT-qPCR primers size (+ intron): 144 (226)			
	Sec (5´---->3´)	**%GC**	**bp** **#N**
Primer up:	CTGAGACCAGTAAGAGATCCAGATA	53	28 29
Primer down:	GCCAGTCAATGCTCCAAAATGTGCTCA	48	27 30
Name: Fatty acid desaturase 8 (*FAD8*)		**locus:** at5g05580	
Process: JA biosynthesis		**targeted:** chloroplast	
PCR primers size (+ intron):485 (579)			
	Sec (5´---->3´)	**%GC**	**bp** **#N**
Primer up:	CAGCCACACTGTTCAGCCTCGG	52	23 1
Primer down:	CCTCTCCCCAGATTCTACCC	56	25 2
RT-qPCR primers size (+ intron): 110 (204)			
	Sec (5´---->3´)	**%GC**	**bp** **#N**
Primer up:	the same as PCR #1	63	22 1
Primer down:	GCCTCTCTACTGGTTCGCTCAA	54	22 31
Name:D-glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)		**locus:** at1g42970	
Process: glycolisis		**targeted:** chloroplast	
PCR primers size (+ intron): 407 (509)			
	Sec (5´---->3´)	**%GC**	**bp** **#N**
Primer up:	CCCCACTCGTTATCATACCAAGC	52	23 3
Primer down:	GGGGACAATGACAACCACACACTCC	56	25 4
RT-qPCR primers size (+ intron): 121 (no intron)			
	Sec (5´---->3´)	**%GC**	**bp** **#N**
Primer up:	same as PCR #3	52	23 3
Primer down:	CGCCTCTTGTCTCTGTTGACTTCA	50	24 36
Name: Light harvesting complex 5 (*LHCA5*)		**locus:** at3g54890	
Process: Chlorophyll binding protein		**targeted:** chloroplast	
PCR primers size(+ intron): 300 (391)			
	Sec (5´---->3´)	**%GC**	**bp** **#N**
Primer up:	GCCATGGATCCGCCAAGTGAGTTGC	60	25 7
Primer down:	CCCAGTCCCGTGGGGTACTTTGCCC	68	25 8
RT-qPCR primers size (+ intron): 172 (572)			
	Sec (5´---->3´)	**%GC**	**bp** **#N**
Primer up:	CCCAGTTTCCATATCCTAATGCTT	41	24 37
Primer down:	GCTTACCTTGACGGTTCTGCTCCT	54	24 38
Name: Ubiquitin 10 (*UBQ10*)		**locus:** at4g05320	
Process: protein degradation (reference house keeping gene)		**targeted:** NO	
rt-qPCR primers (2different sets):			
	Sec (5´---->3´)	**%GC**	**bp** **#N**
Primer up:	GAGGACCAAGTGGAGGGTGGATTCC	60	25 39
Primer Down:	GGATCAGCAGAGGCTTATTTTCGC	50	24 40
Size (+ intron): 100 (no intron)			
	Sec (5´---->3´)	**%GC**	**bp** **#N**
Primer up:	GCAAGAGTTCTGCCATCCTCC	57	22 19
Primer down:	CGGGAAAGACGATTACTCTTGAGG	50	24 20
Size (+ intron): 145 (no intron)			

Name: Fructose bisphosphate aldolase like (*FBP*) **locus:** at2g21330
Process: photosynthesis **targeted:** chloroplast

PCR primers size PCR (+ intron): 443 (526)

Sec (5´---->3´)	**%GC**	**bp**	**#N**
Primer up: CGACTTTGATGCCAGGGACG	60	20	9
Primer down: GGCGTCAAGCACTGCGACTATGC	61	23	10

RT-qPCR primers: No designed

Name: Chalcone synthase (*CHS*) **locus:** at5g13930
Process: flavonoid biosynthesis **targeted:** NO

PCR primers size (+ intron): 441 (527)

Sec (5´---->3´)	**%GC**	**bp**	**#N**
Primer up: ATCTTGGCTATTGGCACTGCTAACCCT	48	27	15
Primer down: GAAGCAACCTTGCTGGTACATCAT	46	24	16

rt-qPCR primers size (+ intron): 131 (217)

Sec (5´---->3´)	**%GC**	**bp**	**#N**
Primer up: CCAGAGAAGGAGCCATGTAAGC	55	22	23
Primer down: CATGACCGACCTCAAGGAGAAG	55	22	24

Name: Asparagine synthetase (*AS*) **locus:** at3g47340
Process: amino acid metabolism **targeted:** NO

PCR primers size (+ intron): 380 (460)

Sec (5´---->3´)	**%GC**	**bp**	**#N**
Primer up: GGAACACGTGCCTCTAGTCC	60	20	11
Primer down: GGCTCACCGGACTTGAAGGC	65	20	12

rt-qPCR: no designed

Name: Phenylalanine ammonia lyase2 (*PAL2*) l**ocus:** at3g53260p
Process: amino acid metabolism targeted: NO

PCR primers size (+ intron): 478 (936)

Sec (5´---->3´)	**%GC**	**bp**	**#N**
Primer up: GGAGTGTGGCAATGTGTGGC	60	20	13
Primer down: CGGGGCACACAAGAGCAACG	65	20	14

rt-qPCR: no designed

Name: Constans (*CO*) **locus:** at5g15840
Process: Flowering

rt-qPCR primers size(+ intron): 118 (353)

Sec (5´---->3´)	**%GC**	**bp**	**#N**
Primer down: GGGACTCACTACAACGACAATGGTT	48	25	27
Primer up: GGGCGTTCTTGGGTGTGAAGCTGTT	56	25	28

Name: Flowering locus T (*FT*) **locus:** at1g65480
Process: Flowering

rt-qPCR primers size(+ intron): 239 (363)

Sec (5´---->3´)	**%GC**	**bp**	**#N**
Primer up: CTTCCTCCGCAGCCACTCTCCCTC	66	24	21
Primer down: CCCTGCTACAACTGGAACAACC	54	22	22

Company list

The addresses of companies which products have been used are listed below.

Roth	Karlsruhe, Germany	www.carl-roth.de
Serva	Heidelberg, Germany	www.serva.de
Sigma	St Louis, USA	www.sigmaaldrich.com
Aldrich	St Louis, USA	www.sigmaaldrich.com
Fluka	Seelze, Germany	www.sigmaaldrich.com/ Brands/Fluka___Riedel_Home.html
Roche	Penzberg, Germany	www.roche-applied-science.com
Invitrogen	Karlsruhe, Germany	www.invitrogen.com
Fermentas	St. Leon-rot, Germany	www.fermentas.de
Covance	California, USA	www.CRPinc.com
Duchefa	Haarlem,The Netherlands	www.duchefa.com
Biorad	Hercules, USA	www.bio-rad.com
Amersham	Uppsala, Sweden	www.amershambiosciences.com
Merk	Darmstadt, Germany	www.merck.de
NewEnglandBiolabs	Frankfurt, Germany	www.neb.com
Eppendorf	Hamburg, Germany	www.eppendorf.com
DIFCO labs	Detroit, USA	www.bd.com
Becton Dickinson	Heidelberg, Germany	www.bd.com
VWR	Darmstadt, Germany	www.vwr.com
Calbiochem	Darmstadt, Germany	www.merckbiosciences.co.uk/html/CBC/ home.html
Applichem	Darmstadt,Germany	www.applichem.de
Quiagen	Hilden, Germany	www.quiagen.com
Licor	Bad Homburg, Germany	www.licor.com
Gigahertz Optik	Puchheim, Germany	www.gigahertz-optik.com

3.1 Blue light induced reporter genes

Light has a very strong effect on gene expression in plants. Multiple transcriptome studies performed with several photoreceptors mutants (Ohgishi M. *et al* 2004; Ma L. *et al* 2001; Tepperman J.M. *et al* 2001; Folta K.M. *et al* 2003), transcription factors mutants (Ma L. *et al* 2002) and light qualities (Ma L. *et al* 2001; Rossel J.B. *et al* 2002) have revealed up to 28 metabolic routes regulated by light.

Blue light activation of gene expression through crys is mainly leaded by cry1. Cry2 has a minor role, as in *Arabidopsis cry2* mutants gene expression under blue light is almost comparable to wild type. However, in a *cry1* mutant *cry2* seems to compensate part of the gene activation, as the mRNA levels of these blue light induced genes in the *cry1* mutant are still higher than in a *cry1cry2* double mutant (Wade H.K. *et al* 2001).

The most important biological output of cry2 is the control of flowering time. Therefore, the way to measure cry2 activity *in vivo*, and to define functional amino acid positions in the photoreceptor, would be to generate mutations in *cry2*, and measure the early or late flowering phenotype. Such work would take too long time, but another approach to measure cry2 activity is still desirable. If an *Arabidopsis* cell culture *cry1cry2* double mutant could be generated, fast transient transformation with different *cry2* mutants would allow checking cry2 activity in a cryptochrome minus background by measuring genes regulated by cry2. Moreover, even genes with not high fold induction should be detected by the use of a sensitive technique like quantitative real time PCR (qrtPCR).

The goal of part of this study is to make the first steps on the establishment of a "cry2 activity measurement system". For that purpose, the available light-inducted gene pool was examined to find genes specifically induced under blue light by cryptochrome action. Genes were chosen from published array papers (Ohgishi M. *et al* 2004; Ma L. *et al* 2001; Tepperman J.M. *et al* 2001; Folta K.M. *et al* 2003; Rossel J.B. *et al* 2002) rejecting transcription factors, components of the circadian clock, as well as proteins of unknown function. Non organelle-targeted genes were preferred; because they will be more useful in the future work with a chloroplast-lacking white cell culture. However, the gene selection proved to be difficult because most of the regulated genes by crys are targeted to organelles, and the cytoplasmatic ones usually show very small induction, or fell in the non elegible category mentioned above.

Anyhow, a set of five cytoplasmatic genes and three chloroplast targeted genes were chosen for characterization (described individually below), first in cell culture and later in *cry1* and *cry1cry2 Arabidopsis* mutants.

All the array studies about light-regulated gene expression have been carried out in *Arabidopsis* plants. However, our goal is to use *Arabidopsis* cell culture instead of the whole plant. Therefore, we first confirmed the existing data *in planta* for reproducibility in cell culture. Thus, PCR with cDNA from both *Arabidopsis* white and green cell cultures was a first control for weakly induced genes. Only when induction was strong enough to be clearly seen in an ethidium bromide stained agarose gel, primers for qrtPCR were designed and used for both, *Arabidopsis* plant and cell culture.

Young etiolated *Arabidopsis cry1* mutant seedlings were used to measure gene expression under different light qualities to compare the effect of *phys* versus *cry2*. An *Arabidopsis cry1cry2* double mutant was also used to confirm that the blue light induction of the studied gene was due to crys and not because of other photoreceptor action.

Primer combinations indicated for each reaction showed refer to the primer list given in the Materials and Methods section. The size of the PCR product and the product with intron are indicated too. Controls were included in every reaction. A negative control (-) without cDNA to test for unspecific amplification, and a positive control, using ubiquitin 10 (*UBQ10*) as house-keeping control gene, were performed. *Arabidopsis* has 14 ubiquitin genes (Bachmair A. *et al* 2001), and *UBQ10* has been used before as control house-keeping gene in other studies (Yanovsky M.J. & Kay S.A. 2002), so it was used here in all reactions for normalization of the data. The ubiquitin primers used in all reactions are primers numbers 19 and 20 in the list given in the Materials and Methods section. Because of the small size of the product and the lack of introns of this gene the same set of primers was used for both, PCR and qrtPCR. In the ethidium bromide stained agarose gels several bands appear in the ubiquitin lane because it is expressed as a repeat of four ubiquitines

3.1.1 Chalcone synthase (*CHS*)

CHS catalyses the first regulatory step in the flavonoid biosynthesis pathway. Amongst the final products of this pathway is anthocyanin, a pigment which can be easily measured, and is routinely used as an indicator of *cry1* activity (Mancinelli A.L. *et al* 1991). *CHS* promoter composition and its regulation is well known in *Brassicaceae* (Kaiser T. & Batschauer A. 1995; Kaiser T. *et al* 1995; Hartmann U. *et al* 1998), being up-regulated under blue light (B) due to *cry1* action (Ahmad M. *et al* 1995). For these reasons, *CHS* was used as a reference for the other blue light induced genes and for comparison and validation of gene expression between cell culture and plant.

As a first control to assert the equivalence between the plant studies and the cell culture, *CHS* gene expression in both green and white cell cultures was tested (Fig. 3.1.1).

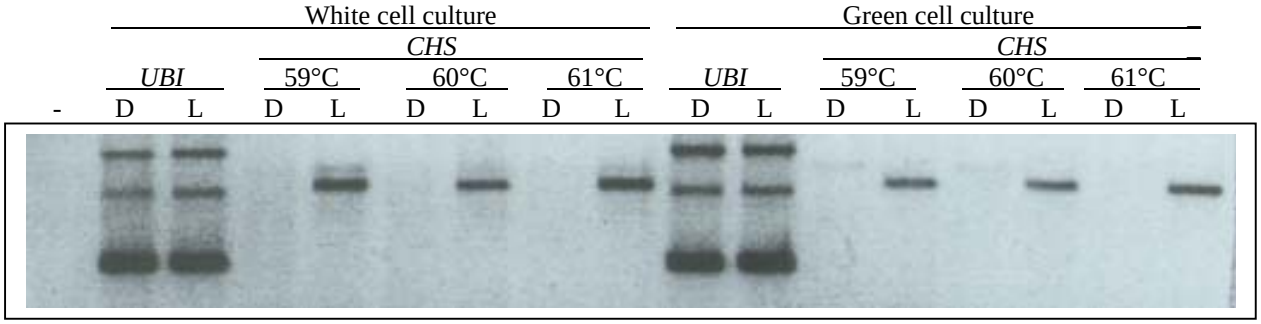


Fig. 3.1.1 *CHS* expression in cell cultures. RNA was isolated from dark-grown white cell cultures (D), or dark adapted green cell cultures kept for 24 h in darkness, or from cell cultures treated with blue light (30 µmol m^{-2} s^{-1}) for 4h (L). cDNA was generated and subjected to 30 cycles of PCR using different annealing temperatures. Negative control reaction without cDNA is indicated as (-). Primers number 15 and 16 for *CHS* from the Materials and Methods list were used. The expected size without intron is 441 bp, or 527 bp with intron.

CHS was clearly up-regulated under blue light without a visible background in darkness in both cell cultures. Its light induction in *Arabidopsis* cell culture had been previously reported (Hartmann U. *et al* 1998), and our results indicate a fast blue light induction. To set a reference time for comparison amongst genes, a light induction kinetic was performed. White cell culture grown in darkness was illuminated for 4, 5, 6 or 7 hours with blue light and the expression levels were analyzed by qrtPCR (Fig. 3.1.2)

The maximum expression of *CHS* in the white cell culture was observed after 5 hours of blue light illumination (Fig. 3.1.2). These data fit very well to the *CHS* induction in wt *Arabidopsis* (Ws ecotype), which showed the maximum induction after 4,5 hours under 15 µmol $m^{-2}s^{-1}$ blue light (Lin C. *et al* 1996), supporting the use of the cell culture as a suitable model for gene expression similar to the whole plant. In order to make the screening easier and to use the

same cDNA in all cases, the expression of all genes was tested after 5 hours of blue light illumination, corresponding to the maximum of *CHS* but not necessarily with the maximum expression for every gene. Therefore, some further improvement should be done by making an induction kinetic for each gene of interest.

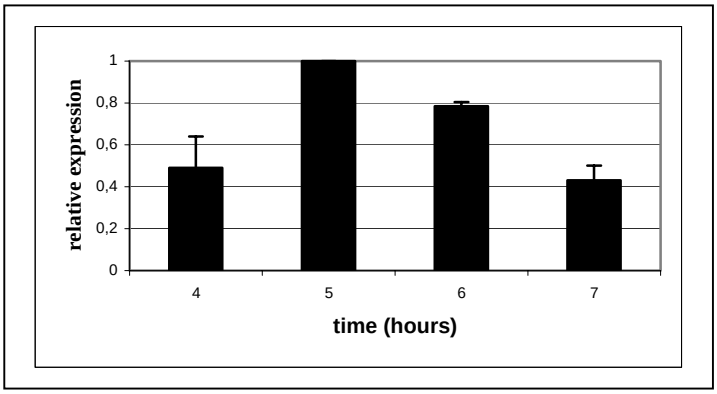


Fig. 3.1.2 Kinetics of *CHS* expression under blue light in white cell culture. White cell culture grown in darkness was illuminated for 4, 5, 6 or 7 hours with blue light (30 μmol $m^{-2}s^{-1}$), total RNA extracted, cDNA generated and qrtPCR performed as described in Materials and Methods. Primers number 23 and 24 from the Materials and Methods list were used.

3.1.2 Omega3 fatty-acid desaturase 8 (*FAD8*)

FAD8 is a member of a gene family enconding enzymes responsible for fatty-acid desaturation in plants. *FAD8* is also essential for JA synthesis (Devoto A. & Turner J.G. 2005). *FAD8* was picked from the quadruple *cry1cry2photphot2* mutant array (Ohgishi M. *et al* 2004) because its up-regulation, although not very high, seemed to be under cry2 control.

It was not possible to detect the PCR product in any of both cell cultures (data not shown). Nevertheless, qrtPCR was carried out on plants to confirm the results shown in the microarray data. Although PCR could not detect *FAD8* in light treated cell culture, the gene product could be detected via qrtPCR (Fig. 3.1.3). This result indicates the greater power of qrtPCR over PCR and validates the data from the published array.

FAD8 was nearly not expressed in darkness. Moreover, there was an induction detectable under red (R) and far-red (FR) light pointing to phytochrome action. However, blue light had a stronger effect than red and far-red on *FAD8* induction in a *cry1* deficient *Arabidopsis* mutant. The blue light up-regulation in the double *cry1cry2* mutant was completely abolished and levels of *FAD8* transcripts were similar to the ones of the *cry1* mutant plant in darkness, indicating that the upregulation seen in the *cry1* mutant under blue light is due only to *cry2* action.

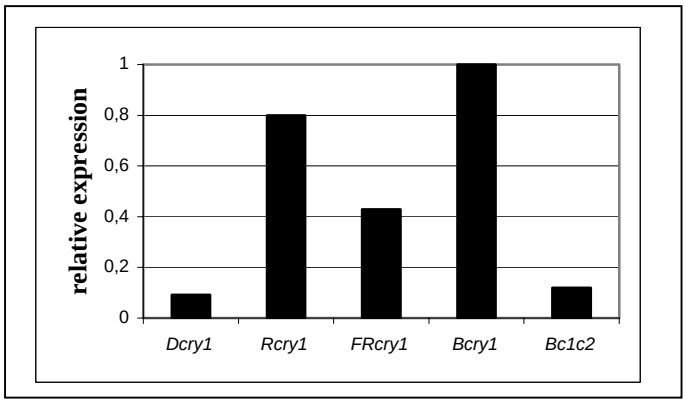


Fig. 3.1.3 *FAD8* levels of *Arabidopsis* mutant plants under different light qualities measured by qrtPCR. 6 days old etiolated seedlings of the indicated genotype were exposed to different light qualities. Total RNA extracted, cDNA generated and qrtPCR performed as indicated on Materials and Methods. Primers number 1 and 31 from the Materials and Methods list were used. *Dcry1*: *cry1* mutant dark control, *Rcry1*: *cry1* mutant 5 h under Red light, *FRcry1*: *cry1* mutant 5 h under Far-red, *Bcry1*: *cry1* mutant 5 h under Blue light, *Bc1c2*: *cry1cry*2 mutant 5 h under blue light.

3.1.3 D-glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)

GAPDH is a chloroplast-targeted gene which catalizes a key step in the Calvin cycle of photosynthesis; its light up-regulation is well known under white, red or blue light (Chan C.S. *et al* 2001). Although phys also regulate *GAPDH*, it was chosen because of its high responsiveness, being 10 fold up-regulated when illuminated with white light versus dark controls, and almost 5 fold when blue light illuminated versus dark control as shown in micro array studies (Ma L. *et al* 2001).

As expected no expression could be detected in the non-photosynthetic white cell culture, but on the green cell culture a clear light dependent up-regulation was proved (Fig. 3.1.4).

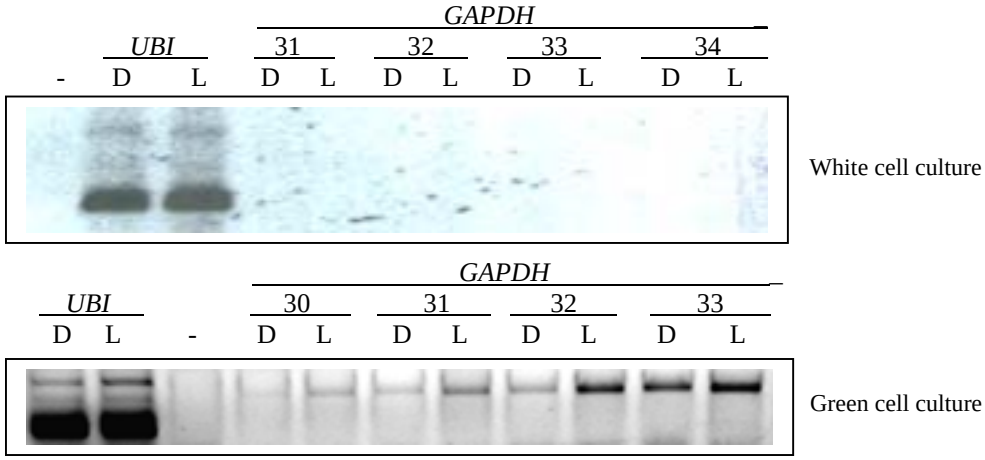


Fig. 3.1.4 *GAPDH* expression in cell cultures. RNA was isolated from dark adapted cell cultures kept for 24 h in darkness (D), or from cell cultures treated with blue light (30 μmol m^{-2} s^{-1}) for 5h (L). cDNA was generated and subjected to PCR, The numbers indicate the number of cycles in the PCR reaction., negative control reaction without cDNA is indicated as (-). *GAPDH* Primers number 3 and 4 from the Materials and Methods list were used. The expected size without intron is 407 bp, or 509 bp with intron.

qrtPCR analysis was performed for *Arabidopsis* mutant plants to check the involved photoreceptors in *GAPDH* expression (Fig. 3.1.5).

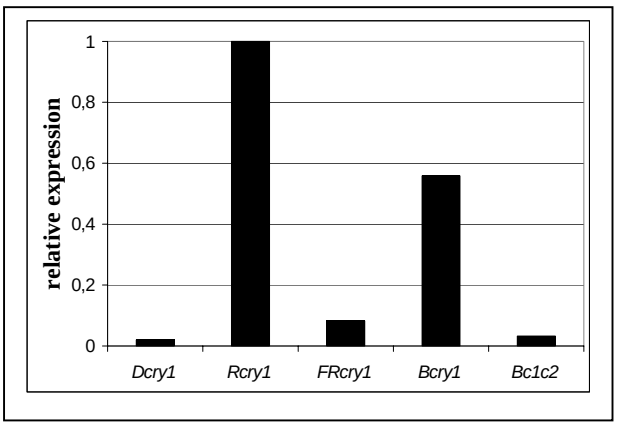


Fig. 3.1.5 *GAPDH* levels of *Arabidopsis* mutant plants under different light qualities measured by qrtPCR. 6 days old etiolated seedlings of the indicated genotype were exposed to different light qualities. Total RNA extracted, cDNA generated and qrtPCR performed as indicated in Materials and Methods. Primers number 3 and 36 from the Materials and Methods list were used. *Dcry1*: *cry1* mutant dark control, *Rcry1*: *cry1* mutant 5 h under Red light, *FRcry1*: *cry1* mutant 5 h under Far-red, *Bcry1*: *cry1* mutant 5 h under Blue light, *Bc1c2*: *cry1cry*2 mutant 5 h under blue light.

GAPDH was not expressed in darkness. However, there was up-regulation under red light as known from previous studies. Lack of expression under FR light excludes phyA from being able to induce *GAPDH* alone. Nevertheless, blue light was able to induce expression in a *cry1* deficient *Arabidopsis* mutant. Moreover, the double *cry1cry2* mutant was completely unable to express *GAPDH* under blue light, indicating that the up regulation seen in the *cry1* mutant under blue light is due to cry2 activity.

3.1.4 Catalase2 (*CAT2*)

Catalase is an enzyme that catalizes the dismutation of H_2O_2 to water and oxygen, working as an antioxidant defence system (Mc Clung C.R. 1997). Its light-dependent up-regulation is not very strong, only 2-fold. However, it is a cytosolic protein, and seems to depend more on cry2 than on cry1 (Ohgishi M. *et al* 2004)

CAT2 was expressed in both, white and green cell culture, although the up-regulation was more evident in the green culture (Fig. 3.1.6)

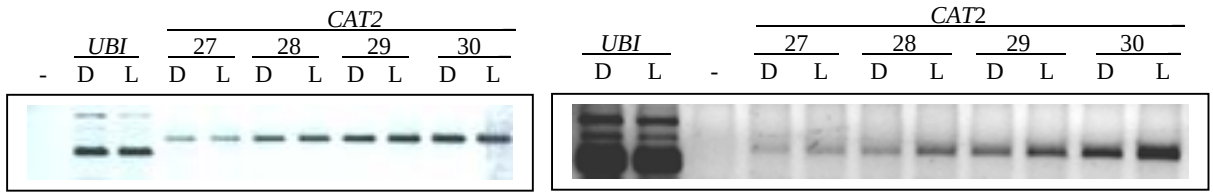


Fig. 3.1.6 *CAT2* expression on cell cultures. RNA was isolated from dark adapted cell cultures kept for 24 h in darkness (D), or from cell cultures treated with blue light (30 µmol m^{-2} s^{-1}) for 5 h (L). cDNA was generated and subjected to PCR, The numbers indicate the number of cycles in the PCR reaction., negative control reaction without cDNA is indicated as (-). Primers number 5 and 6 from the Materials and Methods list were used with annealing temperature of 65°C to amplfy *CAT2* transcrips. The expected size without intron is 297 bp, or 373 bp with intron.

When tested by qrtPCR in *Arabidopsis* (Fig. 3.1.7) *CAT2* showed a very tight light regulation. It was expressed only under the control of cry2

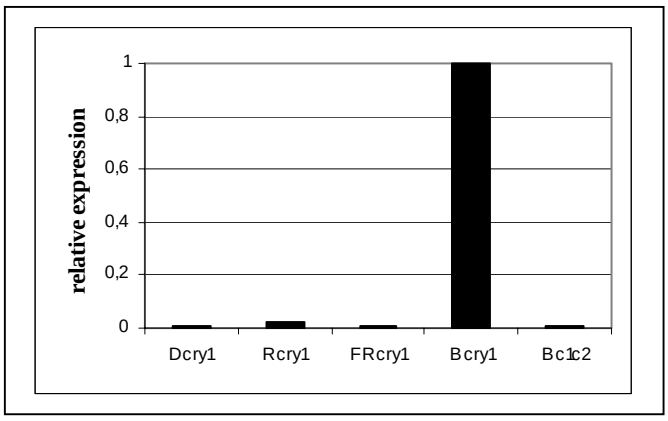
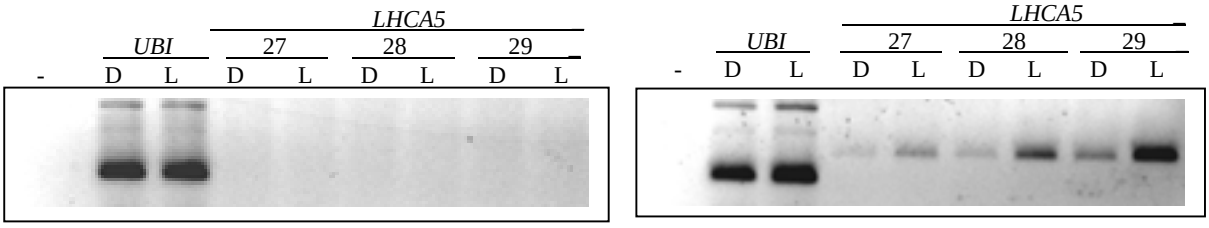


Fig. 3.1.7 *CAT2* levels of *Arabidopsis* mutant plants under different light qualities measured by qrtPCR. 6 days old etiolated seedlings of the indicated genotype were exposed to different light qualities. Total RNA extracted, cDNA generated and qrtPCR performed as indicated on Materials and Methods. Primers number 29 and 30 from the Materials and Methods list were used. *Dcry1*: *cry1* mutant dark control. *Rcry1*: *cry1* mutant 5 h under Red light, *FRcry1*: *cry1* mutant 5 h under Far-red, *Bcry1*: *cry1* mutant 5 h under Blue light, *Bc1c2*: *cry1cry*2 mutant 5 h under blue light.

3.1.5 Light harvesting complex 5 (*LHCA5*)

In *Arabidopsis* there are six members of the *LHCA* family, *LHCA1-4* are down regulated under high light exposure. *LHCA6* is believed not to be expressed at all. Whereas, *LHCA5* is up-regulated under high light in a fluence dependent fashion (Ganeteg U. *et al* 2004). *LHCA5* is a chloroplast-targeted gene. Although phys also regulate *LHCA5* it was chosen for the present study because of its huge responsiveness to light. It was shown in array analysis that it is up-regulated 19 fold when blue light irradiated plants versus dark controls are compared (Ma L. *et al* 2001).



White cell culture Green cell culture

Fig. 3 1.8 *LHCA5* expression in cell cultures. RNA was isolated from dark adapted cell cultures kept for 24 h in darkness (D), or from cell cultures treated with blue light (30 μmol m^{-2} s^{-1}) for 5h (L). cDNA was generated and subjected to PCR. The numbers indicate the number of cycles in the PCR reaction. negative control reaction without cDNA is indicated as (-). Primers number 7 and 8 from the Materials and Methods list were used with annealing temperature of 62°C to amplify *LHCA5* transcripts. The expected size without intron is 300 bp, or 391 bp with intron.

As expected, *LHCA5* expression could be detected only in the photosynthetic green cell culture (Fig. 3.1.8), showing a strong up-regulation by light since PCR products were detected already after cycle number 27, indicates a high amount of transcript.

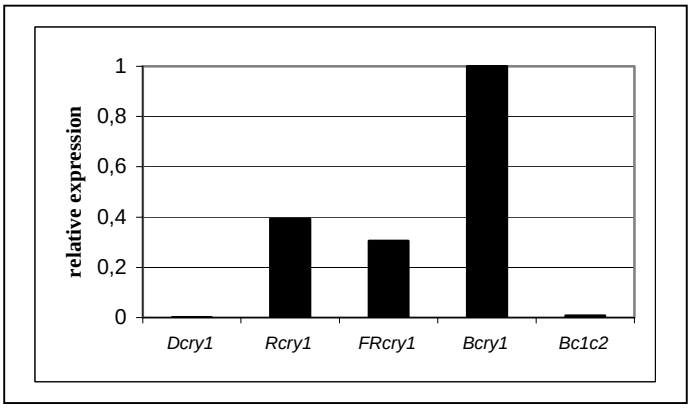


Fig. 3.1.9 *LHCA5* levels of *Arabidopsis* mutant plants under different light qualities measured by qrtPCR. 6 days old etiolated seedlings of the indicated genotype were exposed to different light qualities. Total RNA extracted, cDNA generated and qrtPCR performed as indicated on Materials and Methods. Primers number 37 and 38 from the Materials and Methods list were used. *Dcry1*: *cry1* mutant dark control, *Rcry1*: *cry1* mutant 5 h under Red light, *FRcry1*: *cry1* mutant 5 h under Far-red, *Bcry1*: *cry1* mutant 5 h under Blue light, *Bc1c2*: *cry1cry*2 mutant 5 h under blue light.

When tested by qrtPCR in *Arabidopsis* plants (Fig. 3.1.9) *LHCA5* showed no expression in darkness. There was some up-regulation under red and FR light pointing phytochrome action. Blue light induced much higher expression than R and FR in the *cry1* deficient mutant, an effect completely lost in the double *cry1cry2* mutant, indicating than the up-regulation seen in the c*ry1* mutant under blue light is due only to cry2 action.

3.1.6 Asparagine synthetase (*AS*)

AS is a cytoplasmatic protein involved in nitrogen assimilation by the plant (Oliveira J.C. *et al* 2001). *AS* is down-regulated almost 4-fold under blue light (Ma L *et al* 2001). Because several

cytoplasmatic genes are up-regulated by blue light, a strong and specific down-regulation of a gene could also be useful for monitoring cry activity.

The PCR experiment was performed only in the white cell culture because better candidates were available for the green cell culture. After 33 PCR cycles (Fig. 3.1.10) the signal from the transcript level in the dark sample was not very high. However, a clear down regulation was found after blue light irradiation. This gene was not further tested by qrtPCR.

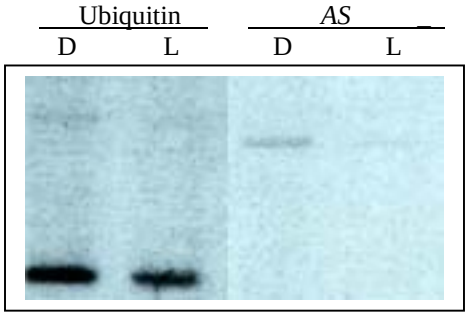


Fig. 3.1.10 *AS* expression in white cell culture. RNA was isolated from dark grown white cell culture (D), or from white cell culture treated with blue light (30 μmol m^{-2} s^{-1}) for 5 h (L). cDNA was generated and subjected to 33 cycles of PCR, The numbers indicate the number of cycles in the PCR reaction.. Primers number 11 and 12 from the Materials and Methods list were used with annealing temperature of 60°C to amplify *AS transcripts*. The expected size without intron is 380 bp, or 460 bp with intron.

3.1.7 Phenylalanine ammonia lyase (*PAL*)

Phenylalanine ammonia lyase is a cytosolic enzyme, producing cinnamic acid, which is the precursor of lignins, coumarins as well as flavonoids. Two *PAL* genes have been described to be regulated by light in *Arabidopsis*. *PAL2* seems to be largely under the control of cry2. Nevertheless, its up-regulation is not very high (Ohgishi M. *et al* 2004). *PAL1* was found to be up-regulated under high light intensities (Rossel J.B. *et al* 2002). *PAL2* was chosen for the same reason as *CAT2*. It encodes a cytosolic protein with small light induction, but seems to be under the control of cry2.

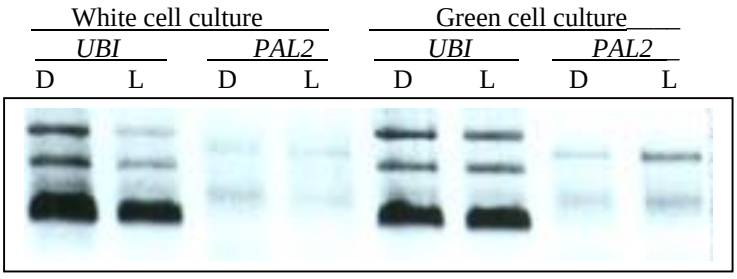


Fig. 3.1.11 *PAL2* expression in cell cultures. RNA was isolated from dark adapted cell cultures kept for 24 h in darkness (D), or from cell cultures treated with blue light (30 μmol m^{-2} s^{-1}) for 5h (L). cDNA was generated and subjected to 33 cycles of PCR. Primers number 13 and 14 from the Materials and Methods list were used with annealing temperature of 60°C to amplify *PAL2* transcripts. The expected size without intron is 478 bp, or 936 bp with intron.

Although *PAL2* is cytosolic and it should be up-regulated in both, white and green cell cultures, only an increase in the green cell culture is detectable (Fig. 3.1.11). It may be

possible that the 5 hour irradiation was not long enough to induce *PAL*, as an expression peak time of around 9 hours was previously reported for parsley protoplasts (Schröder J. *et al* 1979). Having better reporter candidates for the green cell culture no further experiments were conducted and *PAL2* was not tested by qrtPCR.

3.1.8 qrtPCR in green cell culture

After testing all the selected genes by PCR in both cell cultures, it became evident, that the green photosynthetic cell culture gave a better gene induction response under blue light illumination than the white cell culture. For that reason, to corroborate the data obtained with the plant tissue, the studied genes were tested and compared by qrtPCR in the green cell culture (Fig. 3.1.12)

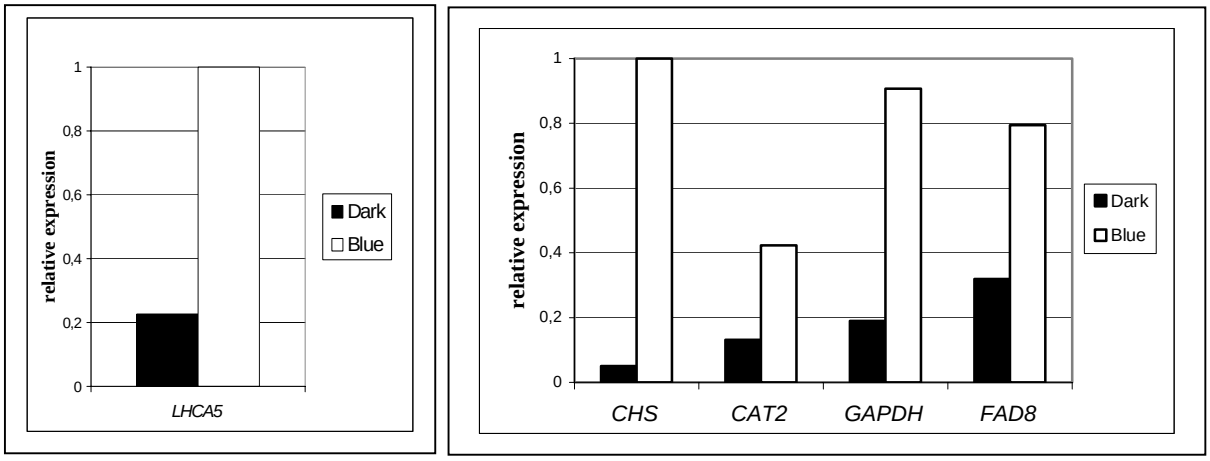


Fig. 3.1.12 Blue light-induction of selected genes in the green cell culture. Total RNA was extracted form dark adapted green cell cultures (Dark) kept for 24 h in darkness, or from green cell cultures treated with blue light (30 µmol $m^{-2}s^{-1}$) for 5 h (Blue), cDNA generated and qrtPCR performed as described in Materials and Methods. The same primers described above for the plant samples were used here.

A blue light up-regulation of all genes tested was obtained in the green cell culture in the same way as it was previously obtained for *Arabidopsis* plants (Figs. 3.1.3, 3.1.5, 3.1.7, 3.1.9). Amongst all of them, *LHCA5* (plotted apart because its higher transcript levels) was the one with the highest mRNA level, showing a 5-fold up-regulation. *CHS* was the gene that gave the most specific results. It had almost no expression in darkness and had a strong up-regulation under blue light. *GAPDH* and *FAD8* showed transcript levels very similar to the ones of *CHS* under blue light. However, the up-regulation was not that strong, being 4.5 fold for *GAPDH* and 2.7 fold for *FAD8*. The mRNA levels of *CAT2* despite being not very high showed a clear light-regulation achieving a blue light induction around 4-fold.

3.2 Effect of green light on cry2 photocycle

Because of the homology between the CNT and its ancestor the CPD PHR, the photocycle of this PHR has been used as a model for plant cryptochrome. However a growing body of evidence points to differences in their activation due to their FAD redox state (Banerjee R. *et al* 2007; Bouly J.P. *et al* 2007). By mean of photoactivation (light exposure) of PHRs fully oxidized (FADox) can be converted to the active fully reduced ($FADH^-$) flavin through a semireduced ($FADH^o$) intermediate (Sancar A. 2003). The optical spectrum of FADox consists in two bands at 450 nm and 380 nm. The intensity of these bands decreases when FAD is semireduced and they vanished when FAD is fully reduced.

Usually FAD from purified PHR is present in the $FADH^-$ active form (Jorns M.S. *et al* 1987) or in a less reduced state like $FADH^o$ or FADox. FAD from purified cry1 is found as FADox. Moreover, determination of the FAD redox state from cry1 and cry2 expressed in insect cells revealed that also FADox is the *in vivo* form of FAD in crys (Bouly J.P. *et al* 2007; Banerjee R. *et al* 2007). Photoactivation of FADox in purified crys is possible, yielding a stable $FADH^o$ (Kottke T. *et al* 2006) which is able to absorb both blue and green light (Lin C. *et al* 1995). Nevertheless, the significance of this green light absorbance was not clear so far.

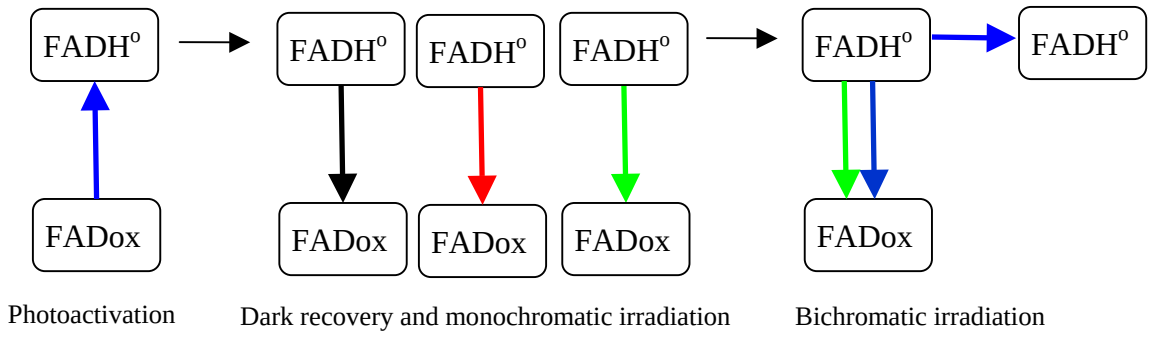


Fig. 3.2.1 *In vitro* photocycle of cry2 (Adapted from Banerjee R. *et al* 2007). The FAD redox state corresponds to the final equilibrium state. Colours indicate wavelengths used: blue, green, red or black for darkness

When FAD containing cry2 purified from insect cells was irradiated with blue light alone accumulation of the FAD cofactor in the $FADH^o$ state could be seen. Transition to darkness of the photo-reduced sample led to dark recovery to the FADox state. The same oxidation was obtained by exposing the photoactivated $FADH^o$ to red light, which can not be absorbed, or to green light that is absorbed by the semireduced flavin (Fig. 3.2.1).

Bichromatic irradiations were also tested. Using blue + red led to the same FADH° accumulation found with blue light, while blue + green light provoked the decrease of the accumulation of $FADH^o$ leading to a final mixture of FADH° and FADox forms (fig. 3.2.1).

The effect of bichromatic blue + green illumination was further tested *in planta* showing the abolishment of blue light-regulated hypocotyl inhibition, anthocyanin accumulation (Bouly J.P. *et al* 2007) and delay of flowering time (Banerjee R. *et al* 2007) in a cry dependent manner.

In long day plants like *Arabidopsis*, flowering is promoted under long day photoperiods with the coincidence model (Suárez-López P. *et al* 2001; Yanovsky M.J. & Kay S.A. 2002). This mechanism relies on the expression of the transcription factor Constants (CO) in the late evening.
In order to achieve correct CO mRNA expression, the putative blue light photoreceptor FKF1 is neccessary but crys or phyA are dispensable (Imaizumi T. *et al* 2003). On the other hand, activated cry2 or phyA are needed to stabilize CO protein, because in the absence of this photoreceptor activity CO will be rapidly degraded (Valverde F. *et al* 2004; Laubinger S. *et al* 2006). CO accumulation allows the expression of the florigen flowering locus T (FT; Yanovsky M.J. & Kay S.A. 2002; Suárez-López P. *et al* 2001) in leave vascular bundles (Takada S. & Goto K. 2003). This signal is transported to the meristem (Huang T. *et al* 2005; Corbesier L. *et al* 2007) where it triggers flower development (Simon R. *et al* 1996).

In order to analyze how green light affects cry2 activity in plants, transcripts of the flowering time genes *FT* and *CO* were measured. Plants were grown under SD, followed by monochromatic light treatments at the end of the light period with blue, green, or blue + green light for 8 h, or were kept in darkness the same time as a control. *CO* and *FT* mRNA expression were analysed by qrtPCR (Fig. 3.2.2).
CO mRNA levels were roughly the same independently of the genotype for each wave band used. Our data show that *CO* expression is the same in wild type or *cry2* mutant plants in either darkness, blue, green, or blue + green, consistent with the previous reported data, which showed no change in *CO* expression in a *cry1cry2* double mutant (Imaizumi *et al* 2003).

When one compares the effect of the different light conditions, it is seen that green and darkness achieved the same *CO* mRNA levels in both the wt and the *cry2* mutant (Fig. 3.2.2 A). However, these levels were somehow enhanced when the plant was exposed to blue light independently of the presence or absence of cry2. This expression enhancement by blue light may be due to FKF1 action (Zikihara K. *et al* 2006). Additionally, the fact that co-irradiation of blue with green light did not abolish *CO* induction points to the fact that FKF1 is not sensitive to green light.

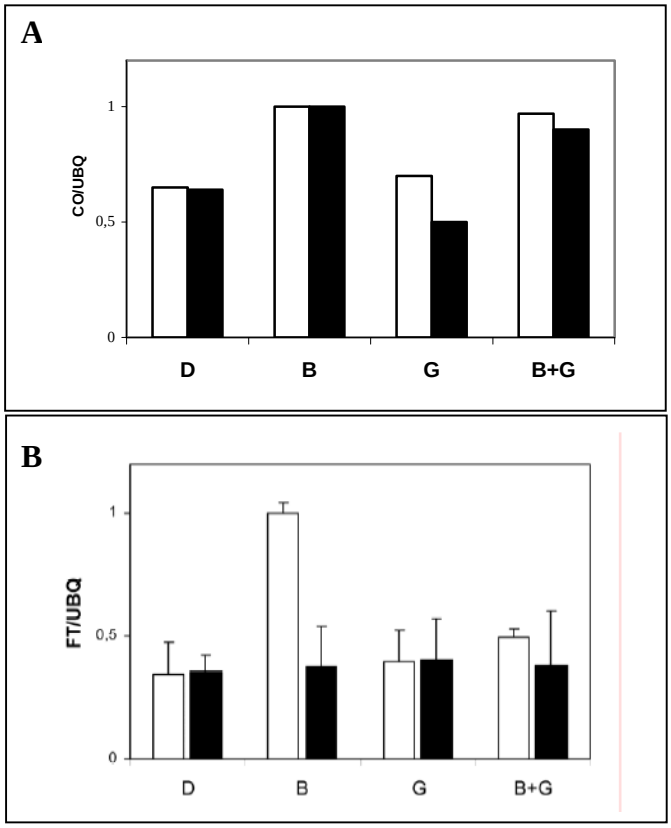


Fig. 3.2.2 Effect of green light on cry2 action. Blue/green light induction of flowering time genes in wild type (open bars) and *cry2* mutant (black bars) seedlings. Plants were grown for 7 days under SD, followed by monochromatic light treatments at the end of the light period with blue (B $\lambda = 470 \pm 10$ nm, fluence rate 2 µmol m^{-2} s^{-1}), green (G $\lambda = 563 \pm 12$ nm, fluence 2 µmol m^{-2} s^{-1}), or blue + green light (B+G same wavebands and fluence rates as indicated before) for 8 h, or were kept in darkness for 8 h as control (D). Light treatments and quantification of transcript levels are described in materials and methods. Data are mean ± SD of 3 independent experiments, normalized to the *UBQ10* control. **(A)** *CO* mRNA expression. **(B)** *FT* mRNA expression.

In any case, *CO* expression was not affected by green light and the delayed flowering time found when plants were irradiated with both blue and green light (Banerjee R. *et al* 2007) can not be explained by *CO* mRNA expression inhibition.

When checking *FT* mRNA expression (Fig. 3.2.2 B) in plants irradiated with blue light, one can note that in the *cry2* mutant the *FT* mRNA levels are impaired compared to wild type plants.

These low *FT* mRNA levels in *cry2* mutant under blue light irradiation correspond to the ones found in either wild type or *cry2* mutant in darkness or under green light. These data are in accordance with the previously known need of active cry2 to promote *FT* expression (Suárez-López P. *et al* 2001; Yanovsky M.J. & Kay S.A. 2002). Irradiation of *cry2* mutant plants with bichromatic blue + green light had, as expected, no effect in *FT* mRNA induction. Nevertheless this irradiation had a great inhibitory effect on wild type plants. Comparing wt plants exposed to blue alone or to blue + green, a reduction of *FT* transcripts to almost basal levels can be observed. Therefore, the flowering delay due to green light co-irradiation can be explained by FT expression inhibition due to cry2 inactivation.

3.3 In planta chromophore composition of *Arabidopsis thaliana* cry2

Studies in chromophore composition of *Arabidopsis* crys expressed in different organisms have revealed the presence of flavin as a primary and indispensable chromophore (Malhotra K. *et al* 1995; Lin C. *et al* 1995a). The presence of a second chromophore, usually MTHF, although a clear fact for *E. coli* photolyase (Johnson J.L. *et al* 1988), is not evident in *Arabidopsis* crys. When *Arabidopsis* crys are expressed in *E. coli* MTHF has been found in addition to FAD (Malhotra K. *et al* 1995). However, when crys have been expressed in a higher eukaryotic system like insect cells, the MTHF could not be detected (Lin C. *et al* 1995a). The lack of this second chromophore seemed not to abolish cry1 biochemical functions, as flavin containing cry1 can undergo autophosphorylation (Bouly J. P. *et al* 2003).

In order to address the question about the presence and identity of a second chromophore in *Arabidopsis* cryptochromes *in planta*, a HiA tagged (Ferrando A. *et al* 2000) version of cry2 was overexpressed in *Nicotiana benthamiana via* transformation with *Agrobacterium tumefaciens* by stomatal infiltration (Voinnet O. *et al* 2003). The HiA tag is composed of a small sequence from the influenza virus hemagglutinin (HA) containing a plant intron (HiA). This system ensures that the expression of the desired tag will be performed in the infiltrated plant but not in the bacteria because only eukaryots can process the intron. The HA tag has also the property of being small, causing little disruption in later biochemical assays once the tagged protein is purified. Moreover commercial, reliable monoclonal antibodies are available against the HA epitope, allowing the specific detection of proteins of interest without the time and money consuming production of specific protein antibodies.

3.3.1 Protein purification controls

Two pull-down elution methods were tested by Western-blot. A mild elution was done by using a pH 3 glycine buffer for 3 hours. Under this condition, the cry2 protein which has a theoretical Ip of 5.8 is expected to be released from the protein-G-agarose beads in a soluble form and with the chromophores still bound. A stronger elution was performed by incubating the beads for 2 hours at 4°C in the presence of native buffer supplemented with TCA to final concentration of 7%. Under these conditions the protein was expected to be denatured and precipitated, releasing the chromophores to the supernatants (Fig. 3.3.1).

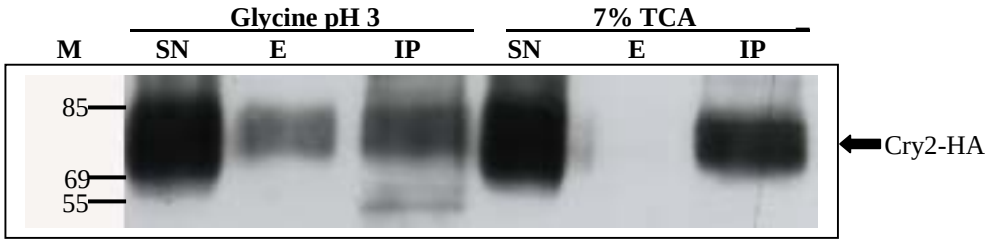


Fig. 3.3.1 Elution controls of cry2-HA immuno precipitation 1 month old *Nicotiana benthamiana* plants were infiltrated with A*grobacterium* carrying the cry2-HiA construct, kept 4 days under LD conditions and afterwards in darkness the day prior to the experiment to enhance cry2 accumulation.
Protein was isolated under safe red light and two immuno precipitation were performed in parallel using an anti HA monoclonal antibody and protein-G-agarose. After removing the supernatant (SN) and washing the pellets, each pull-down pellet was eluted with acid glycine or with TCA buffer as indicated in Materials and Methods. The resultant eluates (E) and the pellet leftovers (IP) were denatured in presence of SDS buffer, loaded on a 10% SDS-PAGE mini-gel and subjected to Western-blot with anti-CCT2 antibody.

As expected, TCA containing buffer precipitates the protein completely, and no protein can be found in the resultant TCA containing supernatant. On the other hand, when a mild elution was used, cry2-HA was found in the eluate fraction, although the elution was not complete as there was still some cry2 remaining in the pellet fraction.

The quality and specificity of the anti-HA antibody (mouse monoclonal anti-HA, clone 12CA5, Roche) was tested by immunoprecipitating protein samples from un-infiltrated and infiltrated (cry2-HA) tobacco samples. The pull-down was performed as described in Materials and Methods using the TCA elution. Protein pellets were analysed by Western-blot and labelled with a specific anti-CCT2 antibody (Fig. 3.3.2).

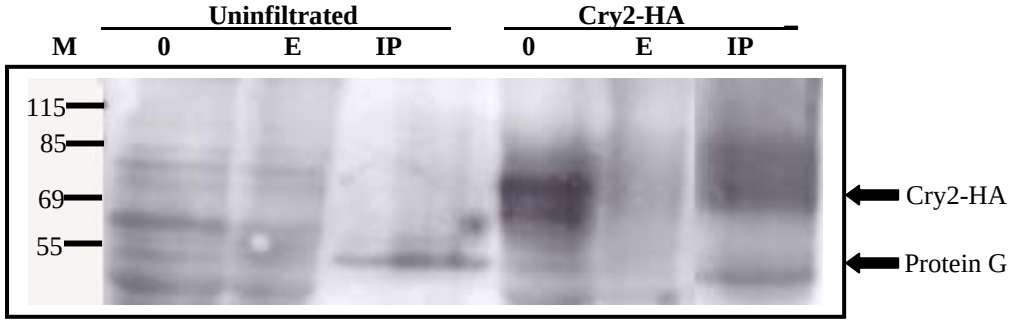


Fig. 3.3.2 Cry2-HA pull-down control. 1 month old *Nicotiana benthamiana* plants were infiltrated with A*grobacterium* carrying the cry2-HiA construct, kept 4 days under LD conditions and afterwards in darkness the day prior to the experiment to enhance cry2 accumulation. Protein extracts were isolated from un-infiltrated and infiltrated (cry2-HA) *Nicotiana benthamiana* plants under safe red light. Two immuno precipitations were performed in parallel using an anti-HA monoclonal antibody and protein-G-agarose. After removing the supernatant and washing the pellets, each pull-down pellet was eluted with TCA buffer as indicated in Materials and Methods. The resultant precipitated protein pellets (IP) and supernatant eluates (E) were denatured in presence of SDS buffer, loaded on a 10% SDS-PAGE minigel, together with a non treated (0) control sample, and subjected to Western-blot with anti CCT2 antibody.

Only one band due to protein G was present in the precipitate from the non-transformed tobacco, indicating that no crossreaction of the anti-HA epitope antibody with other proteins

occurred during the immunoprecipitation. Moreover, the Western-blot showed a complete and specific pull-down of the cry2-HA-tagged protein from the infiltrated tobacco. There was nearly no cry2 in the SN lane but it appears in the pellet. Therefore, this system would be suitable for purification of cry2 from a plant source.

3.3.2 Chromophore studies by fluorescence spectroscopy

3.3.2.1 FAD and MTHF

Although the spectral properties of FAD and MTHF are well known, chromophore stocks were prepared in the TCA buffer used for protein elution described in the Materials and Methods section, and their fluorescence emission and excitation spectra were recorded for a better comparison to the experimental data.

In an emission spectrum the sample is excited with a defined wavelength and the emitted fluorescence is measured for a desired range of the spectrum. Emission spectra were recorded for both FAD and MTHF stock solutions using excitation wavelengths of 380 nm and measuring the fluorescence emission from 400 to 600 nm (Figs. 3.3.3 and 3.3.4, blue line) or exciting at 450 nm and measuring the fluorescent emission from 470 to 600 nm (Figs. 3.3.3 and 3.3.4, black line).
In an excitation spectrum the sample is excited with a range of different wavelengths and the emitted fluorescence light is measured at a defined wavelength. Excitation spectra were recorded for both FAD and MTHF standard solutions measuring fluorescent emission at constant wavelength of 460 nm using exciting wavelength from 320 nm to 440 nm (Figs. 3.3.3 and 3.3.4, yellow line) or using excitation wavelength from 320 nm to 500 nm and measuring fluorescence at 520 nm (Figs. 3.3.3 and 3.3.4, red line).

Oxidized FAD (FADox), but not other redox states of FAD, is fluorescent with an emission maximum at 520 nm and having two excitation peaks, the more intense at 450 nm and the less intense at 380 nm. Consistent with these known properties of FADox, the fluorescence spectra from the FAD standard (Fig. 3.3.3) showed that both emission spectra with excitation at 380 nm (Fig. 3.3.3, blue line) or 450 nm (Fig. 3.3.3, black line) had the maximum emission at 520 nm, with a higher signal when exciting at 450 nm than with excitation at 380 nm.

The excitation spectra measuring emission at 520 nm (Fig. 3.3.3, red line) showed two peaks at 370 nm and 460 nm, very close to the reported maximums, and again being the band at 460 nm more intense than the one at 370 nm. Excitation spectra when measuring emission at 460 nm showed basal levels (Fig. 3.3.3 yellow line).

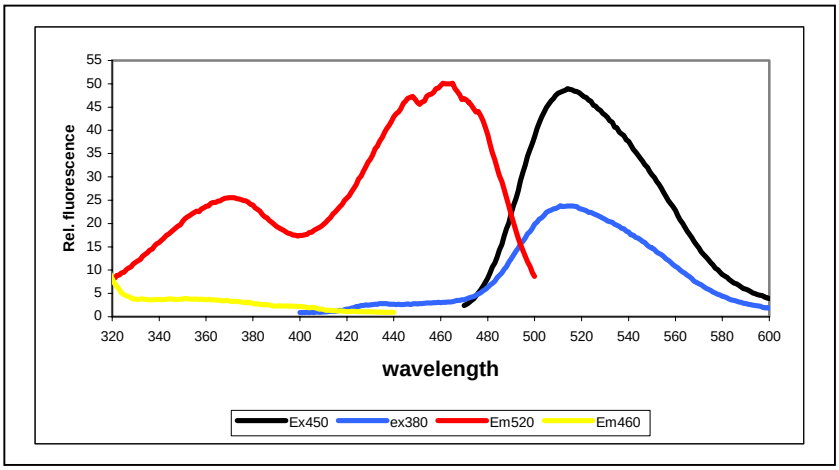


Fig. 3.3.3 FAD standard emission and excitation fluorescence spectra. Emission spectra were recorded for excitation at 380 nm (blue) or 450 nm (black). Excitation spectra were recorded for emission at 460 nm (yellow) or 520 nm (red).

The N^5, N^{10}-methenyl bridge of MTHF, which is responsible for its absorption band at 350-380 nm, is stable only under acidic pH conditions. The maximum of MTHF emission is at 460 nm.

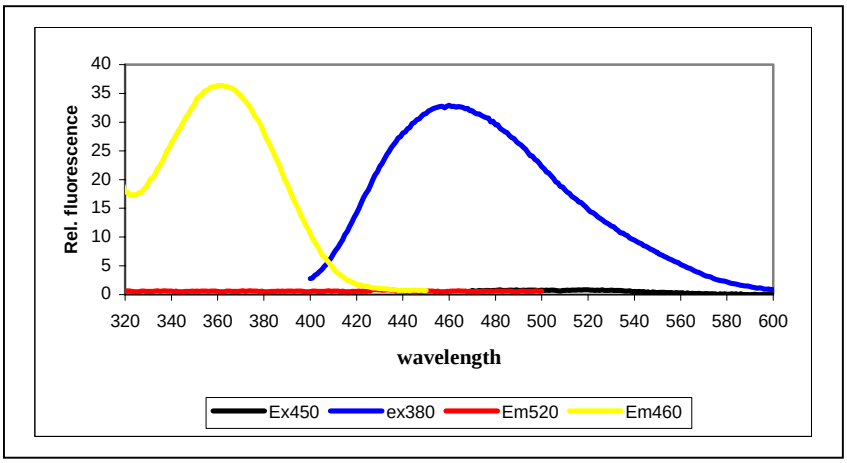


Fig. 3.3.4 MTHF standard emission and excitation fluorescence spectra. Emission spectra recorded for excitation at 380 nm (blue) or 450 nm (black). Excitation spectra were recorded for emission at 460 nm (yellow) or 520 nm (red).

As expected, fluorescent spectra of stock MTHF in TCA buffer (Fig. 3.3.4) showed that the emission spectra for excitation at 380 nm (Fig. 3.3.4, blue line) showed an emission band at 460 nm. Moreover, excitation spectra measuring emission at 460 nm (Fig. 3.3.4, yellow line) had a peak around 360 nm. Both, emission spectra for excitation at 450 nm (Fig. 3.3.4, black

line) or excitation spectra recording emission at 520 nm (Fig. 3.3.4, red line), showed baseline values.

3.3.2.2. Chromophores from plant-purified Atcry2.

To determine *in planta* cry2 chromophore composition, cry2-HA was again expressed in tobacco and purified as described above. Two parallel pull-downs were performed in the same way until elution, when the different elution methods described above were used for each independent pull-down. One sample was eluted using the TCA buffer to completely denature and precipitate the proteins while releasing the chromophores in the supernatant. The second kind of elution was carried out using a pH 3 glycine buffer, described in the Materials and Methods section. Under this condition it was expected to get elution of the cry2 protein from the protein-G agarose beads with the chromophores still bound to the protein.

In order to determine the presence of MTHF, fluorescence spectroscopy recording emission spectra excitation at 380 nm, and excitation spectra measuring emission at 460 nm was performed in both TCA and Gly samples (Fig. 3.3.5).

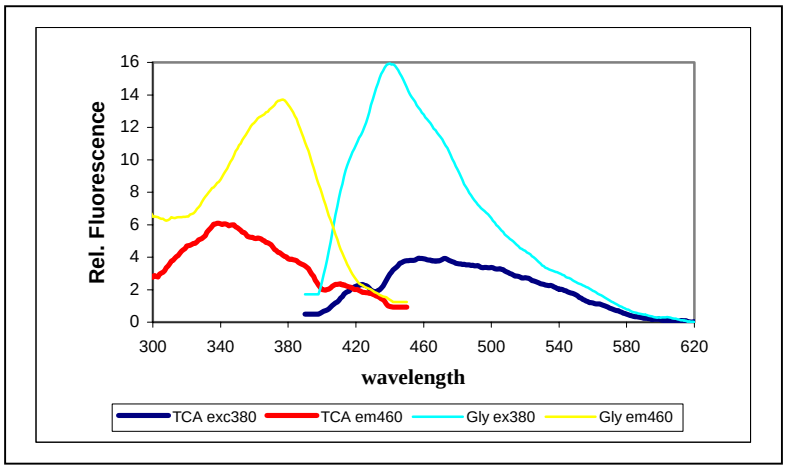


Fig. 3.3.5 Pterine spectra of Atcry2 samples. Fluorescence spectra were recorded for samples containing free (TCA) or cry2 bound (Gly) chromophore. Emission spectra were recorded for excitation at 380 nm. Excitation spectra were recorded for emission at 460 nm.

In the TCA sample, the emission spectrum (Fig. 3.3.5, dark blue line) matched with the one from standard MTHF (Fig. 3.3.4, blue line), with a maximum emission band at 460 nm in both samples. The Gly sample showed a narrow band fluorescence emission, peaking at 440 nm (Fig. 3.3.5, light blue line). Excitation spectra of both TCA (Fig. 3.3.5 red line) and Gly (Fig. 3.3.5, yellow line) samples were similar to standard MTHF (Fig. 3.3.4, yellow line), but

not identical. Standard MTHF had its excitation maximum at 360 nm, whereas the TCA sample, although its spectrum was similar, it had the peak band at 340 nm. On the other hand, in the sample where the chromophore was still bound to the protein had an excitation band shifted to the red region and peaking at 380 nm. Such a red shift of MTHF bound to PHR proteins are common and have been reported before (Sancar A. 2003).

The same TCA and Gly cry2 samples were also analysed for flavin composition by fluorescence spectroscopy recording emission spectra with excitation at 450 nm, and excitation spectra measuring emission at 520 nm (Fig. 3.3.6). The emission spectra of the standard FADox (Fig. 3.3.3, black line), FAD released from cry2 (fig 3.3.6, dark blue line) and FAD bound to cry2 (Fig. 3.3.6, light blue line) coincided with a maximum emission band at 520 nm.

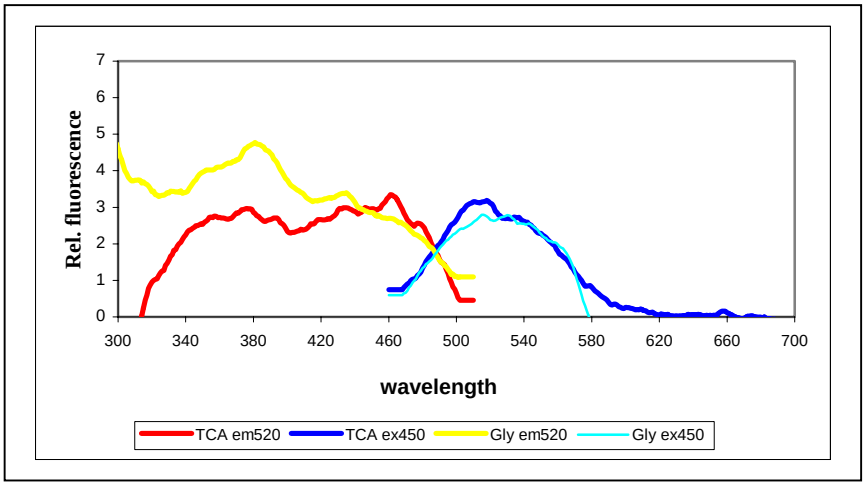


Fig. 3.3.6 Flavin spectra of Atcry2 samples. Fluorescence spectra were recorded for samples containing free (TCA) or cry2 bound (Gly) chromophore. Emission spectra were recorded for excitation at 450 nm. Excitation spectra were recorded for emission at 520 nm.

The excitation spectrum of the TCA sample resembled the one of standard FAD. In both spectra there are two peaks at 380 nm and 460 nm. The standard 460 nm peak is clearly higher than the 370 peak (Fig. 3.3.3, red line), in contrast to the TCA sample (Fig. 3.3.6, red line). The excitation spectrum of the Gly sample, which is supposed to contain both FAD and MTHF bound to cry2, showed the opposite band intensity ratio, being the 380 nm band more intense than the 460 nm band. These results may be explained by an energy transfer from MTHF to FAD. Free FAD can be excited by 380 nm radiation, FAD bound to protein can be excited by Förster resonance energy transfer from MTHF and the total FAD fluorescence emission at 520 nm is higher in latter case.

Additional samples were obtained again by immuno-precipitation as described above to characterize MTHF by other methods. Thin layer chromatography (TLC) of free chromophores and absorption spectra of protein bound chromophores were performed, but gave no clear results (data not shown) very probably because the amounts of protein and chromophores obtained were too small for these assays.

Free chromophore sample was also subjected to MALDI-TOF mass spectrometry, but due to technical problems even fresh diluted standard MTHF could not be detected (data not shown).

3.4 Cry2 dimerization

It has been previously shown that GUS-CCT2 when overexpressed in *Arabidopsis* is constitutively phosphorylated (Shalitin D. *et al* 2002) and active, showing both, a constitutively photomorphogenic (COP) phenotype and early flowering (Yang H.Q. *et al* 2000). Because it is also well known that GUS forms multimers, (Datla R.S. *et al* 1991; Kato A. *et al* 1999) and other photoreceptors like phys (Jones A.M. & Quail P.H. 1986) and phots (Salomon M. *et al* 2004) are dimers, too, the possibility that crys dimerize arose. To address this question a biochemical approach was taken using cry2 tagged versions expressed *in planta.*

This results section is divided in three parts: The first one includes the controls for the plant material, the antibodies, and the light conditions used. The second part describes homodimerization of cry2 and heterodimerization between cry2 and cry1. Finally, *in vivo* effect of cry2 dimerization is explored in the third part.

3.4.1 Controls

Before performing any relevant experiment, controls had to be conducted to check the proper expression of cry2 and truncated versions fused to GFP in our transgenic *Arabidopsis* lines. The antibodies used were also checked by Western-blot using *cry* mutants to ensure that they detect only the desired protein in either *Arabidopsis* or *Nicotiana benthamiana* protein extracts. Finally, light conditions to achieve and detect properly cry2 phosphorylation were set, and *in vitro* stability of phosphorylated cry2 was investigated before using the protein for immuno-precipitation studies.

3.4.1.1 Transgenic *Arabidopsis* cry2-GFP, CCT2-GFP and CNT2-GFP lines

Constructs consistent in full length cry2 or truncated versions (either CNT or CCT) fused to GFP transformed into *Arabidopsis* wt Ler ecotype were available in our laboratory, as indicated in Materials and Methods. The F_2 progeny was analysed for protein expression by Western-blot analysis.

From the six different Cry2-GFP transgenic lines tested (Fig. 3.4.1) only line#7 expressed the construct in significant amount and with the expected molecular mass of 98 kDa.

Strangely, both cry2-GFP and the endogenous cry2 protein run as a double band, except in line#12 where the band close to 69 kDa is lost.

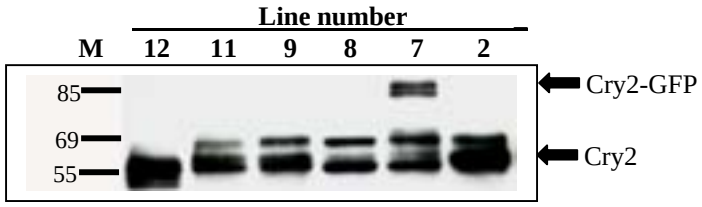


Fig. 3.4.1 Expression of transgenic cry2-GFP. Six different transgenic lines were analysed for cry2-GFP expression. 5 days old etiolated seedlings were harvested under dim red light and subjected to Western-blot analysis labelled with anti-CCT2 antibody.

Four CCT2-GFP and three CNT-GFP F_2 hygromicin-resistant transformants lines were also tested for correct transgene expression (Fig. 3.4.2). Anti-full-length cry2, anti-GFP, and anti-CCT2 antibodies were used to label these blots and detect the CNT2-GFP and CCT2-GFP constructs.

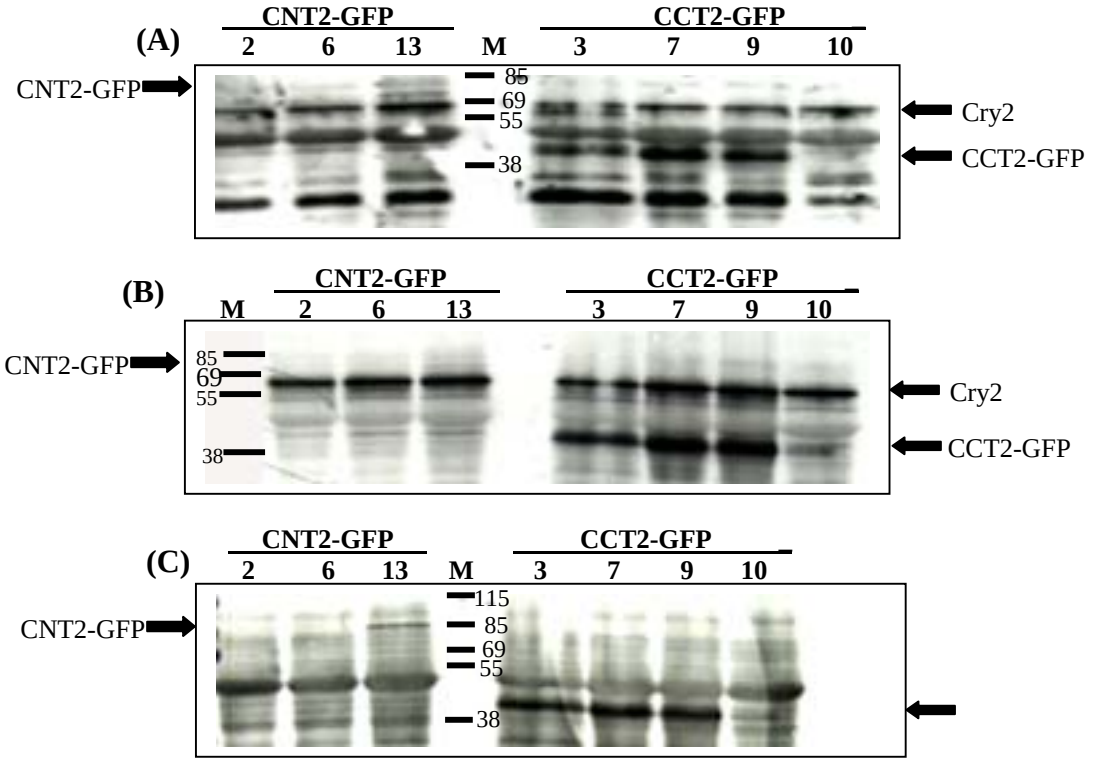


Fig. 3.4.2 Expression of transgenic CCT2-GFP and CNT2-GFP. Three different transgenic lines were analysed for CNT2-GFP expression and four for CCT2-GFP. 5 days old etiolated seedlings were harvested under dim red light and subjected to Western-blot, and labelled with **(A)** anti-cry2 antibody **(B)** striped from A and labelled with anti-CCT2 antibody **(C)** stripped from B and labelled with anti-GFP antibody.

Despite of the strong background signals CNT-GFP line#13 seems to express weakly the construct running close to the expected 89 kDa. From all antibodies used anti-GFP gave the best result. CCT2-GFP is expressed in lines #7, #9 and #3 at the same level as the endogenous cry2.

3.4.1.2 Antibody tests

Because cry2 was detected as a double band (Fig. 3.4.1) the polyclonal anti-CCT2 antibody specificity was tested by Western-blot in etiolated *Arabidopsis* Ler wt, *cry1* and *cry2* mutant seedlings and also in *Arabidopsis* Ler wt seedlings exposed to blue light (Fig. 3.4.3.).

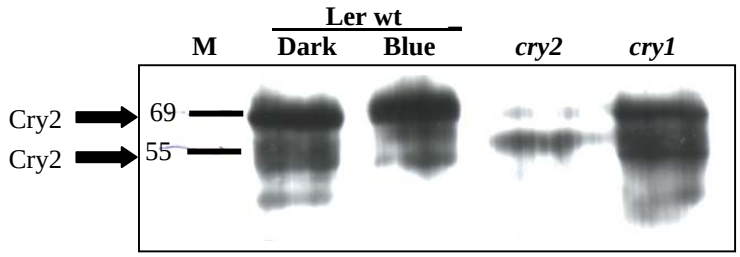


Fig. 3.4.3 Anti-CCT2 antibody control. 5 days old etiolated Ler wt, or *cry1* and *cry2* mutant seedlings were harvested under dim red light. Ler wt were also irradiated with strong blue light (30 µmol m^{-2} s^{-1}) for 10 min. Samples were subjected to Western-blot, with anti-CCT2 antibody.

The first thing to be noted is that cry2 runs as a double band in the wt. Ler wt kept in darkness shows two bands running close to the 55 and 69 kDa marker proteins. When wt seedlings are exposed to blue light the 55 kDa but not the 69 kDa band is depleted. In the *cry2* mutant both, the 55 and the 69 kDa bands are missing, but they are present in the *cry1* mutant, indicating that both bands represent cry2, and they are not a misrecognition of the cry1 protein by the anti-CCT2 antibody.

The anti-CCT1 and the anti-GFP antibodies were also tested to ensure that they could not recognize the cry2 protein. Western-blot was performed with extract from etiolated *Arabidopsis* Ler wt, *cry1, cry2* and *cry1cry2* mutant seedlings (Fig. 3.4.4.).
Anti-CCT1 antibody was able to recognize the endogenous cry1 protein in both *Arabidopsis* wt and *cry2* mutant as a single band running above the 69 kDa marker band (Fig. 3.4.4 A). This cry1 band was not present in either the *cry1* or *cry1cry2* mutants, indicating that the signal does not come from misrecognition of cry2 or other proteins.

It can be noted, that in the *cry1cry2* double mutant a new lower band running above 55 kDa appears. This band corresponds to a truncated *cry1* mutant version.
When the membrane was striped and relabelled with GFP (Fig. 3.4.4 B) some unspecific low molecular weight bands showed up above 38 kDa but no strong band with the size of crys was visible in any sample lane. Some background is visible, probably due to uncomplete stripping of the anti-CCT1 antibody.

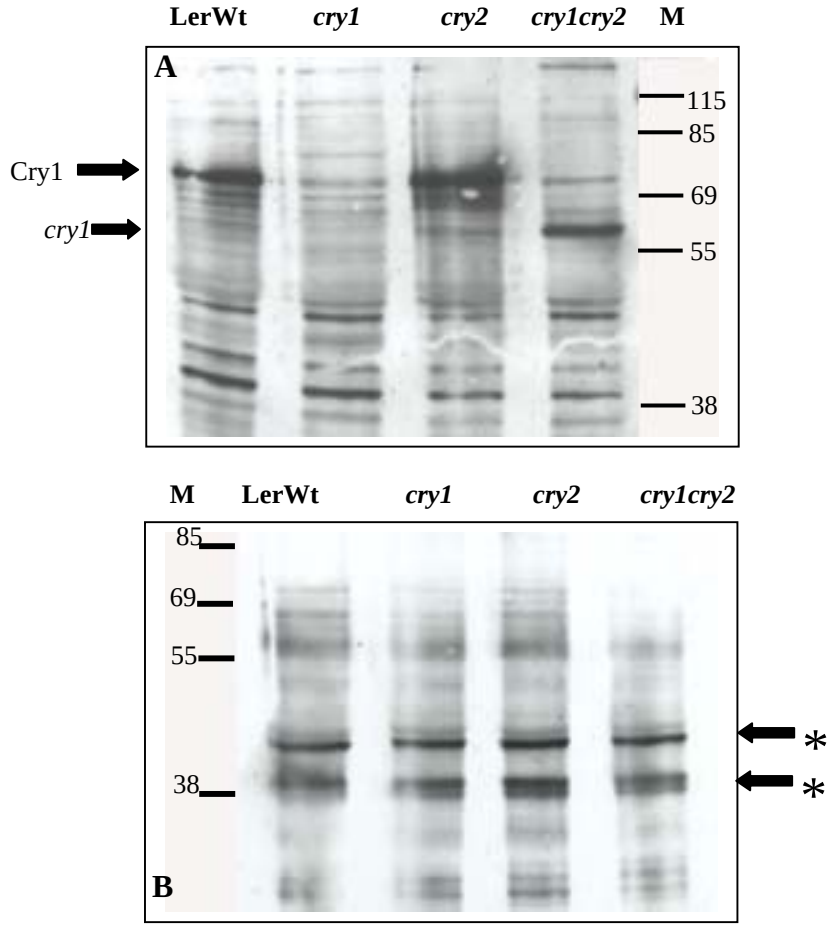


Fig. 3.4.4 Anti-CCT1 and anti-GFP antibodies control. 5 days old etiolated Ler wt, *cry1*, *cry2* and *cry1cry2* mutant seedlings were harvested under dim red light. Protein extracts were obtained and run in a 10% SDS-PAGE gel, subjected to Western-blot, and labelled with **(A)** anti-CCT1 **(B)** stripped from A and labelled with anti-GFP antibody. Cry1 truncated mutant version is indicated as *cry1*. Unspecific crossreacting bands are indicated with asterisks (*)

Antibody specificity and background was tested also in *Nicotiana benthamiana.* Tobacco plants were transformed by *Agrobacterium* infiltration with plasmids for GFP or cry2-GFP expression and were subjected to Western-blot, labelled with anti-GFP, anti-CCT2 and anti HA-antibodies, as control for experiments described below (Fig. 3.4.5).

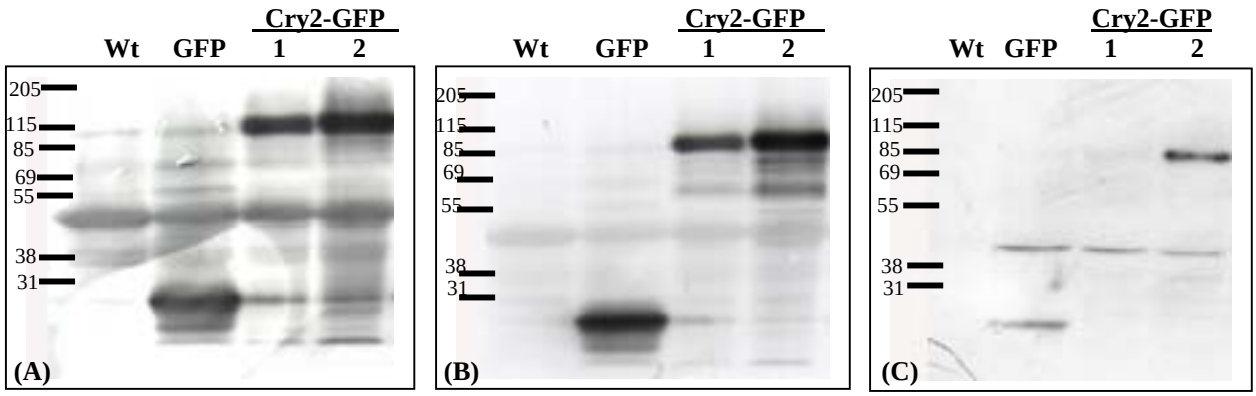


Fig. 3.4.5 Antibody control with tobacco extracts. 1 month old *Nicotiana benthamiana* plants were transformed by the *Agrobacterium* infiltration method with GFP and cry2-GFP plasmids (in duplicate samples 1 and 2). 5 days after infiltration protein extract was obtained and subjected to Western-blot with **(A)** anti-GFP antibody, **(B)** striped from A and labelled with anti-CCT2 antibody, **(C)** stripped from B an labelled with anti-HA antibody.

Anti-GFP antibody although having strong background (Fig. 3.4.5 A) recognizes GFP with calculated molecular weight of 29 kDa running below the 31 kDa marker band, and cry2-GFP of 89 kDa running between 85 kDa and 115 kDa marker bands. On the other hand, although anti-CCT2 antibody recognizes cry2-GFP, and shows less background than the anti-GFP antibody, it also recognizes GFP alone.
The monoclonal anti-HA antibody was also tested in the same blot, showing the lowest background of all three. Unluckily, it recognizes the GFP protein to a low extent.

Anti-HA antibody specificity was further tested in *Nicotiana benthamiana* transformed by *Agrobacterium* infiltration carrying a plasmid for Cry2-HA expression. Samples were subjected to Western-blot, and labelled with anti-HA monoclonal antibody (Fig. 3.4.6).

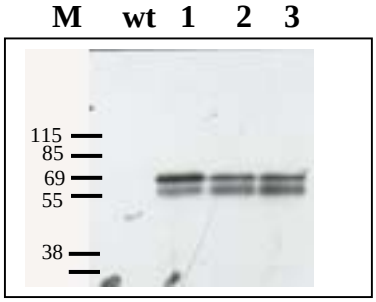


Fig. 3.4.6 Expression control of cry2-HA in tobacco
1 month old *Nicotiana benthamiana* plants in triplicates were infiltrated with *Agrobacterium* carrying the cry2-HiA construct, kept 4 days under LD conditions and kept in darkness the day prior to protein preparation. Samples were subjected to Western-blot with monoclonal anti-HA antibody.

No background was present in the un-infiltrated control (wt) or infiltrated tobacco plants (1, 2 and 3) using the anti-HA antibody (Fig. 3.4.6) indicating that this monoclonal antibody is the most specific one from all tested.
It must be noted that Cry2-HA runs as a double band in tobacco, too, as it was previously seen in *Arabidopsis* (Figs. 3.4.1 and 3.4.3). These bands are not due to background from tobacco as none of them can be seen in the non-transformed samples.

3.4.1.3 Analysis of cry2 phosphorylation

It has been reported that *in vivo* phosphorylation of crys can be seen sometimes as a migration shift in a conventional Lämmli SDS-PAGE gel, or even better detected in a commercial NuPAGE gel. The phosphorylated cry2 protein runs slower than a non-modified cry2 and therefore this band shift can be detected by Western-blot (Shalitin D. *et al* 2002). Thinking in terms of future work on cry2 phosphorylation, it is desirable to reproduce this methodology to have a non-radioactive method to detect phosphorylation of cry2.

Wt Ler *Arabidopsis* seedlings were treated with blue light with suitable fluence rate and time conditions to get maximum phosphorylation (Shalitin D. *et al* 2002, 10 min; 5 µmol m^{-2} s^{-1}). Protein extracts from these plants were obtained in the presence or absence of PPase inhibitors to test the loss of phosphorylation during the protein preparation procedure. Moreover, extracts without PPase inhibitors were subjected to λPPase treatment during different times to revert the protein mobility shift due to phosphorylation. Samples were finally denatured and analysed by Western-blot labelled with anti-CCT2 antibody (Fig. 3.4.7).

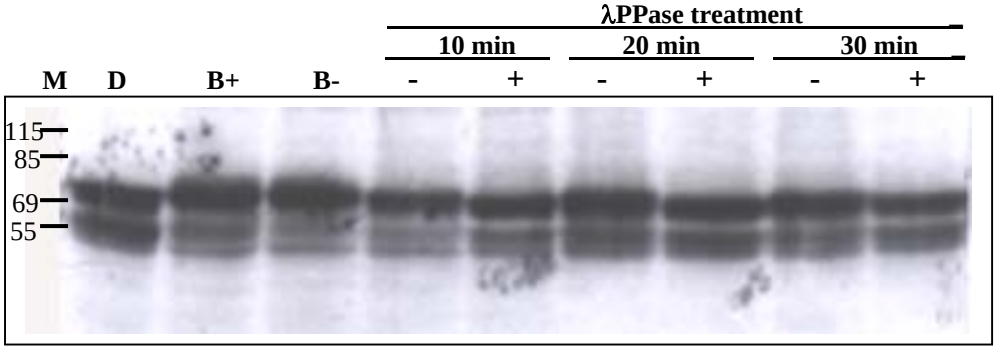


Fig. 3.4.7 Cry2 phosphorylation band shift in Lämmli SDS-PAGE mini-gel. 5 days old *Arabidopsis* wt Ler etiolated seedlings were exposed to blue light (5 µmol m^{-2} s^{-1}, 10 min), or kept on darkness (D) previous to protein extraction. Proteins from blue light irradiated samples were extracted with buffer containing (B+) or lacking (B-) PPase inhibitors. Protein extracts extracted without PPase inhibitors were subjected to λPPase treatment for 10, 20 or 30 min (+), controls (-) manipulated in the same way but without λPPase are included in each case.

Although a little band shift can be seen when dark (D) and blue irradiated (B+ and B-) samples are compared the shift in mobility is not very big. It can be noted, that there is not appreciable difference between samples obtained in the presence (B+) or absence (B-) of PPase inhibitors, as both present the same band mobility shift, and in both the signal from the lower bands close to 55 kDa are weaker than in the non-irradiated dark control (D).

Once treated with λPPase the upper shifted band seems to be lowered, as the treated samples (+) have the cry2 band slightly lower than the phosphorylated untreated controls (-) in all times tested.

3.4.1.4 *In vitro* effect of blue light on cry2 phosphorylation

Cry2 phosphorylation occurs *in vivo* (Shalitin D. *et al* 2002) and cry1 phosphorylation can be conducted also *in vitro* (Bouly J.P. *et al* 2003; Shalitin D. *et al* 2003).

The cry2 phosphorylation state was checked in *Arabidopsis* protein extracts to test if phosphorylation can be obtained *in vitro*, and if blue light has some extra effect on cry2 phosphorylation. For that reason protein extracts from etiolated seedling kept in darkness or

previously exposed to strong blue light, were exposed to strong blue light during times previously reported to be sufficient for cry2 phosphorylation *in vivo* (Fig. 3.4.8).

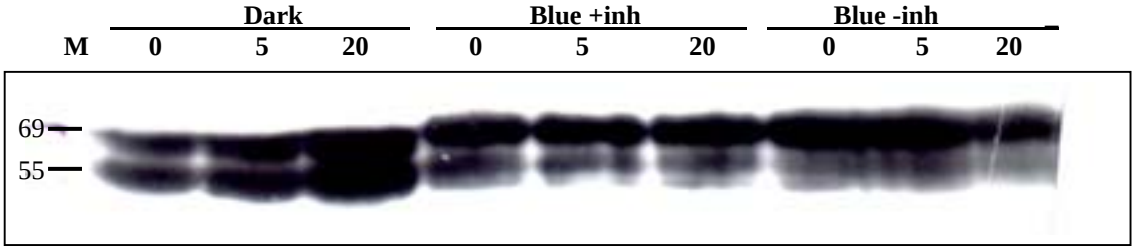


Fig. 3.4.8 *In vitro* effect of blue light on cry2 phosphorylation. 5 days old *Arabidopsis* wt Ler etiolated seedlings were exposed to blue light (5 µmol m^{-2} s^{-1}, 10 min), or kept on darkness (Dark) previous to protein extraction. Protein extraction was performed without PPase inhibitors for (Dark) and (Blue –inh) samples and with PPase inhibitors for (Blue +inh) samples. All of them were exposed to blue light (33 µmol m^2 s^{-1}) for 5 or 20 min. Controls are indicated as time 0 for no light treatment. After light treatment samples were denatured, subjected to Western-blot with anti-CCT2 antibody.

Because phosphorylation needs ATP and this molecule is usually depleted in plant protein extracts. It is not surprising that no phosphorylation *in vitro* was detectable in any protein preparation, because ATP was not added in these assays. Extracts from etiolated *Arabidopsis* seedlings did not show the mobility band shift associated to phosphorylation after blue light-treatment, and no change could be seen in blue light-illuminated extracts from seedlings which had been previously exposed to blue light.

Once more the addition of PPase inhibitors (blue +inh versus blue –inh) had no effect on the phosphorylation state of cry2 as seen before (Fig. 3.4.7).

3.4.1.5 *In vitro* stability of cry2 phosphorylation

Previous experiments showed that the presence of PPase inhibitors had no effect neither in the preparation of phosphorylated cry2 samples nor during the protein extract short incubation time with PPase (Fig. 3.4.7) or blue light (Fig. 3.4.8). Nevertheless, the stability of phosphorylated cry2 was monitored during longer time periods as a control for future immunoprecipitation studies.

Protein extracts from dark or blue light-exposed *Arabidopsis* wt seedlings were incubated in darkness for hours and analysed by Western-blot (Fig. 3.4.9).

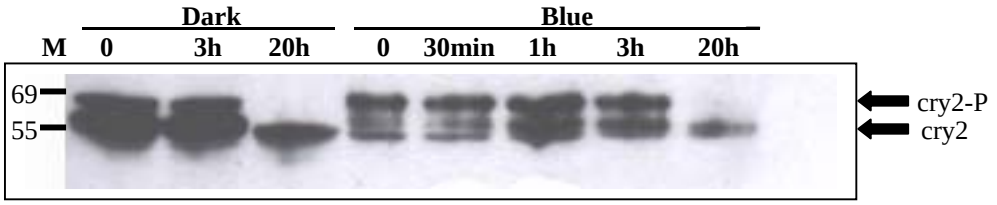


Fig. 3.4.9 *In vitro* stability of cry2 phosphorylation. 5 days old *Arabidopsis* wt Ler etiolated seedlings were exposed to blue light (5 µmol m^{-2} s^{-1}, 10 min), or kept in darkness (Dark) previous to protein extraction. Protein extracts were kept in dark conditions at 4°C during the indicated times, controls are indicated as time 0, before being denatured and subjected to Western-blot with anti-CCT2 antibody.

This time no band mobility shift associated to phosphorylation of the upper cry2 band is visible in the Western-blot, although there is a light effect in the lower cry2 band, as it can be clearly seen that it is depleted in the blue light-treated samples. Cry2 protein is stable when incubated during 3 h in cold and dark conditions, but after 1 h dephosphorylation in the blue light-treated samples was observed as the lower cry2 band appeared again. After long incubation times, like 20 h, cry2 was not stable anymore and a great depletion of the protein was seen in a light independent manner.

3.4.1.6 Blue light fluence dependence of cry2 phosphorylation

One of the main problems of previous experiments was the little separation amongst cry2 bands using the mini gel system. Therefore, to improve electrophoretic resolution a big Lämmli gel system was used for the next experiment. Moreover, the cry2 protein stability and phosphorylation under different blue light fluences has been reported in different papers (Lin C. *et al* 1998, Guo H. *et al* 1999, Shalitin D. *et al* 2002), and an experiment to reproduce all this data to establish our own experimental procedure was needed.

Wt *Arabidopsis* seedlings were illuminated with different blue light intensities and periods, 1 h of low fluence rate (1 µmol m^{-2} s^{-1}), 10 min of medium fluence rate (5 µmol m^{-2} s^{-1}), or 10 min of high fluence rate (30 µmol m^{-2} s^{-1}) were used. Native protein extracts from these samples and dark controls were treated with λPPase and further analysed by Western-blot using a 10% SDS-PAGE big Lämmli gel (Fig. 3.4.10).

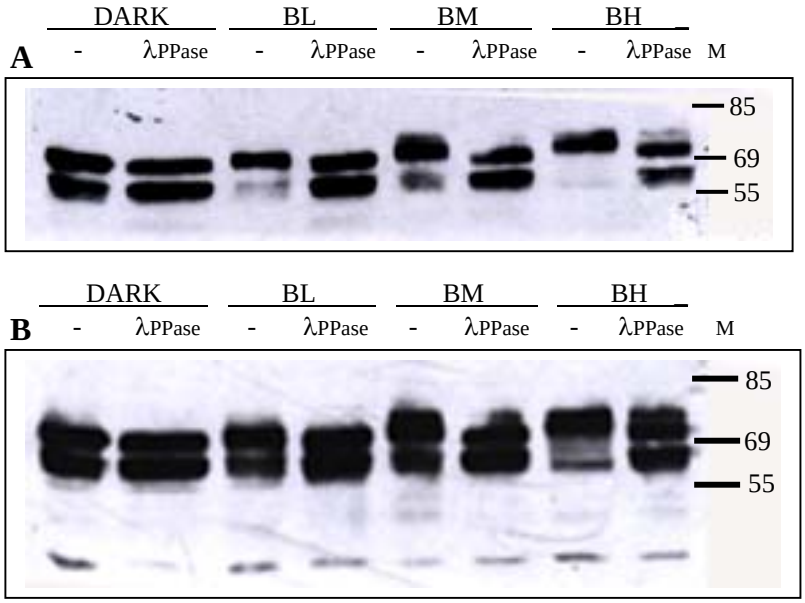


Fig. 3.4.10 Cry2 phosphorylation conditions and detection.
5 days old *Arabidopsis* wt Ler etiolated seedlings were exposed to, 1 h of low (BL) fluence rate (1 µmol m^{-2} s^{-1}), 10 min of medium (BM) fluence rate (5 µmol $m^{-2}s^{-1}$), or 10 min of high (BH) fluence rate (30 µmol m^{-2} s^{-1}) of blue light. Native protein extract from this samples and dark control were subjected to λPPase treatment (λPPase), extract controls without the phosphatase enzyme are included (-). Samples were further analysed by Western-blot with anti-CCT2 antibody and developed using ECL exposure times of (**A**) 1 min (**B**) 5 min.

The big Lämmli SDS-PAGE gel system gave very good results. A clear band separation was obtained, and the mobility band shift associated to phosphorylation of cry2 was also seen.

The cry2 phosphorylation and its reversion due to λPPase treatment can be easily noticed and beacames more evident when stronger blue light fluence rates are used.

Because of the higher separation resolution of this experiment, it can be seen that both cry2 forms already present in the dark samples show a mobility shift when exposed to blue light. The shift is greater in the lower band as was noticed before in the mini gels (Figs. 3.4.7 and 3.4.8), but in the big gels it became evident that the upper band is also shifted up.

In every case, reversion of the mobility band shift in the blue-illuminated samples was achieved with λPPase treatment for both cry2 forms, but the upper band was not converted to the lower due to this treatment. This indicates that the upper band could represent a postranscriptionally modified form of cry2. That result from protein modification by phosphorylation and other processes However, the possibility that some phosphorylated sites in cry2 are not accessible to λPPase in particular after longer light treatments can not be excluded.

In all cases a fraction of cry2 seemed to remain unphosphorylated as can be seen in the blot exposed to long time (Fig. 3.4.10 B).

3.4.2 Cry2 dimerization

3.4.2.1 Cry2 homodimerization

3.4.2.1.1 Immunoprecipitation using cry2-HA and cry2-myc tagged versions

As a first approach to test for cry2 *in vivo* and *in planta* dimerization, immunoprecipitation of tagged version of cry2 expressed in the *Nicotiana benthamiana* system was performed. *Agobacterium* infiltration of tobacco has the advantages of being a fast and high yield protein production method (Voinett O. *et al* 2003). Although transient, the protein production is very strong with the higher levels around 1 week after the transformation. Therefore, this method has an obvious time advantage over the generation of stable *Arabidopsis* transgenic lines, which usually takes months to a year.

Using tagged proteins also gives an advantage, since commercially and reliable antibodies against these small tags are available making possible the detection of the protein of interest by many means. Pull-down of tagged proteins after *Agrobacterium* infiltration has been used before successfully (Ferrando A. *et al* 2000 & 2001) and the same materials described in the literature were used here.
In this immunoprecipitation assay we reasoned that, in case that cry2 dimerizes, when immunoprecipitation is performed against one tagged cry2 protein, it will co-precipitate some of the cry2 fused to the other tag. Therefore, the presence of the second tag can be detected by Western-blot. In order to test this hypothesis, immunoprecipitation was performed with protein extracts from tobacco expressing cry2-HA and cry2-myc (Fig. 3.4.11)

Untransformed tobacco gave no signal when labelled with anti-HA or anti-myc antibodies either in the extract (wt) or in the pull-down negative control (C-). Transformation analog expression efficiency of the different tagged versions of cry2 is not the same. Cry2-HA is much stronger expressed than cry2-c-myc, as can be seen from comparison of the double transformed positive control (+) labelled with anti-HA (Fig. 3.4.11 B) or anti-myc antibody (Fig. 3.4.11 A).
When pull-down was performed with anti-HA antibody and the following Western-blot was labelled with anti-myc antibody, a signal corresponding to the cry2 positive control was obtained (Fig. 3.4.11 B, lane HA P) indicating interaction between cry2-HA and cry2-myc.

The opposite experiment gave positive result, too. A Western-blot from pull-down performed with anti-myc antibody in extracts containing both cry2 tagged versions showed a signal in the pellet fraction when labelled with anti-HA antibody (Fig. 3.4.11 A), pointing once more, to interaction between cry2-HA and cry2-myc.

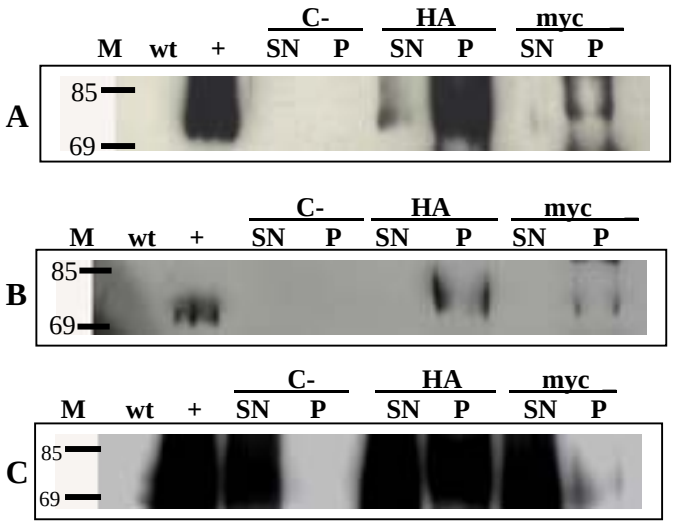


Fig. 3.4.11 Immunoprecipitation of cry2-tagged versions expressed in *Nicotiana benthamiana.*
1 month-old tobacco plants were transformed by infiltration with *Agrobacterium* strains carrying plasmids for cry2-HA and cry2-myc. Double transformations were performed in triplicates, tobacco was left under LD conditions during 4 days and kept under red light the day prior to harvest and protein preparation. Native protein extracts were obtained and subjected to immunoprecipitation with either anti-HA (HA) or anti-myc (myc) antibodies, and protein G-agarose. Native protein extract from untransformed tobacco (negative control, C-) was subjected to the same treatment as the extracts from infiltrated plants, but no antibody was added to check the possible background effect from protein G-agarose. Supernatants (SN) and pellets (P) were denatured, run in a 10% SDS-PAGE minigel. An untransformed tobacco sample (wt) was included as negative control and a double transformed, with cry2-HA and cry2-myc, sample was loaded as positive control. The gel was subjected to Western-blot with **(A)** anti-HA antibody (**B)** stripped and labelled again with anti-myc antibody **(C)** stripped a second time and labelled with anti-CCT2 antibody.

Pitifully, when anti-CCT2 antibody was used to label the same blot, the signal was too strong and black lanes appeared (Fig. 3.4.11 C). The wt untreated control (wt) and the pull-down negative control (C-) pellet remained clear. On the other hand, lanes of samples containing high amounts of cry2-HA (+, HA P) were completely black. However, the pellet from the anti-myc antibody Pull-down (myc P) showed a faint band of the same height and shape like the previously seen when labelled with anti-myc (Fig. 3.4.11 B) or anti-HA antibodies (Fig. 3.4.11 A).

All the supernatant lanes gave a very strong signal. These backgrounds possibly come from the protein G-agarose used in the immunoprecipitation experiment, as the untreated and untransformed negative control sample (wt) remains clear, whereas, the same extract subjected to the same conditions as the rest of the immunoprecipitations (C- SN) gives an unspecific strong signal.

This experiment was repeated several times but in all cases the cry2-myc levels were very low, being difficult to be detected by both, anti-myc and anti-CCT2 antibodies. On the other hand cry2-HA gave always strong signals indicating a good expression of this construct (data not shown).

One reason for this different expression could be that cry2-HA and cry2-cmyc were cloned in different plasmids. Cry2-HiA was cloned in pFGC49 under the control of three copies of the CaMV35S strong promoter, whereas cry2-cmyc was cloned in pPCV812 under the control of two copies of the CaMV35S promoter. Therefore, cry2-cmyc was later cloned in the same way as cry2-HiA, yielding the pFGC49 3xCaMV35S::cry2-myc construct. Nevertheless, when its expression was checked again in tobacco plants the signal did not improve.

Another similar approach could be to use cry2-GFP and cry2-HA for immunoprecipitation but unluckily the anti-HA antibody recognized GFP (Fig. 3.4.5) in a Western-blot.

3.4.2.1.2 Cry2-HA chemical crosslinking

Chemical crosslink allows fixing proteins which interact with partners molecules like DNA or other proteins. To check further the possibility of cry2 dimerization, several crosslinking approaches were used. Glutaraldehyde and formaldehyde at different concentrations, temperatures and incubation times were used as crosslinkers in native protein extracts of tobacco expressing cry2-HA.

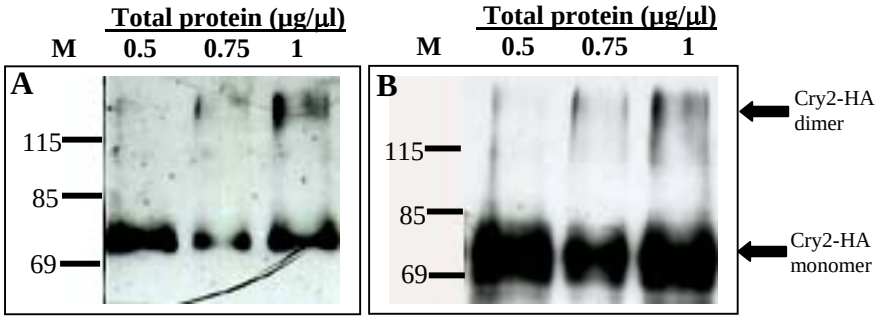


Fig. 3.4.12 *In vitro* Cry2-HA chemical crosslinking. 1 month-old tobacco plants were transformed by infiltration with an *Agrobacterium* strain carrying plasmid for cry2-HA expression. After infiltration tobacco was kept under LD conditions during 4 days and kept under red light the day prior to harvest and protein preparation. Native protein extracts were obtained and subjected to chemical crosslinking using a final concentration of 0.5% (v/v) formaldehyde and increasing protein concentration on each case. Crosslinked samples were denatured, subjected to Western-blot with **(A)** anti-HA antibody **(B)** striped form A and labelled with anti-CCT2 antibody.

The crosslinked extracts were analysed by Western-blot, usually giving too much background in form of many unspecific bands or black blots due to aggressive crosslinking. However,

using formaldehyde with the milder conditions described in Materials and Methods allowed overcoming this problem (Fig. 3.4.12). Increasing amounts of total protein extract were treated with the same final concentration of crosslinker under the same conditions of time and temperature.

Although the expected size of cry2-HA as monomer is 69 kDa, for some reason, when expressed in tobacco cry2-HA monomer runs slower A theoretical cry2 dimer would have a molecular mass of 138 kDa, and in the crosslinked samples a band appeared bigger than the 115 kDa molecular marker when labelled either with anti-CCT2 or anti-HA antibodies. The strength of this upper band increases with the protein concentration, indicating that this may be a dimeric form of cry2-HA.

3.4.2.1.3 Immunoprecipitation of CRY2-GFP

An analogous immunoprecipitation approach as with tagged versions of cry2 expressed in tobacco was performed with transgenic Arabidopsis, using a cry2-GFP *Arabidopsis* transgenic line (transgenic line#7 in Fig. 3.4.1) for immunoprecipitation with anti-GFP antibody. When cry2-GFP and the endogenous cry2 interact, the precipitated cry2-GFP should co-precipitate the endogenous cry2 and further analysis by Western-blot labelled with anti-CCT2 antibody would detect two bands of different size.

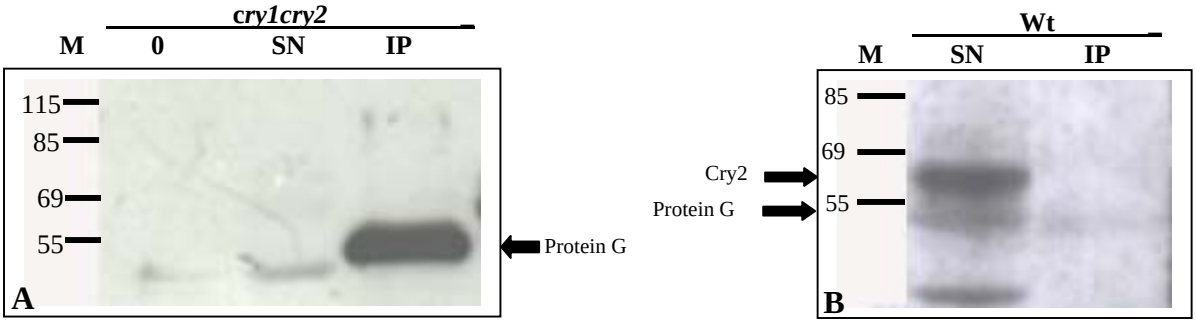


Fig. 3.4.13 Anti GFP immunoprecipitation controls. 5 days-old etiolated *Arabidopsis* wt Ler ecotype and *cry1cry2* double mutant in Ler ecotype background seedlings were used to obtain native protein extracts which were subjected to immuno precipitation with anti-GFP antibody and protein G-agarose. Supernatant (SN) and pellets (IP) were run on a 10% SDS-PAGE minigel, untreated extract negative control for *cry1cry2* mutant was included, indicated as 0. The separated extracts were analysed by Western-blot with anti-CCT2 antibody. **(A)** immunoprecipitation from *Arabidopsis cry1cry2* mutant; **(B)** immuno precipitation from *Arabidopsis* wt ecotype Ler.

Before carrying out this experiment, controls were performed to check that the anti-GFP antibody does not precipitate the endogenous cry2 and to detect unspecific crossreacting bands (Fig. 3.4.13).

Performing immunoprecipitation with protein extracts from *Arabidopsis* wt and *cry1cry2* double mutant revealed a crossreacting band due to protein G-agarose running lower than 55 kDa, and no immunoprecipitation of cry2 by the anti-GFP antibody.

Once controls were obtained, immunoprecipitation was performed with protein extracts from etiolated transgenic *Arabidopsis* cry2-GFP seedlings using an anti-GFP antibody (Fig. 3.4.14).

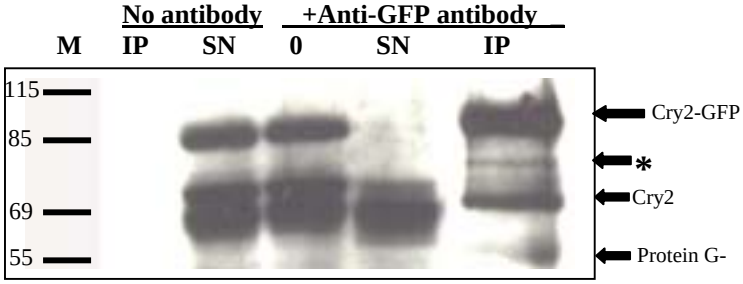


Fig. 3.4.14 Immunoprecipitation of cry2-GFP in extracts from seedlings kept in darkness. 5 days-old etiolated *Arabidopsis* cry2-GFP line#7 transgenic seedlings were harvested and native protein extracts obtained. Immunoprecipitation was performed with anti-GFP antibody and protein G-agarose. A negative control without antibody was carried out at the same time. Supernatants (SN) and pellets (IP) were run on a 10% SDS-PAGE mini gel, untreated extract negative control (0) was included. After the run, the gel was analysed by Western-blot with anti-CCT2 antibody. Unspecific band is labelled as (*)

The pull-down of cry2-GFP was complete, since cry2-GFP band is absent in the SN but present in the precipitated pellet together with the endogenous cry2. Some cry2 is still present in the supernatant probably because cry2-cry2 dimers are also formed which are not precipitated by the anti-GFP antibody. A Control pull-down performed without antibody ensured that the immunoprecipitation effect is no due to unspecific binding of the protein to protein G-agarose, as no band appeared in this pellet and both proteins, cry2 and cry2-GFP, remained completely in the supernatant.

Once determined that cry2 forms homodimers in darkness, the effect of light in dimerization was investigated. The same *Arabidopsis* cry2-GFP transgenic line used before was irradiated with low and high fluence rates of blue light, and the dimerization of cry2 checked again by the same means.

Seedlings exposed to low (Fig. 3.4.15) or high (Fig. 3.4.16) blue light fluence rates showed cry2 homodimerization. In both cases cry2-GFP was completely precipitated, since it did not appear in the supernatant fraction. Cry2 appeared in both, supernatant and pellet, indicating again the formation of different cry2 dimers, being cry2-cry2 dimers not coprecipitated.

In the Western-blot from seedlings exposed to a low fluence of blue light an additional band corresponding most likely with a phosphorylated form of cry2 can be seen in the pellet

fraction (Fig. 3.4.15). This band could not be found in the pellet of seedlings exposed to a high fluence of blue light, and it is also greatly depleted in the supernatant (Fig. 3.4.16).

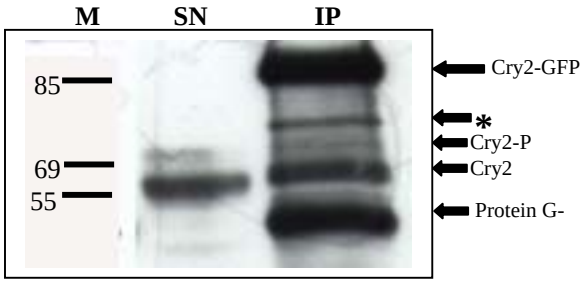


Fig. 3.4.15 Immunoprecipitation of cry2-GFP in extracts from seedlings kept under low blue light irradiation. 5 days-old *Arabidopsis* cry2-GFP line#7 transgenic seedlings were irradiated for 1 h with low fluence rate blue light (1 µmol m^{-2} s^{-1}) before harvest. Native protein extract was obtained and immunoprecipitation performed with anti-GFP antibody and protein G-agarose. Supernatant (SN) and pellet (IP) were run on a 10% SDS-PAGE gel and subjected to Western-blot with anti-CCT2 antibody. Unspecific

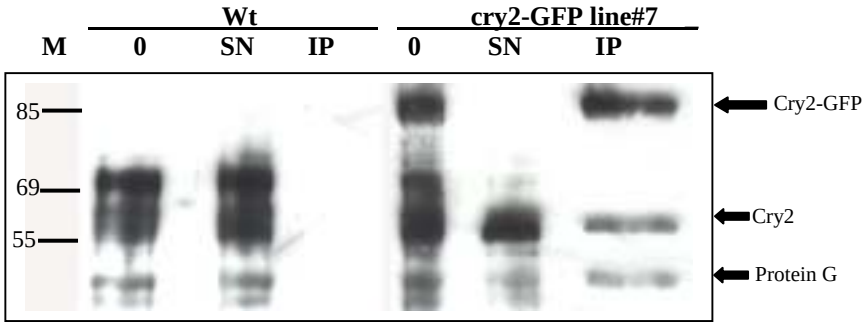


Fig. 3.4.16 Immunoprecipitation of cry2-GFP in extracts from seedlings kept under high blue light irradiation. 5 days-old *Arabidopsis* etiolated wt and cry2-GFP line#7 transgenic seedlings were irradiated for 10 min with high fluence rate of blue light (30 µmol m^{-2} s^{-1}) before harvest. Native protein extracts were obtained and immunoprecipitation was performed with anti-GFP antibody and protein G-agarose. Supernatants (SN) and pellets (IP) were run in a 10% SDS-PAGE gel, untreated extract negative control was included, indicated as 0. After the run, the gel was analysed by Western-blot with anti-CCT2 antibody.

An explanation for this missing phosphorylated cry2 band is the low stability of cry2 under high fluence of blue light Moreover, phosphorylated cry2 showed spontaneous dephosphorylation in the extract (Fig. 3.4.9), and no PPase inhibitors were used in these immunoprecipitations. Thus, is very probable that dephosphorylation occurred in the extract from high blue light fluence-exposed seedlings. Therefore, this immunoprecipitation experiment with was repeated in presence of PPase inhibitors (Fig. 3.4.17).

Coimmunoprecipitation of the endogenous cry2 by cry2-GFP pull-down was successfully reproduced. However, the cry2 phosphorylated form seen in extracts from seedlings kept under low blue light fluence rate was again not found in the pellet fraction. Nevertheless, this time it could be detected in the supernatant. The stability of the cry2 phosphorylated band was increased if compared to the experiment performed without PPase inhibitors (Fig. 3.4.16). However, it was still greatly depleted in comparison to the untreated extract controls (0+ lanes in Fig. 3.4.17).

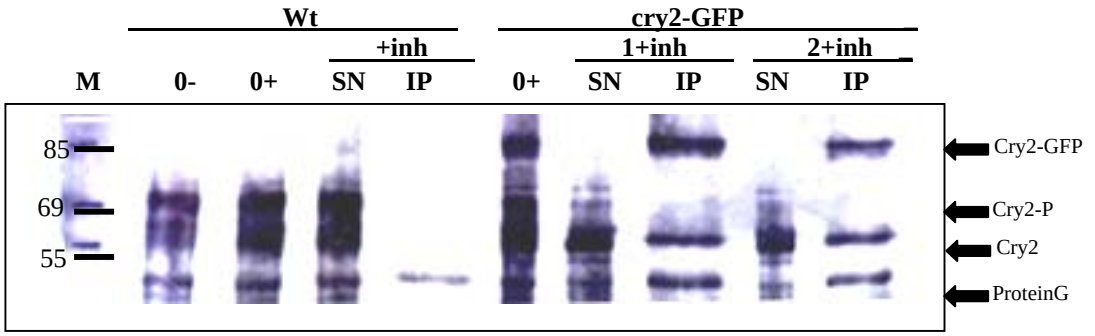


Fig. 3.4.17 Immunoprecipitation of cry2-GFP in extracts from seedlings kept under high blue light irradiation. 5 days-old *Arabidopsis* etiolated wt and cry2-GFP line#7 transgenic seedlings were irradiated during 10 min with high fluence rate of blue light (30 µmol m^{-2} s^{-1}) before harvest. Native protein extract were obtained in presence (+inh) of PPase inhibitors and immunoprecipitation was performed in duplicates (1 and 2) with anti-GFP antibody and protein G-agarose. Supernatants (SN) and pellets (IP) were run on a 10% SDS-PAGE gel, untreated extract negative controls were included, indicated as (0+) when had PPase inhibitors or (0-) when they had not. After the run, the gel was analysed by Western-blot with anti-CCT2 antibody.

3.4.2.2 Domains involved in cry2 homodimerization

Once the question of cry2 homodimerization was positively answered, the next question to address was which are the domains involved in the interaction.

Crys can be divided in their PHR like domain CNT, and their signalling domain CCT. Because *Arabidopsis* transgenic lines expressing either CNT2-GFP or CCT2-GFP were available (Fig. 3.4.2) the same approach of pull-down used with the full-length cry2-GFP version was used.

3.4.2.2.1 Interaction of cry2 with CNT2

Immunoprecipitation was performed with extracts of etiolated transgenic *Arabidopsis* CNT2-GFP and wt seedlings, and with the extracts of CNT2-GFP transgenic seedling lines exposed to high fluence rates of blue light (Fig. 3.4.18).

The CNT2-GFP construct was successfully immunoprecipitated. However, it did not co-precipitate the endogenous cry2 protein which remained in the supernatant in either dark or illuminated conditions. It must be noted than the stripping conditions were not very strong to avoid loss of protein, and cry2 protein was recognized again in the labelling with anti-GFP antibody (band labelled as *) because both anti-CCT2 and anti-GFP are antibodies raised in rabbit, and residual anti-CCT2 bound to cry2 was recognized by the secondary anti-rabbit antibody.

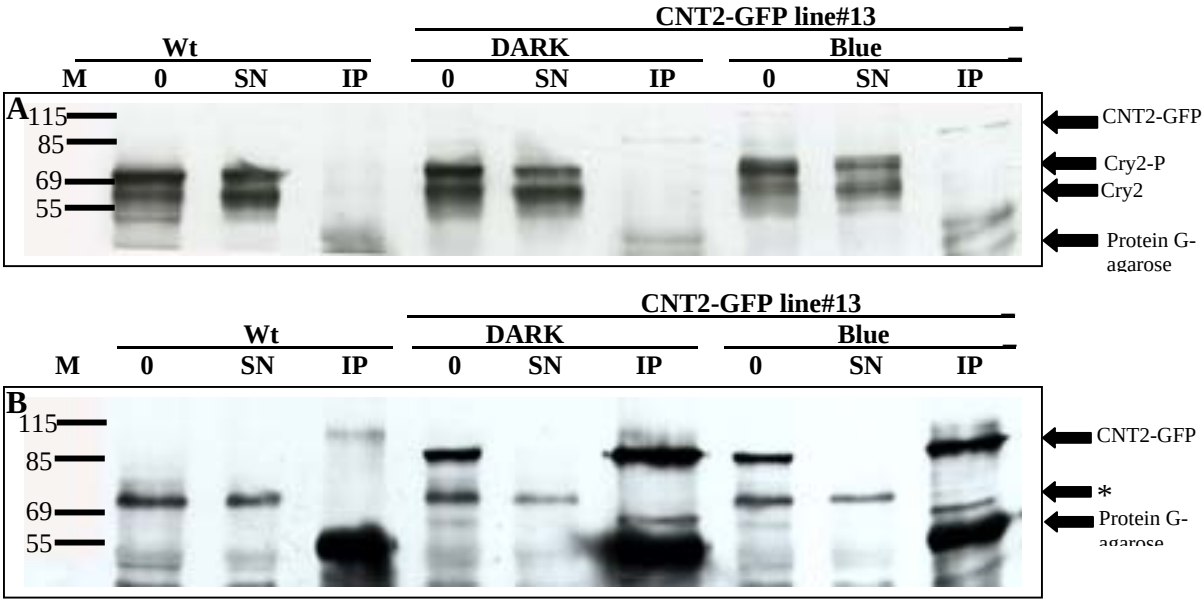


Fig. 3.4.18 CNT2-GFP interaction with cry2 in extracts from seedlings kept in darkness and high blue light irradiation. 5 days-old etiolated *Arabidopsis* wt and CNT2-GFP line#13 transgenic seedlings were harvested under safe dim red light, *Arabidopsis* CNT2-GFP line#13 transgenic seedlings were also exposed to high blue light fluence rate (30 µmol $m^{-2}s^{-1}$) for 10 min before harvest. Native protein extracts were obtained from them in presence of PPase inhibitors and immunoprecipitation was performed with anti-GFP antibody and protein G-agarose. Supernatants (SN) and pellets (IP) were run on a 10% SDS-PAGE gel, untreated extracts were added as negative controls (0). After the run, the gel was analysed by Western-blot with (A) anti CCT2-antibody (B) stripped from A and labelled with anti GFP-antibody.

3.4.2.2.2 Interaction of cry2 with CCT2

Immunoprecipitation was performed with extracts of etiolated transgenic *Arabidopsis* CCT2-GFP and wt seedling (Fig. 3.4.19), and with the same seedling lines exposed to medium and high fluences of blue light (Fig. 3.4.20).

In every case, the CNT2-GFP construct was successfully immunoprecipitated, but it did not coprecipitate the endogenous cry2 protein which remained in the supernatant in either dark or illuminated conditions. Although the protein-protein interaction was not seen, this experiment can be taken as an extra control, proving that the pull-down is not due to an unspecific interaction between the endogenous cry2 and the GFP protein.

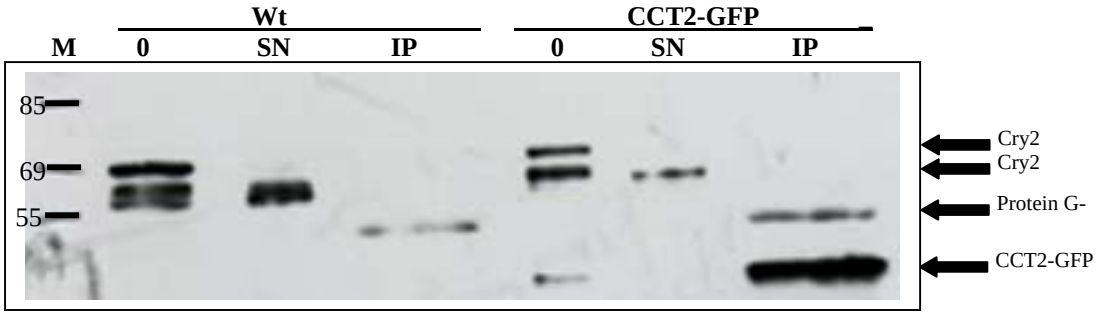


Fig. 3.4.19 Immunoprecipitation of CCT2-GFP in extracts from seedlings kept in darkness. 5 days old etiolated *Arabidopsis* wt and CCT2-GFP line#7 transgenic seedlings were harvested under safe dim red light. Native protein extracts were obtained in absence of PPase inhibitors and immunoprecipitation was performed with anti-GFP antibody and protein G-agarose. Supernatants (SN) and pellets (IP) were run on a 10% SDS-PAGE gel, untreated extracts as negative controls were included (0). After the run, the gel was analysed by Western-blot with anti-CCT2 antibody.

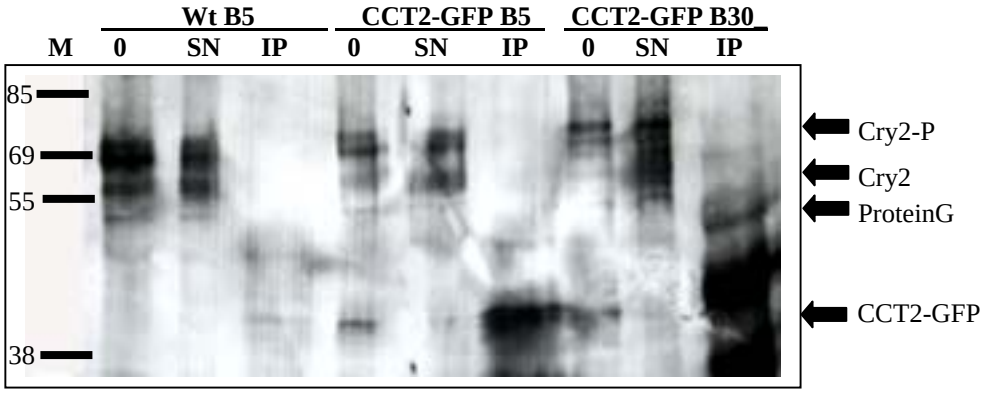


Fig. 3.4.20 Immunoprecipitation of CCT2-GFP in extracts from seedlings kept under blue light. 5 days-old etiolated *Arabidopsis* wt and CCT2-GFP line#7 transgenic seedlings were irradiated for 10 min with high (30 µmol $m^{-2}s^{-1}$) or medium (5 µmol $m^{-2}s^{-1}$) fluence rates blue light before harvest. Native protein extracts were obtained in presence of PPase inhibitors and immunoprecipitation was performed with anti-GFP antibody and protein G-agarose. Supernatants (SN) and pellets (IP) were run in a 10% SDS-PAGE gel, untreated extracts as negative controls were included (0). After the run, the gel was analysed by Western-blot with anti-CCT2 antibody.

3.4.2.3 Heterodimerization

Because cry1 and cry2 share many common structural features, being their PHR-like domain very similar, and common motifs can be found in their specific CCT, the possibility of cry1 cry2 heterodimerization was investigated.

Crys and phys interact physically, and both photoreceptor types interact with COP1 (Fig. 1.3 and references therein). In addition to the known physical interactions, there is a large body of evidence based on genetic studies pointing to the need of phy presence for cry1 activity (Ahmad M. & Cashmore A.R. 1997b; Neff M.M. & Chory J. 1998; Casal J.J. & Mazzella M.A. 1998). A putative cry1-cry2 heterodimerization would add more complexity to the cry-phy dependence, but at the same time it would provide evidence for the existence of a light sensing super-complex, being the cry1-cry2 heterodimer in this scenario a putative physical scaffold for cry1-phyB interaction.

To explore the putative formation of cry1 heterodimers, immunoprecipitation was performed using extracts from etiolated *Arabidopsis* Cry2-GFP transgenic seedlings and wt, and from transgenic cry2-GFP seedlings that were exposed to high fluence rates of blue light (Fig. 3.4.21).

A cry1 signal corresponding with the band seen in the anti-CCT1 antibody controls (Fig. 3.4.4) showed up in all the supernatant (SN) fractions. However, it does not appears in any occasion in the pellet (IP) indicating a lack of interaction between cry2-GFP and the endogenous cry1.

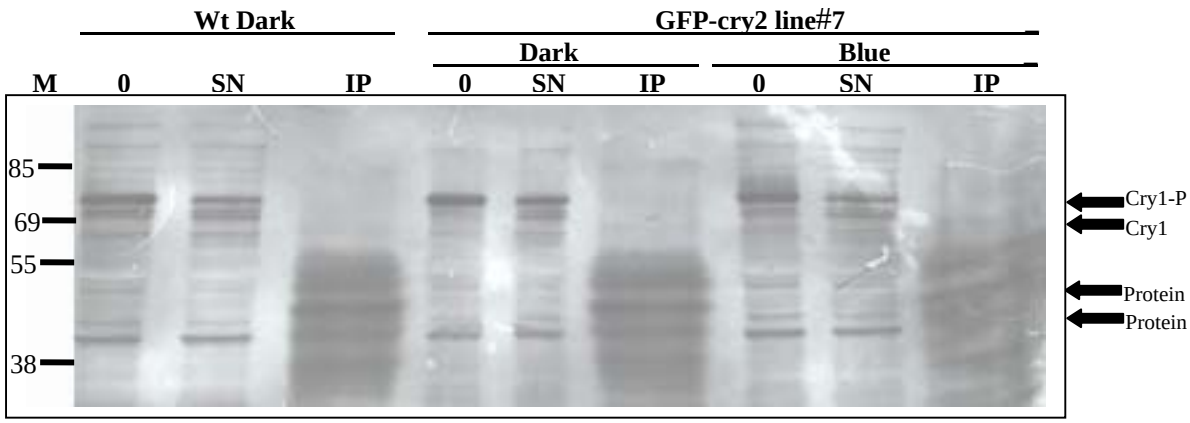


Fig. 3.4.21 Cry2-GFP heterodimerization with cry1. 5 days-old *Arabidopsis* etiolated wt and cry2-GFP line#7 transgenic seedlings were irradiated for 10 min with high fluence rate blue light (30 µmol $m^{-2}s^{-1}$) or kept continuously in darkness before harvest. Native protein extracts were obtained in presence of PPase inhibitors and immuno precipitation was performed with anti-GFP antibody and protein G-agarose. Supernatants (SN) and pellets (IP) were run on a 10% SDS-PAGE gel, untreated extract negative controls were included (0). After the run, the gel was analysed by Western-blot with anti-CCT1-antibody.

For additional prove of lack of interaction, the previous blot from a CNT2-GFP immunoprecipitation (Fig. 3.4.18) was stripped again and labelled with anti-CCT1 antibody (Fig. 3.4.22).

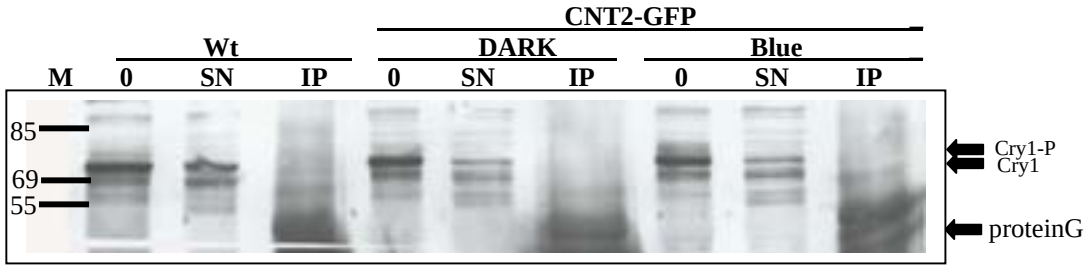


Fig. 3.4.22 CNT2-GFP interaction with cry1. 5 days-old etiolated *Arabidopsis* wt and CNT2-GFP line#13 transgenic seedlings were harvested under safe dim red light, *Arabidopsis* CNT2-GFP line#13 transgenic seedlings were also exposed to 10 min of high blue light fluence (30 µmol m^{-2} s^{-1}) before harvest. Native protein extracts were obtained in presence of PPase inhibitors and immunoprecipitation was performed with anti-GFP antibody and protein G-agarose. Supernatants (SN) and pellets (IP) were run on a 10% SDS-PAGE gel, untreated extracts as negative controls were included (0). After the run, the gel was analysed by Western-blot that had been labelled before, striped a third time and labelled with anti CCT1-antibody.

It was shown before that CNT2-GFP is not able to pull-down the endogenous cry2 protein (Fig. 3.4.18), and neither CNT2-GFP nor cry2-GFP were able to coprecipitate the endogenous cry1.

Interestingly although cry1 is not degraded *in vivo* when exposed to blue light (Shalitin D. *et al* 2003), here we see a clear *in vitro* reduction of cry1 protein during the immunoprecipitation incubation despite the addition of protease inhibitors. This fact points to some instability of cry1 in the extracts, which is however not affected by the light conditions under which the plants were kept before harvest. Moreover, phosphorylation of cry1 under the light conditions used is not clearly detected. Although a smear seems to appear when the seedlings were treated with strong blue light (labelled as cry1-P) there is no evident up-shift as seen for cry2.

Is very probable that cry1 phosphorylation requires longer exposure times than those used in our experiment

To further explore the possibility of cry heterodimerization, different light conditions were used in the seedling treatment prior to immunoprecipitation. In addition to the dark control, wt seedlings were exposed to medium fluence rates blue light, and CNT2-GFP seedlings were exposed to high fluence rates of blue light during long time. Because cry2 is degraded when seedlings are exposed to high intensities of blue light, the CNT2-GFP version was used, as this construct is, based on previous observations, expected not to be degraded. Moreover, under these high light and long time conditions, cry1 is expected to get phosphorylated in an extent enough to be detected

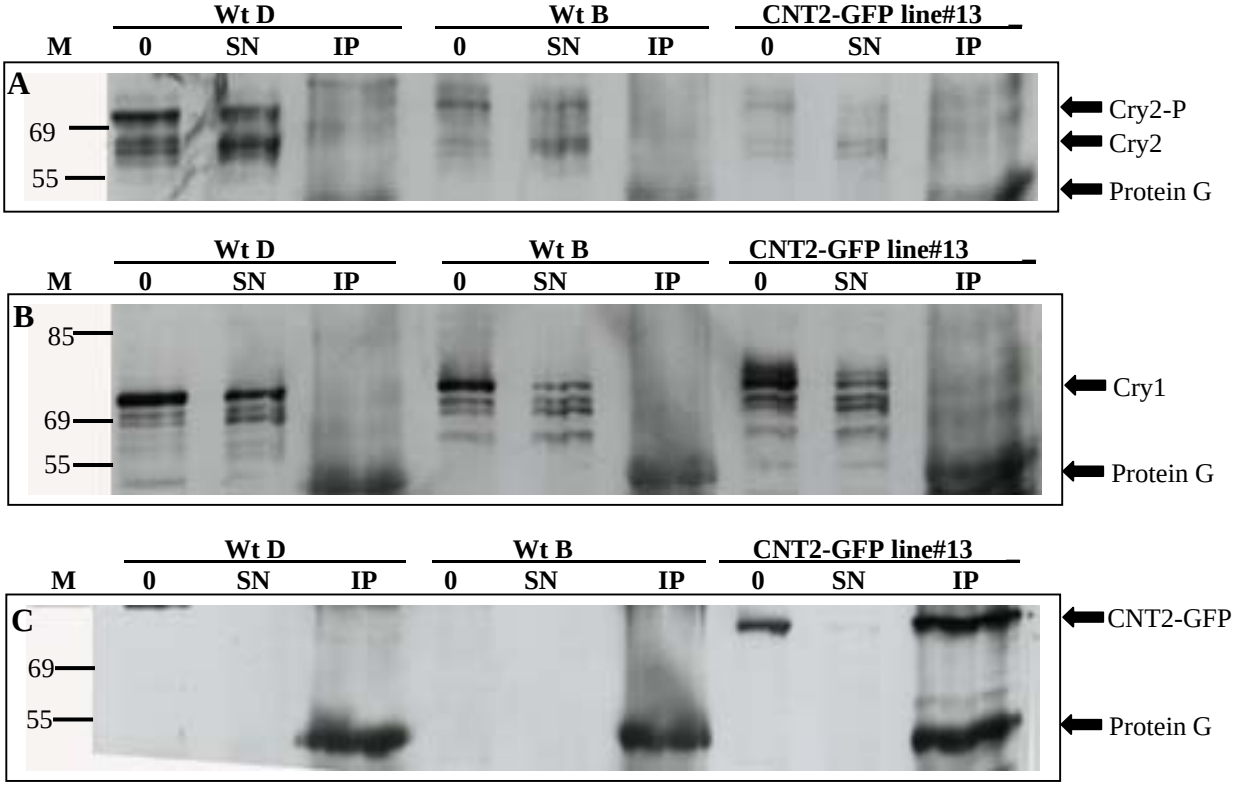


Fig. 3.4.23 Interaction of cry1 with CNT2-GFP. 5 days-old *Arabidopsis* etiolated wt seedlings were harvested under safe dim red light, or exposed for 10 min to medium fluence rate blue light (5 µmol $m^{-2}s^{-1}$) before harvest. Transgenic *Arabidopsis* CNT2-GFP line#13 seedlings of the same age were irradiated for 1 h with high fluence rate blue light (30 µmol $m^{-2}s^{-1}$) before harvest. Native protein extracts were obtained in presence of PPase inhibitors and immunoprecipitation was performed with anti-GFP antibody and protein G-agarose. Supernatants (SN) and pellets (IP) were run on a 10% SDS-PAGE gel, untreated extract negative controls were included (0). After the run, the gel was analysed by Western-blot with **(A)** anti CCT2 antibody, **(B)** striped from A and labelled anti-CCT1 antibody, **(C)** striped from B and labelled anti GFP antibody.

In the resulting Western-blot from this experiment (Fig. 3.4.23), it can be seen that the degradation of the endogenous cry2 depends on the exposure time and fluence rate of blue light (Fig. 3.4.23 A). Once more, CNT2-GFP was not able to coprecipitate the endogenous

cry2 independently of the light conditions used (Figs 3.4.19 and 3.4.20). CNT2-GFP proved to be stable under the strong blue light and long exposure times. It seems to run as a single band, which can be completely immunoprecipitated with the anti-GFP antibody (Fig. 3.4.23 C). However, endogenous cry1 was not coprecipitated with CNT2-GFP (Fig. 3.4.23 B). Additionally, cry1 was not very stable *in vitro* conditions, and considerable amounts of protein were lost during the Immunoprecipitation (signal in supernatants compared to total protein extract). Nevertheless, when seedlings were kept under strong blue light during long times, appreciable band mobility shift could be seen for cry1, indicating cry1 phosphorylation. Looking to all these results together, it is evident that no cry1-cry2 heterodimer formation could be detected despite of the different conditions used.

3.4.3 Biological role of cry2 dimerization

3.4.3.1 Role in phosphorylation

Events associated with cry2 activation are phosphorylation and degradation. For that reason, the first question to be determined is, if cry2-GFP behaves in the same way as the endogenous cry2. Moreover, because of the proven fact of cry2-cry2-GFP heterodimer formation (Figs. 3.4.14-3.4.17), the effect of cry2-cry2-GFP dimerization on the behaviour of the endogenous cry2 was also investigated. For this reasons, a phosphorylation kinetic using long times under high fluence rates of blue light was conducted for wt and cry2-GFP transgenic *Arabidopsis* to compare the two cry2 versions (Fig. 3.4.24).

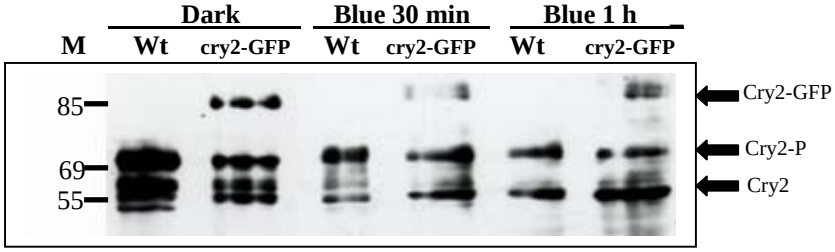


Fig. 3.4.24 Cry2-GFP phosphorylation and degradation kinetics under high fluence rate blue light. 5 days-old etiolated *Arabidopsis* Ler wt, or cry2-GFP transgenic seedlings were grown in continuous darkness, harvested under dim red light (Dark) or treated with high fluence rate blue light (30 µmol $m^{-2}s^{-1}$) for 30 or 60 min before harvest. Samples were subjected to Western-blot with anti-CCT2 antibody. ECL exposure was for 15 min to enhance cry2 detection.

In long time and high blue light fluence treatments, endogenous cry2 from wt and cry2-GFP line seemed to respond equal. The lower band of cry2, running near 55 kD, disappeared after the blue light treatment. Additionally, the upper band of cry2, running close to the 69 kD

marker band, showed the same band mobility shift associated to phosphorylation as in the control experiment (Fig. 3.4.9). Together these data suggest that interaction of cry2 with cry2-GFP does not affect the Phosphorylation. Looking at the cry2-GFP behaviour, it can be noticed that it is also degraded under blue light. It also seems to have some band mobility shift associated to phosphorylation. However, the data are not so clear because of the insufficient band separation due to the high molecular weight of cry2-GFP.

Because cry2 activity *in planta* is activated under low fluence rate of blue light (Lin C *et al* 1998), and to better determine cry2-GFP phosphorylation conditions where the protein is stable, another phosphorylation kinetic was run. Previous reports (Shalitin D. *et al* 2002) and our own data (Fig. 3.4.9) showed a good amount of phosphorylated cry2 when seedlings were exposed for 10 min to medium fluence rate of blue light. Therefore, in this experiment medium irradiances of blue light up to 15 min were used for a better comparison of changes in the band mobility shift associated to phosphorylation of cry2 (Fig. 3.4.25)

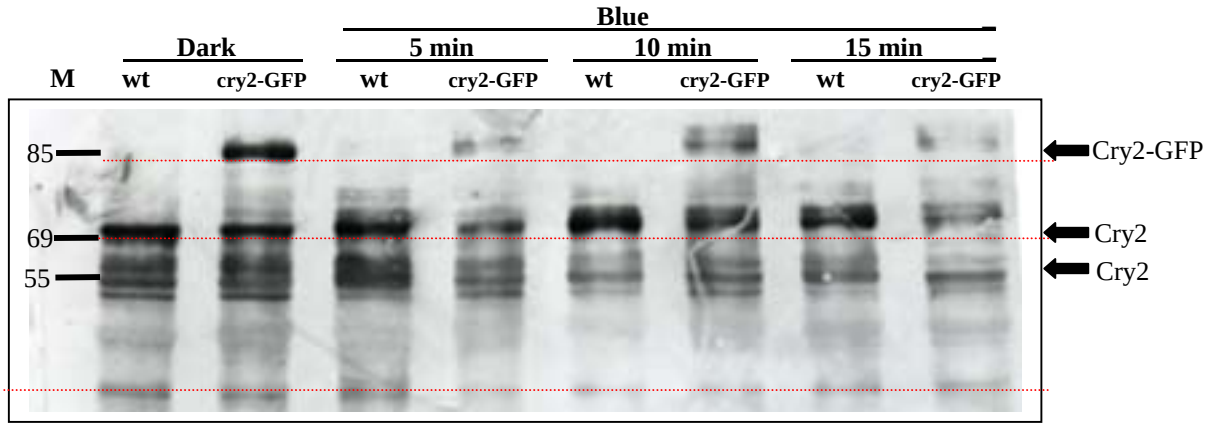


Fig. 3.4.25 cry2-GFP phosphorylation and degradation kinetics under medium blue light fluence. 5 days-old etiolated *Arabidopsis* Ler wt, or cry2-GFP transgenic seedlings were grown in darkness harvested under dim red light (dark) or treated with blue light (5 µmol $m^{-2}s^{-1}$) for 5, 10 or 15 min before harvest. Samples were subjected to Western-blot with anti-CCT2. Red lines are included to facilitate the band shift comparison.

Under these short time and medium fluence conditions, once more, the lower band of cry2, running near 55 kD, disappeared after the light treatment and the higher band of cry2, running close to the 69 kD marker band, showed the band mobility shift associated to phosphorylation. In addition, the cry2-GFP version showed a clear band mobility shift, detected after 10 min of exposure to medium fluence rates blue light.

CCTs of crys are the active domain in regard to signaling. Because CCT alone is very unstable, its expression *in planta* had to be done as a fusion protein, for example with GUS. Overexpression of this GUS-CCT construct could rescue the *Arabidopsis cry1* mutant

(Yang H.Q. *et al* 2000). Moreover, GUS-CCT2 was found to be constitutively phosphorylated (Shalitin D. *et al* 2002). Therefore, once we have shown that cry2-GFP phosphorylation occurs without interference from GFP, phosphorylation of the stable CCT2-GFP monomer was checked in a kinetics under high fluence rate of blue light (Fig. 3.4.26)

The CCT2-GFP monomer did not show the usual band shift associated to phosphorylation under blue light. Moreover, the protein was completely stable, even after 1 h under high fluence of blue light, indicating that the CCT2-GFP is not phosphorylated. Meanwhile, the endogenous cry2 in both wt and the transgenic line showed a clear band mobility shift after 10 min of irradiation. In addition, protein levels of both, cry2 and cry2-GFP are clearly reduced after 30 min of irradiation. This equal behaviour of both endogenous cry2 in the wt and the transgenic plants points to the fact that CCT2-GFP has not any effect on the endogenous cry2 phosphorylation and degradation.

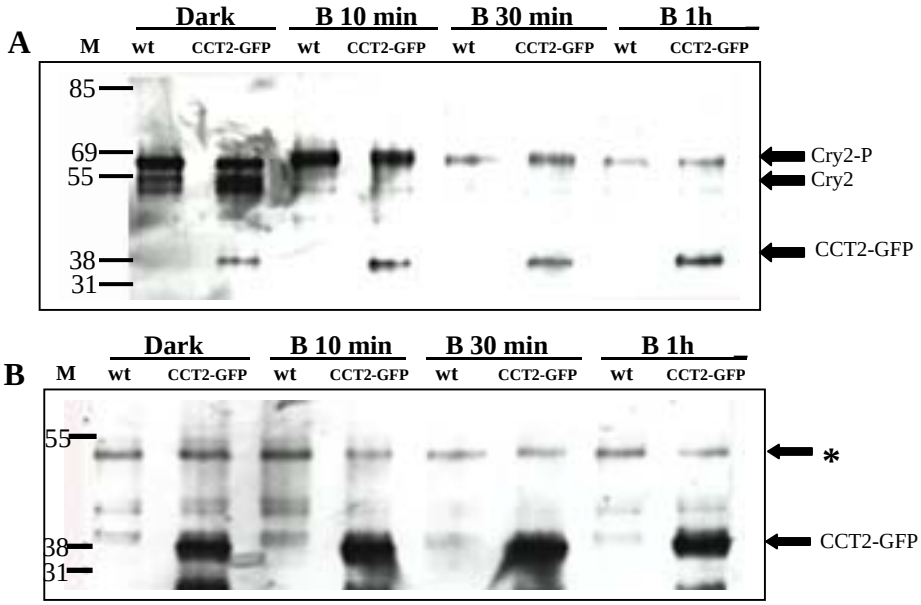


Fig. 3.4.26 CCT2-GFP phosphorylation and stability kinetics under high blue light fluence. 5 days-old etiolated *Arabidopsis* Ler wt and CTT2-GFP line#7 transgenic seedlings were grown in darkness (dark) or treated with high fluence rate blue light (30 µmol $m^{-2}s^{-1}$) for 10, 30 or 60 min before harvest. Samples were subjected to Western-blot, with **(A)** anti-CCT2 antibody, **(B)** stripped from A and labelled with anti-GFP antibody. Unspecific crossreacting bands are indicated with an asterisk (*).

3.4.3.2 Role in flowering time

The major biological role of cry2 is the control of flowering time in the photoperiod pathway. *Arabidopsis* plants flower earlier when the days are long such as 16 hours of light and 8 of darkness.

Cry2-GFP forms dimers (Figs. 3.4.14-3.4.17), is phosphorylated and degraded (Figs. 3.4.24 and 3.4.25). On the other hand, the CNT2-GFP construct did not show dimerization with either cry1 or cry2. Overexpression of CNT2 in *cry1* mutants was not able to revert the wt phenotype (Ahmad M. *et al* 1998b). Moreover, CCt2 alone is sufficient for signalling, as GUS-CCT2 expression in transgenic *Arabidopsis* causes a cop phenotype (Yang H.Q. *et al* 2000).
In order to study any possible adverse effect of CNT2-GFP on the endogenous cry2 activity flowering time of *Arabidopsis* transgenic lines expressing various cry2-GFP constructs was monitored under long photoperiod conditions (Fig. 3.4.27).
If dimerization of cry2-GFP with the endogenous cry2 would affect cry2 activity, or the GFP tag would disrupt some protein interaction a delay in flowering time compared to the wt is expected to occur.

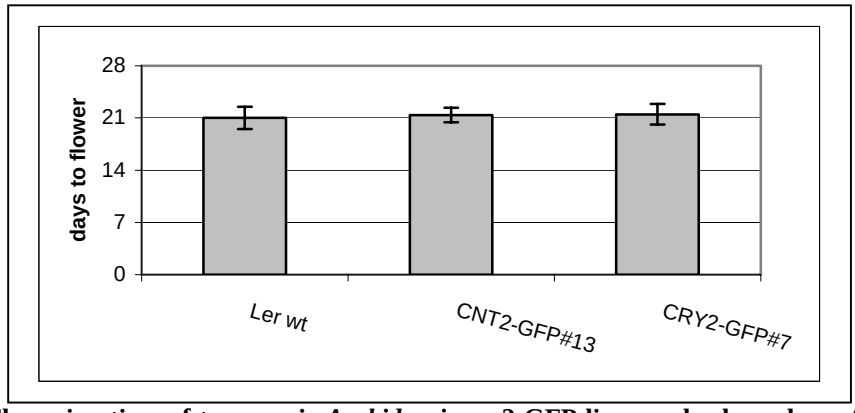


Fig. 3.4.27 Flowering time of transgenic *Arabidopsis* cry2-GFP lines under long days. *Arabidopsis* Ler, CNT2-GFP line#13 and cry2-GFP line#7 transgenic plants were grown on soil as described in Material and Methods under long day photoperiod. The number of days until the first flower was monitored for each line. Error bars represent standard error of a minimum of 15 different plants for each genotype.

Transgenic lines expressing cry2-GFP or CNT2-GFP kept under long photoperiod flowered at the same time as the parental wild type Ler ecotype. Therefore no adverse effect of any of this constructs in cry2 activity or its downstream signalling partners occurred .

As mentioned above, GUS-CCT2 and GUS-CCT1 were able to promote flowering under short days when overexpressed in wt (Yang H.Q. *et al* 2000), as did cry2 in the same conditions (Guo H. *et al* 1998). Trying to reproduce these data, the activity of monomeric and dimeric forms of cry2 was investigated *in vivo*. *Arabidopsis* transgenic lines expressing cry2-GFP line#7, and the three available CCT2-GFP lines#3, #7 and #9 (Fig. 3.4.2) were monitored for flowering time under short day conditions (Fig. 3.4.28).
Ler wt and Cape verde island (Cvi), a natural *Arabidopsis* cry2 overexpressor ecotype with a constitutive early flowering phenotype (El-Assal S.E.D. *et al* 2001), were used as a controls.

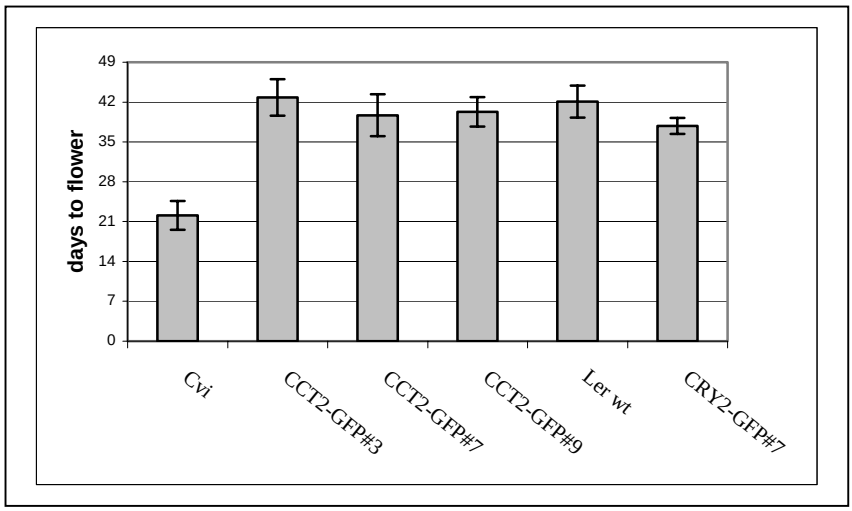


Fig. 3.4.28 Flowering time of transgenic *Arabidopsis* cry2-GFP lines under short days. *Arabidopsis* Ler, Cvi ecotypes, CCT2-GFP lines#3, #7, #9 and cry2-GFP line#7 transgenic plants were grown on soil as described in Material and Methods under short day photoperiod. The number of days until the first flower was monitored for each line. Error bars represent standard error of a minimum of 15 different plants for each genotype.

Ler had the usual late flowering phenotype in SD, flowering after 7 weeks in these conditions. Cvi ecotype showed its early flowering phenotype flowering after 3 weeks under SD photoperiod, the same time as a wt under LD conditions (Fig. 3.4.27). When kept under SD the three transgenic *Arabidopsis* CCT2-GFP lines flowered as late as the wt, indicating that CCT2-GFPs were not active. On the other hand, the transgenic *Arabidopsis* overexpressing cry2-GFP kept under SD flowered slightly earlier than wt. The first flower appeared around 6 days earlier than in Ler wt, pointing to some gain of function due to the presence of extra functional cry2.

3.5 *Arabidopsis* Cry2 phosphorylation

Phosphorylation is an event closely associated to cry activation, although the specific role of phosphorylation or poly-phosphorylation in activation, conformational change, activity or degradation is still unclear. To understand the specific role of phosphorylation for crys it would be desirable to know the phosphorylation sites, generate transgenic *Arabidopsis* plants bearing non phosphorylable cry mutants, and find a phenotype for a non-phosphorylable cry dimer.

Mass spectroscopy (MS) is a method capable of detecting and identifying proteins and their post-transcriptional modifications. MS uses colloidal coomassie stained gels, so all proteins run on the gel can be analysed depending only on their amount (Norregaard-Jensen O. 2004). The use of *Arabidopsis* as cry2 protein source for purification may allow to fish additional interacting proteins which may be coprecipitated during the cry2 purification, detected in the same colloidal coomassie stained gel, and analysed by MS. For this reasons, purification of Atcry2 and further analysis by MS were performed to detect *in planta* phosphorylation sites of cry2 and to find putative cry2 partners.

3.5.1. Purification of Atcry2 from *Arabidopsis* plant

Arabidopsis wild type was the first choice as cry2 protein source, although usage of the polyclonal anti-GFP antibody seems to be a fast and reliable method for cry2-GFP purification. Therefore, the polyclonal anti-CCT2 antibody was tested to check its precipitation efficiency in wt *Landsberg erecta* (Ler) *Arabidopsis* seedlings (Fig. 3.5.1).

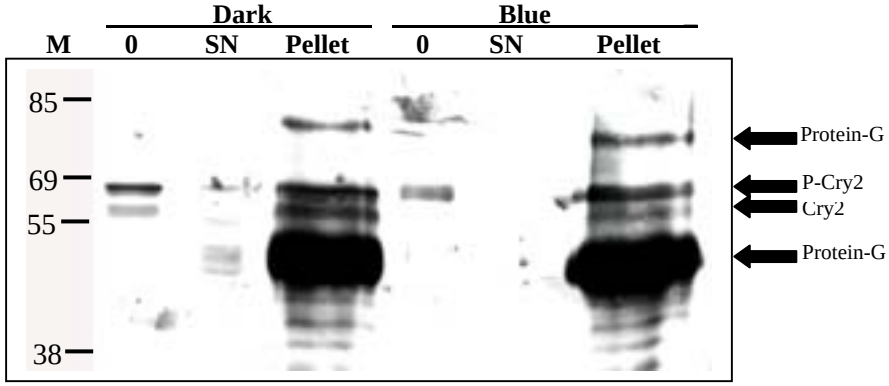


Fig. 3.5.1 Pull-down of endogenous cry2 from *Arabidopsis* wt. 5 days-old etiolated *Langsberg erecta* wild type seedlings were kept continuously in darkness (Dark) or exposed to blue light (33 µmol m^{-2} s^{-1}) for 10 min (Blue) before harvest. Samples were subjected to immuno precipitation using anti-CCT2 polyclonal antibody (2 h incubation) and protein-G agarose (1 h incubation). 20 µg of crude extracts (0), supernatants (SN) and 100 µl of protein containing pellet (pellet) boiled in SDS buffer and separated on 10% SDS-PAGE gel, subjected to Western-blot with the same anti -CCT2 antibody used for pull-down.

The anti-CCT2 antibody showed a complete precipitation in short time of *Arabidopsis* endogenous cry2, similar to the anti-GFP antibody to precipitate cry2-GFP from our transgenic lines. Moreover, the blue light-irradiated seedling sample seems to have an enriched upper band, which corresponds to a phosphorylated form of cry2, indicating that this approach may be suitable to purify phosphorylated cry2. However, the cry2 amount in Ler wt may be not enough for a the following analysis, since cry2 could be detected by Western-blot, but not visualized by staining with colloidal coomassie.

Looking for other cry2 protein sources, *Arabidopsis* Cape verde island (Cvi) ecotype, a known cry2 natural overexpressor, was consi. Cry2 from Cvi bears a mutation in a single amino acid in the FAD binding domain which seems to enhance translation, having Cvi higher cry2 protein levels than wt (El-Assal S.E.D. *et al* 2001).

Cry2 over-expression in *Arabidopsis* may be helpful to purify higher amounts of protein and Cvi cry2 levels were also higher than wt after medium term exposition to light. On the other hand, cry2 Cvi stability under long times of blue light irradiation seems to correspond to Ler wt being both degraded (El-Assal S.E.D. *et al* 2001).

Cvi could be a good source of cry2 protein because of its higher amount, but only if cry2 phosphorylation would be the same in Cvi and the other ecotypes. To address this question a phosphorylation kinetics was run to compare Cvi and Ler cry2 phosphorylation under short times of blue light illumination (Fig. 3.5.2).

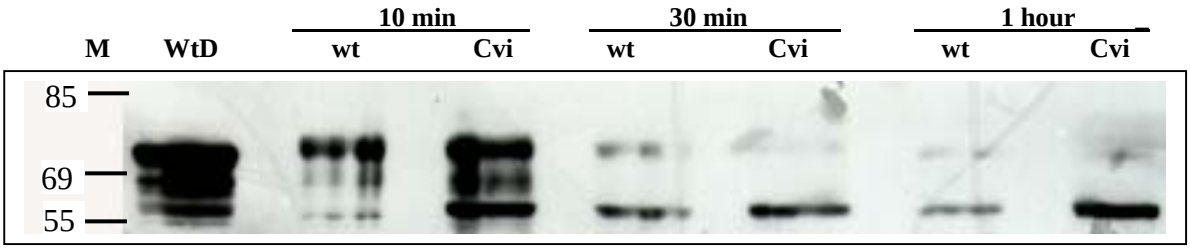


Fig. 3.5.2 Phosphorylation kinetics of *Arabidopsis* Ler and Cvi. 5 days-old etiolated *Landsberg erecta* wild type (wt) or Cape verde island (Cvi) seedlings were exposed to blue light (33 µmol m^{-2} s^{-1}) for the indicated times, a dark control is included for wild type (WtD). 20 mg of each protein sample were subjected to Western-blot analysis with anti CCT2 antibody.

Cry2 from Cvi showed the typical band-shift associated to phosphorylation and it is degraded in the same fashion as in Ler once exposed to blue light, but, as previously reported, there is a larger amount of protein in the Cvi line, making Cvi a good candidate for a cry2 plant source.

Nevertheless, as a first option, Ler wt etiolated seedlings were used to avoid protein loos associated to degradation of phosphorylated cry2 (Shalitin D. *et al* 2002), and to test if cry2

purified from the plant can be detected by colloidal coomassie staining. The protein samples were subjected to immuno precipitation and further staining (Fig. 3.5.3).

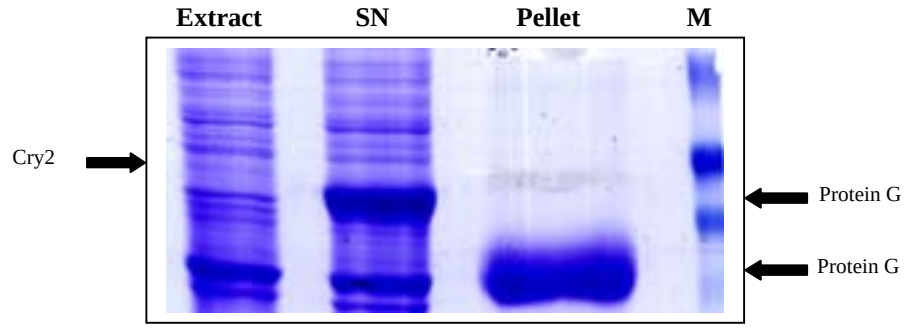


Fig. 3.5.3 Pull-down of endogenous cry2 from *Arabidopsis* wt. Protein extract from 5 days-old etiolated *Arabidopsis* Ler wt seedling was subjected to immunoprecipitation using an anti-CCT2 polyclonal antibody and protein G-agarose (phosphatase inhibitors were included). 30 µg of crude extract (extract), supernatant (SN) and 100 µl of protein containing pellet (pellet) were boiled in SDS buffer, run on 10% SDS-PAGE gel and stained with colloidal coomassie.

As it can be seen in Fig. 3.5.3, there is a tiny band in the Extract lane of around 69 kDa which is not present in the SN, but appeared to be enriched in the pellet (the cry2band in the pellet is missing because it was cut to be sent form analysis), the size of this band correspond to cry2 size seen in Western-blots. Two lower fat bands attributable to the protein-G agarose used in the pull-down can be seen also in the SN and Pellet lanes.

The putative cry2 band present in the pellet was sent for MS analysis but there was not amount enough to be detected. Therefore, the experiment was repeated in triplicate to obtain higher protein amount (Fig. 3.5.4).

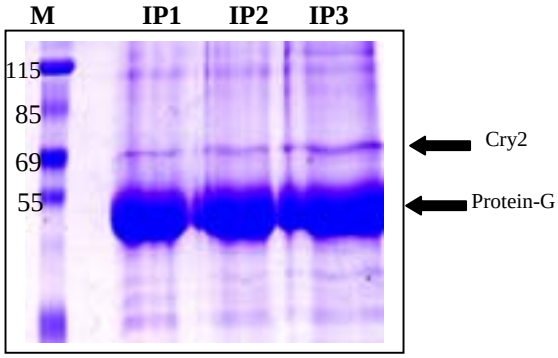


Fig. 3.5.4 Pull-down of endogenous cry2 from Ler wt. Protein extract from 5 days-old etiolated *Arabidopsis* Ler wt seedling was subjected to immuno precipitation using anti-CCT2 and protein G-agarose polyclonal antibody and protein-G agarose. 100 µl of protein containing pellets (IP1-3) boiled in SDS buffer were run on 10% SDS-PAGE gel and stained with Maldi coomassie .

This time, detection by MS was possible but the bands were identified as a conjugate of rabbit BSA (coming from the polyclonal anti-CCT2 antibody raised in Rabbit) and protein-G. Thus, although the pull-down seems to be effective, the cry2 protein of interest is masked by an additional undesired overlapping protein band. Therefore, using the Cvi over-expressor does not seem to be very promising too, as the bands will be also mixed.

Because of this undesired background of the antibody the tobacco expression system was used, expecting a clean pull-down by using the monoclonal anti-HA antibody in the same way as it was used for chromophore identification on section 3.3.

3.5.2 Purification of Atcry2 from *Nicotiana benthamiana*

Cry2 expression in tobacco is possible as shown in this thesis. cry1 overexpression in transgenic tobacco was reported to have a hypersensitive phenotype under blue light (Lin C. *et al* 1995b) and to be degraded (Ahmad M. *et al* 1998b). So it is reasonable to assume that cry2 is also functional in tobacco. To prove Atcry2 phosphorylation when expressed in tobacco a kinetic was performed checking the mobility shift of Atcry2-HA associated to phosphorylation when plants are exposed to blue light (Fig. 3.5.6).

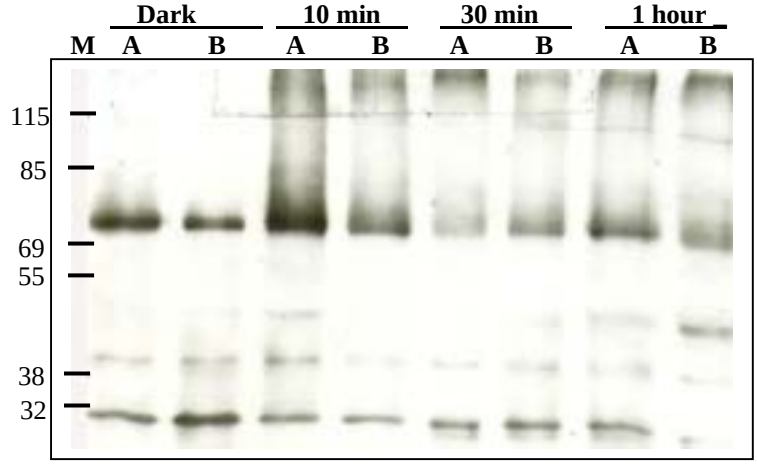


Fig. 3.5.6 Phosphorylation kinetics of Atcry2 expressed in tobacco plants. 1 month-old *Nicotiana benthamiana* plants were infiltrated with *Agrobacterium* carrying the cry2-HA construct, kept 4 days under LD conditions and in darkness the day prior to the experiment to enhance cry2 accumulation. Plants were kept in darkness or exposed to blue light (33 µmol m^{-2} s^{-1}) for the indicated times on duplicates (A and B). Protein samples were subjected to Western-blot with anti-HA antibody.

There is a clear blue light response of cry2-HA in tobacco since illuminated plants, but not dark controls, showed an cry2 shift that is likely due to posttranscriptional modifications of cry2 following degradation. Pitifully, because in each infiltration the transformation efficiency is always different, the s of Phosphorylation and degradation do not seem to correlate with time as good as seen for *Arabidopsis* plants (Fig. 3.5.2). In any case, it seems that Atcry2 expressed in tobacco runs higher than in *Arabidopsis* and is more stable under blue light, as the cry2 signal is reasonably strong after 1 h of high irradiance blue light illumination.

More pull-downs were performed with cry2-HA infiltrated tobacco plants using a monoclonal anti-HA antibody during an overnight incubation, following SDS-PAGE and colloidal coomassie staining, but no bands were found that could be correlated with cry2 (data not shown). Because overnight incubation was used, cry2-HA protein degradation could take place. For that reason cry2-HA stability was tested by incubating the protein extracts overnight at 4°C and comparing those to crude extracts (Fig. 3.5.7).

Fig. 3.5.7 Overnight cry2-HA protein stability. 3 *Nicotiana benthamiana* plants were infiltrated with *Agrobacterium* carrying the cry2-HA construct, kept 4 days under LD conditions and in darkness the day prior to protein extraction. Native protein preparations were kept at 4°C in darkness on a turning wheel overnight (ON), for about 15 h. Crude extracts (0) and overnight incubated extract (ON) were subjected to Western-blot with anti-HA antibody.

As suspected there was a clear degradation of cry2-HA when long incubations were used despite of the presence of protease inhibitors. The overnight incubation is too long. Therefore, shorter times should be used to archive higher protein yield from the pull-downs. Pull-down of new transformed tobacco plants was performed using the anti-HA antibody as indicated above, but reducing the antibody incubation times from overnight to 8 h (Fig. 3.5.8).

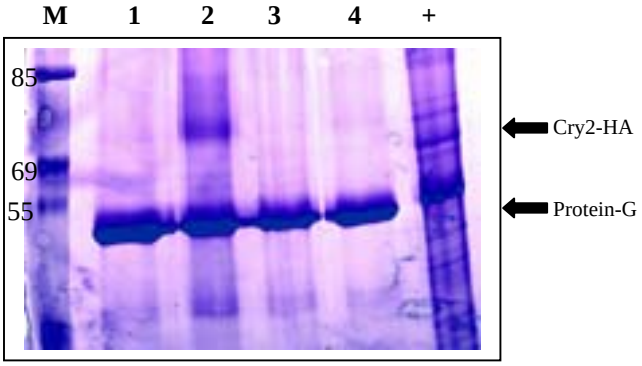


Fig. 3.5.8 Pull-down of cry2-HA from infiltrated tobacco. Four 1 month-old, *Nicotiana benthamiana* plants were infiltrated with *Agrobacterium* carrying the cry2-HA construct, kept 4 days under LD conditions and in darkness the day prior to harvest to enhance cry2 accumulation. Protein extracts were subjected to immunoprecipitation with anti-HA antibody and protein-G during 8 hours. Pellets (1-4) and crude extract (+) were boiled in SDS buffer, run on a 10% SDS-PAGE gel and stained with Maldi Coomassie.

Only 1 of 4 pull-downs seemed to be successful, as a band of the desired molecular weight appeared in the pull-down from plant 2. This putative cry2-HA band was sent for MS analysis but, once again, the protein amount was not enough to give results. Although some improvement was archived by decreasing the incubation time, it is very likely that the 8 hours

incubation time was still too long, and most cry2 was degraded making its detection impossible. For this reason the antibody incubation time was reduced to 4 hours in the next pull-down (Fig. 3.5.9).

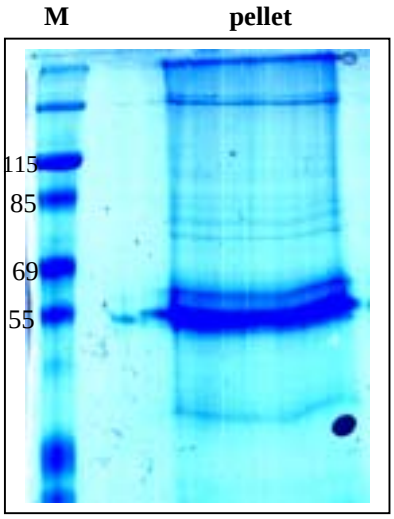


Fig. 3.5.9 Pull-down of cry2-HA from infiltrated tobacco.
1 month old *Nicotiana benthamiana* was infiltrated with *Agrobacterium* carrying the cry2-HA construct, kept 4 days under LD conditions and in darkness the day prior to harvest to enhance cry2 accumulation. Triplicates of protein extracts were subjected to immunoprecipitation using an anti-HA antibody and protein-G during 4 hours. The different pellets were boiled in SDS buffer and the resulting protein containing supernatants were pooled together, giving a final volume of 300 µl, which was run on a 10% SDS-PAGE gel and stained with Colloidal Coomassie

Many bands appeared close to the expected size of cry2, representing most likely protein-G conjugates. The bands were weak probably because the anti-HA antibody is not strong enough to get a complete precipitation within 4 hours. Due to the small protein amount obtained no MS was performed as previous better looking protein bands led to no results by MS-analysis.

3.5.3 phosphorylation of Atcry2 expressed in insect cells

The insect cell expression system is a high-yield recombinant protein production method. Despite being more difficult to handle than the usual *E. coli* bacterial expression, insect cells offer the advantage of being an eukaryotic system and suitable for expression of eukaryotic proteins with posttranscriptional modifications. Purification of crys from *E. coli* had problems of insolubility, whereas insect cell allowed full length cry1 purification for its use in spectroscopy (Lin C. *et al* 1995a; Bouly J.P. *et al* 2007), phosphorylation (Shalitin D. *et al* 2003; Bouly J.P. *et al* 2003; Özgür S. & Sancar A. 2006) and other biochemical studies (Ahmad M. *et al* 1998a; Partch C.L. *et al* 2005). Cry2 expressed in insect cells has been used for spectroscopy (Banerjee R. *et al* 2007) and phosphorylation (Özgür S. & Sancar A. 2006) studies.

Results from these studies highlighted the auto-phosphorylation capacity of Atcry1, in a still unidentified serine residue. It was also noted that the *in vitro* phosphorylation may not

correspond to the *in vivo* situation, as the *in vitro* phosphorylated cry1 does not show the mobility shift when run on SDS-PAGE gels that can be seen for *in vivo* phosphorylated samples (Bouly J.P. *et al* 2003).

On the other hand, no *in vitro* auto-phosphorylation was found for Atcry2 produced in insect cells (Özgür S. & Sancar A. 2006). To this regard, we asked the question whether Atcry2 is already phosphorylated in insect cells. As answer, phosphorylation can no be detected by P^{32}-labelling, as the phosphorylation sites are already occupied. Because the *in vitro* phosphorylated cry does not show gel mobility shift, a two dimension (2D) electrophoresis approach was taken.

By 2D electrophoresis proteins are separated twice, first according to their isoelectric point, (pI) defined as the pH at which the molecule carries no net electrical charge, and second depending in their molecular weight. Even when their molecular mass have only a little change, phosphorylated proteins are easily detected in 2D gels. Phosphorylation replaces neutral hydroxyl groups in serines, threonines or tyrosines with negatively charged phosphates that affect their pI making it lower.

Atcry2 expressed in insect cells was purified under white light as described before (Banerjee R. *et al* 2007), and protein purified from the same batch was analysed by either 2D electrophoresis and colloidal coomassie staining, or 2D electrophoresis and Western-blot (Fig. 3.5.10).

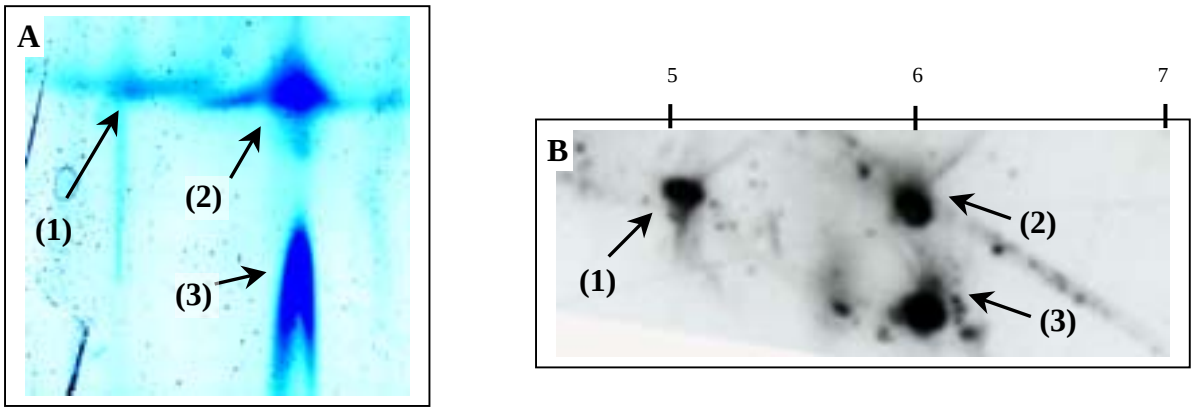


Fig. 3.5.10 2D analysis of Atcry2 expressed in insect cells. 150 µg cry2 from insect cells (500 µl from 0.3 µg/µl stock) were precipitated, resuspended in 2D-buffer and loaded on 18 cm stripe pH 3-11. The stripe was run overnight and a second dimension separation was performed on a 12% SDS-PAGE gel. Two different detection methods were used in two independent experiments. The PAGE size marker run overnight was lost but the gels were free of other spots, avoiding the missrecognition of the Atcry2 protein spots. **(A)** staininig with colloidal coomassie **(B)** Westen-blot with anti-CCT2 antibody

The pattern present in both, colloidal coomassie and Western-blot, for Atcry2 purified from insect cells is the same. The theoretical CRY2 pI is 5.8 and spot number 2 runs at this approximately point. Moreover, spots 1 and 2 run at the same molecular mass, but different

pI. Spot 1 is shifted to lower pI and can be considered as a putative phosphorylated form of Atcry2. Therefore, it may be possible that the insect cells phosphorylates Atcry2, or that autophosphorylation occurred prior to protein labelling. In addition to spots 1 and 2, a third spot is found with the same pI as Atcry2 but with a lower molecular weight. This spot can be due to protein degradation, as it is also present in the control SDS-PAGE performed after purification (data not shown).

4 DISCUSSION

4.1 Blue light induced reporter genes

First of all, as a general conclusion, it can be noted that amongst all the genes tested for their blue light dependent regulation the ones targeted to chloroplast were usually the best responding.

CHS was originally known to be highly up-regulated under cryptochrome control (Ahmad M. *et al* 1995). After all tests, *CHS* showed almost no expression in darkness, but high induction under blue light, in all systems tested (Figs. 3.1.1 and 3.1.2). For these reasons, *CHS* remains as the best choice for a cry action reporter.

LHCA5 was not expressed in cell cultures without chloroplasts (Fig. 3.1.8). Nevertheless, its big transcript levels and high fold up-regulation (Figs. 3.1.9 and 3.1.12) make it a good candidate as a reporter in both, *Arabidopsis* plant and green cell culture.

GAPDH and *FAD8* where up-regulated under blue light, but only in green cell culture or *Arabidopsis* plants (Figs. 3.1.3, 3.1.4 and 3.1.5). The fold induction and the total transcript level of *FAD8* were not as high as in the case of *CHS* (Fig. 3.1.12). Moreover, phys also control *FAD8* and *LHCA5* expression, as induction is observed under red or far-red light (Figs. 3.1.3 and 3.1.9). Therefore, they are recommended to be used as "auxiliary" controls, but not as main reporters.

AS and *PAL2* did not show a very good response. *PAL2* was not light-induced in the white cell culture. Moreover, although there was up-regulation in the green cell culture, the transcript level was low (Fig. 3.1.11). *AS* showed the expected down regulation in the white cell culture, but it did not show a big amount of transcript (Fig. 3.1.10). Therefore, having better candidates, light-induction experiments of these genes were not continued.

CAT2 is the most promising gene found in this study. The catalase family in *Arabidopsis* has 3 members (Mc Clung C.R. 1997). *CAT1* is expressed in flowers, whereas, *CAT2* and *CAT3* are expressed in leaves under circadian control. *CAT2* maximum expression is in the morning (Zhong H.H. *et al* 1994), and *CAT3* has its expression peak in the evening, being out of phase with *CAT2*. In addition, *CAT2* is up-regulated and *CAT3* is down regulated by light. All these data together suggest that the signal from *CAT2* can be easily distinguished from the other members of the family as they are expressed in different tissues, at different time points and regulated by light in different manner. Moreover, the tight blue light dependence of *CAT2* gene expression in both, plant and cell culture (Figs. 3.1.7 and 3.1.12), and its expression in

both, white or green cell culture (Fig. 3.1.6), makes *CAT2* an excellent candidate to be used as a reporter gene for blue light cryptochrome-induced gene expression.

4.2 Effect of green light on cry2 photocycle

Spectroscopic studies performed *in vitro* suggest that cry2 undergoes a photocycle in which FADox represents the ground state and $FADH^o$ the signaling state of this photoreceptor (Bouly J.P. *et al* 2007; Banerjee R. *et al* 2007). Significant accumulation of $FADH^o$ can be achieved upon blue light treatment, and further irradiation with green light, which is efficiently absorbed by the semireduced flavin,leads to a decline in $FADH^o$ concomitant with an increase in FADox (Bouly J.P. *et al* 2007; Banerjee R. *et al* 2007). In cry1 green light induces the further reduction of $FADH^o$ to $FADH^-$, which is unstable and donates its electrons to an unknown acceptor to yield FADox. In the case of cry2 this reduction step has not been recorded, very likely because of the high speed of the process.

Gene expression studies for the key flowering genes *CO* and *FT* revealed a cryptochrome independence of *CO* expression. Moreover, *CO* expression showed absence of sensitivity to green light, as the same *CO* mRNA levels were recorded by irradiating plants with monochromatic blue or bichromatic blue + green light (Fig. 2.1 A). On the other hand, green light had an inhibitory effect on *FT* expression (Fig. 2.1 B), linking the $FADH^o$ form of cry2 to an induction of flowering (Banerjee R. *et al* 2007). Green light inhibition is found only when cry2 is present (no effect on *cry2* mutant) and also when cry2 is activated by simultaneous blue light irradiation.

Together these data strongly suggest that green light inactivates cry2 directly and that $FADH^o$ is the signalling state of cry2 *in vivo*. Further support of this statement are the facts that the *in vivo* redox state of crys expressed in insect cells is FADox (Bouly J.P. *et al* 2007; Banerjee R. *et al* 2007), and that the cry1-dependent hypocotyl growth inhibition action spectra revealed an absorption peak at 450 nm (Ahmad M. *et al* 2002). Moreover, the action spectra for floral induction showed the maximum induction at 455 nm (Brown J.A. & Klein H.W. 1971). These action spectra maximum wavelengths are consistent with the presence of FADox in *Arabidopsis* crys and activation thought blue light absorption. In addition, cry-dependent responses in *Arabidopsis* have been shown to be affected by magnetic fields, which can only result from a mechanism of photoreceptor activation involving radical pair formation, being $FADH^o$ one of them (Ahmad M. *et al* 2007).

Despite the *in vitro* and *in vivo* data for crys fit very well, the effect of green light in other non cry photoreceptors should be taken in account. FKF1 is not affected by green light, as green light had no effect on *CO* transcription. Phytochrome effects under green light can be also discarded, as the adverse effect of green to blue light was seen in *phyAphyB* mutants, but not in cry deficient *cry1cry2* mutant seedlings (Bouly J.P. *et al* 2007). Moreover the *FT* mRNA levels achieved in the *cry2* mutant exposed to blue light are the same as wt in darkness, indicating that under the blue-light low-fluence rates used (2 μmol $m^{-2}s^{-1}$) phyA was not activated. However, this results do not exclude the existence of a, not yet identified, putative green-light-sensing photoreceptor, and the more recently described enhancement of the hypocotyl growth rate and repression of chloroplast gene expression by green light (Folta K.M. 2004; Dhingra A. *et al* 2006) seem not to be caused by the inactivation of the cryptochrome signaling state since these responses were the same in wild type and *cry* mutant plants.

Finally it must be pointed out that the inhibitory effect of green light on cry2 activity may seem to be in conflict with previous data showing an enhanced hypocotyl inhibition response to green light of tobacco seedlings that overexpress *Arabidopsis* CRY1 (Lin C. *et al* 1995b). However, it should be noted that in this study broad-band (490–570 nm) green light was used that extends into the region of cry absorption in its FADox state in contrast to the narrow band-width green light (559 ± 11 nm) applied here to excite specifically the $FADH^{o}$ in cry2.

4.3 *In planta* chromophore composition of *Arabidopsis thaliana* cry2

Atcry2 was purified from *Nicotiana benthamiana* transformed by *Agrobacterium*-mediated infiltration to investigate its chromophore composition. When purified form this plant source FAD from Atcry2 was found in its oxidized state as it had been found before, when Atcry2 had been purified form bacteria (Malhotra K. *et al* 1995) or insect cells (Lin C. *et al* 1995a), and even *in vivo in* crys expressed in insect cells (Banerjee R. *et al* 2007; Bouly J.P. *et al* 2007).

The putative presence of the second cofactor MTHF, which was found in *E. coli* expressed Atcry1 (Malhotra K. *et al* 1995) was confirmed in Atcry2 purified from *Nicotiana benthamiana.* These are the first data showing evidence of *in planta* MTHF presence for Atcry. The lack of MTHF when expressed in insect cells may be attributed to its loss during purification as it happened with the PHR from *Thermus thermophilus* (Ueda T. *et al* 2005) and more recently described for cry from *Drosophila* (Berndt A. *et al* 2007).

In addition, an effect attributable to energy transfer from MTHF to FAD was observed. The fluorescence emission at 520 nm by FAD when it was excited at 380 nm was enhanced in cry2 containing both chromophores, reversing the normal band intensity ratio for FAD excitation spectra. Therefore, these data are consistent with energy transfer from MTHF to FAD as it has been shown before for photolyase (Sancar A. 2003) and Atcry3 (Klar T. *et al* 2007)

4.4 Cry2 dimerization

4.4.1 Cry2 runs in multiple forms on SDS-PAGE

It is known that, once exposed to blue light crys are phosphorylated, and this phosphorylation can be seen in a Western-blot as a band mobility shift (Shalitin D. *et al* 2002 & 2003). In our experiments, full length cry2 (Fig. 3.4.10), and cry2-GFP (Fig. 3.4.1) expressed in a transgenic *Arabidopsis* line were detected as a double band in dark conditions. Both, cry2 and cry2-GFP, bands showed the mobility shift associated to phosphorylation when exposed to blue light. This shift was reverted by lambda phosphatase (λPPase) treatment (Fig. 3.4.10). This double band is not due to misrecognition of the endogenous cry1 by the anti-CCT2 antibody, as both bands are present in the *cry1* mutant but missing in the *cry2* mutant (Fig. 3.4.3). Moreover, this double band in darkness could be also detected for cry2-HA expressed in tobacco (Fig. 3.4.6). Interestingly, the cry2 double band was not seen in truncated cry2 versions (Fig. 3.4.2). Even when resolved in big Lämmli gels, CCT2-GFP (Fig. 3.4.19) and CNT2-GFP (Fig. 3.4.24) run as a single band.

The theoretical molecular mass of cry2 is 69 kDa. When run in a high separation resolution Lämmli gel, the lower putative cry2 band runs a little bit faster than the theoretical cry2 molecular mass, and the upper putative cry2 band runs slower (Fig. 3.4.10). The identity of these cry2 bands is unknown, although a putative posttranscriptional modification can be hypothesized. However, both putative cry2 bands in dark samples are not sensitive to λPPase treatment (Fig 3.4.10), pointing to lack of phosphorylation, or that are phosphorylation sites not accessible for the λPPase protein.

4.4.2 Cry2 is a constitutive dimer

Expression of tagged cry2 versions in tobacco gave evidence for cry2 homodimerization. Coimmunoprecipitation was found in pull-downs from tobacco leaf extracts co-expressing cry2-HA and cry2-myc (Fig. 3.4.11). When a pull-down was performed using anti-HA antibody, and the corresponding Western-blot was developed using anti-myc antibody a signal related to the cry2 control appeared. The same is true when the pull-down was performed with anti-myc antibody and the Western-blot was labelled with anti-HA antibody. Pitifully, the recognition of these bands by the anti-CCT2 antibody was not possible due to a high background caused by the overexpression of cry2-HA protein and by the protein-G agarose used during the experiment.
Further evidence of cry2 homodimerization was obtained when protein extracts from tobacco leaves overexpressing cry2-HA were subjected to chemical crosslinking. After crosslinking a band showed up above the 115 kDa marker band in Western-blot, being the expected size of a cry2 dimer with a calculated mass of 138 kDa. This putative cry2-HA crosslinked dimer could be recognized by both, anti-CCT2 and anti-HA antibodies (Fig. 3.4.12). Moreover, these antibodies have previously shown absolutely no background in Western-blots of non transformed tobacco (Figs. 3.4.5 and 3.4.6), proving that the signal is specific for cry2-HA.

Coimmunoprecipitations performed with transgenic *Arabidopsis* seedlings expressing cry2-GFP as well as the endogenous cry2 protein (Fig. 3.4.1) gave further evidence for cry2 heterodimerization. Pull-down of cry2-GFP with an anti-GFP antibody, which had been previously shown not to recognize the endogenous cry2 (Fig. 3.4.13), successfully coprecipitated the endogenous cry2 from plant samples kept either in darkness (Fig. 3.1.14), or exposed to blue-light low (Fig. 3.4.15) or high (Fig. 3.4.16) fluence rates. Therefore, these data indicate that cry2 forms homodimers in a light-independent way.
In the case of the sample exposed to blue-light low-fluence rates, an additional cry2 band corresponding to a phosphorylated version of cry2 was found in the precipitate. This finding fits with previous data reporting activity and stability of cry2 under blue-light low-fluence rates of blue light (Lin C. *et al* 1998). This phosphorylated cry2 dimer form was not stable in the sample exposed to blue-light high-fluence rates and it could not be detected (Fig. 3.4.16), even in presence of PPase inhibitors (Fig. 3.4.17).

Recent published data from other groups also addressed the question of cry dimerization supporting our results. Cry1 was shown to dimerize in a light-independent way by using gel

filtration, chemical crosslinking, immunoprecipitation, *in vitro* binding of yeast expressed crys, and yeast two hybrid studies (Sang Y. *et al* 2005). Cry2 homodimerization was addressed by *in vitro* binding of yeast expressed protein, yeast two hybrid (Sang Y. *et al* 2005), and also by the same coimmunoprecipitation approach with CRY2-GFP transgenic *Arabidopsis* as used by us (Yu X. *et al* 2007).

4.4.3 Domains involved in Cry2 homodimerization

Once cry2 homodimerization was proven, it was investigates which domains could be responsible for the interaction. *Arabidopsis* transgenic lines expressing CNT2 (amino acids 1-500), and CCT2 (amino acids 501-612) as GFP fusions, in addition to the endogenous cry2 (Fig. 3.4.2), were used to explore the interaction between these domains with the endogenous cry2.
No *in vivo* interaction between CNT2-GFP and cry2 was found either in darkness or under blue light high-fluence rates (Fig. 3.4.18). Additionally, no interaction between CCT2 and full length cry2 was observed in darkness (Fig. 3.4.19), or under blue light medium or high-fluence rates (Fig. 3.4.20).

Previous *in vitro* experiments showed that CNT is able to interact with full length cry. Moreover, CNT-CNT interaction was also found for cry1 and cry2 (Sang Y. *et al* 2005). The fact that the interaction between cry2 and CNT2-GFP was not found *in vivo* can be explained by their different cellular localization. Cry2 is known to be nuclear, but CNT2-GFP is cytoplasmatic (Kleiner O. *et al* 1999a). However, the yeast two hybrid assay found positive interaction amongst CNTs, very probable because this system targets the studied proteins always to the nucleus. In addition, interaction between CNT2-GFP and cry2 in the crude extract was also not detected. The reason for that can be insufficient protein amounts of cry2 and CNT2-GFP produced by the plant

CCT and CNT interaction was successfully conducted *in vitro* using purified protein (Partch C.L. *et al* 2005). However, this interaction was not seen when tested in a yeast two hybrid assay (Sang Y. *et al* 2005). Moreover, pull-downs using extracts from transgenic *Arabidopsis* seedlings showed no interaction between CCT2-GFP and cry2 (Figs. 3.4.19 and 3.4.20). Both, cry2 and CCT2-GFP, are present in the nucleus (Kleiner O. *et al* 1999), so an *in vivo* misslocalization of the truncated version of cry2 is not a valid explanation for the lack of interaction.

An accepted line of thinking argues that, in darkness, the CNT represses the CCT by direct interaction, and only by light activation the CCT is released and active (Cashmore A.R. 2005). Following this model it is reasonable not to find interaction between cry2 and CCT2-GFP in darkness, because in these conditions cry2 is already interacting with its own CCT2 (Fig. 3.4.19). In addition, when cry is exposed to light, a putative conformational change should occur. In concordance to that, small, but appreciable changes in the protein structure of purified CNT1 upon blue-light exposure have been reported (Kottke T. *et al* 2006). Therefore, the lack of interaction amongst CCT-GFP and cry2 in blue light-exposed transgenic seedlings (Fig. 3.4.20) may be explained by conformational changes in the CNT.

4.4.4 Cry1-cry2 heterodimerization

Although heterodimerization between cry1 and cry2 was reported to occur (Sang Y. *et al* 2005) in a yeast two hybrid assay (Rubio V. personal communication), no *in vivo* interaction amongst these proteins was found under any of the light conditions used (Figs. 3.4.21 and 3.4.22). It is known that in darkness, both, unphosphorylated cry1 (Cashmore A.R. *et al* 1999) and cry2 (Kleiner O. *et al* 1999) are in the nucleus. When *Arabidopsis* seedlings were exposed to strong blue-light for 10 min, cry2 but not cry1 was found phosphorylated (Shalitin D. *et al* 2002 & 2003). Nevertheless, when high-fluence rates of blue-light were applied to seedlings during 1 h cry1 phosphorylation was detected (Fig. 3.4.23 B; Shalitin D. *et al* 2003), and cry2 was completely degraded (Fig. 3.4.23 A; Lin C. *et al* 1998). Moreover, cry1 was shown to be translocated from the nucleus to the cytoplasm under long term blue-light irradiation (Wang H. *et al* 2001).

In line with these data, several *Arabidopsis* transgenic lines were used for pull-down assays. Cry2-GFP seedlings were kept in darkness to maintain all crys nuclear and unphosphorylated. In addition, seedlings from the cry2-GFP line were irradiated with blue light of medium-fluence rates to promote both, cry2 and cry2-GFP phosphorylation without inducing its degradation. Seedlings from the CNT2-GFP line were exposed to bluelight of high-fluence rates for long time to induce phosphorylation of cry1, and posterior translocation from the nucleus to the cytoplasm where CNT-GFP is expected to be found unphosphorylated (see Fig. 4.1 for a schematic cartoon).

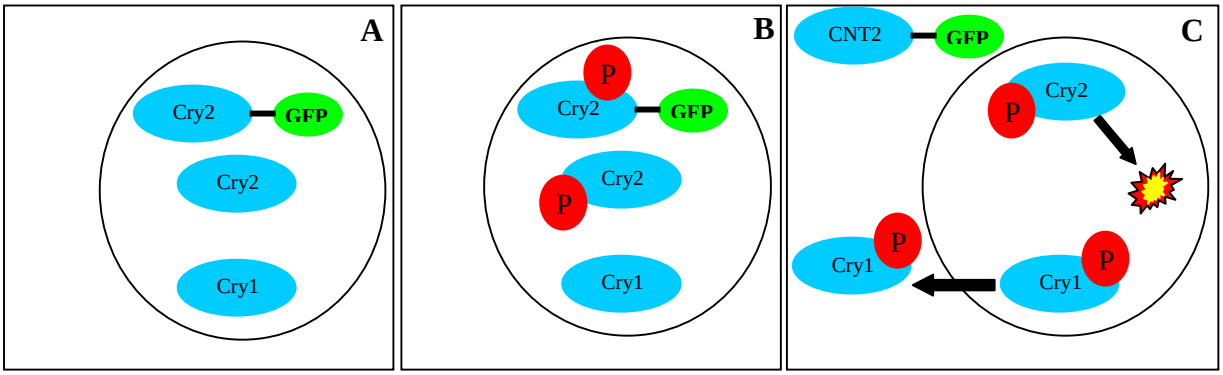


Fig. 4.1 Cryptochrome status in *Arabidopsis* seedlings under different light regimes. (A) cry2-GFP line in darkness, (B) cry2-GFP line irradiated with blue light of medium-fluence rate (5 µmol $m^{-2}s^{-1}$) for 10 min, (C) CNT2-GFP line irradiated with blue light of high fluence rate (30 µmol $m^{-2}s^{-1}$) for 1 h.

No coimmunoprecipitation was obtained by pulling down either cry2-GFP or CNT2-GFP in conditions where crys are localized in the nucleus. In these conditions cry1 was always unphosphorylated, but cry2 was phosphorylated or unphosphorylated depending on the light used. Moreover, no coimmunoprecipitation was found when the light conditions were strong and long enough to induce phosphorylation of cry1. Under these conditions the endogenous cry2 was completely degraded, but cry1 was stable and showed clear phosphorylation. In addition, as expected, under these illumination conditions CNT2-GFP was stable and unphosphorylated. Therefore, it seems like crys did not heterodimerize in any of these experiments. However, the cytoplasmatic localization of cry1 under long time of blue light high-fluence rates was not proven and misslocalization of cry1 and CNT2-GFP can be still the reason for lack of interaction.

4.4.5 Cry2 homodimerization effect on phosphorylation and degradation

GUS-CCT2 constitutive phosphorylation (Shalitin D. *et al* 2002) is now known to be associated to CCT2 dimerization mediated by GUS (Sang Y. *et al* 2005). In order to gain further insight into the effect of dimerization in phosphorylation, the phosphorylation of the CCT2-GFP monomer was investigated.

Phosphorylation and degradation kinetics for CCT2-GFP (Fig. 3.4.26) and cry2-GFP (Figs. 3.4.24 and 3.4.25) were performed under blue light and compared. Cry2 phosphorylation detected as a band mobility shift was visible for both, the endogenous cry2 and cry2-GFP but not for CCT2-GFP. This last one is the smallest protein from the three analysed. Therefore,

phosphorylation modification should have the biggest effect in its mobility. However, no difference was detected pointing to the absence of phosphorylation for the CCT2-GFP monomer. Additionally, the protein stability was also unaffected by blue light. CCT2-GFP was not degraded after 1 h of high-fluence rate blue light irradiation. On the other hand, protein levels of both, cry2 and cry2-GFP were clearly reduced in these conditions. It was previously reported that GUS-CCT2 constructs were stable under blue light. However, it must be noted that GUS is a very stable protein, and its fusion to the N-terminus of full length cry2 enhanced its stability under blue light (Guo H. *et al* 1999). In our results, protein stability was not higher in cry2-GFP than in the endogenous cry2 (Fig. 3.4.24), indicating that GFP did not enhance stability of cry2. These facts, together with the reported instability under blue light of chimeric versions of crys composed by CNT2-CCT1 or CNT1-CCT2 (Ahmad M. *et al* 1998b), and the CNT2-GFP stability under strong blue light irradiation (Fig. 3.4.23), suggest a role of CNT in protein stability. A full length cry bearing either CNT2 or CCT2 is needed for proper recognition by the degradation machinery.

Very recent published data support our findings. Phosphorylation of both GFP-cry2 and GFP-CCT2 expressed in transgenic *Arabidopsis* seedlings was monitored by radioactive P^{32}-labelling (Yu X. *et al* 2007). GFP-cry2 was phosphorylated under blue light, confirming the cry2-GFP phosphorylation observed in our *Arabidopsis* transgenic seedlings. On the other hand, GFP-CCT2 showed a very small light-independent phosphorylation, which was not detected in our CCT2-GFP constructs by band mobility shift. P^{32}-labeling is a more sensitive method than Western-blot. Therefore, is reasonable not to find these very low phosphorylation levels in CCT2-GFP by the band shift assay.

4.4.6 *In vivo* activity

Arabidopsis transgenic lines expressing GUS-CCT constructs of both, cry1 and cry2, showed a constitutive photomorphogenic (COP) phenotype in any light condition, and an early flowering phenotype under SD (Yang H.Q. *et al* 2000). Domain swapping between Atcrys yielding chimeric constructs formed by CNT2-CCT1 and CNT1-CCT2 gave cry1 and cry2 responses, respectively (Ahmad M. *et al* 1998b), indicating once more the importance of the CCT dimerization for cry activity.

Regulation of flowering time is the main biological function for cry2 (Guo H. *et al* 1998). Moreover, it was previously shown that overexpression of cry2 in Wassilewskija (Ws) ecotype was able to promote early flowering under SD (Guo H. *et al* 1998). In Addition, the Cvi ecotype, a natural cry2 overexpressor (El-Assal S.E.D. *et al* 2001), and GUS-CCT2 or GUS-CCT1 expressing transgenic lines in Col ecotype (Yang H.Q. *et al* 2000) flower also earlier than wt under SD. Following these evidences, we wanted to check, if *Arabidopsis* transgenic lines expressing full legth or truncated versions of cry2-GFP would have some effect in the flowering phenotype.

Ler cry2-GFP flowering time was monitored under both SD (Fig. 3.4.28) and LD (Fig. 3.4.27) photoperiods. Ler cry2-GFP flowered at the same time as wt when kept under LD conditions, indicating that the GFP protein does not disrupt the cry2 activity, and that a dimer between cry2 and cry2-GFP is functional. This fact correlates with the data showing the capacity of cry2-GFP to be phosphorylated, and the lack of interference of cry2-GFP with the endogenous cry2 phosphorylation (Figs. 3.4.24 and 3.4.25). Ler cry2-GFP under SD flowered slightly earlier than Ler wt. This slight early flowering phenotype is explained by Ler lacking the flowering locus C (*FLC*) gene (Lee I. *et al* 1994). FLC is a constitutive inhibitor of flowering (Michaels S.D. & Amasino R.M. 1999) acting negatively upon *FT* expression (Mouradov A. *et al* 2002). *FLC* is present in both, *Arabidopsis* Col and Ws ecotypes but not in Ler, and both Col and Ws flower later than Ler under SD (Mockler T. *et al* 2003). For that reason, the presence of additional cry2 in Ler had only a small effect on flower induction. Nevertheless, the early flowering phenotype of Ler expressing cry2-GFP indicates that cry2-GFP is functional.

When three different CCT2-GFP expressing *Arabidopsis* lines were kept under SD, all of them flowered at the same time as wt kept under the same conditions (Fig. 3.4.28). Although CCT2-GFP is nuclear (Kleiner O. *et al* 1999a), and lacks any possible inhibitory effect of the CNT2, it is not active which is consistant with being unphosphorylated and monomeric (Fig. 3.4.27; Yu X. *et al* 2007).

Support for the obserbation that the monomeric CCT2-GFP is not active is the finding that, using an artificial dimerization system, CCT2 dimerization is a required and sufficient condition for its activity (Rosenfeldt G. *et al* 2007). In this study, CCT2 fused either to FKBP and FRB proteins, which strongly dimerize when rapamycin is added, were expressed in *Arabidopsis* protoplasts. Induction of light-regulated genes was observed in darkness when CCT2 dimerization was conducted by adding rapamycin.

Corroborating our results, recent findings form other group showed that expression of cry2-GFP in vascular bundles rescues early flowering of *cry2* mutation in the Columbia ecotype under LD (Endo M. *et al* 2007). However, they did not find the early flowering effect under SD, very probably because they used low-fluence rates of light in this study. To obtain early flowering under SD, in addition to the overexpression of cry2, the use of high-fluence rates of light is also needed. Ws ecotype overexpressing cry2 was illuminated in 9:15 photoperiods with white light of 200 µmol $m^{-2}s^{-1}$ (Guo H. *et al* 1998), and GUS-CCT overexpressing transgenic lines were kept under 8:16 photoperiods of white light with 300 µmol $m^{-2}s^{-1}$ (Yang H.Q. *et al* 2000). We used high-fluence rates of white light (160 µmol $m^{-2}s^{-1}$) in 9:15 photoperiods to achieve early flowering. However, when low fluence rates were used (55 µmol m^{-2} s^{-1}) in 8:16 photoperiods, the early flowering phenotype was not obtained (data not shown). In line with these results are data showing that, although different *cop1* mutants can flower in darkness, they only do it when a certain amount of sugar that is much higher than the minimum required for survival is added (Nakagawa M. & Komeda Y. 2004). Therefore, these data points to photosynthesis and photo-assimilate accumulation as another signals required for flowering regulation.

Further confirmation of our results in flowering time is given by the recently published data showing that GFP-cry2 but not GFP-CCT2 can rescue the late flowering phenotype of *cry1cry2* double mutant in the Columbia ecotype (Yu X. *et al* 2007).

Although GUS-CCT2, GUS-CCT1 (Yang H.Q. *et al* 2000), cry2-GFP (Endo M. *et al* 2007, and this work), CNT2-CCT1, CNT1-CCT2 (Ahmad M. *et al* 1998b), and chemically induced CCT2 dimers (Rosenfeldt G. *et al* 2007) are active, CNT2 is not (Ahmad M. *et al* 1998b). These data indicate that dimerization of two CCTs is necessary for activity. Therefore, it is likely that a dimer formation of CNT2-GFP with cry2 would produce a complex with only one CCT2, equivalent to a non functional CCT2 monomer. Additionally, such a truncated dimer would sequestrate functional endogenous cry2, depleting the amount of functional cry2 dimers present in the nucleus and causing a delay of flowering time. However, the time to flower under LD was the same for both, *Arabidopsis* CNT2-GFP transgenic lines and wt (Fig. 3.4.27), indicating that CNT2-GFP has no adverse effect on cry2 activity. This finding is in line with the data showing lack of coimmunoprecipitation of cry2 when CNT-GFP was pulled-down (Fig. 3.4.18), and the previous known fact that CNT2-GFP is cytoplasmatic whereas the full length cry2 is nuclear (Kleiner O. *et al* 1999). It must be also noted, that the *Arabidopsis* transgenic plants showed CNT2-GFP protein levels comparable to the

endogenous cry2 (Fig. 3.4.18), indicating that a low protein level of the transgene is not the reason for the lack of a phenotype.

4.4.7 Differences between cry1 and cry2 dimerization and further hypothesis

Arabidopsis transgenic lines expressing myc-CNT1 in presence of the endogenous cry1 showed a dominant negative phenotype, losing the response to blue light inhibition of hypocotyl growth (Sang Y. *et al* 2005). In addition to the dimerization and phosphorylation commented above, cry activity is linked to its interaction with COP1 in the nucleus (Wang H. *et al* 2001; Yang H.Q. *et al* 2001). Therefore, the different phenotype of CNT1 and CNT2 can be explained by the different nuclear translocation mechanisms of cry1 and cry2, and the different interaction of CCT1 and CCT2 with COP1.

The current knowledge for *Arabidopsis* crys localization includes nuclear localization for cry2-GFP, CCT2-GFP (Kleiner O. *et al* 1999a), GUS-cry2 (Guo H. *et al* 1999), and GUS-CCT2 (Yang H.Q. *et al* 2000). The same nuclear localization was found for GUS-CCT1 (Yang H.Q. *et al* 2000), GUS-cry1 (Cashmore A.R. *et al* 1999) and GFP-cry1 (Cashmore A.R. *et al* 1999). On the other hand, CNT2-GFP remained cytoplasmatic (Kleiner O. *et al* 1999a), and no data are available about CNT1 localization. Interestingly, GUS-CCT1 was cytoplasmatic when exposed to blue light (Yang H.Q. *et al* 2000). Blue light irradiation degraded cry2 (Lin C. *et al* 1998; Guo H. *et al* 1999). Whereas, this light treatment had no effect on GUS-CCT2, which remained stable and nuclear (Yang H.Q. *et al* 2000).

Translocation of cry2 to the nucleus is dependent on the NLS of its CCT2 (Kleiner O. *et al* 1999a). This NLS is present also in animal crys. It has been proven that mcry2 in an *in vitro* system is actively translocated by alpha/beta importin to the nucleus in 30 min (Sakakida Y. *et al* 2005). Alpha-importin is also present in *Arabidopsis* (Smith M.H. *et al* 1997) and sufficient for protein translocation into the nucleus (Hubner S. *et al* 1999). Therefore, the expected fast translocation of cry2 as a monomer to the nucleus is supported by the lack of phenotype of CNT2-GFP expression in transgenic *Arabidopsis* (Fig. 3.4.27), the lack of interaction of CNT2-GFP with the nuclear endogenous cry2 (Fig. 3.4.18), and the previously known localization of CNT2-GFP in the cytoplasm (Kleiner O. *et al* 1999a).

The *Arabidopsis* cry1 nuclear translocation mechanism is unknown. For that reason the better known animal cry1 is taken as a model in the following discussion.

Some animal crys and plant cry2 have a bipartite NLS in the CCT consisting on a pair of both lysines (K) and arginines (R) residues separated by a stretch of 9-12 random amino acids. *Arabidopsis* cry1 lacks this NLS (Fig. 1.10) in its CCT, and the complete lack of lysines in the whole CCT1 excludes the existence of any other NLS. Therefore, the possibility of transport for *Arabidopsis* CCT1 via a NLS as a mechanism of nuclear localization can be discarded.

Interestingly, in addition to the NLS in the CCT, animal crys have another conserved NLS in the CNT. The FYFKLTDLYKKVKKNSSP sequence can be found in the CNT from zebrafish, bullfrog, mouse, chicken and human and its function for mcry1 nuclear localization is proven (Hirayama J. *et al* 2003). Moreover, further research on mouse cry showed the importance of the basic amino acid clusters KKVKK for mcry1 nuclear translocation in addition of the NLS in the mCCT1 (Sakakida Y. *et al* 2005). In order to check if *Arabidopsis* CNT has a NLS, the protein sequences of both Atcry1 and Atcry2 were screened looking for basic amino acid cluster or bipartite NLS. Some putative and degenerated NLS were found in cry1, but not in cry2 (Fig. 4.2). In addition the basic amino acid cluster RKxKK found in animals was also present in both, *Arabidopsis* cry1 and cry2, showing some degeneration too. This putative NLS sequence ranged from amino acid 232 to 240 in AtCry2 and from amino acid 235 to243 in AtCry1.

```
AtCry1   87 LGTCLITKRSTDSVASLLDVVKSTGA 102
AtCry2   77 LGSDLTLIKTHNTISAILDCIRVTGA 95

AtCry1 173 ESPLLPPKKIISGDVSKCVA 192
AtCry2 166 ESVMLPPPWRLMPITAAAEA 183

Zcry1  262 FYFKLTDLYRKVKKNSSP 282
AtCry2 232         YAKNSKKVV  240
AtCry1 235         YSKNRRKAD  243
```

Fig. 4.2 Degenerated putative NLS in CNT1 and CNT2. Numbers represent the amino acid in the protein primary sequence, bold letters are conserved amino acids in both CNT1 and CNT2, and red letters are for amino acids present in putative NLS. At: *Arabidopsis thaliana*, Z: Zebrafish

When placed in the available CNT1 structure (Brautigam C.A. *et al* 2004), the RKxKK like sequence was found to be located in a loop close to the FAD (Fig. 4.3). Nevertheless, the sequence is degenerated and it was proven that small mutations in the KKVKK sequence disrupted its NLS function (Hirayama J. *et al* 2003). Therefore, this putative NLS is expected not to be functional. However, a further proof is necessary to address this point.

Two degenerated bipartite NLS were also found in both CNT1 and CNT2. AtCry1 sequences from amino acid 87 to 102, and from 172 to 192 (Fig. 4.2) contained a pair of basic residues followed by another basic residue separated both by a stretch of another amino acids. When placed in the CNT1 structure (Brautigam C.A. *et al* 2004), these two sequences showed to be

localized at a place opposite to the FAD binding pocket (Fig. 4.3 A). It is curious that, although these bipartite sequences are very far in the primary protein sequence, they are seen in the CNT1 structure the bipartite sequence in close proximity (Fig. 4.4.3 B and C). Nevertheless, it is likely that, because of their degenerated sequence, they are not functional.

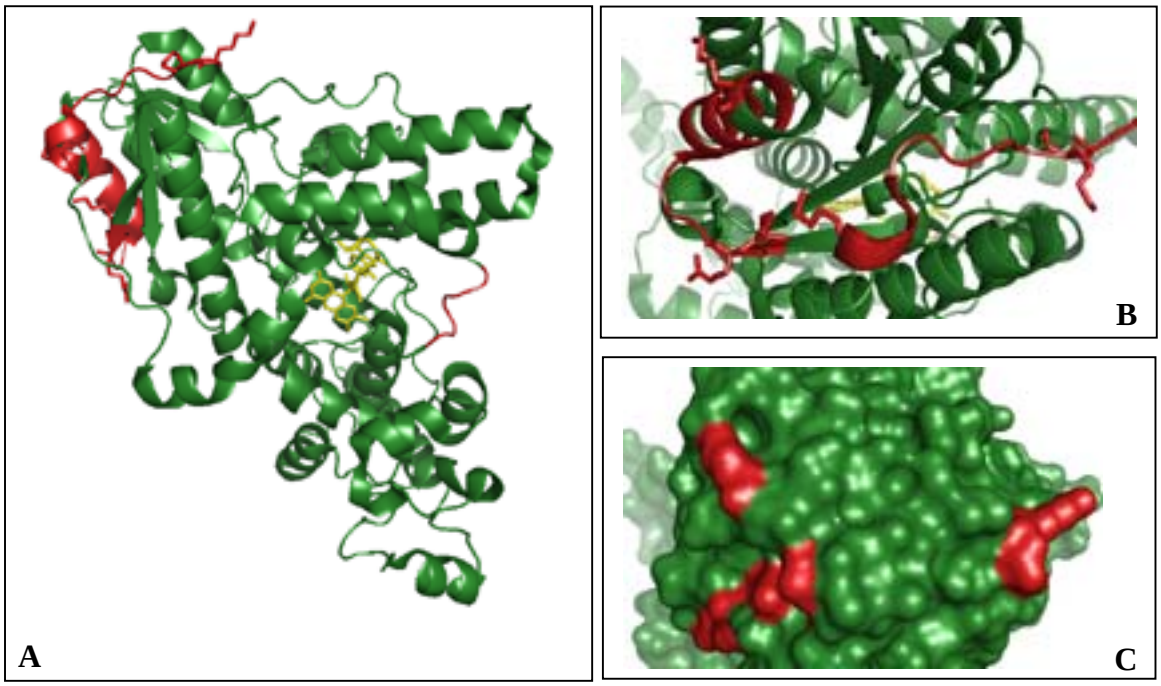


Fig. 4.3 Localization of putative NLS in CNT structure. Putative NLS regions for cry1 are indicated in red, FAD is depicted in yellow. (A) NLS localization in the whole CNT1 molecule, (B) close up of the NLS present in cry1 ranging amino acids 87-102 and 173-192, (C) surface mode of picture (B)

Despite of the possibility of transport of CNT1 via an NLS, a large body of evidence points to cotransport of cry1 by interaction with an actively translocated partner. In the same way as has been shown for mCry2 helping mPER2 to localize in the nucleus (Sakakida Y et al 2005), mCry1 reaches the nucleus due to its interaction with BMAL1 (Chaves I. *et al* 2006). An analogous mechanism has been suggested for Xcry1 (van der Schalie E.A. *et al* 2007). GUS-AtCCT1 lacks any NLS, and is translocated to the nucleus (Yang H.Q. *et al* 2000) indicating that it needs the help of a partner for translocation.

The possibility of a hypothetical Atcry1 co-transport with Atcry2 can be rejected because no heterodimerization was found in our studies (Figs. 3.4.21, 3.4.22 and 3.4.23). Moreover, the cry1 main function, inhibition of hypocotyl growth under blue light, is not disrupted in the *cry2* mutant (Mockler T. *et al* 1999).

On the other hand cotransport of cry1 with COP1 seems to be very probable. GFP-CCT1 co-localizes with COP1 in nuclear speckles and cytoplasmatic inclusion bodies (Wang H. *et al*

2001). Additionally, the nuclear exclusion of both, COP1 (von Arnim A.G. *et al* 1997) and CCT1 (Yang H.Q. *et al* 2000), in light-treated samples was recorded for each protein alone. Moreover, the COP1 nuclear exclusion under blue light seems to depend on cry1 (Osterlund M.T. & Deng X.W. 1998). COP1 translocalization from the nucleus to the cytoplasm upon blue light irridation is slow, taking several hours (von Arnim A.G. *et al* 1997). The cry1 translocation kinetics to the cytoplasm is currently unknown. Therefore, in order to address the question of cry1 cotransport and its speed, it would be interesting to check cry1-GFP, CCT1-GFP, and CNT1-GFP cellular localization under different light regimes, in either *Arabidopsis* wt and in *cop1* mutants that have constitutive cytoplasmatic localization such as *cop1-4* and *cop1-6* (von Arnim A.G. & Deng X.W. 1994).

Further supporting the constitutive interaction between COP1 and cry1 are data showing coimmunoprecipitation of cry1 with COP1-GFP expressed in transgenic *Arabidopsis* in either light or darkness (Yang H.Q. *et al* 2001). In addition, protein-protein interaction has been proven by yeast two hybrid studies between both, full-length cry1 and CCT1, with the WD40 repeats of COP1. On the other hand, CNT1 was not able to interact with the COP1 WD40 repeats (Yang H.Q. *et al* 2001). Therefore, these data suggest that cry1 and CCT1, but not CNT1, interact constitutively with COP1 which has a NLS (Stacey M.G. *et al* 1999) and can cotransport it to the nucleus.

COP1 dimerizes though its coiled-coiled (cc) domain (Torii K.U. *et al* 1998). COP1 dimerization is necessary for its activity (McNellis T.W. *et al* 1996), and a COP1 monomer is not able to interact with a HY5 dimer (Holm M. *et al* 2002). Investigation of which domain in the CCT1 is responsible for WD40 interaction by yeast two hybrid using several CCT1 mutants (Ahmad M. *et al* 1995) revealed strong CCT1-WD40 interaction. Disruption of this interaction by mutations before the DAS motifs, in the A motif, after the D and A motifs and before the S_1 and G motifs of the CCT (Fig. 1.10), indicated that all of them are important for proper cry1-COP1 interaction (Yang H.Q. *et al* 2001). In this line of evidence, a truncated cry2 version consistent of GUS fused to the 80 first amino acids of the CCT2 (including the D and A motifs) has been shown to be functional, although less active than the whole GUS-CCT2 version. Therefore, the rest of the CCT (the S_1 and S_2 motifs) is also important for function (Yu X. *et al* 2007).

In this regard, looking at the yeast two hybrid studies, there were no differences in the interaction strength between full length COP1 with either dimer or monomer CCT1 or CCT2, although the effect of the prey and bait proteins used in the assay in the dimerization is a question not addressed (Yang H.Q. *et al* 2001; Wang H. *et al* 2001). However, when mutations were

introduced in COP1 deleting its cc domain, responsible for dimerization, differences in interaction strength with CCTs appeared. The dimer GUS-CCT1 interacted with almost the same strength as the monomer CCT1 with COP1 versions lacking the cc domain (Yang H.Q. *et al* 2001). On the other hand, the dimer GUS-CCT2 interacted better than the monomer CCT2 with monomeric COP1 versions (Wang H. *et al* 2001). This fact would explain why the non functional CCT-GFP monomers did not yield a negative dominant phenotype as it could be that the affinity for COP1 is greater in a CCT dimer than in a CCT monomer.

The different interaction strength between COP1 and cry1 or cry2 can be further explained by the G motif present in CCT1 but not in CCT2. The CCT1 G motif (Fig. 1.8) resembles a putative WD40 interacting domain (Holm M. *et al* 2001). It has the conserved VPEϕ residues (Fig. 4.4), where VP is known to be essential for interaction with WD40 repeats. In addition, four negatively charged amino acids are necessary to complete the WD40 interaction domain. In this regard, although the serines from the S_1 have no charge, when phosphorylated (Ahmad M. *et al* 1998a) their charge become negative, forming a complete WD40 repeat interaction motif. Interestingly, CCT2 lacks the G domain, but instead has an additional ser rich domain (S_2). Because WD40 repeats are known to bind Ser/Thr phosphorylated amino acids (Yaffe M.B. & Elia A.E. 2001), the interaction of CCT2 with COP1 may strongly rely on the phosphorylation of cry2. Additionally, as it was mentioned above the GUS-NC80 dimer is functional but less than GUS-CCT (Yu X. *et al* 2007). This fact may indicate that the rest of the CCT, which contains the phosphorylable (Ahmad *et al* 1998a) S_1 and S_2 domains, may modulate cry activity by enhancing its interaction with COP1.

```
WD40 interacting sequence          ZZZZ---------VPEϕ--G
At cry1                       S-TAESSSSGRRERSGGIVPEWSPG
At cry2                       S-TAESSS-----SSS--VFFVSQSC--S
```

Fig. 4.4 Comparison of WD40 interacting domains in Atcry1and Atcry2. Domains labelled in red: S_1 (STAESSS), yellow: S_2, green: G. Z represents minus charged amino acids like phosphorylated serines. ϕ stands for an apolar amino acid like W.

Eventually, a model for cry1 and cry2 interaction with COP1 can be proposed (Fig. 4.5). Cry1 is constitutively bound to COP1 due to the G motif present in its CCT1. Because the slow translocation kinetics seen for COP1, CNT1 can dimerize in the cytoplasm with the endogenous cry1, which is constitutively bound to COP1, and the truncated cry1 heterodimer is cotransported to the nucleus together with COP1. On the other hand, cry2 lacks the G motif and is transported fast as a monomer depending on the NLS in its CCT2.This different nuclear translocation and and the difference where cry1 and cry2 monodimerize may explain the negative dominant phenotype seen in transgenic *Arabidopsis* expressing CNT1 plants, but not found in transgenic *Arabidopsis* CNT2 expressing plants.

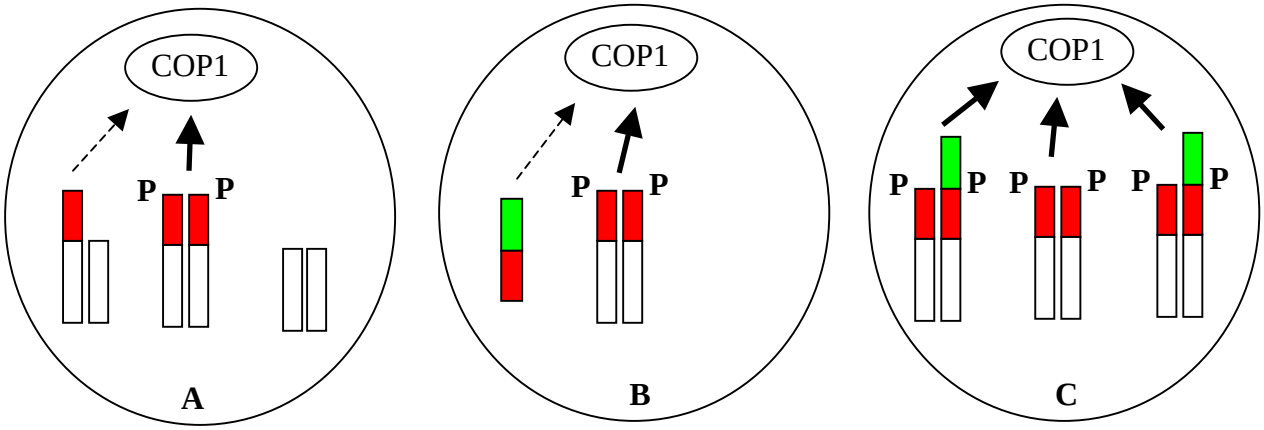


Fig. 4.5 Effect of different cry truncated constructs in dimerization and activity. CCT is represented in red, CNT in white and GFP in green. Bold arrows indicate strong interaction, dashed arrows indicate weak interaction. (A) CNT-cry dimer yield less amount of functional cry dimers (B) CCT-GFP monomer has no effect on cry activity, and it can not effectively compete with a cry dimer for COP1 interaction, (C) cry-GFP produce additional functional dimers with the endogenous cry.

The effect of cry dimerization on COP1 dimerization and activity, and the role of cry phosphorylation in its interaction with COP1 are new open questions waiting to be addressed by future work.

4.5 *Arabidopsis* cry2 phosphorylation

Although purification of cry2 from plant source was a successful approach for chromophore identification, it did not perform well for MS studies.

The use of anti-cry2 antibody for *Arabidopsis* extracts, although successful, had the disadvantage of an unexpected overlapping background band running at the same size as the low expressed cry2 protein (3.5.3 and 3.5.4). The usage of the overexpressing tobacco infiltration system had the problem of a weak tag, which needed long incubations for purification. Because, these long incubations were incompatible with cry2 stability, insufficient amounts of cry2-HA protein were obtained for MS analysis.

As an alternative for these approaches could be the use of cry2-GFP tagged protein. Cry2-GFP in transgenic *Arabidopsis* has been shown to be phosphorylated (Figs. 3.4.24 and 3.4.25) and functional (Fig. 3.4.28; Endo M *et al* 2007). The cry2-GFP line#7 used for the dimerization studies, or infiltration of tobacco with *Agrobacterium* to express cry2-GFP (Fig. 3.4.5) may be good alternatives as GFP performed fast and quantitative immunoprecipitations avoiding the protein degradation associated to long incubations.

In regard to the lack of *in vitro* Atcry2 auto-phosphorylation activity recently reported (Özgür S. & Sancar A. 2006), it was found that spontaneous phosphorylation of Atcry2 occurs when it is expressed in insect cells. Therefore, the lack of *in vitro* P^{32} incorporation into cry2 can be explained by the already occupation of phosphorylable sites by *in vivo* phosphorylation. In order to check the auto-phosphorylation capacity of Atcry2, lambda phosphatase treatment of purified protein samples should be performed previous to the *in vitro* autophosphorylation assay.

Nevertheless, the phosphorylation pattern seen on 2D gels seems to be different for Atcry2 from insect cells and for Atcry2 isolated from *Arabidopsis* cell culture (Djamei A. diploma thesis). 2D Western-blot of Atcry2 purified from plant source shows several spots at the same molecular mass, with different pI, whereas in cry2 isolated from insect cells only 1 extra spot appears (Fig. 3.5.10) pointing to a putative higher phosphorylation in the plant system.

Atcry2 phosphorylation mechanism and mapping of its phosphorylable residues are still open questions to be addressed in future work.

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