Coordination of two different genomes in response to light and stress

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“It always seems impossible until it´s done.”

-Nelson Mandela

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Abstract

About 1.5 billion years ago, a photosynthetic bacteria was engulfed by a eukaryotic cell which initiated a symbiotic relationship and the evolution of the chloroplast as we know it today. The chloroplast has retained its own genome encoding for a number of proteins required for the function of the chloroplast. However, during the evolution most genes were transferred to the nucleus and the chloroplast is thus dependent on the nucleus to provide the majority of proteins necessary for its function. The distribution of genes encoding plastid proteins between two different cellular compartments requires a tight communication in order to coordinate gene expression during different growth conditions. The focus of my PhD studies has been to elucidate signalling pathways between the chloroplast and nucleus that enables the coordination of these two genomes in response to light and stress. The results in this thesis demonstrate that chloroplast retrograde signals triggered by changes in tetrapyrrole levels are important both in response to day-night cycles and during the response to stress. We identified a cytosolic regulatory complex and a novel mechanism that could explain how the tetrapyrrole-mediated signal can be transduced from the plastid to the nucleus and regulate nuclear gene expression in response to changes in the environment. My work further demonstrates that the tetrapyrrole-triggered plastid signalling pathway integrates with the circadian clock in order to fine-tune nuclear gene expression during photoperiodic conditions. These findings provide novel insight into how clock components and plastid signals converge in order to obtain the proper output. I have also examined the regulation of nuclear gene expression in response to redox and early-light signals by identifying transcription factors responding to these signals. My work demonstrates a novel mechanism by which redox-regulation of specific transcription factors directly links cellular redox status to gene regulation. The identified transcription factors were further shown to regulate nuclear genes encoding plastid proteins and they are of particular importance for anterograde control during the early light response and establishment of photomorphogenic growth.

Sammanfattning

Hur två olika genom samarbetar under ljus och stress respons

En av de mest fascinerande sakerna med växter är deras förmåga att överleva stora variationer i växtmiljön fastän de sitter fast och inte kan fly undan skadliga faktorer. Hur växter kan känna av och rätta sig efter förändringar i omgivningen är ett viktigt forskningsområde för att öka vår förståelse om hur växter kan anpassa sig till olika miljöer. Syftet med min avhandling har varit att klargöra vilka mekanismer och signalvägar växter använder för att överleva, anpassa sig, och växa under olika ljus och stress-förhållanden. Kloroplasten är en organell som är specifik för växtceller, där sker den livsviktiga fotosyntesen och produceras det gröna klorofyllet. Solljus är en avgörande faktor för att fotosyntesen ska fungera, men är även väldigt viktigt för andra processer i växtcellen. Olika ljusförhållanden fungerar som information för växten som kan avgöra vilken tid på dagen eller året det är och om det är för mycket eller för lite ljus tillgängligt. Dessa signaler kan bland annat upptäckas av kloroplasten och påverkar hur växten växer, deras utveckling och överlevnad. Mina studier har fokuserat på hur kloroplasten och cellkärnan kan kommunicera med varandra för att kunna växa på bästa möjliga sätt under olika förhållanden. I cellkärnans genom finns det mesta av växtcellens DNA som kodar för och producerar proteiner som cellen behöver. Kloroplasten härstammar från en frilevande fotosyntetisk bakterie som integrerades med en eukaryotisk cell för ungefär 1,5 miljarder år sedan och därefter utvecklades till den växtcell vi känner till idag. Kloroplasten har därför även ett eget bakterie-genom och kan själva producera vissa proteiner, men är beroende av att cellkärnan producerar de allra flesta proteiner de behöver. Det här betyder att växtcellen har två olika organeller som bestämmer vilka kloroplast-proteiner som ska produceras och att det därför är väldigt viktigt att de kan kommunicera med varandra för att koordinera när och vart de olika proteinerna behövs. Genom mina studier har vi identifierat en ny mekanism som förklarar hur signaler kan överföras från kloroplasten till cellkärnan och informera om vilka gener som ska slås på eller stängas av. Den här signalvägen är viktig både under stress för att skydda växten mot exempelvis för mycket ljus och under normala förhållanden för att finjustera vilka proteiner som behövs. Vi har även identifierat specifika proteiner i cellkärnan som påverkas av tidiga ljussignaler och reglerar uttrycket av gener som är viktiga under kloroplastens utveckling från grodd till planta.

List of papers

I Peter Kindgren, **Louise Norén**, Juan de Dios Barajas López, Jehad Shaikhali and Åsa Strand. Interplay between HEAT SHOCK PROTEIN 90 and HY5 controls *PhANG* expression in response to the GUN5 plastid signal. *Molecular Plant; 5(4):901-13, 2012*

II **Louise Norén**, Peter Kindgren, Paulina Stachula, Mark Rühl, Maria E. Eriksson, Vaughan Hurry and Åsa Strand. Circadian and Plastid Signaling Pathways Are Integrated to Ensure Correct Expression of the *CBF* and *COR* Genes during Photoperiodic Growth. *Plant Physiology; 171:1392–1406, 2016*

III Jehad Shaikhali, **Louise Norén**, Juan de Dios Barajas López, Vaibhav Srivastava, Janine König, Uwe H. Sauer, Gunnar Wingsle, Karl-Josef Dietz and Åsa Strand. Redox-mediated Mechanisms Regulate DNA Binding Activity of the G-group of Basic Region Leucine Zipper (bZIP) Transcription Factors in *Arabidopsis*. *The Journal of Biological Chemistry; 287(33):27510-25, 2012*

IV **Louise Norén**, Yan Ji, Kate Howell, Ian Small and Åsa Strand. The role of bZIP16, bZIP68 and GBF1 in early light response. *Manuscript in preparation, 2016*

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The papers will be referred to by their roman numbers in the following text.

Introduction

The animal and plant cells are structurally very similar and they both contain membrane-enclosed compartments such as the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, peroxisomes and lysosomes. Many cellular processes function in the same way in both cell types, however there are a few significant differences that makes the plant cell a plant cell. In addition to the cell wall and a larger vacuole, chloroplasts are structures which generally define plants and enable their function as photosynthetic organisms. Chloroplasts are descended from an ancestral free-living photosynthetic bacteria and have retained their own genome which encodes a number of functional proteins. However, the majority of the ancestral genes were transferred to the nucleus during evolution and the chloroplast is therefore dependent on the nuclear genome to provide necessary proteins for its function. The photosynthetic machinery is thus assembled using proteins encoded both in the chloroplast and nucleus which generates a problem of coordinating the activities of these two different genomes. Despite the importance of chloroplast function and photosynthesis, it is not yet fully understood how the chloroplast and nucleus communicate in order to ensure the correct production and assembly of proteins. During my studies I have focused on signalling pathways between the nucleus and chloroplast, aiming to elucidate how these two different compartments communicate during different growth conditions.

Structure and function of the chloroplast

Features of the chloroplast

The chloroplasts contain the light-absorbing pigment chlorophyll and are responsible for the process of photosynthesis where energy from sunlight is captured and stored in the form of carbohydrates within the cell. Chloroplasts are members of the plastid family in plants which also includes the starch-rich amyloplasts and pigment-storing chromoplasts for example. Amyloplasts are found in non-green tissues such as underground tubers, roots and seeds where they are important for the storage of starch and energy. Chromoplasts accumulate pigments and provide the attractive colours of flowers and fruits (Jarvis and Lopez-Juez 2013). Depending on their developmental stage and surrounding conditions, plastids are involved in many different important processes in addition to photosynthesis. These include the synthesis of amino acids, fatty acids, terpenoids, various pigments and hormones and the assimilation of nitrogen and sulfur

(Solymosi and Schoefs 2010; Pogson and Albrecht 2011; Jarvis and Lopez-Juez 2013).

There are between 20-100 chloroplasts in each cell and the dimensions of a chloroplast resembles that of a bacterium with a diameter of 5-10 µm **(Fig. 1A)** (Jarvis and Lopez-Juez 2013). The chloroplast is surrounded by an envelope membrane consisting of two lipid bilayers with an intermembrane space between the layers **(Fig. 1B)**. Inside the chloroplast there is an extensive internal membrane system with thylakoids packed into grana stacks and interconnected through stroma lamellae. This membrane system provides a compartmentalization between the stroma and the lumen which is necessary for the photosynthetic process **(Fig. 1B)**. The thylakoid membranes contain the chlorophyll molecules and all the components of the photosynthetic electron transport and are thus the site where light energy is captured and converted into chemical energy. The trapped energy from the light is used to drive carbon fixation and synthesis of carbon compounds in the stroma. The carbon compounds can thereafter be exported to the cytosol and used in other cellular processes throughout the plant.

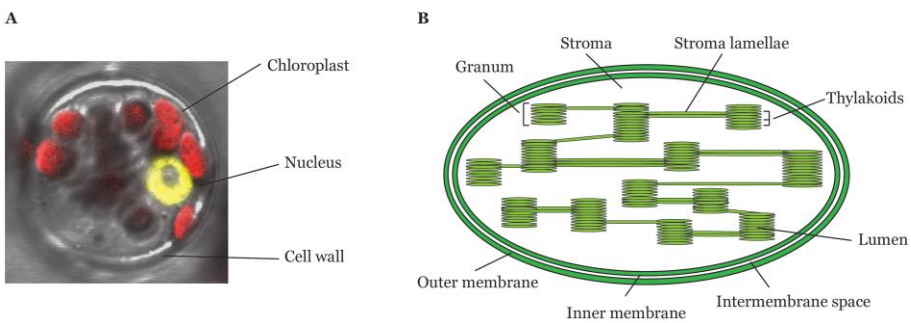

Figure 1. Structure of a cell and chloroplast from *Arabidopsis thaliana*. (A) Confocal image of a cell showing chlorophyll autofluorescence from chloroplasts in red and the nucleus by yellow fluorescence from a nuclear-localized marker protein. **(B)** Illustration of the internal organization and compartments of a chloroplast.

The origin of chloroplasts and their genome

The chloroplast originates from an endosymbiotic event that occurred about 1.5 billion years ago. Even though all details of this complex evolution are not yet established, a great amount of genomic, bioinformatics and experimental data has strongly supported this theory since it was first proposed more than 100 years ago (Schimper 1885; Mereschkowsky 1905; Sandelius and Aronsson 2009). It is believed that a eukaryotic cell, which had already gained the mitochondrion through endosymbiosis of an alpha-proteobacterium, engulfed a free-living photosynthetic cyanobacterial

ancestor. Instead of being digested as “food”, the cyanobacterium was able to survive inside its host and was eventually integrated as the chloroplast we know today **(Fig. 2)** (Bock and Timmis 2008; Green 2011). This symbiotic relationship was of great advantage to the heterotrophic host cell since it provided the possibility of becoming autotrophic and gain its own source of energy from the process of photosynthesis.

The chloroplast and mitochondria have both retained their own genome which are remnants from their genome as free-living bacteria. However, during evolution and the process of becoming an integrated organelle, both genomes have been reduced drastically in size. Phylogenetic studies have shown that the closest homologs to chloroplasts are the cyanobacteria (Douglas and Raven 2003), suggesting that a possible common ancestor could have been the endosymbiont even though the exact lineage of cyanobacteria that gave rise to plastids is unknown. Deusch et al. (2008) identified the *Anabaena variablis* and *Nostoc sp* cyanobacterial genomes as most similar to the plastid genome. These cyanobacteria have a genome size of more than 7 Mb containing around 6000 open reading frames, while the genome of the *Arabidopsis* plastid is 154 kb and contains 87 protein, 4 ribosomal RNA (rRNA) and 37 transfer RNA (tRNA) genes (Sato, et al. 1999). Supposing that the ancestral cyanobacterium had a genome size similar to the present free-living cyanobacteria, the plastid has lost a significant amount of genes. This reduction of the genome size is due to the loss of redundant genes and the transfer of genes to the nuclear genome (Martin, et al. 2002; Bock and Timmis 2008). Even though only 87 proteins are transcribed by the plastids, the total number of different proteins present in the *Arabidopsis* chloroplast is close to 4000 which means that most of the plastid proteins are encoded in the nucleus, translated in the cytosol and thereafter imported into the plastid. The transfer of genes from the integrated organelles to the host nucleus has not been a simple straight forward process, rather it involves transfer of DNA between all the different genetic compartments (Kleine, et al. 2009). Aside from the major transfer of genes from the endosymbionts to the nucleus, other types of intercompartmental DNA transfer have also been observed, for instance from the chloroplast to the mitochondria as in the case of some tRNA genes (Veronico, et al. 1996). There is also evidence for the presence of nuclear DNA in the mitochondria and mitochondrial DNA in the chloroplast **(Fig. 2)** (Kleine, et al. 2009). The transfer of certain organellar genes to the nucleus has been shown to occur also during recent evolutionary events, suggesting that the process has not completely stopped (Kleine, et al. 2009). For instance, the mitochondrial ribosomal protein gene *rps10* was shown to be relocated to the nucleus at 26 instances among 277 analysed angiosperm species (Adams, et al. 2000) and the chloroplast translation initiation factor

1 (*infA*) was lost from the plastid genome in 24 out of 300 angiosperms (Millen, et al. 2001). In at least four of the angiosperm species, the *infA* gene was found in the nuclear genome instead where it is expressed and then imported back into the chloroplast. Despite this ongoing transfer of genetic material within the plant cell, it seems like the process has reached a plateau and that the remaining organellar genes are being kept there for a reason.

Among the ~100 genes that have been retained in the plastid are proteins required for the gene expression machinery and genes encoding components involved in chloroplastic processes such as photosynthesis. Given the extent of gene transfer from the plastid genome, one might question why any genes at all have been retained within the plastid. The majority of the retained photosynthetic genes encode thylakoid membrane-located proteins that are involved in the process of photosynthesis and energy transfer. A common feature of these proteins is that they are highly hydrophobic and one explanation as to why they have been retained could be that their hydrophobicity makes it difficult to import and assembly them correctly. One weak point of this explanation is that other hydrophobic proteins, for instance some light harvesting complex (LHC) proteins of both photosystem I (PSI) and photosystem II (PSII), are encoded and imported from the nucleus (Allen, et al. 2011). However, it has been stated that while LHC proteins can be translated and folded before the addition of chlorophyll molecules, the reaction centres and core antennae proteins of PSI and PSII require co-translational insertion of chlorophyll *a* for correct folding, which might explain the necessity of these proteins to be encoded in the chloroplast (Eichacker, et al. 1996; Sobotka 2014; Yang, et al. 2015). Another hypothesis is based on the fact that the components of the photosynthetic apparatus are involved in reduction-oxidation (redox) processes and their expression has to be tightly regulated. The strong reducing power and potential excess energy in the system could be harmful and thus a rapid adjustment of protein levels is required in response to sudden environmental changes. The location of these genes within the chloroplast enables an on-site efficient regulation of their expression in response to local redox signals (Lopez-Juez and Pyke 2005; Kleine, et al. 2009; Allen, et al. 2011; Jarvis and Lopez-Juez 2013). There is however no feature or function that is common for all plastid gene products explaining why they are still in the plastid genome. Probably, a combination of chance with proposed advantages or disadvantages of plastid versus nuclear location has determined the composition of the genomes (Bock and Timmis 2008).

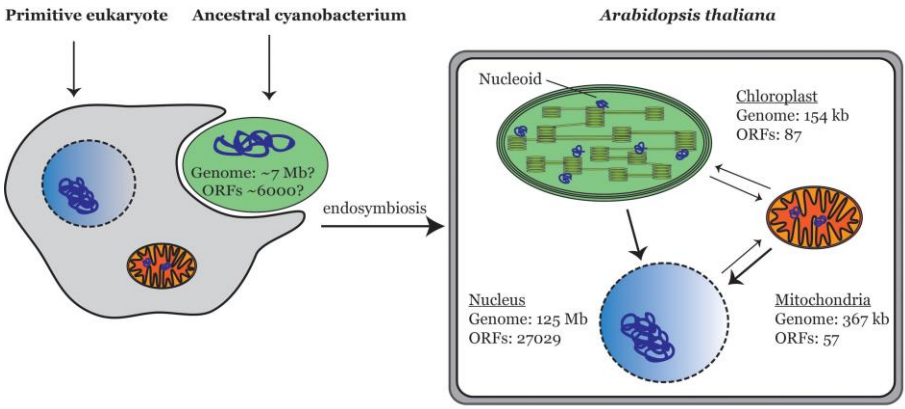

Figure 2. Genome distribution between the nucleus, chloroplast and mitochondria in *Arabidopsis*. The chloroplast originates from a free-living ancestral cyanobacterium which entered a primitive eukaryotic cell through endosymbiosis. This primitive eukaryote already contained the mitochondria which was obtained by endosymbiosis with a proteobacterium. While being integrated with their host cell, most genes from the cyanobacterial and proteobacterial genomes have been lost or transferred to the nuclear genome (illustrated by thick black arrows). Some transfer of genetic material has also occurred from the nucleus to the mitochondria and between the chloroplast and mitochondria (illustrated by thin black arrows).

Plastid transcription and gene regulation

The plastid DNA is organized into structures called nucleoids that are attached to the internal thylakoid membrane system and contain on average 10-20 copies each of the plastid genome. These structures are also associated with the presence of RNA and a variety of proteins involved in DNA replication and repair, transcription and post-transcriptional control of gene expression (Krupinska, et al. 2013; Jensen and Leister 2014). Even though the plastid genome only encodes a limited number of proteins, proper gene expression is crucial for the development and growth of plants. Plastid gene expression mutants display pale phenotypes, abnormal chloroplasts, and can even be embryo-lethal (Pogson and Albrecht 2011; Kindgren, Kremnev, et al. 2012; Kremnev and Strand 2014). The plastid genome of *Arabidopsis* encodes 87 core plastid proteins which are mainly involved in photosynthesis and transcription/translation, and 41 genes encoding rRNAs and tRNAs (Sato, et al. 1999). These genes are transcribed by two types of RNA polymerases; the nuclear encoded plastid RNA polymerase (NEP) and the plastid encoded RNA polymerase (PEP). NEP is a T3-T7 bacteriophage-type single subunit polymerase transcribed in the nucleus and imported to the chloroplast. NEP transcribes the *rpo* subunits of the PEP polymerase and also initiates the transcription of housekeeping genes and some photosynthetic genes (Lopez-Juez and Pyke 2005). PEP is a eubacterial-type of RNA polymerase with a core of four RPO proteins and it requires the

nuclear-encoded sigma factors for promoter recognition and DNA binding (Hanaoka, et al. 2003). There are six sigma factors (SIG1-6) encoded by the nucleus and these determine which genes can be transcribed by PEP in the plastid, providing the nucleus control over the plastid gene expression (Jarvis and Lopez-Juez 2013). There are also several other accessory components of PEP which are transcribed in the nucleus and have different functions such as DNA/RNA binding, linking of the transcription to translation, regulatory proteins and protective proteins (Kindgren and Strand 2015). All of these components together provide a tight control from the nucleus on the plastid gene expression which is necessary for the coordination of the two genomes.

The essence of photosynthesis

Photosynthesis is a biological process in which the light energy from the sun is captured, converted and stored as stable products by an organism. This stored energy is used to provide the power required to drive all other energy-consuming cellular processes within photoautotrophic organisms (Blankenship 2014). The photosynthetic process is not only important for plants and other photosynthetic organisms, but is essential for most life on earth since it provides the foundation for food production and energy resources. The photosynthetic pigments are the keys to photosynthesis since they are the molecules able to capture and transfer light energy. Chlorophyll *a* and *b* are the major pigments present in plants and absorb light in the blue (300-450 nm) and red (600-700 nm) regions of the light spectra. The lack of absorbance of green light gives the characteristic green colour of chlorophyll. Chlorophyll *b* is identical to chlorophyll *a* except that one methyl group is exchanged for a formyl group creating an absorbance shift towards the shorter wavelengths of blue and red light. (Blankenship 2014). This provides a broader spectrum of light absorbance where chlorophyll *b* can absorb and transfer excitation energy to chlorophyll *a*. Other accessory pigments include the carotenoids which absorb light between 400-500 nm. The different pigments are arranged in light harvesting complexes (LHC) found in association with the reaction centres in the photosynthetic electron transport chain as well as in the reaction centres themselves. When photons are absorbed by the pigments, the energy is efficiently transferred between different pigment molecules and funnelled towards the reaction centres in the photosystems where it is used for photochemistry.

The photosynthetic apparatus in plants consists of several multi-subunit complexes and accessory components which enables capture of light energy and transduction into chemical energy. The major protein complexes PSI, PSII, cytochrome b_6f (cyt b_6f) and the ATP synthase are embedded in the

thylakoid membrane. The photosynthetic electron transport (PET) chain involves the transport of electrons from water molecules to the final acceptor $NADP^+$ **(Fig. 3)**. The oxidation of water occurs at the oxygen evolving complex (OEC) associated with PSII. During the process of water oxidation electrons are donated to PSII and oxygen is released together with protons in the thylakoid lumen. The electrons are thereafter transported from PSII through the cytochrome b_6f complex to PSI where they are used to reduce ferredoxin (Fdx) and subsequently $NADP^+$ to NADPH. In addition to this linear electron transport, Fdx is also able to recycle the electrons through the cytochrome b_6f complex in a process called cyclic electron transport. The transport of electrons through cytochrome b_6f is accompanied by the transport of hydrogen ions from the stroma to the lumen, thus, this process promotes the generation of the proton motive force that drives the ATP synthase but does not contribute to the generation of NADPH. The linear and cyclic electron transport are adjusted according to metabolic requirements to balance the need for NADPH and ATP. In summary, the trapped light energy is used to produce reducing power in the form of NADPH and to build up a proton gradient between the stroma and lumen to drive the ATP synthase. NADPH and ATP is used to power the carbon fixation reactions in the Calvin-Benson cycle that takes place in the stroma. In this process, carbon dioxide is reduced to form carbohydrate molecules which are used to synthesize sugars that can be exported from the chloroplast and used as an energy resource to drive different processes throughout the whole plant.

Even though plants are dependent on light energy for photosynthesis and their survival, it is also potentially dangerous to them. In their natural growth conditions, plants face rapid fluctuations in light intensity depending on for instance the time of day, weather and shading from other plants. Under limiting and controlled light conditions, the antennae complexes absorb as much energy as possible in order to drive photosynthesis. However, the strong reducing power in the photosynthetic process could have a toxic effect during high light intensities when more energy is absorbed than can be utilized for photochemistry. During these conditions, the excitation pressure will increase the production of reactive oxygen species (ROS) and cause oxidative stress. ROS molecules can react with other biomolecules such as lipids and proteins and cause damage that affects the whole plant (Foyer and Allen 2003). In order to control the production of ROS, plants have evolved several mechanisms to balance the energy uptake and protect themselves from damage. Non-photochemical quenching (NPQ) involves the dissipation of absorbed light energy as heat and is triggered by acidification of the thylakoid lumen (Niyogi 1999; Jarvi, et al. 2013). This process involves carotenoids present in the LHCII complex which are able to

quench the excited state of chlorophylls via the so-called xanthophyll cycle and prevent the generation of ROS (Niyogi 1999). Carotenoids, other antioxidant molecules and scavenging enzymes such as superoxide dismutase (SOD) are also able to detoxify the different ROS molecules if they are formed (Niyogi 1999). These short-term acclimation mechanisms act in concert and provide a way for plants to quickly respond to fluctuations in the environment and protect themselves from photo-oxidative damages. However, if the exposure to high irradiances is prolonged, long-term responses are required to adjust the LHC antennae sizes and photosynthetic capacity. Since photosynthesis involves proteins that are expressed both in the nuclear and plastid genomes, the response to high light requires gene regulation in both compartments. The involvement of two different genomes demands a reliable communication between the nucleus and chloroplast to ensure that the photosynthetic gene expression is properly coordinated.

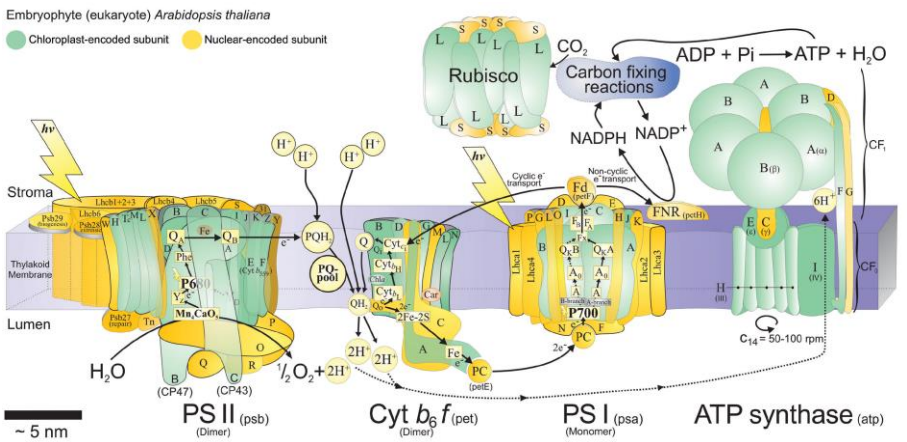

Figure 3. Components of the photosynthetic apparatus in *Arabidopsis thaliana*. Schematic illustration of the major photosynthetic complexes in plants and their accessory components located within the chloroplast. Photosystem II (PS II), cytochrome b6f (Cyt b6f), photosystem I (PS I) and the ATP synthase are embedded in the thylakoid membrane while Rubisco and the carbon fixing reactions takes place in the stroma. Protein subunits encoded by genes located in the chloroplast are coloured green and protein subunits encoded by genes located in the nucleus are coloured yellow. (Allen, et al. 2011. Reprinted with kind permission of Elsevier Ltd.)

Nuclear events and regulation of gene expression that impact upon chloroplast function

The issue of genome coordination in plants

During the establishment of the cyanobacterium as an organelle inside the eukaryotic cell it had to be integrated with the cellular processes of its host cell. By transferring the majority of the cyanobacterial genes to the nucleus, the host cell could gain control over the development and function of the endosymbiont (Sandelius and Aronsson 2009). Today the chloroplast is dependent on the nucleus to provide the majority of the proteins required for its function. In order to provide these proteins at the right time and in the right amount, the nucleus is dependent on information from the chloroplasts about their developmental stage and requirements during different growth conditions. This necessitates a tight communication between the different compartments and a coordinated regulation of the gene expression from the two genomes. One example that clearly demonstrates the importance of this coordination is the photosynthetic complexes. These are composed of subunits encoded by genes from both the nuclear and plastid genome. The core subunits of PSI, PSII, cyt b_6f and the ATP synthase are encoded by plastid genes while the peripheral subunits are encoded by nuclear genes. Also, the large subunit of Rubisco is plastid encoded while the small subunit is nuclear encoded **(Fig. 3)** (Allen, et al. 2011). In order to assemble these complexes stoichiometrically and to ensure that they function correctly when the growth conditions change, the expression of the different proteins must be tightly regulated. This regulation also allows for the reorganization and adjustment of photosynthetic activity in response to environmental changes (Blankenship 2014). In order to coordinate their activities, the nucleus is able to control gene expression in the chloroplast through so-called anterograde signalling. Nuclear components respond to cellular and environmental conditions and adjust gene expression of the critical components for the plastids accordingly. The chloroplast is also able to coordinate nuclear gene expression with its developmental and metabolic state through retrograde signals.

Nuclear transcription and gene regulation

The control of nuclear gene expression is a complex process involving different layers of regulation. Firstly, the structure of chromatin determines the accessibility of transcription, allowing or preventing the transcriptional machinery access to gene sequences. The chromatin structure is altered by chromatin remodelers, histone modifiers and DNA methylating/ demethylating factors (Meier and Brehm 2014). Chromatin modifications

can be transient, for instance induced during stress responses to activate gene expression and thereafter removed and restored. Epigenetics provide another layer of gene regulation where heritable, stable or long-term chromatin modifications confer altered levels of gene expression and activity even in the absence of a stimulus (Meier and Brehm 2014). Secondly, the regulation of exposed genes is dependent on the promoter sequences located upstream of the transcriptional start. The promoter region contains *cis*-acting elements which are short DNA sequences that function as the binding site for specific proteins acting as transcriptional regulators. The promoters of eukaryotic genes include common *cis*-elements such as the TATA-box and CAAT-box. These *cis*-elements bind proteins that form a complex and promote transcription by guiding the RNA polymerase and additional components to the transcriptional start site. The formation and activity of the transcriptional machinery complex is however dependent on transcription factors that can act as activators or repressors of the complex. There are a number of different *cis*-elements that can be found within different gene promoters and also a variety of transcription factors that specifically bind certain *cis*-elements to regulate gene expression. The transcription factors themselves are also regulated at different levels (i.e. at transcriptional, translational and posttranslational levels) by signals that are communicated within the cell and adjust gene expression according to the state of development or surrounding environmental conditions such as light, temperature and time of the day.

Light signals can have major effects on the regulation of gene expression in plants. Several microarray experiments have shown that the expression of a large number of nuclear-encoded genes is changed in response to growth under different light conditions. Studies of *Arabidopsis* seedlings grown in dark versus light have revealed that up to one third of the nuclear-encoded genes are either induced or repressed in response to light signals (Ma, et al. 2001; Jiao, et al. 2005). Multiple studies have also shown that exposure to high light induce a rapid response to adapt the expression of a large number of genes (Rossel, et al. 2002; Kimura, et al. 2003; Kleine, et al. 2007). A study by Kleine et al. (2007) identified a total of 992 genes that were either induced or repressed in the response to 3 hours high light treatment. For instance, early light-inducible protein 1 and 2 (*ELIP1* and *ELIP2*) and ascorbate peroxidase 2 (*APX2*) were strongly induced while the light-harvesting chlorophyll *a*/*b*-binding protein 2.4 (*LHCB2.4*) gene was strongly repressed. These genes are all involved in photoprotection and are considered marker genes for light stress responses. Kleine et al. (2007) also performed a bioinformatics study of 500 bp promoter sequences of the identified high light responsive genes and identified several enriched *cis*-elements. These included the G-box (CACGTG), G-box variants (NACGTN),

I-box (GATAAGR), MYC/MYB binding site (CATGTG) and the CryR1 and CryR2 (GnTCKAG, ACATAwCT) elements. The G-box and I-box elements were first characterized in studies of the promoter region of the light regulated ribulose 1,5-bisphosphate small subunit (*RBCS*) gene (Giuliano, et al. 1988) and the G-box element has since been reported in the promoter regions of numerous plant genes. Many of these genes are responsive to light signals, like *RBCS* and the *LHCB* genes, but the G-box is also found within promoters of genes responsive to, for instance, anaerobiosis and hormones such as ABA, ethylene and methyl jasmonate (Guiltinan, et al. 1990; McKendree and Ferl 1992; Eyal, et al. 1993; Mason, et al. 1993; Reinbothe, et al. 1994; Menkens, et al. 1995). Kleine et al. (2007) identified the G-box *cis*-element in promoters of light responsive genes that were both induced and repressed in the response to high light, suggesting interactions with transcription factors acting both as activators and repressors. Both bZIP (basic region/leucine zipper) and bHLH (basic helix-loop-helix) transcription factors are known to interact with the G-box *cis*-element in plants (Siberil, et al. 2001). bZIPs are known to also bind other *cis*-elements, especially with an ACGT core, whereas bHLH rather bind to the CANNTG consensus sequence (Siberil, et al. 2001; Jakoby, et al. 2002).

The function of bZIP transcription factors

In *Arabidopsis*, close to 6% of the entire genome is dedicated to encode for more than 1500 transcription factors, demonstrating the high need and importance of gene regulation (Riechmann, et al. 2000). Almost 50% of these transcription factors are specific to plants while the remaining transcription factors can be found in many different organisms. The bZIP transcription factors are present in all eukaryotes ranging from yeast to humans but are more abundant in plants compared to other organisms (Riechmann, et al. 2000; Llorca, et al. 2014). The bZIP protein domain consists of a α-helix comprising a basic region and a leucine zipper. The basic region includes a nuclear localizing signal followed by a motif that is necessary to allow DNA binding (Jakoby, et al. 2002). The leucine zipper contains a heptad repeat of leucine residues and is responsible for the formation of homo- and heterodimers **(Fig. 4A and B)** (Siberil, et al. 2001; Jakoby, et al. 2002; Llorca, et al. 2014). There are 75 members of the bZIP family in *Arabidopsis* and these transcription factors are involved in the regulation of a variety of important processes such as light and stress signalling, hormone signalling, plant development and pathogen defence (Siberil, et al. 2001; Jakoby, et al. 2002; Llorca, et al. 2014). The bZIP family in *Arabidopsis* has been clustered into 10 subgroups (A-I and S) according to their sequence similarities of the basic region, size of the leucine zipper and presence of other common domains **(Fig. 5)** (Jakoby, et al. 2002). This

subdivision of the bZIPs is not strictly phylogenetic but members of the same group probably recognize similar *cis*-elements and regulate similar processes due to their conserved basic region and other properties (Jakoby, et al. 2002).

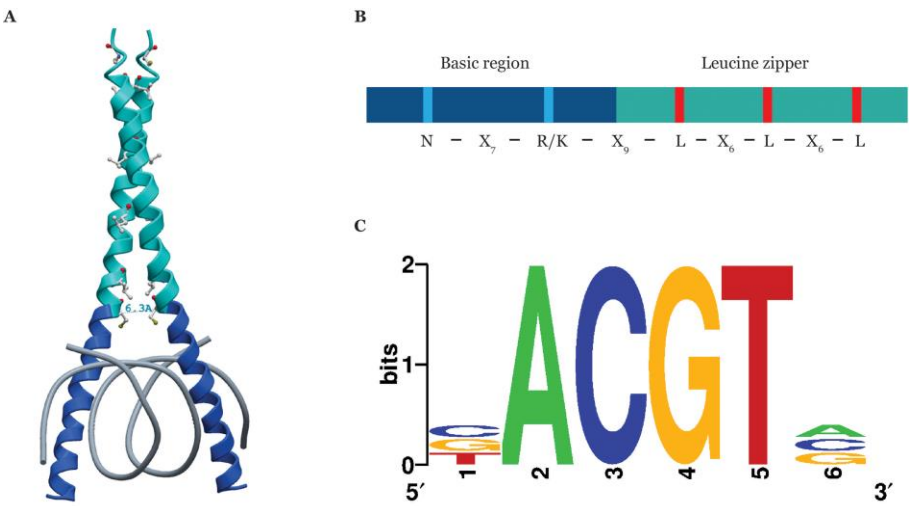

Figure 4. ***Arabidopsis thaliana*** **bZIP transcription factors. (A)** Theoretical three-dimensional structure retrieved by comparative modelling of the bZIP domain of bZIP16 bound to DNA (adapted from Shaikhali, Noren, et al. 2012). Two subunits interact through hydrophobic regions in the leucine zipper (coloured in cyan) to form a dimer and binds DNA via the basic region (coloured in dark blue). **(B)** Primary structure of the bZIP protein domain which consists of a basic region with the DNA binding domain (N-X7-R/K) and a leucine zipper with repeated leucine residues (L). Conserved residues are shown in light blue and red boxes and a consensus sequence is stated below (adapted from Jakoby, et al. 2002). **(C)** Consensus sequence logo of the bZIP binding cis-elements (G-box, C-box and A-box) demonstrating the ACGT core element. The logo was created at: http://weblogo.berkeley.edu using standard settings.

The bZIP transcription factors have been shown to preferentially bind to *cis*-elements with an ACGT core such as the G-box (CACGTG), C-box (GACGTC) and A-box (TACGTA) present in a variety of different gene promoters **(Fig. 4C)** (Foster, et al. 1994; Jakoby, et al. 2002; Llorca, et al. 2014). The binding specificity of bZIPs to DNA is a result of both variations of the *cis*-element and its flanking nucleotides, and variability of the binding region of the protein (Llorca, et al. 2014). Certainly many of the bZIPs recognize the same DNA sequences and have redundant functions in regulating some processes, however, extensive regulation of the bZIP proteins themselves give rise to specific functions and responses to certain signals. The regulation of bZIP proteins includes for instance transcriptional/translational control, dimerization properties and post-translational control. The expression levels of bZIP genes are dependent on the tissue type, developmental stage and stress conditions (Llorca, et al. 2014). For instance, several members of the S-group are repressed in response to sucrose and various abiotic stresses

such as salt and cold (Weltmeier, et al. 2009). The bZIP proteins are also regulated by alternative splicing and the initiation of translation can be adjusted according to regulatory signals (Zou, et al. 2007; Llorca, et al. 2014). Aside from the transcriptional/translational control, the bZIP transcription factors are highly regulated at the post-translational level. This regulation is especially generated through the formation of dimers. Monomeric bZIP proteins can form dimers through interactions between residues in their leucine zipper motifs. Either homo- or heterodimers may be formed depending on the specificity of the leucine zipper residues. Thus, the composition of the leucine zipper determines if an interaction between two monomers is possible or not, and it is more likely that closely related proteins interact than two distantly related proteins (Llorca, et al. 2014). Hydrophobic forces between the leucine residues or other hydrophobic amino acids in the leucine zipper drive the formation of dimers, but the position of the remaining amino acids determines if the interaction is possible. Depending on whether attractive or repulsive residues are exposed to each other, i.e. charged or polar amino acids, it is more or less likely that dimerization will take place (Llorca, et al. 2014). The formation of both homo- and heterodimers has been shown for instance within the G-group (Shen, et al. 2008; Shaikhali, Noren, et al. 2012) and the H-group of bZIP transcription factors (Holm, et al. 2002). Also, interactions between members of different groups has been shown for the G-box binding factor 4 (GBF4) A-group transcription factor and members of the G-group (Menkens and Cashmore 1994). In addition, the H-group members elongated hypocotyl 5 (HY5) and HY5 homolog (HYH) can interact with the G-group member G-box binding factor 1 (GBF1) (Singh, et al. 2012; Ram, et al. 2014). Singh et al. (2002) showed that the interactions between GBF1, HY5 and HYH play a role during light-regulated gene expression and photomorphogenesis. Each of these proteins is able to form homodimers which can bind to the G-box element and regulate gene expression. However, the formation of heterodimers between GBF1 and HY5 increases their binding affinity to DNA, while the GBF1-HYH heterodimer is unable to bind to the G-box. Thus, the specific dimer composition has an impact on DNA binding and transactivation properties which adds a layer of complexity to the functional roles of the bZIP transcription factors.

The regulation of bZIP proteins by phosphorylation events controls the monomer activity in several ways. Residues of both the leucine zipper and the basic region can be phosphorylated which affects the dimerization possibilities and DNA binding by introducing negative charges (Deppmann, et al. 2003; Kirchler, et al. 2010; Lee, et al. 2010). Phosphorylation may also induce conformational changes, affecting bZIP activity or protein turnover rates. For instance, HY5 protein degradation is prevented by

phosphorylation of residues which prevent the interaction with the E3-ubiquitin-protein ligase constitutive photomorphogenic 1 (COP1) (Hardtke, et al. 2000). The subcellular localization of bZIP proteins in the cytosol or nucleus is another way of controlling their activity (Llorca, et al. 2014). Taken together, the different levels of regulation of the bZIPs provide a highly flexible and dynamic system where transcription factors can be specifically adjusted in order to respond to different processes.

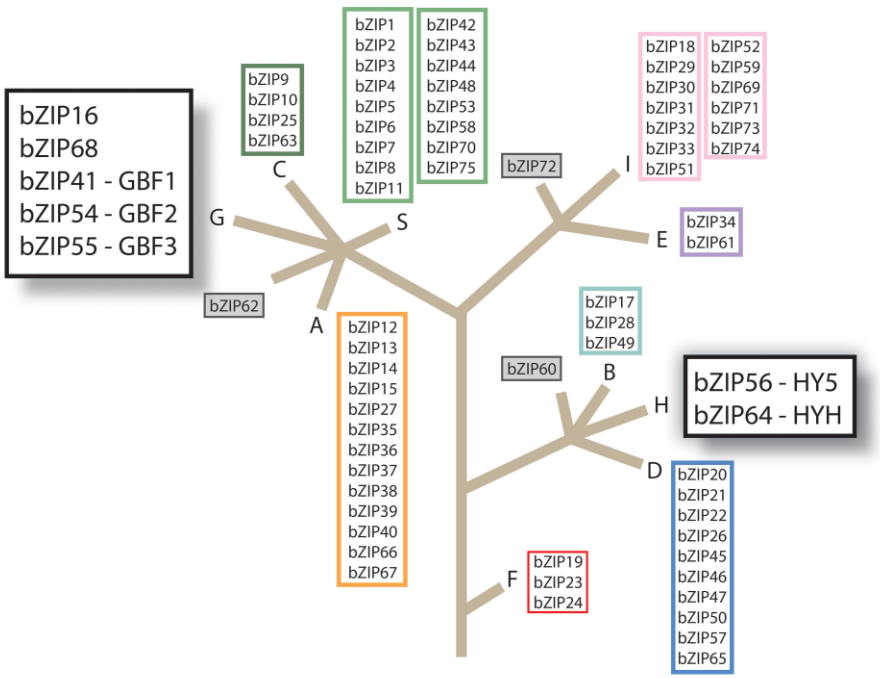

Figure 5. Classification and evolutionary relationships of bZIP transcription factors in *Arabidopsis*. The evolutionary analysis was performed using the full length protein sequences of all 75 bZIP proteins. The analysis was conducted in MEGA7 (http://www.megasoftware.net/) using the UPGMA method. Recommended settings were used and the evolutionary distances were computed using the Poisson correction method. The tree is not drawn to scale. The bZIP proteins were classified into ten groups (A-I and S) as previously described by Jakoby et al. (2002), this classification was based on sequence similarity of the basic region and additional conserved domains using the MEME tools (http://meme-suite.org/). Three genes did not fit in any group and are placed in the tree solely according to the phylogeny (in grey).

The impact of light signalling

Light provides plants with important information to regulate many different processes such as seed germination, photomorphogenesis, development, photosynthesis, shade avoidance, phototropism and circadian rhythms (Fankhauser and Staiger 2002; Chen, et al. 2004; Galvao and Fankhauser 2015). In order to obtain information to regulate these processes, plants use photoreceptors which are able to sense the intensity, direction, duration and wavelength of light to induce signalling pathways accordingly. These photoreceptors include the red/far-red absorbing phytochromes (phy), the blue light/UV-A absorbing cryptochromes (cry), phototropins (phot) and ZTL-type photoreceptors, and the UV-B absorbing UVR8 photoreceptor (Galvao and Fankhauser 2015). Light signals from the photoreceptors converge with other signalling pathways such as the circadian clock and hormones to mutually regulate and adapt nuclear gene expression according to changing light conditions (Casal and Yanovsky 2005).

The development of a seedling into a plant with true leaves is dependent on light. In the absence of light, for instance under the soil surface, the seedling keeps the apical hook closed and elongates its hypocotyl in order to reach a light source. An etiolated seedling is pale since the undifferentiated proplastid cannot develop into a mature chloroplast in the absence of light, but instead forms a dark-grown intermediate called an etioplast. When the seedling is exposed to light, chlorophyll biosynthesis is activated and the etioplast subsequently develops into a functional chloroplast (Pogson and Albrecht 2011; Jarvis and Lopez-Juez 2013). In the response to light exposure, about one third of the nuclear encoded genes change their expression pattern in order to promote chloroplast development (Ma, et al. 2001). Many of the target genes encode chloroplast-localized proteins that are up-regulated in the response to light and promote chloroplast development (Ma, et al. 2001; Pogson and Albrecht 2011). The chloroplast is for instance dependent on the nucleus to transcribe NEP and the sigma factors to induce plastid gene expression. NEP in turn transcribes the PEP genes in the plastid and induces the expression of photosynthetic genes. The light-mediated anterograde control of chloroplast development also includes the expression of components of the protein import machinery, chlorophyll biosynthesis and photosynthetic genes **(Fig. 6)** (Jarvis and Lopez-Juez 2013). The early light signals are mediated through the photoreceptors which in turn affects a variety of transcription factors and their target genes. HY5 is a positive regulator of photomorphogenesis that binds to the G-box *cis*-element present in many light regulated genes. During early light signalling in seedlings, about 60% of all genes regulated by phytochromes contained HY5 binding targets (Lee, He, et al. 2007). Both phytochrome and

cryptochrome light signalling contributes to higher levels of HY5 and the induction of target genes. In darkness, inactive cryptochromes are bound to COP1 in the nucleus and promote the degradation of HY5 by the 26S proteasome. Meanwhile, the negative regulator phytochrome interacting factor 3 (PIF3) is bound to the G-box or PBE-box element of the target genes and suppresses transcription. Blue light photoactivation of cryptochromes leads to the export of COP1 from the nucleus and thus allows HY5 to accumulate. Also, the activation of phytochromes by red light provides their translocation from the cytosol to the nucleus where they interact with PIF3. PIF3 is then targeted for degradation and allows HY5 to bind the G-box and promote gene expression (Osterlund, et al. 2000; Stephenson, et al. 2009; Waters and Langdale 2009).

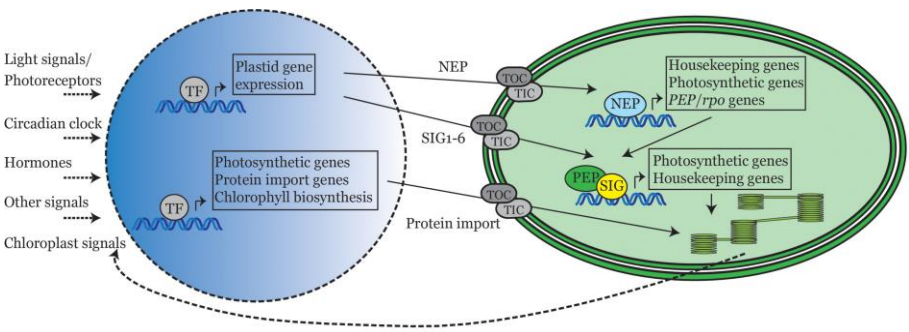

Figure 6. Coordination of gene expression in the nucleus and chloroplast. Nuclear gene expression is dependent on the activity of transcription factors which repress or activate the transcription of genes in response to light and other signalling pathways. Anterograde control of the plastid gene expression involves the transcription of the NEP protein and the sigma factors which are imported to the chloroplast. NEP transcribes the *PEP* genes in the chloroplast which functions together with the sigma factors and promotes the expression of housekeeping and photosynthetic genes necessary for the functional chloroplast. The nuclear genome also provides proteins for the protein import machinery, chlorophyll biosynthesis and photosynthetic process. The nuclear gene expression is dependent on retrograde signals from the chloroplast to coordinate the different genome activities.

Circadian regulation of nuclear events

The expression of light-regulated nuclear genes that encode chloroplast localized proteins are also controlled by the circadian clock. For instance, *LHCB* expression is strongly induced by light during the subjective day but not during subjective night demonstrating that both light and circadian signals converge to control its gene expression (Millar and Kay 1991, 1996). Since many processes are dependent on light it is important for plants to anticipate daily changes and optimize the usage of different resources during the day and night cycles. The circadian clock consists of a self-sustainable central oscillator, input pathways integrating the clock with environmental cues and output pathways that regulate diverse processes (Harmer 2009;

Eriksson and Webb 2011). This system provides a robust 24-hour internal rhythm by using a mechanism of components in the central oscillator with interlocked transcriptional feedback loops (Pokhilko, et al. 2012). More than 20 clock-associated components have been identified in *Arabidopsis* (Hsu and Harmer 2014), and the complexity of the interactions within the circadian clock is not fully unravelled. Also, the transcriptional interactions between these components are coupled with other layers of regulation, such as post-transcriptional, post-translational and chromatin remodelling (Nagel and Kay 2012). Recent results demonstrate that the plant clock is a highly interconnected and complex regulatory network in which distinct feedback loops are very difficult to identify (Fogelmark and Troein 2014; Hsu and Harmer 2014). In the scope of this thesis, a simplified model including the core components is described **(Fig. 7)**.

In *Arabidopsis*, the circadian clock consists of a set of interconnected pathways that can be viewed as morning and evening loops. The morning components late elongated hypocotyl (LHY) and circadian clock associated 1 (CCA1) are co-expressed at dawn and act redundantly to regulate several other clock components. The pseudo-response regulators PRR5, 7 and 9 are activated by LHY and CCA1 accumulation in the early morning. This activation could be accomplished through direct transcriptional activation by CCA1/LHY (Pokhilko, et al. 2012) or alternatively as a result of CCA1/LHY repression of PRR repressors (Fogelmark and Troein 2014). CCA1 and LHY repress the evening component timing of CAB expression 1 (TOC1) and also suppress the expression of the evening complex (EC) components lux arrhythmo (LUX), early flowering 3 and 4 (ELF3 and ELF4) (Pokhilko, et al. 2012). During the day, LHY and CCA1 expression will subsequently be repressed by the PRRs and also by TOC1 until the following morning when the EC down-regulate the expression of TOC1 and the PRR proteins (Pokhilko, et al. 2012). Gigantea (GI), zeitlupe (ZTL) and heat shock protein 90 (HSP90) are cytosolic components involved in post-translational regulation of clock components. ZTL is a F-box protein responsible for target recognition of the SCF^{ZTL} E3 complex that facilitates ubiquitination of proteins targeted for proteasomal degradation (Kim, et al. 2003). In addition to the F-box domain, ZTL protein also contains a LOV (Light, Oxygen, Voltage) domain allowing ZTL to act as a blue light-dependent regulator of clock associated proteins (Kim, et al. 2007; Kim, et al. 2011). ZTL and GI are co-stabilized in the cytosol in a blue light-dependent manner (Kim, et al. 2007; Kim, et al. 2013). The stability of ZTL protein is also dependent on HSP90 that acts in the same stabilization pathway as GI (Kim, et al. 2011). HSP90 proteins are constitutively expressed while GI expression is regulated by CCA1 and LHY (Pokhilko, et al. 2012). ZTL functions in the circadian clock by targeting the components TOC1 and PRR5 for proteolytic

degradation (Mas, et al. 2003; Kiba, et al. 2007). GI was also shown to be involved in the regulation of the EC components together with the E3 ubiquitin-ligase COP1 (Pokhilko, et al. 2012).

The circadian clock is a self-sustainable mechanism and the biological rhythms persist even in constant conditions in the absence of environmental cues (Harmer 2009). However, components of the circadian oscillator receive environmental input signals to allow entrainment of the clock. These signals adjust the transcription or activity of clock components to synchronize the oscillator phase to the environmental time (Bell-Pedersen, et al. 2005; Atkins and Dodd 2014). The input signals are mainly mediated through light signals perceived by photoreceptors and temperature fluctuations, but sugar signals have also been shown to regulate the circadian oscillator (Haydon, et al. 2013; Dodd, et al. 2015). These input signals are important to mediate temporal information to the output processes and to regulate for instance seasonal processes.

Circadian regulation in *Arabidopsis* has been shown to be important for increased photosynthetic activity, growth, survival, seed viability and competitive advantage (Green, et al. 2002; Dodd, et al. 2005). The process of photosynthesis is controlled by the circadian clock by synchronizing light harvesting, photosynthetic electron transport and CO_2 fixation with the light and dark cycles (Dodd, Kusakina, et al. 2014). Up to 70% of the chloroplast genome and 36% of the nuclear genome have been shown to be circadian regulated, including many of the photosynthetic genes (Harmer, et al. 2000; Schaffer, et al. 2001; Michael and McClung 2003; Michael, et al. 2008). The necessity for interaction between the circadian clock and the chloroplast is obvious and several mechanisms and signalling pathways that connect the circadian clock to the regulation of chloroplast transcription have been proposed. Expression of all nuclear-encoded sigma factors (*SIG1* to *SIG6*) which control the PEP-mediated gene expression in chloroplasts can be circadian regulated (Noordally, et al. 2013) and it has been suggested that the SIGs control timing of chloroplast transcription. Oscillations in the abundance of transcripts of the sigma factor and its chloroplast target genes has been shown to match for *SIG1* and *psaA*, *SIG3* and *psbN*, *SIG4* and *ndhF* and *SIG5* and *psbD*, and SIG5 was specifically shown to mediate the circadian gating of light input to the chloroplast and optimize photosynthesis (Noordally, et al. 2013; Atkins and Dodd 2014). It has been suggested that the chloroplast itself is able to regulate circadian rhythms of nuclear encoded genes through retrograde signalling. A number of studies have shown that circadian regulation is closely connected to processes within the chloroplast, such as photosynthesis and its metabolic products (Dodd, et al. 2015). Sugars produced by photosynthesis have been demonstrated to trigger

retrograde signals and adjust the expression of circadian oscillator genes (Haydon, et al. 2013). Also, a chloroplast protein associated with plastid ribosomes (chloroplast RNA binding, CRB) and PET signals have been proposed to alter circadian transcripts and alternative splicing (Hassidim, et al. 2007; Petrillo, et al. 2014). Close interactions between the chloroplast and circadian clock could provide a mechanism for fine-tuning the expression of specific genes according to the timing of the day.

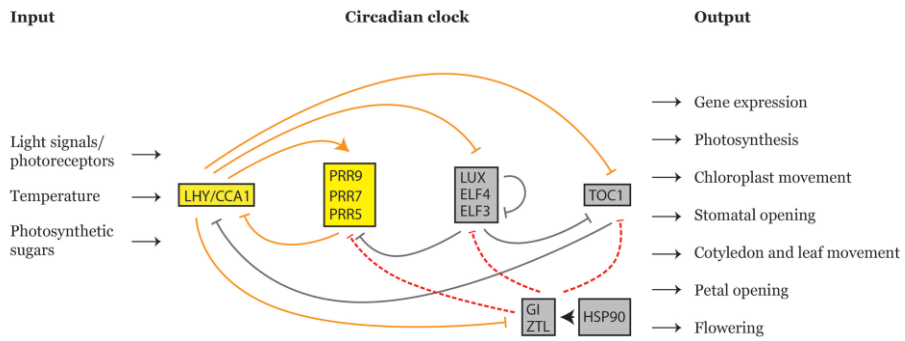

Figure 7. Overview of the circadian clock in *Arabidopsis*. In the morning, late elongated hypocotyl (LHY) and circadian clock associated 1 (CCA1) act redundantly to repress the evening component timing of cab expression 1 (TOC1). LHY and CCA1 also repress the expression of the evening complex (EC) consisting of lux arrhythmo (LUX), early flowering 3 and 4 (ELF3 and ELF4) as well as gigantea (GI). The expression of the pseudo-response regulators PRR5, 7 and 9, is activated by LHY and CCA1, which in turn repress LHY and CCA1 expression. In the evening, TOC1 repress the expression of LHY and CCA1, and the EC subsequently mediates the repression of the PRR proteins, TOC1 and the EC itself. Post-translational regulation of TOC1 and PRR5 by zeitlupe (ZTL) and the EC components by GI is shown by red dashed lines. Transcriptional regulation by morning components are shown in yellow and by evening components in grey.

Photoprotection and light stress

Besides the important role of light during plant development and regular growth conditions, light can also cause massive damage to the cell. As mentioned before, prolonged exposure to high irradiances cause oxidative stress and induce harmful redox reactions within the cell. In order to protect themselves and avoid these damages, plants are able to sense when photon fluency exceeds the utilization capacity of the chloroplast and adjust nuclear and chloroplast gene expression in response to these signals. For instance, the changes in gene expression triggered by exposure to excess light reduce the size of the antennae complexes to minimize photon uptake, adjust the photosynthetic capacity and increase the levels of photoprotective components (Kleine, et al. 2007). Several signalling pathways are involved in the response to high irradiances, including signalling components of the chloroplast, cytosol and nucleus. As the site of photosynthesis, chloroplasts have a distinct role in sensing and communicating light information which will be discussed in the next section. However, it has also been shown that

cytosolic and nuclear components play an important role in mediating the high light response independently of the chloroplasts. It has been suggested that photoreceptors are able to sense light intensity and mediate a response to excess light. The cryptochrome 1 (CRY1) was shown to be required for the regulation of a significant number of genes responsive to high light, including genes encoding proteins associated with stress responses and transcriptional regulation. The transcription factor HY5 was identified to act downstream of CRY1 and regulates a subset of genes containing the G-box *cis*-element in the response to CRY1-mediated high light signalling (Kleine, et al. 2007). The CryR1 *cis*-element was also found to be enriched in the promoters of CRY1 responsive genes and subsequently, zinc finger protein expressed in inflorescence meristem like 1 and 2 (ZML1 and 2) were identified to specifically bind this element (Shaikhali, de Dios Barajas-Lopez, et al. 2012). ZML1 and ZML2 form heterodimers and interact with the CryR1 *cis*-element to induce the expression of photoprotective genes in response to CRY1-mediated signals. The exact mechanism of how CRY1 affects the transcription factors is not known and no direct interaction could be detected between CRY1 and the ZML proteins. CRY1 might act through some unknown component to promote the formation or activation of ZML1 and ZML2 heterodimer complexes, or modify protein levels or phosphorylation status (Shaikhali, de Dios Barajas-Lopez, et al. 2012). It is not known how CRY1 could act as a light intensity sensor. However, the chromophore cofactor flavin adenine dinucleotide (FAD) has been shown to exist in three interconvertible redox forms that could possibly mediate information of the light environment (Bouly, et al. 2007). The oxidized state of FAD accumulates in the dark. Upon blue light-illumination, an excited state of FAD ($FADH^{\cdot}$) has been described to be formed while light of longer wavelengths in the blue-green region promotes the formation of reduced FAD (FAD-) (Bouly, et al. 2007). This CRY1/FAD photocycle could possibly be responsible for structural changes in the conformation of the photoreceptor and allow or block interactions with downstream signalling partners (Kottke, et al. 2006; Bouly, et al. 2007).

Redox signalling is another important system in plants which is highly responsive to light stress and the accumulation of ROS molecules. Redox changes within the plant cell are continuously buffered through the actions of a variety of compounds such as antioxidants, ascorbate, glutathione and the redox-regulating proteins thioredoxin (Trx), glutaredoxin (Grx) and protein disulfide isomerase (PDI) (Chi, et al. 2013). Trx, Grx and PDI belong to the same superfamily of thioredoxins and are involved in the reduction of disulfide bridges (Meyer, et al. 2009). Thiol/disulfide redox modifications is a posttranslational modification that regulates the formation or breakage of a bond between cysteine residues in proteins. These modifications affect the

conformation and activity of many different target proteins in the response to redox changes (Marchal, et al. 2014). Photosynthetic organisms have a large number of Trx and Grx proteins which have been found to be located in almost all cellular compartments and regulate diverse processes. As one of the main sites of ROS production due to the photosynthetic process, the chloroplast contains several Trx proteins regulating chloroplast development, carbon metabolism and antioxidant stress responses. Other types of Trx proteins have been localized to the mitochondria, cytosol and nucleus (Vieira Dos Santos and Rey 2006; Marchal, et al. 2014).

Some Trx proteins have been shown to localize to the nucleus in the response to oxidative stress in wheat (Serrato and Cejudo 2003). In addition, a functional nuclear Trx system has been demonstrated in wheat (Pulido, et al. 2009) and recently also in *Arabidopsis* (Marchal, et al. 2014). Nucleoredoxins (NRX) are potential nuclear Trx proteins that are found in most eukaryotic organisms. Marchal et al. (2014) showed that the two *Arabidopsis* NRX genes, NRX1 and NRX2, have disulfide reduction capacities and are dually localized to the cytosol and the nucleus. The cytosolic thioredoxin reductase NTR is also partially localized to the nucleus, suggesting a complete NTR/NRX thioredoxin system in the plant nucleus. The DNA-binding activity of nuclear transcription factors have been shown to be altered by redox-mediated modifications and a nuclear thioredoxin system could have a key role in controlling transcription under oxidative stress conditions (Vieira Dos Santos and Rey 2006). A few redox sensitive transcription factors have been identified where the activity is dependent on the formation of disulfide bonds between regulatory cysteine residues. The DNA-binding activity of the R2R3 MYB transcription factor and the homeodomain proteins HAHR1 and HAHB10 is regulated in a redox-dependent manner. Under non-reducing conditions, two cysteines of the R2R3 domain form a disulfide bond and prevents DNA-binding (Heine, et al. 2004; Hernandez, et al. 2004). Similarly, in the oxidized state HAHR1 and HAHB10 proteins form intermolecular disulfide bonds resulting in inefficient DNA-binding (Tron, et al. 2002) and the DNA-binding activity of these proteins is induced by breaking the regulatory disulfide bonds. Also, the activity of the transcription factor Rap2.4a was shown to be regulated by both reducing and oxidizing conditions, and its homodimeric structure is stabilized by an intermolecular disulfide bond (Shaikhali, et al. 2008).

Plastid events and signalling pathways

Plastid retrograde signalling

The presence of genes in three different compartments within the plant cell presents the complex problem of coordinating the activities of these genomes. Considering the great control the nucleus holds over the chloroplasts and mitochondria by producing most of the proteins they need, one might think that the organelles have nothing to say. This is however not the case since the nucleus is equally dependent on signals from these organelles in order to receive information about their developmental stage and changes in environmental conditions. Retrograde (organelle-to-nucleus) signalling coordinates the expression of nuclear genes encoding organellar proteins with the developmental and metabolic state of the chloroplasts and mitochondria (Barajas-Lopez Jde, et al. 2013; Blanco, et al. 2014). In contrast to plastid signalling, less is known about the mitochondria retrograde regulation (MRR) in plants (Blanco, et al. 2014). However, similarly to plastid signalling, MRR has been shown to be triggered by perturbations in mitochondrial processes such as disruption of the electron transport and accumulation of ROS (Rhoads and Subbaiah 2007; Ho, et al. 2008; Blanco, et al. 2014). Plastid signals are of great importance to the plant during the initial developmental stages but also in their adult stage to sense environmental changes and respond to different stresses. As the chloroplast is the site of several metabolic processes, it is also sensitive to environmental changes that can affect these processes. These can include changes in light quality and intensity, drought or cold. The different chloroplastic processes initiate different responses to specific conditions all controlling different sets of genes, demonstrating the need for multiple signalling pathways. Several plastid signals have been described as influencing the transcription of nuclear genes encoding plastid-localized proteins and an increasing number of signalling molecules have been reported (Barajas-Lopez Jde, et al. 2013; Bobik and Burch-Smith 2015; Chi, et al. 2015; Singh, et al. 2015; Chan, et al. 2016). To date, more than 40 components are known to participate in chloroplast retrograde signalling pathways regulating genes associated with photosynthesis, redox-responsive genes and other defence genes (Chan, et al. 2016).

The plastid retrograde signalling pathways can broadly be classified into four groups: signals related to (1) tetrapyrrole biosynthesis, (2) photosynthesis and the plastid redox state, (3) plastid gene expression and (4) plastid metabolism **(Fig. 8)**. The signalling pathways involving tetrapyrrole intermediates and photosynthesis/redox signals will be discussed in more detail in the following text. Perturbations of the plastid gene expression has

been shown to trigger retrograde signals both at the transcriptional and translational levels (Woodson, et al. 2013; Tiller and Bock 2014). Inhibition of plastid gene expression or translation has a strong effect on the expression of nuclear genes during the early stages of seedling development (Oelmuller, et al. 1986). Also, redox regulation of PEP activity via plastid redox insensitive 2 (PRIN2) has been shown to generate a retrograde signal to regulate the expression of nuclear encoded photosynthesis genes (Kindgren, Kremnev, et al. 2012). Genome uncoupled 1 (GUN1) is a chloroplast localized pentatricopeptide repeat (PPR) protein that has been implicated in sensing or mediating signals related to plastid gene expression (Koussevitzky, et al. 2007). The exact function of GUN1 is not known, but GUN1 is also known to respond to tetrapyrrole and photosynthetic signals, implying that GUN1 acts as an integrator of signals within the chloroplast (Koussevitzky, et al. 2007; Waters and Langdale 2009).

Several metabolites have been shown to trigger retrograde signalling pathways. Sugars, such as sucrose generated in the photosynthetic process, have a regulatory effect on the expression of nuclear encoded photosynthetic genes. In general, low sugar conditions promotes upregulation of photosynthetic genes, whereas high sugar conditions downregulate photosynthetic genes and instead promote growth and storage (Rolland, et al. 2002; Hausler, et al. 2014). Phosphoadenosine phosphate (PAP) is a signalling metabolite involved in drought and light stress responses. PAP is regulated in the chloroplast by dephosphorylation to AMP by SAL1 phosphatase. Drought or high light stress inhibits the activity of SAL1 and promotes accumulation of PAP. It is proposed that PAP is able to travel from the chloroplast to the nucleus where the expression of *APX2* and *ELIP2* is induced (Estavillo, et al. 2011). Derivatives of the carotenoid biosynthetic pathway has also been shown to act as signalling molecules. Oxidation of β-carotene induced by light stress results in β-cyclocitral (β-CC) which in turn affects $^1O_2$-responsive genes (Ramel, et al. 2012). Cleavage products from some intermediates, for instance ζ-carotene and phytofluene, has been proposed to act as putative signalling molecules, even though the exact nature of these molecules are not known (Avendano-Vazquez, et al. 2014). Accumulation of methylerythritol cyclodiphosphate (MEcPP), a precursor of carotenoids, cause induction of a subset of stress-related genes upon wounding and exposure to high light intensities (Xiao, et al. 2012).

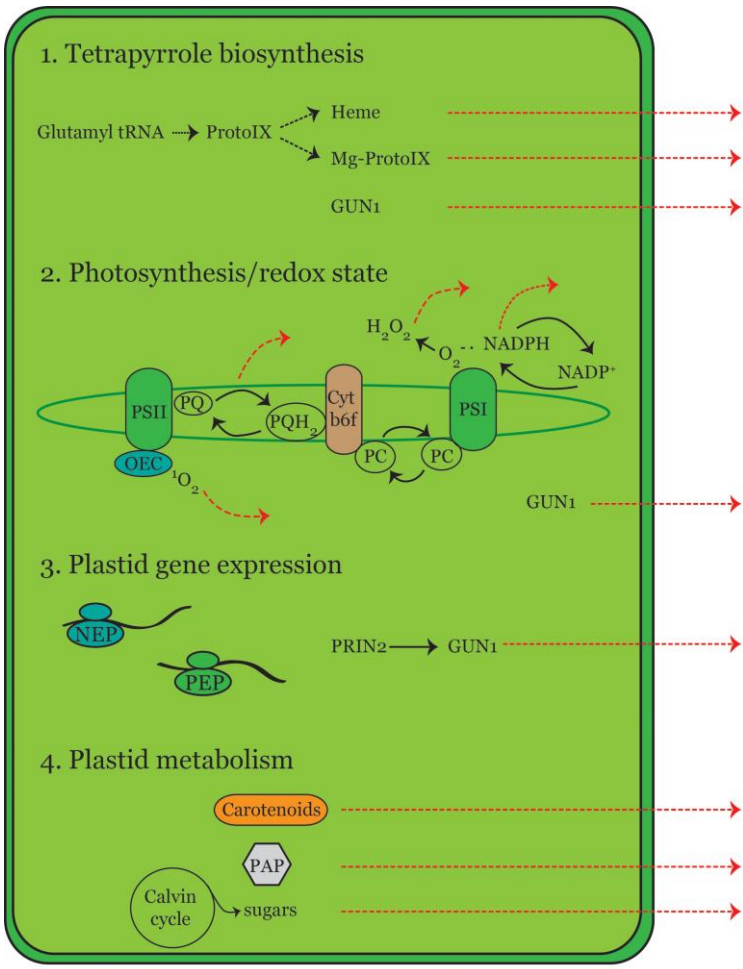

Figure 8. Plastid processes that trigger retrograde signals. Proposed components of chloroplast retrograde signalling originating from (1) tetrapyrrole biosynthesis, (2) photosynthesis and plastid redox state, (3) plastid gene expression and (4) plastid metabolism. Red arrows indicate the source of signalling molecules which are able to trigger retrograde signalling towards the nucleus.

Chlorophyll biosynthesis

The major tetrapyrroles in plants, chlorophyll, heme, phytochromobilin and siroheme, are synthesized through a common biosynthetic pathway **(Fig. 9)** (Tanaka and Tanaka 2007). The synthesis takes place inside the chloroplast and starts from the molecule glutamyl tRNA which is converted into the precursor 5-aminolevulinic acid (ALA). The enzymes responsible for the first reaction steps, from glutamyl tRNA to the formation of protoporphyrinogen IX, are all located in the stroma. The synthesis of siroheme branches off from the intermediate uroporphyrinogen III. The synthesis steps to

protoporphyrin IX are common for the other tetrapyrroles. The formation of protoporphyrin IX and subsequent heme and chlorophyll synthesis takes place in the thylakoid and inner envelope membranes **(Fig. 9)** (Mochizuki, et al. 2010; Zhang, et al. 2015). Protoporphyrin IX constitutes a branch point where the insertion of either Mg^{2+} by Mg-chelatase or Fe^{2+} by Fe-chelatase directs protoporphyrin IX either into the chlorophyll or heme biosynthetic pathway (Tanaka and Tanaka 2007). In the heme branch, protoheme and subsequently the heme and phytochromobilin molecules are formed. In the chlorophyll branch, Mg-protoporphyrin IX (Mg-ProtoIX) is further modified by the Mg-proto IX methyltransferase (CHLM) and Mg-ProtoIX monomethylester cyclase (CRD) to form protochlorophyllide. In the following steps, the light-dependent enzyme NADPH:protochlorophyllide oxidoreductase (POR) forms chlorophyllide *a* which then is converted into chlorophyll *a* or chlorophyll *b* depending on the demand.

Chlorophylls and siroheme molecules are maintained within the chloroplast where they carry out their functions, chlorophylls in light harvesting and photosynthesis, and siroheme in nitrogen and sulfur assimilation (Tripathy, et al. 2010). Phytochromobilin is the chromophore of phytochrome photoreceptors and is assembled with phytochromes in the cytosol (Terry, et al. 1993; Quail 2002). Heme plays essential roles both within the chloroplast and mitochondria in the process of photosynthesis and respiration, respectively, and several heme-binding proteins have also been found in the cytosol, ER and peroxisomes (Mochizuki, et al. 2010). The transport mechanism used to shuttle tetrapyrroles from the chloroplast to other parts of the cell is completely unknown. Transport of phytochromobilin and heme to other cell compartments is evident and it has also been proposed that protoporphyrinogen IX can be transported to the mitochondria where protoporphyrinogen oxidase and ferrochelatase activity has been demonstrated, suggesting that the last steps of heme synthesis can occur both in the chloroplast and mitochondria (Smith, et al. 1993; Watanabe, et al. 2001; Tanaka and Tanaka 2007). In addition, catabolites from chlorophyll degradation are transported from the chloroplast to the vacuole (Hortensteiner 2006).

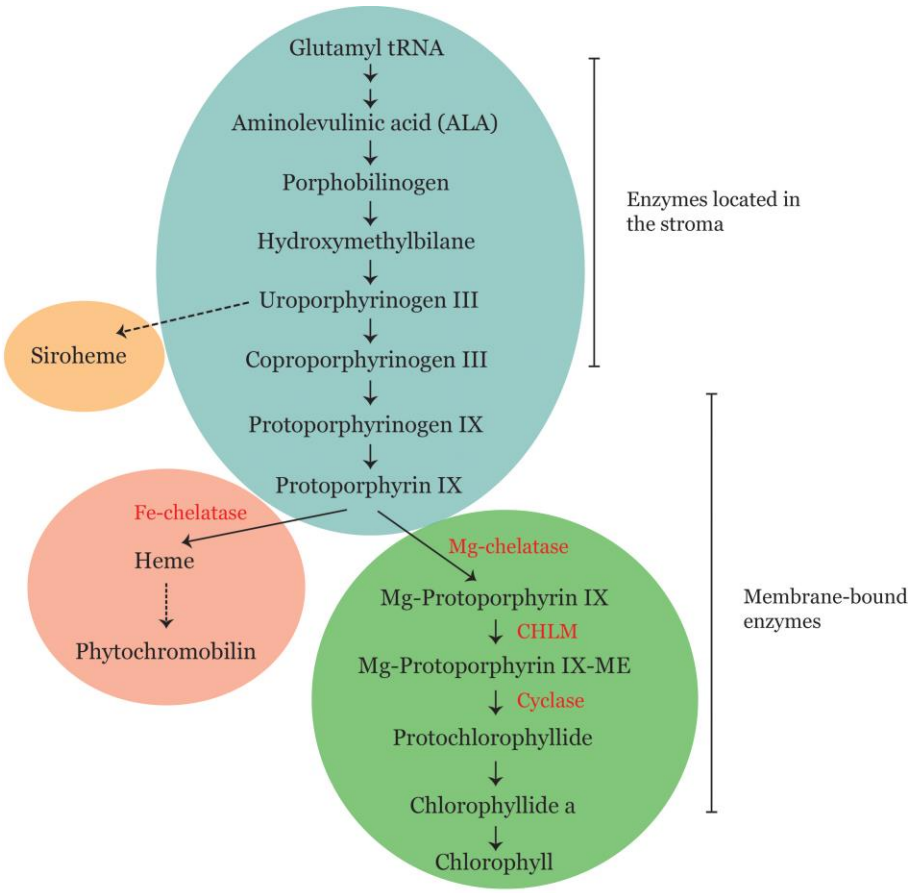

Figure 9. Tetrapyrrole biosynthesis in chloroplasts. The four major tetrapyrroles in plants, siroheme, heme, phytochromobilin and chlorophyll, are synthesized through a common pathway starting with the precursor glutamyl tRNA. The biosynthesis takes place in the stroma until the step where protoporphyrinogen IX is formed, the subsequent enzymes are membrane-bound. Siroheme synthesis branches from the uroporphyrinogen II intermediate while the heme and chlorophyll pathways branch at the protoporphyrin intermediate. Important enzymes discussed in the text are shown in red.

Tetrapyrrole-mediated signalling

Considering the wide range of cellular functions of the tetrapyrroles and the changing demand on specific tetrapyrroles determined by tissue type and developmental stage, it is clear that the regulation of the tetrapyrrole biosynthesis pathway is important. In addition, free tetrapyrrole intermediates generate highly reactive radicals and ROS, causing photo-oxidative damage. Thus, the enzymatic reactions must be tightly controlled to avoid accumulation of substrates or products at any biosynthesis step. Perturbations of the biosynthesis of tetrapyrroles have been shown to trigger large changes in nuclear gene expression (Strand, et al. 2003; Ankele, et al.

2007; Pontier, et al. 2007; Mochizuki, et al. 2008; Moulin, et al. 2008; Zhang, et al. 2011). In *Arabidopsis*, the involvement of tetrapyrrole intermediates in retrograde signalling has mainly been studied by the analysis of genome uncoupled (*gun*) mutants. These mutants were isolated in a genetic screen where oxidative stress was used to trigger retrograde signalling and photosynthetic associated nuclear gene (*PhANG*) expression was used as a marker in the nucleus. Wild type plants exposed to oxidative stress show a strong repression of *PhANG* expression, while the *gun* mutants were identified by maintained *PhANG* expression (Susek, et al. 1993).

Four of the isolated *gun* mutants (*gun2* to *gun5*) have lesions in enzymes involved in different steps of the tetrapyrrole biosynthetic pathway **(Fig. 10)**. The *gun2* and *gun3* mutants have lesions in enzymes of the heme branch and are unable to further convert heme into phytochromobilin, resulting in accumulation of heme (Susek, et al. 1993; Barajas-Lopez Jde, et al. 2013). The accumulation of heme will negatively regulate the first step in the pathway and reduce the formation of ALA (Goslings, et al. 2004). GUN4 and GUN5 are part of the Mg-chelatase complex in the chlorophyll branch responsible for the formation of Mg-ProtoIX. GUN5 is the H-subunit (CHLH) of the enzyme which binds tetrapyrroles and GUN4 is a cofactor activating the enzyme (Mochizuki, et al. 2001; Larkin, et al. 2003). Another *gun* mutant, *gun6-1D*, is a gain-of-function mutant in which the Fe-chelatase 1 (FC1) expression and activity is increased (Woodson, et al. 2011). The increased flux through the heme branch results in a similar phenotype as the other *gun* mutants suggesting heme could act as a positive regulator of nuclear gene expression (Woodson, et al. 2011). All *gun2* to *gun6* mutants have perturbations in tetrapyrrole biosynthesis and are unable to accumulate Mg-ProtoIX in response to oxidative stress (Strand, et al. 2003; Barajas-Lopez Jde, et al. 2013). The copper response defect (*crd*) mutant has impaired activity of the cyclase complex converting Mg-ProtoIX-ME to protochlorophyllide. As a result of this lesion, the *crd* mutant over-accumulates Mg-ProtoIX-ME and the upstream Mg-ProtoIX molecule (Tottey, et al. 2003; Bang, et al. 2008). However, this mutant does not show a *gun* phenotype, suggesting that the accumulation of Mg-ProtoIX and/or Mg-ProtoIX-ME is indeed required for the initiation of tetrapyrrole-mediated retrograde signalling.

The *gun* mutants have provided strong evidence for the involvement of tetrapyrroles in retrograde signalling between the chloroplast and nucleus. In *Arabidopsis* wild type, several studies have shown that Mg-ProtoIX and Mg-ProtoIX-ME accumulate transiently in response to oxidative stress and this accumulation coincides with changes in nuclear gene expression (Strand, et al. 2003; Stenbaek, et al. 2008; Kindgren, et al. 2011; Zhang, et

al. 2011). The accumulation of these tetrapyrroles has also been shown to regulate nuclear gene expression in the green algae *Chlamydomonas reinhardtii* (Johanningmeier and Howell 1984; Jasper, et al. 1991; Kropat, et al. 1997, 2000; Vasileuskaya, et al. 2004), barley seedlings (La Rocca, et al. 2001) and cress seedlings (Kittsteiner, et al. 1991). Feeding of Mg-ProtoIX to *C. reinhardtii* cell cultures was shown to induce the expression of nuclear-encoded heat shock 70 (HSP70) proteins (von Gromoff, et al. 2008). In *Arabidopsis* protoplasts, Mg-ProtoIX feeding inhibited the expression of *LHCB* genes whereas feeding with other tetrapyrroles (porphobilinogen, ProtoIX and heme) had no effect (Strand, et al. 2003).

Even though a considerable amount of data indicates that the chlorophyll precursors Mg-ProtoIX and Mg-ProtoIX-ME are responsible for regulation of nuclear gene expression, their role as signalling molecules has been questioned and the mechanism and exact nature of the tetrapyrrole-mediated signal have been widely debated. The reservations mainly come from two studies where no correlation between Mg-ProtoIX accumulation and *LHCB* expression could be reported (Mochizuki, et al. 2008; Moulin, et al. 2008). In these studies, no tetrapyrrole accumulation could be detected in plants exposed to oxidative stress caused by norflurazon (NF) treatment, even though *PhANG* expression was repressed. NF inhibits the phytoene desaturase (PDS) enzyme in the carotenoid biosynthetic pathway, causing photooxidation in the chloroplasts and complete destruction of the thylakoid membranes. This has been a common treatment to induce oxidative stress in plants. However, this treatment generates an artificial condition of severe stress, resulting in pleiotropic effects and difficulties to reproduce specific responses. Zhang et al. (2011) showed that short-term NF treatment (less than 96 h) results in inhibited activities of the CRD and POR enzymes, accumulation of Mg-ProtoIX/Mg-ProtoIX-ME and repression of *LHCB* expression. However, after 96 h of NF treatment, high variations of tetrapyrrole levels between individual plants were observed even though the *LHCB* repression was still maintained. Treatment with methyl viologen (MV) is a more controlled way to induce oxidative stress in plants. MV is an inhibitor of photosynthetic electron transport and following treatment the photoreduction of O_2 generates $O_2^{\cdot-}$ (superoxide) and H_2O_2. Mg-ProtoIX and Mg-ProtoIX-ME levels increased gradually and peaks after 3 h treatment following MV treatment, thereafter the levels decreased towards control levels (Kindgren, Noren, et al. 2012). This transient nature of tetrapyrrole accumulation following oxidative stress could explain the reported discrepancies between different studies, depending on different treatment methods and sampling conditions. Since free tetrapyrroles are harmful to the cell due to their potent photoreactive properties, it is reasonable that accumulation would be transient in order to induce the signalling response

and the accumulation thereafter being actively removed by a scavenging system.

Despite the controversy about the signalling role of Mg-ProtoIX, no other evident explanation of the origin of the tetrapyrrole-mediated signalling has so far been presented. Accumulation of ROS, changes in the flux through the biosynthetic pathway and the activity of Mg-chelatase has been suggested as alternative signal initiators (Mochizuki, et al. 2008; Moulin, et al. 2008). Expression of marker genes for ROS was investigated in the *gun5* mutant and as the expression of those genes was not different in *gun5* compared to wild type it was concluded that the *gun5* phenotype is not related to an altered accumulation of ROS (Voigt, et al. 2010). In addition, *LHCB* repression cannot be fully reversed by ROS quenchers (Zhang, et al. 2011). However, as a complement to the negative signal triggered by Mg-ProtoIX/Mg-ProtoIX-ME, a positive signal triggered by the accumulation of heme was suggested (Woodson, et al. 2011). Heme is known to regulate gene expression in animal and yeast cells (Zhang and Hach 1999; Ogawa, et al. 2001) and the feeding of heme and Mg-ProtoIX was shown to trigger global changes in gene expression in *C. reinhardtii* (Voss, et al. 2011). Studies of the gain-of-function mutant *gun6-1D* has shown that induced heme synthesis by FC1 heme synthase up-regulates *PhANG* expression (Woodson, et al. 2011). Interestingly, heme synthesis by the second heme synthase in plastids, FC2, does not affect *PhANG* expression, suggesting that heme produced by the different enzymes could contribute to different pools destined for either signalling or other purposes (Woodson, et al. 2011). The suggested presence of both a positive (heme) and negative (Mg-ProtoIX) regulator of *PhANG* expression could imply that the gene expression output is dependent on the balance between these molecules. Changes in the biosynthetic pathway that favours either the accumulation of heme or Mg-ProtoIX would determine if a positive or negative signal is being sent towards the nucleus.

Cytosolic interactions and nuclear targets of tetrapyrrole-mediated signalling

Even though great advances have been made in identifying components involved in retrograde signalling (Chan, et al. 2016), the cytosolic interactions and components have been considered a “black box”. Many studies have identified the processes in the chloroplast which trigger retrograde signals and their targets in the nucleus, but how these signals are transduced through the cytosol and reach the nucleus to induce a response have remained elusive. The tetrapyrrole-mediated retrograde signalling is dependent on the accumulation of specific intermediates, Mg-ProtoIX/Mg-ProtoIX-ME and heme. These molecules are not considered to be long-

distance signalling molecules since they are very photoreactive and hydrophobic. Even though it is not known how tetrapyrroles are transported from the chloroplast, it is clear that heme, heme precursors, phytochromobilin and chlorophyll degradation products are exported from the chloroplast (Smith, et al. 1993; Terry, et al. 1993; Watanabe, et al. 2001; Quail 2002; Hortensteiner 2006; Tanaka and Tanaka 2007; Mochizuki, et al. 2010). Furthermore, *in vivo* visualization of Mg-ProtoIX in *Arabidopsis* showed that Mg-ProtoIX accumulates both in the chloroplast and in the cytosol following oxidative stress (Ankele, et al. 2007; Zhang, et al. 2011). This suggests that there is a transport mechanism for Mg-ProtoIX to exit the chloroplast in response to stress conditions and transmit the signal to the cytosol.

In order to identify cytosolic interacting partners of Mg-ProtoIX which could subsequently be involved in mediating the signal to the nucleus, a proteomic study was performed by Kindgren et al. (2011). In this study, several interacting proteins were identified including a large group of stress-related proteins and proteins involved in the scavenging and degradation of tetrapyrroles, which strengthened the support for presence of Mg-ProtoIX in the cytosol and its role as a signalling molecule. Furthermore, interactions between Mg-ProtoIX and the heat shock protein 90-type (HSP90) and HSP70 proteins were identified. This result was especially interesting since in yeast, HSP90 proteins have been shown to form a chaperone complex together with HSP70 proteins and the transcription factor heme-responsive transcriptional activator 1 (HAP1). The activity of this complex is regulated by heme, promoting the transcription of genes required for oxygen utilization and protection (Zhang and Hach 1999). It was thus proposed that a similar complex, involving HSP90 proteins and tetrapyrroles, could be conserved in plants to regulate gene expression. The presence of a HSP90-Mg-ProtoIX complex in the cytosol was subsequently confirmed and it was demonstrated that this complex regulates the transcription factor HY5 which in turn controls *PhANG* expression **(Fig. 10)** (Kindgren, Noren, et al. 2012).

The GUN1 protein has been suggested to be involved both in generation and integration of multiple plastid signals, such as tetrapyrrole-mediated signals, plastid gene expression-mediated signal and ROS signals **(Fig. 8)** (Koussevitzky, et al. 2007; Zhang, et al. 2011). The exact function of GUN1 is not known, but it has been shown to act upstream of the abscisic acid insensitive 4 (ABI4) transcription factor that binds to the ACGT motif of light- and ABA-responsive genes (Koussevitzky, et al. 2007). The expression of *ABI4* is regulated by a PHD type transcription factor with transmembrane domains (PTM), located in the outer envelope membrane of the chloroplasts. PTM has been suggested as a putative signalling component linking the

chloroplast to the nucleus. The GUN1-mediated signal activates a proteolytic mechanism which cleaves the N-terminal part of the PTM protein. The N-PTM peptide is transferred to the nucleus where it induces expression of the *ABI4* transcription factor and thereby mediates repression of *PhANG* expression **(Fig. 10)** (Siberil, et al. 2001; Sun, et al. 2011). ABI4 is also a key component in ABA signalling and the *gun1* mutant is more sensitive to ABA compared to wild type plants, implying a role for GUN1 also in ABA signalling (Cottage, et al. 2010).

The *cis*-elements found in the promoters of genes responsive to plastid retrograde signalling largely overlap with light-responsive elements (Brown, et al. 2005; von Gromoff, et al. 2006; Koussevitzky, et al. 2007). The best characterized *cis*-elements in plants responding to both light and retrograde signals are the G-box, CUF1 and GATA elements. ABI4 inhibits the expression of *LHCB* by binding to the G-box element in the promoter region in response to both plastid and ABA signalling (Koussevitzky, et al. 2007). The bZIP transcription factor HY5 is a key regulator in light response and has also been shown to bind the G-box of *LHCB* promoters (Chattopadhyay, et al. 1998). In addition, HY5 has been shown to respond to plastid signals (Ruckle, et al. 2007; Kindgren, Noren, et al. 2012; Noren, et al. 2016). This demonstrates that integration of light and plastid signalling plays an important role for the proper output of gene expression. In addition, competition between different transcription factors for the same *cis*-elements is probably a major regulatory mechanism determining the final gene expression output.

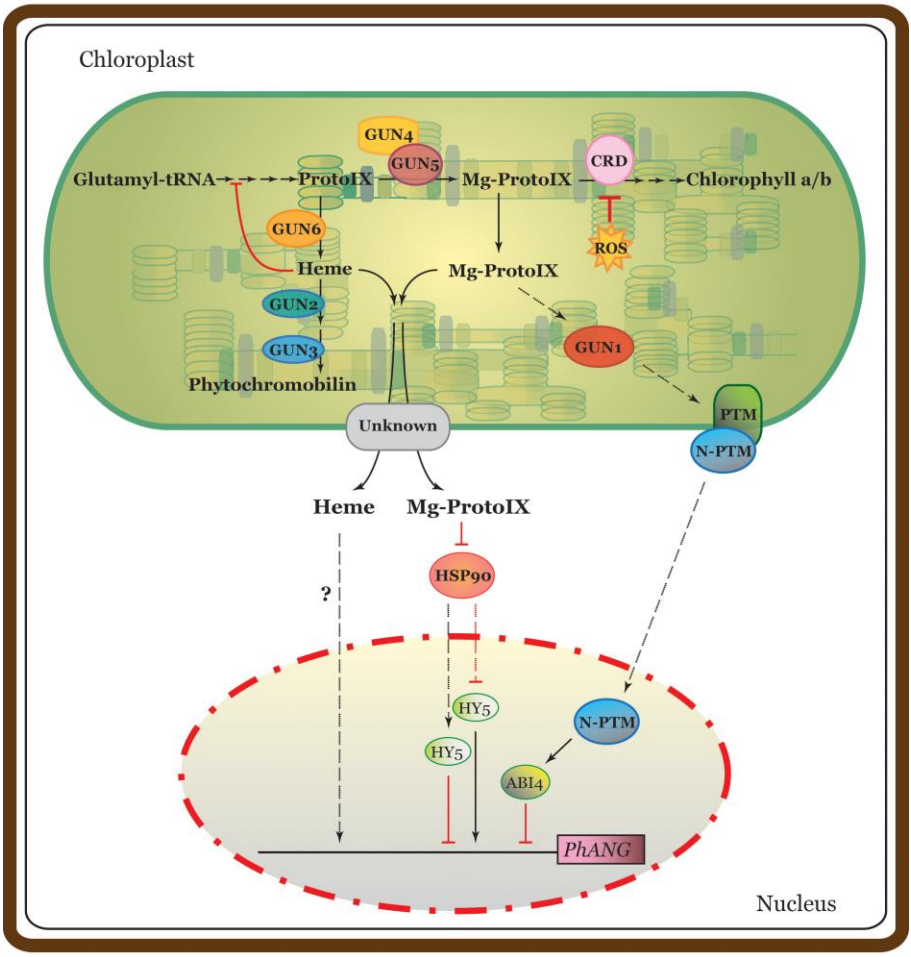

Figure 10. Pathways of tetrapyrrole retrograde signalling. The tetrapyrroles heme and Mg-ProtoIX have been identified as metabolites responsible to initiate retrograde signals. The transport mechanism of these tetrapyrroles out from the chloroplast is unknown. Heme has been reported to act as a positive regulator of *PhANG* while Mg-ProtoIX is a negative regulator. Oxidative stress results in inhibited flux through the tetrapyrrole biosynthetic pathway due to inhibition of the CRD cyclase system and results in accumulation of the Mg-ProtoIX intermediate. Interaction between Mg-ProtoIX and HSP90 in the cytosol inactivates the activity of HSP90 causing a repression of *PhANG* through HY5 by either activating HY5 repression or inhibiting HY5 activation of gene expression. GUN1-mediated signalling activates the stress sensor PTM in the envelope membrane. The N-terminal part of the PTM protein is then released and travels to the nucleus where it activates the ABI4 transcription factor. The identified GUN1-6 proteins and their involvement in tetrapyrrole biosynthesis are indicated. Adapted from Barajas-López, et al. 2013.

Redox-mediated signalling

Perturbations in the photosynthetic process can cause redox changes within the chloroplast and trigger retrograde signalling pathways. These perturbations can be caused by environmental factors such as high light,

drought and changes in temperature. Redox signals are responsible for the coordination of expression of photosynthetic genes encoded in the nucleus and chloroplast and are important both during the greening process of young seedlings and for adult plants to adapt to changing growth conditions (Barajas-Lopez Jde, et al. 2013). The accumulation of specific ROS molecules, the redox state of the plastoquinone (PQ) pool and the acceptor availability at PSI have all been suggested as possible sources for the plastid redox signal(s) **(Fig. 8)**. Environmental changes disrupt the balance between ROS production and scavenging, causing a transient accumulation of ROS due to overreduction of the electron transport chain and transfer of electrons to oxygen (Elstner 1991; Asada 2006). At PSII, excited chlorophyll molecules can react with oxygen and produce singlet oxygen ($^1O_2$). Oxygen can also be reduced at the acceptor side of PSI, producing super-oxide ($O_2^{\bullet-}$) (Niyogi 1999). $O_2^{\bullet-}$ is rapidly converted to hydrogen peroxide (H_2O_2) (Mullineaux and Karpinski 2002; Apel and Hirt 2004). $^1O_2$ and $O_2^{\bullet-}$ are very reactive and have short half-lives, which makes it unlikely that these compounds would move within the cell and act as signalling components themselves (Kim and Apel 2013). H_2O_2 on the other hand, is more stable and is thought to move across membranes through aquaporins (Bienert, et al. 2007; Petrov and Van Breusegem 2012). Thus, H_2O_2 could itself function as a retrograde signal and directly interact with cytosolic signalling components. Also, it has recently been shown that H_2O_2 relocates from the chloroplast to the nucleus via stromules under stress conditions (Caplan, et al. 2015), possibly enabling direct effects on nuclear targets.

Even though the different ROS molecules cause similar cellular damage, they have been shown to activate distinct signalling pathways. H_2O_2 regulates the expression of genes involved in plant development and stress responses, in particular the *APX2* gene encoding a ROS scavenger which responds to H_2O_2 accumulation (Pnueli, et al. 2003; Vanderauwera, et al. 2005). The accumulation of $^1O_2$ has been shown to primarily activate genes involved in cell death and a minor set of genes encoding plastid located proteins (Wagner, et al. 2004; Danon, et al. 2005), suggesting that $^1O_2$ is rather used for general stress response than the coordination of genome activities (Barajas-Lopez Jde, et al. 2013). Executer 1 and 2 (EX1 and EX2) have been identified as two chloroplast localized proteins involved in the $^1O_2$ mediated signalling (Lee, Kim, et al. 2007) but their exact role is still elusive.

The redox status of the photosynthetic electron transport chain has been shown to be correlated with changes in gene expression in both the nucleus and chloroplast (Brautigam, et al. 2009; Foudree, et al. 2010; Kindgren, Kremnev, et al. 2012). The redox state of the PQ pool can be modified by two different inhibitors, DBMIB (2,5-dibromo-3-methyl-6-isopropyl-

benzoquinone) and DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). DBMIB inhibits the electron transfer from PQ to the cyt b_6f complex, resulting in a reduced PQ pool, while DCMU inhibits the electron transfer from PSII to PQ, leaving PQ oxidized. These treatments were shown to correlate with changes in *PhANG* expression, DBMIB treatment decreased *LHCB* expression whereas DCMU increased *LHCB* expression, suggesting that the redox state of the PQ pool is sensed and triggers retrograde signals (Escoubas, et al. 1995). It is probable that in addition to the PQ pool, other components of the electron transport chain or coupled components in the stroma are able to sense and mediate information about the chloroplast redox status (Brautigam, et al. 2009; Barajas-Lopez Jde, et al. 2013). The acceptor side of PSI has also been shown to be a site for initiation of redox-mediated signals. The redox state of components on the reducing side of PSI was shown to affect nuclear gene expression, particularly at early redox signalling within 1 to 3 hours after changes in environmental conditions (Piippo, et al. 2006). The redox-mediated signals are all triggered by perturbations in the photosynthetic process and their signalling pathways are most likely interconnected and converge in order to induce the proper response to different conditions.

Aim

The overall aim of this thesis was to understand the interaction and communication between the chloroplast and the nucleus during different growth conditions in *Arabidopsis thaliana*.

Two main questions have been addressed in this thesis:

1. What mechanism(s) and which components are involved in the tetrapyrrole-mediated plastid signalling pathway under photoperiodic growth and during oxidative stress conditions?

2. Which transcription factors regulate nuclear gene expression in response to light and cellular redox changes?

Results and discussion

The main goal of this thesis was to elucidate signalling pathways between the chloroplast and the nucleus used to coordinate gene expression from the two genomes. It becomes more and more evident that the nucleus and chloroplast are equally dependent on each other in order to implement the correct responses, and also that different signalling pathways converge in order to fine-tune gene expression. My approach was to study different pathways and to analyse their importance during various growth conditions. My work demonstrates how plants are able to sense and respond to excess light and oxidative stress **(Paper I and III)**, but also how they grow and adjust processes to day-night changes during photoperiodic conditions **(Paper II and IV)**. This work demonstrates further that tetrapyrroles play an important role in the response to oxidative stress and we have identified an interaction with a previously unknown cytosolic component, providing a mechanism for how the tetrapyrrole-mediated signal can be transduced from the plastid to the nucleus and regulate nuclear gene expression **(Paper I)**. An increasing number of reports state that the convergence of different signalling pathways within the cell is extensive and that this view has to be considered in order to fully understand the output of different stimuli. Here we also show that the tetrapyrrole-mediated signalling pathway integrates with circadian signalling in order to fine-tune nuclear gene expression during photoperiodic conditions **(Paper II)**. The regulation of nuclear gene expression in the response to high light and redox changes has been analysed by identifying transcription factors which responds to these signals **(Paper III)**. These transcription factors were shown to regulate nuclear genes encoding plastid proteins and are of particular importance for anterograde control during the early light response and establishment of photomorphogenic growth **(Paper IV)**.

Tetrapyrrole-mediated retrograde signalling

Accumulation of tetrapyrroles during different growth conditions

The connection between tetrapyrroles, retrograde signalling and regulation of nuclear gene expression is commonly accepted, however, the exact mechanisms involved and the components that mediate this signal have been widely debated over the years. The chlorophyll intermediates Mg-ProtoIX and Mg-ProtoIX-ME have been shown, by different groups, to accumulate when plants are exposed to various treatments causing oxidative stress (Strand, et al. 2003; Wilson, et al. 2003; Aarti, et al. 2006; Ankele, et

al. 2007; Kindgren, et al. 2011; Zhang, et al. 2011). In wild type plants, oxidative stress causes a transient accumulation of Mg-ProtoIX/Mg-ProtoIX-ME and a subsequent repression of the nuclear *PhANG* genes (Strand, et al. 2003; Kindgren, et al. 2011; Zhang, et al. 2011). The enzymatic activity of Mg-ProtoIX monomethylester cyclase is highly sensitive to ROS and oxidative stress both in *Arabidopsis* and cucumber, and could explain the rapid accumulation of tetrapyrroles (Aarti, et al. 2006; Stenbaek, et al. 2008). Seedlings grown on norflurazon (NF) have been shown to accumulate high amounts of Mg-ProtoIX and Mg-ProtoIX-ME and NF treatment has commonly been used to study the response to oxidative stress. However, this treatment is harsh and large variations in the tetrapyrrole levels were reported, both within and between different studies (Susek, et al. 1993; Strand, et al. 2003; Mochizuki, et al. 2008; Moulin, et al. 2008; Kindgren, et al. 2011; Zhang, et al. 2011). The demonstrated correlation between Mg-ProtoIX accumulation following NF treatment and changes in nuclear gene expression (Strand, et al. 2003) could not be reproduced in two studies and it was therefore postulated that Mg-ProtoIX is not responsible for initiation of plastid signalling (Mochizuki, et al. 2008; Moulin, et al. 2008). Strand et al. (2003) reported several independent experiments with consistently higher tetrapyrrole levels following NF treatment but the increase compared to control varied between 4-15 fold, and Zhang et al. (2011) demonstrated a high variation in tetrapyrrole levels between individual seedlings after 96 h of NF treatment. It is reasonable that the accumulation of Mg-ProtoIX is transient following stress in order to initiate a signalling response and that the molecules thereafter are sequestered. However, it is clear that the conditions used to study this pathway must be chosen with great care in order to detect the true response.

In order to address the uncertainties associated with the NF treatment, we have used alternative conditions to manipulate the tetrapyrrole biosynthesis pathway to induce accumulation of Mg-ProtoIX/Mg-ProtoIX-ME and consequently trigger the tetrapyrrole-mediated signalling pathway. Methyl viologen (MV) treatment triggers oxidative stress by blocking the photosynthetic electron transport and generates a more controlled and physiological accumulation of ROS compared to the NF treatment. Mg-ProtoIX and Mg-ProtoIX-ME have been shown to accumulate following MV treatment with a peak after 3.5 h exposure and thereafter the levels decline again (Aarti, et al. 2006; Kindgren, et al. 2011). We could confirm the accumulation of Mg-ProtoIX/Mg-ProtoIX-ME following 3.5 h of MV treatment **(Paper I, Table 1)**. We used both the NF and MV treatments in our studies and showed that both treatments induce a repression of *LHCB1.1*, *LHCB2.4* and *RBCS* expression in wild type plants **(Paper I, Fig. S2A and B)**. To further analyse the involvement of Mg-ProtoIX in plastid-

mediated repression of *PhANG* expression, Mg-ProtoIX feeding experiments were performed. During these experiments, plants were kept in constant low light to avoid diurnal patterns and ROS production, enabling analysis of Mg-ProtoIX accumulation itself without stress or other side-effects. The feeding experiments showed that Mg-ProtoIX accumulated following 12 h feeding **(Paper I, Table 2)** and that *PhANG* expression was repressed **(Paper I, Fig. S2C)**. Another alternative treatment to trigger the tetrapyrrole-mediated signal was geldanamycin (GDA), an inhibitor of HSP90, one of the identified down-stream components discussed below. In a similar manner to Mg-ProtoIX feeding, 12 h GDA treatment mimics the induction of tetrapyrrole signalling and repression of *PhANG* expression **(Paper I, Fig. S2D)**.

In addition to stress conditions and feeding experiments, accumulation of Mg-ProtoIX and Mg-ProtoIX-ME has also been shown to follow a strong rhythmic pattern during non-stressed photoperiodic conditions. In tobacco, it was shown that the levels of ProtoIX, Mg-ProtoIX and Mg-ProtoIX-ME increase rapidly in the beginning of the light period with a peak at mid-day and thereafter the levels decrease until dusk (Papenbrock, et al. 1999). The diurnal oscillation of these tetrapyrroles is immediately and completely abolished if the plants are transferred to constant conditions such as continuous light or dark. This is consistent with the enzyme activity patterns of Mg-chelatase and Mg-ProtoIX monomethylester cyclase which are also inhibited during constant conditions (Papenbrock, et al. 1999). Diurnal accumulation of tetrapyrroles in *Arabidopsis* was confirmed to be dependent on daily light and dark changes to maintain the oscillation pattern **(Paper II, Fig. 2B and C)**. We demonstrated here that the rhythmic pattern of Mg-ProtoIX accumulation during photoperiodic growth conditions triggers the tetrapyrrole-mediated chloroplast-to-nucleus communication and generates input to the regulation of nuclear encoded genes.

In the work presented here, different approaches and treatments are being used to analyse the tetrapyrrole-mediated plastid signalling. Specifically two questions were addressed with the aim to shed light on the exact involvement of Mg-ProtoIX in retrograde signalling; (1) How could the Mg-ProtoIX-mediated signal be transduced to the nucleus? **(Paper I)** and (2) Are there any physiological relevant conditions where Mg-ProtoIX plays a role in plastid signalling? **(Paper I, II)**. The results presented here once more point to that the Mg-ProtoIX molecule itself is indeed involved in the tetrapyrrole-mediated retrograde signalling.

Interaction between Mg-ProtoIX and cytosolic HSP90 proteins

One of the constraints for tetrapyrrole intermediates acting as signalling molecules is the fact that they are highly reactive in their free state. As a response, the levels of free tetrapyrroles are tightly regulated since accumulation of these molecules would eventually result in ROS production and severe oxidative damages. However, a transient accumulation of Mg-ProtoIX and Mg-ProtoIX-ME has been shown to occur in the response to stress and is believed to mediate a signal to the cytosol before the molecules are scavenged (Kindgren, et al. 2011; Zhang, et al. 2011). This tetrapyrrole-mediated signal has been shown to repress nuclear gene expression in response to stress, but how the signal is transduced and which components are involved has remained elusive and has given rise to the expression of the cytosol as the unknown "black box". In order to address this question, Kindgren et al. (2011) performed a proteomic study in which cytosolic interaction partners of Mg-ProtoIX were identified. Three cytosolic HSP90 type proteins were found among the identified proteins. In *Arabidopsis*, there are seven HSP90 proteins: four cytosolic (HSP90.1-4), one chloroplastic (HSP90.5), one mitochondrial (HSP90.6) and one located in the ER (HSP90.7) (Krishna and Gloor 2001). The function of HSP90 proteins is essential and evolutionary conserved among fungi, animals and plants, even though specific substrates may vary among the different species (Sangster and Queitsch 2005). HSP90 proteins interact with proteins in their near native state and have been shown to be essential for the activity of numerous signalling proteins (Young, et al. 2001) and for preventing aggregation of newly translated chloroplast pre-proteins (Fellerer, et al. 2011). HSP90 functions in complexes together with various co-chaperones that modulate its ATPase activity and interaction with client proteins (Sangster and Queitsch 2005). HSP90 proteins are abundant under physiological conditions but are also induced, and particularly important, during different stress conditions (Frydman 2001).

The identified interaction between Mg-ProtoIX and HSP90 proteins by Kindgren et al. (2011) was confirmed *in vitro* by analysing the fluorescent spectra of different tetrapyrroles incubated with expressed HSP90.2 protein. We showed that both Mg-ProtoIX and Mg-ProtoIX-ME specifically bind the HSP90.2 protein **(Paper I, Fig. 1)**. The protein folding mechanism of HSP90 proteins is ATP dependent and energy derived from ATP hydrolysis is necessary for its activity. The N-terminal of HSP90 contains the ATP binding site (Prasad, et al. 2010) and the compound GDA has been shown to specifically bind this site (Whitesell, et al. 1994) and inhibits the ATPase activity of HSP90 (Avila, et al. 2006). Due to the conservation of HSP90 proteins between species, GDA has been shown to function as a specific

inhibitor in a variety of different organisms, including *Arabidopsis* (Queitsch, et al. 2002). Inhibition of the ATPase activity prevents HSP90 from retrieving energy and performing its chaperone actions, leading to unstable complexes and protein degradation (Queitsch, et al. 2002). In order to test the possibility that the binding and action of tetrapyrroles could resemble the effect of GDA, we performed a coupled enzymatic assay in which the ATPase activity of HSP90 can be determined (Avila, et al. 2006). The ATPase activity of HSP90.2 was shown to be strongly inhibited when incubated with GDA or Mg-ProtoIX **(Fig. 11A) (Paper I, Table 3)**. This result confirms the binding of Mg-ProtoIX to HSP90.2 and further demonstrates that Mg-ProtoIX inhibits the ATPase activity of HSP90. In addition to GDA, other inhibitory compounds of HSP90 have been identified (Avila, et al. 2006) and even structurally unrelated molecules have been shown to bind to the same nucleotide-binding pocket of HSP90 (Roe, et al. 1999; Queitsch, et al. 2002), implying the possibility that also Mg-ProtoIX could have this function **(Fig. 11B)**. Exactly how tetrapyrroles interact with proteins is not known. No binding motif could be detected among the proteins identified in the interaction study and it seems like the binding depends on a specific fold rather than an amino acid motif (Kindgren, et al. 2011). Crystal structure information for GUN4, another protein identified to interact with Mg-ProtoIX (Larkin, et al. 2003) revealed a three dimensional fold with a “cupped hand” shape where binding of Mg-ProtoIX occurs and this fold was also suggested to have a protective role in tetrapyrrole trafficking (Verdecia, et al. 2005).

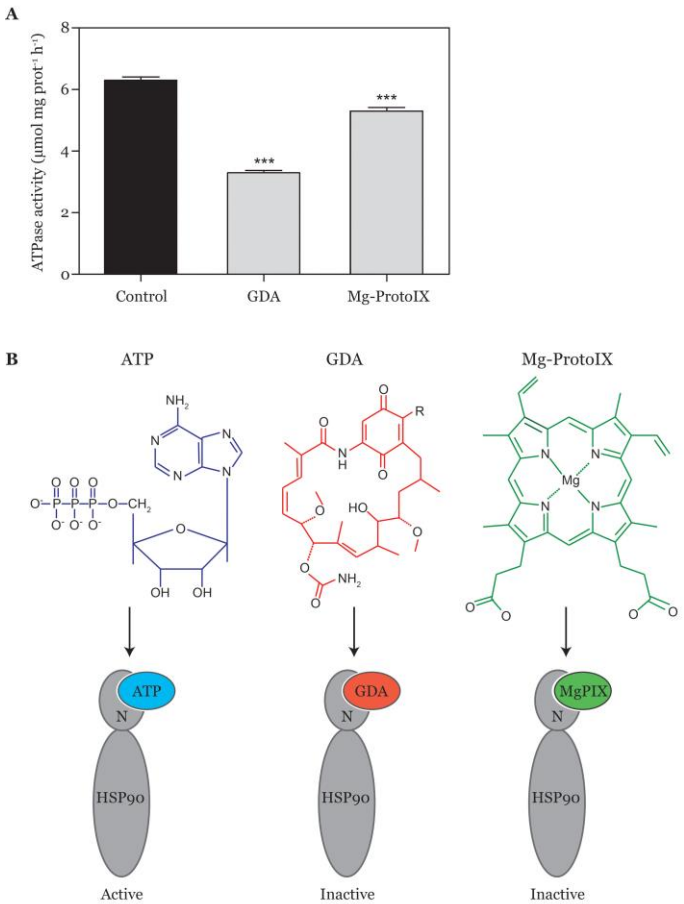

Figure 11. ATPase activity for HSP90. (A) Expressed HSP90.2 was incubated with 40 µM GDA or 20 µM Mg-ProtoIX and the ATPase activity was measured in a coupled enzymatic assay. Each data point represents the mean (±SD) of at least four independent biological replicates. The HSP90 ATPase activity following GDA and Mg-ProtoIX treatments was significantly different from the control, as demonstrated by Student´s t-test: ***P<0.001. **(B)** Schematic representation of the ATP, GDA and Mg-ProtoIX molecules and their interaction with HSP90 proteins. The binding of Mg-ProtoIX to the ATP binding site is putative.

HY5 responds to the Mg-ProtoIX/HSP90 signal and regulates PhANG expression

A chaperone complex composed of the HAP1 transcription factor, HSP90, HSP70 and some additional co-chaperones has been identified and well characterized in yeast (Zhang and Hach 1999; Lan, et al. 2004). This complex is regulated by the tetrapyrrole heme which enhances the interaction between HAP1 and HSP90 under aerobic conditions and promotes HAP1 activation (Lan, et al. 2004). In addition to the HSP90 proteins, Kindgren et al. (2011) also identified HSP70 and HSP70-

interacting protein 1 (HIP1) as Mg-ProtoIX interacting proteins. This suggests the possibility that a complex similar to the one in yeast is conserved in plants and controls photosynthetic gene expression in response to changes in the environment. To test whether the interaction between Mg-ProtoIX and HSP90 proteins has a biological function in the tetrapyrrole-mediated signalling, we constructed RNAi lines targeting all four cytosolic HSP90 proteins. Wild type Col-0 and the *gun5* were transformed with this construct **(Paper I, Fig. 2)** and analysed for *PhANG* expression following oxidative stress. The *Col-hsp90* RNAi lines showed a strong repression of *PhANG*, similar to or stronger than Col-0, following both NF and MV treatment **(Paper I, Fig. 3 and 4)**. While the *gun5* mutant displayed no *PhANG* repression, the *gun5-hsp90* RNAi lines showed significantly lower *PhANG* expression compared to *gun5* **(Paper I, Fig. 3 and 4)**. Thus, the *gun5* phenotype was suppressed in the *gun5-hsp90* RNAi lines following both NF and MV treatment. These results demonstrated that cytosolic HSP90 is required for *PhANG* expression in the *gun5* mutant and mediates the GUN5 signal during oxidative stress.

In order to transmit the Mg-ProtoIX-mediated signal towards the nucleus and induce changes in gene expression, the inactivation of HSP90 proteins in the cytosol should eventually affect some transcription factor(s). There is no ortholog of the yeast HAP1 transcription factor present in the *Arabidopsis* genome. However, the transcription factor HY5 is known to bind to the G-box element present in many of the *PhANG* promoters and has been shown to be converted from a positive to a negative regulator of *LHCB* in response to plastid signals (Larkin, et al. 2003; Lee, He, et al. 2007). If HY5 would respond to the GUN5/HSP90-mediated signal, the *hy5* mutant should display a *gun* phenotype similar to *gun5* following oxidative stress. Indeed, *PhANG* expression was not affected in the *hy5* mutant following MV treatment and the expression was maintained at control levels **(Paper I, Fig. 5 and S7)**. To further confirm the involvement of Mg-ProtoIX in the regulation of *PhANG* expression, Col-0, *gun5* and *hy5* plants were fed Mg-ProtoIX and analysed for *PhANG* expression. Following 12 h feeding, Col-0, *gun5* and *hy5* all accumulated similar levels of Mg-ProtoIX **(Paper I, Table 2)**. Wild type Col-0 and the *gun5* mutant both showed a strong repression of *LHCB1.1* and *RBCS* expression following Mg-ProtoIX feeding, while the *hy5* mutant did not respond to the feeding **(Paper I, Fig. 6)**. This result demonstrated that the *gun5* phenotype can be explained by the inability to accumulate tetrapyrroles and is therefore rescued by exogenously applied Mg-ProtoIX. Despite the initiation of the tetrapyrrole-mediated signal following MV treatment and Mg-ProtoIX feeding, the *hy5* mutant is unable to respond and adjust the *PhANG* expression. This suggests that there is a link between GUN5 and HY5, and that HY5 acts downstream of the

tetrapyrrole accumulation in order to repress *PhANG* expression. HY5 could thus be the transcription factor that is responding to the activity of HSP90 proteins in plants.

To further demonstrate the involvement of cytosolic HSP90 and confirm that HSP90 and HY5 are components of the same pathway, we treated Col-0, *gun5* and *hy5* plants with GDA for 12 h and analysed *PhANG* expression. GDA does not induce any stress response but specifically inhibits the activity and action of the HSP90 component (Nishizawa-Yokoi, et al. 2010). Similarly to the Mg-ProtoIX feeding, GDA treatment resulted in a strong repression of *PhANG* expression in wild type Col-0 and the *gun5* mutant while the *hy5* mutant did not show any repression **(Paper I, Fig. 7)**. This further indicates that Mg-ProtoIX and GDA could function in the same manner and inhibit the activity of HSP90 proteins. The observation that *hy5* is insensitive to GDA demonstrates that HSP90 and HY5 are regulatory components in the same pathway. Taken together, the results indicate that Mg-ProtoIX, HSP90 and HY5 are part of the same signalling pathway responding to oxidative stress in order to repress *PhANG* expression. However, no direct interaction could be detected between HSP90.2 and HY5 in a yeast-two-hybrid (Y2H) interaction analysis **(Paper I, Fig. S10)** or Co-IP in *Arabidopsis* protoplasts (unpublished data). This suggests that HY5 is associated to the cytosolic HSP90 complex through another component, potentially HSP70 or HIP1, or that the interaction requires some specific condition not yet detected in order to occur. It has also been shown that promoter binding of HY5 is not sufficient to regulate transcription, but that HY5 requires other factors in order to induce or repress gene expression (Lee, He, et al. 2007; Larkin and Ruckle 2008). It is possible that the HSP90 complex regulates the activity of another factor that in turn affects HY5 activity. There are thus two possible mechanisms by which the *PhANG* expression could be regulated upon oxidative stress. Accumulation of Mg-ProtoIX inhibits the activity of HSP90 which could trigger changes to HY5 via an unknown protein and subsequently activate repression of *PhANG*, or, inhibited activity of HSP90 could cause impaired maturation of positive HY5 cofactors and therefore inhibit *PhANG* activation **(Paper I, Fig. 8)**.

The role of tetrapyrroles during oxidative stress

Oxidative stress in plants may be associated with several different stress conditions such as high light, low temperature and other factors affecting the photosynthetic electron transport rate. The accumulation of ROS during these conditions is the main reason for decreased productivity, damages and eventually cell death, and plants have thus evolved several mechanisms to protect themselves against excess ROS. The CRD cyclase complex is

extremely sensitive to ROS (Aarti, et al. 2006; Stenbaek, et al. 2008) and early ROS-mediated inhibition of CRD may explain the observed rapid accumulation of tetrapyrroles following oxidative stress (Strand, et al. 2003; Wilson, et al. 2003; Aarti, et al. 2006; Ankele, et al. 2007; Kindgren, et al. 2011; Zhang, et al. 2011; Barajas-Lopez Jde, et al. 2013). The activity of CRD and diurnal oscillation of tetrapyrrole levels could thus provide a system for sensing changes in the environment and for adjustment of *PhANG* expression. High accumulation of ROS levels activate several different signalling pathways such as H_2O_2 and $^1O_2$-dependent signalling pathways, however, the tetrapyrrole-mediated pathway does not seem to integrate with these ROS signals (Voigt, et al. 2010). Cross-talk between the different ROS-mediated signals has been proposed to provide fine-tuning of the response to oxidative stress (Laloi, et al. 2007), and possibly tetrapyrrole-mediated signalling could integrate with other metabolite or light signalling pathways. Redox-mediated retrograde signals might be viewed as a network of factors sensitive to different environmental and developmental changes to adjust the intensity of the message from the chloroplast (Barajas-Lopez Jde, et al. 2013), and tetrapyrroles could provide an input to this network in order to fine-tune gene expression.

Retrograde signalling during photoperiodic conditions

We demonstrate that a rapid and transient accumulation of tetrapyrroles following various oxidative stress conditions triggers plastid signalling and regulates nuclear gene expression. In addition to accumulation in response to oxidative stress, tetrapyrrole levels follow a strong diurnal oscillation pattern. The *crd* mutant which has impaired activity of the cyclase complex converting Mg-ProtoIX-ME into protochlorophyllide, overaccumulates the tetrapyrroles Mg-ProtoIX and Mg-ProtoIX-ME (Tottey, et al. 2003; Bang, et al. 2008). The daily oscillation pattern of Mg-ProtoIX and Mg-ProtoIX-ME in *crd* was shown to follow the same rhythm as wild type, but with a much higher amplitude **(Paper II, Fig. 2B and C)**. Similar to wild type, the tetrapyrrole levels in *crd* decreased drastically and were maintained at low levels in constant light conditions. Several nuclear genes encoding chloroplastic proteins have been shown to be repressed in the *crd* mutant, such as genes encoding LHC, PSI, PSII and chlorophyll biosynthesis proteins (Bang, et al. 2008), suggesting that the defect in *crd* mediates a retrograde signal to the nucleus. Taking advantage of the *crd* mutant and investigating retrograde signalling during diurnal conditions, we have been able to demonstrate a role for Mg-ProtoIX-mediated signalling also during non-stressed photoperiodic growth.

Many of the nuclear genes encoding chloroplast-localized proteins have been shown to follow a rhythmic daily expression pattern during photoperiodic conditions, such as several *LHCA/B*, *PSI* and *PSII* genes (Harmer, et al. 2000). The expression levels of these genes peak around the middle of the subjective day and ensures that the usage of available resources are optimized according to the day and night cycles (Green, et al. 2002; Dodd, et al. 2005; Dodd, Dalchau, et al. 2014). *LHCB* and *RBCS* are commonly used as marker genes when studying retrograde signalling, however, these genes have also been described to be regulated by the circadian clock, light/photoreceptors, hormones and various stresses in addition to plastid signals (Pfannschmidt 2010). This could make it difficult to distinguish the effect between different signalling pathways and make them unsuitable for more detailed studies. The identification of novel marker genes with less crosstalk between different signalling pathways is thus important for the analysis of plastid signalling (Pfannschmidt 2010). In addition to photosynthetic genes, other categories of genes responding to plastid signals have also been identified, such as genes within the category metabolism, in which new marker genes could be identified (Fey, et al. 2005; Bang, et al. 2008; Brautigam, et al. 2009; Pfannschmidt 2010). Bang et al. (2008) showed that a variety of *non-PhANG* genes were misregulated in the *crd* mutant and among these three cold responsive (*COR*) genes were identified. *COR15a* was strongly repressed in the *crd* mutant during control conditions and we show here that *COR15a* and the upstream C-repeat binding factor (*CBF*) genes can be used as marker genes for retrograde signalling during photoperiodic conditions **(Paper II)**.

Diurnal oscillation of tetrapyrroles regulates nuclear gene expression

Expression of the nuclear-encoded CBF transcription factors (TFs) CBF1, CBF2 and CBF3 follows a daily rhythm and is circadian regulated. The expression of these TFs has been shown to be induced in the beginning of the light period and peak around mid-day before the levels decline again towards the dark period (Harmer, et al. 2000; Bieniawska, et al. 2008; Kidokoro, et al. 2009; Dong, et al. 2011). The CBF TFs are important for cold acclimation and freezing tolerance in *Arabidopsis* and regulates the expression of more than 100 downstream *COR* genes (Fowler and Thomashow 2002; Park, et al. 2015). These *COR* genes have also been shown to follow a circadian rhythm during the day and peaks just after the *CBFs* (Dong, et al. 2011). During exposure to low temperatures, the CBFs are transient and strongly induced by upstream regulators inducer of CBF expression (ICE) and calmodulin-binding transcription activator 3 (CAMTA3), among others, and mediate the cold-responsive pathway by expressing the cryoprotective *COR* genes (Lee, et

al. 2005; Doherty, et al. 2009) and growth-repressing DELLA proteins (Achard, et al. 2008). However, the expression pattern of *CBFs* under normal warm growth conditions propose that they also play a role during control conditions. For instance, overexpression of *CBF3* has been shown to affect the vegetative growth and flowering time in plants (Liu, et al. 1998; Kasuga, et al. 1999; Gilmour, et al. 2000), suggesting that CBF3 is involved in additional processes to cold acclimation. This is further demonstrated by the downstream *COR* genes that in addition to cold also respond to other stimuli. COR15a is a cryoprotective protein targeted to the chloroplast stroma where it decreases the propensity of plastid membranes in response to cold and protects stromal proteins from aggregation under cold stress (Steponkus, et al. 1998). In addition to low temperature and the circadian clock, *COR15a* expression has also been shown to be regulated by conditions that affect the functional state of the chloroplast (Lin and Thomashow 1992; Kleine, et al. 2007) and *COR15a* expression is altered in plants with defective chloroplasts and altered levels of tetrapyrroles (Nakayama, et al. 2007; Bang, et al. 2008; Dong, et al. 2011). The mechanism by which chloroplasts can mediate signals to regulate nuclear *COR15a* expression has remained unknown. However, a few possible mechanisms have been proposed to function in the regulation of *CBFs* during warm growth conditions. These include activation by the circadian clock components LHY and CCA1 (Dong, et al. 2011), and repression by PRR5, PRR7 and PRR9 (Nakamichi, et al. 2009). The transcription factors phytochrome interacting factor 4 and 7 (PIF4 and PIF7) were shown to repress *CBF* expression under circadian control in long-day conditions (Kidokoro, et al. 2009; Lee and Thomashow 2012).

We analysed the expression levels of two *COR* genes, *COR15a* and *COR47*, in wild type Col-0 and the *crd* mutant during photoperiodic short-day conditions. In accordance with previous reports, expression of the analysed *COR* genes increased following the onset of light and peaked just before dusk at 9 h into the light period (ZT 9) **(Paper II, Fig. 1)** (Dong, et al. 2011). *COR* expression in the *crd* mutant, which overaccumulates Mg-ProtoIX and Mg-ProtoIX-ME, showed the same rhythmic pattern as in wild type but with strongly repressed levels, as previously reported by Bang et al. (2008). In order to investigate if the misregulation of the *COR* genes in *crd* is an effect of misregulation of the upstream *CBF* genes, we also analysed *CBF1-3* expression. *CBF* expression is under circadian control and the oscillation of *CBF* expression was shown to peak at ZT 6, 3 h before the expression peak of the *COR* genes **(Paper II, Fig. 2A and S1)**. The *crd* mutant displayed repressed levels of *CBF1-3* expression compared to wild type, specifically at ZT 6. This result suggests that the repression of *COR* expression in *crd* is most likely caused by the misregulation of the CBF1-3 transcription factors,

rather than a direct effect on the *COR15a* and *COR47* genes. The demonstrated rhythms of tetrapyrroles and the *CBF* expression indicated that the daily accumulation peak of tetrapyrroles triggers a plastid signal to repress *CBF* expression during photoperiodic conditions. The strong *CBF* repression in the tetrapyrrole overaccumulating *crd* mutant further confirmed that tetrapyrrole levels are most likely involved in the regulation of *CBFs*.

Plastid and circadian signals converge to regulate nuclear gene expression

CBF expression has been proposed to be regulated by light signals and components in the clock in addition to cold temperatures (Bieniawska, et al. 2008; Kidokoro, et al. 2009; Nakamichi, et al. 2009; Espinoza, et al. 2010; Dong, et al. 2011). Our results suggest that a tetrapyrrole-mediated plastid signal contributes to the circadian regulation of *CBF* expression. The daily oscillation pattern of Mg-ProtoIX and Mg-ProtoIX-ME levels requires light and dark changes to be maintained and is not regulated by any clock mechanism (Papenbrock, et al. 1999). By transferring plants to constant light, the diurnal oscillation of tetrapyrroles is completely abolished and it is thus possible to dissect the circadian and diurnal signalling pathways. We analysed *CBF3* expression in wild type Col-0 and *crd* plants following a two days shift to constant light. In wild type, the circadian oscillation of *CBF3* expression was maintained, but displayed a slight shift in the peak and a lower amplitude **(Paper II, Fig. 2A)**. This is commonly observed following shifts to constant conditions for clock-regulated genes and also for the *CBF3* and *COR* genes (Harmer 2009; Dong, et al. 2011). The circadian rhythm of *CBF3* expression was also maintained in the *crd* mutant, however, the observed difference in expression levels between the two genotypes, wild type and *crd*, during photoperiodic conditions was diminished **(Paper II, Fig. 2A)**. In these plants, the levels of Mg-ProtoIX were undetectable and the levels of Mg-ProtoIX-ME were decreased to the amount detected in darkness and showed no oscillation pattern during the subjective day when the *CBF3* expression levels were determined **(Paper II, Fig. 2B and C)**. This demonstrates that when the tetrapyrrole oscillation pattern is lost, *CBF3* expression is regulated only by the circadian clock without any input from the plastid and therefore, no difference is observed between wild type and the *crd* mutant. However, during diurnal conditions, the accumulation of tetrapyrroles contributes to the circadian control of *CBF3* expression.

The most likely location where the plastid and circadian pathways could converge in order to regulate *CBF* expression is in the cytosol. Therefore, we investigated if the identified cytosolic interaction between Mg-ProtoIX and

HSP90 proteins demonstrated to be important during oxidative stress also could have a function during photoperiodic conditions and regulate *COR* and *CBF* expression. We used two of the *Col-hsp90* RNAi lines described earlier **(Paper I, Fig. 2)** with 30% left of the wild type *HSP90* expression levels and analysed *CBF3* and *COR15a* expression. Both *CBF3* and *COR15a* expression were significantly lower in the *Col-hsp90* RNAi lines compared to wild type, supporting a role for HSP90 in the regulation of these genes **(Fig. 12) (Paper II, Fig. 5A and 7)**. In addition, we used the inhibitor GDA to reduce the activity of HSP90 in Col-0 and *crd* and analysed *CBF3* expression. If our theory was correct, GDA treatment of wild type Col-0 should resemble the phenotype of the *Col-hsp90* RNAi lines, and this was also observed. Col-0 plants showed a strong repression of *CBF3* expression following GDA treatment and similar to the wild type, *crd* also showed a clear repression of *CBF3* **(Paper II, Fig. 5B)**. This strongly supports that HSP90 proteins have a function downstream of the tetrapyrrole accumulation and are also involved in the regulation of *CBF3*.

The HSP90 complex could also provide a link to the circadian signalling pathway since HSP90 proteins have been reported to play a role in the circadian clock. HSP90 has been shown to interact with and control the maturation and stability of the evening-phased F-box protein ZTL, providing a rhythm of ZTL protein levels and contributing to the oscillation of the clock components TOC1 and PRR5 (Kim, et al. 2011). *Col-hsp90* RNAi lines with reduced HSP90 levels were previously reported to have reduced ZTL protein levels (Kim, et al. 2011). This was also observed in our lines and confirms the involvement of HSP90 in ZTL stability **(Paper II, Fig. S5)**. In order to test the putative involvement of ZTL in tetrapyrrole-mediated plastid signalling, we analysed ZTL protein levels in wild type Col-0 and the *crd* mutant during photoperiodic growth conditions. It has previously been shown that ZTL protein levels are post-translationally regulated by circadian degradation and oscillate with a peak at the end of the light period (Kim, et al. 2003). Gigantea was shown to be essential for the oscillation pattern of ZTL by direct protein-protein interaction (Kim, et al. 2007) and it was later demonstrated that also HSP90 is involved in the stabilization of ZTL (Kim, et al. 2011). In wild type, ZTL protein levels showed an oscillation pattern also under our experimental conditions with the highest levels at ZT 9 and 12, as described in earlier reports **(Paper II, Fig. 6A)**. This pattern negatively correlates with the diurnal oscillation of tetrapyrroles and shows that the ZTL levels increase when tetrapyrrole levels decrease **(Paper II, Fig. 2 and 6A)**. If ZTL is linked to the plastid signal, the accumulated levels of tetrapyrroles in the *crd* mutant should reduce the levels of ZTL due to inactivation of HSP90. A significant reduction of ZTL protein levels was indeed also observed in the *crd* mutant compared to wild type at all time-

points during the day **(Paper II, Fig. 6A and B)**. Our data suggest that the diurnal oscillation of tetrapyrrole levels contributes to the levels of ZTL via the inactivation of HSP90 activity and subsequent destabilization of ZTL. HSP90 protein levels are high in the cell under physiological conditions and can account for up to 1% of the total cellular protein (Taipale, et al. 2010). *HSP90.1* expression has been shown to be stress-induced while *HSP90.2* to *4* are described as constitutively expressed (Yabe, et al. 1994; Krishna and Gloor 2001). However, Kim et al. (2011) were unable to detect any variations in total cytosolic HSP90 levels under photoperiodic conditions or constant light. Despite this, the actions of HSP90 follows a circadian pattern, suggesting that the activity of HSP90 is regulated post-translationally. Specific phosphorylation sites of HSP90 has been suggested to affect the interaction with co-chaperones and client proteins (Mollapour, et al. 2010; Mollapour and Neckers 2012). However, our results provides an additional explanation to how the activity of HSP90 proteins can be regulated during the day by the oscillation pattern of tetrapyrroles and their inhibition of the ATPase activity of HSP90 (**Paper I**).

We showed that under photoperiodic conditions, HSP90 is involved in the regulation of *CBF* and *COR* expression and that ZTL protein levels are dependent on the tetrapyrrole-mediated regulation of HSP90 proteins. In order to examine if the HSP90-dependent regulation of *CBF* and *COR* is dependent on ZTL protein levels, we analysed the expression levels in the *ztl* mutant and a *ZTL-OX* line. In the *ztl* mutant, *CBF1-3* expression was very low, indicating that ZTL is required for expression of *CBFs* during photoperiodic conditions **(Fig. 12) (Paper II, Fig. 6D and S4)**. In addition, the downstream *COR15a* expression and protein level were also strongly repressed in the *ztl* mutant **(Paper II, Fig. 7)**. In contrast, the *ZTL-OX* line displayed higher *CBF3* expression and further confirms that ZTL is involved in the regulation of *CBF* expression. Furthermore, we produced a *ztl-crd* double mutant and analysed *CBF3* and *COR15a* expression. The expression levels of both genes in *ztl-crd* is similar to *ztl* and does not show any enhanced repression, supporting that CRD and ZTL are genetically linked and that ZTL has a function downstream of the accumulation of tetrapyrroles **(Fig. 12) (Paper II, Fig. 6D and 7)**.

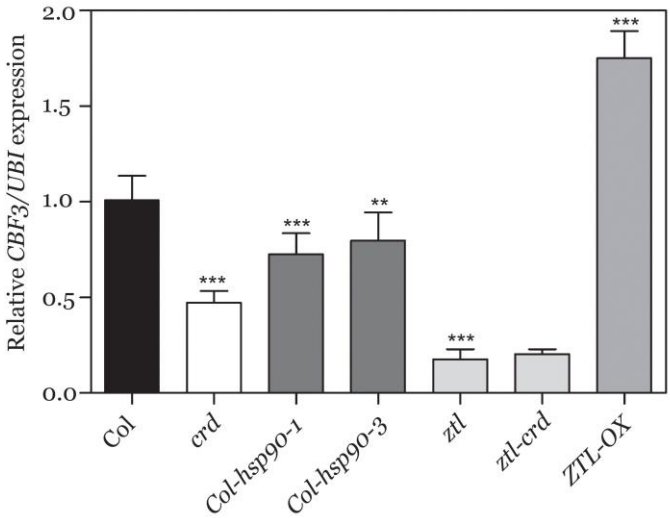

Figure 12. CRD, HSP90 and ZTL are involved in *CBF3* regulation. Plants were grown in short-day conditions and 3-week-old plants of Col, *crd*, two independent *Col-hsp90* RNAi lines, *ztl*, *ztl-crd* and *ZTL-OX* were analysed for *CBF3* expression 6 h into the light period. Gene expression was normalized to ubiquitin-like protein (UBI) and related to the amount present in wild type Col. *CBF3* expression was significantly different in *crd*, the *Col-hsp90* lines, *ztl* and *ZTL-OX* compared to Col, but not in *ztl-crd* compared to *ztl*, as demonstrated by Student´s t test: *** P<0.001, ** P<0.01.

HY5 regulates nuclear gene expression during photoperiodic conditions

In Paper I, we showed that the transcription factor HY5 responds to the activity of the cytosolic HSP90 complex and regulates *PhANG* expression is response to the tetrapyrrole-mediated signal during oxidative stress **(Paper I, Fig. 8)**. We have also demonstrated that *CBF* and *COR* expression is dependent on tetrapyrrole accumulation and the activity of HSP90 proteins. Therefore we wanted to investigate if HY5 could mediate the regulation of *CBF* and *COR* expression also in response to diurnal changes of tetrapyrroles. HY5 is known to bind ACGT-containing *cis*-elements, such as the G-box present in the promoters of many *PhANG* genes. The promoters of *CBF1* and *CBF2* contain G-boxes (CACGTG) (Kidokoro, et al. 2009) close to the transcriptional start while *CBF3* has two Z-boxes (TACGTG). The promoter of *COR15a* contains several G-boxes, C-repeat elements and also the binding sites for the CBFs (Baker, et al. 1994). The *CBF* promoters show high sequence similarity and a 65-bp fragment (-113 to -47 from the transcriptional start) has been shown to be responsible for the circadian and cold regulation of *CBF2* (Kidokoro, et al. 2009). In order to study if HY5 interacts with the promoters of *CBFs* and *COR15a*, HY5 protein was incubated with 50-bp fragments of each promoter and analysed by

electromobility shift assays (EMSA). Interaction between HY5 and the promoter fragments were observed for all genes **(Paper II, Fig. 3)**, demonstrating that HY5 is able to recognize the promoters of *CBF1-3* and *COR15a*. However, a stronger binding affinity was observed for *CBF1* and *CBF3* **(Paper II, Fig. 3B)** and competitor experiments with either unlabelled *CBF1* or *CBF3* probe revealed that HY5 preferentially binds to the *CBF3* promoter *in vitro* **(Paper II, Fig. 3D and E)**.

The preferred binding of HY5 to the *CBF3* promoter was confirmed *in vivo* by analysing *CBF1-3* expression in the *hy5* mutant. In concert with the binding assay data, the *hy5* mutant displayed higher expression of *CBF3* compared to wild type but no effect was observed for *CBF1* or *CBF2* expression **(Paper II, Fig. 4B and S4)**. Thus, HY5 seems to function as a repressor of *CBF3* expression only and the interaction between HY5 and the *CBF1* and *CBF2* promoters identified *in vitro* has no or little effect *in vivo*. A consequence of the higher *CBF3* expression was an increased *COR15a* expression in the *hy5* mutant compared to wild type **(Paper II, Fig. 7)**. In order to examine if HY5 regulates the *CBF* genes in response to the tetrapyrrole-mediated plastid signal we performed feeding experiments with Mg-ProtoIX in wild type Col-0 and *hy5* plants. In the wild type plants, a strong repression was shown for the *CBF1-3* and *COR15a* genes following incubation with Mg-ProtoIX **(Paper II, Fig. 4A and S3C)**. In contrast, *hy5* did not display any repression of *CBF3* or *COR15a* in response to the tetrapyrrole accumulation, however, *CBF1* and *CBF2* expression levels were identical to those in the wild type **(Paper II, Fig. 4A)**. In addition, *CBF3* expression in the *hy5* mutant was insensitive to GDA treatment, but again not *CBF1-2* expression **(Paper II, Fig. 5B and S3D)**. This supports that HY5 acts downstream the tetrapyrrole accumulation and HSP90 in order to specifically regulate *CBF3* expression. Furthermore, we generated a *hy5-crd* double mutant to test the genetic interaction between CRD and HY5. If HY5 acts downstream of the tetrapyrrole accumulation, the *hy5-crd* mutant should release the suppressed levels of *CBF3* and *COR15a* expression observed in the *crd* single mutant. The *hy5-crd* mutant showed significantly higher expression levels compared to the *crd* single mutant **(Paper II, Fig. 4B and 7)**. However, *hy5-crd* is not completely epistatic to *crd*, suggesting that HY5 is not the only component regulating *CBF3* expression. We also analysed *CBF3* expression in the *hy5* mutant following exposure to constant light for 2 days. When the oscillation of tetrapyrrole levels were diminished, the *hy5* mutant displayed *CBF3* expression similar to wild type, as in the case with the *crd* mutant **(Paper II, Fig. 4C)**. Taken together, these results propose that HY5 specifically represses *CBF3* expression in response to the diurnal accumulation of tetrapyrroles.

HY5 is regulated by the HSP90-ZTL complex

We have demonstrated that both ZTL and HY5 are linked to the tetrapyrrole-mediated plastid signal and to the activity of HSP90 proteins in the cytosol. Possibly, ZTL could be the link between HSP90 and HY5 and explain how the plastid signal and circadian clock may converge in the photoperiodic control of *CBF* and *COR* expression. To determine whether there is a physical interaction between ZTL and HY5, we performed an *in vivo* co-immunoprecipitation (Co-IP) assay in an *Arabidopsis* cell culture system. HY5 was found to directly interact with ZTL, demonstrating a connection between these two components **(Paper II, Fig. 6C)**. ZTL is responsible for the proteasome-dependent degradation of the clock components TOC1 and PRR5 (Mas, et al. 2003; Kiba, et al. 2007) and, possibly, HY5 could also be a target for ZTL-mediated degradation. We analysed HY5 protein levels in etiolated seedlings of wild type Col-0, *ztl* and the *ZTL-OX* line treated with cyclohexamide (CHX) to determine the HY5 protein half-life (Kiba, et al. 2007). CHX blocks the translation of proteins and it is thus possible to measure the rate of degradation in the different genotypes following incubation with CHX. In the wild type plants, HY5 levels were significantly reduced following 9 and 12 h of CHX incubation compared to the control at 0 h **(Paper II, Fig. 8)**. In *ztl*, the half-life of HY5 was extended and no significant degradation could be detected during the full experiment. In contrast, the *ZTL-OX* line showed enhanced degradation of HY5 with significantly lower levels already after 6 h of CHX incubation **(Paper II, Fig. 8)**. This result suggests that HY5 is a possible target for ZTL-mediated degradation. Kiba et al. (2007) showed similar results for the ZTL-mediated degradation of PRR5, but for PRR5 the effect was stronger than what we show for HY5. This suggests that HY5 protein level is possibly also regulated by other factors in addition to the proposed ZTL-mediated regulation. For instance, the regulation of HY5 during light-regulated development has been shown to be mediated by COP1. During these conditions, COP1 accumulates within the nucleus in the dark and targets HY5 for degradation, while in light, COP1 is exported from the nucleus and allows HY5 to accumulate and promote photomorphogenesis (Ang, et al. 1998; Osterlund, et al. 2000). Thus, the proposed regulation of HY5 by ZTL could provide a fine-tuning of HY5 levels during photoperiodic conditions in order to properly regulate nuclear gene expression.

Photoperiodic regulation of CBF expression

We show that the diurnal accumulation of tetrapyrroles contributes to the circadian regulation of *CBF1-3* expression through Mg-ProtoIX interaction with the HSP90-ZTL cytosolic complex. HSP90-ZTL-HY5 interaction

specifically mediates the regulation of *CBF3* expression. However, the repression of *CBF3* is not completely recovered in the *hy5-crd* double mutant compared to the *crd* single mutant **(Paper II, Fig. 4B)** suggesting that other factors might be involved. ZTL is known to mediate the degradation of TOC1 and PRR5 in the circadian clock (Mas, et al. 2003; Kiba, et al. 2007). No effect on *CBF1-3* expression has been reported in the *toc* mutants and thus it is unlikely that TOC1 would be involved in the *CBF* regulation. However, PRR5 was shown to mediate repression of the *CBF* genes (Nakamichi, et al. 2012). We confirmed that the *prr5* mutant show higher expression levels for the *CBF1-3* expression and thus that PRR5 is a repressor of *CBF* expression **(Paper II, Fig. 6D and S4)** and subsequently the downstream *COR15a* **(Paper II, Fig. 7)**. Therefore, the phenotype in *ztl* with strongly repressed expression of *CBFs* could be an effect of higher levels of the repressors PRR5 and HY5. The analysis of *CBF3* expression in the double mutant *ztl-hy5* and *ztl-prr5* further demonstrates that they are both involved in *CBF3* regulation since none of them are completely epistatic to *ztl* **(Paper II, Fig. 6D)**. Future analysis of a *ztl-hy5-prr5* triple mutant could reveal if HY5 and PRR5 are the only regulators of *CBF3* or if more factors are involved.

The regulation of *CBF* expression during warm growth conditions has previously been proposed to involve repression by PRR5, PRR7, PRR9 (Nakamichi, et al. 2009) and PIFs (Kidokoro, et al. 2009; Lee and Thomashow 2012) and activation by CCA1 and LHY **(Fig. 13)** (Espinoza, et al. 2010; Dong, et al. 2011). The circadian morning components CCA1 and LHY were shown to positively regulate *CBF* expression through binding to CCA1-binding sites and evening elements (EE) in their promoters (Espinoza, et al. 2010; Dong, et al. 2011). PIF7 was shown to repress *CBF1-2* expression in plants exposed to constant light but not during photoperiodic conditions (Kidokoro, et al. 2009; Lee and Thomashow 2012). Unlike other PIFs, PIF7 is stable in constant light conditions and could explain the specific function of PIF7 during these conditions, while in photoperiodic long-day conditions, PIF4 and PIF7 were shown to act redundantly and possibly together with other PIFs to repress *CBF* expression (Lee and Thomashow 2012). Nakamichi et al. (2009) showed that the *prr9-prr7-prr5* triple mutant has elevated expression of *CBF1-3*, suggesting that also PRR7 and PRR9 could be involved in the repression of *CBF* during photoperiodic conditions. However, this is contradictory since significant amounts of PRR7 and PRR9 are present in the morning when the induction of *CBFs* by CCA1/LHY is highest. Possibly, the phenotype in the *prr9-prr7-prr5* mutant might be explained by high levels of CCA1/LHY, since the PRRs are repressors of *CCA1* and *LHY* (Eriksson and Webb 2011). HY5, PRR5 and PIFs could thus potentially interact as repressors of *CBF* expression during photoperiodic conditions, or

play more specific roles depending on specific growth conditions. In addition, CCA1 and HY5 physically interact (Andronis, et al. 2008) and it is possible that HY5 impacts on the CCA1-mediated regulation of *CBF*, or *vice versa*. During the night, the low levels of CCA1 and LHY allows for repression mediated by either HY5, PRRs or PIFs, or a combination of these components **(Fig. 13)**.

Our results propose a model for integration between plastid and circadian signalling pathways for the regulation of *CBF* expression during photoperiodic growth **(Paper II, Fig. 9)**. Tetrapyrrole levels follow a strong rhythmic pattern and when the levels are elevated, a plastid signal is triggered and inhibits the activity of HSP90 in the cytosol. HSP90 inactivation causes a significant reduction in ZTL protein levels and consequently increased levels of HY5 and PRR5. HY5 and PRR5 act in concert to repress *CBF3* expression and PRR5 also repress *CBF1-2* expression. Thus, the plastid signal contributes to the rhythm of *CBF* and *COR* expression during photoperiodic growth. The tight regulation of *CBFs* during warm control conditions proposes that they have important functions in addition to cold responses. CBF3 overexpression was shown to result in growth retardation (Liu, et al. 1998; Gilmour, et al. 2000), late flowering and reduced seed yield (Gilmour, et al. 2000). The regulation of *CBF* levels during photoperiodic growth conditions is thus important in order to properly induce expression of downstream genes and at the same time avoid accumulation of CBFs and subsequent negative effects.

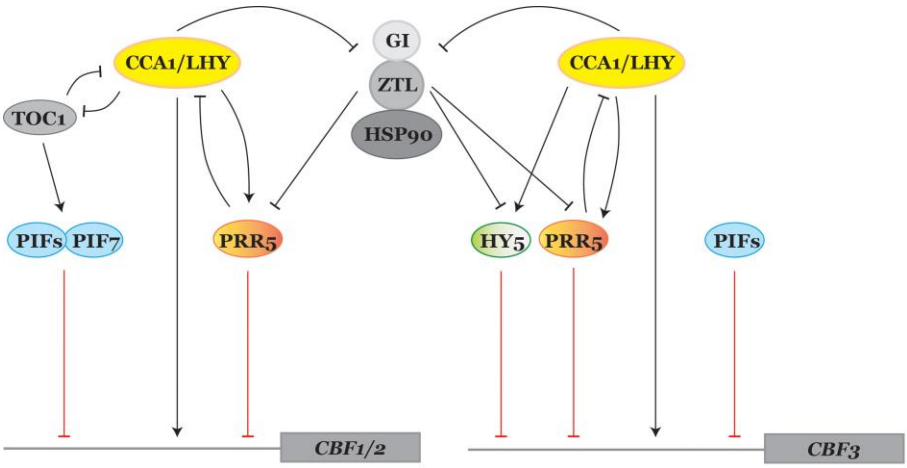

Figure 13. Overview of proposed components involved in the photoperiodic regulation of *CBF* expression. CCA1 and LHY promotes the expression of *CBF1-3* expression in the morning while a combination of HY5, PRR5 and PIF activities mediates the repression of *CBF1-3*. PIF7 activity has been shown to be regulated by its binding partner TOC1. HY5 and PRR5 are positively regulated by CCA1/LHY and negatively regulated by ZTL. The figure is drawn based on data provided in this thesis and additional published data concerning *CBF* regulation.

Summary tetrapyrrole-mediated retrograde signalling

The work presented in this thesis strengthens the suggested function of tetrapyrroles in plastid retrograde signalling. Inconsistent data from different groups questioned the importance of tetrapyrroles and created a heated debate within the research field a few years ago. The main critic against a function for tetrapyrroles was based on the fact that there was no proposed mechanism for how the signal may be transmitted and no experimental condition identified other than NF treatment where tetrapyrroles were shown to play a role. We demonstrate that Mg-ProtoIX interacts with cytosolic HSP90 proteins both in the response to oxidative stress of more physiological nature and following diurnal accumulation of tetrapyrroles **(Fig. 14)**. The accumulation of tetrapyrroles is a trigger for the initiation of plastid signalling and the interaction with HSP90 proteins mediates the response further to transcription factors and changes in nuclear gene expression. This provides a previously unknown mechanism for how tetrapyrroles may transduce information to the nucleus. We show that both *PhANG* and *non-PhANG* genes are regulated by tetrapyrrole-mediated signalling. HY5 mediates the repression of *PhANG* in response to oxidative stress **(Fig. 14)** and has also been shown to be required for the circadian expression of *LHCB* genes together with the clock component CCA1 during control conditions (Andronis, et al. 2008). My work on tetrapyrrole-mediated signalling during photoperiodic control conditions established that

daily oscillation of tetrapyrroles contributes to the fine-tuning of nuclear gene expression. It is thus also possible that the diurnal oscillation of tetrapyrroles contributes to the HY5-mediated circadian regulation of *LHCB* during control conditions. The *CBF* genes were identified as novel *non-PhANG* marker genes for tetrapyrrole-mediated signalling during photoperiodic growth. The tetrapyrrole signal was shown to integrate with the ZTL clock component and regulate the activity of HY5 and PRR5 in order to fine-tune the expression of *CBFs* **(Fig. 14)**. The convergence of different signalling pathways is extensive within the cell and ensures that the proper output is acquired during different conditions. For instance, recent reports demonstrates that chloroplast and light signals are highly dependent on each other during photomorphogenesis and that their integration is important for the fine-tuning different responses (Martin, et al. 2016; Xu, et al. 2016). This is an important aspect to be considered in future studies and a greater understanding of light responses will be significant to fully understand retrograde signalling pathways.

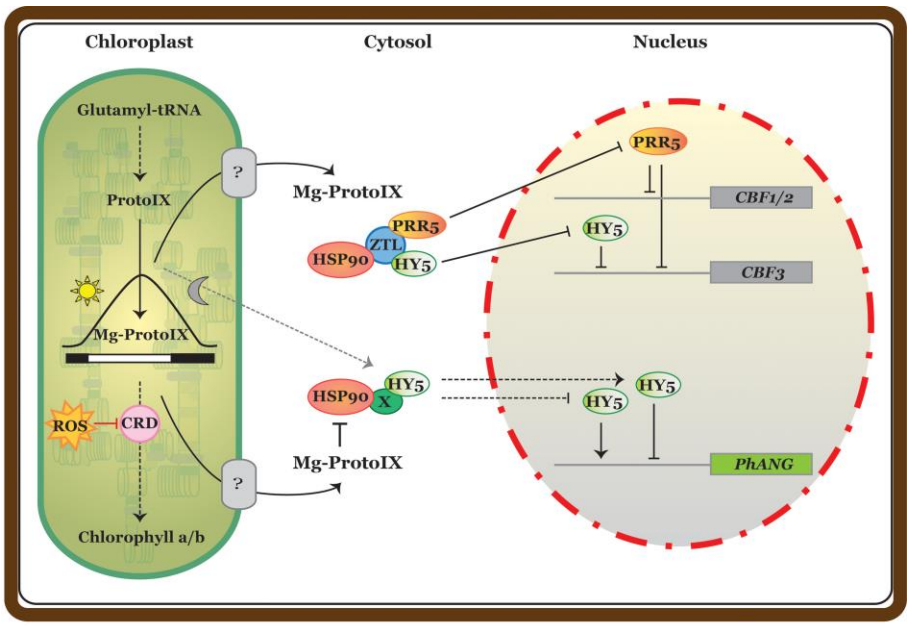

Figure 14. Model of tetrapyrrole-mediated signalling pathways during oxidative stress and photoperiodic conditions. During oxidative stress, the accumulation of Mg-ProtoIX/Mg-ProtoIX-ME is caused by ROS-mediated inactivation of the cyclase system. Mg-ProtoIX is transported to the cytosol by an unknown mechanism and inactivates the activity of HSP90. The inactivation of HSP90 affects the transcription factor HY5 through an unknown factor X, resulting in repression of *PhANG* by either activating HY5 repression or inhibiting HY5 activation of gene expression. Under photoperiodic conditions, the accumulation of tetrapyrroles demonstrates a strong rhythmic pattern and elevated levels following mid-day triggers the signal and inactivates HSP90. HSP90 inactivation reduces the levels of ZTL protein and releases the ZTL-mediated reduction of HY5 and PRR5 levels. HY5 and PRR5 act in concert to repress *CBF3* expression and PRR5 also repress *CBF1* and *CBF2* expression. The daily accumulation of tetrapyrroles may also have an impact on HY5 for the circadian regulation of *LHCB* (grey dashed arrow).

Light signalling and nuclear events

G-group bZIP proteins interact with the light responsive G-box cis-element

Light is not only the primary energy source for plants but it also provides them with information to modulate growth and developmental processes. Light signals have been shown to induce dramatic changes in the nuclear transcriptome to adjust gene expression according to different growth conditions (Ma, et al. 2001; Jiao, et al. 2005). In response to light of high irradiances, a specific set of genes including ROS scavenging proteins is induced in order to protect the plant from harmful redox reactions and oxidative damage of macromolecules (Mittler, et al. 2004). Several regulatory *cis*-elements have been identified in the promoters of high light responsive genes, such as the CryR1/CryR2 elements and the G-box and its

variants containing the ACGT core element (Kleine, et al. 2007). However, little is known about how the gene expression is regulated in response to excess light. In order to identify nuclear components responding to changes in light intensity, we performed a biochemical DNA affinity trapping experiment using some of the identified high light regulatory *cis*-elements. In this approach, a DNA fragment containing the *cis*-element of interest is tagged with biotin and linked to magnetic beads through streptavidin interactions. Thereafter, nuclear proteins prepared from plants are incubated together with the DNA fragment and following washing steps to eliminate unspecific binding, the DNA binding proteins are eluted and identified using mass spectroscopy **(Paper III, Fig. 1A)**. This approach was previously shown to be successful to isolate transcription factors in bacterial and yeast systems (Gabrielsen, et al. 1989; Rey, et al. 2003), and we could show that it is also possible to isolate DNA binding proteins from the more complex plant system using this method. In the analysis of G-box interacting proteins, we used a 144 bp fragment containing two G-box elements from the promoter of *LHCB2.4*. The *LHCB2.4* gene encodes a protein in the PSII light harvesting complex and the gene is representative for genes responding to high light and cellular redox changes (Kleine, et al. 2007). The G-box containing fragment was incubated with nuclear proteins prepared from wild type plants and by using the described approach, we identified the bZIP16 transcription factor and another protein with unknown function (At4g25210) as G-box binding proteins **(Fig. 15)**. We focused our further studies on the bZIP16 protein which belongs to the G-group of bZIP transcription factors together with bZIP68 and GBF1-3 **(Fig. 5)** (Jakoby, et al. 2002).

The identified interaction between bZIP16 and the G-box *cis*-element was confirmed by EMSA experiments. Expressed bZIP16 protein incubated with the 144 bp fragment or a shorter 21 bp sequence containing the G-box element of the *LHCB2.4* promoter both demonstrated a clear binding **(Paper III, Fig. 1B and C)**. Furthermore, the 21 bp fragment was used to produce fragments with mutagenized half-sites of the G-box. The G-box is a palindromic motif composed of two half-sites (CACGTG) and the mutagenized fragments were produced by altering either the first (ACAGTG) or the second (CACACA) half-site. The binding of bZIP16 protein to the 21 bp fragment was completely abolished when either of the half-sites were mutagenized, demonstrating a high specificity of the binding between bZIP16 and the ACGT core sequence of the G-box **(Paper III, Fig. 1D)**. The bZIP domain of the five G-group bZIP members share more than 85% amino acid sequence similarity **(Paper III, Fig. 3A)**. In addition, bZIP16 shares 78% overall sequence similarity with bZIP68, 48% with GBF1 and less than 40% with GBF2-3 **(Paper III, Fig. 3B)**. Considering the high similarity

between bZIP16, bZIP68 and GBF1, it is likely that they bind the same *cis*-elements and could have similar function. Therefore, we also wanted to analyse the binding activity of bZIP68 and GBF1. Similar to bZIP16, both bZIP68 and GBF1 specifically bound the 21 bp G-box containing fragment but not the mutagenized half-sites **(Paper III, Fig. 4A and B)**. Taken together, these results show that bZIP16, bZIP68 and GBF1 are able to specifically recognize and bind the ACGT core of the G-box *cis*-element.

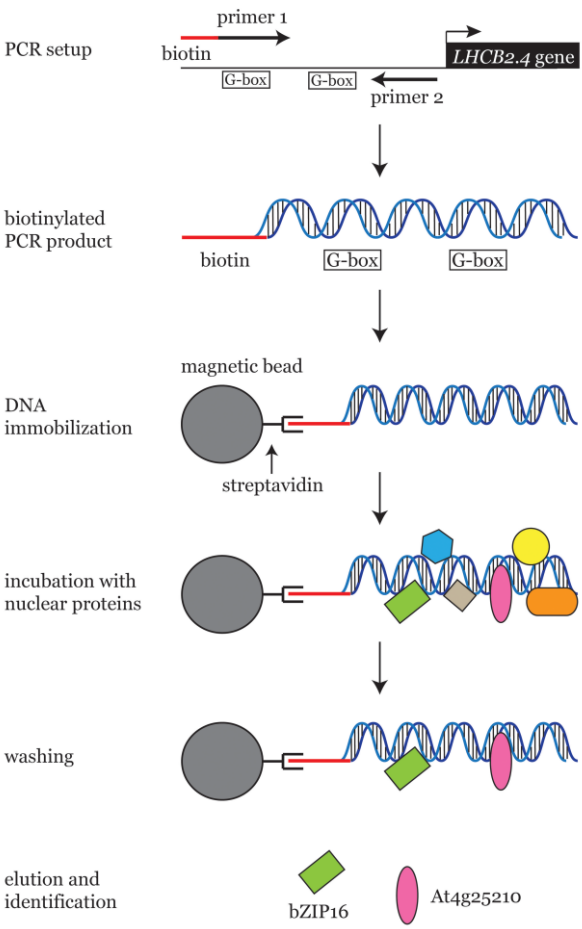

Figure 15. Schematic representation of the DNA affinity trapping method. PCR amplification was performed from the *LHCB2.4* promoter to generate a biotinylated DNA fragment containing two G-box *cis*-elements. The DNA fragment was immobilized by interaction with streptavidin-coated magnetic beads. Nuclear proteins were prepared from plants and incubated together with the DNA fragment to allow binding. Following washing to remove unspecific binding the G-box interacting proteins were eluted and identified by MS. Adapted from Shaikhali, Noren, et al. 2012.

DNA-binding activity of bZIP16, bZIP68 and GBF1 is redox-regulated

The G-box *cis*-element is present in the promoter of genes responsive to high light and redox changes (Kleine, et al. 2007). We identified bZIP16, bZIP68 and GBF1 as proteins interacting with the G-box element and as potential regulators of light-responsive genes such as *LHCB2.4*. The activity of regulatory proteins is often dependent on posttranslational modifications in the response to changing conditions and also bZIP proteins have been described to be highly regulated at the posttranslational level (Llorca, et al. 2014). The DNA binding activity of human bZIP proteins have been shown to be regulated for instance by phosphorylation or redox modifications (Amoutzias, et al. 2006). Phosphorylation of specific serine residues within the DNA binding region has been shown to add a negative charge and inhibit interaction with the negatively charged DNA (Mahoney, et al. 1992; Deppmann, et al. 2003; Kirchler, et al. 2010; Lee, et al. 2010). Reduction/oxidation of cysteine residues within the DNA binding region of human bZIPs has been shown to block or allow DNA binding (Abate, et al. 1990; Xanthoudakis, et al. 1992; Amoutzias, et al. 2006) and some proposed mechanisms include reversible formation of sulphenic acid, a disulphide bond or S-glutathiolation (Klatt, et al. 1999; Marshall, et al. 2000). The formation or breakage of disulfide bonds between cysteine residues in proteins is a key modification for responses to redox changes and for changing protein activity (Amoutzias, et al. 2006; Marchal, et al. 2014). The sequence analysis of the G-group bZIP proteins revealed the presence of a conserved cysteine residue among bZIP16 (Cys^{330}), bZIP68 (Cys^{320}) and GBF1 (Cys^{247}) in the basic region of the bZIP domain **(Paper III, Fig. 3A)**. In addition, bZIP16 and GBF1 have a second conserved cysteine residue in the bZIP domain (Cys^{358} and Cys^{275}, respectively) while bZIP68 has a second cysteine located upstream of the bZIP domain (Cys^{182}).

The presence of conserved cysteine residues suggests that bZIP16, bZIP68 and GBF1 could form disulfide bonds and that these bonds could potentially be subject to redox regulation. We therefore investigated the possibility that the identified interaction between the bZIP proteins and the G-box *cis*-element can be regulated by different redox conditions. We determined the DNA binding activity by EMSA using binding reactions treated with fixed amounts of the reducing agent dithiothreitol (DTT) or the oxidizing agent hydrogen peroxide (H_2O_2). The results showed that the DNA binding activity of bZIP16, bZIP68 and GBF1 is significantly enhanced following DTT treatment compared to the control condition and H_2O_2 treatment **(Paper III, Fig. 5B, 7A and C)**. This suggests that cysteines in the reduced state in these bZIP proteins could be important for efficient DNA binding *in vitro*. To

further confirm the involvement of cysteines in the DNA binding activity of the bZIP proteins, we produced mutant proteins in which each of the cysteine residues were exchanged for leucines. The cysteine residue conserved among all three proteins were exchanged (bZIP16-C330L, bZIP68-C320L and GBF1-C247L) and the mutant proteins showed a significantly enhanced DNA binding activity compared to the wild type proteins and to the mutant forms of the other cysteine residues **(Paper III, Fig. 5D, 7B and D)**. This suggests that the conserved cysteine residue found in bZIP16, bZIP68 and GBF1 is important for the redox regulation of DNA binding *in vitro*.

Redox changes affect the quaternary structure and DNA binding activity of bZIP16

The conventional model for DNA binding of bZIP proteins suggests that dimerization is a requirement for DNA binding and recognition of specific sites (Gentz, et al. 1989; Ellenberger, et al. 1992). However, an alternative pathway has been proposed since it was shown that also the monomeric form of some bZIPs can recognize and bind DNA, forming a bZIP monomer-DNA complex. Subsequently, a second bZIP monomer is bound and a functional bZIP dimer-DNA complex is formed on the DNA (Metallo and Schepartz 1997; Berger, et al. 1998; Kohler, et al. 1999; Cranz, et al. 2004). The monomer pathway was proposed to provide rapid and specific recognition of DNA binding sites and efficient assembly of the bZIP dimer-DNA complex (Kohler, et al. 1999). To analyse if the observed redox regulation of cysteine residues could have an impact on the quaternary protein structure, we performed analyses of bZIP16 subjected to different reducing or oxidizing conditions using denaturing polyacrylamide gels (SDS-PAGE). During non-reducing control conditions, several bands were detected corresponding to the monomeric, dimeric and oligomeric forms of the bZIP16 protein **(Paper III, Fig. 5A)**. Following increasing concentrations of DTT, the oligomeric and dimeric forms were reduced into monomers **(Paper III, Fig. 5A and S4)**. In contrast, incubation with increasing concentrations of H_2O_2 demonstrated a gradual loss of monomers and subsequently also the other forms, indicating the formation of very high molecular mass oligomers **(Paper III, Fig. 5A)**. Thus, different redox conditions have an impact on the quaternary structure of bZIP16 protein and reduced cysteines promote the formation of monomers. Furthermore, we demonstrated that the mutation of Cys^{330} in the bZIP16 protein results in conversion into the monomeric form even under non-reducing conditions, while the mutation of Cys^{358} does not show this effect **(Paper III, Fig. 5C)**. This demonstrates the importance of Cys^{330} for the formation of disulfide bonds and that the redox regulated disulfide formation is specifically

targeted to the cysteine at this position. Taken together, these results suggest that reducing conditions breaks the intermolecular disulfide bond and promotes the conversion of bZIP16 into the monomeric form *in vitro*. According to the analysis of DNA binding activity, the reduced form of bZIP16 also enhanced DNA binding **(Paper III, Fig. 5B)**. To confirm that bIZP16 is able to bind DNA as a monomer, we used a truncated form of bZIP16 containing the basic DNA binding region but without the leucine zipper domain and performed DNA binding analysis. This truncated form of bZIP16 was shown to bind the DNA **(Paper III, Fig. 5E)**. Thus, we suggest that the models for dimeric and monomeric binding of bZIP16 to DNA are both possible and at this point we do not know which mechanism is the primary one.

The molecular structure of the bZIP16 protein could reveal if the critical Cys^{330} is located in a position that could support a possible role in the regulation of DNA binding activity. While crystallization experiments were not successful, a theoretical model of the bZIP domain from bZIP16 was generated by comparative modelling of free and DNA-bound protein **(Paper III, Fig. 8)**. In this model, Cys^{330} was positioned at the domain between the leucine zipper and the basic DNA-binding region, just outside the DNA contact sites. In the DNA-free dimeric form generated through the leucine zipper, the Cys^{330} of each monomer is located sufficiently close to each other to allow the formation of a disulfide bond (5.5 A). Upon DNA binding, there was a predicted spatial separation of the two Cys^{330} residues (6.3 A) and this distance between the residues most likely do not allow disulfide bond formation. Thus, the theoretical models propose a critical position for the Cys^{330} residue in the bZIP16 domain. When the disulfide bond is formed, the conformation of the zipper may not be open or be flexible enough to allow DNA binding, while breakage of the bond during reducing conditions would open the zipper and stimulate DNA binding.

Redox-regulation of bZIP proteins is important for their activity in planta

The performed experiments *in vitro* suggest that the binding activity of bZIP16, bZIP68 and GBF1 to the G-box *cis*-element is regulated by the redox state of a conserved cysteine residue. However, the *in vitro* conditions used during our experiments might be very different from the actual *in vivo* conditions, and the result does not prove what is really going on *in planta*. In order to test if the *in vitro* results are biologically relevant, we performed *in planta* experiments using bZIP16 overexpression lines of the wild type bZIP16 protein (*bZIP16-WTOX*) and lines where the cysteines were mutated, *bZIP16-C1OX* (Cys^{330}) and *bZIP16-C1C2OX* (Cys^{330} and Cys^{358}). GBF1 has

previously been described to play a role during photomorphogenesis and we therefore used conditions similar to the published GBF1 studies (Mallappa, et al. 2006) to analyse the bZIP16-OX lines (5 days old seedlings grown in 10 µE constant white light). During the response to early light, hypocotyl elongation is inhibited and *LHCB* expression is induced in order to promote photomorphogenesis. Analysis of *bZIP16-WTOX* plants revealed that this line displayed significantly longer hypocotyls and lower *LHCB2.4* expression levels compared to wild type plants **(Paper III, Fig. 9B and C)**. Our results suggest that bZIP16 functions as a negative regulator of inhibition of hypocotyl elongation and as a repressor of *LHCB2.4*. However, the overexpression lines with cysteine mutations (*bZIP16-C1OX* and *bZIP16-C1C2OX*) displayed no phenotype and showed similar hypocotyl length and *LHCB2.4* expression as was observed in wild type. These results indicate a biological relevance for bZIP16 in photomorphogenesis and light regulated gene expression. In addition, the results support the importance of a redox regulation of Cys^{330} for the activity of bZIP16 *in planta*. We also analysed *LHCB2.4* expression in the T-DNA single mutants *bzip68* and *gbf1*. The elimination of these proteins resulted in decreased expression of *LHCB2.4* compared to wild type when grown under the low light (10 µE constant white light) implying that in contrast to bZIP16, bZIP68 and GBF1 both function as activators of *LHCB2.4* expression **(Paper III, Fig. 9D)**. To further analyse the importance of these three bZIP proteins, we generated a triple mutant and analysed *LHCB2.4* expression during photomorphogenesis. The *bzip triple* mutant displayed a strong repression of *LHCB2.4* expression compared to wild type plants **(Fig. 16)**. This result demonstrates that in the absence of bZIP16, bZIP68 and GBF1, *LHCB2.4* expression is not properly induced during photomorphogenesis.

Previous studies have shown that GBF1 plays a role during photomorphogenesis, lateral root formation and senescence (Mallappa, et al. 2006; Smykowski, et al. 2010). GBF1 has been shown to bind the G-box and Z-box elements containing the ACGT core element and function as a repressor of *RBCS* and *CATALASE2* expression and as an activator of *LHCB* expression as also our data demonstrates **(Paper III, Fig. 9D)** (Mallappa, et al. 2006; Smykowski, et al. 2010). GBF1 was further shown to be a repressor of blue light-mediated inhibition of hypocotyl elongation (Mallappa, et al. 2006). We show that also bZIP16 is a negative regulator of inhibition of hypocotyl elongation during photomorphogenesis which was subsequently shown to be red light-dependent by Hsieh et al. (2012). bZIP16 was also implicated to play a role during seed germination and hormone signalling (Hsieh, et al. 2012). These studies are in concert with our data suggesting that the bZIP16, bZIP68 and GBF1 proteins do not have redundant functions despite the high similarity and common regulatory

mechanism. The specific functions of these proteins could possibly be explained by their putative differences in localization or degradation during different conditions. For instance, GBF1 was shown to accumulate in light and to be degraded in dark (Mallappa, et al. 2008) while bZIP16 mainly functions as a transcriptional repressor in darkness (Hsieh, et al. 2012). In addition, limitation of nuclear access could also be an important control of the activity. In parsley, the transport of GBFs to the nucleus was shown to be light-dependent (Harter, et al. 1994) and by analysing GBF fusion proteins in *Arabidopsis* it was shown that GBF4 is mainly localized in the nucleus, GBF2 accumulates in the nucleus in blue light and that GBF1 is distributed between the cytosol and nucleus (Terzaghi, et al. 1997). In our study, bZIP16 was exclusively localized to the nucleus following protoplast transfection in light **(Paper III, Fig. 2)**. Thus, the spatial and temporal distribution of these bZIPs could contribute to their specific functions and future studies elucidating degradation patterns and localization during different conditions could reveal important information about their functions. bZIP proteins are dimeric and have been described to form both homo- and heterodimers, preferable within but also between different groups (Llorca, et al. 2014). Interactions between the G-group members have previously been described (Shen, et al. 2008; Llorca, et al. 2015) and we also demonstrated that bZIP16 interacts with bZIP68 and GBF1 in an yeast two hybrid (Y2H) experiment **(Paper III, Fig. 4C)**. This provides a potential mechanism for generating additional levels of regulation of these bZIP proteins and depending on their interactions, different outputs can be generated. The combinatorial interactions among bZIP16, bZIP68 and GFB1 could thus provide a flexible system regulating redox-responsive gene expression.

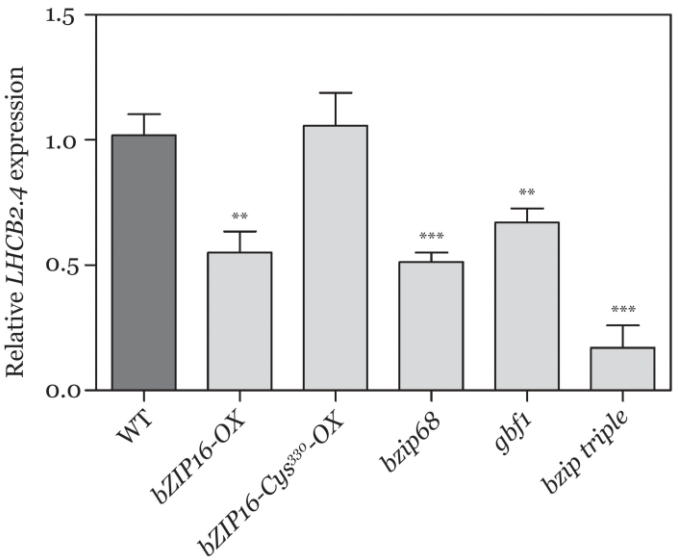

Figure 16. bZIP proteins are involved in the regulation of *LHCB2.4* expression. Plants were grown in 10 µE constant white light for 5 days and analysed for *LHCB2.4* (At3g27690) expression. Gene expression was normalized to ubiquitin-like protein (UBI) and related to the amount present in wild type Col. The mean ± S.E. (error bars) of at least three biological replicates is shown. The expression was significantly different from wild type in *bZIP16-OX*, *bzip68*, *gbf1* and *bzip triple* mutant as demonstrated by Student's t test: **, $p < 0.01$ and ***, $p < 0.001$.

The effect on nuclear gene expression by high light, stress and redox changes within the cell is well established. Microarray analyses have revealed that a large number of genes are either repressed or induced during these conditions (Rossel, et al. 2002; Kimura, et al. 2003; Vanderauwera, et al. 2005; Kleine, et al. 2007). The repression of certain genes aims to minimize the photon uptake and adjust the photosynthetic capacity while expression of genes encoding photo-protective proteins is induced. The induction of protective genes includes the expression of antioxidant genes, ROS scavenging enzymes, chaperones, genes involved in anthocyanin biosynthesis as well as several unknown genes with possible regulatory and metabolic functions (Rossel, et al. 2002; Kimura, et al. 2003; Vanderauwera, et al. 2005; Kleine, et al. 2007). However, the exact mechanisms of how oxidative stress caused by a variety of environmental conditions impacts upon gene expression are largely unknown. In our work we provide a mechanism for redox regulation of nuclear transcription factors providing a direct link between oxidative stress and changes in gene expression. A conserved cysteine residue within the bZIP16, bZIP68 and GBF1 proteins was shown to regulate DNA-binding activity in response to changes in redox conditions. This cysteine residue was proposed to be located at a critical position in the bZIP domain just outside the DNA binding region which

could possibly control the conformation of the bZIP proteins and allow or restrict DNA binding activity. A sequence comparison among all plant bZIP proteins revealed that the cysteine residue at this specific location is only present in the G-group members bZIP16, bZIP68 and GBF1 and in the previously ungrouped bZIP62 protein **(Fig. 17A)**. This cysteine residue is not conserved among the other two G-group members GBF2 and GBF3 or any of the other bZIP proteins. The presence of this cysteine residue suggests that these four bZIP proteins could be specifically regulated by this redox-mediated mechanism and thereby provide a specific role for these transcription factors. The bZIP62 protein was left unclassified by Jakoby et al. (2002) since it did not fit into any of the proposed groups of bZIP proteins. A phylogenetic analysis of all plant bZIP proteins revealed that the closest homologues of bZIP62 are the G-group proteins **(Fig. 17B)**. The demonstrated homology and conservation of the critical cysteine residue possibly suggests that bZIP62 could be classified together with the G-group members, but this needs to be confirmed by further analyses.

High light can be sensed both by the chloroplasts and the photoreceptors in the cytosol/nucleus which mediate the information to the nucleus to adapt gene expression (Kleine, et al. 2007; Brautigam, et al. 2009; Foudree, et al. 2010; Kindgren, Kremnev, et al. 2012). A nuclear redox system would be required for the redox regulation of bZIP proteins and also for other identified redox-regulated transcription factors (Tron, et al. 2002; Heine, et al. 2004; Hernandez, et al. 2004; Vieira Dos Santos and Rey 2006; Shaikhali, et al. 2008). Marchal et al. (2014) demonstrated the presence of a functional NTR/NRX thioredoxin system in the nucleus of *Arabidopsis* that possibly could be involved in the redox regulation of transcription factors. Another possible mechanism in which chloroplasts may mediate redox changes to the nucleus is through the formation of stromules; stroma-filled tubules extending from the chloroplast. Exactly how and why these structures are formed is not well known, but it has for instance been shown that numerous stromules surround the nucleus during pathogen defence. The stromules-nucleus contact sites were shown to provide transport of a chloroplast-localized defence protein and H_2O_2 from the chloroplast to the nucleus (Caplan, et al. 2015). Thus, it is possible that these structures are formed during oxidative stress conditions and provide a mechanism to transfer redox signals from the chloroplast to the nucleus (Bobik and Burch-Smith 2015).

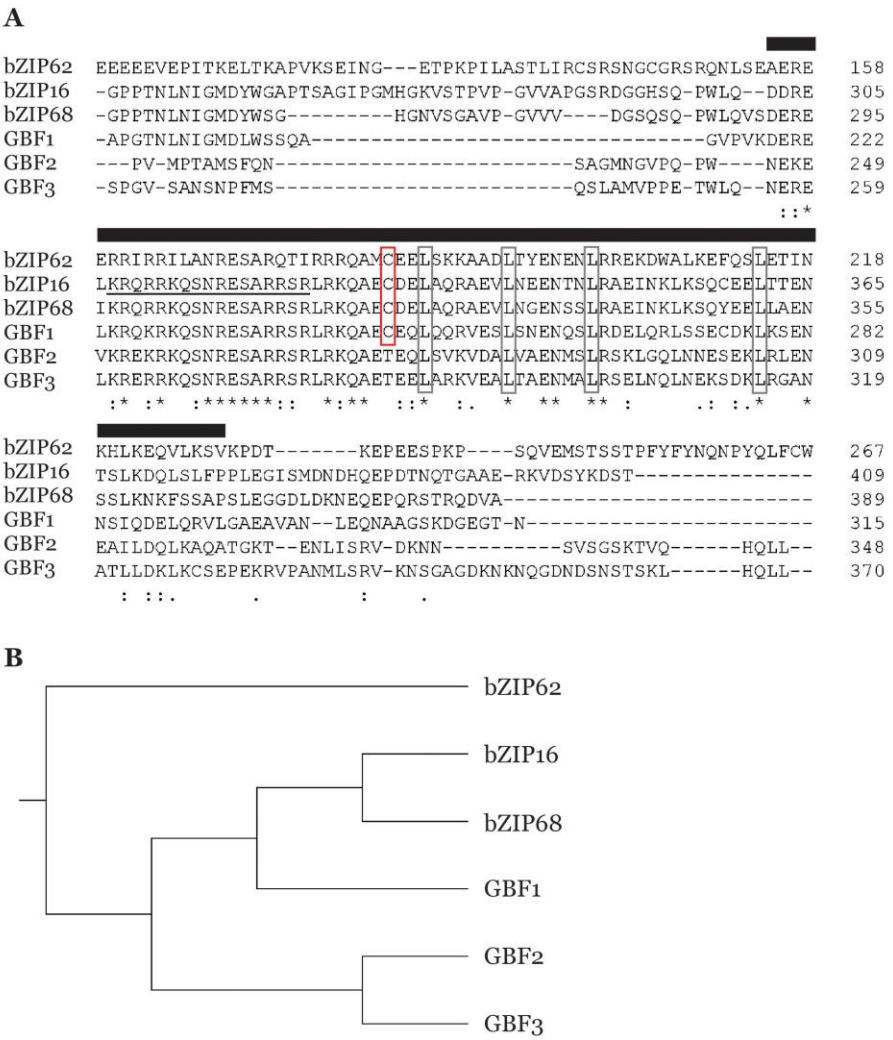

Figure 17. Sequence comparison of the G-group and bZIP62 transcription factors in Arabidopsis. (A) Alignment of the predicted protein sequences of bZIP62 (At1g19490), bZIP16 (At2g35530), bZIP68 (At1g32150), GBF1 (At4g36730), GBF2 (At4g01120) and GBF3 (At2g46270). The protein region containing the bZIP domain was cut out for simplicity and shown in the figure. The asterisk (*) indicates fully conserved amino acid residues, colon (:) indicates conservation between groups of strongly similar properties, period (.) indicates conservation between groups of weakly similar properties and dashes (-) indicate gaps introduced to maximize the alignment. The bZIP domain is indicated by a black bar, the conserved Cysteine residue is indicated by a red box and conserved Leucines in the zipper are indicated by grey boxes. **(B)** A phylogenetic tree of all 75 plant bZIP proteins was generated using MEGA7 software with recommended settings as described in figure 5. The G-group bZIPs and bZIP62 were cut out for simplicity and shown in the figure.

bZIP16, bZIP68 and GBF1 are involved in deetiolation

The bZIP16 and GBF1 proteins have been shown to play a role during photomorphogenesis and our data suggests that together with bZIP68, they are involved in the regulation of light responsive genes **(Paper III)** (Mallappa, et al. 2006; Hsieh, et al. 2012). In order to further understand the role of these bZIP proteins we characterized the *bzip16-bzip68-gbf1* triple mutant (*bzip triple*) and examined the phenotype during dark-to-light transition. Dark-grown seedlings use their energy to reach for the soil surface by elongating their hypocotyls and the cotyledons are pale since they have undeveloped chloroplasts. The development of the proplastid, or dark-grown etioplast, to a photosynthetically active chloroplast is dependent on light (Pogson and Albrecht 2011). Following transition to light, deetiolation and photomorphogenic growth is promoted and the energy is used to develop functional chloroplast to enable light capture for photosynthesis. During this developmental process, nuclear and plastid activities are dependent on each other to ensure the correct expression of proteins required for chlorophyll biosynthesis and photosynthesis. In order to examine the involvement of bZIPs in the regulation of components required during photomorphogenic growth, the *bzip triple* mutant was grown for 5 days in darkness and thereafter analysed following transition to light. In wild type plants, deetiolation was observed already after 12 h in light as indicated by the separation of the cotyledons and visual accumulation of chlorophyll **(Paper IV, Fig. 1 and 2)**. Following 24 and 48 h in light wild type plants displayed fully expanded cotyledons and an increased dark green colour due to strong accumulation of chlorophylls. In contrast, the *bzip triple* mutant displayed a delayed deetiolation process compared to the wild type plants. Following 12 h in light, the *bzip triple* displayed only a slight separation of the cotyledons and no chlorophyll accumulation **(Paper IV, Fig. 1 and 2)**. After 24 and 48 h in light the *bzip triple* mutant displayed separation of the cotyledons and a pale green phenotype as indicated by the initiation of chlorophyll biosynthesis. The strong accumulation of chlorophyll in wild type following 48 h light exposure was not observed in the *bzip triple* mutant, but the *bzip triple* mutant rather reached the levels observed in wild type at 12 h of light **(Paper IV, Fig. 1)**. This indicates that the deetiolation process in the *bzip triple* mutant following dark-to-light transition is delayed compared to wild type plants.

Nuclear and plastid transcripts are repressed in the bzip triple mutant

Several transcription factors such as HY5 and PIFs have been shown to bind promoters of light responsive genes such as *LHCB2.4* and be involved in the

regulation of nuclear gene expression during photomorphogenesis (Lee, He, et al. 2007; Leivar and Monte 2014). In order to examine if the delayed deetiolation phenotype of the *bzip triple* mutant could be an effect of misregulated gene expression, we analysed the expression of *LHCB1.1* during 24 h following dark-to-light transition. LHCB1.1. is a chlorophyll *a*/*b* binding protein associated with PSII and contains the light-regulated G-box *cis*-elements in the promoter. In the wild type plants, *LHCB1.1* expression was strongly induced after 12 h in light and displayed high expression levels at 24 h **(Paper IV, Fig. 3A)**. This is in concert with the observed chlorophyll levels at 24 and 48 h and demonstrates that the expression of *LHCB1.1* and assembly of the light harvesting complexes after 24 h light exposure also stimulates the biosynthesis and accumulation of chlorophyll **(Paper IV, Fig. 1 and 3A)**. The *bzip triple* mutant on the other hand did not display a strong induction of *LHCB1.1* expression following 24 h light exposure, but rather a slight increase in expression levels **(Paper IV, Fig. 3A)**. This result suggests that the delayed deetiolation and chlorophyll accumulation in the *bzip triple* mutant could be an effect of misregulated expression of key components required for chloroplast development. The development of chloroplasts is dependent on the coordinated expression of nuclear genes with chloroplastic genes to ensure the correct assembly and function of the photosynthetic machinery. During this process, anterograde and retrograde signals are required to communicate information between the two different compartments, and thus, an effect on nuclear components and gene expression may also affect plastid activities. In order to examine if plastid gene expression is affected in the *bzip triple* mutant, we analysed the expression of *psaA* encoding a PSI protein in the chloroplast. *psaA* expression was rapidly induced in wild type after 9 h in light and increased further with exposure to light **(Paper IV, Fig. 3B)**. However, the *bzip triple* mutant did not show any induction of *psaA* in response to light exposure during 24 h. In addition, we analysed the expression of two other plastid encoded genes, a PSII associated protein (*psbA*) and the large subunit of rubisco (*rbcl*). These two genes were also strongly repressed in the *bzip triple* mutant compared to wild type plants **(Paper IV, Fig. S3G-H)**. In order to examine the complete impact on the plastid genome in the *bzip triple* mutant, we performed a plastid transcriptome analysis of wild type and *bzip triple* exposed to 24 h light. The analysis of 78 chloroplastic genes demonstrated significantly lower expression levels of the plastid-encoded genes in the *bzip triple* mutant compared to wild type following the dark-to-light transition **(Paper IV, Fig. 4 and Table S1)**. More than 90 % of the analysed genes were significantly down-regulated in the *bzip triple* mutant compared to wild type, including genes transcribed by both NEP and PEP polymerases. The strong effect on the plastid genome expression during deetiolation in the *bzip triple* mutant could possibly be an effect of a missing

positive anterograde signal due to misregulation of key components such as *LHCB1.1* or other crucial factors. Transcription by PEP is dependent on the nuclear-encoded sigma factors (SIG1-6) which are induced in the response to light to promote plastid gene expression and development (Allison 2000). All *SIGs* except *SIG5* showed suppressed expression levels in the *bzip triple* mutant following the dark-to-light transition **(Paper IV, Fig. S3A-F)**, however, further experiments are required in order to examine if the bZIP proteins directly controls the expression of genes required for PEP or NEP activity, or if the phenotype is an effect of a missing positive anterograde signal. The observed defect in nuclear and plastid gene expression in the *bzip triple* mutant during early light exposure would restrict the onset of photomorphogenic growth and thus result in the demonstrated delayed deetiolation phenotype.

Despite the initially strong phenotype in the *bzip triple* mutant following a dark-to-light shift, the plants eventually manage to recover and induce photomorphogenic growth as indicated by the phenotype after 48 h in light **(Paper IV, Fig. 1 and 2)**. Thus, it indicates that the bZIP16, bZIP68 and GBF1 proteins play a role during the early light signalling where they are important for correct gene regulation, but that other factors also are involved and could complement their activity. In order to examine if the bZIP function is specific to dark-to-light transition and deetiolation, or also during other early light conditions, we further analysed the *bzip triple* mutant grown under constant light and long-day conditions. Following 5 days in constant low light (10 μE) the *bzip triple* mutant displayed a pale green phenotype and strongly repressed nuclear gene expression (*LHCB1.1*, *LHCB2.4*) but no effect on plastid gene expression (*psaA*) was observed compared to wild type plants **(Paper IV, Fig. 5A-B)**. When the plants were grown for 5 days in 150 μE constant light, the *bzip triple* phenotype was weaker **(Paper IV, Fig. 5C-D)**, and when grown in normal long-day condition, there was no observed difference between the *bzip triple* mutant and wild type plants **(Paper IV, Fig. 5 E-F)**. Taken together, these results indicate that the function of bZIP16, bZIP68 and GBF1 is mainly required during early light signalling and at low light intensity levels, and that they primarily function by regulating nuclear gene expression.

The function of bZIP16, bZIP68 and GBF1 during early light signalling

Our data proposes a role for bZIP16, bZIP68 and GFB1 during early light signalling and photomorphogenesis **(Paper IV)**. During dark-to-light transition and continuous low light, the elimination of these proteins cause a strong repression of nuclear-encoded genes resulting in an impact on the

establishment of functional chloroplasts. However, chloroplast development and photomorphogenesis is not completely blocked, implying cross-talk with other factors. HY5 and PIFs are major players shown to be involved in the response to light (Lee, He, et al. 2007; Leivar and Monte 2014). PIFs accumulate in the dark and promote skotomorphogenesis by repressing the expression of photomorphogenic genes (Leivar and Monte 2014). In response to light, the inactive form of phytochrome (Pr) is changed to the active Pfr form and enters the nucleus where it binds to and targets PIF3 for degradation. This action releases the repression of light induced genes and promotes photomorphogenesis. PIF3 was shown to directly regulate the expression of PEP-associated proteins (PAPs) and the sigma factors, but not the expression of *PhANGs* such as *LHCBs* and *RBCS* (Dubreuil, et al. unpublished). It was thus suggested that *PhANGs* are rather secondary targets of PIF3, regulated by other TFs controlled by PIF3 action **(Fig. 18)**. The bZIP TF HY5 plays a major role during light signalling and could be one of the targets of PIF3 regulation that is induced during light, and possibly bZIP16, bZIP68 and GBF1 are also targets of PIF3, or alternatively downstream targets of HY5. PIF3 binds to the PBE-box (CACATG) and the G-box (CACGTG) (Zhang, et al. 2013), and HY5 to the G-box (CACGTG) in promoter sequences (Chattopadhyay, et al. 1998). The promoter regions of bZIP16, bZIP68 and GBF1 all contain both the PBE and G-box core sequences, making the three bZIPs potential targets of both PIF3 and HY5. HY5 was assigned close to 4000 binding sites in the genome, including several TFs and genes encoding plastid proteins, and GBF1 was identified as a target of HY5 regulation (Lee, He, et al. 2007). In addition, HY5, HYH and GBF1 were shown to interact and to play a role during light-responses and photomorphogenesis (Singh, et al. 2012) implying that an interplay between several different bZIP proteins may be important during early light signalling and regulation of *PhANG* expression. The initial expression of *PAPs*, *SIGs* and *PhANGs* upon illumination provides positive anterograde signalling to the chloroplast to promote the conversion from skotomorphogenic to photomorphogenic growth. However, this anterograde control is not enough to complete the development of chloroplasts. Following the initiation of plastid transcription and photosynthetic activity, the chloroplast sends a positive retrograde signal to the nucleus to promote a second phase of strongly induced expression of the plastid-localized proteins to fully ensure chloroplast development **(Fig. 18)**. If this retrograde signal is missing, or a negative signal is sent instead, chloroplast development is blocked. This has been demonstrated by for instance lincomycin experiments. Lincomycin specifically inhibits chloroplast translation and was shown to subsequently inhibit the second phase of induced nuclear gene expression and the development of chloroplasts (Sullivan and Gray 1999; Martin, et al. 2016). The misregulation of *LHCB1.1* expression in the *bzip*

triple mutant could potentially eliminate the positive anterograde signal which is required to initiate chloroplast development and subsequently to trigger the positive retrograde signal. This would result in the reduced expression of *SIGs* and the plastid encoded genes, and the delayed chloroplast development observed in the *bzip triple* mutant. The establishment of chloroplasts and the photomorphogenic growth is a complex process involving two different compartments and genomes and the interactions between the nucleus and chloroplast during this process is not well understood. We identified the bZIP TFs as regulatory factors of key components involved in the early light response, but further experiments has to be performed in order to fully understand their role during different conditions, time frames and light quantity and quality.

Summary light signalling and nuclear events

The development of a seedling into a photosynthetically active plant is a crucial and complex process in the plant life cycle. Despite its importance, we do not fully understand the regulatory mechanisms behind the establishment of functional chloroplasts and photosynthetic reactions in response to light. The work in this thesis provides new insight into light signalling and regulation of nuclear genes by identifying the function and regulation of bZIP transcription factors. Being such an important process, photomorphogenesis is likely regulated on many different levels and by cross-talk between several different factors. PIF3 has been shown to be a major repressor of photomorphogenesis and HY5 has been demonstrated as a major activator of photomorphogenesis (Lee, He, et al. 2007; Leivar and Monte 2014). Up to one third of the nuclear transcriptome is changed upon illumination (Ma, et al. 2001) and most likely it involves the action of several different transcription factors. The described redox-regulation of bZIP16, bZIP68 and GBF1 and their involvement in early light signalling suggest that they may play an important role during certain conditions and could provide new insight into the interactions between nuclear and plastid activities during deetiolation **(Fig. 18)**. There are several different environmental conditions that are associated with changes in cellular redox status, such as high light, changes in light quality/quantity, and different stresses. Depending on the conditions, different signals and responses are induced to regulate gene expression and adapt to the changing condition. However, the mechanism behind redox regulated gene expression is not known. My work demonstrates a model where redox regulation of specific transcription factors directly links cellular redox status to gene regulation. A conserved cysteine residue identified in the specific group of bZIP transcription factors suggests these transcription factors may be regulated by redox changes and thereby have specific functions. During dark-to-light transition and early

light signalling, the seedlings face changes in redox status that potentially could affect the bZIP16, bZIP68 and GBF1 proteins. The mechanism of redox-regulation of these proteins could be the prerequisite to their important role during early light signalling. It is clear that these proteins are involved in the regulation of key components required for the establishment of photosynthetically active chloroplasts and further analyses could provide a better understanding about the regulatory mechanisms behind seedling development.

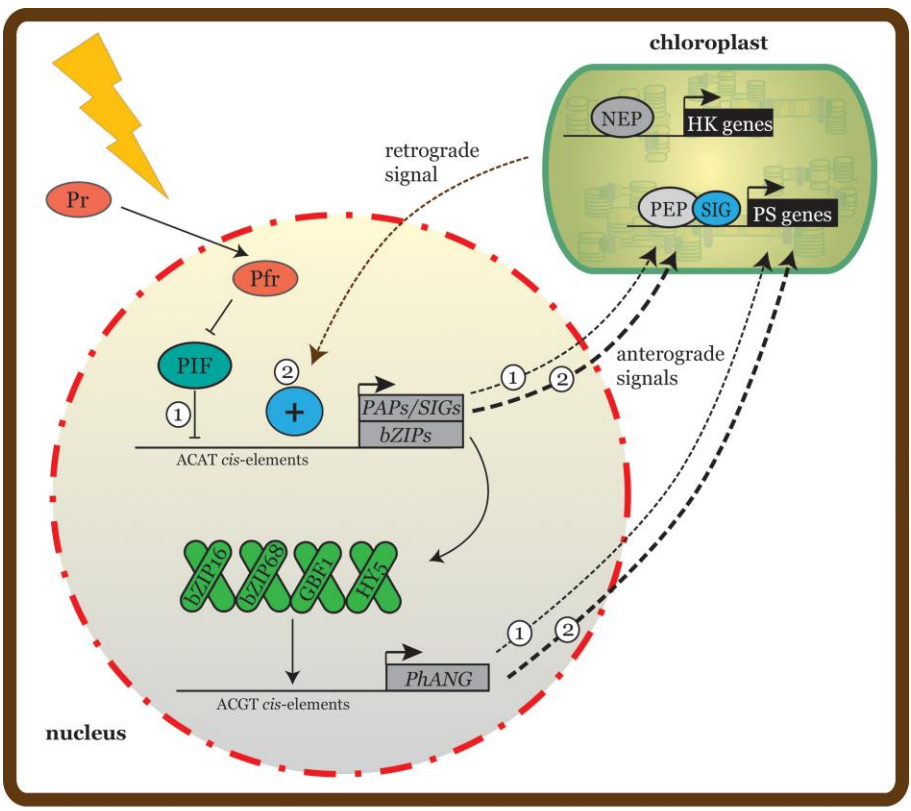

Figure 18. Working model of bZIP function during early light signalling. Following exposure to light, the inactive form of phytochrome (Pr) is converted to the active form (Pfr) which enters the nucleus and promotes degradation of PIF3. PIF3 degradation enables induced expression of PEP associated proteins (PAPs), sigma factors (SIGs) and bZIP transcription factors. The bZIPs subsequently induce the expression of *PhANG* genes. PAPs/SIGs and *PhANGs* provide the initial positive anterograde signals to the chloroplast (1) to promote photomorphogenic growth. Following correctly activated transcription in the chloroplast and the initiation of photosynthetic activity, a positive retrograde signal is sent to the nucleus to initiate a second phase of strongly induced expression of the plastid proteins (2) and fully promote chloroplast development.

Conclusions and future outlook

The work in this thesis considers how plants perceive different environmental signals and how these signals are transferred within the plant cell in order to control gene expression. The chloroplast and the process of photosynthesis are naturally of great importance for plants but the chloroplast also functions as a major sensor of changes in the growth environment. Communication between the chloroplast and the nucleus is crucial both during development and in response to changes in the environment to ensure the coordination of the activities of their respective genomes. So far, the research on chloroplast and nucleus communication has defined several processes within the chloroplast where signals can be induced to affect the nucleus, and also nuclear events that control chloroplast activity. However, our understanding about how these signals are actually transduced between the two different compartments and which components are involved is still very poor. This thesis work provides important findings which provide a better understanding of how plants manage to adapt to different growth conditions, and also opens up the field for more questions and future challenges. In order to study the interactions between the chloroplast and the nucleus I have been working with two main goals: (1) to examine the mechanism(s) and components involved in tetrapyrrole-mediated plastid signalling and (2) to identify nuclear transcription factors involved in the regulation of genes encoding proteins essential for chloroplast development and function.

Perturbations of the tetrapyrrole biosynthetic pathway in the chloroplast and accumulation of the intermediates Mg-ProtoIX and Mg-ProtoIX-ME have previously been implicated to trigger retrograde signals, but this pathway has also been strongly questioned within the research field. Here we strengthen the role of Mg-ProtoIX as a plastid signalling molecule by providing a previously unknown mechanism for how the induced signal is mediated to the nucleus to regulate gene expression, both during physiological stress and photoperiodic control conditions. HSP90 proteins were identified as the first known cytosolic interacting partner of Mg-ProtoIX that forms a regulatory complex in the cytosol. Accumulation of tetrapyrroles following oxidative stress or daily oscillations was shown to inhibit the activity of HSP90 and subsequently affect downstream transcription factors and nuclear gene expression **(Paper I and II)**. This mechanism is analogous to the well-described HSP90-HSP70-HAP1 regulatory complex in yeast that is regulated by heme levels and controls the switch between anaerobic and aerobic growth (Zhang and Hach 1999). In yeast, heme accumulation is an indicator of changes in the environment and

controls the oxidative response by interacting with and activating the HSP90-HSP70-HAP1 complex and downstream target genes. Our results suggest that the HSP90 regulatory complex is evolutionary conserved and that in plants, tetrapyrrole levels function as an indicator of environmental changes affecting HSP90 activity and nuclear gene expression.

The HSP90 complex likely involves additional components and does not necessarily provide a simple linear pathway. HSP90 Co-IP experiments during specific conditions could reveal additional components involved in the cytosolic complex and elucidate if different proteins interacts with the complex during certain growth conditions. We further demonstrate that the clock component ZTL is involved in the HSP90 complex during photoperiodic conditions and provides the convergence of plastid and clock input on nuclear gene regulation **(Paper II)**. Possibly, different F-box proteins or other regulatory proteins could interact with HSP90 during specific conditions to mediate signals to the nucleus. Chloroplast retrograde and light signalling networks utilizes common *cis*-elements and transcription factors in the response to changing conditions and cross-talk between these networks could possibly occur through the HSP90 complex. Further investigation of the HSP90 complex could reveal if it functions as a cytosolic hub to integrate different signalling pathways to fine-tune the expression of nuclear genes.

In our studies, we demonstrated that the bZIP transcription factor HY5 is regulated by the activity of the cytosolic HSP90 complex both in the response to oxidative stress and during photoperiodic conditions. HY5 has been described as an important hub responding to a variety of stimuli such as light, plastid, circadian, temperature and hormone signals, and HY5 regulates the expression of more than 9000 genes (Zhang, et al. 2011). HY5 binds to G-box *cis*-elements present in gene promoters but requires other co-factors in order to induce or repress gene expression (Lee, He, et al. 2007; Larkin and Ruckle 2008). Other G-box binding factors, such as HYH and GBF1, have been shown to interact with and affect the activity of HY5 during light-responses and photomorphogenesis (Singh, et al. 2012). We aimed to identify additional nuclear transcription factors regulating genes encoding for chloroplast-localized proteins in response to light, and isolated bZIP16 and bZIP68 together with GBF1 as G-box binding proteins **(Paper III)**. We further demonstrated a mechanism regulating these specific bZIP proteins in a redox-dependent manner which provides new insight into how transcription factors can be regulated in order to adapt gene expression according to the current growth conditions. bZIP16, bZIP68 and GBF1 were shown to play important roles specifically during photomorphogenesis and early light signalling at low light intensities **(Paper III and IV)**.

The G-box *cis*-element is a shared binding site for several transcription factors such as bZIP and bHLH proteins and is found in a variety of gene promoters. How the different proteins cooperate during different conditions to ensure correct gene regulation is not fully understood. bZIP16, bZIP68 and GBF1 single mutants display misregulation of gene expression during certain conditions and thus do not have completely redundant functions. At this point we do not know the relationship between these proteins and if they have completely individual roles or are dependent on each other. Possibly, the competition for the same binding element together with other factors, such as HY5, is dependent on the abundancy of each regulatory component at the specific condition. The localization and abundance of these proteins during different experimental conditions could reveal how their specific functions are maintained. Heterodimerization between bZIP proteins is another important factor that potentially could provide a mechanism to regulate and fine-tune gene expression during changing conditions. ChIP experiments with the specific bZIP proteins in mutant backgrounds of bZIP16, bZIP68 and GBF1 would reveal if their binding patterns are dependent on interactions with each other or not. In summary, further analyses of known transcription factors and their relation to each other during different conditions could provide a better understanding of the gene regulatory network in plants.

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