Philipps Universität Marburg

Gene expression in *Phycomyces blakesleeanus* after light and gravity stimulation

Dissertation

zur Erlangung des Doktorgrades der
Naturwissenschaften (Dr. rer. nat.)

dem Fachbereich Biologie
der Philipps-Universität Marburg

vorgelegt von

Norman-Ditmar Seger
geboren in Bochum

Marburg / Lahn 2011

Vom Fachbereich Biologie der Philipps-Universität Marburg
als Dissertation angenommen am: 10.10.2011

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Tag der mündlichen Prüfung: 09.12.2011

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Abbreviations

Aa	Amino acid	HSF	Heat shock transcription factor
AM	Ante meridiem	HSP	Heat shock proteins
APEs	*al-3* proximal elements	J	Joule
ASF	Anti-silencing factor	JGI	Joint Genome Institute
ATP	Adenosine triphosphate	kb	Kilo-base pairs
ß-Me	ß-Mercaptoethanol	kcal	Kilocalorie
Bp	Pulse of blue light	kDa	Kilo Dalton
HLH	Basic helix-loop-helix	LED	Light-emitting diodes
bp	Base pair(s)	LIAC	Light-induced absorbance changes
BRp	Pulse of blue then red light	LOV	Light, oxygen, voltage
cB	continuous blue light	LRE	Light regulating elements
ccg	Clock controlled genes	M	Mol per liter
CLG	Complex of lipid globules	m	Meter
cm	Centimeter	mad	Max Delbrück
cR	Continuous red light	MAT	Mating type
c(RB)	Continuous red/blue light	mb	Mega-base pairs
cry	Chryptochrome	min	Minute
C_T	Threshold cycle	ml	Milliliter
Ø	Diameter	mm	Millimeter
°C	Degree Celsius	mM	Millimol
D	dark	µg	Microgram
E. coli	*Escherichia coli*	µl	Microliter
EDTA	Ethylenediaminetetraacetic acid	NTE	N-terminal extension
EMSA	Electrophoretic mobility shift assay	NTG	Nitrosoguanidine (N-methyl-
EST	Expressed sequence Taq		N-nitrosoguanidine)
et al.	And others (et alli)	NTQ	4-nitroquinoline-1-oxide
FAD	Flavin adenine dinucleotide	OD	Optical density
Fig.	Figure	ORF	Open reading frame
FMN	Flavin mononucleotide	p.	page
G	Gibbs free energy	pp.	pages
g	Gram	PAS	Period, ARNT, SIM
g	Gravity of earth	PAZ	PIWI, Argonaute, Zwille
GAF	cGMP-specific phoshodiesterases,	PCR	Polymerase chain reaction

	cyanobacterial adenylate cyclases, formate hydrogen lyase transcription activator FhlA
GHKL	Gyrase, Histidine Kinase, MutL
h	Hour
HAT	Histone acetyltransferase
HK	Histidine-kinase domain
HMG	High mobility group
HSE	Heat shock elements
REC	Response-regulator domain
RISC	RNA-induced silencing complex
rpm	Revolutions per minute
RT	Reverse transcriptase
SI	Stage-I
SVI	Stage-VI
s	Second
seq.	sequence
TAE	Tris-acetate buffer
u	Unit
UV	Ultraviolet
V	Volt
v/v	Volume/volume
W	Watt
wc	White collar
WCC	White collar complex
WT	Wildtype (NRRL1555 (-))
w/v	Weight/volume
Znf	Zinc finnger
pH	Hydrogen ion activity
phot	Phototropin
phy	Phytochrome
PHY	Biliverdin chromophore domain
PM	Post meridiem
qRT-PCR	Quantitative real time polymerase chain reaction
Rp	pulse of red light
RBp	pulse of red then blue light

Chapter 1. INTRODUCTION

1.1 The model organism *Phycomyces blakesleeanus*

1.1.1 Taxonomy

Phycomyces blakesleeanus is a filamentous fungus which belongs in the order Mucorales of the division Zygomycota. It grows saprophytically as a mycelium mass of filamentous hyphal cells which produce aerial vegetative fruiting bodies so called sporangiophores. Since more than a century ago, *P. blakesleeanus* is studied as a model organism for sensory physiology.

1.1.2 Life cycle

Phycomyces has two kinds of life cycle; asexual life cycle and sexual life cycle. Asexual life cycle permits the rapid dispersion of organism. It starts from the germination of the spores caused by heat shock, certain chemicals or appropriate nutrients. A spore generally has two germination tubes which are grown at the tip and form the coenocytic mycelium by repeated branching of hyphae. Sporangiophores which are specialized unbranched hyphae grow aerially and vertically. The nuclei from the mycelium migrate through the developing sporangiophore. Three or four nuclei are packed into a spore inside a sporangium formed at the apical tip of a sporangiophore. Around 10^5 spores which liberate from a sporangium geminate and form new mycelia. There are two types of sporangiophore depending on the environmental conditions; microphores and macrophores. Under innutritious or low temperature conditions, microphores develop. Microphores are generally 1 mm and contain around one thousand spores (Rudolf, 1958; Thorton, 1972; Gutiérez-Corona, 1985). On the other hand, macrophores which only develop under the right environmental cues or induced by blue light (Corrochano and Cerdá-Olmedo, 1988) are around ten centimeters and contain around 10^5 spores.

Sexual life cycle permits the genetic recombination. *P. blakesleeanus* is categorized into two types of sex: (+) and (-) without morphological distinction (Blakeslee, 1904). Genetically, Idnurm et al., 2008, identified sex genes in the mating type loci (MAT) of *Phycomyces*, *sexP* mating type (+) and *sexM* mating type (-) specific genes, encoding for α-box proteins, homeodomain, HMG-domain

transcription factors.

Sexual reproduction occurs only when hyphae of opposite mating type grow near to each other. The recognition via pheromones leads to physiological and morphological changes like enhanced bright yellowish coloration by increased accumulation of β-carotene (sexual carotenogenesis) and formation of the zygospores. The fusion of two haploid nuclei of opposite sex forming diploid nuclei which undergo the meiosis and postmeiotic mitosis take place in the zygospore. After a period of dormancy the zygospore germinates to produce the germsporangium with 10^{-4} haploid germspores which can initiate a new life cycle (Cerdá-Olmedo, 1975; Eslava *et al.,* 1975a, b).

1.1.3 General behavior

1.1.3.1 Mycelia

P. blakesleeanus spores germinate after a heat shock with usually two or three germ tubes which grow only on their tip (hypha) with a rate of ~ 10 μmh^{-1}, but branch lateral tubes forming a hyphal pad, the Mycelia. Whole mycelia spread out about 2 cm per day on solid medium. It also can grow in liquid medium. Mycelia of *P. blakesleeanus* were subject in cell biology as well as in physiology studies of secondary metabolism like lipid and ß-carotene synthesis.

The best study of the influence of light on secondary metabolism is the photocarotenogenesis, meaning mycelia grown under a continuous light fluence of at least 1 Wm^{-2} show a tenfold increase of ß-carotene against the dark grown control (Bergmann et al., 1973; Sandmann & Hilgenberg, 1978). In continuous blue light the threshold of the photocarotenogenesis in wildtype of *P. blakesleeanus* is 10^{-5} Wm^{-2}, and so 10^{-4} times higher then the photogravitropic threshold (López Díaz & Cerdá-Olmedo, 1980). Jayaram et al., (1979) show that the dose response curves of photocarotenogenesis has sigmoidal shape, indicating different photoregulatory elements for low and high light fluencies.

1.1.3.2 Sporangiophores

The phorogenesis or sporangiophorogenesis is the growth change of mycelia by forming sporangiophores, cylindrical, unbranched tubes which grow straight up

(negative gravitropic) into the air. The size of the sporangiophores depends strongly on the environmental conditions but generally there are two strongly distinct types, the ~ 1 mm long microphores (Carnoy, 1870) and the > 10 cm long macrophores. There are five distinct developmental stages of the macrophores described by Carnoy (1870) and Errera (1884). Stage I (SI) is the first apical elongation with clockwise rotation up to 15 mm. In stage II (SII) accumulation of ß-carotene and radial growth occurs, where in SIII growth stops but sporangiophores become deeply yellow to dark brown in SIV with intercalary growth, SIVa counterclockwise, SIVb clockwise, SIVc counterclockwise. SV refers to old non growing, black sporangiophores.

This single-celled giant (~100 µm Ø, up to > 10 cm in length) sporangiophore of *P. blakesleeanus* reacts to a wide range of external stimuli by changes in growth speed and direction. The sporangiophores grow with a rate of 2-3 mmh^{-1} at the stimuli sensitive or reactive growing zone 0.1-3 mm below the tip. Thus sporangiophores are used in physiological experiments, which allow the analysis of the signaling pathways depending on the different tested stimuli. The sporangiophores show tropisms (Greek *trope* or turn) to mainly three types of stimuli.

Photogravitropism:

The movement (bending of sporangiophores) toward unilaterally given blue light (positive) or away from the UV-light source (negative), which occurs always under the earth gravitational field (~1 x g) why photogravitropism is the more accurately term. This tropism, the light-growth response and the dark adaptation, is related to the transient modulation of growth rate after changes of the irradiation (intensity or direction), like a pulse of light or a step-up, step-down of photon fluencies. Whereas growth responses are adaptive, the phototropism is not.

Gravitropism:

Negative gravitropism is the growth against the earth gravitational field (1 x g). The straight growth as well as the bending upward of horizontally placed sporangiophores assumes the ability to sense the gravity equally in dark and light. The sporangiophores contain octahedral protein crystals of high density (1.27 g cm^{-3}) in the central vacuole which can sediment (gravisusceptors) and an apical complex of lipid globules (CLG) with lower density than the cytoplasm (0.79 g cm^{-3}), buoyancy (Wolken, 1969; Schimek et al., 1999; Grolig et al., 2000). Both cell components are involved in the gravisusception. For the complex interaction of gravity and light stimulus is still little understood. An empirical relationship was

observed (Galland et al., 2002), where a linear increase of g results in an exponential increase of the threshold fluence rate ("exponential law"). On a molecular level there is no convincing evidence regarding the graviperception mechanism neither in *P. blakesleeanus* nor in other fungi.

Other response:

The sporangiophores show several responses to enclosure, gases, wind and barriers which are all manifested through growth and tropism. A curios phenomenon is the avoidance response of the sporangiophores to obstacles but not to other sporangiophores. It was first described by Elfving in 1881 based on observations of Sachs (1879). Shropshire rediscovered it in 1962 and studied most of these responses, but it is still unclear how the sporangiophores can bend away from nearby (0.1 – 4 mm) obstacles before or without touching them (Cerdá-Olmedo & Lipson, 2001, pp. 135-137).

1.1.4 Genome

The first characterization of the Phycomyces genome was made by the DNA reassociation technique by Dusenbery, 1975. But at that time the genetic analyses were focused on the characterization of isogenic strains and mating types, as well as complementation and recombination analyses. A genetic map was made for 24 markers which are localized into eleven linkage groups (Centromere) (Eslava et al., 1975; Orejas et al., 1987a; Álvarez et al., 1993).

Based on the sequence of 251 genomic DNA fragments obtained after *MspI* digestion, Avalos et al., 1996 determined the genomic guanine and cytosine (G + C) content of 35 % and 50 % in coding sequence. They estimated a genome size of 30 mega bases (Mb), containing 10 % of repetitive DNA and short tandemly repeated sequences named *PrA*1 and *PrA*2 of 31 bp in 5 % of total genome.

The Joint Genome Institute sequenced in 2006 the genome of *Phycomyces blakesleeanus* wildtype strain NRRL1555 (-) (40 Mb). The 8x assembly of the *P. blakesleeanus* genome is finished and the automatic annotation started in January of 2007 and still goes on.

In total a set of 26,880 cDNA clones has been characterized and about 53,000 ESTs have been obtained. Currently there are 14,792 gene models predicted and functionally annotated.

1.2 Mutants

1.2.1 *mad* mutant strains

The *mad* mutant strains were isolated by their reduced phototropic sensitivity (Bergman et al., 1973) by coworkers of Nobel laureate Max Delbrück and to honor his name, the abbreviation mad was used. At this time there are ten different types of *mad* mutants isolated, *madA-J*. They were all genetically characterized by complementation analyses. The location of the corresponding genes is known and shown in the genetic map (Orejas et al., 1987a; Álvarez et al., 1993). All of these *mad* mutants are morphologically indistinguishable from the wildtype. Only *madA* and *madB* genes are presently isolated, sequenced and molecularly characterized (Idnurm et al., 2006; Sanz et al., 2009). The other *mad* genes are not isolated and therefore corresponding protein and function in signaling pathways of photoresponses remain unclear.

Classically *mad* mutants are classified into two major groups according to their phototropical behavior. Class-1 mutants have an elevated photogravitropic threshold but show normal gravitropism. This group includes *madA, madB, madC* and *madI*.

Class-2 mutants have a strongly reduced gravitropism and also an elevated photogravitropic threshold. This group includes *mad D, E, F, G,* and *J*. In the *madH* mutant strain all tropic responses are enhanced (hypertropic) whereas the action spectra for phototropic equilibrium as well as the one for the light growth response are similar to the once of the wildtype.

In this work mainly class-1 *mad* mutant *madA, madB* and *madC* were studied. They have an elevated photogravitropic threshold in common and in general show abnormal photoresponses in comparison to the wildtype of *P. blakesleeanus*, which are listed in Tab. I-IV (Appendix, pp. 1-3). The *madC* mutant shows the unique and puzzling feature that the phototropic threshold for blue light is about 100-fold lowered by giving red light above 600 nm, which itself is phototropically ineffectual (Galland et al., 1995; Delbrück et al., 1976). Neither the wildtype nor other *mad* mutants display this behavior. A red light absorbing intermediate of the photoreceptors involved in photoresponses in wildtype and with an elevated level in *madC* mutant is presumed from that observation (Galland et al., 1995).

1.3 Photoreceptors

Light is one of the most powerful abiotic factors since it lead to oxygen accumulation on earth produced by photosynthetic organism. But more than this it is used nearly in all evolutionary branches for the orientation in time and space. It serves in fungi which are non photosynthetic to trigger a multitude of developmental key aspects of their biology and lifestyle.

Best and detailed studied are the blue light responses in ascomycete *Neurospora crassa.* The photoreceptor proteins of *N. crassa* shown in Fig. 1.3.1, are given as en example of the set of known fungal photoreceptors involved somehow in photoresponses, whereas the development of the reproductive organs, photogravitropism and the activation of the secondary metabolism like the biosynthesis of ß-carotene are the most common and studied examples of light regulation not only in *N. crassa* (Corrochano 2007, Herrera-Estrella & Horwitz 2007).

1.3.1 Opsins

An ancient and large group of highly conserved photoactive proteins among all organism and maybe best known photoreceptor are the opsins. They are structurally composed by seven membrane spanning domains, where on a conserved lysine within the seventh transmembrane domain the chromophore retinal is bound. After light absorption and the initial isomerization of the chromophore (11-cis → all-trans for rhodopsin) a nonreversible sequence of dark reactions, in which ultimately the chromophore detaches from the protein, activates the photoreceptor. Opsin encoding genes are presented in the genomes of all organisms supporting a model of convergent evolution throughout microbial organisms to animals. Functionally they can serve as photoresponding ion pumps (H^+, Cl^-) mainly in archaea and photosensors in the eye of animals. The first fugal opsin characterized was NOP-1 of *N. crassa*. The isolated protein gives no evidence of H^+ pumping and shows green light-absorbance (Bieszke et al., 1999 a, b). It is involved in the regulation of the genes *al-1, con10* and *con13* (Bieszke et al., 2007). The expression of these genes is up regulated in the mutant strain with the deletion of *nop-1,* whereas its role in light-dependent regulation on gene expression remains for further investigations (Olmedo et al., 2010). In *P. blakesleeanus* the opsins as part of the photoreceptor

system were early excluded due to the observation that lack of carotene and thus lack of retinal does not affect the phototropic sensitivity (Bergmann et al., 1973).

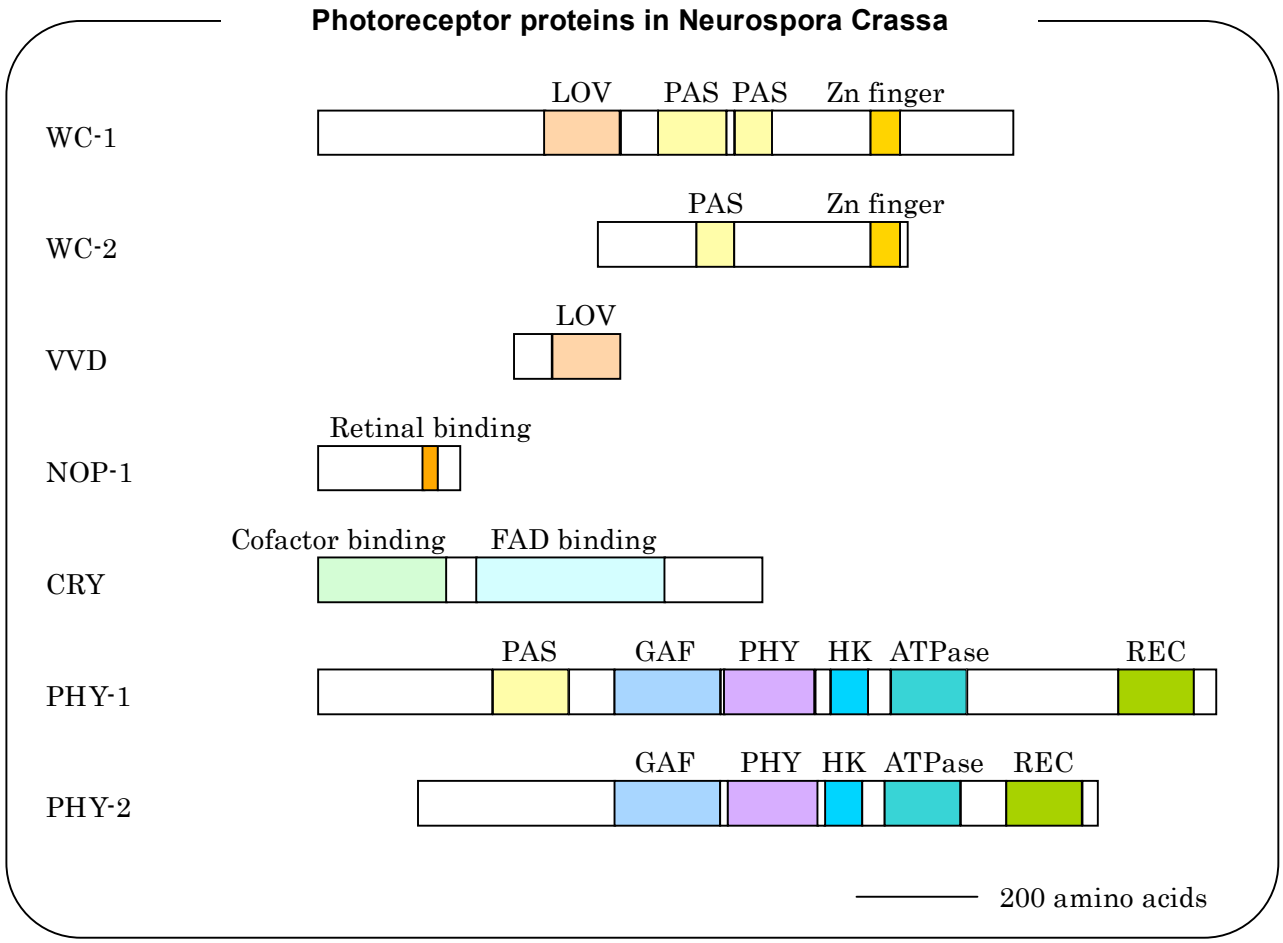


Fig. 1.3.1: Summary of photoreceptor proteins of *Neurospora crassa*. The shown conserved domain structure was obtained by using SMART and CD Search. Taken from Corrochano 2007, with modifications.

1.3.2 Phytochromes

Phytochromes are a widespread group of red/far-red light absorbing photoreceptors, first discovered in plants (Buttler et al., 1959). They are present in prokaryotes, fungi and plants. Their function is studied bests in plants where they are involved closely in all developmental processes and growth, from seed germination to the regulation of flowering (Josse et al., 2008). In fungi their role is much less clear due to their recently identification. All phytochromes have in common the covalently attached linear tetrapyrrole chromophore group which has

the ability to interconvert between two forms (Z→E isomerization). Thus red light can reversibly convert the Pr ~650 nm absorbing form into the active Pfr form, whereas absorption of far-red light (> 730 nm) by Pfr converts it back to Pr, the thermal stabile form. The photoconverted Pfr show, in the absence of light, a dark reversion into the Pr form.

The domain structure of the phytochromes varies among the organisms, thus they can be classified into subfamilies. An example for fungal phytochromes of *N. crassa* is shown in Fig. 1.3.1. In general one can distinguish an N-terminal photosensory region and a C-terminal response regulatory region. The photosensory part contains three domains. The PAS domain serves as protein interaction. It contains a cryptic NLS for the nuclear location and is important for light mediated signaling (Cheng et al., 2003). A GAF (cGMP-specific phoshodiesterases, cyanobacterial adenylate cyclases and formate hydrogen lyase transcription activator FhlA) domain is found in other signaling and sensory proteins. The PHY domain is important for the binding of the biliverdin chromophore to undergo a complete photocycle (Froehlich et al., 2005; Rockwell et al., 2006). The fungal phytochromes have in comparison to those of plants an additional N-terminal extension (NTE) which is important for their characteristic red light absorption spectrum. The deletion of the NTE leads to an absorption also in the blue spectrum. The C-terminal response regulator domain is composed of a histidine-kinase domain (HK) and a (REC).

Phytochrome genes are identified in the model organisms of various fungal phyla like *N. crassa* and *A. nidulans* (ascomycota), *Ustilago maydis* and *Cryptococcus neoformans* (basidiomcota) and in *P. blakesleeanus* (zygomycota). Only very recently the predicted phytochrome orthologue gene was manually annotated. But as a result of the lack of a clear phenotype of *fphy* deletion mutant strains, little is known about their involvement in physiological processes in the whole fungal kingdom.

The expression of the two *phy* genes in *N. crassa* is not regulated by light but under the control of the circadian clock, whereas the *phy* gene products are light-dependent phosphorylated. They are localized in the cytoplasm, thus their function in gene regulation remains unclear (Froehlich et al., 2005).

Aspergillus nidulans shows a different picture in which the PHY proteins form dimers interact and shows the typical photoreversibility between Pr (707 nm) and Pfr (754 nm). In contrast to *N. crassa* these proteins are detected in the cytoplasm as well as in the nucleus where they can interact in a pictorial manner with other

proteins like the blue light photoreceptors (WC-1, WC-2) and other important regulatory proteins (VEA, VELB). A direct involvement in gene regulation could not be shown at the time but a strong light-dependent autophosphorylation was found (Brandt et al., 2008; Calvo, 2008; Purschwitz et al., 2009; Bayram et al., 2010).

1.3.3 Cryptochrome/photolyases

In fungal genomes, sequence homologies for blue light photoreceptors chryptochrome, evolutionary derived from DNA-repair photolyases, are found and in *N. crassa* as well as *A. nidulans* identified. They bind FAD (not via a LOV domain) and show the N-terminal extension typical for cry-DASH type sensory proteins (Fig. 1.3.1). The *cry* gene of *N. crassa* is light regulated in a *wc-1* dependent manner and under circadian clock control in continuous darkness. But DNA-repair-activity, typical for photolyases, is not shown (Froehlich et al., 2010). More insight in comparison to the one of *N. crassa* comes from the studies in *A. nidulans.* In the *cryA* deletions mutant increased amounts of mRNA of developmental genes (*veA, nsdD, rosA*) were detected. Thus CRYA seems to trigger developmental processes like the other photoreceptors as well (Bayram et al., 2008; Corrochano, 2007; Herrera-Estrella & Horwitz, 2007). In the genome sequence of *P. blakesleeanus* only one putative *cryA* gene could be found, which is currently under investigation (Rodriguez-Romero, personal comment).

1.3.4 *white collar* type blue light photoreceptors

The WC-1 and WC-2-like blue light photoreceptors are identified in the genomes from the Ascomycota, Basidiomycota and Zygomycota phyla (Corrochano, 2007). But their involvement in photoregulation of fungal development, sex, growth, secondary metabolism and the entrainment of the circadian clock is still best studied in *N. crassa*, in which *wc* genes first were identified. The molecular structure of these photoresponsive transcription factors of the WC-1 family is characterized by three conserved domains; LOV-domain, PAS-domain and the C-terminal zinc-finger (Znf) domain (Ballario et al., 1996).

The WC-2 family contains an amino-terminal PAS domain and a zinc-finger at the carboxyl-end (Linden & Macino, 1997). The N-terminal LOV-domain (light, oxygen and voltage) is responsible for light sensing through interaction of

non-covalently bound chromophore flavin FAD/FMN and the highly conserved cystein of a protein pocket forming the cysteinyl-adduct (He et al., 2002; see Fig. 1.4.2.1). The PAS-domain generally mediates physical protein-protein interaction as shown for WC-1 and WC-2 of *N. crassa* (Ballario et al., 1998; Denault et al., 2001). The PAS acronym itself was suggested in 1991 by a sequence motif in Period, aryl hydrocarbon receptor nuclear translocator (ARNT), and Single-minded (SIM), three eukaryotic proteins that are more closely related to each other than to the broader PAS family (Hoffman et al., 1991). Members of the PAS family are now known to share important structural and functional features like the basic helix-loop-helix (bHLH) transcription factors or in serine-threonine kinase, like in plant phototropins.

The typical zinc finger DNA binding domain (C-X2-C-X18-C-X2-C) in the C-terminal region of WC-1 allow the recognition of the consensus sequence (A/T) GATA (A/G) and transcriptional regulation of client genes like *al-1, al-3* or *con-10* (Ballario et al., 1996; Linden & Macino, 1997). Both, WC-1 and WC-2 form a heteromeric photoresponsive transcription factor complex (WCC) which binds transiently to the promoter region of client genes after blue light exposure.

In comparison to the highly conserved domain structure of the WC-type photoreceptors among different fungi their involvement in photoresponses shows a wide variation. In contrast, in other fungi multiple *wc* genes are present.

Another blue light photoreceptor of *N. crassa* is a small protein VIVID which contains only a FAD/FMN binding domain (LOV). This photoreceptor is located in cytoplasm and mainly involved in adaptation of the circadian clock and light responses. Photospectrometric research is most advanced among the *wc*-type photoreceptors using the LOV domain of vivid (Zoltowski & Crane, 2008).

1.3.5 Photoreceptors of *P. blakesleeanus*

The recently sequenced *madA* and *madB* genes of *P. blakesleeanus* are homologue to the *wc-1, wc-2* genes of the ascomycete *N. crassa*. The *P. blakesleeanus* genome contains two other *wc*-1 type photoreceptor genes *wcoA* and *wcoB* (white collar one) and four *wc-2* like, *madB*, *wctB, wctC* and *wctD* (white collar two) (Idnurm et al., 2006; Sanz et al., 2009).

There are three *madA* mutant strains containing different mutations in its gene; C21, C47 and A893. The *madA* mutant strain C21 contains a splicing mutation. Mutation in the LOV domain of the strain C47 leads to a reduced FAD binding

capacity by amino acid substitution (Asn142Ser; AA**C**→A**G**C). The *madA* mutant strain A893 lacks the zinc-finger domain due to a premature stop codon (TG**G** →TG**A**; Trp-434). In all 16 *madB* mutant strains the same mutation leads to a lack of Znf domain (Idnurm et al., 2006; Sanz et al., 2009) (Fig. 1.4.2.1; see also Tab. 3.2.6.1.1, p. 65).

Photoresponses in *P. blakesleeanus* are mainly regulated by the MAD complex. This complex is formed by the gene products of *madA* and *madB* and homologue to the blue-light responsive WHITE COLLAR complex (WCC) of *N. crassa* (Sanz et al., 2009). The MAD complex is responsible for several physiological blue light responses including tropism, carotenogenesis and gene regulation (Cerdá-Olmedo 2001; Corrochano 2007; Corrochano & Garre 2010). The MAD complex receipts blue light most likely via FAD non-covalently bound on Cys144 in the LOV domain of WC-1 type MADA, similar as shown for the WC complex of *N. crassa* (He et al., 2002). Further spectroscopic characterizations of WCC of *N. crassa* or any other fungi are not available, neither in vivo nor in vitro.

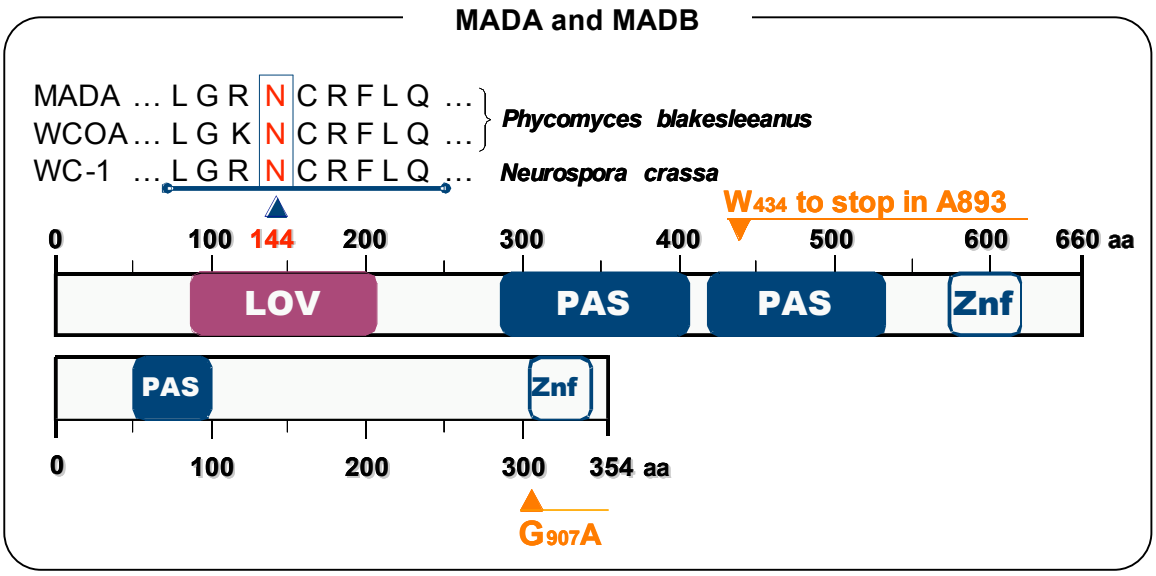


Fig. 1.4.2.1: Domain structure of WC-1, WC-2 photoreceptors of *P. blakesleeanus*
Conserved consensus sequence of LOV domain important for FAD/FMN binding (top), conserved functional domains of MADA and MADB proteins with the position of mutations.

In *P. blakesleeanus* light-induced absorbance changes (LIACs) measurements in the growing zone of sporangiophores were performed with a rapid-scan spectrophotometer. Under moderate white or blue light irradiations the absorbance increases around 450 nm and at 610 nm (Fig. 1.4.2.2; Schmidt & Galland, 1999).

The biological relevance of the LIACs is given by the fact that the maxima of the in vivo measured LIACs coincide with those of the action spectra of dark adaptation and light growth response in the wildtype of *P. blakesleeanus*. In the *madA* to *madC* mutants the LIACs were not detected thereby underlying their biological function and linking them up to the blue light photoreceptors of *P. blakesleeanus*.

The LIACs are also absent, when measured under red light irradiation, indicating a red light absorbing intermediate of blue light photoreceptors in the wildtype (Schmidt & Galland 1999). More evidence for a red light absorbing intermediate of blue light photoreceptors is given by the fact that the photogravitropic threshold for blue light in the *madC* mutant strain is lowered 100 fold in the presence of red background light (3 x 10^{-2} Wm^{-2}, 644 nm). Another red light effect is the complete loss of the far-UV and blue light antagonistic photogravitropic behavior of stage-IVb sporangiophores of the wildtype under less than 5 Wm^{-2} broadband red light. The fluence-rate response and resulting action spectra for subliminal light controlled photogravitropic adaptation show beside the major peaks around 450 and 500 nm an extension from 570 to 670 nm, meaning subliminal red light accelerates the recovery of sensitivity for blue light by increasing the rate of adaptation (Cheng et al., 1993; Galland et al., 1995; Galland, 1998). On the other hand is the quantum effectiveness above 500 nm is lowered about 10^{-8} compared to that at 450 nm (Delbrück et al., 1976). The action spectra of photogravitropism and light growth response show also a sharp drop of for wavelength above 500 nm (Delbrück & Shropshire, 1960; Galland 1983).

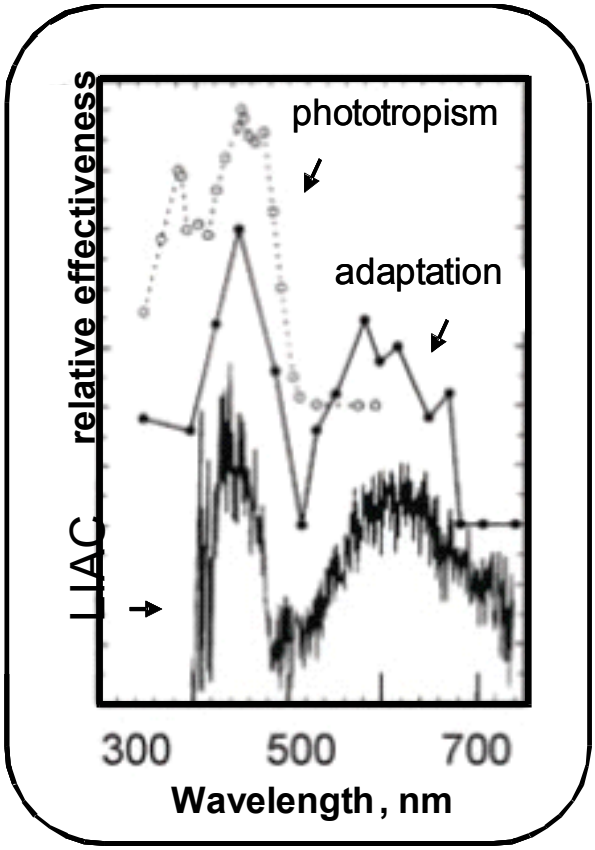


Fig. 1.4.2.2: Action spectra

Other *mad* mutants (*D-J*) with alterated photoresponses and gravitropism

indicate that there are more regulatory compounds in photosignaling pathways and that this pathway somehow is linked with other sensory pathways like the gravitropical or heat shock (Rodriguez-Romero & Corrochano, 2006). On the molecular level their function remains still unclear due to their unidentified genomic sequences.

Very recently two other photoreceptor genes were found in the genomic sequence of *P. blakesleeanus*. One gene encoding for a predicted cryptochrome/photolyase (*cryA*) and another for a putative phytochrome orthologue gene (*phyA*) are manually annotated.

1.4 Genes

The survival of single cells and organisms depends on the continuity of all functional compounds of the cell. Adaptation to the environment is a specific modulation of single compounds with the aim to maintain the general stability of all essential functions. At the end all these processes end up in changes of gene expression. Cells developed different mechanisms to maintain the stability of their protein assembly by transcriptional and translational control. This control mechanism comprising the detection of specific environmental stimuli via receptor and adequate responses is regulated via complex molecular networks, including modulations on transcriptome as well as on proteome.

In *P. blakesleeanus* several good results accumulated in sensory physiology over the past 50 years. But molecular approaches are less advanced. Light leads to the adaptation of gene expression which can also be followed by secondary transcriptional regulation via transcription factors. Thus all chosen genes in this work encode for predicted proteins directly involved in transcriptional control or secondary metabolism. In the following these chosen genes will be presented.

1.4.1 Housekeeping genes

The use of reference genes in gene expression studies is a general consensus from the postgenomic era. Since housekeeping genes are required for cellular survival, it is assumed that they are stably expressed. Their use has often a long tradition and so they are still used without validating their suitability. Moreover, a reference gene with stable expression for a set of experiments can vary considerably

under different experimental conditions.

For expression studies in *P. blakesleeanus* the genes encoding for the nucleotide biosynthesis orotidine-5´-phosphate-decarboxylase *pyrG* (Díaz-Minguez et al., 1990) and the cytoskeleton constituent actin, *actA* (AJ287184; Voigt & Wöstemeyer, 2001) were mainly used as reference genes for internal control (Blasco et al., 2001; Idnurm et al., 2006; Rodriguez-Romero & Corrochano 2004, 2006; Almeida & Cerdá-Olmedo 2008).

In this work also a fragment of actin gene was used as well as a fragment of one beta-tubulin gene (AY944795). Furthermore a gene (*rib*) encoding a predicted ribosomal protein was used.

1.4.2 Photoreceptor genes

The previous physiological and genetic description of *madA* and *madB* mutant strains can be extended since their sequences are available. It opens the door to further biomolecular characterization of the interaction and involvement of these WC-1, WC-2 type photoreceptors in signaling pathways. It is the basis to close the gap between physiological observations and the genetic network behind them allowing a comprehensive view of experimental observations and predictions. Thus, for the gene expression studies in this work all three *wc-1* and four *wc-2* type genes of *P. blakesleeanus* were chosen.

1.4.3 ß-carotene synthesis genes *carB* and *carRA*

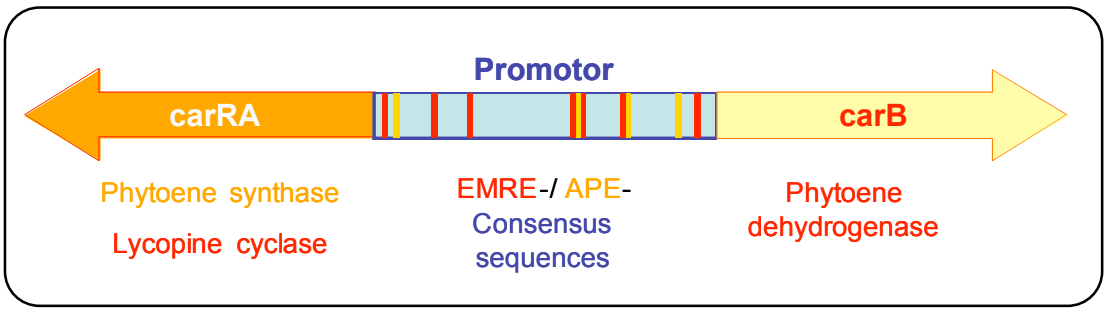


Fig. 1.4.3.1: Organization of β-carotene biosynthesis genes *carB* and *carRA* of *P. blakesleeanus*

The influence that is studied best of blue light on secondary metabolism is the β-carotenogenesis. Blue light induces the transcription of the genes *carB* and *carRA*

(Ruiz-Hidalgo et al., 1997; Blasco et al., 2001; Rodriguez-Romero & Corrochano, 2004, Sanz et al., 2010). The transcriptional regulation of the *carB* and *carRA* genes via a 1379 bp long bidirectional promoter sequence between them remained for a long time unclear. There are several consensus sequences of regulatory elements, like four light regulating elements (LRE) known from *N. crassa* as well as *al-3* proximal elements, (APEs), (Carattoli et al., 1994), eight magnetic field regulating elements (EMRE) or c-myc binding sites (Lin et al., 1999, 2001) and gravitropic response elements (Moseyko et al., 2002). For a specific 315 bp fragment of the promoter or intergenic region protein binding was demonstrated by gel mobility shift experiments (EMSA). Interestingly however, the shifted fragment contains no regulating consensus sequences apart of one GATA-box. The binding occurs in dark grown mycelia and eight minutes after light exposure. These eight minutes after irradiation correlate with a "drop down" of *carB* and *carRA* transcript (Sanz et al., 2010).

From the observation that the blue light induced accumulation of *carB* mRNA is accompanied by an increase of degradation of *carB* transcript, other specific features of the photoregulation of the carotene pathway have to be postulated (Blasco et al., 2001).

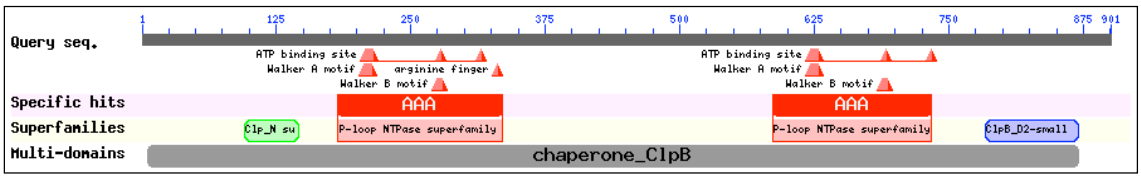
1.4.4 Heat shock proteins (HSP)

In response to heat the group of heat shock proteins is a part of the protection mechanism and thus their genes are highly expressed after a heat shock. They are classified according to their molecular weight. In this work only genes of the group of *hsp90* and *hsp100* were used.

1.4.4.1 *hspA*, the *hsp100* of *P. blakesleeanus*

HSP100´s belong to the superfamily of AAA+ ATPases (ATPases Associated with a wide variety of cellular Activities), an ancient group of ATPases. As the name reflects, members of this superfamily represent a wide range of cellular functions like molecular chaperons for thermo tolerance and disaggregation of stress-damaged proteins, ATPase subunits of proteases, helicases, or nucleic-acid stimulated ATPases. The HSPA protein of *P. blakesleeanus* shows two AAA+ domains and the typical short amino terminal ClpB domain, which is why it is

classified as HSP100/ClpB (Corrochano 2002). This molecular chaperone does not act as a protease, but rather serves to disaggregate misfolded and aggregated proteins



and is involved as well as in the regulation of gene transcription and heat tolerance (Schirmer et al., 1996; Maurizi & Xia 2004).

Fig. 1.4.4.1: Conserved domain structure of *hspA* (AJ418044), obtained by using CD Search

Transcription of the *hspA* gene can be activated by heat or even just blue light. The promoter of this gene shows therefore the highly conserved heat shock elements (HSE) or consensus sequence 5´-nGAAn-3´ for the heat shock transcription factor (HSF) binding. A heat shock of 34 °C or 42 °C can stimulate the transcription of *hspA* 160 times. Apart of this, HSEs contains the promoter light regulating elements (LRE) known from *N. crassa* as APEs (al-3 proximal elements). These consensus sequences are also found in the promoter of *carB/carRA* and other photoinducible genes of *P. blakesleeanus* and *Mucor circinelloides*. With a threshold of 1 J/m^{-2} the *hspA* gene is 10 times induced by blue light (Rodríguez-Romero & Corrochano, 2004). The photoinducibility is transient in a MADA and MADB dependent manner and thereby separated from heat induced gene expression. In *madA* and *madB* mutant strains no change in *hspA* gene transcription after blue light treatment is detectable, but they show the same heat induction of the *hspA* gene as the wild-type. The *madC* mutant shows a 30-fold higher *hspA* gene expression than the wildtype after a heat shock, as well as after blue light treatment. As the *madC* mutation seems to be involved not only in photoregulation but also in heat shock, the *madC* gene product might be a general transcription regulator.

1.4.4.2 heat shock protein 90

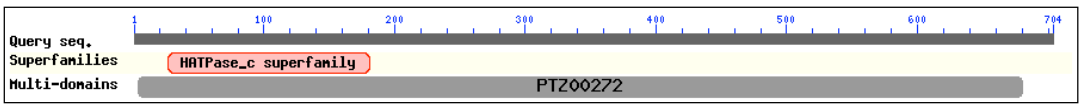


Fig. 1.4.4.2: Conserved domain structure of *hsp90* obtained by using CD Search

The ancient and evolutionarily conserved group of heat shock proteins 90 is

one of the first studied molecular chaperones, found in eubacteria and all branches of eucarya (Chen et al., 2005; 2006). It is one of the most highly expressed cellular proteins across all species. The amount of the HSP90s increases from 1–2 % up to 4–6 % of total cellular proteins, when stressed by heat, as their name implies (Crevel et al., 2001). The HSP90s are key components of the quality control machinery in cells involved in folding and maintenance, or in degradation of misfolded or destabilized proteins. It forms large complexes with different sets of substrates, cofactors or other chaperones regulating a wide variety of cellular pathways implicating normal cell development, physiology, diseases and transcriptome modulating processes (Csermely et al., 1998).

The HSP90s form a constitutive homodyne. The proteins has a highly conserved domain structure with the N-terminal HATPase_c domain for ATP binding also found in members of the ATPase/kinase GHKL (Gyrase, Histidine Kinase, MutL) superfamily (Fig. 1.4.4.1). It has a middle domain for substrate interaction and the C-terminal domain, which provides a strong dimerization interface which is essential for its function (Prodromou et al., 2000; Harris et al., 2004).

HSP90s is also a capacitor for morphological development as shown in *Drosophila*, where the reduction of HSP90 levels concealed phenotypic variations (Rutherford & Lindquist, 1998).

Other functions of HSP90s are nucleus-associated. HSP90 and p23 physically interact with telomerase (Holt et al., 1999) and a reduction of *hsp90* expression level affects the telomerase length (Grandin & Charbonneau 2001). Interaction of HSP90 and DNA helicases or chromatin remodeling complexes including SAGA, Swi/Snf and SWR-C are found. A comprehensive proteomic screen in yeast resulted in the identification of about 600 potential HSP90 cofactors and substrates out of 6000 ORFs (Zhao et al., 2005). Finally HSP90 is an important target in cancer because it participates in many key processes in oncogenesis such as self-sufficiency in growth signals, stabilization of mutant proteins, metastasis and angiogenesis. The development or discovery of new drugs with inhibitory effects on HSP90 got into the focus of pharmacological and clinical research (Taldone et al., 2009).

Members of the *hsp90* gene family can be classified in four distinct groups found in the genome of eucarya which are highly conserved among them (70 % identical). Two isoforms are present in cytoplasm (human *hsp90α* and *hsp90β*, 78 % identical) and in yeast (*Saccharomyces cerevisiae*) virtually identically termed *hsp82*, which is heat shock induced, and *hsc82*, which is constitutively expressed. They

arose by gene duplication very early in the evolution of eukaryotic cells. A third member is localized in the lumen of the endoplasmic reticulum (*grp94*) and a fourth member in mitochondria *trap1* (Csermely et al., 1998). The importance of this gene family is underlined by the high genomic copy number of *hsp90* genes in humans, 17 and in *Arabidopsis thaliana* six, and in *P. blakesleeanus* four.

The four annotated *hsp90* genes found at the time in *P. blakesleeanus* genome sequence were all used in this work:

a) *hsp90:* *hsc82 Saccharomyces c.* (yeast) homologue and constitutively expressed, located in cytoplasm, *P. blakesleeanus* predicted protein show 70-80 % identity from fungi, plant to vertebrata
b) *hsp9020:* *hsp82 Saccharomyces c.* (yeast), heat shock induced, located in cytoplasm and predicted protein show 70-80 % identity from fungi to plant
c) *hsp9036:* *trap1* homologue located in mitochondria and *P. blakesleeanus* predicted protein show around 50 % identity from fungi, plant to vertebrata
d) *hsp901-3:* *grp94* homologue located in endoplasm and *P. blakesleeanus* predicted protein show around 50 % identity from fungi, plant to vertebrata

1.4.5 RNA modification genes

1.4.5.1 Argonaute and Dicer

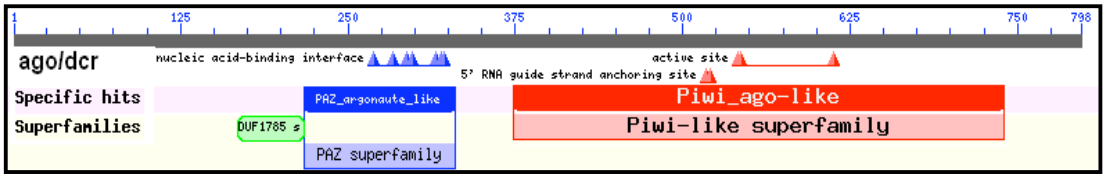


Fig.1.4.5.1: Conserved domain structure of ARGONAUTE and DICER, obtained by using CD Search.

RNA interference (RNAi) is a general eukaryotic mechanism to regulate gene transcription. It includes the endonucleases DICER and AGONAUTE (*dcr, ago*), which are central components of a RNA-induced silencing complex (RISC). They cleave double stranded RNAs (dsRNA), generated by RNA hairpin structures (small interfering RNAs, siRNA) or encoded micro RNAs (miRNA), into short fragments of around 20 nt. One of the two strands, the so called guide strand, can anneal to the complementary sequence of the mRNA, forming dsRNA, which again is recognized

by the endonuclease forming an autocatalytic post-transcriptional gene silencing process (Pratt & MacRae 2009).

AGONAUTE and DICER share two conserved domains, the C-terminal domain PIWI consists of two sub domains, one of which provides the 5' anchoring of the guide RNA and the other, the catalytic, endonuclease site for splicing. The N-terminal PAZ domain, named after the proteins PIWI, Argonaute, Zwille recognizes characteristic 3' overhangs in siRNAs. In the genome sequence of *P. blakesleeanus* two genes could be found with a high homology to the above mentioned criteria. Both are used in this work for the gene expression studies. It was suspected that they are up regulated after light treatment because the *carB* mRNA showed high degradation after its photoinduction (Blasco et al., 2001).

1.4.5.2 Histone acetyltransferases (HAT) and histone chaperone ASF

Another mechanism linked to transcriptional activation is the histone acetylation by histone acetyltransferases (HAT). The bromodomain containing Gnc5-like subfamily mediates acetylation of histones at a conserved lysine by catalyzing the transfer of the acetyl group from acetyl-Coenzyme A to the primary amine of histone H3, ε-N-acetyl-lysine. This transfer neutralizes the positive charge of histone. The bromodomain is a 110 amino acid long protein-protein-binding domain composed of a bundle of four α-helices, found in many chromatin-associated proteins.

One of these GCN5-related N-acetyltransferases (GNAT, where N is any NTP), is NGF-1 from *N. crassa.* It acetylates transiently the residue K14 of histone H3 which is associated to the photoinducible *albino-3* promoter in a WC-1 depending manner (Grimaldi et al., 2006). One homologue of *ngf-1* could be found in the genome sequence of *P.* blakesleeanus. It can be speculated that the *ngf* homologue of *P. blakesleeanus* shows photoinducibility or that its expression differs from the wildtype in the *mad* mutant strains.

At the beginning of this work there were three genes automatically annotated encoding HAT enzymes. These genes were used in this work (*hat10, hat50, hat53*). At this time there are nine genes annotated.

Another important protein which is involved in the regulation of gene expression, cellular responses to DNA damage and nucleosome assembly is the conserved histone chaperone ASF. It can interact directly with the histone H3/H4 complex, modulating nucleosome assembly and disassembly reactions as well as

with sequence specific complexes modifying the transcriptional activity of its target genes, e.g. silencing specific target genes.

1.4.6 Transcription factors

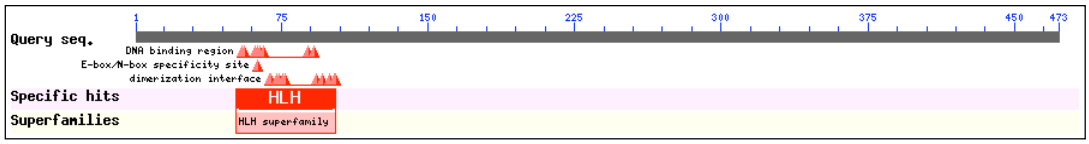


Fig. 1.4.6.1: Conserved domain structure of helix-loop-helix transcription factor (*myc*: fgeneshPB_pg.35__62) using CD Search.

The transcriptional regulation in eukaryotic organism can be mediated by transcription factors. These proteins are in general directly involved in the transcriptional regulation of gene expression by binding specific sequence elements in the promoter region of their interacting target genes. The transcription factors can be classified by their molecular structure which is linked to the consensus sequences of their conserved domains. The molecular structure of *myc* is based on a helix-loop-helix which forms a left handed bundle of four helices by dimerization (bHLH type). This type for DNA-binding recognizes the consensus sequence 5′-CANNTG-3′ (E-box), which can be found in upstream sequences of almost all light regulated genes in this work, like *cryA*, *carB* and *carRA* and *madA, madB* as well as *wctB* and *wctC*.

Therefore expression studies of the *myc* gene may reveal very interesting aspects of light regulated gene expression. Via the photoreceptors MADA and MADB (MAD complex) the light signal can be transferred to the regulation of gene expression. Further signal amplification can occur via transcription factors.

1.5 Aim of this work

Among the different fungi investigated in biosciences *Phycomyces blakesleeanus* is one of the model organisms for photosensoric physiology since more than 50 years. Light plays a central role in morphological development, growth, photogravitropical sensations and secondary metabolism with the β-carotene production as the most famous example. The *mad* mutant strains, classically isolated by their reduced phototropic sensitivity (Bergmann et al., 1973), have been subject to more detailed investigations of the signal transduction of light in *P. blakesleeanus.*

Since molecular biology advanced, the investigations were focused more and more on molecular regulatory processes and were facilitated after whole genome sequences of *P. blakesleeanus* became available and *madA* and *madB* were identified as homologues to the *wc-1*, *wc-2* blue light photoreceptor genes of the ascomycete *Neurospora crassa*.

To study gene regulatory mechanisms more profoundly, quantitative real time RT-PCR (qRT-PCR), as the currently most powerful and sensitive method, became appropriate, to perform gene expression analyses after a wide variety of external stimuli.

The versatile responses to light, particularly blue light, of *P. blakesleeanus* were for a long time subject of investigation but the molecular mechanisms of light transduction remained still to be illuminated. Thus the qRT-PCR method was applied to characterize transcriptional behavior of *P. blakesleeanus* after blue and red light treatments as well as gravitropic stimulation to move a step deeper in molecular signal transduction. Therefore several genes encoding five different functional groups from important cellular processes and signaling were searched manually by successive alignments and investigated.

I sought to characterize the gene expression in the wildtype and *mad* mutant strains in darkness, after continuous and a pulse of blue and red light. Bichromatic and double pulse experiments with red and blue light should be performed to investigate their influence on gene transcription on the one hand and on the other, to analyze the chromatic and photopic nature of the photoreceptors involved in transcription.

During the period of this work multiple *wc* genes in *P. blakesleeanus* were identified as well as the formation of the MAD complex by *madA* and *madB* gene products (Sanz et al., 2009). Thus the participation of the photoreceptor/transcription factor complex MAD in transcriptional regulation of the here chosen genes after gravity-stimulation became into the focus of the investigations.

Chapter 2. MATERIAL AND METHODS

2.1 Strains of *Phycomyces*, growth conditions, light treatment

2.1.1 Strains

The bacterial strain *Escherichia coli* DH5α were used for the plasmid transfection.

The strains of *Phycomyces blakesleeanus* used in this work are listed in Tab.2.1.1.1. The standard wildtype (WT) strain of *P. blakesleeanus* (Burgeff) NRRL1555 (-), mating type (-), was originally obtained from the Northern Regional Research Laboratories, Peoria, IL., USA. From the wildtype, NRRL1555 (-), mutant strains were obtained by chemical mutagenesis with N-methyl-N´nitro-N-nitrosoguanidine (NTG), 4-nitroquinoline-1-oxide (NTQ) or by crossing and complementations (Bergman et al., 1973). The prefix of the mutant strains identifies there origin. A: Prof. Arturo P. Eslava, University of Salamanca, Spain; C: Prof. Max Delbrück, California Institute of Technology, Pasadena, CA, U.S.A.; L: Prof. Edward D. Lipson, Department of Physics, Syracuse, NY, U.S.A.

2.1.2 Growth conditions

All strains of *P. blakesleeanus* were grown on SVI solid minimal medium (Sutter, 1975) which contains per liter demineralized water:

20	g	D(+)-glucose	2	g	citric acid
2	g	L-Asparagine·H_2O	1.5	g	$Fe(NO_3)_3 \cdot 9H_2O$
5	g	KH_2PO_4	1	g	$ZnSO_4 \cdot 7H_2O$
0.5	g	$MgSO_4 \cdot 7H_2O$	0.3	g	$MnSO_4 \cdot H_2O$
30	g	Bacto Agar	0.05	g	$CuSO \cdot 5H_2O$
2	ml	$CaCl_2$ (0,1 M)	0.05	g	$Na_2MoO_4 \cdot 2H_2O$
1	ml	Thiamin·HCl (0.1 %)			
0.1	ml	concentrated supplement			pH is around 4.2

Media were autoclaved, 25 ml or 4 ml were filled in Petri dishes (9.2 or 3.5 mm diameter, Sarstedt, Nürmbrecht, Germany) and stored at 4 ° C.

Tab.2.1.1.1: Strains of *P. blakesleeanus* used in this work and strains from which they were derived. Gene designation based on the nomenclature of Demerec et al., (1966): *mad* abnormal phototropism, whereas *madA* and *madB* encode a *wc-1* and *wc-2* type photoreceptor respectively. Furthermore *geo* abnormal geotropism, *car* abnormal carotene synthesis, (-) / (+) mating type, *pde-1* cAMP phosphodiesterase 1.

Strains	Genotype	Origin/mutagen	Reference
NRRL1555	*(-)*	wild-type	Bergmann et al., 1973
C21	*madA7, pde-1 (-)*	NRRL1555 (-), NTG	-"-
C47	*madA35 (-)*	NRRL1555 (-), NTG	-"-
A893	*madA403 (-)*	NRRL1555 (-), NTG	Campuzano et al., 1994
C111	*madB103 (-)*	NRRL1555 (-), NTG	Bergmann et al., 1973
A202	*madC469 (-)*	A56 x B24	
L51	*madA7, madB103 (-)*	C303 x C21	Lipson & Terasaka, 1980
L52	*madA7, madC119 (-)*	L2 x C21	-"-
L57	*madB103, madC119 (-)*	C303 x C148	Campuzano et al., 1990
L72	*madA7, madB103, madC119 (-)*	L2 x L51	-"-
L163	*madE726 (-)*	NRRL1555 (-), NTG	-"-
L161	*madF724 (-)*	NRRL1555 (-), NTG	-"-
A282	*madG131 (+)*	A56 x C307	Orejas et al., 1987
A909	*madJ407 (-)*	NRRL1555 (-), NTG	Campuzano et al., 1994
C5	*carB10, geo-10 (-)*	NRRL1555 (-), NTG	Bergmann et al., 1973
C2	*carA5 (-)*	NRRL1555 (-), NTG	-"-
C9	*carR21 (-)*	NRRL1555 (-), NTG	-"-
C171	*carA30, carR21 (-)*	C9, NTG	Ootaki et al., 1973
A98	*carC652 (-)*	NRRL1555 (-), NQO	

All experiments with *P. blakesleeanus* mycelia or sporangiophores were done in temperature controlled chambers at 21 °C.

Petri plates (92 mm or 35 mm) were inoculated with about 500 or 75 heat activated spores respectively (10 min at 49 °C; Rudolph H., 1960).

Inoculated Petri dishes were transferred to transparent plastic boxes (340 x 220 x 90 mm, Kobe-Laborbedarf, Marburg, Germany) and kept under fluorescent lamps (L40W/19 Tageslicht, Osram, Munich, Germany) providing white light of moderate fluence rate (0,7 W/m^2).

To get homogeneous SI-sporangiophores the transparent plastic boxes were

closed additionally with parafilm surrounding their lid and kept for 4-5 days under continuous light conditions.

Inoculated Petri dishes for light pulse experiments were placed for 70 h in continuous darkness in black boxes (400 x 250 x 100 mm, workshop Philipps-Universität Marburg, Germany).

Inoculated Petri dishes (35 mm) for gravitropical stimulation were raised for 5-6 days in the transparent plastic boxes under moderate white fluorescent lamps (see above).

2.1.3 Light treatment

In addition to the light source of the growth-chamber with continuous fluorescent white light 0.7 W/m^2, all light treatments were made with an overhead array of 10 LED´s (light-emitting diodes, Dotlight, Jülich, Germany) with a peak wavelength of 475 nm (blue) or 630 nm (red) and a half band width of 11 nm and 17 nm respectively, determined with the USB 2000-VIS spectrometer (Ocean Optics, Dunedin, Fla., USA). For a homogeneous illumination of 10 cm Ø the LED-array was placed on a plastic holder about 23 cm over the samples in the illumination box (350 x 300 x 400 mm).

Fluence rates were measured with a UV-enhanced photodiode (Meßkopf BN-9102-4; Gigahertz-Optic, Puchheim, Germany) and a calibrated readout instrument (Optometer P-9201, Gigahertz-Optic, Puchheim, Germany).

The LED arrays provide a fluence rate of 16 Wm^{-2} 475 nm blue and 15 Wm^{-2} 630 nm red light. Red and blue together were adjusted to 15 Wm^{-2} with their power supplies.

All 70 h dark grown mycelia were irradiated separately with blue, red, blue and red together or sequenced blue immediately followed by red or red immediately followed by blue light pulses with 40 J/m^2 (25 s) and then replaced into dark. After 5, 10, 20, 80 min Mycelia were taken out of the Petri dish, separated from the SIV solid medium, frozen in liquid nitrogen and stored at -80 °C. The harvest of samples was performed under very week indirect red (630 nm) light.

2.1.4 Gravitropic Stimulation

P. blakesleeanus wildtype NRRL1555 (-), *madC* mutant strain A202 and triple mutant strain L72 (*madABC*) sporangiophores were raised under moderate white light (0.7 Wm^{-2}, see above) and kept four hours before and during the experiment in darkness. For gravitropic stimulation, five days old SIVb-sporangiophores were oriented horizontally at time 0 in a black plastic container (600 x 455 x 350 mm, workshop Philipps-Universität Marburg, Germany). The samples were harvested two, one and 0.5 hours before the gravitropical stimulation and then each 30 minutes later by freezing in liquid nitrogen and by separation of sporangiophores from mycelia and placing the sporangiophores for further treatment in 2 ml reaction tubes (Sarstedt, Nürmbrecht, Germany) while frozen.

2.2 Gene expression studies

2.2.1 Gene sequences

Selected genes investigated in this work are listed in Tab. 2.5.1. The sequences of genes of interest were searched and downloaded from JGI (Joint Genome Institute http://genome.jgi-psf.org/annotator/servlet/jgi.annotation.Annotation?pDb=Phybl1). All further sequence analyses were performed by the program Vector NT10 (Invitrogen, Karlsruhe, Germany). The intron positions were manually determined by an alignment of genomic and coding sequence of predicted genes and signed in the coding sequence.

2.2.2 Primer design

For qRT-PCR the primers were designed with Vector NT10 for optimal annealing temperature of 60 °C (T_M 65 °C ± 3 °C) with a length of 20-30 bp, excluding palindromes and repeats. The C and G nucleotides should be uniformly distributed and the primer should not be self-complementary or complementary to any other primer in the reaction. To minimize spontaneous and nonspecific formation of dimers or hairpin loops their theoretical ΔG should be less then -1 kcal/mol. The amplicon length of around 200 bp was chosen close to the 3´end of transcripts for amplification of all probable splicing variants. If possible, intron

spanning primer pairs were preferred. All primers were generated by MWG Biotech, Ebersberg, Germany. Specificity of the primer sequences was controlled by alignment against the whole Phycomyces genome sequence (http://genome.jgi-psf.org/ cgi-bin/searchGM?db= Phybl1) and by melting curve analyses after a usual qRT-PCR with 40 amplification cycles using only one pair of primer without template DNA. Intron spanning primer pairs were controlled by melting curve analyses of qRT-PCR with genomic and cDNA templates. The amplification product generated by using genomic DNA as template on the other hand should have a higher melting point than using cDNA as template. Additional control consists in gel-electrophoresis (2 % w/v agarose gels) of qRT-PCR products with a 1 kb-DNA-standard-ladder (Fermentas, St. Leon-Rot, Germany) and visualization by ethidium bromide staining.

2.2.3 Gel electrophoresis

Agarose (1 %, w/v Roth, Karlsruhe, Germany) gel electrophoresis was performed in 20 mM Tris–acetate, 2 mM EDTA, pH 8.2 (TAE) in a Wide Mini Cell GT with Powerpac 300 (BioRad, Hercules, USA) with 4-7 V/cm. Gels were stained with Ethidium bromide (0.5 µg/ml TAE buffer) for 0.5 h and destained for 1 h in TAE buffer. Imaging was carried out with the TF-20 (Vilbert Lourmat, Marne la Vallee, France) or with a UV transilluminator Gel Doc 1000 (BioRad, Hercules, USA).

2.2.4 DNA Restriction

All restriction enzymes supplied from Fermentas (St. Leon-Rot, Germany) were used according to the manufacturers' protocol.

2.2.5 Cloning of DNA

DNA-fragments were cloned using CloneJET (Fermentas, St. Leon-Rot, Germany) following the manufacturers instruction.

2.2.6 RNA-preparation

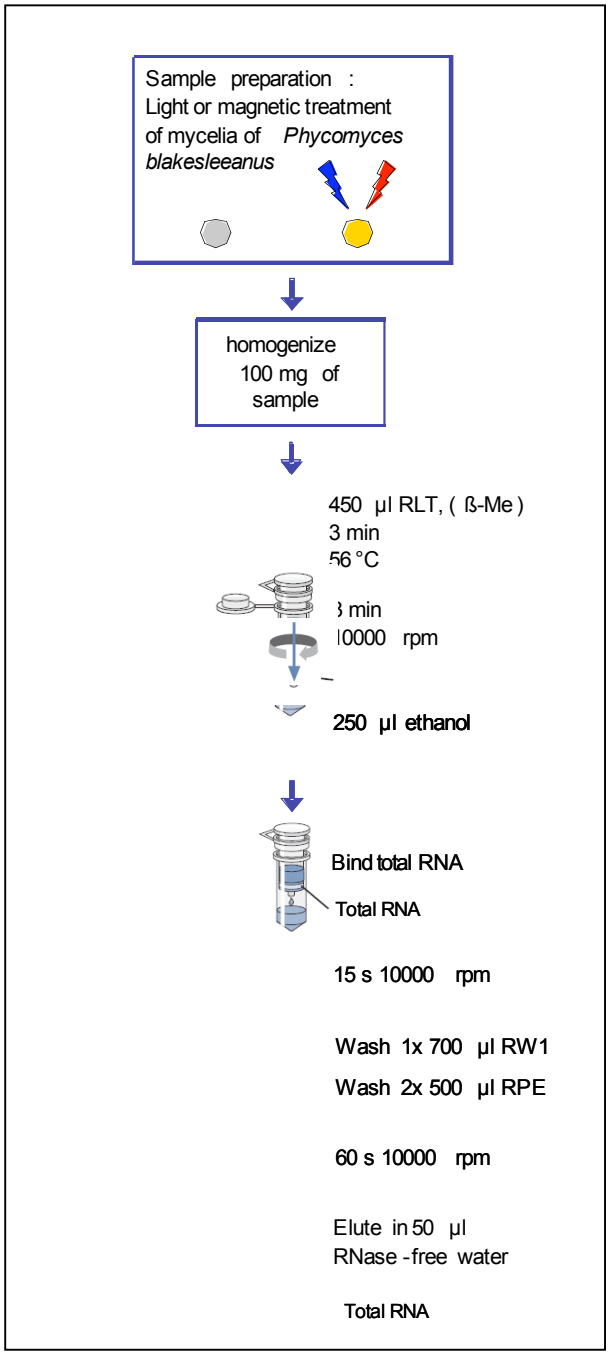


Fig. 2.2.6.1 Flow chart of RNA isolation with RNeasy miniprep columns (Qiagen)

100 mg of frozen samples, the mycelia of sporangiophores of *P. blakesleeanus* wildtype as well as the *mad* mutant strains collected after all types of experiments, were grounded with pestle and mortar while frozen in liquid nitrogen.

The total RNA was extracted using the RNeasy miniprep columns (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The integrity of the RNA was electrophoretically verified by ethidium bromide staining of 2 % (w/v) agarose gels. The concentration of the total RNA was determined by the absorption ratio of OD_{260}/OD_{280} nm > 1.95, measured with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). After the quantification a 10 µl aliquot was kept on ice for further treatment (see below), meanwhile the rest was frozen and stored at -80 °C.

2.2.7 DNase I Digest and cDNA Synthesis

To obtain DNA-free RNA, 2 µg of each RNA sample were digested with [1u/µl] DNase I (Fermentas, St. Leon-Rot, Germany) in 15 µl at 37 °C for 15-30 min. Enzyme was inactivated by incubation at 70 °C for 10 min with 1 µl 25 mM EDTA. At this temperature the secondary structure of the RNA also melts, which is why this step was used for primer annealing. 1 µl Oligo$(dt)_{18}$ primer [0.5 µg] (Fermentas, St. Leon-Rot, Germany) was added after 5 min and after 5 additional minutes probes were immediately cooled on ice.

For the following cDNA first strand synthesis M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (Promega, Madison, USA) was used according to the manufactures instructions in the same tube as DNase I digestion: 5 µl 5x MLV Reaction Buffer, 1.25 µl of each dNTP [10 mM], 25 units Ribonuclease inhibitor [40 u/µl](RiboLock, Fermentas, St. Leon-Rot, Germany) and nuclease-free water adding up to a final volume of 25 µl were added and the whole reaction was preheated at 42 °C before 200 units M-MLV RT [200 u/µl] were added. After 60 min at 42 °C the reverse transcriptase was heat inactivated for 10 min at 70 °C. Probes were cooled on ice and stored at 4 °C or directly used in qRT-PCR.

2.3 Polymerase Chain Reaction (PCR)

2.3.1 Standard PCR

Standard PCR reactions were performed on MJ mini (BioRad, Hercules, USA). The initial denaturation step at 95 °C was held for 5 min, followed by 25-35 cycles consisting in a denaturation step at 95 °C for 30 s, an annealing step in which primer specific temperature was chosen for 30 s and an elongation step at 72 °C for between 30 s up to 3 min (usual polymerase amplifies 1 kb/min) followed by a final elongation step at 72 °C for 8 min using rTaq DNA polymerase (Amersham, Uppsala, Sweden).

The reaction mix with a final volume of 25 µl contains 2.5 µl 10x PCR buffer, 5 µl 25 mM $MgCl_2$ [2.5 mM], 0.5 µl 2 mM dNTP Mix, 1µl of each primer [1 µM] and 0.2 µl (1 u) Taq DNA polymerase and x µl template DNA (0.1-2 µg) in nuclease-free water making up the difference to the final volume.

2.3.2 Real-Time qRT-PCR Conditions

Real-time-polymerase chain reaction was performed on the Mastercycler ep realplex (Eppendorf, Hamburg, Germany) with the following standard thermal profile (Fig. 2.3.2.1), used for all qRT-PCR reactions. The initial hot start at 95 °C for 15 min to release and activate the polymerase is followed by 40 cycles of 15 s denaturation at 95 °C, 30 s annealing at 60 °C and a 30 s elongation step at 72 °C. At the end of each cycle the fluorescence of the cyanine dye SYBR Green I, which intercalated in the minor groove of double stranded DNA (Zipper et al., 2004), was measured (absorption max. at 488 nm, emission max. at 522 nm). Melting curves were recorded after cycle 40 by heating from 60 °C to 95 °C with a ramp speed of 1.7 ° C min^{-1} with continuous fluorescence measurement.

The qRT-PCR reactions were mixed in 96-well plates (Sarstedt, Nürmbrecht, Germany) containing 12.5 µl 2x ABsolute QPCR SYBR Green Mix (ABgene, Epsom, UK), 1.5 µl each primer [1.5 µM], 2 µl cDNA and 7.5 µl PCR grade water, closed with optically transparent adhesive sealing tapes (Sarstedt) and shortly centrifuged to avoid air bubbles.

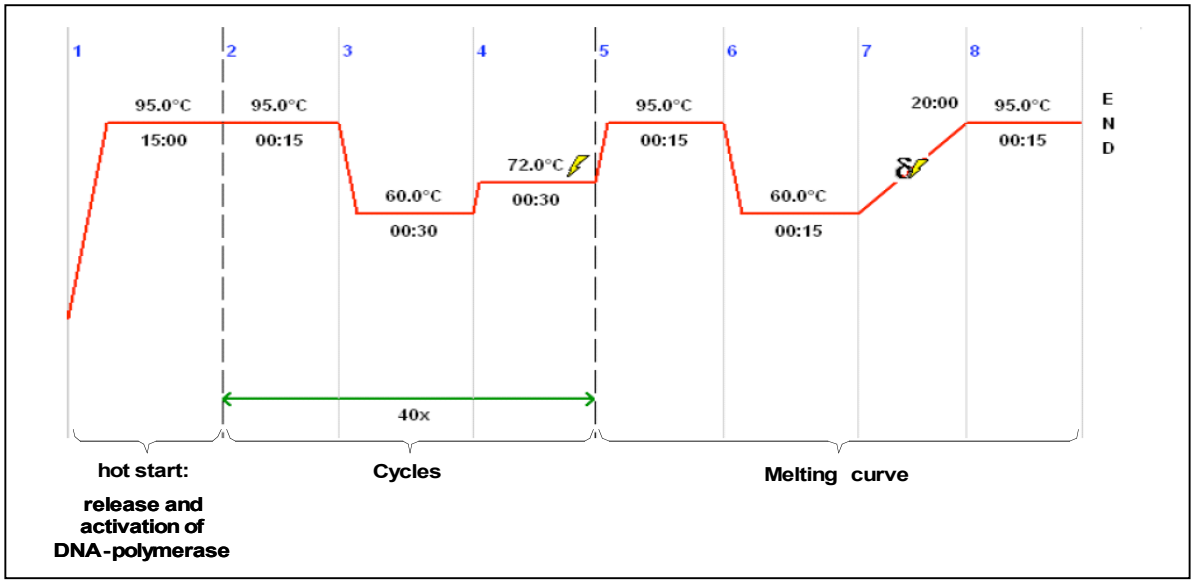


Fig.2.3.2.1: Standard qRT-PCR thermal profile: 1 hot start, 2-4 40 amplification cycles with plate read out at the end of step 4 and amplicon dissociation curve 5-8. Time is given in minutes. (Based on Eppendorf, 2005 with modification).

2.3.3 Real-Time qRT-PCR Analysis

Background and dye calibration were made following manufactures instruction. The qRT-PCR assays data are fluorescence intensity as a function of the number of cycles and for this reason they are log-linear plotted. The C_T (threshold cycle) is the cycle in which the fluorescence rises significantly (10 times the standard deviation) above the noise of the baseline (threshold). Thus C_T is an exponential term which was converted by the term 2^{-CT}. For all amplification plots C_T values were obtained by using a baseline individually calculated for every sample (automatic Baseline setting) with drift correction and an adjusted threshold (1000). All of these C_T values were converted with the term 2^{-CT} for statistical analyses (Prophet Cambridge, Massachusetts, USA.) and graphical presentations in Origin8, (OriginLabs, Corporation, Northampton, USA).

All corresponding data obtained by qRT-PCR after the different irradiations in the wildtype as well as the *mad* mutant strains were first converted by the term 2^{-CT} before the average calculations and graphical presentation.

2.4. Software

The following software was used for statistical and graphical calculating and representation:

Prophet6 (Prophet Cambridge, Massachusetts, USA.)
Origin8 (OriginLabs, Corporation, Northampton, USA)
Microsoft Excel
Sequence analyses and manipulation were performed using:
Vector NTI (Invitrogen, Karlsruhe, Germany)
Sequence tool for P. blakesleeanus genome sequence of the Joint Genome Institute http://genome.jgipsf.org/annotator/servlet/jgi.annotation.Annotation?pDb=Phybl1
BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi
CD SEARCH: Conserved Domain Structure
http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; (Marchler-Bauer et al., 2009; Marchler-Bauer; Bryant, 2004)
SMART: http://smart.embl-heidelberg.de; (Schulz et al., 1998; Letunic et al., 2006, 2009)

2.5 Analyzed genes

Tab. 2.5.1: Summary of all analyzed genes:

symbol	gene name	Annotation number	Function
rib	Ribosomal protein	estExt_Genewise1.C_150170	ribosome
act	*actin-1*	AJ287184	constituent of cytoskeleton
tub	*beta-tubulin*	AY944795	constituent of cytoskeleton
carB	*phytoene dehydrogenase*	X78434	carotene biosynthesis
carRA	*phytoene synthase* *lycopene cyclase*	AJ278287	carotene biosynthesis
hspA	*heat-shock protein A*	AJ418044	chaperone activity
hsp90	*heat shock protein 90*	estExt_Genewise1Plus.C_10701	chaperone activity
hsp90 20	*heat shock protein 90*	estExt_fgeneshPB_pm.C_20158	chaperone activity
hsp90 1-3	*heat shock protein 90*	e_gw1.3.60.1	chaperone activity
hsp90 36	*heat shock protein 90*	estExt_Genewise1.C_360085	chaperone activity
ago	*argonaute*	estExt_fgeneshPB_pg.C_20543	RNA silencing
dcr	*dicer*	gw1.9.12.1	RNA silencing
asf	*anti silencing factor*	e_gw1.20.240.1	histone chaperone
ngf	*N-acetyltransferase*	fgeneshPB_pm.54__1	chromatin remodeling
hat10	*histone acetyltransferase*	fgeneshPB_pg.2__1035	RNA-processing protein
hat 50	*histone acetyltransferase*	estExt_fgeneshPB_pg.C_50068	RNA-processing protein
hat 53	*histone acetyltransferase*	estExt_Genewise1.C_530073	RNA-processing protein
myc	*c-myc*	fgeneshPB_pg.35__62	Transcription regulator
cryA	*cryptochrome A*	JLR_estExt_Genewise1Plus.C_11049	DNA photolyase
madA	*madA*	DQ229146	wc-1-like photoreceptor
wcoA	*white collar one A*	DQ229145	wc-1-like photoreceptor
wcoB	*white collar one B*	FM178798	wc-1-like photoreceptor
madB	*madB*	FM178799	wc-2-like photoreceptor
wctB	*white collar two B*	FM178800	wc-2-like photoreceptor
wctC	*white collar two C*	FM179475	wc-2-like photoreceptor
wctD	*white collar two D*	FM178801	wc-2-like photoreceptor

Tab. 2.5.2: Oligonucleotides used in this work:

	gene symbol	gene name	universal / reverse primer (5´->3´)
1	*act*	*actin-1*	actu: GTTACGCCCTCCCCCACG
			actr: GACCACGCTCAGCAAGGATACG
2	*tub*	*beta-tubulin*	tubu: GATGTTGTCCGCAAGGAGGCTGA
			tubr: GATACAGAAGGTGGCATCGCAGTTC
3	*hspA*	*heat-shock protein A*	hspAu: TGAACTGGAGAAGGCACACAAGGATGT
			hspAr: TCTGGGCTAAAGTGCTGGCGGACA
4	*hsp90*	*heat shock protein 90*	90u1: CGAAGAAGATGAAGATGAAAAGAAGGCG
			90r1: GGAAGAGTCACGAAGAGCCTGGG
5	*hsp90 20*	*heat shock protein 90*	20_90u: GCTCTCCGTGACTCTTCCATGTCCTC
			20_90r: AACCATGCGGTGAATGCGGT
6	*hsp90 1-3*	*heat shock protein 90*	1-3u: GAATGGAAAGGAACAAGATGCCATGC
			1-3r: GAAGCACCGACGCCCTTACG
7	*hsp90 36*	*heat shock protein 90*	36_90u: TGATGCAAGGTTCTGCAAAGTTGG
			36_90r: GGAACGAGGATCATCCATCACACC
8	*carB*	*phytoene dehydrogenase*	Bu: CAACACGGATGACAAGGGCAAGAAGG
			Br: GAGCGACCAGTAGAAGGAGATAGAAGAAGAGG
9	*carRA*	*phytoene synthase lycpene cyclase*	RAu: CGACCTTGTCTACTACTCTGCCTGCGTGGC
			RAr: GTGTCGATGAGCAAGAAGAAGAGG
10	*ago*	*agronaute*	agou: CTTCCGTTGCTGCCCTTACCG
			agor: CTTCACCATCGAGGACCTGCTTG
11	*dcr*	*dicer*	dcru: GAGGATGGTCAGGAGTACACCGTTG
			dcrr: GAATGCTTGCAGATAAGGGTTGG
12	*asf*	*anti silencing factor*	asfu: CGACAAGACCCACGACCAAGTTC
			asfr: CGGGAGGTTCAAGACGCATTTC
13	*ngf*	*N-acetyltransferase*	ngfu: CTATCCAGAGAAGAGCAGTGCATGAG
			ngfr: CATCCATTTCTGGTGTCCAGCCCG
14	*hat10*	*histone acetyltransferase*	10HATu: ACCTCCAGTCTCGCCACATATCAGC
			10HATr: CGCTTGGATTTATCTCACGAGAAGGG
15	*hat 50*	*histone acetyltransferase*	50HATu: CGTGGAGCAACAGAAGAGAATCG
			50HATr: CATAATAATCAGCTCCATAAGAGCCTTG

	gene symbol	gene name	universal / reverse primer (5´->3´)
16	*hat 53*	*histone acetyltransferase*	53HATu: TGAAGAGGAGAATGGTCTGGCACG
			53HATr: TCAGCATACTTTAGACAGACAATACGCAC
17	*myc*	*c-myc*	mycu: TTGGCTAACTGACAATTTGGCTGG
			mycr: GAGTCGATGGTGTTTGGTTTAATGG
18	*cryA*	*cryptochrome A*	cryu: GGATCTTTATATCGACTGGAGAATGGGTGCA
			cryr1: GGACACCAAAGCTTGACATAATTGCC
19	*madA*	*madA*	madu: GCCCAGATGCTTCAAAGTAATGAACATCAAGCCAGTATTATC
			madr: CCAACAAAGAACGCGACCTCATTAAATTCATTGC
20	*wcoA*	*white collar one A*	wcu: GAATCTGCTGGAATACGAACCTGAAG
			wcr: GCGGCTCTTGCTTTGGTCC
21	*wcoB*	*white collar one B*	wcoBu: CACTACTACTACTATTGCTGCTGCCCG
			wcoBr: GTTGAGCCTGGCAATCTGCGTAAAC
22	*madB*	*madB*	wctAu: TGCGATACGACCTGGGAGAGCG
			wctAr: CAGATCTTTGGGGCTTCAACCTCGATAC
23	*wctB*	*white collar two B*	wctBu: CGCCAAACTTTATCAAGCTGGGC
			wctBr: CCTTTTCTCCATTCTGGTGCGTTC
24	*wctC*	*white collar two C*	wctCu: GGAGCCGGGTTTGGATGAAAATAC
			wctCr: CAGCGCACACAAACTCATCTGAACCCT
25	*wctD*	*white collar two D*	wctDu: CCTCAGCCGCCGACCTTTG
			wctDr: CCATTTCTCTTTGCGTTCAGGTG
26	*opcA*	*octahedral crystals*	PKrc: CCGAACGATCAAGAGAGGGTGACATAAGC
			Pkuc: CGATGTGGACACCCATATCCAAGG

Chapter 3. RESULTS

3.1 Search of genes

3.1.1 Alignments (search for red light photoreceptor genes)

The sequencing genome project of *P. blakesleeanus* wildtype, NRRL1555 (-), was approved in 2006 by the Joint Genome Institute and so first sequencing results became available several month later, before the automated annotation started (http://genome.jgi-psf.org/Phybl1/Phybl1.home.html). Several genes were searched manually by successive alignments with sequences of known genes of other organisms or only with the consensus sequences of conserved functional protein domains, like the FAD binding site of blue light photoreceptor cryptochrome *cry* and white collar *wc-1*. The search for the red light photoreceptor phytochrome (*phy*) homologue genes were performed with phytochrome consensus sequences of cyclic GMP phosphodiesterase/adenyl cyclase/FhlA (GAF) domain which is surrounded by a PAS and PHY domain, which clearly distinguishes phytochrome from other GAF-containing proteins (Froehlich et al., 2005; Corrochano 2007). The most probable identified sequence from scaffold 70 around the position 45978-54372 is now manually annotated by Antonio G. Pisabarro (fgeneshPB_pg.70_15). The analyzed genes in this work are listed in Tab. 2.5.1. Due to the extent of experiments, their comprehensive results and the later identification of the predicted phytochrome orthologue gene of *P. blakesleeanus*, it was not included in the experiments of this work. Another reason is that the *phy* gene expression in *N. crassa* is not regulated by light (Froehlich et al., 2005).

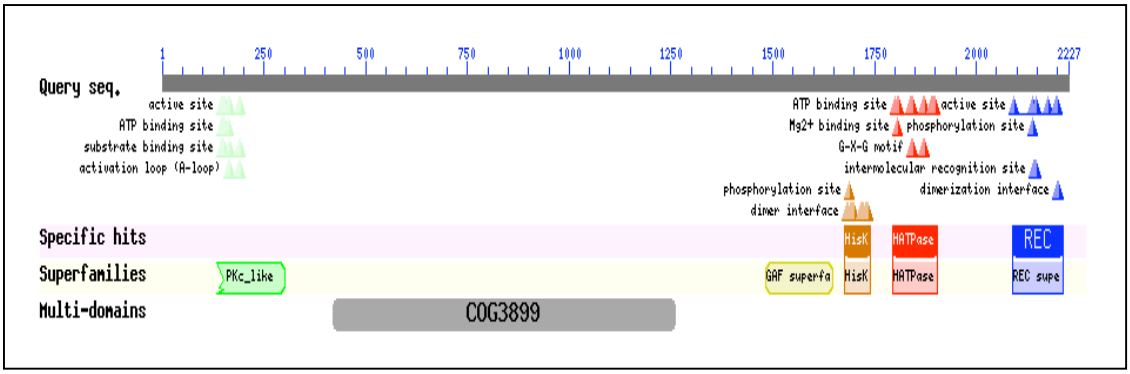


Fig. 3.1.1.1: Conserved domain structure of predicted phytochrome orthologue of *P. blakesleeanus* using CD search (fgeneshPB_pg.70_15).

3.2 Gene expression

3.2.1 Gene expression in continuous darkness

Quantitative real time RT-PCR (qRT-PCR) is currently one of the most powerful and sensitive techniques for transcriptome and gene expression analyses. For the first time this method was used in *P. blakesleeanus* to study gene expression over a time-course of 11.5 hours, in dark grown mycelia of wildtype. The time-course was chosen from hour 62 up to 73.5 after spore inoculation. This is on one hand the usual working time from 7 AM to 6:30 PM and on the other hand it is the time in which mycelia are grown enough to harvest them. Furthermore the time-course lies within the competence period of all known physiological and morphological developments, like carotenogenesis and photomorphogenesis (Bejerano et al., 1991). It also covers an additional hour, the period in which sporangiophore development starts, usually 72.5 hours after spore inoculation under the experimental conditions in which detectable SI-sporangiophore formation becomes macroscopically notable.

The dark grown mycelia were harvested each 30 minutes from 7 AM to 6:30 PM, i.e. 62 - 73.5 hours after spore inoculation by freezing them in liquid nitrogen. Transcript levels of which were examined by qRT-PCR.

The results of the gene expression during the time-course of 11.5 hours are shown in Fig. 3.2.1.1 - Fig. 3.2.1.7. The genes were therefore grouped by the presumable biological function of the predicted proteins. In Fig. 3.2.1.1 the classical housekeeping genes *act, tub* and *rib* are summarised together with *carB* and *carRA* which encode important enzymes of the β-carotene biosynthesis, the phytoene dehydrogenase and the bifunctional enzymes phytoene synthetase/lycopene cyclase. The expression patterns of the *hsp* gene family members, which encode for the molecular chaperones, the heat shock proteins, are shown together in Fig. 3.2.1.2. The genes coding for RNA endonucleases argonaute and dicer (*ago, dcr*), central constituents of the RNA–induced silencing complex RISC, are subsumed in Fig. 3.2.1.3. The chromatin-modulating enzymes, the acetyltransferases, encoded by the genes *hat10, hat50, hat53* and *ngf* as well as the ant silencing factor gene *asf* are shown together in Fig. 3.2.1.6. The photoreceptor genes are separated in the *wc-1* type genes *madA, wcoA, wcoB* and the predicted cryptochrome/photolyase orthologue gene *cryA* shown in Fig. 3.2.1.4 and the *wc-2* type photoreceptor genes *madB, wctB, wctC and wctD* shown in Fig. 3.2.1.5.

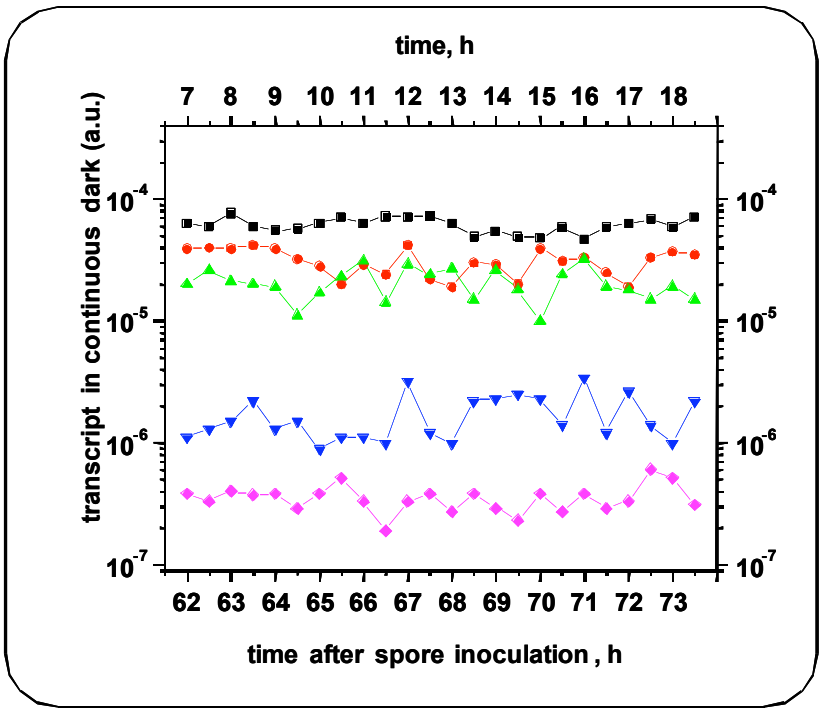


Fig. 3.2.1.1: 11.5 h time-course studies: gene expression levels in mycelia of the wildtype NRRL1555 (-), grown 62 to 73.5 hours in continuous darkness. From 7 AM to 6:30 PM two mycelia were harvested every 30 minutes and mRNA levels were examined by qRT-PCR. The plots show average transcript levels of the genes ■*rib,* ●*act,* ▲*tub,* ▼*carB* and ◆*carRA.*

The results of the 11.5 hour time-course reveal different expression levels for the chosen genes, which cover in total three orders of magnitude (10^{-8}-10^{-5} a.u.). With an average value of 6.1 x 10^{-5} a.u. the *rib* gene shows the highest level of transcript. This is twice as much as *act* or *tub*, which have also high levels around 3 x 10^{-5} a.u., followed by *hsp9020* with 1.4 x 10^{-5} a.u. (Fig. 3.2.1.1, 3.2.1.2 and Tab. 3.2.1.1). An order of magnitude lower than the housekeeping genes are the genes *asf* and *hat53* expressed with 1.1 and 1.7 x 10^{-6} a.u. respectively (Fig. 3.2.1.6), followed by the *carB* and *madB* gene, 2 x 10^{-6} a.u. and the gene *ago* with 3 x10^{-6} a.u. (Fig. 3.2.1.1, 3.2.1.3, 3.2.1.5). The expression levels of the majority of genes lies within the range of 10^{-7} a.u. (Tab. 3.2.1.1). Whereas seven genes have expression values from one to five x 10^{-7} a.u. like the *wc-2* type genes *wctC* and *wctD* show with 1 and 1.7 x 10^{-7} a.u. lower levels, which are the same as *ngf* and *hat50* both 1.5 x 10^{-7} a.u., and *dcr* and *wcoA* both around 3 x 10^{-7} a.u. (Fig. 3.2.1.4, 3.2.1.5, 3.2.1.6). The genes *carRA, wcoB* and *cryA* and *madA* display an average value of their transcripts of 5, 6.3, 7 and 9.3 x 10^{-7} a.u., respectively, within the upper half of 10^{-7} (Fig. 3.2.1.1,

3.2.1.4). The genes *hat10* and *wctB* show with 1.2 and 2.3 x 10^{-8} a.u. and finally *hsp9036* with 8.2 x 10^{-8} a.u. show the lowest detected expression levels (Fig. 3.2.1.2, 3.2.1.5, 3.2.1.6 and Tab. 3.2.1.1).

The expression patterns of the different genes obtained for the 11.5 hours time-course do not only differ by the level of expression, but also by the stability of transcription. There are genes like *rib* or *hsp9036* which are very stably transcribed during the time-course. Their expression values show just a "straight line" (Fig. 3.2.1.1, 3.2.1.2). Other genes like the housekeeping genes *act* and *tub* used by other investigators previously, or the genes *hat50* and *hat53* as well as *madB* and *hsp9020* are expressed nearly stable, even though the range of each measurement is not as constant as for the gene *rib*. These genes might be considered as housekeeping or internal reference genes for expression analyses as shown in Fig. 3.2.1.8.

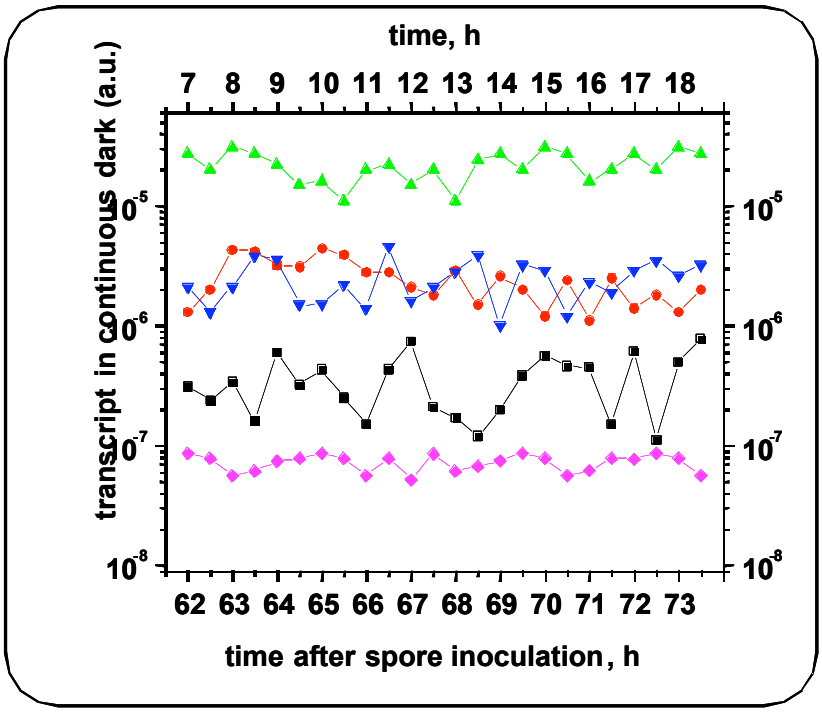


Fig. 3.2.1.2: 11.5 h time-course study: gene expression levels in mycelia of the wildtype NRRL1555 (-), grown 62 to 73.5 hours in continuous darkness. From 7 AM to 6:30 PM two mycelia were harvested every 30 minutes and mRNA levels were examined by qRT-PCR. The plots show average transcript levels of the genes ■ *hspA,* ● *hsp90,* ▲ *hsp9020,* ▼ *hsp901-3* and ◆ *hsp9036.*

The transcription level of some other genes show a disproportionately large, supremely diverse and not homogeneous "up and down" as measured for the genes

hspA or *hsp901-3*, shown in Fig. 3.2.1.2, for the genes *ago, ngf* and *dcr* (see below) or the *madA* and *wctB* genes shown in Fig. 3.2.1.4 and Fig. 3.2.1.5. Among these genes only the expression of the genes *ago* and *dcr* show somehow a rhythmical transcription in phase from 10:00 AM or 65 hours after spore inoculation. Thus their expression level is most widely spread (Fig. 3.2.1.7). The expression levels of the *madA* gene show lowest values at 12:00, 15:30 and 16:30 PM and the highest value at 13:00 PM. The expression of the *ngf* gene shows interestingly transient weak minima at 8:00 and 10:00 AM and a more pronounced one at 14:30 PM (63, 65 and 69 hours after spore inoculation) whereas its expression shows no outstanding maxima. The highest expression value was measured at 12:30 PM. The latest transcriptional minimum (14:30 PM) of the amount of *ngf* gene transcript coincides with the transient reduction of the *madB* transcript level which reaches its minimum at 15:00 PM or 70 hours after spore inoculation. Thus the strongest transient changes on the transcriptional levels for each gene were measured predominantly in the afternoon. Interestingly, the *madA* or *madB* do not show an outstanding transient change of their transcription, which could suggest a circadian rhythmicity of their gene expression.

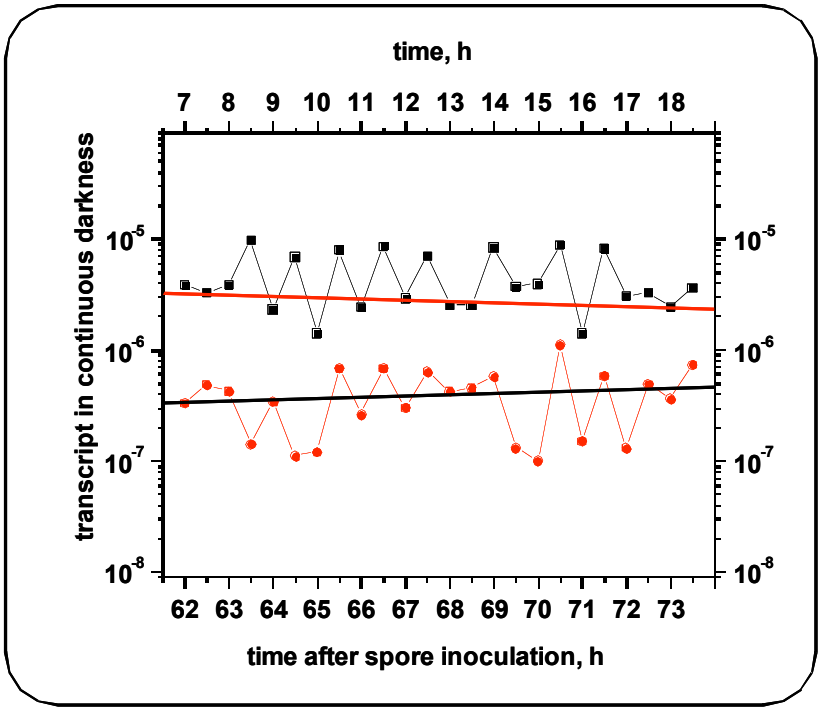


Fig. 3.2.1.3: 11.5 h time-course study: gene expression level in mycelia of the wildtype NRRL1555 (-) grown 62 to 73.5 hours in continuous darkness. From 7 AM to 6:30 PM two mycelia were harvested every 30 minutes and mRNA levels were examined by qRT-PCR. The plots show average transcript levels of the genes ■ *ago* and ● *dcr* with best fit line.

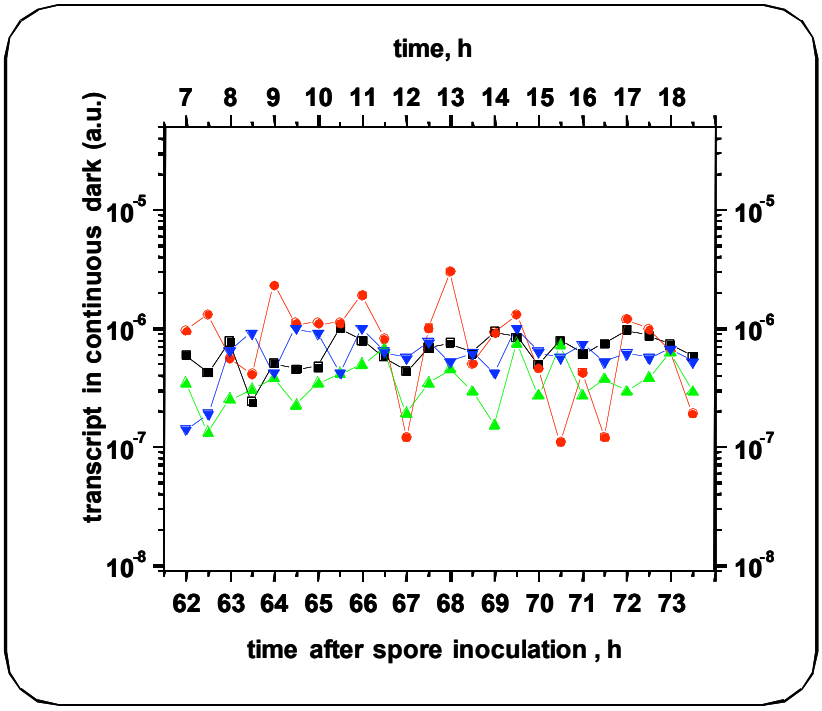


Fig. 3.2.1.4: 11 ½ h time-course study: gene expression level of the genes ■*cryA,* ●*madA,* ▲*wcoA* and ▼*wcoB*. For detailed description see legend below.

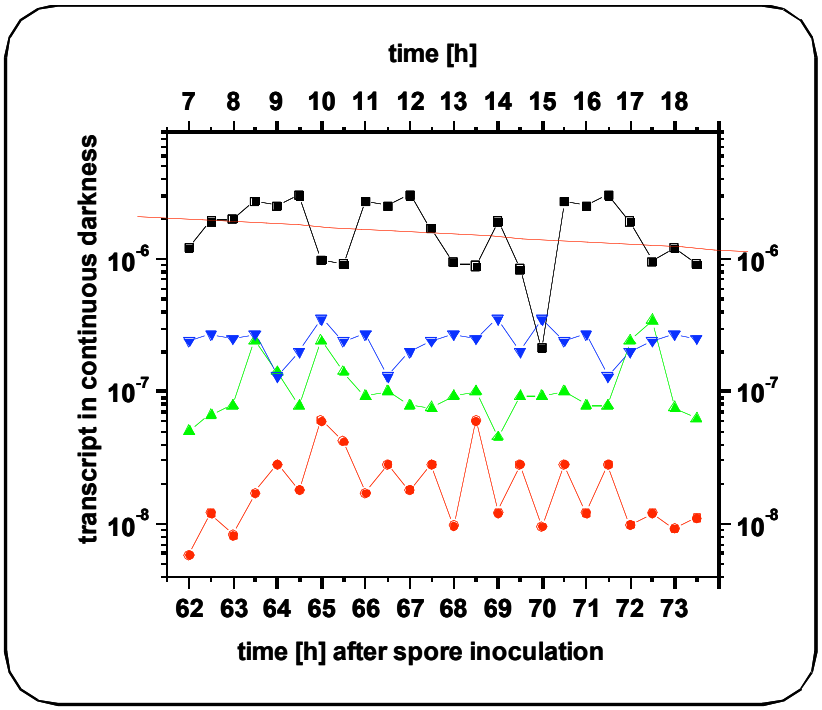


Fig. 3.2.1.5: 11.5 hours time-course study: gene expression level genes ■ *madB* with best fit line, ● *wctB,* ▲ *wctC* and ▼ *wctD*. For detailed description see legend below.

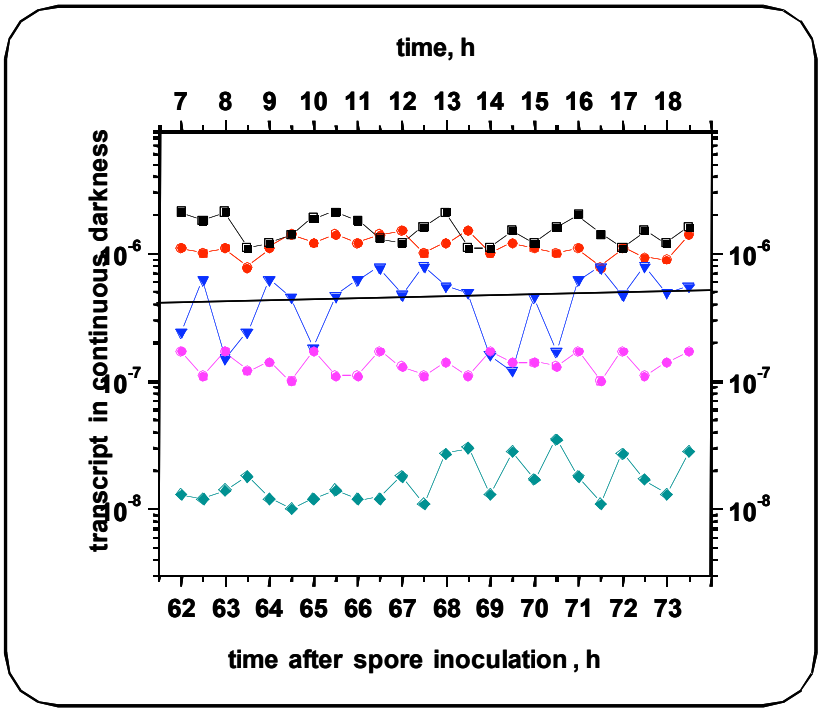


Fig.3.2.1.6: 11 ½ h time-course study: gene expression levels in mycelia of the wildtype NRRL1555 (-) grown 62 to 73.5 hours in continuous darkness. From 7 AM to 6:30 PM two mycelia were harvested every 30 minutes and mRNA levels were examined by qRT-PCR. The plots show average transcript levels of the genes ■ *hat53,* ● *asf,* ▼ *ngf* with best fit line,b ● *hat50* and ◆ *hat10.*

All analyzed genes are sorted in ascending order by the average of their expression levels in Tab. 3.2.1.1. All analyzed genes encoding for proteins are important for the viability of the cell. Thus it is conspicuous that the expression levels of all these genes are so widely spread from 10^{-8}-10^{-5} a.u. in *P. blakesleeanus*. The *hsp9020* is the only gene with a level of transcription within the same range as the housekeeping genes *act, tub* and *rib*, whereas all other genes are transcribed at a lower level. Most interestingly the transcription levels of the majority of them lies within 10^{-7} a.u. The expression levels of the *hsp* gene family members are in general most diverse among the analyzed functional groups. They cover the whole range (Fig. 3.2.1.2 and Tab. 3.2.1.1). This means the transcription level of the gene *hsp9036,* encoding the predicted mitochondrial located HSP90, is lowest within 10^{-8} a.u. transcribed, the gene *hspA* encoding a HSP100 is within 10^{-7} a.u. expressed, the genes *hsp901-3*and *hsp90,* encoding the predicted endoplasm and cytoplasm located HSP90, are within 10^{-6} a.u. and the gene *hsp9020,* encoding a cytoplasmatic located HSP90, is highest within 10^{-5} a.u. expressed. Another group in which the expression

levels of each member diverge includes the genes encoding for predicted RNA-modifying proteins or the histone acetyltransferases. Among them the gene *hat10* is lowest expressed which is one order of magnitude below the level of the genes *hat50, ngf* and *dcr* (10^{-7} a.u.). Within 10^{-6} a u. the gene *asf* is closely a third less expressed than the gene *ago*. The transcription level of the *wc-1* type photoreceptor genes are more homogeneous within 10^{-7}a.u., even though the gene *wcoA* is three times and *wcoB* 30 % less transcribed than the *madA* gene. This suggests a more important role of the *madA* gene product or a greater demand for the *madA* mRNA due to a higher turn over of the MADA. The transcription level of the *wctB* gene is the lowest within the group of *wc-2* type photoreceptors with a level of 2.3 x 10^{-8} a.u. The genes *wctD* and *wctC* are expressed an order of magnitude higher. The *madB* gene shows the highest transcription level which is 1000 times higher than that of *wctD* gene and 100 times higher as that of *wctB* gene. Surprisingly the *carB* gene shows four times higher expression than the *carRA* gene, even though both are regulated by the same bidirectional promoter.

Tab. 3.2.1.1: Range of gene expression values from the 11.5 hours time-course.

Genes expression values (av, n = 48) are sorted in ascending order by their expression level in mycelia of wildtype NRRL1555 (-), 62 -73.5 hours grown in continuous darkness. Two mycelia were harvested every 30 minutes by freezing in liquid nitrogen. mRNA level determined by qRT-PCR. Bold genes are very stably expressed during the time-course.

10^{-8}		**10^{-7}**		**10^{-6}**		**10^{-5}**	
8.2	***hsp90 36***	9.3	*madA*	2.9	*ago*	**6.1**	***rib***
2.3	*wctB*	7	*cryA*	2.2	*hsp90 1-3*	**3.2**	***act***
1.2	*hat 10*	6.3	*wcoB*	2	*madB*	**2.9**	***tub***
		5	*carRA*	1.9	*carB*	**1.4**	***hsp90 20***
		3.2	*dcr*	1.9	*hsp90*		
		3.1	*wcoA*	**1.7**	***hat 53***		
		2.3	*hspA*	1.1	***asf***		
		1.5	*ngf*				
		1.5	***hat 50***				
		1.7	*wctD*				
		1	*wctC*				

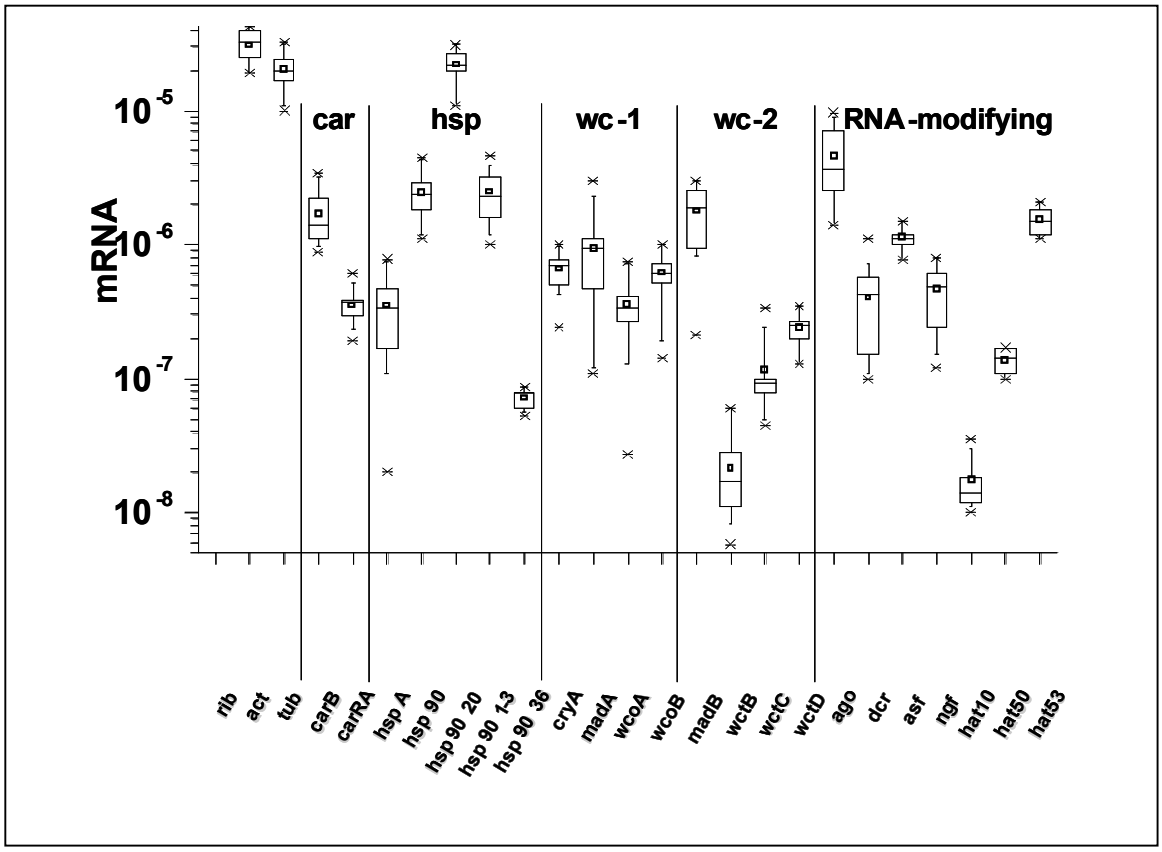


Figure 3.2.1.7: Stability and levels of gene expression in continuous darkness.
Box plots include all data from 11.5 h time-course study. Boxes show the upper and lower quartile of expression values (2^{-CT}) of chosen genes from mycelia of wildtype NRRL1555 (-) grown in continuous darkness. From 7 AM to 6:30 PM or 62 -73.5 hours after spore inoculation every 30 minutes two samples were taken (n = 48). Whiskers represent the 1st and 99th percentiles, mean (□) and median (—) are indicated. Xs indicate minimum and maximum values.

Some of the genes show stable transcription similar to the one of housekeeping genes like *act* and *tub*. The gene expression during a time course or after specific stimuli like chemical components, abiotic stress or different light quantities or qualities can be analysed by qRT-PCR. Housekeeping or reference genes are generally used as an internal control. Nevertheless the stability of their expression should be verified for each kind of applied stimulus to validate their suitability. In *P. blakesleeanus* the cytoskeleton constituent actin, *actA* gene (AJ287184; Voigt & Wöstemeyer, 2001) was mainly used in gene expression experiments without any validation. The results of the 11.5 hour time course revealed not only different expression levels but also an enormous variation in the stability of gene expression.

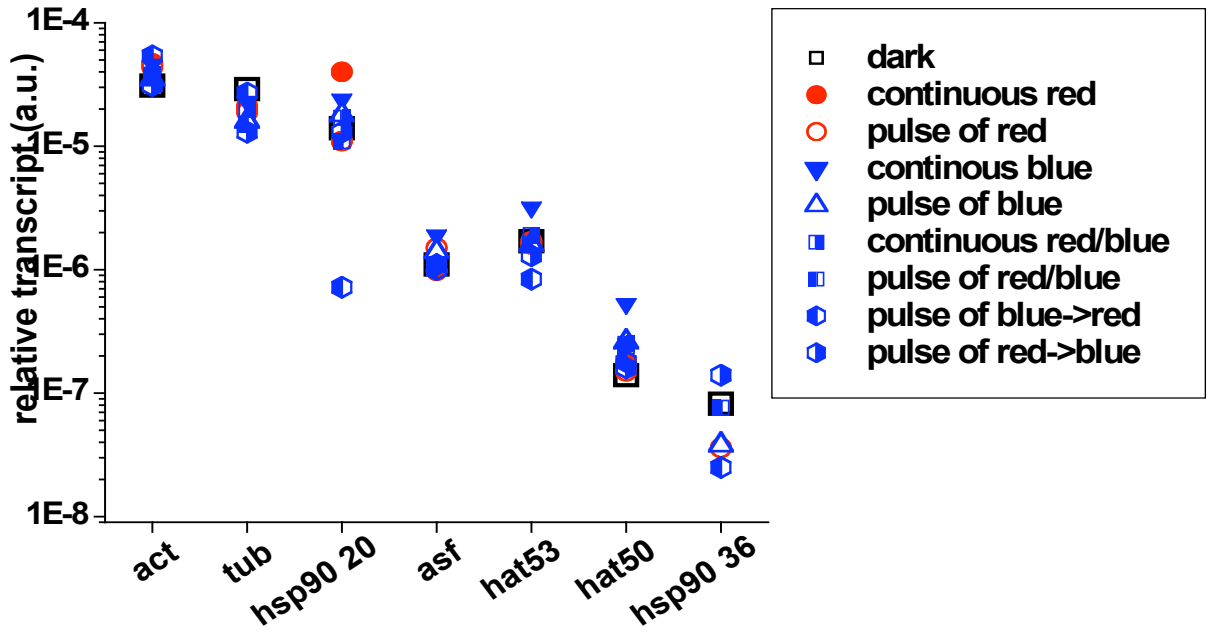


Fig. 3.2.1.8: Stability of gene expression after different irradiations

Gene expression pattern in 70 hours old mycelia of wildtype NRLL1555 (-), rose in continuous darkness, red_{630} (15 Wm^{-2}), $blue_{475}$ (16 Wm^{-2}) or bichromatic red and blue light (15 Wm^{-2}). Transcript levels were also assayed 20 minutes after a pulse of different wavelength as indicated or a double pulse of red then blue or blue then red light (40 Jm^{-2}, 25 s) in dark grown mycelia.

The differences in the range of detected expression values due to the stability of transcription during the 11.5 hours of *rib* and *dcr* as shown in Fig. 3.2.1.7 is remarkable, where expression range of *dcr* span almost one whole order of magnitude. The most stably expressed genes of the time course are shown in Fig. 3.2.1.8 in descending order of their expression ranges of continuous darkness with their amounts of transcript measured after different light exposures.

The gene expression pattern of *act* is most stable under all light treatments in comparison to the other genes. Only in continuous red and blue light or after a double pulse of red then blue light the expression of the gene *act* is higher (about 1.5 - 1.7-fold) compared to controls grown in dark. Therefore it is always enhanced after different light treatments, if not equal compared to the dark-grown samples. The gene *tub* shows the opposite pattern, which is slightly suppressed by different light treatments. The gene *asf* displays the same stability of transcription as the gene

act. Only upon continuous blue light its expression level is about 1.7 times enhanced. The expression of the gene *hat50* is 3.5-fold, that of *hat53* is twice enhanced under continuous blue light but 1.5-fold increased after continuous, and a pulse of bichromatic red and blue light. Other light exposures are not affecting the expression of *act* nor *asf* or *hat50*. The *tub* gene is up to 50 % suppressed by all kinds of light treatments. The *hsp9020* expression is about three times enhanced after continuous red light but strongly reduced (20-fold) after a double pulse of blue then red light. The gene with the lowest expression level in continuous darkness, *hsp9036*, is affected by all light treatments with the exception of a pulse of bichromatic red and blue light.

From the seven most stably transcribed genes in continuous darkness only *act* and *asf* are also very stably expressed under all different light exposures and therefore ideal candidates for the use as reference or internal control genes under these experimental conditions. Nevertheless the transcriptional variation of the gene *act* is about 1.7-fold after different light treatments. In the case of the 1.7-fold enhancement of the *act* mRNA it is around 40 % of the enhancement of the *carB* transcript measured in this work, which was also obtained and published by Sanz et al., 2010, thus further expression data will be presented in the scale of arbitrary units ($2e^{-CT}$).

3.2.2 Gene expression in continuous, and after a pulse of blue light

Blue light can trigger morphological development like the phorogenesis, cause tropism or induce gene expression. On the molecular level blue light enhances the expression of several genes in *P. blakesleeanus.* The blue light induced gene expression was first studied on genes for β-carotene biosynthesis *carB* and *carRA* (Ruiz-Hidalgo et al., 1997), later complemented by expression studies of heat shock protein *hspA* and the *wc-1* and *wc-2* like photoreceptor/transcription factor genes *madA* and *madB.* In dark grown mycelia of *P. blakesleeanus* all these genes show a common maximum increase of transcript during the first half hour by exposure to blue light for variable times up to one hour (Rodriguez-Romero & Corrochano, 2004; 2006; Idnurm et al., 2006; Sanz et al., 2009).

In this study the main focus lies on the primary blue light induced gene expression. Under the published experimental conditions where gene expression was measured variable times after blue light exposure, secondary transcriptional and

translational regulation is possible. To avoid this, a protocol was developed where the shortest light activation by a pulse of actinic light with intensities above the threshold of the photoreceptor/transcription factor system gave the highest, non saturating response on gene expression. Best results on induction of gene transcription were obtained 20 minutes after a short pulse of blue light (25 s, 475 nm, 40 Jm^{-2}). To monitor how photoresponsive the genes selected in this work are (Tab. 2.5.1, p. 30), and to characterize in more detail their blue light dependent regulation, mRNA levels were determined 10, 20 and also 80 minutes after a pulse of blue light.

A summary of gene expression levels of all analyzed genes in mycelia of wildtype grown in darkness, continuous blue light (16 Wm^{-2}), and 20 minutes after a 25 s pulse of blue light (40 J m^{-2}) is given in Fig. 3.2.2.1. The gene expression values after continuous or a pulse of blue light are plotted against the corresponding values of mycelia grown in continuous darkness. All genes respond somehow to blue light, with the vast majority responding with enhanced expression. The strength of photoinducibility differs widely between genes. The transcription of some genes is slightly enhanced, about two fold, while others show a strong increase of up to several hundred-fold. Only two of the genes have close to the same transcription values in continuous dark and blue light. Four others show very small responses to a pulse of blue light. These genes are less than 5 % affected by light exposure. On the other hand, a suppression of transcription can be observed, which is not as strong as the positive effect of blue light.

Around 60 % of the analyzed genes are photoinducible by blue light with different levels of transcription enhancement (3 to 300-fold) in comparison to the ones of dark-grown mycelia. Changes in gene transcription refer in the following to the corresponding amount measured in dark-grown mycelia. Six genes show reduced expression values after blue light exposure. The *hsp9036* gene is 55 % less expressed after both blue light treatments. The classical housekeeping gene tubulin (*tub*) is 20 % less expressed in continuous blue light and up to 50 % less after a pulse of blue light. After continuous blue light exposures the *madA* transcript is about 50 % lower. The mRNA of the *hat10* gene is reduced by 25 % after a pulse and twenty fold enhanced after continuous blue light. The genes *hat53* and *hsp901-3* show a reverse effect of suppressed transcription after a pulse and increased expression after continuous blue light. Both genes show less than 10 % suppression after a pulse and an increase of the amount of its mRNA (twice- and sixfold, respectively) after continuous blue light exposure. All other genes show moderate or strong photoinduction.

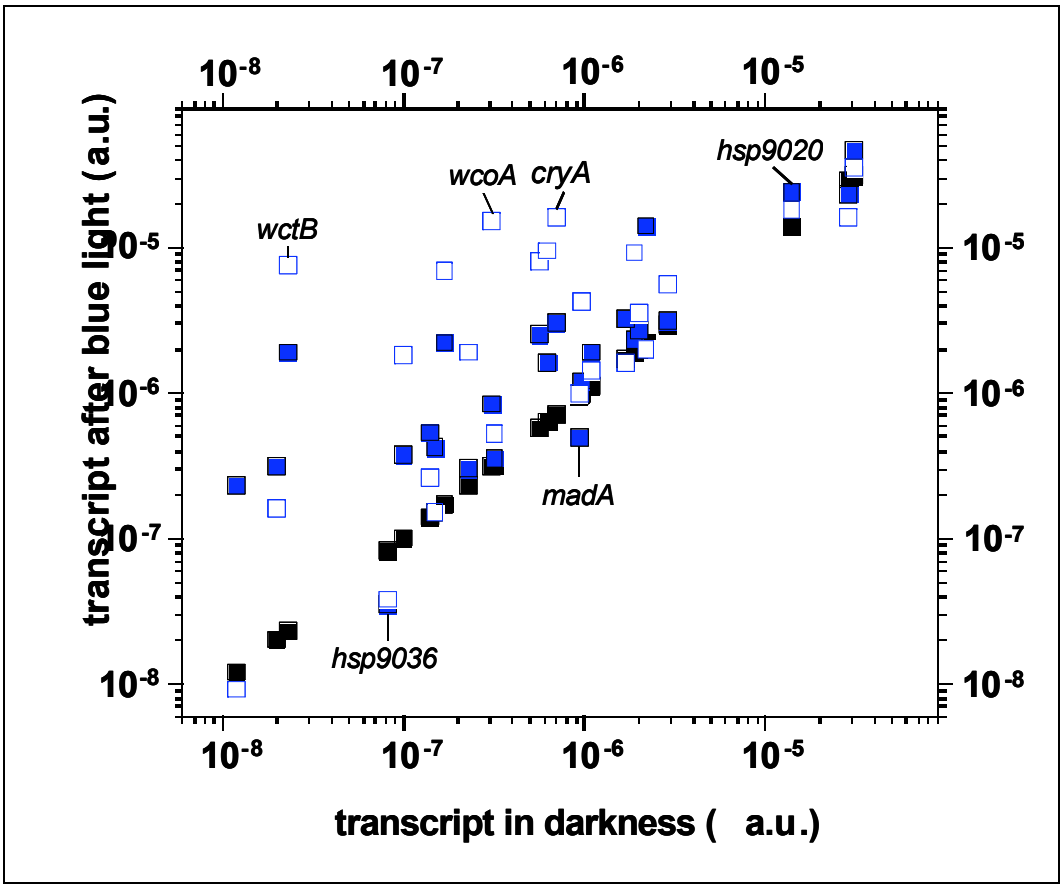


Fig. 3.2.2.1: Summary of blue light effect on gene expression.

Levels of gene transcript in 70 hours old mycelia of wildtype NRRL1555 (-), plotted against the corresponding expression values in continuous darkness. Gene expression values in dark (■), continuous blue$_{475}$ (16 Wm^{-2}, ■) and 20 minutes after a pulse of blue$_{475}$ light (40 Jm^{-2}, 25 s, □). mRNA levels measured by qRT-PCR.

The genes *act, ago, asf* and *dcr* as well as *madB* show weak effects in response to blue light. Their expression is less than twice enhanced in continuous or after a pulse of blue light. There are only a few genes like *ngf, hat50* and *hat10* that show enhanced expression in continuous blue but not after a pulse of blue light. Their induction by light is also moderate with the exception of the 20-fold enhanced expression value of the *hat10* gene. The expression of the genes *carB, hspA* and *hsp90* are not effected by continuous blue light but after a pulse of blue light their expression is moderately around five to eight fold enhanced. The rest of the analyzed genes show increased amounts of transcript for both continuous and a pulse of blue light. The genes *carRA, wcoB, wctC, cryA* and *wcoA* are with four- to fivefold moderately enhanced in continuous blue light. A pulse of blue light can

enhance the expression of the genes *carRA* and *wcoB* 14 times, of *wctC* 18, *cryA* 23 and *wcoA* about 50 times. The genes *wctD* and wctB show with 14- and 83-fold respectively, increased expression values in continuous blue light, and a 40- and 325-fold enhancement after a pulse of blue light. The increase of expression levels after a pulse of blue light is three to sixfold with respect to their expression levels under continuous blue light, with the exception of the 16-fold increase of the *wcoA* mRNA. The gene *myc* is the only one which is twice as strongly (16-fold) expressed in continuous blue light than after a pulse of it (8-fold). In general, the photoinducibility of gene expression is higher after a pulse of blue light than in continuous light. The gene expression constantly and transiently increases 20 minutes after a pulse of blue light as shown for the moderately induced genes *hsp90* and *carB* in Fig. 3.2.5.2 and Fig. 3.2.5.4 and the strongly induced *cryA* and *wcoA* genes in Fig. 3.2.5.3 on page 61. The amount of mRNA of these genes, 80 minutes after a pulse of blue light, is on the level of dark grown mycelia independent of how strong the enhancement was. The transcript level of the *madA* gene is most pronounced with a six fold photosuppression 80 minutes after a pulse of blue light (Fig. 3.2.5.4, p. 62).

As shown above, more than half of the investigated genes are strongly photoregulated. As an empirical rule, the increase of transcript after blue light exposure is higher, the lower the dark expression level is, and none of the photoinduced genes show higher mRNA levels after blue light exposure than the housekeeping genes *act* or *tub*. It is remarkable that the detected range of transcript amounts in both, dark grown or blue light treated mycelia is within 10^{-9}-10^{-5} (a.u.), based on the 2 µg total RNA for the cDNA-synthesis (0.08 µg /qRT-PCR reaction).

3.2.3 Gene expression after red light exposure

Previous studies in *P. blakesleeanus* mainly examined the effects of blue light on morphological development, secondary metabolism, and tropical behavior and on the molecular level the enhanced expression of several genes. Less attention was paid to red light, due to the absence of clear effects and maybe supported by early results showing extremely lowered quantum effectiveness in red light of about 3 x 10^{-8} compared to that at 450 nm (Delbrück et al., 1976). Nevertheless, several observations of red light effects in *P. blakesleeanus* accumulated over the last decades. To test if red light (> 600 nm, continuous or a pulse of it) has any effect

on the photoregulation of gene expression in mycelia of *P. blakesleeanus* wildtype, four different experimental conditions were chosen.

The four different experimental conditions of red light treatment include the growth of mycelia for 70 hours in continuous red_{630} light (15 Wm^{-2}, 630 nm) and darkness. The protocol for a pulse of blue light was also used with red light by harvesting 70 hours old dark grown mycelia 20 minutes after a pulse of red_{630} light (40 Jm^{-2}, 25 s). In a third experiment the photon fluence dependency on gene expression was observed after a 10-fold lowered and more then 20 fold higher pulse of red light (0.4 and 950 Jm^{-2}, 25 and 250 s, respectively) in 70 hours dark grown mycelia. To investigate the effect of red light on gene transcription in 70 hours old dark grown mycelia in more detail, the time dependent red light effect on gene expression levels were examined by harvesting five, ten and twenty minutes after a pulse of red_{630} light (40 Jm^{-2}, 25 s). The transcription levels of different genes were obtained by qRT-PCR.

A summary of gene expression levels of all analyzed genes (Tab. 2.5.1, p. 30) in mycelia of wildtype grown in darkness, continuous red-light and 20 minutes after a pulse of red light is given in Fig. 3.2.3.1. The expression levels after continuous red light or a pulse of red light are plotted against the corresponding values of mycelia grown in complete darkness.

For most genes the expression level after red-light treatment is close to the control value, over the entire rage from 10^{-8}-10^{-5} (a.u.) or for all genes from *hat10* to *act*, resulting in a diagonal distribution of the datapoints. In comparison to the gene expression pattern after blue light exposure only few genes show strong responses to the red light treatment. Among these genes are mainly the members of the heat shock family *hsp90*, the predicted transcription factor *myc* and the *wc-2* type photoreceptor genes *wctB* and *wctC* (Fig. 3.2.3.2; Fig. 3.2.3.2).

Upon continuous red light the member of the heat shock family *hsp9020* gene, the *wct-2* type photoreceptor gene *wctB* and the transcription factor gene *myc* show an increased expression in mycelia of wildtype of about three times against the dark grown. Around 50 % higher expression values were detected for the housekeeping gene *act* and for the gene *carRA*, more than 30 % for the genes *madA*, *madB, wcoB, carB* and *hat50* and around 20 % for *ago, wctC, ngf* and *wcoA*. Interestingly, an equilibrated proportion of up and down regulation was detected. The *tub* gene shows 30 %, the genes *hsp90* and *hsp901-3* 20 % and the genes asf, *wctC, hat10, dcr, cryA and hspA* around or less than 10 % lowered transcript values against dark (Fig. 3.2.3.1 and Fig. 3.2.3.2).

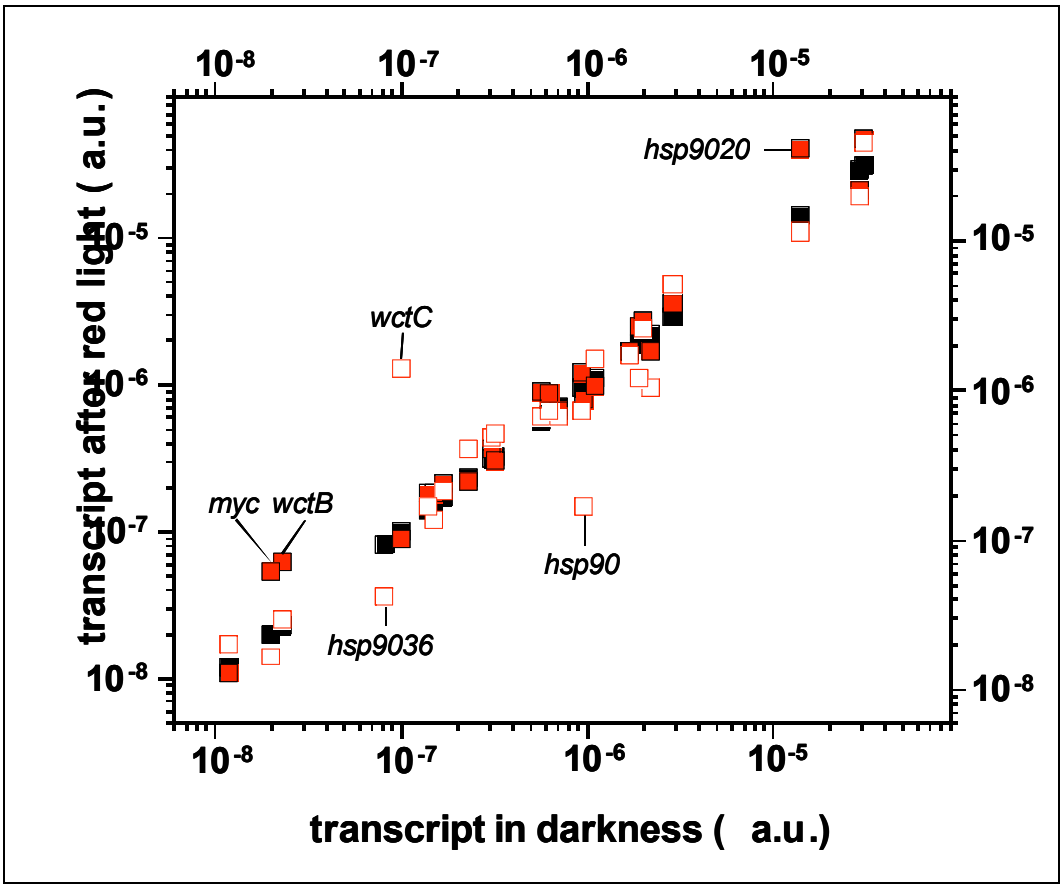


Fig. 3.2.3.1: Summary of red light effect on gene expression.
Levels of gene transcript in 70 hours old mycelia of wildtype NRRL1555 (-), plotted against the corresponding expression values of continuous darkness. Gene expression values in dark (■), continuous red_{630} light (15 Wm^{-2}, ■) and 20 minutes after a pulse of red_{630} light (40 Jm^{-2}, 25 s, □). Transcript values were examined by qRT-PCR.

In general the positive effect of a pulse of red light has less strength in comparison to the blue light response except for the thirteen fold increase of the *wctC* gene transcript. The genes *ago* and *hspA* show 60 % and *hat10, wcoA, dcr*, asf and *act* 40 % enhanced amounts of mRNA. All *wc-2* genes are between 10 and 20 % induced after a pulse of red light. On the other hand, the strongest reduction of expression values was observed by the members of the heat shock family, 7-fold for the gene *hsp90* and 55 % for *hsp901-3* and *hsp9036*. The genes *tub*, *carB, madA, myc* show between 30-40 % reduced amounts of mRNA and the genes *hat53, cryA, ngf* and hsp9020 are less than 10-20 % expressed. The positive enhancement and the inhibitory effect of red light on the regulation of gene expression is numerically the same. The strongest red light dependent changes on gene expression are pointed out

in Fig. 3.2.3.2.B. Due to the slight effect of red light exposure on gene transcription of most of the other genes; the average of expression values was normalized against the corresponding average values from mycelia grown in continuous darkness as shown in Fig. S.3.2.2.1 and Fig. S.3.2.3.1 (Appendix B, p. 4).

The moderate effects on the gene expression pattern after red light exposure reveal a remarkable numerical balance of induced and suppressed genes. Interestingly both effects can occur in the same gene depending on the light treatment. After blue light treatment only three genes show a reverse effect of reduction in their levels of mRNA after a pulse of actinic blue light and an increase after continuous light exposure compared to the one of darkness. The *madA* gene shows the opposite reverse effect. Ten genes show a reverse expression pattern after red light treatment. The genes *hsp9020*, *carB*, *madA*, *ngf* and *myc* are photoinduced by continuous, but suppressed 20 minutes after a pulse of red light. The opposite pattern was detected for the genes *asf*, *dcr*, *hspA*, *wctC* and *hat10.* They all are between 10 and 20 % less transcribed after continuous, but show differing expression values after a pulse of red light against the one grown in dark. This reverse expression pattern can not be observed after a 0.4 and 950 Jm^{-2} pulse of red light (Fig. 3.2.3.3).

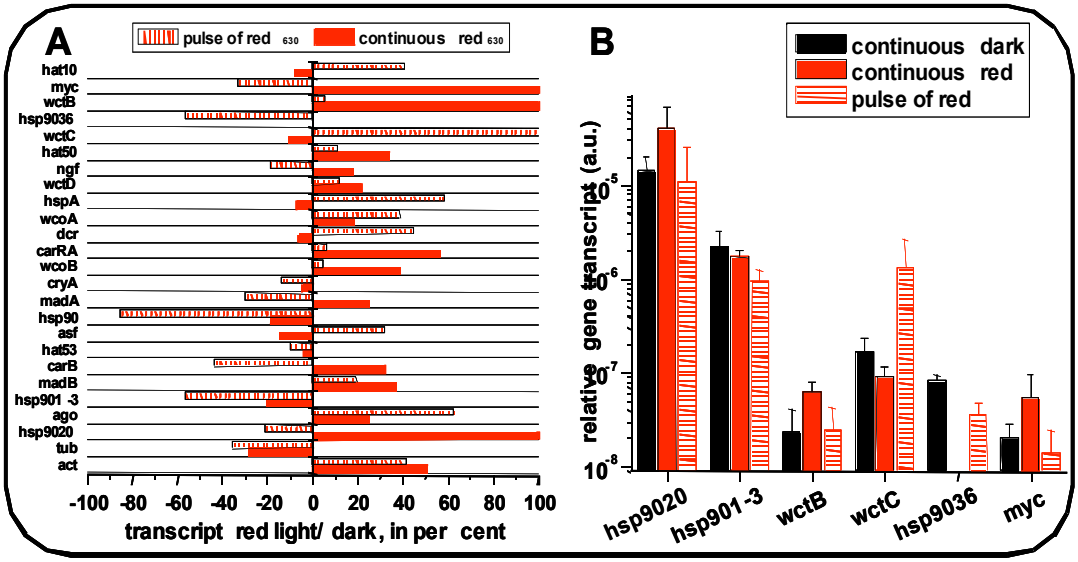


Fig. 3.2.3.2: Gene expression after continuous and a pulse of red_{630} light.

Gene transcript in 70 hour old mycelia of wildtype NRRL1555 (-) grown in continuous darkness, continuous red_{630} light (15 Wm^{-2}) and 20 minutes after a pulse of red_{630} light (40 Jm^{-2}, 25 s). Mycelia were harvested by freezing in liquid nitrogen. A. Gene transcript after red light exposure normalized against the corresponding expression values for dark-grown samples. B. Gene expression levels measured by qRT-PCR (a.u.).

Changing the photon fluence of the red light by a pulse of 0.4 Jm^{-2} or 950 Jm^{-2} all genes show less amount of mRNA compared to the one of dark with the exception of three genes (Fig. 3.2.3.3). The red light-dependent strength of the suppression of gene expression can be taken as a criterion to categorize the genes. Nine genes, all of the *wc-2* type genes, the *madA, hat50, carB, wcoA, and hsp9020* genes show the strongest reduction after a pulse of red light at the highest photon fluency used. Among these genes *hsp9020* and *carB* are the strongest suppressed (22-fold), the gene *wctD* sevenfold, the gene *hat50* sixfold and the two to fourfold the gene *act* and the other *wc-2* type genes.

Four genes, the *hsp90, hsp901-3, hsp9036* and *myc* are most strongly suppressed after a 40 Jm^{-2} pulse of red light as described before. There are six genes which show the strongest reduction of their transcript after a pulse of red light with the lowest given photo fluency. Their reduction is not as pronounced, twofold for the genes *ngf* and *hat53,* threefold for the gene *wcoB* and fourfold for the genes *ago* and *dcr* but fivefold for the gene *tub*. Examples of different expression patterns are given in Fig. 3.2.3.3.A whereas some are pointed out in Fig. 3.2.3.3.B.

Most of the genes show an expression pattern similar to the one of the *madB* gene, where 20 minutes after a 40 Jm^{-2} pulse of red light the transcript is enhanced, but after a pulse of both other photon fluencies reduced. The gradual reduction of the *carB* and *cryA* transcript with the strongest photosuppression after the highest given red fluency is not an exception. About 40 % of the genes show also the strongest reduction of their transcript after the highest applied red-light intensity. The gene *madA* shows an exceptional expression pattern. The *madA* gene transcription is suppressed after a 0.4 and 40 Jm^{-2} pulse of red light equally but strongest after a 950 Jm^{-2} pulse of red light. Another exception is the expression pattern of the gene *hsp901-3*, which shows a three and fourfold positively enhanced amount of transcript after a pulse of 0.4 and 950 Jm^{-2} red light but twice reduced amount after a 40 Jm^{-2} pulse of red light. The *wcoA* gene shows enhanced amounts of its transcript after all red light treatments, but the strongest induction of 133 % after the lowest given photon fluency (0.4 Jm^{-2}) as well as the *asf* gene (60 %). Among fourteen genes which show an elevated amount of transcript after the pulse of 40 Jm^{-2} red light are nine with a clear revers expression pattern because the amount of their transcript after a pulse of the other red light intensities is reduced. The reduction of the gene transcript is in some cases almost as strong as the increase after the blue light treatment and strongest after the highest given red light fluence (950 Jm^{-2}).

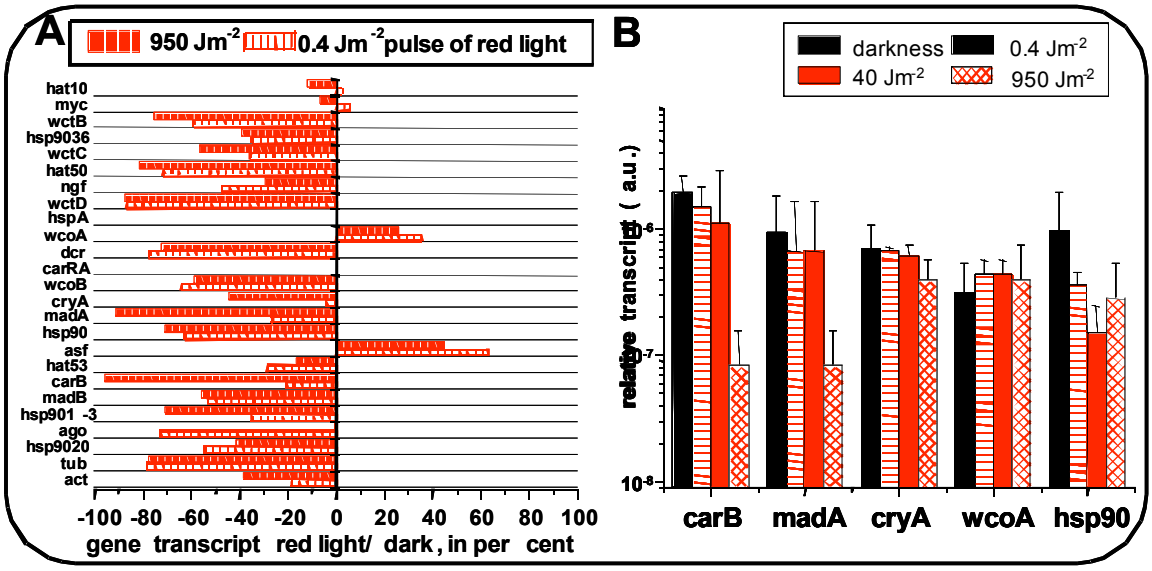


Fig. 3.2.3.3: Gene expression after a pulse of different photon fluencies of red$_{630}$ light. Gene expression level in 70 hours old mycelia of wildtype NRRL1555 (-) grown in continuous darkness and 20 minutes after a pulse of red light (0.4, 40 and 950 Jm^{-2}, 25 and 250 s, respectively). Mycelia were harvested by freezing in liquid nitrogen. Gene expression levels measured by qRT-PCR (a.u.). A. Gene transcript after red light exposure normalized against the corresponding expression values for dark. B. Gene expression level measured by qRT-PCR [a.u.].

As shown in the previous chapter, gene expression constantly and transiently increases after a pulse of blue light. For the genes *hsp90, madA, cryA, wcoA* and *carB,* the time dependency of their expression was measured five, ten and twenty minutes after a red light pulse of 40 Jm^{-2} (25 s). The resulting expression patterns of these genes are shown in Fig. 3.2.5.2 to Fig. 3.2.5.4 (pp. 58 – 64). The responses to a 40 Jm^{-2} (25 s) pulse of red light after five, ten and twenty minutes underline the tendency of suppression of the expression. A moderate and constant reduction of transcription by 30 – 40 % was measured for the *madA* gene and about 20 % for the *cryA* gene. With a reduction of about 40 % after five minutes, the *wcoA* gene is stronger reduced but its amount of mRNA increases up to 20 and 40 % compared to the dark control within ten and twenty minutes. The strongest suppression was detected for the amount of *hsp90* mRNA which is reduced about four, two and seven times after five, ten, and twenty minutes, respectively (Fig. 3.2.5.2). The *carB* gene also shows an alternating pattern of stronger and weaker suppression of transcript amounts. Additionally it shows a revers effect with a sixfold reduction after five minutes and then close to twice the increase after ten minutes followed by

a twofold reduction of its mRNA after twenty minutes against the dark value (Fig. 3.2.5.4).

The results demonstrate a substantial effect of red light on the gene transcription in *P. blakesleeanus* mainly by modulation of the expression pattern. This modulation includes gene specific revers changes of the amount of mRNA. Its complexity is given by the dynamic dependency on the quality and quantity of the light treatment and also by the moment of observation. The effects of red light are generally not as homogenous and strong as the ones observed after blue light treatment.

3.2.4 Gene expression after bichromatic blue and red light exposure

In the previous two chapters the effects of blue and red light on gene expression in *P. blakesleeanus* were presented. The results raise the question of the type of interaction between red and blue light on gene expression. As shown, the red light modulate moderates and reversely whereas the blue light mainly induces the gene transcription. In the following the interaction of both light qualities should be studied in more detail by bichromatic red and blue light exposure. The previous protocols for the light treatments were taken as a means to an end. To compare the light dependent changes on gene expression or their pattern the mycelia of wildtype were raised for 70 hours in continuous dark or under bichromatic red_{630} and $blue_{475}$ light with 15 Wm^{-2} or harvested 20 minutes after a pulse of bichromatic red and blue light (40 Jm^{-2}, 25 s).

The results of the bichromatic red-blue light exposure are summarized in Fig. 3.2.4.1, where the expression levels after the light exposure are plotted against the corresponding values of mycelia grown in continuous darkness. Surprisingly the datapoints reveal a pattern closer to the one of blue light than red light treatment. None of the prominent red light dependent transcriptional changes, e.g. the three times promoted expression of *hsp9020, myc* or *wctB* after continuous red light exposure, nor the suppressed expression of the genes *hsp90* or *hsp9036* after a pulse of red light, occur after the bichromatic light treatment. The increased amount of *wctC* transcript is somehow constant after a pulse of light, 13-fold after a pulse of red, 18-fold after a pulse of blue and 15-fold after a pulse of bichromatic red and blue light.

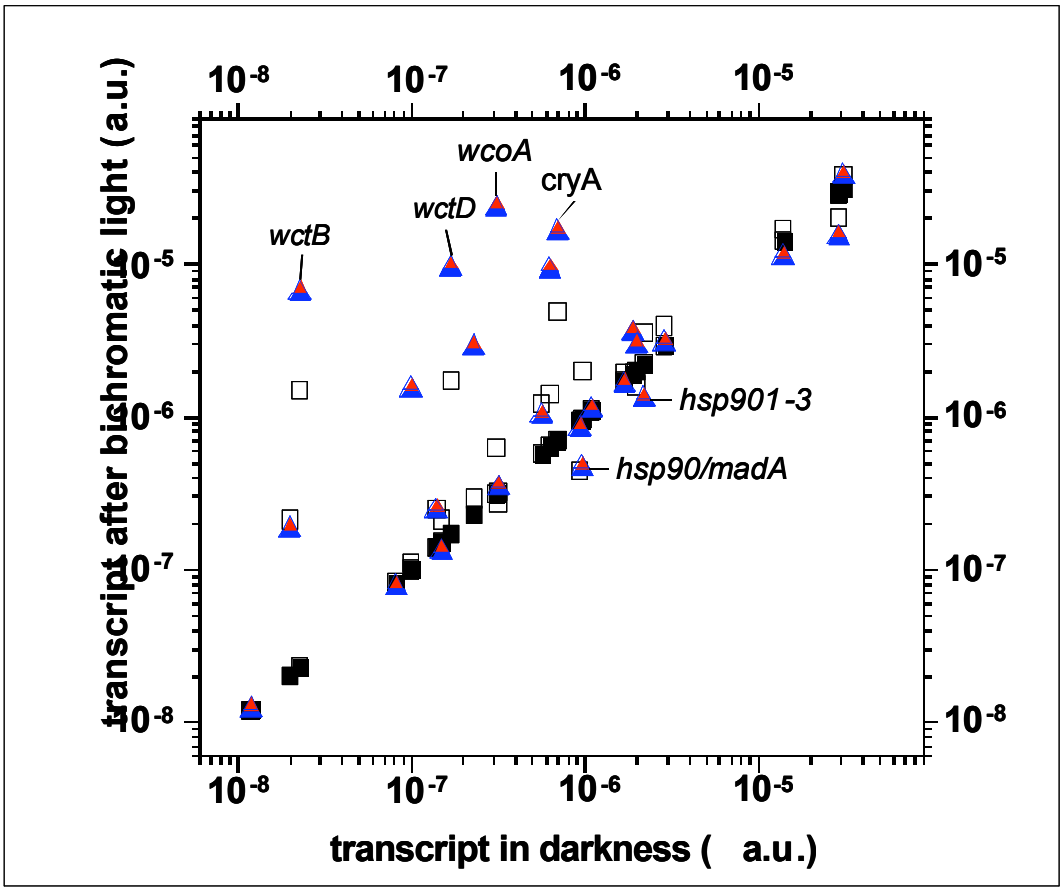


Fig. 3.2.4.1: Summary of bichromatic red and blue light effect on gene expression.
Levels of gene expression in 70 hours old mycelia of wildtype NRRL1555 (-), plotted against the corresponding expression values in continuous darkness. Gene expression values raised in dark (■), continuous bichromatic red_{630} and $blue_{475}$ light (15 Wm^{-2}, □) and 20 minutes after a pulse of bichromatic red and blue light (40 Jm^{-2}, 25 s, ▲). mRNA levels measured by qRT-PCR (a.u.).

On the other hand the strongest blue light induced genes show a very similar response to the bichromatic red and blue light irradiations. The transcription of the *wctB* gene shows a 65-fold increased amount of its mRNA after continuous bichromatic light exposure which is a decrease of 20 % compared to the one after only blue light. The genes *myc* and *wctD* are about ten times and the *cryA* sevenfold stronger transcribed compared to the dark control. Around two times photoinduced are the genes *hsp90*, *carRA, hat50* and the *wc-1* type photoreceptor genes *wcoA* and *wcoB*. The *madA* gene transcript is twice reduced similar to the *hspA* gene. With 30 % reduced amount of the *tub* mRNA and about 20 % reduced amounts of transcript of the genes *dcr* and *madB* they show a less pronounced suppression.

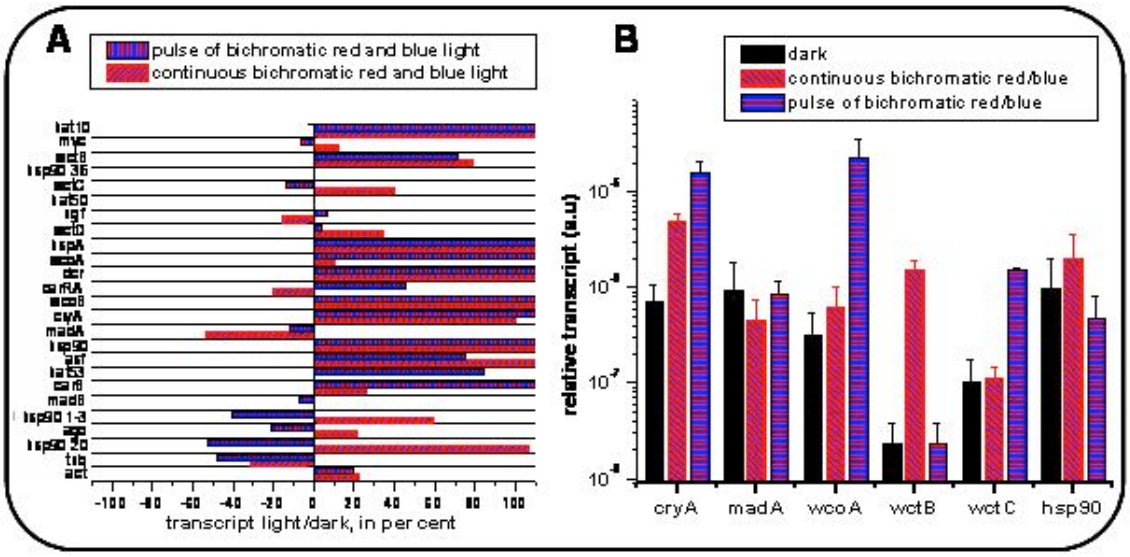


Fig. 3.2.4.2: Gene expression after continuous and a pulse of bichromatic light.
Gene transcript in 70 hours old mycelia of wildtype NRRL1555 (-) grown in continuous darkness, continuous bichromatic $blue_{475}$ and red_{630} light (15 Wm^{-2}) and 20 minutes after a pulse of bichromatic light (40 Jm^{-2}, 25 s). Mycelia were harvested by freezing in liquid nitrogen. A. Gene transcript after blue and red light exposure normalized against the corresponding expression values for dark-grown samples. B. Gene expression level measured by qRT-PCR (a.u.).

The gene expression is also affected by a pulse of bichromatic light (Fig. 3.2.4.2). In general the photoreceptor genes show a strong photoinduction of their transcription except for the *madA* gene which is slightly suppressed (10 %) and *madB* gene which is only 36 % enhanced. The *wctB* gene shows the highest photoinduction with a 280-fold increase. Similar to its elevated expression upon continuous bichromatic irradiations the photoactivation after simultaneous red and blue light is 25 % less pronounced than after a pulse of blue light alone. But still, for both light treatments it is the highest detected photoenhancement among all analyzed genes. The *wcoA* gene with a 75-fold and the *wctD* with a 55-fold increase of their transcript show a strong photoactivation compared to the dark control. The genes *wcoB* and *wctC* show a 15 times higher amount of their transcript. The *cryA* gene is with a 22-fold enhancement equally expressed than after a pulse of blue light, whereas the *hspA* gene is with a 12-fold increased amount of its transcript about 40 % enhanced but the gene *myc* is with a an ten-fold increased amount of transcript compared to the dark control about 50 % less transcribed than after a pulse of only blue light. The *carB* and *carRA* genes show both less than twofold photoinduction

which is equal to 40 and 60 % of their blue light photoactivation. The genes *act, ago* and *dcr* are less than 20 % photoinduced. A down regulation was observed for the housekeeping gene *tub* and for the members of the heat shock family *hsp90* and for the *hsp9036*, *asf* and *hst53* less than 10 %. The genes *hsp9020, hsp901-3, hsp90, hat53* and *ngf* show a reverse effect of increased amounts of transcript after continuous and reduced ones after a pulse of bichromatic light exposure in comparison to their amounts of dark-grown mycelia. The genes *madB* and *dcr* show the opposite reverse effect with increased amounts after continuous and reduce amounts after a pulse of bichromatic light exposure compared to the corresponding ones of dark-grown mycelia. In total there are more genes up than down regulated after simultaneous red and blue light exposure in comparison to their expression in dark. The highest expression is always within 10^{-5} (a.u.), the same as after the exposure to blue light.

The gene expression after bichromatic irradiations shows notable differences in comparison to each, blue and red light treatment. One of them is that the photoactivation after a pulse of bichromatic light treatment is for some genes (*hspA, wcoA* and *wctD*) stronger but for most of the genes less pronounced compared to the one after blue light exposure. In other words, the red light can cause both a synergistic transcriptional enhancement but also an antagonistic effect by a suppression of the blue light promoted effect on gene transcription. Another difference is that more genes show a reverse expression pattern than after only blue light but this is still less compared to the red light exposure. Among 22 blue light responsive genes are 14 genes affected by bichromatic red and blue light treatments. The photoinduction of the *hat10* gene expression show an exceptional 20-fold and the one of the hsp901-3 a sixfold increase of their transcript only after continuous blue light but not after the bichromatic irradiations. Furthermore upon continuous bichromatic irradiations less genes show an enhancement of their expression level compared to the ones after blue irradiations. Among them are the *wctC* gene as well as the above referred genes *hat10* and *hsp901-3*. Most interestingly the *cryA* gene shows a transcriptional suppression only after continuous bichromatic irradiations in comparison to the blue light exposure whereas its transcription is equal after the pulses of both blue light alone as well as bichromatic irradiations. These results manifestly show a synergistic as well as antagonistic interaction of blue and red light suggesting a multichromophoric as well as multimeric photoreceptor complex for the transcriptional control.

3.2.5 Gene expression after a double pulse of blue-red or red-blue light

The obtained results on gene expression after bichromatic irradiation opened a new field of investigation. The differential effect of red and blue light on the gene expression in *P. blakesleeanus* can be studied in more detail by double pulse experiments. To test how blue and red light affect the photoregulation of gene expression in mycelia of *P. blakesleeanus* wildtype, three different experimental conditions were chosen. 70 hour dark-grown mycelia were harvested 20 minutes after the pulses. The double pulses consist in a 40 Jm^{-2} pulse of $blue_{475}$ light (25 s) followed immediately by a 40 Jm^{-2} pulse of red_{630} light (25 s). The same conditions apply with an alternate sequence of red followed by blue light. As shown in the previous chapters, gene expression constantly and transiently increases after a pulse of blue light but not after red light exposure. For the genes *hsp90, madA, cryA, wcoA* and *carB,* the time dependency of their expression was measured five, ten and twenty minutes after the double pulses of red and blue. In a third experiment the effect of $green_{545}$ and $blue_{475}$ double pulses on gene transcription was studied.

A summery of gene expression 20 minutes after a double pulse of blue and red light is given in Fig. 3.2.5.1. The expression levels are plotted against the corresponding values obtained from mycelia grown in continuous darkness. The resulting pattern shows a strong photoactivated transcription like after a pulse of blue light but also an increased suppression compared with the previous light treatments. When a pulse of blue light is followed by red light the induction of gene transcription is more pronounced than after a pulse with the opposite order (red-blue). The *wcoA* gene is 60-fold up regulated, the *cryA* gene 22-fold and *wctD* 11-fold after a double pulse of blue followed by red light. The *carB* transcript is seven and the *hspA* mRNA is five times increased. The gene transcript of *myc*, *dcr* and *ago* is around three times and *madA* twice elevated. A 22-fold reduced amount of transcript against the dark control was measured for the *hsp9020* gene and around three times less for all the other *hsp90* genes and *ngf* as well. The *hat10*, *carRA* and *hat53* genes show about 50-60 % reduced gene transcript. The genes *tub*, *asf* and *wctC* show a less than 10 % reduced amount of mRNA against the dark control. After a double pulse of red then blue light the *wctD* gene is 40 and *wcoA* 33 times higher transcribed in comparison to the dark-grown mycelia. The *cryA* gene is tenfold up regulated, the *myc* gene fivefold and *hspA, carB* and *ago* show a two and a half fold increased transcription. The mRNA of *hsp9036* is 70 %, *wctC* 50 %, *hat50* and *dcr* are around 15 % enhanced. The genes *hsp90* and *hsp901-3* are three

times, *tub* and *ngf* twice reduced in expression. The transcription of the genes *hat10* and *madA* is about 40 %, *hat53* 25 % suppressed.

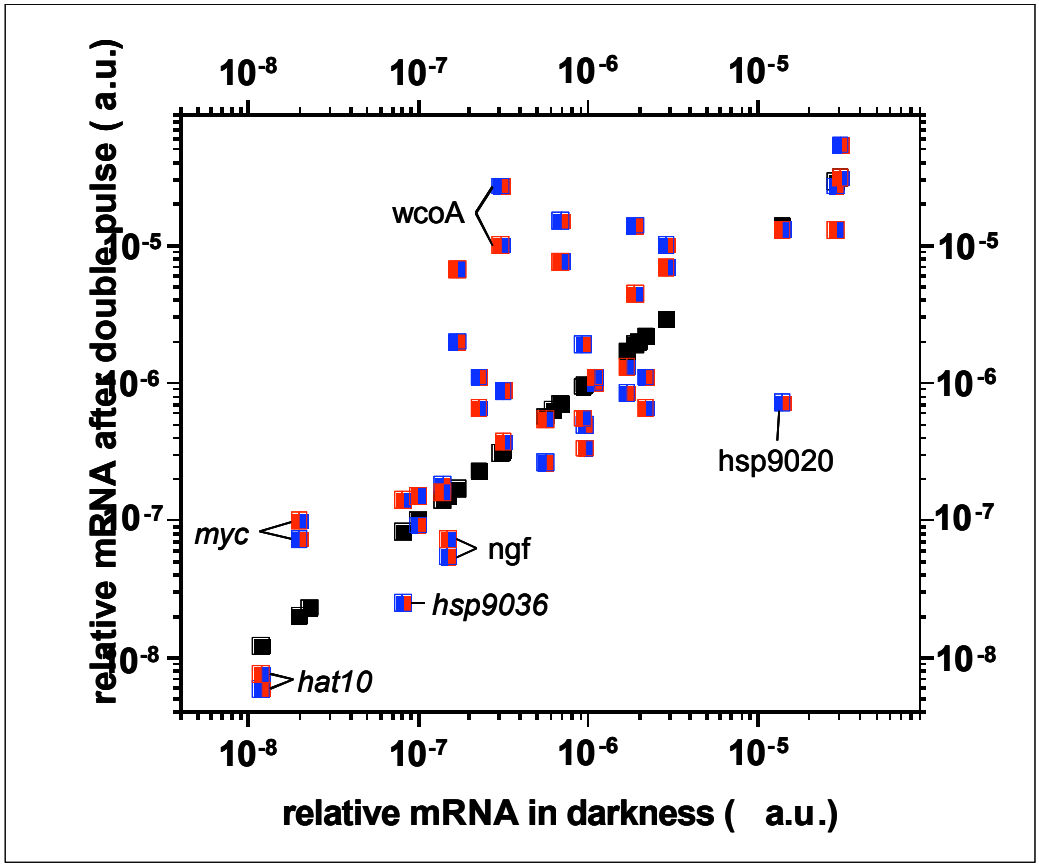


Fig. 3.2.5.1: Summary of gene expression pattern after a double pulse of red-blue light. Gene transcript in 70 hours old mycelia of wildtype NRRL1555 (-) plotted against the corresponding expression values in continuous darkness. Gene expression levels in dark (■), 20 minutes after a double pulse of red_{630} then $blue_{475}$ (■) or blue then red light (■) (40 Jm^{-2}, 25 s). Transcript levels measured by qRT-PCR (a.u.).

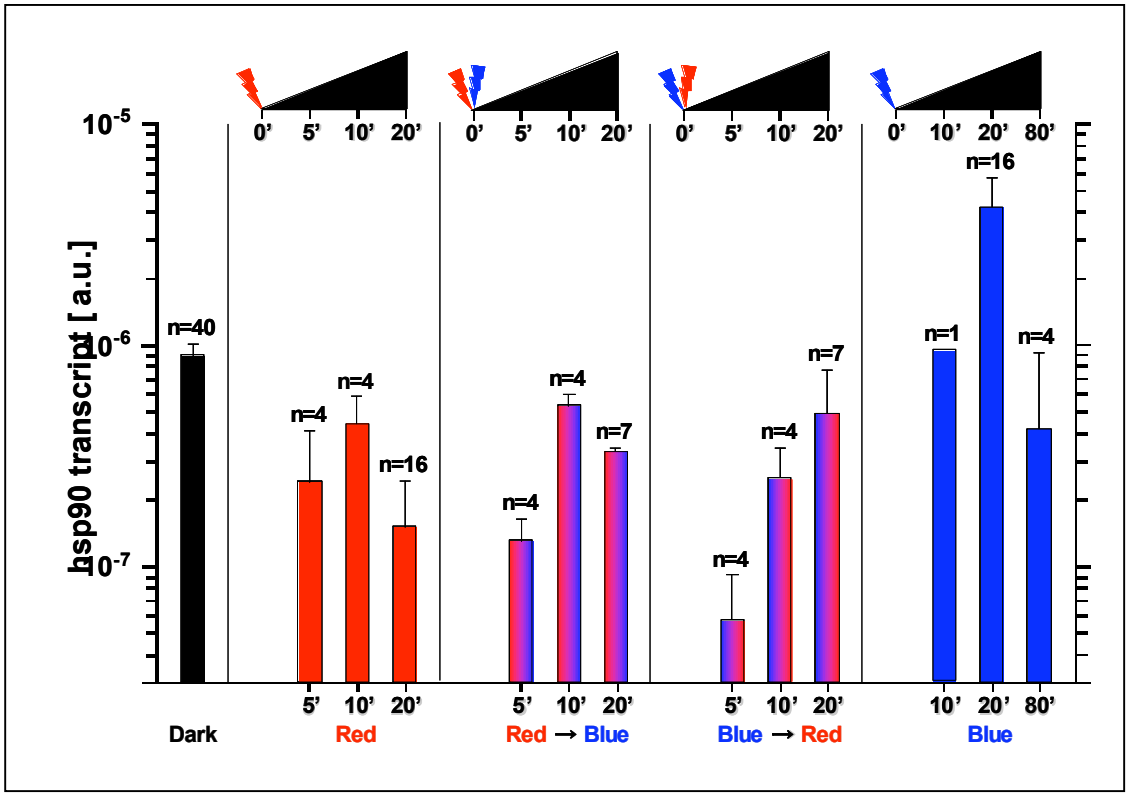


Fig. 3.2.5.2: Time dependent *hsp90* expression after a pulse of light (40 Jm^{-2})

Mycelia of wildtype NRRL1555 (-) were grown in continuous darkness for 70 hours. Light treatments consisted in a 25 s pulse of red_{630} or $blue_{475}$ light, a double pulse red-blue or blue then red light. Mycelia were kept in darkness for indicated times (5, 10, 20 or 80 minutes) before the harvest by freezing in liquid nitrogen. Transcript levels in which were measured by qRT-PCR. Plots show the average and SD of indicated n.

The gene *hsp9036* and *madA* show a photoreversible expression pattern twenty minutes after the double pulses of light. The *hsp9036* gene is after the pulse of blue-red light three times suppressed but twice promoted after a pulse of red-blue light against the dark one. The transcription of the *madA* gene shows the opposite pattern with an enhanced expression while blue light was given first and a 40 % reduced transcription when the double pulses were given reversely. An alternating revers expression pattern was found for the photoregulation of the *carB* gene five, ten and twenty minutes after a pulse of red light with a six fold reduced, two times increased and again two fold reduced amount of its transcript against the one of darkness (Fig. 3.2.5.4). The time dependent expression of the *carB* gene after a pulse of red-blue light is two and three times increased compared to the one of dark-grown mycelia.

During the first five minutes after the pulses of light it reaches a more or less stable expression level measured ten and twenty minutes after the light treatments.

This is a unique pattern which no other gene shows. By changing the order of light pulses the expression is five minutes later fivefold suppressed compared to the dark-grown mycelia, after ten minutes twice but twenty minutes later seven fold enhanced. The reduction of the amount of gene transcript after a double pulse of blue-red light and a gradual increase up to a higher expression level than after a pulse of blue light alone is also found in other genes. In general, all analyzed genes show the same expression pattern after a double pulse of blue followed by red light. The lowest expression levels were detected five minutes after the pulses. The transcription enhanced gradually and twenty minutes after the pulses reached its maximum, the highest detected expression levels measured during the period of observation. The time dependent expression pattern for all genes after a double pulse of red followed by blue light shows also the lowest expression levels first (5 minutes), but the maximum is reached earlier, after ten minutes. During the following ten minutes (up to 20 minutes) the expression can be reduced or be the same than after ten minutes.

Five minutes after the pulses of blue then red light the amount of transcript of the *hsp90* gene is 17 times and for the *madA* gene six fold reduced. The *cryA* gene is eight times and the *wcoA* gene 15 % enhanced against the corresponding one of the dark-grown mycelia. The following gradual enhancement of their transcript is different in strength. Ten minutes after the pulses the gene transcript of *hsp90* is four times and madA three times reduced. The amount of *cryA* mRNA is 12-fold and the one of *wcoA* sixfold enhanced. Twenty minutes after the double pulse the *hsp90* gene shows a twofold reduction and the *madA* gene a twofold enhanced amount of their transcript compared with the levels of dark-grown mycelia. The enhancement of mRNA was 20- and 85-fold for the *cryA* and *wcoA* gene.

The expression pattern after the double pulse of red then blue light shows for the *hsp90, cryA* and *madA* gene first, five minutes after the pulses a reduction of their transcript, followed by an enhancement with a maximum ten minutes after the pulses. The expression level of *hsp90* is among these genes with above sevenfold most strongly reduced; *cryA* shows a 50 % and *madA* a 12 % reduction against their corresponding dark levels. Ten minutes after the pulses the transcriptional maximum for the *hsp90* gene is reached at twofold, the lowest, reduced amount of mRNA against the one of darkness. The *cryA* gene shows a 15 times and the *madA* gene a 60 % increase and with this the highest expression levels during the time course. Twenty minutes after the double pulses the amount of transcript of the *hsp90* gene is three times and *madA* gene 40 % reduced and the *cryA* gene tenfold increased

against their corresponding dark ones. The *wcoA* gene shows a different pattern of the time dependent transcription with an early and fast increase (7-fold) five minutes and 30-fold ten minutes after the double pulse of red then blue light. Twenty minutes after these pulses the 30-fold elevated amount of *wcoA* gene transcript was also measured (Fig. 3.2.5.3).

The time dependent gene expression after a double pulse where red light was given first followed by a pulse of blue light shows for all genes higher amounts of their transcript after five and ten minutes, but lower ones after twenty minutes in comparison to the double pulses of blue, followed by red light.

Comparing the expression values five, ten and twenty minutes after a double pulse of red, then blue light with the double pulse where blue light is followed by red light, the *carB* gene shows the strongest difference in its expression levels. Thirteen-fold and seven fold increased and threefold reduced after five, ten and twenty minutes. The difference in the amount of *wcoA* mRNA is a six- and fivefold increase and threefold decrease, five, ten and twenty minutes after the pulses. The transcription level of the *madA* gene shows after five minutes a fivefold and after ten minutes a fourfold increase, but after twenty minutes a threefold reduction. For the *hsp90* gene the difference is uniformly twofold. Five minutes after the pulses the cryA gene made an exception with a 17-fold reduced amount of its mRNA. The difference is less then twice after ten and twenty minutes.

The expression of the five analyzed genes show a maximum ten minutes after the double pulses of red followed by blue light. These maxima transcription levels are twice less compared to the ones twenty minutes after the double pulse (blue-red) or a pulse of blue light. The level of *hsp90* gene transcript is an exception which is eightfold reduced compared to the one after a pulse of blue light. The gradually increasing expression levels after the double pulse of blue then red light reach their maximum after twenty minutes. For the *carB*, *wcoA* and *madA* genes these levels are twice as much compared to the one after a pulse of blue light. For the *cryA* gene it is closely the same and only the *hsp90* gene shows an eight fold reduced expression level. Summarizing the time dependent gene expression after a double pulse (blue-red) shows the gradually increasing amount of transcript, the typical pattern for the blue light induced gene transcription, but it starts with a reduction in mRNA amounts. Specifically the expression pattern after a pulse of red light shows for the *carB* and *hsp90* gene an alternating up and down regulation of gene transcript after five, ten and twenty minutes. The same pattern is given after a double pulse red then blue light, also for the *madA* gene.

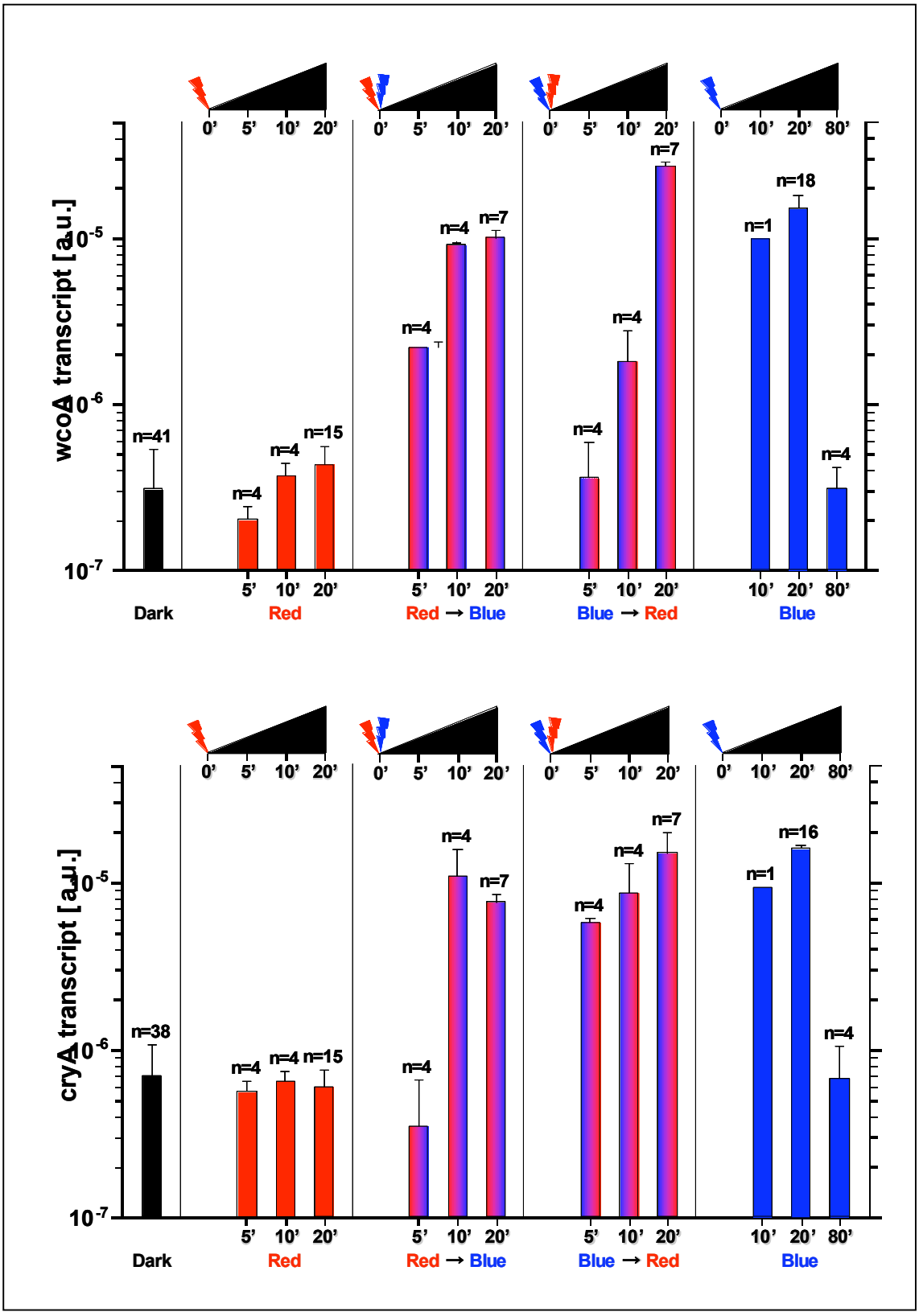


Fig. 3.2.5.3: Time dependent *wcoA* and *cryA* expression after a pulse of light (40 Jm^{-2})

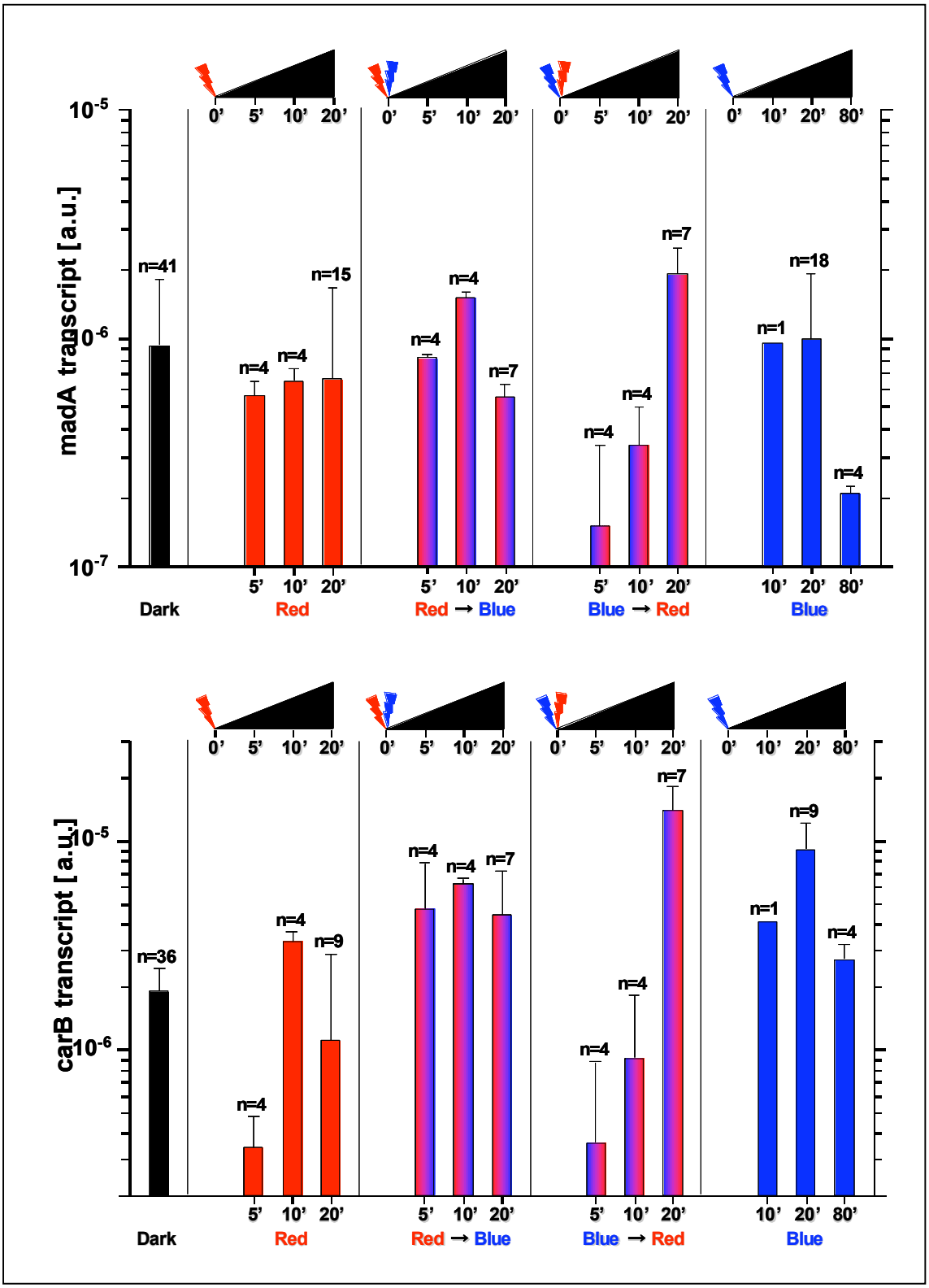


Fig. 3.2.5.4: Time dependent *madA* and *carB* expression after a pulse of light (40 Jm^{-2})

Fig. 3.2.5.3-4: Mycelia of wildtype NRRL1555 (-) were grown in continuous darkness for 70 hours. Light treatments consisted in a 25 s pulse of red_{630} or $blue_{475}$ light, a double pulse red-blue or blue then red light. Mycelia were kept in darkness for indicated times (5, 10, 20 or 80 minutes) before the harvest by freezing in liquid nitrogen. Transcript levels in which were measured by qRT-PCR. Plots show the average and SD of indicated n.

The double pulses of green and blue light have a promoting effect on the transcription of the *cryA* and *wcoA* gene and a suppressing effect on the expression of the *carB* and *madA* gene, independent in which order the different light qualities were given. The suppressing effect is pronounced after the double pulse of green, then blue light. The amount of *carB* transcript is 26 times and the one of the *madA* gene 44 times reduced in comparison to the one in dark-grown mycelia. After the double pulse of blue light followed by green light the *carB* gene transcript is 18 times and the *madA* mRNA 27 times reduced compared to the ones of darkness. The positive effect on the expression of the *cryA* gene shows a 34-fold increase after the pulses of green followed by blue light and 95-fold after the double pulse where blue light was given first followed by green light. The *wcoA* gene also shows an enhancement of its transcription after the pulses but with opposite maxima as for the *cryA* gene. The double pulse of green then blue light induces the *wcoA* gene expression sevenfold. A threefold increase was measured after the pulses of blue followed by green light compared to the one of dark-grown mycelia. The expression pattern of the *hat10* gene shows a photoreversible effect. The amount of *hat10* mRNA after the double pulse green then blue light is five times enhanced but about 20 % reduced after the pulses of first blue light then green light.

After comparing the results of all different light treatments it is obvious that the differential transcriptional regulation strongly depends on the gene of interest. In Fig. 3.2.5.5 some examples are summarized. The gene expression of the *madA* gene shows a red light dependent suppression and only blue followed by red light shows a promoting effect. Both green light treatments result in the same transcriptional reduction. It is tempting to take this as an empirical rule for all genes, red light suppresses and blue light activates the transcription. But the genes *cryA* and *wcoA* disprove that rule. Both genes are after all different irradiations photoactivated whereas red light alone shows the weakest effect. The expression of the *carB* gene is heterogeneous with suppression after a pulse of red as well as both green light treatments and a photoactivation after all other irradiations. The gene *hat10* is suppressed after almost all combinations of different wavelength and only positively

affected by a double pulse of green followed by blue light.

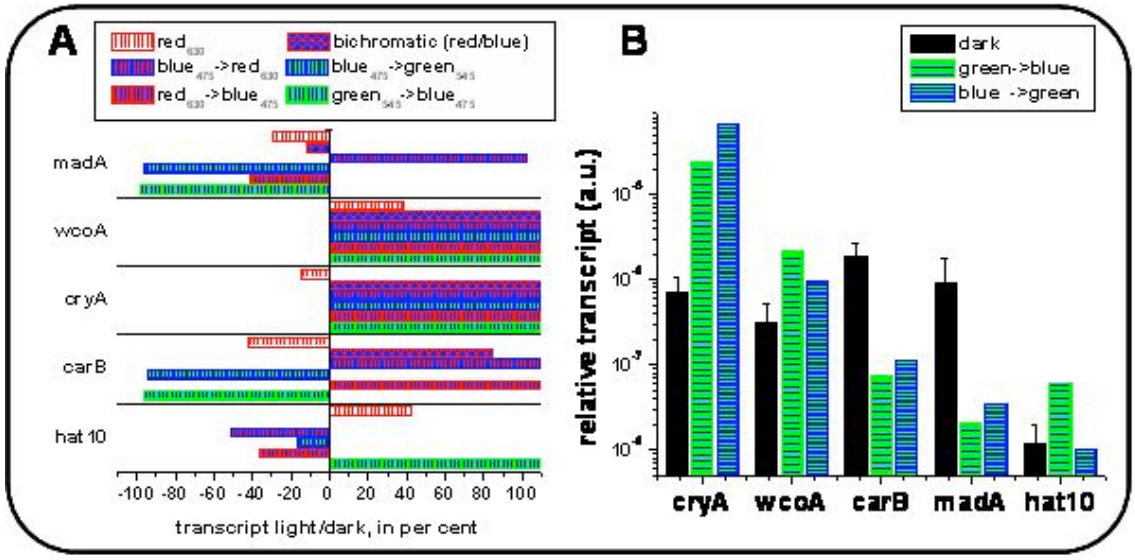


Fig. 3.2.5.5: Gene expression after a double pulse of light (40 Jm^{-2}).
Gene expression level in mycelia of wildtype NRRL1555 (-) grown 70 hours in continuous darkness and 20 minutes after pulses of different wavelength. Mycelia were harvested by freezing in liquid nitrogen. A. Gene transcript after a pulse and double pulse of indicated light qualities. The negative ordinate displays the inhibitory effect of irradiation (dark/light). The positive ordinate displays the promotive effect of irradiation (light/dark). B. Gene expression level after a double pulse (green-blue) and (blue-green) measured by qRT-PCR (a.u.).

3.2.6 Gene expression in different *madA, madB, madC* mutant strains

The induction of gene expression in wildtype of *P. blakesleeanus* after a pulse of blue light, as presented in the previous chapters, shows more than 60 % of the analyzed genes are photoactivated, where a moderate and strong photoactivation could be distinguished. In this chapter the involvement of the *madA, madB* and *madC* mutations in photoinduced gene expression after a pulse of different light qualities will be presented.

The *madA* and *madB* genes encode the WC-1 and WC-2 type blue light photoreceptors/transcription factors which form the MAD complex (Sanz et al., 2009). The molecular structure of the photoresponsive transcription factor WC-1 is characterized by three conserved domains; PAS-domain, LOV-domain, and

C-terminal zinc-finger (see Fig. 1.4.2.1, p. 11). Thus the WC-1 type protein can be structurally defined in a photosensory and a response/regulatory domain like other photoreceptors. The PAS (Per/Arnt/Sim)-domain serves for protein-protein-interaction. The chromophore flavin FAD or FMN is bound in a specific highly conserved protein pocket within the LOV-domain. The C-terminal (GATA) zinc finger (Znf) can bind the corresponding conserved DNA consensus sequences of light-inducible genes like *wcoA* or *hspA* (Idnurm et al., 2006; Rodriguez-Romero & Corrochano, 2006). The molecular structure of WC-2 type photoreceptors is characterized by only the PAS-domain and C-terminal zinc-finger (see Fig. 1.4.2.1, p. 11). Thus a direct transformation of light signals to the photoactivation of gene transcription via the MAD complex can be suspected.

3.2.6.1 Structural analyses of the *mad* mutations

Tab. 3.2.6.1.1: Summary of intact (+) or loss (-) of functional MAD-protein domains.
LOV- (FAD) and zinc-finger domain in different WC-1 type *madA* and WC-2 type *madB* mutant strains and wildtype of *P. blakesleeanus*. (Double and triple mutant strains carry the *madA7*, *madB103* and the *madC119* alleles).

Protein / Strains	MADA = WC-1		MADB = WC-2	MADC
	FAD	zinc-finger	zinc-finger	
wildtype	+	+	+	+
C21 *madA*	-	-	+	+
C47 *madA*	-	+	+	+
A893 *madA*	+	-	+	+
C111 *madB*	+	+	-	+
A202 *madC*	+	+	+	-
L51 *madAB*	-	-	-	+
L52 *madAC*	-	-	+	-
L57 *madBC*	+	+	-	-
L72 *madABC*	-	-	-	-

In the *madA* mutant strains C21, C47 and A893 different mutations in the *madA* gene were detected which can cause a loss of functional protein. The *madA* allele in the strain C21 (genotype *madA7 pde-1* (-)) contains a nucleotide exchange resulting in a changed splicing side producing an aberrant mRNA. A nucleotide exchange

leads to an amino acid substitution, resulting in a diminished FAD/FMN binding capacity in the strain C47 (genotype *madA35* (-)). In the strain A893 (genotype *madA403* (-)) a nucleotide exchange generates a premature stop codon resulting in the loss of the zinc-finger domain. In all 16 *madB* mutant strains the same mutation was detected which leads to a lack of the zinc-finger domain (Sanz et al., 2009). The *madA madB* double mutant strain L51 as well as the *madA madB madC* triple mutant strain L72 contains both the same mutated *madA* allele as the strain C21 (*madA7*).

So far *madA* mutation can be distinguished in FAD "minus" (C47, C21, L51, L52 and L72), zinc-finger lacking (A893, C21, L51, L52 and L72) and "knock out" or "loss of function" mutant strains (C21, L51, L52, L72). The *madA madB* double mutant strain L51 which is characterized by a loss of *wc-1* and *wc-2* type zinc-finger may still form a complex but not respond to light by increasing the expression of their corresponding genes like the *wcoA* and *hspA* (Idnurm et al., 2006; Rodriguez-Romero and Corrochano, 2006). Currently, the investigations on the *wc-1* and *wc-2* type genes and proteins are focused on the functional aspects of their participation in the photobiology of *P. blakesleeanus*. Little attention was paid on their involvement on gene expression in dark. A possible changed transcription in the *mad* mutant strains in comparison to the wildtype would underscore their function in the transcriptional regulation as key global transcription factors in *P. blakesleeanus*. Through it the gene expression in dark-grown mycelia of the *mad* mutants can be enhanced, indicating a repressor function, or even suppressed, indicating an enhancer function of the photoresponsive transcription factors of the *wc-1*, *wc-2* type of *P. blakesleeanus.*

A summary of the *mad* mutant strains and their specific mutation is given in Tab. 3.2.6.1.1. The WC-1 type photoreceptor protein MADA has intact (+) LOV- and Znf-domain in wildtype, *madB* mutant strain C111, in *madC* mutant strain A202 and in *madB madC* mutant strain L57. A loss of functional protein domains is indicated by (-). The *madA madB madC* triple mutant strain L72*,* has neither an intact WC-1 type LOV and WC-1, WC-2 type Znf domain nor intact *madC* gene, whereas the *madC* mutation on genetic and molecular level still remains unknown. Due to these mutations the photoactivation of gene expression should be changed, reduced or even abolished.

The *P. blakesleeanus* genome contains beside the *madA* and *madB* (*wctA*) genes two other *wc-1* type blue light photoreceptor genes; *wcoA* and *wcoB* and four *wc-2* type genes *madB* (*wctA*), *wctB*, *wctC*, and *wctD.* Furthermore a

cryptochrome/photolyase orthologue gene *cryA* is manually annotated and its light activated transcription was shown in the previous chapters whereas function of its gene product is currently under investigation. Another manually annotated photoreceptor gene is a phytochrome orthologue. There are additionally ten genes of the *rhodopsin-GPCR* superfamily automatically annotated.

To characterize in more detail how the MADA and MADB photoreceptors are involved in the regulation of gene transcription, and the photoinduced gene expression found in 70 hour dark grown mycelia of the wildtype, the different *mad* mutant strains were exposed to each, blue, red light but also to bichromatic and double pulses, following the same protocols as described previously.

3.2.6.2 Gene expression in *mad* mutant strains after a pulse of blue light

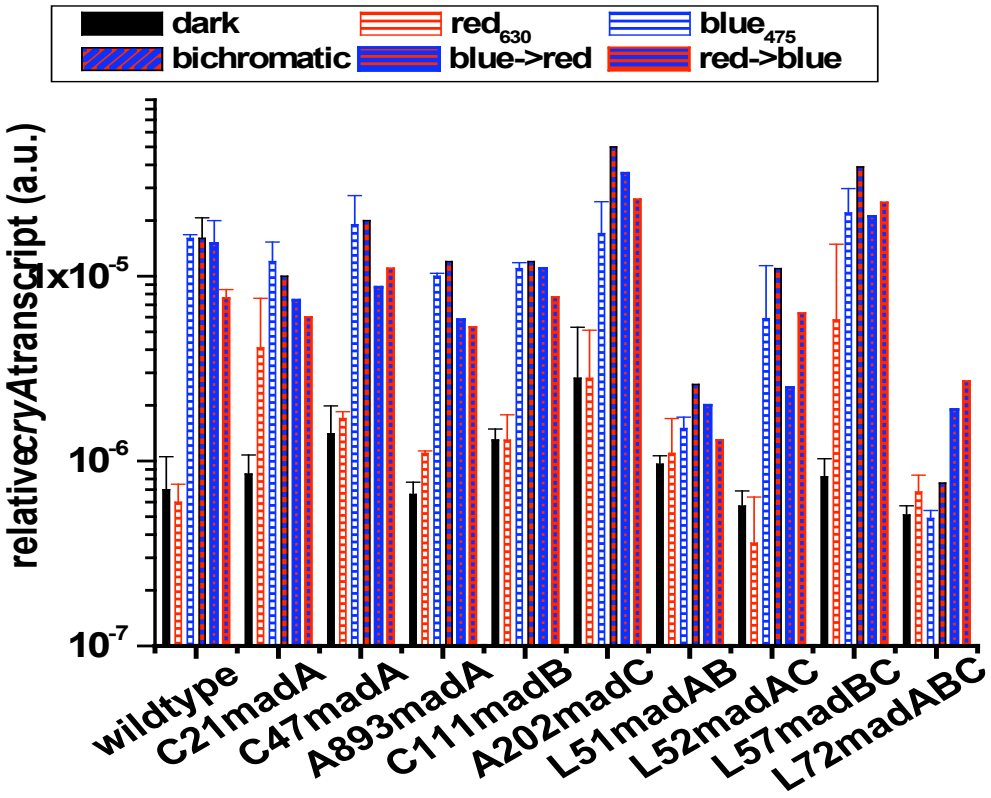


Fig. 3.2.6.2.1: *cryA* gene expression after different light treatments, (40 Jm^{-2}).
Mycelia of *P. blakesleeanus* wildtype NRRL1555 (-) and *mad* mutant strains were raised for 70 hours in continuous dark. Light treatments consisted in a 25 s pulse of red_{630} or $blue_{475}$ light, a double pulse of red followed by blue light or blue then red light. Mycelia were kept in darkness for 20 minutes before the harvest by freezing in liquid nitrogen. Transcript levels in which were measured by qRT-PCR. Plots show the average and SD.

In all tested *mad* mutant strains the resulting expression levels of the analyzed genes are in both, dark-grown and light exposed mycelia, different to the corresponding ones in wildtype. Several observations are in agreement with the predicted loss of photoinducibility. The strongly photoactivated gene expression of the *wc-1* and *wc-2* type genes, the *cryA* and *carRA* gene, and the less pronounced ones of the genes *carB, hspA, myc* and *hsp90* is 20 minutes after a pulse of blue light in the *madA madB* double mutant strain L51 and the *madA madB madC* triple mutant strain L72 abolished (Fig. 3.2.6.2.2). In the triple mutant strain L72 the amount of the *carB* and *hsp90* mRNA is 30 and 10 % reduced compared to the

corresponding dark control. In the double mutant strain L51 the *carB* transcript is 40 % and the one of the gene *hspA* 20 % reduced compared to the corresponding dark control. In comparison to the expression levels of wildtype the genes *hsp90* and *myc* show a 50 and 30 % reduction in the strain L51.

The results demonstrate clearly that the photoactivation by blue light of all these genes is under the transcriptional control of MADA, MADB and/or MADC. The expression pattern of the *carB* gene supports this hypothesis. It shows in comparison to the fivefold enhanced transcription in the wildtype a uniform slightly suppressed expression after a pulse of blue light in all single *madA* and *madB* mutant strains (Tab. 3.2.6.2.1). Only in the *madC* mutant strain A202 the *carB* gene expression is photoactivated after a pulse of blue light like in the wildtype. Interestingly, none of the single mutant strains show such suppression after blue light exposure as detected in the triple mutant strains L72, neither a photoactivation similar to that of the wildtype. The *cryA* gene for example is 23-fold in wildtype and still about 15-fold in the *madA* mutant strains, tenfold in the *madB* strain and sixfold in the *madC* strain photoinduced (Fig. 3.2.6.2.1). The transcription of the *wcoA* gene is not at all abolished in the single *mad* mutant strains as well (Tab. 3.2.6.2.1).

The photoactivation of gene transcription in the *madA* mutant strain C21 despite of the splicing mutation is still detectable for the genes *cryA, hsp90, wcoA* and *wctC* and most strongly for the gene *myc*. In the strain C21 the amount of the *myc* transcript shows an exceptional 120-fold enhancement which is the strongest among all genes and it is 15 times more than in the wildtype. In contrast to the suppression of the *hsp9036* gene expression in wildtype the strain C21 shows a sixfold photoinduction. In the strain C47, carrying the *madA* allele with reduced FAD-binding ability, only the photoactivated transcription of the *cryA* and *wcoA* genes was detected. On the other hand the amount of *hsp9020* transcript is 13-fold reduced. The loss of *madA* Znf-domain in the strain A893 results in a blue light induced gene expression pattern most similar to the one in the strain C21 for the genes *wcoA, myc* and *cryA* with the additional sevenfold induction of the *hsp9020* gene transcription. Thus among the single *mad* mutant strains the blue light induced gene expression is most pronounced in the strain A893. Despite the loss of zinc-finger domain in the *madB* mutant strain C111 photoactivation of the *wcoA, cryA, hsp9020* and *hsp90* gene transcription was detected. The *carB* and *madA* gene expression are three and fivefold suppressed, respectively. The *madC* mutation in the strain A202 yields also a diminished photoactivation of gene transcription except for the 70-fold induction of the gene *myc*. The involvement of the *madC* gene

product on gene expression is furthermore obvious by the reduced transcription of the genes *hsp90, myc* and *wctB* in the strain L51$_{madAB}$ in comparison to the wildtype, and their abolished photoactivation in the triple mutant strain L72. The observation that the blue light induced gene expression in the single *mad* mutant strains is still detectable but abolished in the double and triple mutant strains, suggests that other blue light photoreceptors should be involved. This hypothesis is emphasized by the observation of the strongly enhanced expression of the gene *myc* after a pulse of blue light in the single *mad* mutant strains. Its expression level in the *madA* mutant strain C21 is 120 times, in the strain A893 52 times and in the *madC* mutant strain A202 70 times elevated. As this blue light induced gene expression is reduced in the double and abolished in the triple *mad* mutant strains, an intact MADA and MADB protein is however required for the photoactivation of the gene transcription. Most interestingly the observed photoactivation in the *madC* mutant strain A202 is ninefold higher than the one of the wildtype suggesting a participation of the MADC in the formation of the MAD complex or at least in the regulation of the *myc* gene expression.

Tab. 3.2.6.2.1: Transcription in the wildtype and *mad* mutant strains after a pulse of blue light (40 Jm^{-2}). Expression level determined by qRT-PCR of dark grown mycelia and 20 minutes after a pulse of blue light in 70 hours old mycelia of wildtype NRRL1555 (-) and *mad* mutant strains. Negative values display the inhibitory effect of irradiation (dark/light). Positive values display the promotive effect of irradiation (light/dark).

		madA			***madB***	***madC***	***madAB***
blue	**WT**	**C21**	**C47**	**A893**	**C111**	**A202**	**L51**
carB	4.8	-1.4	-1.4	-1.7	-2.8	5.9	-1.7
madA	1	1.3	-2.4	1.9	-5.2	3	-1.0
wcoA	48.4	6.4	5.1	37.5	6	20	2.2
hsp90	4.3	1.9	-1.5	4.8	3.6	1.8	2.0
hsp9020	1.3	-2.2	-13.5	7.2	5.1	-2.3	4.2
hsp901-3	-1.1	-1.3	-2.4	2.9	-1.6	1.2	1.2
hsp9036	-2.2	6	-3.4	2.8	1.4	-1.3	3.9
myc	8	118.6	3.1	52.1	2.6	69.4	5.8
cryA	22.9	13.8	14.1	15.9	11.5	6.2	1.6
wctB	326.1	-	-	-	20	264.9	4.3
wctC	18	6.8	-1	1.2	1.4	2	1.1

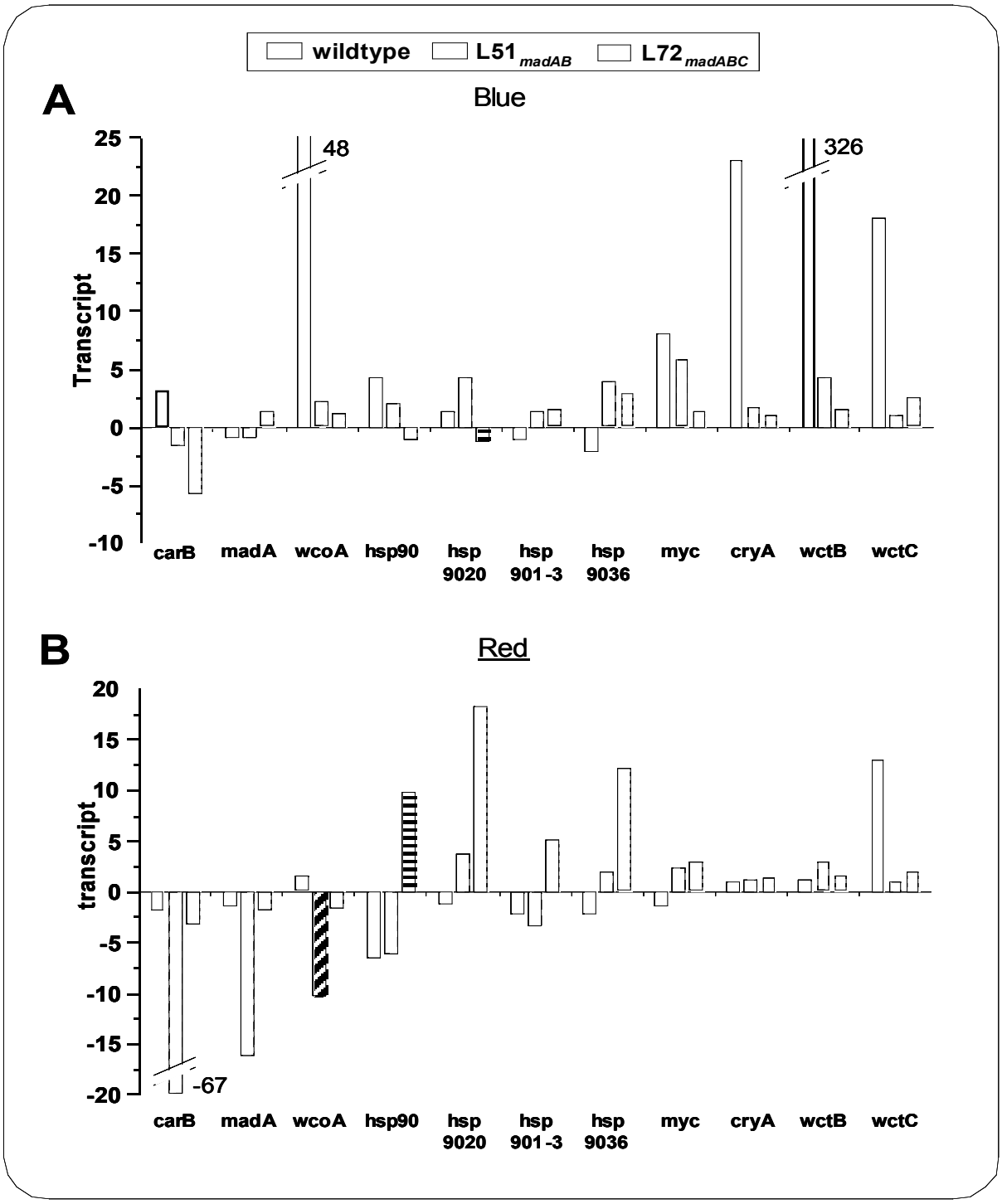


Fig. 3.2.6.2.2: Gene expression after a pulse of light (40 Jm^{-2}, 25 s).

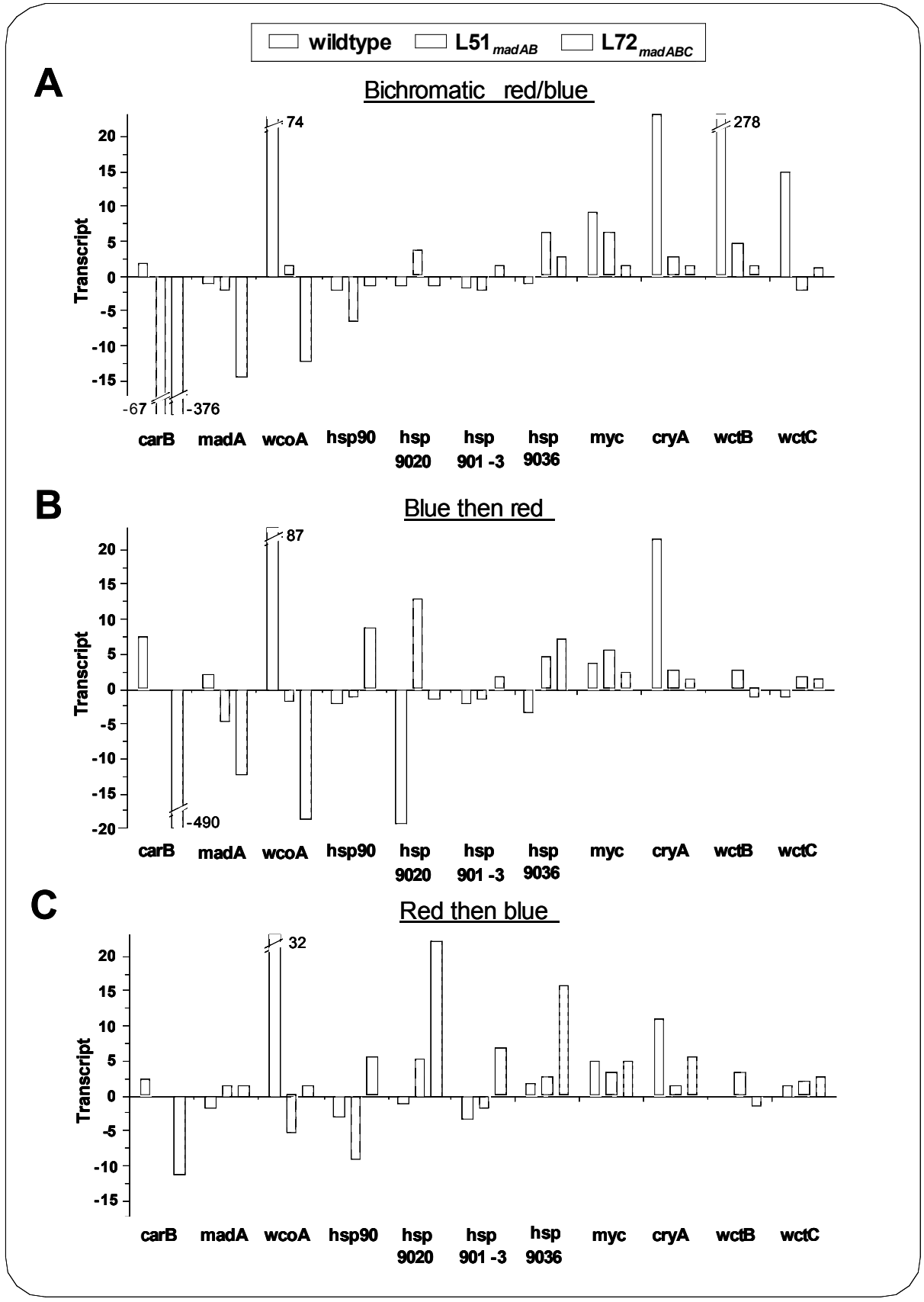


Fig. 3.2.6.3.1: Gene expression after a pulse of light (40 Jm^{-2}, 25 s).

Fig. 3.2.6.2.2/3.2.6.3.1: Gene expression after a pulse of light (40 Jm^{-2}, 25 s).
Gene transcript in 70 hours old mycelia of wildtype NRRL1555 (-), $L51_{madAB}$ and $L72_{madABC}$ mutant strains grown in continuous darkness. Mycelia were harvested by freezing in liquid nitrogen 20 minutes after a pulse of $blue_{475}$ (A), red_{630} light (B), p. 71; bichromatic red and blue (A), double pulse blue then red (B) and red then blue (C), p. 72. Gene expression levels were determined by qRT-PCR. The negative ordinate displays the inhibitory effect of irradiation (dark/light). The positive ordinate displays the promotive effect of irradiation (light/dark).

3.2.6.3 Gene expression in *mad* mutant strains after a pulse of red light

The discovery of the moderate changes in gene expression after a pulse of red light in wildtype extends the complexity of photoregulation of transcription. It seems that the predicted phytochrome (*phy*) photoreceptor is presumably involved in the light-dependent regulation of transcription in *P. blakesleeanus*. To investigate the participation of *mad* mutations in the red light depending gene regulation, the abundance of mRNA was determined in all *mad* mutant strains 20 minutes after a pulse of red light (40 Jm^{-2}, 25 s).

The gene expression levels obtained in the mutant strains show in general the same tendency as in the wildtype, but for some genes a more pronounced reduction of their transcripts was measured (Fig. 3.2.6.2.2; Tab. 3.2.6.3.1). Best results were obtained for the *wc-1* photoreceptor genes *madA, wcoA*, the member of the *hsp90* gene family and the gene *carB*. Most interestingly, the inhibitory effect of red light on gene expression of these genes becomes obvious in the *madA madB* double mutant strain L51 but not in the triple mutant strain L72 (Fig. 3.2.6.2.2). The most strongly reduced amount of transcript compared to the corresponding one in dark-grown mycelia was measured for the genes *carB* (67-fold)*, madA* (16-fold) and *wcoA* (10-fold) in the strain $L51_{madAB}$ whereas in the strain $L72_{madABC}$ the amount of their transcript is equal to the ones in wildtype. A reduction of the same magnitude of mRNA of these genes was also determined in the single mutant strains A202 (*madC*) and A893 (*madA*) but not in the other strains (only two- to fourfold), except the 28-fold reduced amount of *carB* mRNA in the *madB* mutant strain C111. Only for these genes however, the red-light suppression in the strains A893, A202 and L51 is comparable in its strength to the promoted effect of blue light in wildtype obtained on the strongly photoinducible genes like *wctB* or *wctC*. Interestingly the strong suppression is not the reversal of the photoactivation by blue light.

Tab. 3.2.6.3.1: Transcription in wildtype and mutant strains after a pulse of red light (40 Jm^{-2}). Expression level determined by qRT-PCR of dark grown mycelia and 20 minutes after a pulse of blue light in 70 hours old mycelia of wildtype NRRL1555 (-) and *mad* mutant strains. Negative values display the inhibitory effect of irradiation (dark/light). Positive values display the promotive effect of irradiation (light/dark).

		madA			**C111**	**A202**	***madBC***
red	**WT**	**C21**	**C47**	**A893**	***madB***	***madC***	**L57**
carB	-1.7	-1.9	-2.1	-159.5	-28.1	-63	-1.1
madA	-1.4	-3.9	-1.9	-12.9	-2.1	-7.3	-4.3
wcoA	1.4	-2.3	1	-8.6	1.5	-11.2	-1.5
hsp90	-6.5	-9.1	-1.1	-4.9	4.1	-8.1	-4.6
hsp9020	-1.3	-40.3	-1.8	-1.7	27.6	-6.4	-1.3
hsp901-3	-2.3	-10.2	-2	-7.3	-7.5	-1.5	-1.2
hsp9036	-2.3	-6.7	-1.2	1.7	4.3	-1.5	1.2
myc	-1.4	4.2	1.2	1.7	1.2	2.4	1.6
cryA	0.9	4.9	1.2	1.6	1.3	1	7.1
wctB	1.1	-	-	-	1.3	1	31.1
wctC	13	-1.4	-1.3	-2.2	1.1	-1.6	1.6

A different pattern was obtained for the members of the *hsp90* gene family. The expression of the *hsp90* gene with the strongest suppression in wildtype (6.5-fold) after a pulse of red light for example, is although equally suppressed in the single mutant strains C21, A893, A202 and the double mutant strain L51 and L57, ten fold enhanced in the triple mutant strain L72 and four fold in the strain C111, whereas its transcription is ineffectual by red light in the strain C47. Interestingly, only in the strains C47 and L57 the expression of all *hsp90* genes is equal to the wildtype, whereas their expression is promoted in the strain L72 and strongly reduced in the strain C21. The gene *hsp9020* in comparison to the corresponding dark control is enhanced 28-fold in the *madB* mutant strain, 18-fold in the triple mutant strain and 3.5-fold in the strain L51, but 40-fold and sixfold reduced in the strains C47 and A202 respectively. The amount of the *hsp901-3* gene transcript is fivefold elevated in the strain L72, tenfold reduced in the strain C47, about seven times in the strains A893 and C111 and 3.5 times reduced in the double mutant strain L51. The expression of the gene *hsp9036* is about seven times suppressed in the strain C21 but five and 12 times enhanced against the corresponding dark control in the strains C111 and L72 respectively. The strongest

positive detected effect after a pulse of red light in mycelia of the wildtype, the 13-fold enhancement of *wctC* transcript is abolished in all *mad* mutant strains. This uniform effect is an exception. A promoted gene transcription against the corresponding dark controls was measured for the genes *myc* and *cryA* in the *madA* mutant strain C21 (fourfold) and in the strains A202, L51 and L72 only for the *myc* gene (2.5-fold). The *madB madC* double mutant strain L57 shows the strongest positive effect of red light by a sevenfold enhanced expression of the *cryA* gene and a 31-fold elevated amount of *wctB* mRNA, which underlines the regulatory role of MADC (Tab. 3.2.6.3.1).

The influence of red light on gene expression in *P. blakesleeanus* becomes most surprisingly more obvious in the analyzed blue light photoreceptor mutant strains which underline their important role in the transcriptional regulation not only after blue but also after a pulse of red light. The suppressed expression of all *hsp90* genes in the single *madA* mutant strains and the genes *wcoA* and *carB* in the double mutant strain L51 underscores the important role of MADA and MADB in the transcriptional regulation after both, blue and red light irradiations. Furthermore, the results give rise to the suspicion that a separate red light photoreceptor is also involved in the regulation of gene expression, whereas the photoresponsive transcription factor (MAD) complex is essential for its signal-integration on the transcriptional level. Based on the comparison of the promotive and suppressive effects of each light quality on transcription in wildtype versus the double mutant strain L51, the genes can be categorized into three groups (Fig. 3.2.6.2.2). First, those in which transcriptional photoactivation by blue light in the wildtype is abolished in the double mutant strain and red light is ineffectual (*myc, cryA, wctB* and *wctC)*. Second, where the promotive effect of blue light in the wildtype is abolished or converted in the double mutant strain and red light shows a suppressive effect in the wildtype, that is even more pronounced in the strain L51 (*carB, madA, wcoA*). Third, those in which transcription is slightly affected both positively and negatively by blue and by red light in wildtype as well as in the mutant strain L51 (all *hsp90* genes). Interestingly, the expression patterns of all these genes are extremely diverse in the single *madA* and *madB* mutant strains, which suggests a more complex regulatory mechanism of gene expression by other blue light photoreceptors and a red light photoreceptor and maybe other regulatory elements as well. But it also underlines the integrative participation of the MADA and MADB in the red-light dependent transcription which should be based on their DNA-binding ability (Znf). The gene expression in the strain C47 (-FAD) is in fact among all *mad*

mutant strains less affected after a pulse of red light, while the strong photosuppressions were not observed.

The maintenance of a wildtype-like expression of the genes *carB, madA* and *wcoA* for example, is most strongly affected by a loss of Znf-domains of *madA* (A893) and *madB* (C111) gene products after red light exposure. The suppressed expression of these genes in the *madC* mutant strain A202 is surprisingly very similar to the ones obtained in the strains A893 and L51$_{madAB}$. On the other hand the expression of the *hsp90* genes is most strongly reduced in the *madA* mutant strain C21 whereas the loss of Znf-domains in the strains A893 and C111$_{madB}$ can cause an antagonistic (*hsp90*) or synergistic (*hsp901-3*) effect after red-light exposure (Tab. 3.2.6.3). These exceptionally diverse gene expression patterns among the mutant strains demonstrate clearly that regulation by light is specifically individual for each gene. The *madC* gene product displays an important and central role in the transcriptional regulation after both blue and red light irradiations. The reduced expression of the genes *carB, madA* and *wcoA* in the *madC* mutant strain A202 and the enhanced transcription of all *hsp90* genes in the triple but not double mutant strains after a pulse of red light underscore the regulatory role of MADC, as shown in Fig. 3.2.6.2.2 most impressively.

3.2.6.4 Gene expression in *mad* mutant strains after bichromatic light exposure

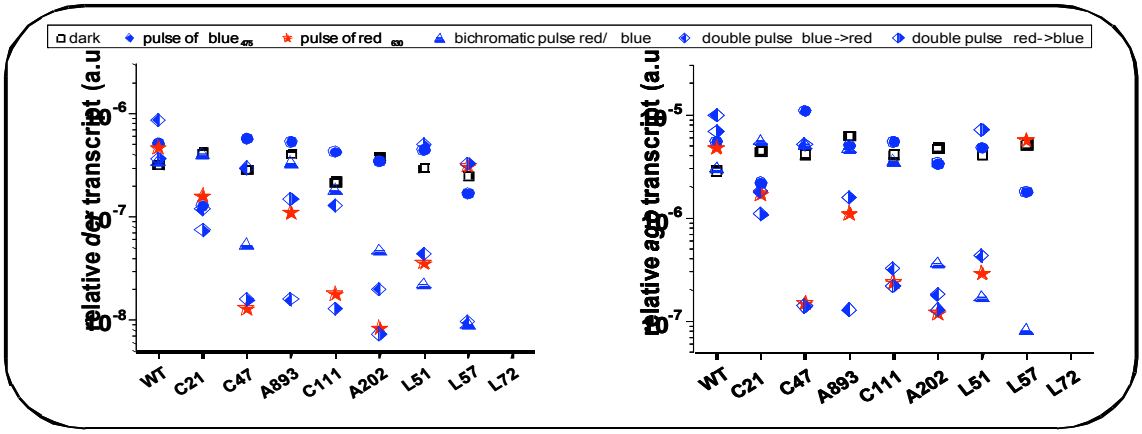


Fig. 3.2.6.4.1: Expression of *dcr* and *ago* in *mad* mutant strains after different irradiations. Relative transcript 20 minutes after different light treatments as indicated (red$_{630}$, blue$_{475}$ each 40 Jm^{-2}, 25 s) in 70 hours dark grown mycelia of the wildtype NRRL1555 (-) and *mad* mutant strains. Mycelia were harvested by freezing in liquid nitrogen and expression levels were determined by qRT-PCR.

As a result of the aforementioned findings it seems imperative to have a closer look at the gene expression after bichromatic red and blue light exposure (Tab. 3.2.6.4.1; Fig. 3.2.6.3.1). The expression of the *cryA, myc* and *wc-2* type genes which were not affected by red light show, as expected, after a pulse of bichromatic red and blue light the tendency toward the pattern obtained after a pulse of blue light with nearly equal expression values in the wildtype as well as in the single, double and triple *mad* mutant strains (Fig. 3.2.6.3.1/3.2.6.2.2; Tab. 3.2.6.4.1/3.2.6.3.1). The amount of the *wctB* gene is reduced after a bichromatic irradiation (278-fold) in comparison to the one after blue light exposure (326-fold) in the wildtype, but about three times enhanced in the strain $A202_{madC}$ and $L57_{madBC}$ (Tab. 3.2.6.4.1). Interestingly the *cryA* gene shows a similar enhancement in these strains as well, whereas its transcription is equal in the wildtype after a pulse of both blue and bichromatic irradiations. The strong photoactivation after a pulse of blue light of the *myc* gene (119-fold) in the strains C21 is abolished, reduced in the strain A893, whereas its expression is more pronounced in the strain A202 (107-fold) after bichromatic light exposure.

Surprisingly, the strong red light effects on transcription of the *hsp90* genes in the mutant strains are abolished after bichromatic red and blue light exposure. Thus the antagonistic effect of blue and red light is compensated by bichromatic red and blue light treatment in the wildtype as well as in the mutant strains. In the wildtype the *hsp90* gene for example is fourfold enhanced after a pulse of blue, 6.5-fold reduced after red and shows a twice reduced expression after a pulse of bichromatic red and blue light. The mutant strains also show this compensatory effect of bichromatic irradiation on gene transcription, except for the sixfold suppression of the *hsp90* gene expression in the strain $L51_{madAB}$.

The genes *carB, madA* and *wcoA* show diverse expression patterns after a pulse of bichromatic red and blue light in the wildtype as well as in the mutant strains. The expression of the *carB* gene seems to be “compensated” in the wildtype but ineffectual by bichromatic irradiation in the strains C21, C47 and L57. The strong reduction of its transcript after red light exposure is abolished in the strain A893 and A202, less pronounced in the strain C111, equal in the double mutant but strongly pronounced in the triple mutant strain. The amount of the *madA* mRNA is only reduced in the strains L57 (sixfold) and L72 (14-fold) but not in the other strains. In the wildtype the *wcoA* gene expression is more pronounce after bichromatic than after blue irradiation but not in the mutant strains. Only the strain L72 shows a reduction (12-fold). In the strain A893, A202 and L51 the suppressive effect of red

light on the *wcoA* transcription is reverse after bichromatic light exposure.

Tab. 3.2.6.4.1: Transcription in wildtype and *mad* mutants after a pulse of bichromatic red and blue light (40 Jm^{-2}). Expression level determined by qRT-PCR of dark grown mycelia and 20 minutes after a pulse of blue light in 70 hours old mycelia of wildtype NRRL1555 (-) and *mad* mutant strains. Negative values display the inhibitory effect of irradiation (dark/light). Positive values display the promotive effect of irradiation (light/dark).

		madA			***madB***	***madC***	***madBC***
red/blue	**WT**	**C21**	**C47**	**A893**	**C111**	**A202**	**L57**
carB	1.8	-2.4	1.1	4.9	-9.2	1.6	-2.9
madA	-1.1	-2.3	-1.7	-1.2	-2.2	-2.9	-6.4
wcoA	74.2	3.7	8.7	10.7	3.7	17.5	1.3
hsp90	-2.1	-1.4	-1.7	-1.3	4.5	-2.2	-1.9
hsp9020	-1.3	-1.6	-1.3	3.8	47.3	-1.0	-2.6
hsp901-3	-1.7	-2.0	-1.2	-3.6	-4.3	1.9	-9.1
hsp9036	-1.1	-1.6	-1.3	2.1	3.2	1.8	-1.2
myc	9.0	4.0	12.3	34.0	8.8	107.5	4.7
cryA	22.9	11.8	14.6	18.7	12.6	18.1	47.8
wctB	278.3				15.8	648.6	196.7
wctC	15.0	-1.1	-1.2	-1.2	-1.3	1.9	1.0

3.2.6.5 Gene expression in *mad* mutant strains after double pulses

For further analyses of the interaction of red and blue light dependent changes of transcription the abundance of mRNA was measured 20 minutes after a double pulse of red followed by blue or blue then red light in all *mad* mutant strains (Fig. 3.2.6.3.1). The group of genes (*myc, cryA, wctB* and *wctC)* with transcriptional photoactivation by blue light in the wildtype is abolished in the double mutant strain and red light is ineffectual, showing the same blue-light like expression pattern after the double pulses in all *mad* mutant strains as after a pulse of bichromatic irradiation. The expression of these genes is in general more pronounced after the double pulse blue followed by red than red followed by blue light. The group of genes (*carB, madA, wcoA*) shows a diverse expression pattern after the double pulses as well as after a pulse of simultaneous red and blue light treatment. The effect of the double pulse blue followed by red light suggests a synergistic effect of both wavelengths on gene expression, predominantly in the strain $L72_{madABC}$. The transcription of these genes is stronger photodepressed than after each, blue and red light alone.

Tab. 3.2.6.5.1: Gene expression in wildtype and *mad* mutant strains after a double pulse of blue then red light (40 Jm^{-2}). Expression level determined by qRT-PCR of dark grown mycelia and 20 minutes after a pulse of blue light in 70 hours old mycelia of wildtype NRRL1555 (-) and *mad* mutant strains. Negative values display the inhibitory effect of irradiation (dark/light). Positive values display the promotive effect of irradiation (light/dark).

		madA			***madB***	***madC***	***madBC***
B->R	**WT**	**C21**	**C47**	**A893**	**C111**	**A202**	**L57**
carB	7.4	-1.0	-6.2	-3.7	-285.7	-3.1	-1.0
madA	2.0	-1.5	-12.1	-1.6	-10.5	-9.4	-3.0
wcoA	87.1	4.3	1.6	11.9	-1.7	1.9	2.1
hsp90	-2.0	-1.0	-1.7	3.3	-2.4	-11.2	1.6
hsp9020	-19.4	-1.1	-2.9	14.0	3.3	-7.1	-1.6
hsp901-3	-2.0	-1.8	-3.8	-1.8	-19.1	1.1	-6.5
hsp9036	-3.3	-1.2	-2.5	4.1	1.2	1.9	1.3
myc	3.7	18.4	5.4	38.5	3.0	24.9	2.5
cryA	21.4	8.7	6.3	12.5	11.5	13.0	25.8
wctB					27.5	486.5	147.5
wctC	-1.1	-1.0	-4.3	-1.3	1.3	3.0	1.2

Tab. 3.2.6.5.2: Gene expression in wildtype and mad mutant strains after a double pulse of red then blue light (40 Jm^{-2}). For detail description see legend above.

		madA			***madB***	***madC***	***madBC***
R->B	**WT**	**C21**	**C47**	**A893**	**C111**	**A202**	**L57**
carB	2.3	-2.6	-9.1	-22.2	-26.5	-11.9	2.2
madA	-1.7	-2.4	-12.1	-12.8	-1.9	-30.8	-1.7
wcoA	32.3	2.7	1.3	2.2	2.2	1.5	3.8
hsp90	-2.9	-3.7	-1.5	-1.9	2.5	-14.2	2.3
hsp9020	-1.1	-24.9	-3.0	1.3	15.6	-10.5	4.8
hsp901-3	-3.4	-2.5	-3.8	-9.2	-6.5	-2.1	-1.7
hsp9036	1.7	-2.3	-2.3	1.0	3.3	-1.1	2.1
myc	5.0	8.8	5.1	6.2	5.9	19.7	12.9
cryA	10.9	7.0	7.7	8.1	7.8	9.5	29.8
wctB					15.8	170.3	113.1
wctC	1.5	-1.1	-3.1	-3.0	-1.0	-2.3	1.4

The synergism shown before depends on the order of the different wavelength. The double pulse red followed by blue light does not lead to the same strong suppression of these genes in the strain L72$_{madABC}$. In the single mutant strains the expressions of these genes suggest a higher compensatory effect of both wavelengths, whereas the light quality given first is more dominant than the second one (Tab. 3.2.6.4.1; Tab. 3.2.6.5.1/3.2.6.5.2).

The group of *hsp90* genes also show expression patterns after the double pulses in which the first given light quality is dominant over the second one. Mainly in the strain L72, but also in the single mutant strains, the enhanced expression of the *hsp90* genes after a pulse of red light is nearly equal after the double pulse where red light was given first followed by blue light but not after the double pulse blue then red light. In the strain C21 the transcriptional suppression of *hsp90* genes after red light exposure is also present only after the double pulse red then blue but not after the double pulse blue then red. On the other hand, the expression patterns after a double pulse blue then red light show the same tendency as the patterns after a pulse of blue light. There are some exceptions where it seems that red light is predominant. In the strain L72 the genes *hsp90* and *hsp9036*, in the strain A202 the genes *hsp90* and *hsp9020* and in the strain C111 the gene *hsp901-3* all show after a double pulse of blue then red light an effect like after red light alone (Fig. 3.2.6.3.2/3.2.6.3.3; Tab. 3.2.6.3.1/3.2.6.5.1/3.2.6.5.2).

However the interaction of red and blue light should be discussed also. All light treatments in which red light was involved show clear effects on the same genes which underline their reliability. The numerous red light effects most obvious in the *mad* mutant strains suggest once more that a separate red-light photoreceptor participates in transcriptional regulation in *P. blakesleeanus*. The enormous effects of red light in the strains L72 and A202 on the expression of the genes *carB* and *wcoA* underscore the importance of MADC as a transcriptional regulator.

All the above mentioned interpretations of the transcriptional photoregulation in a WC-1 and WC-2 dependent manner are furthermore supported by the expression patterns obtained for the genes *dcr* and *ago* (Fig. 3.2.6.4.1). For these genes nearly no photoinduction neither photosuppression of their transcription could be detected in the wildtype of *P. blakesleeanus* but in all *mad* mutant strains strong effects could be measured. The expression pattern of these genes underscores once more as well the importance of the *madC* mutation by the strongest transcriptional suppression in the strain A202 after the pluses of red and red then blue light.

Another aspect of the involvement of the MAD complex in gene regulation is

given by the fact that there are nine genes, the transcription of which in wildtype is only positively affected after light treatments. The genes *act, ago, dcr* and *madB* are not as strongly photoinduced than the genes *wcoA, wcoB, wctB, wctD* and *cryA* in the wildtype. They never show reduced amounts of transcript after a pulse of all different wavelengths in comparison to the dark control. Among these genes only the *cryA* and *wctB* genes show also elevated amounts of their mRNA after a pulse of different light qualities in all *mad* mutants.

3.2.6.6 Gene expression in *mad* mutant strains in dark

After all the presented results of light treatments and the shown involvement of *mad* mutations e.g. *wc-1*, *wc-2* type photoreceptors in *P. blakesleeanus,* one important aspect should not be missed. The initially presented expression level of all genes of dark-grown mycelia of the wildtype should be compared further to the ones of the *mad* mutant strains.

Tab. 3.2.6.6.1: Gene expression in darkness.

Expression level determined by qRT-PCR of 70 hours dark grown mycelia of wildtype NRRL1555 (-) and *mad* mutant strains. The average gene expression values from the *mad* mutant strains were normalized against the corresponding ones of the wildtype. Negative values display the suppressive effect on gene transcription by the *mad* mutations (WT-dark/*mad*-dark). Positive values display the promotive effect (*mad*-dark/WT-dark)

		madA			***madB***	***madC***	***madAB***
dark	**WT**	**C21**	**C47**	**A893**	**C111**	**A202**	**L51**
carB	1.0	1.9	1.0	-1.9	2.2	-2.4	-5.7
madA	1.0	1.5	3.8	-4.9	2.5	1.1	1.1
wcoA	1.0	1.5	1.9	-1.6	-1.6	2.1	-1.7
hsp90	1.0	1.2	1.9	1.1	-2.0	3.4	1.0
hsp9020	1.0	1.7	2.0	-8.3	-31.6	-1.5	-73.7
hsp901-3	1.0	0.9	1.0	1.1	3.2	-3.6	-3.6
hsp9036	1.0	1.9	2.3	-3.5	-2.4	1.5	-4.3
myc	1.0	-3.2	-1.7	-5.4	-6.6	-6.3	-11.2
cryA	1.0	1.2	2.0	-1.1	1.4	4.0	1.4
wctB	1.0				5.2	1.6	-5.0
wctC	1.0	1.3	3.7	1.3	1.0	-1.3	-2.3

Several genes show different transcript levels in dark-grown mycelia of the *mad* mutant strains in comparison to the wildtype (Fig. 3.2.6.6.1; Tab. 3.2.6.6.1). The members of the *hsp90* gene family as well as the genes *myc*, *ngf, wctB* and *carB* show lowered expression levels in dark-grown mycelia of the double and triple mutant strains than in the wildtype. The gene *hsp9020* in the strain L51$_{madAB}$ and in the strain L72$_{madABC}$ shows the strongest lowered amounts of its transcript compared to the one of the wildtype (73-fold and 11-fold respectively). The genes *carB and myc* show the same pattern of lowered transcript levels in the strain L51 compared to L72. The amount of the *wctB* and *wctD* transcript is also lowered strongest in the strain L51 but enhanced in the strain L72. The genes *madA* and *hsp90* show the reverse pattern, slightly reduced amounts in the strain L72, but equal amounts of their mRNA in the strain L51 compared to the ones of the wildtype. Only the genes *hsp901-3* and *ngf* show less amounts of their mRNA in the strain L72 (six- and ninefold, respectively) than in the strain L51 (four- and eightfold).

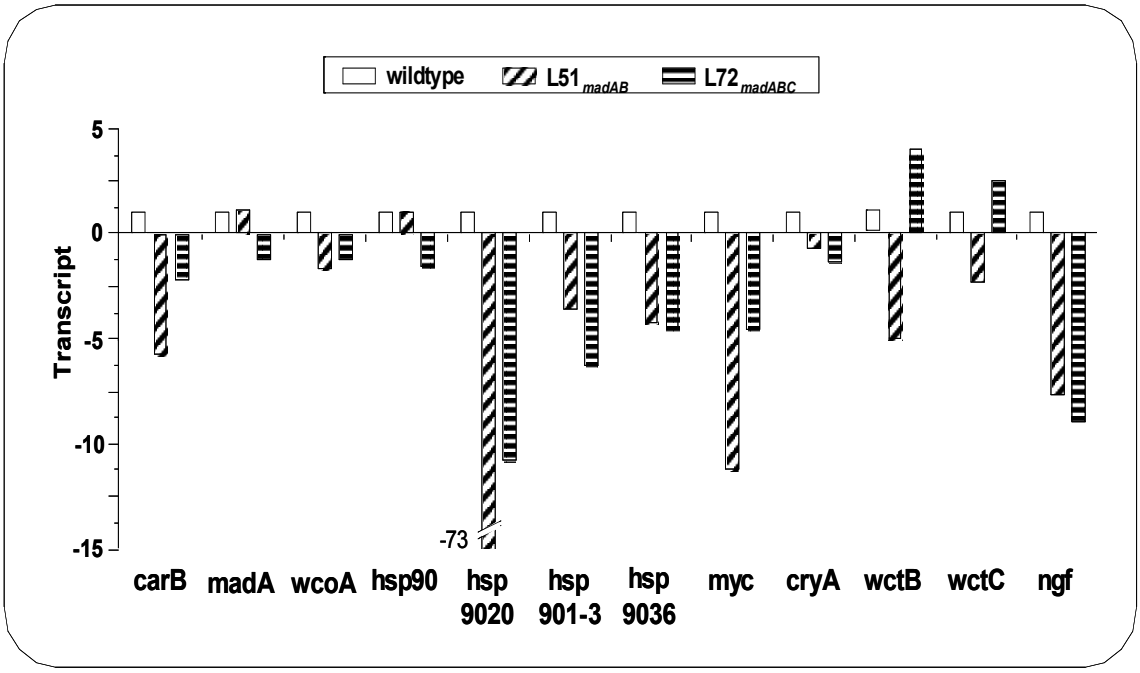


Fig. 3.2.6.6.1: Gene expression in dark-grown mycelia

Gene transcript in 70 hours old mycelia of wildtype NRRL1555 (-), L51$_{madAmadB}$ and L72$_{madABC}$ mutant strain grown in continuous darkness. Mycelia were harvested by freezing in liquid nitrogen. Gene expression levels were determined by qRT-PCR and normalized against the corresponding values of wildtype. The negative ordinate displays the inhibitory effect of the *mad* mutations on gene expression (WT-dark/*mad*-dark). The positive ordinate displays the promotive effects on gene expression (*mad*-dark /WT-dark).

These results together suggest the participation of *madA, madB* and *madC* gene products in the transcriptional regulation also in the dark. The different gene expression patterns of the strain L51 and L72 underline the importance of MADC for the gene regulation not only after light exposure but also in darkness. This hypothesis is emphasized by the observation of the fourfold enhanced transcript levels of the genes *cryA* in the dark only in the *madC* mutant strain A202 compared to the wildtype (Fig. 3.2.6.2.1; Tab. 3.2.6.6.1).

3.2.6.7 The *madC* mutation

The importance of the *madC* gene product was pointed out several times. Another remarkable correlation is the fact that the expression values of some genes are equal in the *madC* mutant strain A202 and $L51_{madAB}$ and/or the single *madA, madB* mutant strains. The *myc* gene in the strains A893, C111 and A202 is equally suppressed (~sixfold) and somehow synergistically 11-fold in the double mutant strain L51 in dark grown mycelia. The gene *hsp901-3* is threefold suppressed in the strain A202 and L51 and synergistically sixfold in the triple mutant strain. Most interestingly the *carB* gene is equally suppressed in the strain A893 and A202 (2.5-fold) and 5.7-fold in the double mutant strain.

Such a correlation was also observed after irradiations of the genes *carB, madA, wcoA* and *hsp90*. The transcriptional suppression after a pulse of red light and after the double pulse red followed by blue light is equal or similar between the strains L51, A893 and A202. After bichromatic light treatment this correlation occurs only on the gene *wcoA* and after the double pulse blue followed by red only the gene *carB* is equally suppressed in the strain A893 and A202.

The genes *cryA* and *myc* show a similar correlation of transcriptional behavior between the strains A893 and A202 after a pulse of blue light as well as after all other light treatments. The photoinduction of these genes is after all different irradiations equal or similar between these three strains. Most interestingly the double mutant *madB madC* strain L57 shows the same tendency with lowered values compared to the other strains.

The suppression of the *hsp90* and *hsp9020* gene after red light and both double pulses in the strain A202 is a unique feature.

According to these correlations the MADC is most properly a *wc*-1-like blue light photoreceptor like *madA*. There are two possible candidates known, the *wcoA* gene encodes a *wc*-1 like photoreceptor as well as the *wcoB* gene. It is remarkable

that the *madC* mutant strain A202 shows correlations of expression patterns of some genes with the *madA* mutant strain A893 but not other *mad* mutant strains. The mutation in the *madA* allele (*madA403*) of the strain A893 is listed in Tab. 3.2.6.1.1 on page 65. A nucleotide exchange generates in this allele a premature stop codon resulting in the loss of the zink-finger domain of the MADA (Fig. 1.4.2.1, page 11) (Idnurm et al., 2006). Thus, the MADC should be a *madA* photoreceptor without the zink-finger domain. Only the gene *wcoB* encodes a protein which serves this purpose. The *wcoB* gene arose via gene duplication thus it shares four of five introns with both others *wc-1*-like genes, *madA* and *wcoA*. At this time no *wcoA* or *wcoB* phenotype is known.

3.3 Gene expression after gravitropic stimulation

For the first time changes in transcript abundances were monitored over a course of five hours of gravity-stimulated SIVb-sporangiophores of *P. blakesleeanus*. In response to the gravitropic stimulation, by placing the sporangiophores horizontally, some of the analyzed genes show specific changes in their transcript levels. The genes *carB* and *carRA* show a gradually reduced and the genes *madA* gene a slightly increasing drift of its expression during the time course of gravitropic stimulation (Fig. 3.3.1, Fig. 3.3.2). The *wc-1* and *wc-2* type genes show different expression patterns with specific up and down regulations in the wildtype (Fig. 3.3.3). In the previous chapters the results of the light exposure experiments as well as the expression pattern analyses of the *mad* mutants suggest a more general function of *madA* and *madB* gene products in transcriptome modulation. To investigate whether the gravity specific gene expression pattern of the wildtype is related to the MADA/MADB protein complex, or to the shown repressor function of *madC* mutation, the changes in the abundance of gene transcripts during the period of gravitropic stimulation were also monitored in the *madC* strain A202 and the triple mutant strain L72.

The amounts of the *carB* and *carRA* gene transcript lie at about 2×10^{-6} a.u. and show a gradual down regulation during the course of time. The level of the *carB* gene transcript in the wildtype is quickly reduced in gravitropic response within the first half an hour and slightly increased after one and a half hours before it decreases until the fifth hour. The total reduction of the *carB* transcript from time zero to the fifth hour is 14 times (1.8×10^{-7} a.u.). The *carRA* gene shows the same tendency

with less reduction of its amount of transcript than the *carB* gene. During the first hour of gravitropic stimulation the amount of the *carRA* gene mRNA is reduced threefold (5 x 10^{-7} a.u.), then increases slightly and shows a threefold reduction at the fourth hour again in comparison to time zero. At the fifth hour its amount of transcript is twice reduced. The *madC* mutant strain A202 and the triple mutant strain L72 show different expression patterns without the gradual suppression of *carB* and *carRA* mRNA. In the *madC* mutant strain the transcription level of the *carB* gene is the same as in wildtype at the beginning of the time course, increases to a maximum of 5 x 10^{-6} a.u. after one and a half hours and decreases until the end of the stimulation to the same level as in the beginning. The amount of *carRA* transcript is reduced (7 x 10^{-7} a.u.) in comparison to the wildtype but very stable during the whole time course.

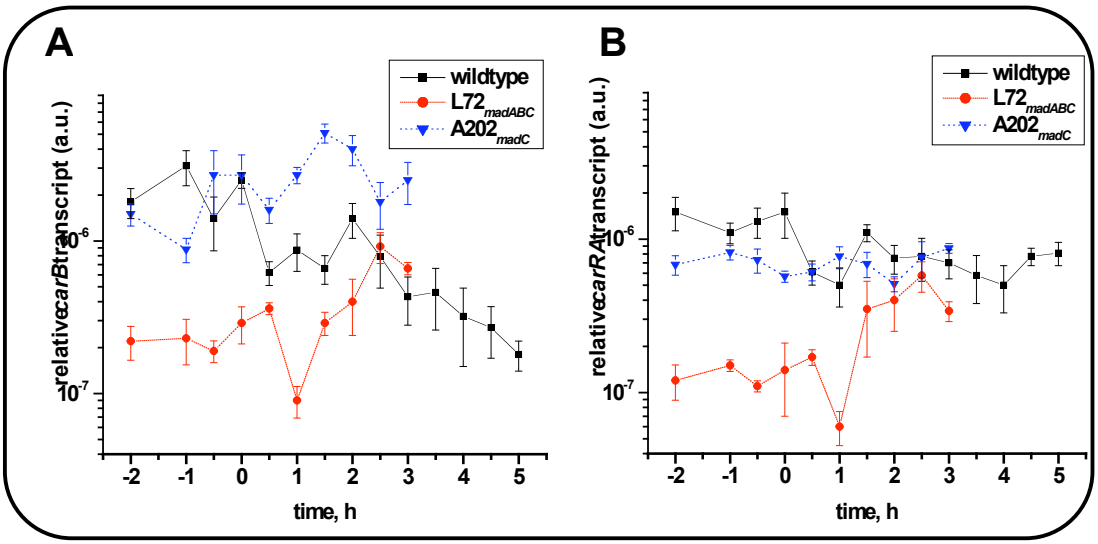


Fig. 3.3.1: Expression pattern of the genes *carB, carRA* after gravity-stimulation.
Gene transcription levels during a five hour time course of gravitropic stimulation. For stimulation, five days old SIVb-sporangiophores were oriented horizontally at time 0. *P. blakesleeanus* wildtype NRRL1555 (-), *madC* mutant strain A202 and triple mutant strain L72 (*madABC*) sporangiophores were raised under moderate white light (0.7 Wm^{-2}) and kept four hours before and during the experiment in darkness. The samples were harvested two, one and 0.5 hours before the gravitropical stimulation and then each 30 minutes by freezing in liquid nitrogen. Levels of mRNA were determined by qRT-PCR. A. *carB* gene expression pattern. B. *carRA* gene expression pattern. Plots show the average and SD of n=3 (-2, -1 and -0.5 h), n=9 (0-3 h) and n=6 (< 3 h) (wildtype), n=4 (both mutant strains).

In the triple mutant strain L72 the *carB* and *carRA* gene expression patterns are very similar. Their transcript levels are initially lower than in wildtype (~2 x 10^{-7} a.u.). It decreases transiently to 9 and 6 x 10^{-8} a.u. respectively during the first hour and increases gradually one order of magnitude (9 and 6 x 10^{-7} a.u.) after two and a half hours of gravitropical stimulation.

The expression patterns of the *wc-1* type genes in the wildtype are not uniform. The transcription of the *madA* gene shows a drift of its transcription with a slight increase of 1.5 times during the entire period of observation and a transient threefold reduction of its mRNA (9.7-3.1 x 10^{-7} a.u.) after the first hour. The *wcoA* gene shows the same tendency but with a maximum of its expression after the first and the second hour with a threefold and a fivefold elevated amount of its transcript.

In both analyzed mutant strains the expression pattern of the *madA* gene shows a gradual suppression of transcription similar to the one of the *carB* gene. The level of *madA* mRNA is slightly reduced in the strain A202 but strongly in the strain L72 (fivefold) at the fifth hour. Another similarity between the *madA* and *carB/RA* transcription is the lowered initial level of *madA* transcript in the triple mutant strain L72 in comparison to the one in wildtype (6.5-fold reduced). The *wcoA* gene transcription shows the opposite effect by a fivefold elevated initial level and a stable expression of the *wcoA* gene in the strain L72 during the time course. In the *madC* mutant strain A202 the level of *wcoA* mRNA is most strongly reduced after one hour (30 times), increases gradually to the initial level during the following two and a half hours, followed by a second reduction at the third hour (sixfold). In the wildtype of *P. blakesleeanus* the *wc-2* type genes show a maximum of gravity induced expression after two and a half hours, except for the ineffectual transcription of the *madB* gene. The amount of the *wctB* gene is first gradually diminished until one and a half hours into the stimulation period, and then shows a twofold increase and a stronger gradual suppression with a sevenfold reduced amount of its transcript after three and a half hours followed by a slight increase up to the fifth hour. The genes *wctC* and *wctD* show a similar expression pattern. Their level of transcript is seven and fourfold elevated after one hour and 15 and 10-fold increased after two and a half hours in comparison to the ones at time zero.

In the mutant strain L72 the expression pattern of the *wctD* gene is similar to the one of the wildtype with a lowered maximum (fivefold). In the *madC* mutant strain the initial amount of *wctD* mRNA is a magnitude of order reduced compared to the one in wildtype. After a weak decrease during the first 30 minutes it increases

gradually until the end of the time course up to three times compared to the level in the beginning.

The expression of the *cryA* gene shows in the wildtype, after an initial slight suppression, a twofold increase after one, and one and a half hours (shoulder), and a further increase to a peak of a fourfold elevated amount of its mRNA two hours after stimulation. This is followed by a decrease close to its initial level of mRNA. Beginning at the third hour until the end of the gravitropic stimulation the level of *cryA* mRNA is more or less stable (~8.5 x 10^{-7} a.u.), which is still a twofold increased transcription level compared to the one at time zero. In both mutant strains the *cryA* expression is first slightly enhanced (1.5-fold) compared to the one in wildtype and then during the following hour suppressed. In the strain A202 the transcription of the *cryA* gene increases to the initial level two hours after stimulation followed by a slight decrease. In contrast its expression in the strain L72, a twofold maximum increase after two and a half hours is followed by a reduction to the initial level at the end of the time course.

Among the gene family which encodes histone modifying enzymes (*hat*) the *hat10* gene shows strong changes in its transcription after gravitropical stimulation in wildtype but not in the *madC* mutant strain A202. The expression pattern of the *hat10* gene in the wildtype shows a 28 and tenfold reduction of its mRNA amount one half and one and a half hours after gravity stimulation. A threefold increase was measured after three and three and a half hours and a ten to 20-fold reduction again during the following two hours before its transcript level increases at the fifth hour. The initial expression level of the *hat10* gene in both mutant strains is about four times reduced. In the *madC* mutant strain A202 the amount of *hat10* transcript is slightly diminished after 30 minutes of gravitropical stimulation until the third hour. In the triple mutant strain L72 the level of *hat10* transcript increases fivefold compared to the initial level during the first 30 minutes of the stimulation, decreases twice during the following hour and increases gradually again until the end of the time course to a fivefold elevated transcriptional level.

In order to complement the knowledge of photogravitropic responses in *P. blakesleeanus* on a transcriptional level the changes in transcript abundance of different genes were monitored after gravitropical stimulation of dark adapted SIVb-sporangiophores. The gene expression in wildtype and the *mad* mutant strains reveal a complex pattern of specific gravity regulation and confirm our conjecture that the *madA, madB* and *madC* gene products are not only exclusively involved in photo- but also in graviregulation.

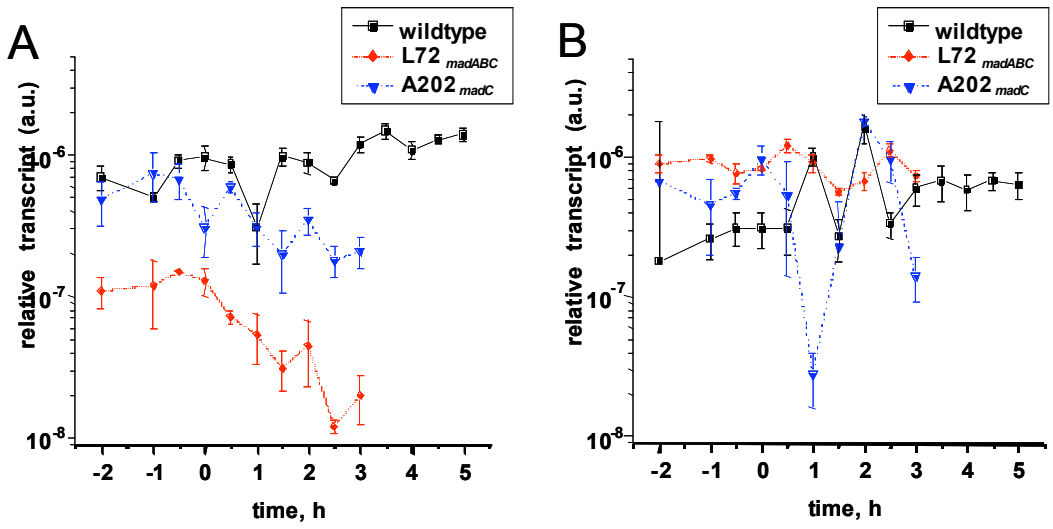


Fig. 3.3.2: Expression pattern of the genes *madA* and *wcoA*

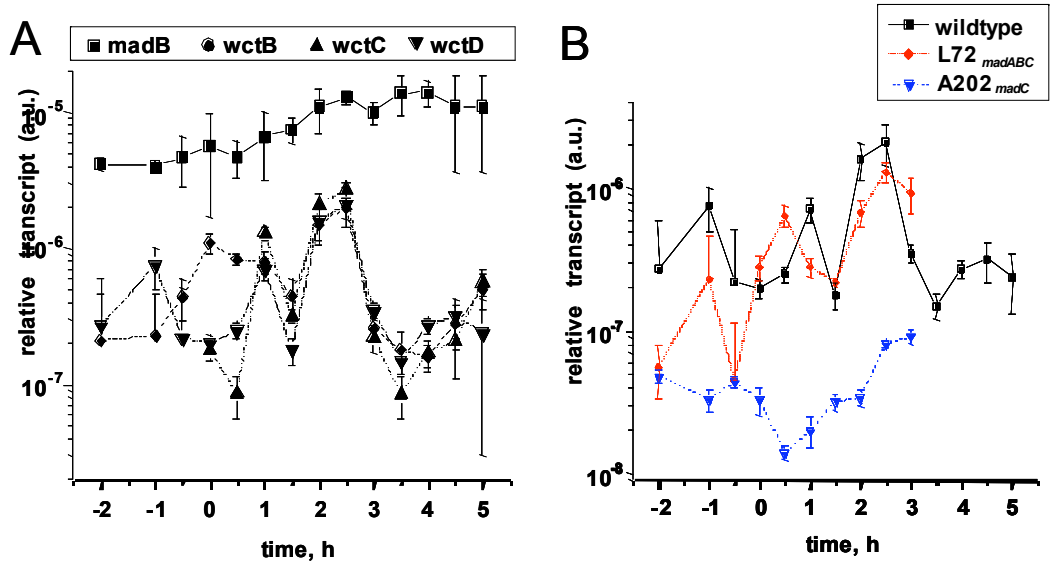


Fig. 3.3.3: Expression pattern of the *wc-2 type* genes

Fig. 3.3.2/3.3.3: Expression pattern of the *wc-type* genes after gravity-stimulation

Gene transcription levels during a five hour time course of gravitropic stimulation. Fig. 3.3.2.A/B transcription of the *wc-1* type genes *madA*/*wcoA* and *wc-2* type gene *wctD* in Fig. 3.3.3.B monitored in wildtype and *mad* mutant strains A202 and L72. Fig. 3.3.3.A shows the expression pattern of *wc-2* type genes in wildtype. Experimental procedure and detailed data description see legend of Fig. 3.3.1.

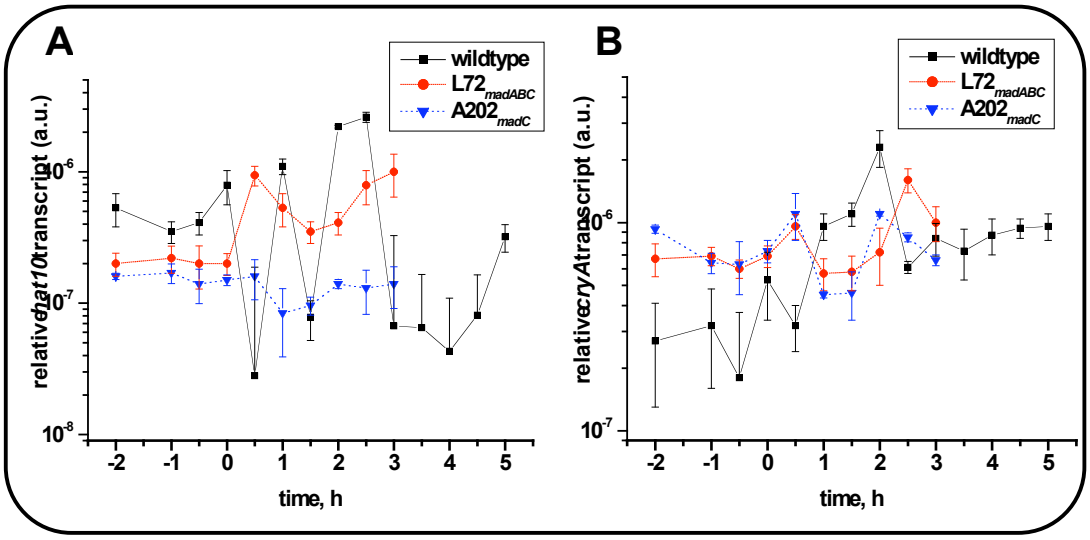


Fig. 3.3.4: Expression pattern of the genes *hat10, cryA* after gravity-stimulation

Gene transcription levels during a five hour time course of gravitropic stimulation. A. *hat10* gene expression pattern. B. *cryA* gene expression pattern. Experimental procedure and detailed data description see legend of Fig. 3.3.1.

To prove the hypothesis that the *wc-1* and *wc-2* gene products as photoresponsive transcription factors are not only integrating the light, but also the gravity signal as well by modulating the gene expression, their transcript levels were determined after gravity stimulation. The obtained results show changes in the direction of gravity is accompanied with differently expressed genes in the sporangiophores of *P. blakesleeanus*. The transcriptional changes detected in the wildtype are either a slight but constant drift, a transient suppression, or enhancement of gene expression during the five hours of gravitropic stimulation. Most interestingly these gravitropic responses are strongly alterated in the *mad* mutant strains L72$_{madABC}$ and A202$_{madC}$. This suggest the participation of the *mad* gene products, thus the MAD complex is a global key regulator of transcription in *P. blakesleeanus*.

Chapter 4. DISCUSSION

Light is one of the most powerful abiotic factors which trigger a multitude of developmental key processes of fungal biology and lifecycle. The filamentous fungus *Phycomyces blakesleeanus* is a model organism in sensory physiology since more than 50 years. Thereby a linear flow of light information from sensors to effectors was assumed as shown in the model of the sensory pathway of *P. blakesleeanus* in Fig. 4.1.1.

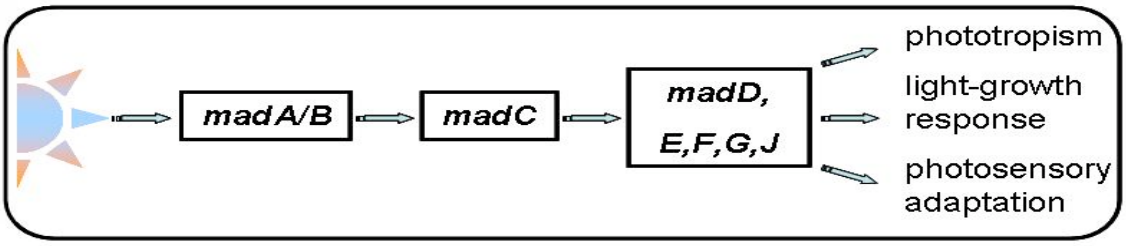


Fig. 4.1.1 Linear sensory pathway of light stimuli of *P. blakesleeanus*.
Light stimuli transduction chain from the receptors via transducer to the responses (modified after Bergmann et al., 1973 and Cerda-Olmedo, 2001).

Since the isolation and characterization of behavioral *mad* mutant strains and moreover the recent identification of the set of *wc*-photoreceptor genes (*madA, madB*) molecular approaches of photophysiology can advance. The counterparts of *N. crassa* White Collar-1/-2 photoreceptors/transcription factors are in *P. blakesleeanus* the MADA and MADB proteins which form a photosensitive transcription factor complex (MAD complex) to govern many light-controlled processes in the fungus (Sanz et al., 2009). These complexes have been identified in ascomycetous and basidiomycetous fungi as well, suggesting the WC complex as a highly conserved fungal regulatory mechanism of photoresponses (Herrera-Estrella and Horwitz, 2007).

In this work the method of qRT-PCR was used to move a step deeper in light dependent gene expression to gain new insights about the regulatory role of the *wc*-type photoreceptor mutant *madA, madB and madC*. Therefore, a protocol was developed that consisted in a pulse of actinic light gave a non saturating transcriptional response. Not only the characterization of the blue light responses in both wildtype and *mad* mutant strains came into the focus of these approaches, but also the search for red light responses on a transcriptional level.

4.1 Gene expression after blue light exposure

4.1.1 Housekeeping genes

In the wildtype of *P. blakesleeanus* after blue light exposure, continuous light as well as a pulse of it, many of the analyzed genes show a response. These photoresponses are mainly a transcriptional activation but a suppression of gene expression was detected as well. Among the genes which show very weak changes after blue light irradiation and the most stable expression in darkness, monitored during an 11.5 hour time course of mycelia growth, the genes *act, tub, hsp9020, asf, hat53, hat50, and hsp9036* could be considered as housekeeping genes (Fig. 3.2.1.8, p. 42). Use of constitutively expressed genes as an internal control or reference, when analyzing stimulus dependent transcriptional changes, is basic for the general consensus. Therefore a stable expression for these genes should be assumed. To validate the suitability of the above referred genes their expression after all different irradiations was compared with the one of dark-grown mycelia. The obtained expression pattern revealed that not all of them are equally expressed after irradiations. Most interestingly the *act* gene shows only enhanced and the *tub* gene only reduced amounts of mRNA after the different light treatments, whereas the photosuppression of the *tub* gene can be more than twice, and therewith it is stronger than the transcriptal change of the gene actin. Only the genes *act* and *asf* show a very stable expression during the time course of mycelia growth and development as well as after light exposure (Fig. 3.2.1.7). Their expressions show a less than twofold variation after the different light treatments in comparison to the drak-grown mycelia. Thus these genes are suitable candidates for the use as reference genes with respect to the given stimuli. On the other hand the measured 1.5-1.7-fold elevated expression of these genes equals 25-50 % of the photoactivation compared to the moderate photoresponsive genes *carB* or *hspA*.

4.1.2 Gene expression upon continuous versus a pulse of blue light

The transcriptional photoresponses in *P. blakesleeanus* wildtype, obtained in this work, can be separated into a strong and moderate (> 10-fold) enhancement of gene expression. Under continuous blue light the transcription of most of the genes is moderately enhanced whereas after a pulse of blue light the opposite ratio was

determined. In *N. crassa* the light signal can be transduced very fast by the early-light induced genes like the carotene genes *al-1* and *al-2* or with a delayed induction of the late-light responding genes such as the clock-controlled genes *ccg-4* and *ccg-6* (Sommer et al., 1989). In *P. blakesleeanus* none of the analyzed genes show a late-light response; this holds for the wildtype and the *mad* mutants. The strength of the transcriptional enhancement correlates with the dark expression level. The lower a gene is transcribed in dark the stronger it can be induced after blue irradiation (Fig. 3.2.2.1, p.45). On the other hand, equal dark-expression levels do not necessarily result in the same photoactivation. Thus the transcriptional control is gene specific and should consist in more constituents than only the MAD complex. The genes *myc* and *wctB* for example, displayed the lowest dark expression (~2 x 10^{-8} a.u.). After a pulse of blue light the *myc* gene is eightfold but the *wctB* gene, however, 326-fold photoinduced. In *P. blakesleeanus* the photoactivation of the *hspA* or the *wc-1, wc-2* genes is transient and show the same kinetic but reaching different, gene specific maxima (Rodriguez-Romero & Corrochano, 2006; Sanz et al., 2009). On the other hand the inhibitory effect of blue light on transcription is very weak (> 2-fold) and occurs after both continuous and a pulse of blue irradiation only on the genes *hsp9036* and *tub* (Fig. 3.2.2.1, p. 45). The expression of the *wc-1* type photoreceptor gene *madA* is reduced only in continuous blue light. This photosuppression takes place on a lager time scale as only shown for the *madA* gene. It is twice depressed after more than one hour and up to fourfold during the following three hours of blue-light exposure. Thus the transcriptional photorepression of the *madA* gene is slower and more moderate than the photoactivation of the remaining *white collar* genes or the *carB/RA* genes (Rodriguez-Romero & Corrochano, 2006; Sanz et al., 2009; 2010). In contrast to the transcriptional photoactivation the photosuppression is less investigated. The photosuppression of the *madA* gene and a presumable depletion of MADA on the promoters of its target genes could be a central component in photoadaptation (Fig. 4.2, p. 99).

4.1.3 Transcriptional regulation of the *carB* gene

One example for the multifactorial regulation of gene expression and a more profound study of transcriptional photoactivation was very recently published for the genes *carB* and *carRA* (Sanz et al., 2010). The kinetics of photoinduction show

the characteristic increase of transcription of these genes directly after light exposure. Subsequently there is a "drop down" of *carB*/*carRA* mRNA eight minutes after irradiation which correlates with the binding of proteins in a specific promoter region shown by electrophoretic mobility shift assays (EMSA). It demonstrates how important DNA-binding in gene regulation is and confirms the common results of *carB* expression obtained in three different laboratories by two different methods, the Northern blot and qRT-PCR technique. On the other hand formation of the binding complex is not altered in the single *madA*, *madB* and *madC* mutant strains. (Ruiz-Hilgado et al., 1997; Sanz et al., 2010; this work, chapter 3.2.2). In contrast, it was found in this work that the blue-light activation of the *carB* gene is in all the *mad* mutants abolished or suppressed (Fig. 3.2.6.2.2, p. 71; Tab. 3.2.6.2.1, p. 70). This indicates that the MAD complex should interact with other transcriptional regulators (Fig. 4.1.2.1, p. 99). Most interestingly the fragment of the *carB/carRA* intergenic region which shows the shift in the EMSA experiments contains only a GATA-box but none of the other light regulating elements (LREs, APEs, see also Fig. 1.4.3.1, p. 14). It can be concluded that the binding complex should consists in GATA-transcription factors and that most probably the MAD complex interacts on different positions of the *carB/carRA* intergenic region.

4.1.4 Transcriptional regulation of the genes *ago* and *dcr*

Another observation of the transcriptional photoactivation is that none of the genes show higher expression levels than the cytoskeleton constituent *act* and *tub*. On the other hand none of the genes show lower expression levels than the lowest expressed gene *hat10* not even after the strongest transcriptional photodepressions (Fig. 3.2.2.1, p. 45). The regulatory mechanism, which conducts the transcriptional level within this range (10^{-9}-10^{-5} a.u.) is still unclear and remains cryptic. One initial explanation was a suspected involvement of the RNA-induced silencing complex RISC with the catalytic constituent AGRONAUTE and DICER (Ribonuclease III). This conjecture is supported by the observation that the photoinduction of the *carB* gene is accompanied by an enormous increase of its degraded transcript (Blasco et al., 2001). It can be assumed, that enzymes like DICER and AGRONAUTE are involved in the transcriptional regulation. This is supported by the investigations in *Mucor circinelloides* in which the content of 21-nt and 25-nt siRNAs after photoactivation of the transgenic *carB* reporter gene increases (Nicolás et al., 2003).

It can be suspected that the transcriptional level of photoactivated genes is regulated via the RNA-silencing mechanism. This would be once more emphasized when the orthologues genes *ago* and *dcr* of *P. blakesleeanus* would be photoresponsive. But after all different irradiations no photoinducibility of these genes was detected. Their expression level in dark is nearly equal in all *mad* mutant strains in comparison to the ones of the wildtype. This observation does not exclude the regulatory participation of the gene products of *ago* and *dcr* but on the other hand neither supports the principal conjecture that their participation in the transcriptional photoregulation correlates with the photoresponsiveness of their own expression. Instead of this a remarkable particularity of their expression pattern was found in the wildtype. These genes show a unique wide spread expression level in dark-grown mycelia during the 11.5 hour time course experiment which span almost a whole order of magnitude. Most surprisingly, these genes are rhythmically in phase up and down (Fig. 3.2.1.3, p. 37). Evidence was found in *N. crassa* that antisenese RNA of the core oscillator *frq* cycles in antiphase with the *frq* mRNA upon free-running conditions, suggesting that the *frq*-antisense RNA has a clock function. Mutant strains, in which the usual light-induced *frq*-antisense RNA expression is abolished, show a delayed circadian rhythmicity and a dramatically enhanced phase response to light pulses in comparison to the wildtype (Kramer et al., 2003). These results might explain why the genes *ago* and *dcr* in *P. blakesleeanus* are not photoresponsive to blue light. Their expression might be under the control of the endogenous clock or moreover they are central components of a rhythmicity generating mechanism. Most surprisingly their expression is dramatically suppressed after a pulse of red and equally after a double pulse of red then blue light in all the mad mutant strains. This photoresponse is specific to red light and moreover only detectable in the *mad* mutant strains but not in the wildtype (Fig. 3.2.6.4.1, p. 76), suggesting that their transcription might be under control of the MAD complex directly or indirectly by other still unknown proteins (Fig. 4.1.2.1, p. 99). However, this is a very interesting field which requires more detailed investigations.

4.1.5 Transcriptional regulation of the *hat* genes

A different mechanism of gene regulation, mainly for the activation of gene expression, is the light-dependent modulation of chromatin. In *N. crassa* the

photoactivation of the gene *al-3* is accompanied with the histone H3 acetylation by NGF-1 in a WC-1 dependent manner, thus chromatin modifications are a general aspect of transcriptional photoresponse. After a pulse of blue light none of the analyzed genes coding for histone modifying enzymes show the excepted transcriptional changes in *P. blakesleeanus*. Most interestingly the *ngf*, *hat50* and *hat10* genes show slightly enhanced expression levels only under constant blue light (Fig. 3.2.2.1, p. 45). It can be speculated that these genes encoding acetyltransferases are specific for light-responses. Evidence is given by the fact that the genes *ngf* and *hat10* are slightly suppressed in the wildtype after the double pulses but stronger suppressed after all different irradioations only in the *madC* mutant strain A202 and most strongly after the double pulse red then blue light (Fig. 3.2.6.2.2, p. 71). It might be that these genes are regulated in a MADA, MADB and MADC dependent manner upon continuous light exposure (Fig. 4.1.2.1, p. 99). To proof this, further experiments should be done by monitoring the transcriptional level of their mRNA in the *mad* mutant strains, grown in continuous light. But there remain more open questions about the gene regulation. The histone acetylation still can not explain the maintenance of the dark expression level. For that reason, additional sophisticated studies remain to be completed.

4.1.6 Transcriptional behavior of the *mad* mutant strains

Nevertheless, all the photoresponses found in this work are under control of MADA and MADB because their photoactivation is abolished in the *madAB* double mutant strain L51$_{mad\text{AB}}$. This confirms the suggestion that MADA and MADB are primary for all blue light dependent changes on gene expression. It is consistent with the results of previous studies (Idnurm et al., 2006; Rodriguez-Romero & Corrochano, 2006; Sanz et al., 2010). These class-1 mutants have an elevated photogravitropic threshold in common and show in general aberrant photoresponses in comparison to the wildtype (for more details see also Appendix A). On the transcriptional level several genes are only photosuppressed in the strain L51$_{mad\text{AB}}$ but in the strain L72$_{madABC}$ their photoactivation is completely abolished. Thus the *madC* gene product should participate in the MAD complex involved in the transcriptional regulation of these blue light responding genes in the mycelia of *P. blakesleeanus*. Even if this proposal is not conform to the previous studies in which intact *madA* and *madB* genes, but not the *madC* gene, are required for the

photophorogenesis, photosporogenesis and photocarotenogenesis (Russo & Galland, 1980). In this work the gene expression after blue light exposure is strongly reduced in single *mad* mutant strains but not abolished like in the *madA madB* double nor the *madA, madB, madC* triple mutant strains which suggest that the remaining *wc-1* (*wcoA, wcoB*) and *wc-2* (*wctB-D*) gene products should be involved in the transcriptional control of gene expression most likely by their participation in the formation of a multimeric MAD complex (Fig. 4.1.2.1, p. 99). A puzzling feature displays *madB madC* double mutant strain in comparison to the other mad mutant strains.

For a long time a complex photoreceptor system was postulated by different investigators to explain the results of their photosensory experiments to explain the prodigious light intensity range of ten orders of magnitude (10^{-9}-10 Wm^{-2}) to which *P. blakesleeanus* wildtype can respond, including the photomorphogenesis, phototropic responses, as well as the action spectra of light growth responses, and dark adaptation (Galland 1983; Galland & Lipson 1984, 1987). With the identification and characterization of the *wc* genes present in the genome sequence of *P. blakesleeanus* these previous predictions are validated. But, except for the *madA* and *madB* mutant strains, there are no clear phenotypes available for all the *wc* genes which is the reason that their role remains an open field of speculation. This situation presents itself quite differently in other fungi where distinct *wc-1* gene products are involved in specific photoresponses. In *Mucor circinelloides* three *wc-1* type genes are identified (*mcwc1a-c*). They share the same conserved domain structure of WC-1 as well as in other fungi but regulate different light responses. The *mcwc-1a* gene product is mainly involved in the phototropism under blue and green wavelengths and the transcriptional photoactivation of *mcwc-1c* by only blue irradiation whereas the *mcwc-1c* gene product controls the photocarotenogenesis in a blue light dependent manner in *M. circinelloides* (Silva et al., 2006). Here I show on transcriptional level evidence that the distinct *wc* gene products of *P. blakesleeanus* are involved in the photocontrol of gene expression by forming a multimeric MAD complex (Fig. 4.1.2.1, p. 99). The expression pattern of the *cryA, myc* and *wcoA* genes support this assumption. These genes are only photosuppressed in the single *mad* mutant strains but their transcriptional photoactivation is abolished in the strains $L51_{madAB}$ and $L72_{madABC}$. Thus at least for these genes the other *wc-1* and *wc-2* gene products should be participating in the formation of the MAD complex which still demands more detailed knowledge. From the observation of the different transcriptional behavior of *wc* genes after irradiation it can be assumed that

they serve to adjust the light signal on a transcriptional level. Upon continuous blue light the *madA* gene is twice less expressed than in continuous darkness. This photosuppression is accompanied with an enhanced expression of the other *wc-1* gene *wcoA* (threefold). Its transcription level is upon continuous blue light up regulated exactly to the same level as the *madA* gene in darkness. Therefore the *madA* gene product appears the primary factor for an adequate transcription in darkness, but then after light exposure it gets in part substituted or supplemented by the *wcoA* gene product which might be the primary photoreceptor/transcription factor for the photoresponses. Thus its transcription is strongly photoactivated after "short" light treatments as also shown by other groups (Idnurm et al., 2006; Sanz et al., 2009).

4.1.7 The *wc-1* genes and the function of their predicted gene products

In addition to the dark-control of all *wc* genes via the MAD complex in *P. blakesleeanus* their transcriptional photoactivation depends also primarily on the *madA* and *madB* gene products. Most surprisingly the *wcoB* gene is photoactivated as well upon continuous light exposure. Because its gene product misses the Znf-domain and it can not function as a transcription factor, but it seems nevertheless important in photoregulation. One possible explanation is that the *wcoB* gene product, after light activation, might be involved in the adaptation of the light responses similar to VVD of *N. crassa* (Schwertefeger & Linden 2001). In contrast to the findings in other fungi no distinct photoresponse could be detect for the *wc-1* gene products at the time. The observed variations in the expression pattern of some genes in the different *mad* mutant strains suggest that the transcriptional regulation in *P. blakesleeanus* might be controlled by the constituent WC-1 of the MAD-complex. The *wcoA* gene product of the ascomycete *Fusarium fujikutoi* for example shows the typical conserved domain structure of WC-1 but is neither photoactivated nor involved in the regulation of photocarotenogenesis. The *wcoA* mutant strains show aberrant fusarin, bikaverin and giberillin production as well as different conidiation rates in comparison to the wildtype (Estrada & Avalos, 2007). Among all these different functions of the *wc-1* gene products in other fungi in *P. blakesleeanus* the WC-1-type photoreceptor MADA is still the global key regulator of a wide range of cellular functions from photogravitropic responses to transcriptional control by forming a MAD complex with MADB (Appendix A).

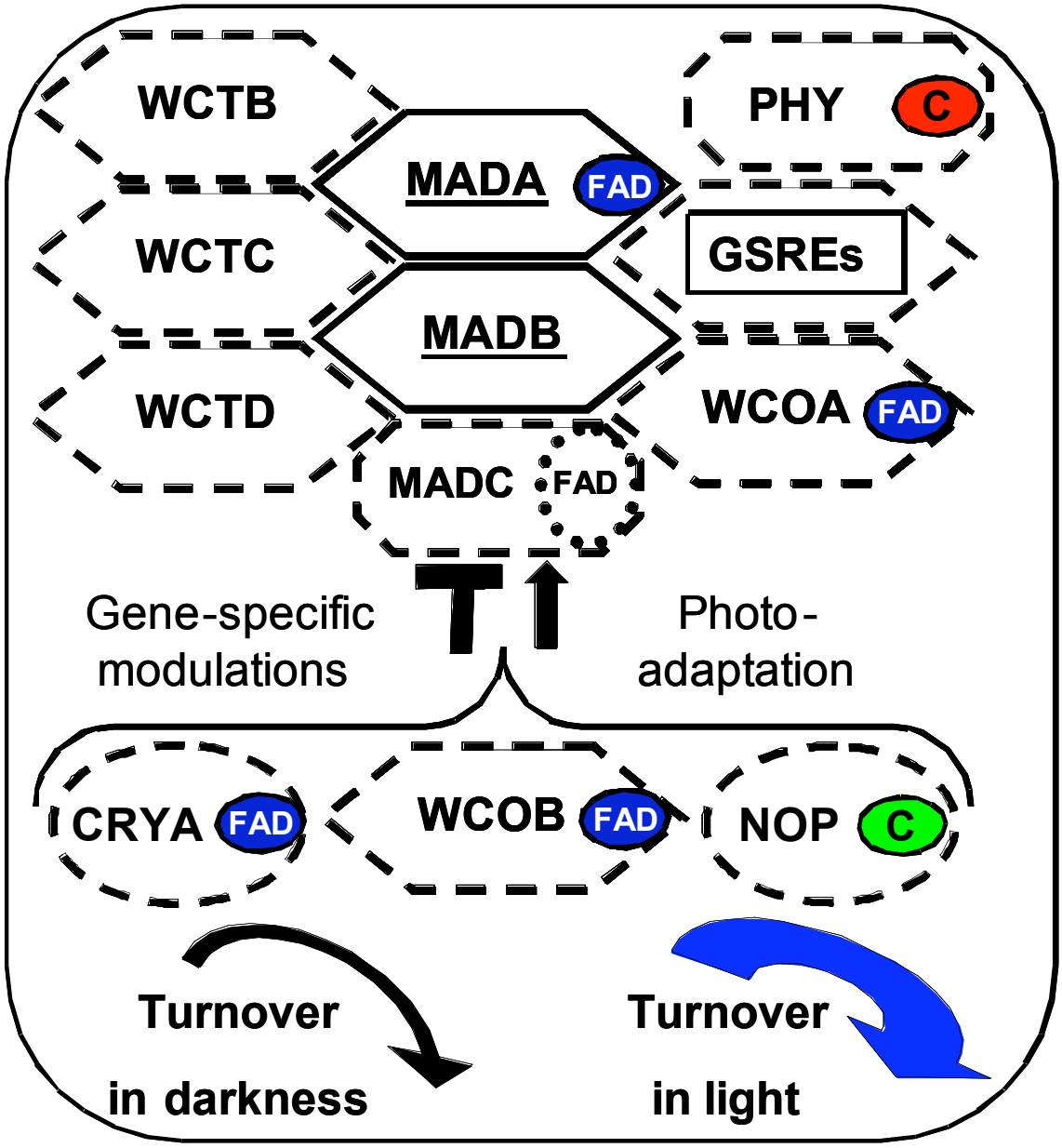


Fig. 4.1.2: Multimeric photoreceptor/transcription factor complex of *P. blakesleeanus*

The MAD complex regulates gene specific transcription in darkness and light. The central and essential components, MADA and MADB, form with WCOA, the other WC-2 type proteins (WCTB, C, D) and MADC the MAD complex. Phytochrome (PHY) interacts with the MAD complex through associated gene specific regulator elements (GSREs). The secondary photoreceptors WCOB, CRYA, NOP and the GSREs might modulate the light responses in a gene specific manner. Each component has a turnover in dark to maintain a basal transcription which is activated by MADA in the presence of light. Protein interaction and components which are yet to be determined marked with dashed lines. Small circles indicate the chromophore (C) and FAD in the wavelength color they absorb.

The remaining *wc-2* (wctB-D) gene products should be involved specifically in some of these photoresponses as their transcription is also photoactivated in a MADA, MADB depending manner. The *wctB* gene, among the *wc-2* genes, shows the strongest enhanced expression upon both continuous and a pulse of blue light. It is maybe a counterpart of the *wcoA* gene product which supplements the MADB after light treatment, or it might interact via its PAS-domain with still unknown additional proteins. Among the *wc* genes the *wctB-D* genes show the lowest dark-expression level which might insinuate their specifics in light-dependent responses in *P. blakesleeanus*. Most interestingly the *wctC* gene is not affected by continuous light exposure as the *wctB* and *wctD* genes, but nearly equally photoactivated by red and blue light which underscores the predicted specific role of the distinct *wc-2* gene products in light-regulated processes.

Among all analyzed genes the *wc-1* genes *wcoA, wcoB*, the *wc-2* genes *wctD* and *wctB*, the transcription factor gene *myc*, the histone acetylase genes *hat10* and n*gf*, the heat shock protein gene *hsp901-3* (endoplasm) and the *carRA* gene as well as cryptochrome/photolyase gene *cryA* show an enhanced transcription upon continuous light in the wildtype. After a pulse of blue light, photoactivation of the genes *hat50*, *hat10, ngf* and *hsp901-3* was not detected. In addition to the above mentioned genes the heat shock protein genes *hspA* and *hsp90* as well as the genes *wctC* and *carB* are photoactivated, which underscores the distinguishable transcriptional regulatory activation mechanism in *P. blakesleeanus*. Further investigations should focus on the involvement of MADA, MADB and MADC in the transcriptional regulation under continuous blue light.

4.1.8 The cryptochrome orthologue *cryA* gene

The photoactivation of the gene *cryA* after light treatments, continuous and a pulse, suggests that CRYA is also involved in light-dependent processes. But this gene and its product and the *wc-1/-2* genes are very diverse in their photoactivation and function among the fugal phyla. In *A. nidulans* CRYA, except for the photolyase function as DNA-repair enzyme, is moreover involved in light dependent gene regulation and thereby a regulator of sexual development indicating photoreceptor functions typical for chryptochromes. In *N. crassa* the *cry* gene product can bind double- and single-stranded DNA as well as RNA indicating a functional photolyase whereas no transcriptional regulatory activity could be found

at the time. On the other side the *cry* gene is photoinducible in a *wc-1* dependent manner and its expression is clock controlled (Bayram et al., 2008, 2010; Froehlich et al., 2010). The expression pattern of the *P. blakesleeanus cryA* gene is photoinducible by a pulse of all tested wavelength, controlled by the MADA/B/C complex similar to *N. crassa*. On the other hand no rhythmicity in its expression could be observed during the 11.5 hour time course. Thus next to the results of the transcriptional behavior of the *cryA* gene reported in the previous chapters of this work, a detailed knowledge about the regulation and function of the *cryA* gene and its gene product maybe anticipated which are still under investigation (Rodriguez-Romero, personal comment).

4.2 Gene expression in darkness

In most of the photosensory experiments the results were related to the dark "steady state" as a control. All physiological reactions of the organism are based on transcription to provide the appropriate enzyme syncrisis. In this work for the first time the gene expression was monitored during an important morphologically and physiologically developmental period of 11.5 hour in dark-grown mycelia, from hour 62 up to 73.5 after spore inoculation. This time course experiment was mainly made to determine the transcriptional level of analyzed genes, but furthermore shed light on the stability of gene expression, on possible rhythmical changes in transcription or even expression change due to the competence period of physiological developments. The results should serve also to exclude possible interference of the different light treatments.

The expression levels of chosen genes cover in total three orders of magnitude from 10^{-8}-10^{-5} a.u. whereas the expression levels of half of the genes lie in the range of 10^{-7} a.u. and none of the genes show higher expression levels than the housekeeping genes (Tab. 3.2.1.1, p. 40). These results can be useful in further experiments characterising new genes of interest and helpful for searching strongly inducible genes. They also show that different regulatory mechanisms take effect in darkness (Fig. 4.1.2.1, p. 99).

More evidence for this is given by the diversity of dark-expression levels of some genes in the *mad* mutant strains compared to the ones of the wildtype (Tab. /Fig. 3.2.6.6.1, p. 81, 82). For example, the most strongly lowered amounts of mRNA were exclusively found in the double and triple mutant strains$L51_{madAB}$ and

$L72_{madABC}$ for the *hsp90* genes, the *carB* and *wc-2* type genes. Thus the formation of the MAD complex by the *madA, madB* and *madC* gene products suggests their primary role in the maintenances of the dark-expression of their target genes (Fig. 4.1.2, p. 99). The dark-expression monitored during the time course show besides the gene specific variation of transcriptional levels, shows also that the stability of expression differs in a gene specific manner. This underscores the prediction of gene specific regulator elements associated with the MAD complex also in darkness and presumes a turnover of each component.

Among the *hsp90* genes *hsp9020* and *hsp9036* show most stable expression during the course of time. In continuous darkness these genes show the most strongly lowered mRNA amounts in the strains $L51_{madAB}$ and $L72_{madABC}$ in comparison to the wildtype. On the other hand in these mutant strains the genes *hsp901-3* and *ngf* show also a strongly lowered expression level in continuous darkness but their transcription during the time course is not as stable as the one of the genes *hsp9020* and *hsp9036*. In addition, only in the wildtype hsp901-3 shows a moderate enhanced expression level upon continuous blue light in comparison to the one in darkness. However, the regulation of dark-expression seems to be governed by the MAD complex and the discriminative stability of the dark-expression in the wildtype might be due to a turnover of the cis-acting regulatory elements (Fig. 4.1.2, p. 99). Most interestingly, the MAD complex controls not only the photoactivation of the remaining *wc* genes, but also their expression in dark (Fig. 3.2.6.6.1, p. 82). The presence of the MAD complex in darkness posed to activate its target genes directly after irradiations was mentioned and discussed by Sanz et al., 2010 as well.

The time course experiment was performed with the expectancy finding an endogenously rhythmic expression pattern of some genes indicating a circadian rhythm as in *N. crassa.* The circadian system known from *N. crassa* provides the primary model among the filamentous fungi for understanding circadian rhythm. Such endogenous cellular timekeepers are an ubiquitous biological example how a small number of genes can underlay a complex interactions controlling a wide variety of physiological and molecular key processes. Circadian rhythms share three basic properties. First an endogenous period of ~24 h (from lat. circa and dies: about a day) which persist upon constant conditions such as removed light/dark cycles. Second the rhythm is entrainable (reset) by external signals such as light or temperature. Third the period length of the rhythm should remain relatively constant with respect to temperature (temperature compensation). Circadian rhythms in fungi have been known for some time and most profoundly studied in *N. crassa*, primarily

because of its well-known genetics and biochemistry (Bell-Pedersen et al., 1996; Loros and Dunlap 2001; Dunlap and Loros 2006). The mechanism of the input and output components as well as the core oscillator the *frq* gene product and the WCC have been extensively studied (Lee et al., 2000; Liu et al., 2000). According to Dunlap and Loros (2006), the two photoreceptors, WC-1 and WC-2 of *N. crassa* generate the circadian rhythm via a transcriptional feedback loop with the core oscillator frequency (*frq*). As *P. blakesleeanus* has WC-1 and WC-2 type photoreceptors, a circadian rhythm similar to *N. crassa* was suspected.

Among the 25 analyzed genes most surprisingly only the genes *ago* and *dcr* show a rhythmical "up and down" in phase of their mRNA during the time course (Fig. 3.2.1.3, p. 37) which is discussed in 4.1.4, p. 94. No further transcriptional rhythmicity could be observed on the remaining analyzed genes. Thus circadian rhythm in *P. blakesleeanus* remains once more an area where further research is required since there has not been enough research on circadian rhythm in gene expression.

4.3. Red light absorbing intermediate of *wc*-type versus red light photoreceptor

4.3.1. Red light and its antagonistic effect on gene expression

The moderate changes on gene expression after continuous and a pulse of red light are the first red-light effects detected in the wildtype of *P. blakesleeanus*. All previous physiological red-light responses were only found in combination or related to blue light. In contrast to these observations red light itself affects the gene expression in the mycelia of *P. blakesleeanus* wildtype both, positively and negatively, when applied continuously as well as after a pulse of it (Fig. 3.2.3.1, p. 45). Thereby for some genes similar expression patterns as after blue light exposure were found. The strongest effects, the 13-fold enhancement of the *wctC* gene transcript, the 6.5-fold reduction of the *hsp90* gene transcript and a twofold photosuppression of the remaining *hsp90* genes were obtained after a pulse of red light. Under continuous red light the effects are less in strength similar to those after blue light treatment, whereas after red irradiation the strongest effects are a threefold photoactivation of the genes *hsp9020, wctB* and *myc* and the photosuppression is even weaker, generally only 10-20 %. This suggests very similar regulatory mechanisms for the photoregulation of the gene expression in response to both

wavelengths. But all the transcriptional red-light responses are moderate in comparison to the ones after blue light. Beside the above referred changes of gene expression most of the remaining red-light photoresponses are so slight which is why they seem to be negligible compared to the blue light effects. In contrast to the blue light treatments the transcriptional suppression preponderates and a remarkable numerical balance of induced and suppressed genes was observed. Both effects can occur at different strength on the same genes depending on the light treatment, continuous or a pulse of red, indicating a complex photoreceptor system, or rather complex regulatory mechanisms. Most interestingly all conceivable combinations of antagonistic effects of the different irradiations were found. The *hat10* gene expression for example is stronger enhanced after a pulse of red light than reduced after blue light, but upon continuous light exposure a revers expression was observed corroborating the prediction of a complex photoreceptor/transcription factor system.

So far the antagonistic and complex effect of red light is consistent with the previous experimental approaches in *P. blakesleeanus* wildtype. The antagonism of the negative photogravitropic bending away from far-UV light (280 nm) and the positive bending towards the blue light (454 nm) of the sporangiophores upon bilateral bichromatic irradiations is greatly reduced when red light was given from above (< 600 nm, 5 Wm^{-2}). Also the photogravitropic bending of ether far-UV or blue light is reduced by simultaneously applied overhead red light, depending on the ratio of the photon fluencies of both wavelengths, whereas red light itself is photogravitropically ineffectual (Galland et al., 1997). The interpretation of these results pointed always to a red light absorbing intermediate of the blue light photoreceptor(s).

4.3.2. The red-light photoreceptor paradigm

One example for red light intermediates of the LOV-domain containing blue light photoreceptors, is the Phot1-LOV1 domain of the green alga *Chlamydomonas reinhardtii*. A detailed characterization of its photocycle was provided by spectroscopic investigations (Kottke et al., 2003a). Blue light absorption of the chromophore FMN leads to an excited singlet state and fast intersystem crossing (ISC) in a few nanoseconds (ns) which leads to an excited triplet state with two red light absorbing forms (715 nm). The red-shifted intermediates then decay in 800 ns

and ~ 4 μs into a blue absorbing species (390 nm) that represents the flavin-cysteinyl adduct between the C4a atom of FMN and the sulfur of a nearby cysteine residue, Cys57. The adduct converts in several hundred seconds back to the original dark state depending on pH and salt concentration (Fig. 4.3.1, p. 106). According to this photocycle all previously obtained red-light effects in *P. blakesleeanus* and the postulated dichroic photoreceptor(s) are explainable with the ancillary presumption that the red-absorbing species are capable of photoreversal. In favor of the obtained changes on transcriptional level a red-shifted FAD/FMN species already presented or accumulating in darkness should be additionally presumed. Thus red light exposure can trigger both, suppression of the gene expression by shifting the red species back to the ground state which blocks or locks the dark form in place which is why a reduction of transcript occurs. On the other hand, transcriptional activation via completing the photocycle by formation of the cycteinyl adduct can also occur. This model is able to explain why the observed red light effects are less in strength compared to the ones of blue light. In the case of the transcriptional red-dependent suppression, the pool of red-absorbing intermediates already present in darkness would be shifted back to the ground state, or in the case of the red-dependent transcriptional activation, only the already in darkness existing red-intermediates could be converted to the active form. Thus the red light effects would reflect the pool of red-species in darkness. Bichromatic red and blue irradiations should result in a faster and/or more efficient generation of the signaling state, the cycteinyl adduct, leading to a pronounced gene expression (synergism) or in contrast a red/blue antagonism where red light forces the dark form resulting in a reduced expression in comparison to blue light alone. A non linear or not directly related transcriptional change after both red and bichromatic red and blue light treatments could be explained by the steady state of dark and red-shifted photoreceptor forms. Most interestingly a methionine exchange of the reactive cysteine (C57M) results in dramatic changes of the spectrometric and structural Phot-LOV1 (*C. reinhardtii*) properties. Instead of the red-shifted intermediates (715 nm a/b) and the following cycteinyl adducts (390 nm) after excitation, the formation of an irreversible neutral flavin radical, which attaches covalently at the position N5 to the methionine side chain (C-S-C) of the apoprotein with an absorption maximum at 675 nm could be assigned. This C57M-675 is extremely stable. It decayed back into the ground state on a time scale of hours (Kottke et al., 2003b). Thus maybe due to the codon usage bias of *P. blakesleeanus* the *wc-1* gene products consist also in part in a C144M-Δred which could explain the complex transcriptional photoresponses after

each blue, red and bichromatic red and blue light. Indeed this conforms to the results of the experimental gene expression studies for some genes.

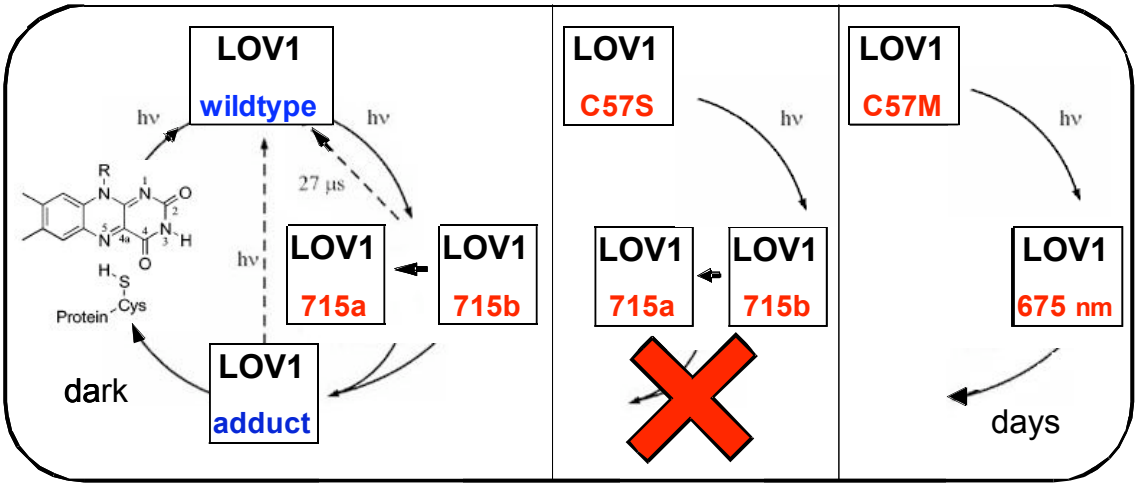


Fig. 4.3.1: Photocycle of wildtype Phot1-LOV1 with mutations of *C. reinhardtii*
The photocycle of wildtype and mutant phototropin Phot1- LOV1 domain from Clamydomonas reinhardtii deduced from time-resolved absorption spectroscopy. Adapted from Kottke et al. 2003a, 2003b, 2006. Upper boxes correspond to the dark form $LOV1_{447}$; LOV1-715, to the excited triplet state of the FMN-chromophore; LOV1-675, to the neutral flavoprotein radical and LOV1 adduct to the flavin-cyctein57-adduct (390 nm).

The gene expression after bichromatic red and blue irradiations is by its strength in general closer to the one of blue light, indicating similar or same regulatory mechanisms including the slight to moderate enhancement after continuous light, and the pronounced photoactivation of gene expression after a pulse. Referring to the above mentioned mechanism it seems coherent that neither any of the prominent red-light effects could be detected and that the strength of the blue-light depending transcriptional changes differs after both continuous and a pulse of bichromatic irradiations. Furthermore the obtained compensatory expression pattern of the genes *hsp90* and *carB* after a pulse of bichromatic light exposure supports this hypothesis. The red and blue antagonism is compensated by bichromatic light exposure meaning their transcriptional suppression after red and the enhancement after blue light yields an equal sum of both after a pulse of bichromatic irradiations. But in comparison to the photoactivation by blue light the bichromatic light exposure shows an antagonistic effect of both wavelengths similar

to the expression pattern of the genes *wctB* and *wctC* (see below). The genes *hspA*, *wcoA* and *wctD* show a positively synergistic red and blue light effect at about 30-50 % enhanced expression values after a pulse of bichromatic irradiation in comparison to the one of blue light alone. This would indicate a faster and more efficient formation of the signaling form according to the above proposed mechanism. On the other hand the genes *wctB* and *wctC* show a 25 % lower transcriptional enhancement after bichromatic rather than blue light treatment, suggesting a preferable photoreversion of the red-absorbing species towards the ground state by red light and therefore a reduction of the active form (Fig. 4.3.1, p.106). Other genes like the *cryA*, *wcoB* and *myc* show equal photoactivation after a pulse of blue and bichromatic light exposure suggesting an individually specific mechanism of photoregulation. This conjecture is conform with the diverse gene expressions after continuous light exposure compared to the ones after a pulse of light, as well as the ones after the different irradiations and it demonstrates once again the complexity of the transcriptional photoresponses. The exceptional photoactivation of the genes *hat10* and *hsp90* only under continuous blue light is one example. Another is, the unique transcriptional behavior of the *wctB* gene, which is equally activated under continuous and after a pulse of blue and bichromatic light treatments. The other genes presented above show either, a positive synergism or antagonism after continuous bichromatic irradiations in comparison to each, red or blue light. Interestingly, none of the genes show a compensatory effect as obtained for the gene expression of the *carB* and *hsp90* gene. For all these transcriptional photoresponses the Phot1-LOV1 photocycle of *C. reinhardtii* provides a substantial model bearing in mind the initial ancillary presumption (Fig. 4.3.1, p.106).

Double pulses are appropriate to prove in more detail the interaction of red and blue light on gene expression. According to the model and to the obtained individual gene specific transcription, the group of genes which shows a positively synergistic effect after bichromatic irradiations (*hspA, wcoA, wctD*) should also show a more pronounced expression after the double pulse blue followed by red light. Witch is why the red-shifted intermediate after excitation is also converted into the active form by red light, whereas the double pulse red then blue light, should show a lower enhancement due to the low concentration of the red-absorbing intermediate in dark (Fig. 4.3.1, p.106). Surprisingly only the *hspA* and *wcoA* gene expression is conform to the prediction whereas the *wctD* gene possesses more of a pronounced expression after the double pulse red followed by blue light.

For the group of genes which shows an antagonistic effect of red and blue light by a lowered expression after the bichromatic irradiations than after blue light alone (*wctB, wctC, carB, hsp90*) the transcriptional photoactivation should be stronger after the double pulse red followed by blue light. The pool of the red-absorbing species would be converted by red light expanding the pool of the dark form thus the following blue light pulse could be more efficient whereas the opposite order of wavelengths would expand the pool of red-shifted intermediate which is directly converted into the ground state by the following red light pulse. Among the group of these genes only the *carB* gene expression also shows the predicted pattern. The *hsp90* is equally suppressed, the *wctC* is ineffectual and due to technical problems no data are available for the *wctB* gene expression after the double pulses. Most surprisingly the *cryA* gene which shows an equal expression after blue and bichromatic irradiations is also equally transcribed after the double pulse blue then red light but reduced when red is given first followed by blue light. The *myc* gene expression after the double pulses is also affected but opposite in comparison to the *cryA* gene. Its transcription is more pronounced after the double pulse of red then blue light. The *hsp9020* is exceptionally photosuppressed by only blue then red light and the gene expression of the genes *ago* and *hsp9036* show a uniquely more pronounced effect after blue then red light than after the double pulse red then blue light. The transcriptional photoresponses after the double pulses underscore the complex and gene specific regulatory and photosensory mechanism in the wildtype of *P. blakesleeanus* (Fig. 4.1.2, p.99).

4.3.3 Time dependent gene expression

A similarly complex interaction of red and blue light was previously observed by Löser and Schäfer for the photogravitropism after bichromatic irradiations (450 and 605 nm). Red shows an antagonistic effect to blue light by a reduction of the phototropic response of low-intensity blue light only when given in a distinct time window of 2, 20 and 48 seconds after the pulse of blue light. In a later study they could demonstrate that red light also reduces the photogravitropic equilibrium angles when it was given 2 and 20 seconds immediately before the pulse of blue light.

This is consistent with the results of the time dependent gene expression after red light and the double pulses. Among the five analyzed genes, red light was most

effective five minutes after a pulse of it and the double pulse blue-red, except for the cryA gene, which is strongest suppressed only after the double pulse red-blue. This supports on the one hand a gene specific transcriptional regulatory mechanism and on the other hand it suggests a multichromophoric photoreceptor system with at least two separate photoreceptors. The "kinetics" of gene expression after red light exposure show the strongest transcriptional suppression after five minutes, followed by a gradually increase of *wcoA* transcript during the following 15 minutes, except for the genes *carB* and *hsp90* that show a maximum after ten minutes, and a decrease again after 20 minutes. This alternating expression pattern of the *carB* and *hsp90* genes was also observed after the double pulse red then blue light and additionally for the *madA* and less pronounced for the *cryA* gene. Whereas for all genes after the double pulse blue-red light the lowest expression level after five minutes increases gradually in the following time of observation, which is very similar to the photoactivation by blue irradiation alone, except for the initial suppression. These results imply a red-absorbing antagonistic photoreceptor constituent as stipulated earlier by many investigators.

4.3.4 Gene expression after varying the photon fluencies

An experimental approach to characterize further the red-absorbing photoreceptor component via the gene expression is a variation of the red photon fluency. Against the expectation, variations of the red light intensities affect the gene expression more than the initially chosen pulse of 40 Jm^{-2}. Thereby a ten times lower as well as a < 20 times higher photon fluence of red leads to a stronger suppression of gene expression than the pulse of 40 Jm^{-2}, affirming a complex photosensory system. Among all genes, 50 % are affected by the different red photon fluencies. Basically the *wc* and *hsp90* genes, as well as the *carB*, *ago* and *dcr* genes, show the strongest transcriptional effect after a pulse of the highest photon fluency is applied. Interestingly, the obtained results are somehow similar in strength to the ones after the blue light treatment, only in reverse. But for an individual gene the photoreversal effects, the transcriptional red-light suppression and the blue-light activation, are not correlated in strength. Only the *carB* gene shows an equal activation after blue than suppression after the lowest red light intensity. Unfortunately there is no dose response curve available for the blue light photoactivation of the *carB* gene. But most interestingly the strongest suppression of

the *carB* transcription was obtained after five minutes (40 Jm^{-2}) whereas five minutes later the reverse effect of a twofold induction compared to the dark control was measured. The photoinduction after blue light shows after eight minutes a sharp "drop down" of *carB* mRNA as published by Sanz (Sanz et al., 2010). Thus the time dependent expression pattern after red light is reverse to the one after blue light, suggesting a different regulatory mechanism which should include a separate red light photoreceptor beside the MAD complex. But before discussing this hypothesis more profoundly, the results of the gene expression in the mad mutant strains after the different irradiations need to be considered.

4.3.5 The red light photoreceptor paradigm versus MAD complex

This work provides clear but mostly divers changes on gene expression after red light treatments in the wildtype as well as in the analyzed *mad* mutant strains of *P. blakesleeanus*. A simple model of transcriptional photoregulation does not suffice to explain these results. Phytochromes are a widespread group of red light photoreceptors. In fungi their functions are less clear due to their recent identification. During the period of this work one phytochrome orthologue gene was manually annotated in *P. blakesleeanus* which is still under investigation. Thus the involvement of such a red light photoreceptor in transcriptional regulation remains theoretical. Among the highly conserved protein domains of the phytochromes none is capable to bind DNA. They can interact with other proteins by their C-terminal response regulator domain (Fig. 1.3.1, p. 7). That is another reason why their involvement on physiological processes and gene regulation remains to be elucidated for the whole fungal kingdom. Supposing their participation on gene expression based on the results of this work, additional interaction partners have to be postulated. These proteins may interact with the photoreceptor/transcription factor MAD complex as gene specific regulator elements, shown in Fig 4.1.2 (p. 99), or independently of the MAD complex with other cis-acting elements on the promoter regions.

On the other hand, the wide variety of reverse, antagonistic and reversible or irreversible red light effects on gene expression in *P. blakesleeanus* were indented to be explained by a red light absorbing flavin intermediate (FAD/FMN) of the WC-1 type blue light photoreceptor MADA. An analog profoundly studied, is given by the photocycle of the blue light photoreceptor phototropin Phot1-LOV1 of *C.*

reinhardtii (Fig. 4.3.1, p.106). The noncovalently bound flavin mononucleotide (FMN) of the Phot1-LOV1 domain after irradiation undergoes a photocycle forming a covalent photoadduct of a cysteine residue and the FMN. Mutational Exchanges of the reactive thiol group of the $cysteine_{144}$ residue of the apoprotein, closely posed to the chromophore, by serine or methionine lead to red light absorbing intermediates or a covalently attached neutral flavin radical and so to an impaired photocycle (Kottke et al., 2003a, 2003b, 2006).

Several presented changes in gene expression after red irradiations underline this proposal favorably. It gets more substantial with the ancillary presumption, that due to the codon usage of *P. blakesleeanus*, the *wc-1* gene products consist in part in a photoreversible C144M-Δred intermediate. This proposal holds for several obtained results and implicates the MAD complex as the dominant transcriptional photoregulator (Fig. 4.1.2, p. 99).

For some genes the changes in their transcript abundances after a pulse of red light are so pronounced or diverse from the ones of the wildtype that the above mentioned appear unlikely. Best examples are the strongly lowered mRNAs of the genes *carB* and the *wc-1* type *madA* and *madB* in the investigated single, double and triple *madA/B/C* mutant strains after a pulse of red light (Fig. 3.2.6.2.2, p. 71; Tab. 3.2.6.3.1, p. 74). These are some lowest expression levels detected after red light which underscore the central role of the MAD complex, the participation of other photoreceptors than only *madA/madB* and the axiomatic involvement of the *madC* gene product in transcriptional red-light regulation. According to the above mentioned model it implies a high ratio of exited triplet and/or neutral radical flavoprotein still presented in darkness which seems impossible, because the red-light absorbing intermediates are generated in consequence to actinic irradiation (Fig. 4.3., p.106).

It seems impossible to find a satisfactory explanation for all the obtained expression patterns even more taking into account the effects of green light on transcription (Fig. 3.2.5.5, p. 64). Green irradiation shows a synergistic effect after the double pulses green/blue and a more pronounced one after blue/green on the expression of the *cryA* gene in comparison to blue light alone. The transcription of the *carB* and *madA* genes shows after both double pulses strongly lowered mRNA levels similar to the ones obtained in the double mutant strain $L51_{madAB}$. The expression of the *wcoA* gene shows an antagonistic effect of green wavelength after the double pulses by reduced transcriptional levels than after only blue light.

The interpretation of these results follows the same pattern as for the one of red light. About green light sensing photoreceptors still less is known in fungi. In contrast to the deluge of sequence information, e.g. the findings of countless opsin orthologues in many of the fungal genome sequences there is no comparative model available that helpful in would be explaining the here found transcriptional green light effects. The NOP-1, first fugal opsin, characterized in *N. crassa* might be a strong candidate for a green light photoreceptor. Its gene is up regulated in the nop-1 deletion mutant strain after light exposure but its role in light-dependent transcriptional regulation remains to be investigated further (Olmedo et al., 2010). In the multimeric MAD complex model (Fig. 4.2.1, p. 99) the NOP-1 is for that reasons little separated from the MAD complex than the phytochrome. Even so, the results of this work are strongly evident with a closer interaction. Taken in account the findings of the *wc-1* gene *mcwc-1a* of *M. circinelloides* which show the involvement of its protein mainly in phototropism after blue and green wavelength, it is maybe not such separated from MAD complex as shown in Fig. 4.1.2. The interpretation of the red-absorbing intermediate should hold for green light as well as it was shown for other FAD containing blue light photoreceptors such as the *cry1* of *Arabidopsis thaliana* (Bouley et al., 2007).

4.4 MAD complex and gravitropic stimulation

The sporangiophores of the wildtype and the *madA-C* mutant strains of *P. blakesleeanus* show in response to the earth's gravitational acceleration or to centrifugal acceleration (g) negative gravitropism. The *madA-C* mutants are altered in phototropism only but not gravitropism. On a molecular level all the above discussed results show impressively that the gene products of the analyzed *mad* mutants are strongly involved in the photoregulation of gene expression. Although gravitropism in *P. blakesleeanus* has been studied for a long time, there is no convincing evidence regarding the transcriptional mechanism of graviperception.

The *madA* and *madB* gene products seem to be key components of the transcriptional photoactivation and also in the maintenance of distinct expression level in darkness. These results suggest that the MAD complex is a global key regulator of transcription in *P. blakesleeanus*. To investigate more profoundly its participation in gene regulation, for the first time, the transcription of the *wc-1* and

wc-2 type genes were monitored in the wildtype and the *mad* mutant strain $L72_{madABC}$ and $A202_{madC}$ after gravitropic stimulation.

The change in the direction of gravity is accompanied with differently expressed genes in the sporangiophores of *P. blakesleeanus*. The transcriptional changes detected in the wildtype are either a slight but constant drift, a transient suppression, or enhancement of gene expression during the five hours of gravitropic stimulation. Most interestingly the responses to gravity show some certain similarity to the transcriptional behavior after blue light irradiation. Especially in response to gravity, a suppressive drift of the *carB* and *carRA* transcription is remarkable. But, their mRNA level is during the first half an hour after gravitropic stimulation transiently lowered as quickly, as enhanced after a pulse of blue light. The *wc-1* type genes *madA* and *wcoA* respond to gravity-stimulation as well. Both genes in contrast to the *carB/RA* genes show a slightly increasing transcription during the entire period of observation. But their transient expression change at the same time after gravity-stimulation is also comparable to their photoresponsiveness after a pulse of blue light. After the first hour of stimulation the transient gravisuppression of the *madA* gene is twice, and the gravienhancement of the *wcoA* gene is a tenth as strong as after blue-light exposure. The *wcoA* gene shows an additional, more pronounced maximum of its transcription after the second hour of gravity-stimulation. Most interestingly, except for the ineffectual expression of the *madB* gene, the remaining w*c-2* genes are graviresponsive as well as the *wc-1* type genes, but not as compared to their photoresponsiveness. Their expression increases after the first hour closely to the same transcriptional level with a similar expression pattern as the *wcoA* gene. All of them show a second, more pronounced enhancement. Also the transcription of the *cryA* gene shows a first increase after one hour followed by an enhancement at the second hour after gravitropic stimulation. This kind of expression pattern, shown by four different genes, suggests the typical response for the transcriptional gravity-activation in the wildtype of *P. blakesleeanus*. On the contrary, the expression of these genes increases exponential during 15 – 20 minutes up to a saturated maximum under constant blue-light exposure (Sanz et al., 2009). Thus the transcriptional mechanisms after light- and gravity-stimulation are diverse. The fact that the *wc-1* and wc-2 type genes show a response to gravitropic changes suggests strongly an involvement of their gene products in both signal transduction chains. Most interestingly the changes of the above discussed expression patterns are alterated in the analyzed *mad* mutant strains. This underscores once more the

conjecture that the MAD complex and the *wc* genes are global key regulators in P. blakesleeanus.

These results show impressively an unexpected complex involvement of the *wc* type gene products in transcriptional regulation after photoactivation by different irradiations and gravity-stimulation. Thus it seems clear that the photoreceptor/transcription factor complex MAD has a central role in gene regulation of *P. blakesleeanus*. It is to be expected that these results are helpful understanding the molecular mechanism of transcription, although further investigations have to be completed.

Summary

Light is one of the most powerful abiotic factors triggering a multitude of developmental key processes of fungal biology and lifecycle. The filamentous fungus *Phycomyces blakesleeanus* has been a model organism in sensory physiology since more than 50 years. Therefore a linear flow of light information from sensors to effectors was assumed.

Since the isolation and characterization of behavioral *mad* mutant strains and even more since the recent identification of the set of *wc*-photoreceptor genes (*madA, madB*) molecular approaches of photophysiology were able to advance. The counterparts of *N. crassa* White Collar-1/-2 photoreceptors/transcription factors in *P. blakesleeanus* are the MADA and MADB proteins which form a photosensitive transcription factor complex (MAD complex) to govern many light-controlled processes in the fungus (Sanz et al., 2009).

In this work 25 genes encoding mainly five different functional protein groups, the *wc*-type photoreceptors, heat shock proteins, RNA-/chromatin-modulating enzymes, the ß-carotene enzymes and constituents of the cytoskeleton were used to move a step further in light dependent gene expression to gain new insights about the regulatory role of the *wc*-type photoreceptor mutants *madA and madB*. Therefore, a protocol was developed that consisted of a pulse of actinic light, which gave a non saturating transcriptional response, measured by the method of qRT-PCR. Not only the characterization of the blue light responses in both wildtype and *mad* mutant strains came into the focus of these approaches, but also the search for red light and gravity responses on a transcriptional level.

The gene expression in dark grown mycelia of the wildtype of *P. blakesleeanus* measured during an 11.5 hour time course shows different expression levels for each gene. The levels of expression are not correlated with a functional group of genes, indicating that the genes are individually regulated even in darkness. In the *mad* mutant strains the gene expression levels of more than half of the genes are different to the ones in the wildtype supporting this hypothesis and demonstrating the importance of the photosensitive transcription factor complex MAD. In contrast to the findings of *N. crassa* none of the genes show an endogenous rhythmicity of their expression.

In the wildtype of *P. blakesleeanus* many of the analyzed genes (60 %) show a response after exposure to continuous or a pulse of blue light. These photoresponses are mainly a transcriptional activation but a suppression of gene expression was

detected as well. Continuous light conditions can enhance the expression of 11 genes moderately, about three to 20-fold. After a pulse of it, the transcription of 15 genes is increased. The photoactivation after a pulse of blue light can be divided into a strong (20-80-fold) or moderate increase of mRNA in the wildtype which do not correlate with a functional group of genes, indicating that the genes are individually regulated. As an empirical rule, the increase of transcript after blue light exposure is higher, the lower the dark expression level is. Four genes show a moderate decrease of their transcript after both blue light treatments. After a pulse of blue light none of the single *mad* mutant strains show a complete suppression of the photoactivation but strongly diverse mRNA levels of the light induced genes compared to the wildtype. Only in the double *madAB* or triple *madABC* mutant strains an abolished photoactivation of transcription could be detected. It shows the dominant role of the MAD complex on the one hand and suggests a more complex regulatory mechanism on the other.

In addition to the blue light effects the influence of red light on transcription was investigated. Red light moderately modulates the gene expression, both positively and negatively. Some genes show photoreversible expression patterns after a pulse and continuous blue and red light. The time dependent red light effects on transcriptional level are different to the ones of blue light and for some genes show reverse patterns in the wildtype. For some genes mutation of the *wc*-type photoreceptors results in a more pronounced suppression after red than activation after blue light in the wildtype or increased transcription in the mutant strains while unaffected in the wildtype after a pulse of red light. The results demonstrate the complex regulatory mechanisms of gene expression in *P. blakesleeanus* suggesting the interaction of more than only the *madAB* photoreceptors.

The expression patterns after bichromatic light treatments support the notation of other than *wc*-type photoreceptors being involved in transcriptional regulation.

Gravity stimulation, for some genes, results in specific or transient expression changes in the wildtype, dramatically different to the ones in the *madC* and *madABC* triple mutant strains. These results underlie the central role of the *madAB* photoreceptors/transcription factors not only by integrating the light, but also the gravity signal as well and suggest the interaction of more than only the *madAB* photoreceptors in a gene regulation complex. A model of the gene regulation in *P. blakesleeanus* is presented with a set of proposal, as other photoreceptors and gene specific regulating elements.

Zusammenfassung

Licht zählt zu den wichtigsten abiotischen Faktoren. Es steuert eine Vielzahl von Schlüsselprozessen der pilzlichen Biologie und des Lebenszyklus. Der filamentöse Pilz *Phycomyces blakesleeanus* ist seit mehr als 50 Jahren ein Modellorganismus der sensorischen Physiologie. Eine lineare Signaltransduktionskette vom Lichtrezeptor zu den Effektoren wurde bisher postuliert.

Durch die Isolierung und Charakterisierung von Verhaltensmutanten (*mad)*, aber besonders durch die Identifizierung der Gruppe der *wc*-Fotorezeptorgenen (*madA, madB*) konnten große Fortschritte in der molekularen Photophysiologie erzielt werden. Das Pendant der White Collar-1/-2 Fotorezeptoren/Transkriptionsfaktoren in *N. crassa* sind in *P. blakesleeanus* die MADA- und MADB-Proteine, welche einen photosensitiven Transkriptionsfaktor-Komplex (MAD-Komplex) bilden, um multiple lichtkontrollierte Prozesse der pilzlichen Genese zu steuern (Sanz et al., 2009).

In dieser Arbeit wurden, aus fünf differenten funktionellen Proteingruppen wie den *WC*-Typ Fotorezeptoren, Hitzeschock-Proteinen, RNA-/Chromatik-Modulierenden Enzymen, Zytoskelettkomponenten und Enzyme der ß-Karotinbiosynthese, 25 Gene verwendet, um die Rolle der WC-Typ Fotorezeptoren in der fotoregulierten Genexpression näher zu untersuchen. Dazu wurde ein Protokoll entwickelt, in dem eine nicht saturierte transkriptionelle Änderung nach einem Puls von aktinischem Licht, mittels der qRT-PCR-Methode gemessen, determiniert wurde. Dabei stand nicht nur die blaulichtabhängige Transkriptionsänderung des Wildtyps und der mad-Mutanten im Vordergrund, sondern vor allem die Erforschung der Genexpression nach Rotlicht- und gravitropischer Stimulation.

Die Genexpression von in Dunkelheit gewachsenen Myzelien des Wildtyps von *P. blakesleeanus* wurde über einen Zeitraum von 11,5 Stunden gemessen. Sie zeigt differente transkriptionelle Niveaus der Gene. Diese korrelieren nicht mit ihren funktionellen Gruppen, was eine spezifische Genregulation postulieren lässt. In den *mad*-Mutanten ist die Transkription von mehr als der Hälfte der Gene verschieden von derjenigen des Wildtyps, was die Hypothese einer genspezifischen Regulation und die Wichtigkeit des photosensitiven Transkriptionskomplexes MAD untermauert. Im Gegensatz zu *N. crassa* konnte keine endogene Genexpressionsrhythmik nachgewiesen werden.

Im Wildtypstamm von *P. blakesleeanus* ist nach einem Blaulichtpuls oder

konstanterer Blaulichtbestrahlung die Transkription der meisten Gene (60 %) verändert. Dabei wurden hauptsächlich Expressionsaktivierungen, aber auch Inhibitionen festgestellt. Konstante Blaulichtbestrahlung führt bei 11 Genen zu einer moderaten, (3 bis 20-fachen), und bei 15 Genen zu einer starken (20 bis 80-fachen) transkriptionellen Induktion im Wildtyp. Diese unterschiedlichen Expressions-aktivierungen korrelieren nicht mit den funktionellen Gengruppen, was eine individuelle Genregulation vermuten lässt. Dabei gilt als empirische Regel, dass die Gene, mit dem geringsten Expressionsniveau in Dunkelheit, nach Blaulicht den höchsten Anstieg ihrer mRNA zeigen. In keinem der einfachen *mad*-Mutanten konnte nach einem Blaulichtpuls eine komplette Expressionsinhibition gemessen werden, vielmehr wurden stark vom Wildtyp abweichende Transkriptionsniveaus festgestellt. Nur in den Doppel-*madAB*- und Triple-*madABC*-Mutanten blieb eine Photo- aktivierung aus. Dies demonstriert die dominante Rolle des MAD-Komplexes einerseits und lässt einen komplexeren Regulationsmechanismus andererseits vermuten.

Zusätzlich zu den transkriptionellen Blaulichteffekten wurde die Genexpression nach Rotlichtbestrahlung erforscht. Rotlicht moduliert die Transkription sowohl positiv als auch negativ. Einige Gene zeigen photoreversible Expressionsänderungen nach einem Rotlichtpuls oder nach konstanterer Rotlicht-bestrahlung. Die zeitabhängigen transkriptionellen Rotlichteffekte sind im Wildtyp abweichend von denen nach Blaulichtbestrahlung, wobei einige sogar gegenteilige Änderungen zeigen. Mutationen an den WC-Typ Fotorezeptorgenen führen nach Rotlichtbestrahlung bei einigen Genen zu einer stärkeren Inhibition der Genexpression als der entsprechenden Aktivierung nach Blaulicht im Wildtyp. Diese Ergebnisse verdeutlichen einmal mehr die Komplexität der Genregulation in *P. blakesleeanus*, welche die Beteiligung von mehr als nur den *madAB* Fotorezeptoren nahe legt. Die Expressionsänderungen nach bichromatischer Bestrahlung unterstreichen die Vermutung, dass weitere Fotorezeptoren in der transkriptionellen Regulation involviert sind.

Gravitropische Stimulation führt bei einigen Genen im Wildtyp zu einer transienten Expressionsänderung, welche sich von der in den *mad*-Mutanten deutlich unterscheidet. Diese Ergebnisse unterstreichen die zentrale Rolle des MAD-Komplexes für die Genregulation nicht nur bei der Integration des Lichtsignals, sondern auch bei der des Schwerereizes, und deuten auf weitere regulatorische Elemente hin. Ein Genregulationskomplex-Modell mit genspezifischen regulatorischen Elementen und anderen Fotorezeptoren wird diskutiert.

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Appendix A: *Mad*-mutant strains

Tab. I: Photoresponses of the class I *madA*-mutant strain of *P. blakesleeanus.*

Photoresponses	*madA*-mutant	Reference
photogravitropic threshold	10^{-4} elevated	Bergmann et al., 1973; Galland 1983
photogravitropic epuilibrium	like wt	Galland 1983
threshold light-growth response	± wildtype	Foster & Lipson 1973
dark –adaptation	slower	Galland & Russo 1984a
light-adaptation	alterated	"
UV sensitivity		Campuzano et al., 1996
photodifferentiation microphores	elevated	Galland & Russo 1979; Russo & Galland 1980; Russo et al., 1980;
……..macrophores		Corrochano & Cerdá-Olmedo 1991; Jayaram et al., 1980;
red-light response	no LIACs	Schmidt & Galland 1998
negative gravitropism	normanl, like wt	Russo 1977
avoidence response	normanl, like wt	"
ethylene response	normanl, like wt	"
contain riboflavin	only 10%	Hohl et al., 1992b
decrease of ADH	similar to wt	Garcés & Medina 1985
malatdehydrogenase	not tested	
photoaccumulation of ß-carotene	strongly reduced	Jayaram et al., 1980
gene photoinduction		
carB/carRA	stronly reduced	? This work
hspA	stronly reduced	Rodriguez-Romero & Corrochano 2006
wc-1	stronly reduced	Idnurm et al., 2006; Sanz et al., 2009
wc-2	stronly reduced	Sanz et al., 2009

Tab. II: Photoresponses of the class I *madB*-mutant strain of *P. blakesleeanus.*

Photoresponses	***madB*-mutant**	**Reference**
photogravitropic threshold	10^{-5} elevated	Bergmann et al., 1973
photogravitropic epuilibrium	alterated	Galland & Lipson 1985b
threshold light-growth response	10^{-6} elevated	Foster & Lipson 1973
dark –adaptation	slower	Galland & Russo 1984a
light-adaptation	slower	
UV sensitivity	no prominent peak	Campuzano et al., 1996
Photodifferentiation: microphores	similar/> to madA	Galland & Russo 1979; Russo & Galland 1980; Russo et al., 1980; Corrochano & Cerdá-Olmedo 1991; Jayaram et al., 1980
photodifferentiation: macrophores	similar/> to madA	
red-light response	no LIACs	Schmidt & Galland 1998
negative gravitropism	normal, like wt	Russo 1977
avoidence response	normal, like wt	"
ethylene response	normal, like wt	"
riboflavin/pterine	alterated pterin pattern	Hohl et al., 1992b
decrease of ADH	similar to wt	Garcés & Medina 1985
Malate dehydrogenase	50% < than wt	Rodriguez-Aparico et al., 1987
photoaccumulation of ß-carotene	strongly reduced	Jayaram et al., 1980
carB/carRA	stronly reduced	? This work
hspA	stronly reduced	Rodriguez-Romero & Corrochano 2006
wc-1	stronly reduced	Idnurm et al., 2006; Sanz et al., 2009
wc-2	stronly reduced	Sanz et al., 2009

Tab. III: Photoresponses of the class I *madC*-mutant strain of *P. blakesleeanus.*

Photoresponses	*madC*-mutant	Reference
photogravitropic threshold	10^{-6} elevated	Bergmann et al., 1973
photogravitropic epuilibrium	alterated	Galland & Lipson 1985b
threshold light-growth response	10^{-6} elevated	Foster & Lipson 1973
dark –adaptation	slower	Galland & Russo 1984a
light-adaptation	slower	"
UV sensitivity	no prominent peak	Campuzano et al., 1996
Photodifferentiation: microphores	uneffected	Galland & Russo 1979; Russo & Galland 1980; Russo et al., 1980
Photodifferentiation: macrophores	uneffected	Corrochano & Cerdá-Olmedo 1991; Jayaram et al., 1980;
red-light response	no LIACs	Schmidt & Galland 1998
negative gravitropism	normal, like wt	Russo 1977
avoidence response	normal, like wt	"
ethylene response	normal, like wt	"
riboflavin/pterine	alterated pterin pattern	Hohl et al., 1992b
Photoaccumulation of β-carotene	normal, like wt	Jayaram er al. 1980
decrease of ADH	similar to wt	Garcés & Medina 1985
photoaccumulation of ß-carotene		Jayaram et al., 1980
carB/carRA	stronly reduced	This work
hspA	elevated/higher	Rodriguez-Romero & Corrochano 2006
wc-1	± wildtype	Idnurm et al., 2006; Sanz et al., 2009
wc-2	± wildtype	Sanz et al., 2009

Tab. IV: Photoresponses of the class I double *mad* mutant strains of *P. blakesleeanus.*

Double mutants		Reference
madAmadB	completely blind	Lipson & Terasaka, 1981
madBmadC	completely blind	"
madAmadC	madC is epistatic over madA	Galland and Lipson, 1985b
madAmadBmadC	completely blind	

Appendix B: Blue/Red light effects

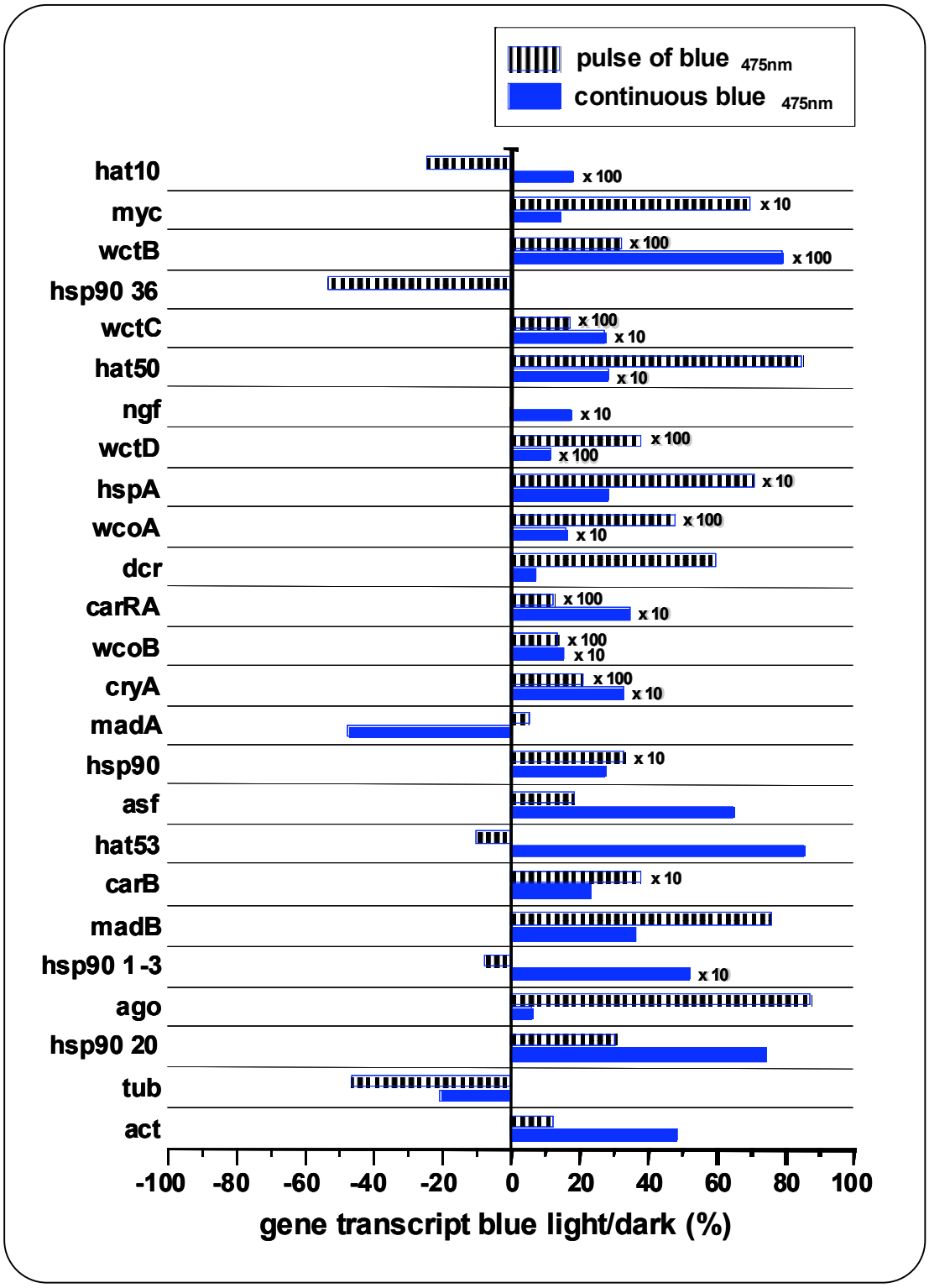

Fig. S.3.2.1.1: Summary of blue light effects on gene expression in per cent Gene transcript after continuous and 20 minutes after a pulse of blue$_{475}$ light normalized against the corresponding expression values for darkness. Gene expression was examined by qRT-PCR in 70 hours old mycelia of the wildtype NRRL1555.

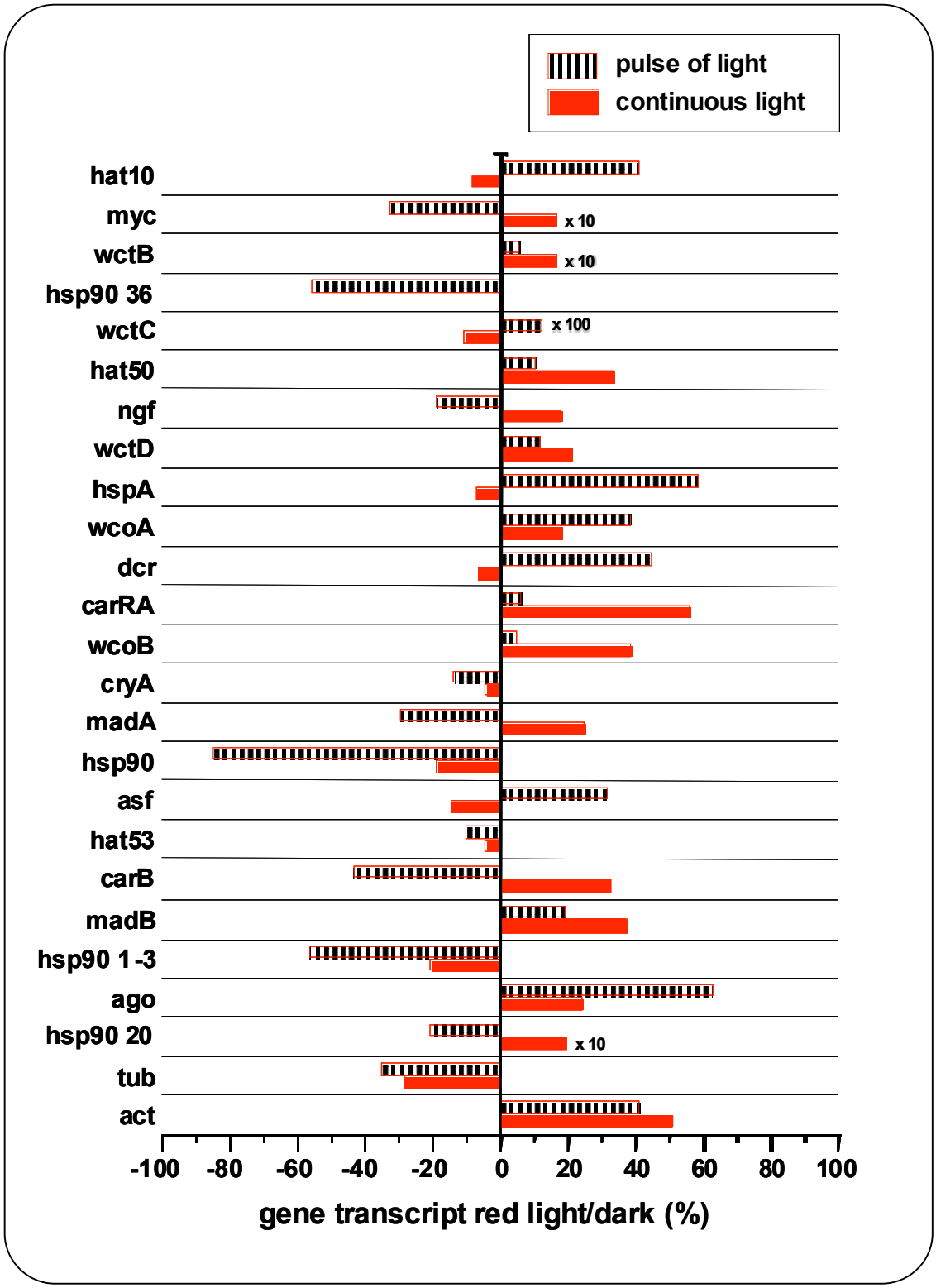

Fig.3.2.3.1: **Summary of blue light effects on gene expression in per cent** Gene transcript after continuous and 20 minutes after a pulse of $blue_{475}$ light normalized against the corresponding expression values for darkness. Gene expression was examinated by qRT-PCR in 70 hours old mycelia of the wildtype NRRL1555.

Appendix C: Expression values of all genes (wildtype)

Tab. S.1: Average values of gene expression levels (a.u.) in 70 hours dark grown mycelia (n) of *P. blakesleeanus* wildtype NRRL1555 (-), 20 minutes after different irradiations (40 Jm^{-2}, 25 s).D-dark, c-stands for continuous and p for pulse of R = red_{630}, B = $blue_{475}$, (RB) = bichromatic red and blue, BR = double pulse of blue followed by red and RB = double pulse red then blue light.

act	D n=39	cR n=7	Rp n=12	cB n=12	Bp n=15	c(RB) n=8	(RB)p n=8	BRp n=7	RBp n=7
av	$3.2x10^{-5}$	$4.7x10^{-5}$	$4.4x10^{-5}$	$4.6x10^{-5}$	$3.5x10^{-5}$	$3.8x10^{-5}$	$3.7x10^{-5}$	$5.4x10^{-5}$	$3.1x10^{-5}$

tub	D n=40	cR n=8	Rp n=12	cB n=12	Bp n=15	c(RB) n=8	(RB)p n=8	BRp n=7	RBp n=7
av	$2.9x10^{-5}$	$2.1x10^{-5}$	$1.9x10^{-5}$	$2.3x10^{-5}$	$1.6x10^{-5}$	$2.0x10^{-5}$	$1.5x10^{-5}$	$2.7x10^{-5}$	$1.3x10^{-5}$

hsp 90	D n=40	cR n=7	Rp n=14	cB n=11	Bp n=15	c(RB) n=7	(RB)p n=7	BRp n=7	RBp n=7
av	$9.7x10^{-7}$	$7.9x10^{-7}$	$1.5x10^{-7}$	$1.2x10^{-6}$	$4.2x10^{-6}$	$2.0x10^{-6}$	$4.6x10^{-7}$	$4.9x10^{-7}$	$3.3x10^{-7}$

hsp90 20	D n=9	cR n=3	Rp n=9	cB n=3	Bp n=6	c(RB) n=3	(RB)p n=1	BRp n=3	RBp n=3
av	$1.4x10^{-5}$	$4.0x10^{-5}$	$1.1x10^{-5}$	$2.4x10^{-5}$	$1.8x10^{-5}$	$1.7x10^{-5}$	$1.1x10^{-5}$	$7.2x10^{-7}$	$1.3x10^{-5}$

hsp 1-3	D n=13	cR n=3	Rp n=9	cB n=3	Bp n=10	c(RB) n=3	(RB)p n=6	BRp n=3	RBp n=3
av	$2.2x10^{-6}$	$1.7x10^{-6}$	$9.5x10^{-7}$	$1.4x10^{-5}$	$2.0x10^{-6}$	$3.5x10^{-6}$	$1.3x10^{-6}$	$5.9x10^{-7}$	$1.0x10^{-6}$

hsp90 36	D n=6	cR	Rp n=6	cB	Bp n=3	c(RB)	(BR)p n=3	BRp n=3	RBp n=3
av	$8.2x10^{-8}$		$3.6x10^{-8}$	$3.8x10^{-8}$	$3.8x10^{-8}$		$7.6x10^{-8}$	$2.5x10^{-8}$	$1.4x10^{-7}$

hspA	D n=32	cR n=4	Rp n=5	cB n=8	Bp n=8	c(RB) n=4	(RB)p n=1	BRp n=4	RBp n=4
av	$2.3x10^{-7}$	$2.2x10^{-7}$	$3.7x10^{-7}$	$3.0x10^{-7}$	$1.9x10^{-6}$	$2.9x10^{-7}$	$2.8x10^{-6}$	$1.1x10^{-6}$	$6.5x10^{-7}$

carB	D n=36	cR n=5	Rp n=9	cB n=9	Bp n=8	c(RB) n=5	(RB)p n=5	BRp n=7	RBp n=7
av	1.9×10^{-6}	2.5×10^{-6}	1.1×10^{-6}	2.3×10^{-6}	9.1×10^{-6}	1.9×10^{-6}	3.5×10^{-6}	1.4×10^{-6}	4.4×10^{-6}

carRA	D n=9	cR n=3	Rp n=6	cB n=3	Bp n=10	c(RB) n=3	(RB)p n=6	BRp n=3	RBp n=3
av	5.0×10^{-7}	8.9×10^{-7}	6.0×10^{-7}	2.5×10^{-6}	7.9×10^{-6}	1.2×10^{-6}	1.0×10^{-6}	2.6×10^{-7}	5.4×10^{-7}

cryA	D n=38	cR n=8	Rp n=15	cB n=11	Bp n=16	c(RB) n=7	(RB)p n=7	BRp n=7	RBp n=7
av	7.0×10^{-7}	6.7×10^{-7}	6.0×10^{-7}	3.0×10^{-6}	1.6×10^{-5}	4.8×10^{-6}	1.6×10^{-5}	1.5×10^{-5}	7.6×10^{6}

madA	allD n=41	cR n=8	Rp n=15	cB n=12	Bp n=18	c(RB) n=8	(RB)p n=7	BRp n=7	RBp n=7
av	9.4×10^{-7}	1.2×10^{-6}	6.6×10^{-7}	4.9×10^{-7}	9.8×10^{-7}	4.4×10^{-7}	8.3×10^{-7}	1.9×10^{-6}	5.5×10^{-7}

wcoA	D n=41	cR n=8	Rp n=15	cB n=12	Bp n=18	c(RB) n=8	(RB)p n=7	BRp n=7	RBp n=7
av	3.1×10^{-7}	3.7×10^{-7}	4.3×10^{-7}	8.3×10^{-7}	1.5×10^{-5}	6.2×10^{-7}	2.3×10^{-5}	2.7×10^{-5}	1.0×10^{-5}

wcoB	allD n=13	cR n=3	Rp n=6	cB n=3	Bp n=7	c(RB) n=3	(RB)p n=3	BRp	RBp
av	6.3×10^{-7}	8.7×10^{-7}	6.6×10^{-7}	1.6×10^{-6}	9.4×10^{-6}	1.4×10^{-6}	9.0×10^{-6}		

madB	D n=11	cR n=3	Rp n=7	cB n=3	Bp n=3	c(RB) n=3	(RB)p n=3	BRp	RBp
av	2.0×10^{-6}	2.7×10^{-6}	2.4×10^{-6}	2.7×10^{-6}	3.5×10^{-6}	1.6×10^{-6}	2.9×10^{-6}		

wctB	D n=11	cR n=3	Rp n=5	cB n=3	Bp n=5	c(RB) n=3	(RB)p n=3	BRp	RBp
av	2.3×10^{-8}	6.2×10^{-8}	2.5×10^{-8}	1.9×10^{-6}	7.5×10^{-6}	1.5×10^{-6}	6.4×10^{-6}		

wctC	D n=11	cR n=3	Rp n=9	cB n=3	Bp n=6	c(RB) n=3	(RB)p n=6	BRp n=3	RBp n=3
av	1.0×10^{-7}	9.0×10^{-8}	1.3×10^{-6}	3.7×10^{-7}	1.8×10^{-6}	1.1×10^{-7}	1.5×10^{-6}	9.2×10^{-8}	1.5×10^{-7}

wctD	D n=11	cR n=3	Rp n=9	cB n=3	Bp n=8	c(RB) n=3	(RB)p n=5	BRp n=3	RBp n=3
av	1.7×10^{-7}	2.1×10^{-7}	1.9×10^{-7}	2.2×10^{-6}	6.8×10^{-6}	1.7×10^{-6}	9.3×10^{-6}	2.0×10^{-6}	6.7×10^{-6}

ago	D n=33	cR n=5	Rp n=9	cB n=9	Bp n=12	c(RB) n=5	(RB)p n=5	BRp n=7	RBp n=7
av	2.9×10^{-6}	3.6×10^{-6}	4.8×10^{-6}	3.1×10^{-6}	5.5×10^{-6}	3.9×10^{-6}	3.0×10^{-6}	1.0×10^{-5}	6.9×10^{-6}

dcr	D n=33	cR n=5	Rp n=9	cB n=9	Bp n=12	c(RB) n=5	(RB)p n=5	BRp n=7	RBp n=7
av	3.2×10^{-7}	3.0×10^{-7}	4.7×10^{-7}	3.5×10^{-7}	5.2×10^{-7}	2.7×10^{-7}	3.4×10^{-7}	8.7×10^{-7}	3.7×10^{-7}

asf	D n=13	cR n=3	Rp n=9	cB n=3	Bp n=10	c(RB) n=3	(RB)p n=6	BRp n=3	RBp n=3
av	1.1×10^{-6}	9.8×10^{-7}	1.5×10^{-6}	1.9×10^{-6}	1.4×10^{-6}	1.1×10^{-6}	1.1×10^{-6}	1.0×10^{-6}	1.1×10^{-6}

ngf	D n=13	cR n=3	Rp n=9	cB n=3	Bp n=10	c(RB) n=3	(RB)p n=6	BRp n=3	RBp n=3
av	1.5×10^{-7}	1.8×10^{-7}	1.2×10^{-7}	4.2×10^{-7}	1.5×10^{-7}	2.1×10^{-7}	1.3×10^{-7}	5.4×10^{-8}	7.3×10^{-8}

hat 10	D n=9	cR n=3	Rp n=9	cB n=3	Bp n=6	c(RB) n=3	(RB)p n=5	BRp n=3	RBp n=3
av	1.2×10^{-8}	1.1×10^{-8}	1.7×10^{-8}	2.3×10^{-7}	9.1×10^{-9}	1.2×10^{-8}	1.2×10^{-8}	5.9×10^{-9}	7.6×10^{-9}

hat 50	D n=9	cR n=3	Rp n=9	cB n=3	Bp n=6	c(RB) n=3	(RB)p n=6	BRp n=3	RBp n=3
av	1.7×10^{-7}	1.8×10^{-7}	1.5×10^{-7}	5.3×10^{-7}	2.6×10^{-7}	2.5×10^{-7}	2.4×10^{-7}	1.8×10^{-7}	1.6×10^{-7}

hat 53	D n=9	cR n=3	Rp n=9	cB n=3	Bp n=6	c(RB) n=3	(RB)p n=6	BRp n=3	RBp n=3
av	1.7×10^{-6}	1.7×10^{-6}	1.6×10^{-6}	3.2×10^{-6}	1.6×10^{-6}	1.9×10^{-6}	1.6×10^{-6}	8.4×10^{-7}	1.3×10^{-6}

myc	D n=9	cR n=3	Rp n=9	cB n=3	Bp n=6	c(RB) n=3	(RB)p n=5	BRp n=3	RBp n=3
av	2.0×10^{-8}	5.4×10^{-8}	1.4×10^{-8}	3.1×10^{-7}	1.6×10^{-7}	2.1×10^{-7}	1.8×10^{-7}	7.3×10^{-8}	9.9×10^{-8}

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Danksagung

Ich danke Herrn Prof. Dr. Paul Galland für die offene und freie Gestaltung des interessanten Themas. Besonderer Dank gilt seiner Großzügigkeit für die Umsetzung und die zahlreichen, immer wieder neuen Anregungen, sowie seiner ständigen Diskussionsbereitschaft.

Herrn Prof. Dr. Alfred Batschauer danke ich für die Zweitkorrektur dieser Arbiet und der freizügigen Bereitstellung der verschiedensten Geräte, was besonders in der Anfangsphase dieser Arbeit äußerst hilfreich war.

Ich danke Herrn Dr. Franz Grolig für seine Anregungen und Ratschläge, sowie die entspannte Arbeitsatmosphäre.

Ich danke Herrn Peter Gäbelein für das Korrekturlesen meiner Arbeit und den ermutigenden Gesprächen.

Marco Göttig und Sigrid Völk danke ich für die Herstellung der Sporenstammlösungen, der Medien sowie ihrer Hilfeleistung bei der praktischen Versuchsdurchführung.

Bei Herrn Prof. Dr. Uwe Maier bedanke ich mich für die Benutzung des Mastercyclers. Bei Herrn Dr. Stephan Zauner bedanke ich mich für die Sequenzierungen.

Ich danke Herrn Dr. Keram Pfeiffer für seine hilfreichen Anregungen, Vorschläge und praktischen Tipps zur Auswertung der Versuchsergebnisse. Ganz besonder Dank gilt vor allem seiner ständigen Hilfsbereitschaft und moralischen Unterstützung.

Ich danke Tomoko Oizumi ganz herzlich für ihre geduldige und stets aufbauende Unterstützung und ihrer Fähigkeit all den temporären Rückschlägen und meinen Verstimmungen den Respekt der Herausforderung abzugewinnen.

Meinen lieben Freunden danke ich für ihre Begleitung und Rücksichtsnahme in dieser nicht immer einfachen Zeit. Für all die schönen Momente und Unternehmungen.

Mein ganz besonderer Dank glilt meiner Familie, meinen Eltern und Geschwistern. Sie haben mir nicht nur mit finanzieller Hilfe das Studium ermöglicht, sondern vor allem haben sich mich in all den Jahren mir den Rücken gstärkt.

Erklärung

ich versichere, dass ich meine Dissertation

”Gene expression in *Phycomyces blakesleeanus*
after light and gravity stimulation“

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

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen
Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg:_____ _____

(Ort/Datum) (NormanDitmar Seger)