THE CHLOROPLAST TALKS

- Insights into the language of the chloroplast in Arabidopsis

Peter Kindgren

Akademisk avhandling

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The chloroplast originates from an endosymbiotic event 1.5 billion years ago, when a free living photosynthetic bacteria was engulfed by a eukaryotic host. The chloroplastic genome has through evolution lost many genes to the nuclear genome of the host. To coordinate the gene expression between the two genomes, plants have evolved two types of communication, nucleus-to-plastid (anterograde) and plastid-to-nucleus (retrograde) signalling. This thesis will focus on retrograde communication with emphasis on redox and tetrapyrrole mediated signalling.

In this thesis, we establish the tetrapyrrole Mg-ProtoIX as an important retrograde negative regulator of nuclear encoded plastid proteins. We show that Mg-ProtoIX accumulates in both artificial and natural stress conditions, and that the accumulation is tightly correlated to regulation of nuclear gene expression. Using confocal microscopy, we could visualize Mg-ProtoIX in the cytosol during stress conditions. In addition, exogenously applied Mg-ProtoIX stayed in the cytosol and was enough to trigger a signal to the nucleus. The results presented here indicate that Mg-ProtoIX is transported out of the chloroplast to control nuclear gene expression. Mg-ProtoIX mediated repression of the nuclear gene, COR15a, occurs via the transcription factor HY5. HY5 is influenced by both plastid signals and the photoreceptors. Here, we show that photoreceptors are part of Mg-ProtoIX mediated signalling as well as excess light adaptation. We identified the blue light receptor, CRY1, as a light intensity sensor that partly utilizes HY5 in the high light response. To further understand the high light regulation of nuclear genes, we isolated a mutant with redox insensitive (rin) high light response. The rin2 mutant has a mutated plastid protein with unknown function. Characterization of the rin2 mutant revealed that the protein is important in regulating plastid gene expression as well as nuclear gene expression. The rin2 mutant is the first characterized rin mutant and could prove important in elucidating the cross-talk between redox mediated coordination between the plastid and the nuclear genome.

Keywords: Arabidopsis thaliana, chloroplast, photosynthesis, retrograde communication, Mg-ProtoIX, oxidative stress, gene expression.
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**Författare:** Peter Kindgren  
**Titel:** Kloroplasten pratar - En inblick i kloroplastens språk i *Arabidopsis*

**Sammanfattning:** Kloroplasten kommer ursprungligen från en endosymbiotisk händelse som skedde för ungefär 1,5 miljarder år sedan. En fotosyntetiserande bakterie blev en del av en eukaryot värdcell. Kloroplastens genom har under evolutionens gång förlorat många av dess gener till värdens nukleära genom. För att koordinera genuttrycket i båda genom har växtcellen utvecklat två typer av kommunikation, kärn-plastid (anterograd) samt plastid-kärn (retrograd) signalering. Den här avhandlingen fokuserar på retrograd kommunikation med tonvikt på redox- och tetrapyrrol-signalering.

June 1\textsuperscript{st} 2004 – October 15\textsuperscript{th} 2010

Why study chloroplast- to nucleus signaling? That was the question I asked myself after my first four days of work. After starting a fire in the sterile hood and placing 10000 Arabidopsis seeds, one by one, with a toothpick, on plates for our first mutant screen; I wasn’t sure that doing a PhD was my thing. Well, after a summer working at a warehouse, I was full of enthusiasm to start my journey, first as a master- and then as a PhD student. Being a PhD student has been compared to a roller coaster ride. I agree. Some days are full of sunshine and you achieve good results, but some days are dark and bleak and not even a simple PCR reaction is working. Now, in the end of my time as a PhD student, I realize that all those hard times served a purpose. You learn from your mistakes and that is what forges you into the person you are today. One of the best things of being a PhD student is that you are able to go around the world on conferences to meet fellow researchers and defend your research. In its heart, that is what science is all about. You can not sit in your lab and hide, you must be able to go out and talk to people and be able to present your ideas, make other people understand. These last 5 years of work have meant trips to Australia, USA, Spain, Finland and Denmark. I was fortunate enough to spend part of my PhD in Perth, Australia. An amazing city and country, I learned a lot there. The journeys and interactions with people are not something that will be evident in this thesis, but I would like to stress that without the people in Sweden and Australia, I would not be the man I am today. This thesis would not be the same. Thank you. The roller coaster ride is soon at its end. There have been so many good memories and moments. In the end though, the best moment of the PhD ride is these last months, when you start to see the final product, the thesis. I know that I am proud of it. I hope you will enjoy it.

Peter Kindgren

Umeå, September 2010
LIST OF PAPERS

I  Elisabeth Ankele*, Peter Kindgren*, Edouard Pesquet* and Åsa Strand
   * These authors contributed equally
   In vivo visualization of Mg-Protoporphyrin IX, a coordinator of photosynthetic gene expression in the nucleus and the chloroplast.
   *Plant Cell, Vol. 19, 2007*

II  Peter Kindgren, Juan de Dios Barajas López, Jehad Shaikhali, Catherine Benedict, Anasuya Mohapatra, Simon P. Gough, Mats Hansson, Thomas Kieselbach and Åsa Strand
   Interaction between Mg-protoporphyrin IX and HEAT SHOCK PROTEIN 81 is essential for regulation of LHCB expression during plant stress response
   *Submitted*

III  Peter Kindgren, Mats-Jerry Eriksson and Åsa Strand
   Expression of COR15a is regulated by an interplay between tetrapyrrole accumulation and HY5 in Arabidopsis thaliana
   *Manuscript*

IV  Tatjana Kleine, Peter Kindgren, Catherine Benedict, Luke Hendrickson and Åsa Strand
   Genome-wide gene expression analysis reveals a critical role for CRYPTOCHROME1 in the response of Arabidopsis to high irradiance.
   *Plant Physiology, Vol. 144, 2007*

V  Peter Kindgren, Dmitry Kremnev, Juan de Dios Barajas López, Mats-Jerry Eriksson, Christian Tellgren-Roth, Tatjana Kleine, Ian D Small and Åsa Strand
   RIN2, a novel chloroplast protein involved in retrograde signaling in response to excess light.
   *Manuscript*

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The papers will be referred to by their Roman numbers in the text.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Sammanfattning</td>
<td>iv</td>
</tr>
<tr>
<td>June 1st 2004 – October 15th 2010</td>
<td>v</td>
</tr>
<tr>
<td>List of papers</td>
<td>vii</td>
</tr>
<tr>
<td><strong>PREFACE</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>3</td>
</tr>
<tr>
<td><strong>THE CHLOROPLAST</strong></td>
<td>3</td>
</tr>
<tr>
<td>Evolution</td>
<td>3</td>
</tr>
<tr>
<td>From proplastid to chloroplast</td>
<td>4</td>
</tr>
<tr>
<td>Plastid protein import</td>
<td>6</td>
</tr>
<tr>
<td>Photosynthesis</td>
<td>7</td>
</tr>
<tr>
<td>Tetrapyrrole biosynthesis</td>
<td>9</td>
</tr>
<tr>
<td><strong>RETROGRADE SIGNALLING:</strong></td>
<td>12</td>
</tr>
<tr>
<td>From the chloroplast to the nucleus</td>
<td>13</td>
</tr>
<tr>
<td>What triggers retrograde signalling?</td>
<td>15</td>
</tr>
<tr>
<td>Chlorophyll intermediates</td>
<td>18</td>
</tr>
<tr>
<td>Plastid gene expression</td>
<td>19</td>
</tr>
<tr>
<td>Plastid protein import</td>
<td>19</td>
</tr>
<tr>
<td>Photosynthesis related signals</td>
<td>22</td>
</tr>
<tr>
<td>Cytosolic components</td>
<td>24</td>
</tr>
<tr>
<td>Nuclear targets</td>
<td></td>
</tr>
<tr>
<td><strong>AIM</strong></td>
<td>27</td>
</tr>
<tr>
<td><strong>RESULTS AND DISCUSSION</strong></td>
<td>28</td>
</tr>
<tr>
<td><strong>Mg-ProtoIX MEDIATED SIGNALLING:</strong></td>
<td>28</td>
</tr>
<tr>
<td>Accumulation and transport to the cytosol</td>
<td>28</td>
</tr>
<tr>
<td>Mg-ProtoIX accumulates during stress conditions</td>
<td>28</td>
</tr>
<tr>
<td>Cytosolic activity of Mg-ProtoIX</td>
<td>30</td>
</tr>
<tr>
<td>Transport of Mg-ProtoIX to the cytosol</td>
<td>31</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Cytosolic interaction partners to Mg-ProtoIX</td>
<td>33</td>
</tr>
<tr>
<td>Isolation of putative Mg-ProtoIX interacting partners</td>
<td>33</td>
</tr>
<tr>
<td>HSP90 binds to Mg-ProtoIX and suppresses the <em>gun</em> phenotype</td>
<td>34</td>
</tr>
<tr>
<td>Mg-ProtoIX involvement in the activated methyl cycle</td>
<td>37</td>
</tr>
<tr>
<td>Transport and degradation of Mg-ProtoIX</td>
<td>40</td>
</tr>
<tr>
<td><strong>Nuclear gene regulation</strong></td>
<td>41</td>
</tr>
<tr>
<td>Expression of photosynthesis associated nuclear genes</td>
<td>41</td>
</tr>
<tr>
<td>Regulation of <em>COR15a</em></td>
<td>42</td>
</tr>
<tr>
<td>Mg-ProtoIX regulates <em>COR15a</em> via HY5</td>
<td>43</td>
</tr>
<tr>
<td>Photoreceptors</td>
<td>45</td>
</tr>
<tr>
<td>Model of Mg-ProtoIX mediated <em>COR15a</em> regulation</td>
<td>47</td>
</tr>
<tr>
<td>Summary: Mg-ProtoIX mediated signalling</td>
<td>48</td>
</tr>
<tr>
<td><strong>HIGH LIGHT REGULATION OF GENES</strong></td>
<td>49</td>
</tr>
<tr>
<td>Transcriptome changes in response to high light</td>
<td>49</td>
</tr>
<tr>
<td><em>cry1</em> and <em>hy5</em> mutants show impaired high light response</td>
<td>50</td>
</tr>
<tr>
<td>Impact of CRY1 and HY5 after high light exposure</td>
<td>50</td>
</tr>
<tr>
<td>CRY1, an important component in high light adaptation</td>
<td>51</td>
</tr>
<tr>
<td><strong>ISOLATION OF REDOX INSENSITIVE MUTANTS</strong></td>
<td>52</td>
</tr>
<tr>
<td>The isolation and positional cloning of <em>rin2</em></td>
<td>53</td>
</tr>
<tr>
<td>Localization of RIN2</td>
<td>53</td>
</tr>
<tr>
<td>Plastid expression profile in <em>rin2</em> implies</td>
<td>53</td>
</tr>
<tr>
<td>involvement in regulating of PEP activity</td>
<td>54</td>
</tr>
<tr>
<td>Light irradiance sensitivity in <em>rin2</em> seedlings</td>
<td>55</td>
</tr>
<tr>
<td>RIN2, a mediator of redox signals?</td>
<td>56</td>
</tr>
<tr>
<td>Redox regulation of PEP</td>
<td>57</td>
</tr>
<tr>
<td>RIN2 and retrograde signalling</td>
<td>58</td>
</tr>
<tr>
<td>Summary: <em>rin2</em></td>
<td>59</td>
</tr>
<tr>
<td><strong>CONCLUSIONS AND FUTURE PERSPECTIVES</strong></td>
<td>59</td>
</tr>
<tr>
<td><strong>ACKNOWLEDGEMENTS</strong></td>
<td>62</td>
</tr>
<tr>
<td><strong>REFERENCES</strong></td>
<td>64</td>
</tr>
</tbody>
</table>
PREFACE

Imagine yourself on a walk; it’s early morning in the beginning in September. The Swedish winter is approaching and cold winds from the north bring the temperature below zero. Rime covers the ground and the trees’ branches. The sun starts to climb up on the horizon. Sun rays hit your face and you can still feel that there is some heat left. In what seems to happen in a second, the temperature rise. A new day has arrived. You slowly complete your walk and head inside to warm up with a cup of hot chocolate.

Nice text? I hope you felt the cold in your face and the warmth of going inside. As a plant scientist, however, you could interpret the short text in a somewhat different way. Described above are several environmental conditions that plants face during their development and growth. While we humans have the option to go inside to warm up, plants are forced to stay behind, outside. Described above are also conditions that are within the scope of this thesis; the transition between dark and light, cold temperatures and exposure to high light intensities. To be able to survive, plants have evolved a complex network of regulatory processes to adapt to environmental variations. One type of regulatory signals originates in the chloroplast. The chloroplast is the organelle within the plant cell that produces energy via photosynthesis. Signals from the chloroplast regulate gene expression in the nucleus and these signals are the focus of this thesis. This thesis presents new exciting results in plastid-to-nucleus signalling and many new threads of ideas that researchers have to tackle in the future.
INTRODUCTION

THE CHLOROPLAST

Chloroplasts are organelles in plant and algal cells that perform photosynthesis. Chloroplast derives from the Greek words chloro (green) and plast (form or entity) and they belong to an organelle family called plastids. Chloroplasts main role in plant cells is photosynthesis, a process where captured sun light is converted to energy that the plant can use. In addition, chloroplasts have important functions in amino acid and phospholipid biosynthesis (Waters and Langdale, 2009).

Chloroplasts are contained by two phospholipid layers, the outer and inner membranes that form a robust barrier against the rest of the plant cell. There are a number of compartments inside the chloroplasts (Figure 1). The internal milieu of chloroplasts, the stroma, contains, among other things, the circular genome. The photosynthetically active membranes are called thylakoids and are found within the stroma. Thylakoids form a continuous membrane network enclosing a single chamber, the lumen. Several thylakoids stacked on top of each other is called a granum. The inclusion of plastids into the pro-eukaryotic cell was a crucial evolutionary step for land based life on Earth but when and how did it happen?

![Figure 1. Internal structure of a chloroplast from the model plant Arabidopsis thaliana. (A) TEM image of a chloroplast. Bar represents 1μm. (B) Compartments inside the chloroplast.](image)

Evolution

The evolution of the photosynthetic plant cell began 1.5 billion years ago. Ancestral eukaryotes already possessed nucleus and mitochondria and had evolved separate from photosynthetic cyanobacteria. The origin of plastid evolution started in an event called endosymbiosis, where a cyanobacterium was engulfed by a pro-eukaryotic cell (Martin et al., 2002). The idea that contemporary cyanobacteria
share the same ancestor as chloroplasts was first described in the early 20th century (Mereschkowsky, 1905). The endosymbiont hypothesis was proposed after observations highlighting the similarities between free living cyanobacteria and plastids, both structurally and how they divide.

Chloroplast genomes today contain only 5-10% of the genes found in cyanobacterial genomes. Molecular genetic studies show, on one hand, that plastid genomes comprise 60-200 genes whereas genomes of free living cyanobacteria have 1000-5000 genes (Martin et al., 2002). Proteomic studies, on the other hand, show that chloroplasts contain roughly the same amount of proteins as their free living cousins (Martin et al., 2002). Thus, chloroplasts have to import a majority of their proteins, but from where and what has happened to the earlier chloroplastic genes?

The explanation is found in the nuclear genome. Most genes originating from the endosymbiont can today be found in the nucleus where they are expressed and the translated proteins later imported into the chloroplasts. The once free-living endosymbiont therefore became dependent on their host for proper function and evolved as organelles inside the host. The driving force of gene transfer between the chloroplast and nucleus is believed to be a combination of lower mutation rate, improved repair mechanisms and the sexual reproduction for nuclear genes in addition to difference in amino acid composition of plastid proteins compared to nuclear proteins (Howe et al., 2003).

Thus, evolution has formed chloroplasts to be dependent on their host and as a consequence, communication between the nucleus and plastids, anterograde signalling, has become essential (Nott et al., 2006). Signals that originate in the plastids with targets in the nucleus (retrograde signals) have also been shown to play a crucial role in plant development and survival (Bradbeer et al., 1979). Both anterograde and retrograde signals are important for the developing chloroplast, particularly when the seedling experiences light for the first time.

**From proplastid to chloroplast**

Proplastids in the developing seedling differentiate to chloroplasts in response to light. The differentiation is an important part of a process called photomorphogenesis. Proplastids originate maternally during formation of plant zygotes and are normally maintained undifferentiated in meristematic cells of developing plants (Mullet, 1988). To differentiate into mature chloroplasts, non-photosynthetic proplastids must receive the correct signals from the surrounding environment, both intra- and inter-cellular (Bräutigam et al., 2007). These signals include light, but also gene products from the nucleus (Mullet, 1988). Without
light, proplastids develop into etioplasts. The inner envelope of the proplastid invaginates to form prolamellar bodies, later used for primary internal membranes. Upon illumination, etioplasts differentiate by forming the network of thylakoids seen in mature chloroplasts (Wellburn and Wellburn, 1971).

In plastid genomes there are essentially two types of genes, photosynthetic and transcription/translation related genes (Sato et al., 1999). In proplastids, the transcription/translation genes are transcribed at faster rates than the photosynthetic genes. In contrast, mature chloroplasts have more active expression of photosynthetic genes (Baumgartner et al., 1993; DuBell and Mullet, 1995). This is not surprising, since photosynthesis requires light. To achieve a controlled expression of plastid genes, plants and algae have evolved something ingenious; two different transcription systems, responsible for expressing the two types of genes. Photosynthetic genes are primarily transcribed by a plastid encoded polymerase (PEP) whereas a nuclear encoded polymerase (NEP) generally expresses the transcription/translation genes (Maliga, 1998).

Although the core of PEP is encoded in the plastid, it still requires nuclear encoded cofactors called sigma factors (Allison, 2000). The sigma factors are believed to specify promoter recognition for PEP (Allison, 2000; Lysenko, 2007). The six sigma factors found in Arabidopsis are basically similar to each other, but recent findings suggest that at least some of them have distinct functions in plant development and proliferation (Lysenko, 2007). The genes for NEP have been cloned in a number of plant species and it encodes a single-subunit bacteriophage-type enzyme (Lerbs-Mache, 1993; Hedtke et al., 1997; Chang et al., 1999; Ikeda and Gray, 1999). There are three different NEP genes (RPOTs), each encoding a functional enzyme. One protein is targeted to the plastid (RPOTP), one to mitochondria (RPOTM) and the third protein can be found in both plastids and mitochondria (RPOTMP) (Bräutigam et al., 2007).

It is generally assumed that NEP is more active in proplastids and etioplasts and that PEP takes over in developing and mature chloroplasts (Maliga, 1998; Bräutigam et al., 2007). When PEP acquires a more prominent role in mature chloroplasts, it recruits additional components to regulate transcription (Pfannschmidt and Liere, 2005), allowing a tighter control of plastid gene expression. All nuclear encoded plastid proteins, including NEP and the sigma factors, have one thing in common; they all have to be translocated from the cytosol, over the chloroplast envelope membranes, to their final chloroplastic destination. Plants have therefore been forced to evolve an active import system that recognises the nuclear encoded plastid proteins.
Plastid protein import

The recognition signal was first found in the small subunit of Rubisco (RBCS) (Schmidt et al., 1979), a nuclear encoded plastid protein that together with the plastid encoded large subunit (RBCL), form the Rubisco complex that fix CO$_2$ in plants. Observations in *Chlamydomonas* showed that the precursor of RBCS was larger then the mature form (Dobberstein et al., 1977). Experiments done on isolated chloroplasts confirmed that an amino terminal extension, later cleaved off, was required for import into the chloroplast (Chua and Schmidt, 1978; Highfield and Ellis, 1978). The vast majority of nuclear encoded plastid proteins have the amino terminal extension, called the transit peptide (TP) (Cline and Dabney-Smith, 2008). Intriguingly, the sequence of the TP varies greatly between different proteins (Jarvis, 2008). However, they share a common, conserved secondary structure; they all have the capacity to form helices with one positive side and one hydrophobic side (Jarvis, 2008). The secondary structure of the TP is believed to be crucial for the import into the chloroplast.

Chaperones in the cytosol recognize the TP and guide the polypeptide to the chloroplast and two multi-subunit import complexes in the envelope, the TOC (translocation at the outer chloroplast envelope) and the TIC (translocation at the inner chloroplast envelope) (Figure 2). A few important proteins are worth to mention for future understanding in the results and discussion part. The TOC159 and TOC34 proteins function as receptors on the surface of the envelope (Jarvis, 2008). TOC75 is the protein that forms the pore through the membrane (Cline and Dabney-Smith, 2008). The TIC channel is formed by TIC110 and TIC20. It is important to remember that the polypeptide is still in an unfolded state through the whole translocation process (Jarvis, 2008). In order to stay unfolded, chaperones assist in translocating plastid polypeptides. HEAT SHOCK PROTEINS (HSPs) function as chaperones and are important components in all steps of translocation. HSP90 and HSP70 assist in guiding the proteins to the envelope, HSP70 is localized in the inter membrane space to bridge the distance between TOC and TIC, and finally HSP93 and HSP70 sits in the stroma to take care of the translocated proteins (Jarvis, 2008).

When the proteins finally reach the stroma, the TP is cleaved off and depending on where the proteins’ final destination is, the protein is either folded or transported to the correct compartment within the chloroplast. A major function for the import machinery is to translocate nuclear encoded photosynthetic proteins when etioplasts differentiate to mature chloroplasts. In order to grow, the plant needs to start its own large scale production of energy, via photosynthesis.
Figure 2. A simplified model of the import machinery of chloroplasts. Heat shock proteins (HSP) guides the unfolded pre-protein to the TOC/TIC (translocation at the outer/inner chloroplast envelope) machinery. TOC159 and TOC34 function as receptors in the outer membrane and TOC75 forms the channel through the membrane. HSP70 bridge the distance between TOC and TIC in the inter membrane space. TIC110 and TIC20 form the pore through the inner membrane and HSP93 and HSP70 assist in the folding or, if the protein is localized to other compartments in the chloroplast, the further transport. (Modified from Jarvis, 2008)

Photosynthesis
The main energy in a plant comes from photosynthesis. In photosynthesis, photons are absorbed and their energy is converted into chemical energy that the plant can use for growth (Vernon and Avron, 1965). The assembly of the photosynthetic apparatus is a good example of the difficulties that the plant faces with nuclear- and chloroplast genomes. Most proteins found in the cores of the photosystems are plastid-encoded whereas peripheral proteins are transcribed in the nucleus and later imported to the chloroplast (Figure 3). Absorption of light occurs in the thylakoid membrane. Light harvesting antenna complexes containing nuclear encoded plastid proteins (LHCB and LHCA) which bind chlorophyll relay the captured energy to the photosynthetic reaction centres in two multi subunit complexes, photosystem I (PSI) and photosystem II (PSII) (Figure 3) (Cheng and Fleming, 2009).

The first photosystem, PSII, contains a specialized chlorophyll molecule in its reaction centre. The energy from the antenna complex adjacent to PSII is relayed and used to excite this chlorophyll molecule. The excited chlorophyll molecule is now a strong reducing agent that is able to donate an electron to an acceptor. The electron transfer traps part of the energy from the absorbed photons as redox
energy. Immediately after the electron transfer, the reaction centre chlorophyll is reduced again by a secondary electron donor. The donated electrons originate from water split in the core of PSII (Pushkar et al., 2008). Splitting water releases protons and oxygen, a vital side product for life on Earth. The core of PSII consists of two proteins, PSBA and PSBD that bind a number of electron carriers (Barber, 1998). Pheophtin accepts the electrons from the reaction centre chlorophyll, and delivers electrons to the first of two quinone electron acceptors, QA. QA, in turn, reduces QB (Barber, 1998). The plastoquinone (PQ) pool shuttles the trapped energy from QB to a protein complex, the cytochrome b6f complex. The cytochrome b6f complex transports protons from the stromal to the luminal side of the thylakoid membrane. Between cytochrome b6f and PSI a luminal component, plastocyanin (PC), carries the trapped energy (Vernon and Avron, 1965). PC now functions as an electron donor to the reaction centre chlorophyll in PSI. The core of PSI consists of a heterodimer of the proteins PSAA and PSAB (Golbeck, 1992). In the reaction centre of PSI, specialized chlorophyll molecules get excited by energy transferred from antenna complexes close to PSI and reduce electron acceptors, similar to the reactions in PSII. The trapped redox energy is transferred between electron carriers and end up in an iron-sulphur centre (Golbeck, 1992). The soluble protein ferrodoxin then directs the energy to ferrodoxin-NADP+ reductase (FNR) to reduce NADP.

Figure 3. The photosynthetic machinery of higher plants. Excitation energy is trapped in the photosystems and an electron transport chain shuttles the trapped redox energy between them. During photosynthesis, protons are pumped over the thylakoid membrane to create a proton gradient that drives an ATP synthase. Reducing power, NADPH, is formed to enable the incorporation of CO2. Fixated CO2 generates carbohydrates used in plant growth and development. Plastid encoded proteins are in green and nuclear encoded proteins are in yellow. (Herrmann & Westhoff (Adv. Photosynth. & Resp.), 2001. Reprinted with kind permission of Springer Science and Business Media)
In summary, the trapped energy is used for two things; (1) to build up a proton gradient over the thylakoid membrane that drives an ATP synthase and (2) to create reducing power in the form of NADPH that primarily is used to fix carbon in the Calvin cycle. Photosynthesis provides a strong reducing power that could have potential toxic effects for the plant. This happens especially under high irradiances, when more energy is trapped then the photosystems can utilize. The production of an excess of excited molecules is due to the fact that electron acceptors, such as the pool of quinones, PQ or PC, already are fully reduced (electron binding) and cannot accept more. Excited molecules can react with nearby molecules, particularly oxygen, and produce toxic compounds or react with proteins in the photosystems. As a consequence, proteins will be damaged and unable to perform photosynthesis. In response, photosynthetic organisms have evolved different protection mechanisms.

There are short- and long-term acclimation strategies for a plant. Short-term strategies include molecules that absorb the energy from excited molecules, carotenoids (Siefermann-Harms, 1987), and proteins to scavenge toxic compounds (Asada, 2006). The plant can also redistribute the excitation energy between the photosystems, a process called state transition (Dietzel et al., 2008). Long-term strategies include regulation of nuclear and chloroplastic genes (Dietzel et al., 2008). How this regulation occurs is discussed later in this chapter. A number of strategies to protect the plant from damage involve the synthesis and degradation of the light harvesting pigment in plants, chlorophyll. Chlorophyll is an end product in a well studied biosynthetic pathway that produces several important molecules, called tetrapyrroles.

**Tetrapyrrole biosynthesis**

Higher plants synthesize two different forms of chlorophyll, chlorophyll $a$ and $b$ (Tanaka and Tanaka, 2007). Both are localized in the thylakoid membrane where they sit adjacent to photosystem I and II. Chlorophyll absorbs blue and red light causing the green colour of plants. In addition to chlorophyll, plants also synthesize three other groups of tetrapyrroles, namely heme, siroheme and phytochromobilin (Tanaka and Tanaka, 2007). Heme has a vital role in different biological processes including photosynthesis and respiration.

Contrary to chlorophyll that incorporates magnesium, heme and siroheme contain an iron atom. Siroheme functions in the assimilation of nitrogen and sulphur (Tripathy et al., 2010). The linear tetrapyrrole, phytochromobilin, is a chromophore that is bound by phytochromes, the red and far-red sensing photoreceptors in plants (Quail, 2002). Plastids are the major site for tetrapyrrole
synthesis in plants, but all enzymes are nuclear-encoded. It is only the last steps of heme biosynthesis that are suggested to occur both in the plastid and the mitochondria (Tanaka and Tanaka, 2007).

All tetrapyrroles originate from a common starting molecule, glutamyl-tRNA. The steps to protoporphyrin IX are common for chlorophyll, heme and phytochromobilin, whereas siroheme synthesis branch off from uroporphyrinogen III (Figure 4). At the protoporphyrin IX branch point, two enzymes decide the fate of the porphyrin ring. Magnesium chelatase uses ATP to insert magnesium in the first unique step of chlorophyll synthesis, and the second enzyme, ferrochelatase, inserts iron for a continuation in the heme/phytochromobilin pathway (Tanaka and Tanaka, 2007).

Depending on the demand for each end product, there is a tight regulation in each of the branch points in addition to a more general regulation of the entire pathway. The first regulatory point is the formation of 5-Aminolaevulinic acid (ALA). The formation of ALA creates an important regulatory point since all tetrapyrroles downstream of ALA are phototoxic and accumulation of tetrapyrroles is a possible threat to the plant (Tanaka and Tanaka, 2007). By the regulatory mechanisms so far discovered, feedback regulation—especially by heme and protochlorophyllide—is suggested to be important (Meskauskiene et al., 2001; Goslings et al., 2004).

Another regulatory point is the above mentioned branch point between the chlorophyll and heme pathways. Magnesium chelatase (Mg-Chl) is a protein complex with three subunits, H, D and I where CHLH is the subunit that binds the tetrapyrrole (Masuda, 2008). Activity of Mg-Chl is stimulated by: the GUN4 protein (Davison et al., 2005) and the Mg^{2+} concentration (Reid and Hunter, 2004); regulated by: the chloroplastic redox state (Jensen et al., 2000) and the ATP/ADP ratio (Reid and Hunter, 2004). The ATP/ADP ratio has also been found to regulate Ferrochelatase (Cornah et al., 2002) (Figure 4).

The redox regulation of Mg-Chl is probably responsible for decreasing the production through the chlorophyll branch during dark periods in photoperiodic conditions (Papenbrock et al., 1999). In addition, the dark induced block of chlorophyll synthesis at the reduction step of protochlorophyllide directs the tetrapyrroles to the heme branch resulting in heme synthesis also in the dark (Papenbrock et al., 1999). The tightly regulated tetrapyrrole pathway is vital for plant development and growth, and is another example of the complex network of processes that occurs in a plant.
Figure 4. The tetrapyrrole biosynthesis pathway of higher plants. The common starting point, glutamyl tRNA, is synthesised to four different end products; chlorophyll, siroheme, heme and phytochromobilin. The pathway has two major regulation points, feedback regulation from heme and protochlorophyllide, and regulation of Mg- and Fe-chelatase.
RETROGRADE SIGNALLING:
FROM THE CHLOROPLAST TO THE NUCLEUS

What probably is obvious by now is the requirement for communication between the nucleus and plastid and vice versa. All the processes described above have components that are synthesised or encoded both in the nucleus and the plastid. Intra-cellular communication is divided into anterograde (nucleus-to-plastid) and retrograde (organelle-to-nucleus) communication. While anterograde signals originate in the nucleus to control plastid development and activity, retrograde signals communicate with the nucleus what gene products are needed in the chloroplast. Anterograde signals can be assigned into four functional classes. The categories include: (1) import, assembly and sorting of plastid proteins, (2) transcriptional/translational regulation of the plastid genome, (3) plastid division and (4) modification of plastid enzymes (Bräutigam et al., 2007).

An emerging theory termed ‘control by epistasy of synthesis’ (CES) might explain how plastid encoded proteins are regulated in response to the import of nuclear encoded components (Pogson et al., 2008). As the name hints, CES denotes that the synthesis of one subunit of a multi-subunit complex is dependent on the synthesis of the other. CES is exemplified in higher plants by the assembly of Rubisco where the nuclear encoded RBCS have been found to regulate the translation of the plastid encoded RBCL (Wostrikoff and Stern, 2007). To what extent higher plants use CES to regulate subunits of other protein complexes is still unknown.

Although CES is an elegant way to explain how plastid encoded proteins are regulated and confirms that anterograde signals are crucial for a plant, the work in this thesis will focus on how the plastid communicates with the nucleus. As illustrated by the above mentioned photosystems, the coordination of multi subunit complexes that consist of proteins from both the plastid and nucleus demands a tight retrograde communication to fine tune the nuclear gene expression.

Communication between the plastid and the nucleus was first proposed about 30 years ago (Bradbeer et al., 1979). In these studies, white sectors in the leaves of albostrians and saskatoon mutants in barley (Hordeum vulgare) were shown to have reduced expression of photosynthetic nuclear-encoded genes (Bradbeer et al., 1979). Later results suggested that a plastid-encoded component was required for light induction of these genes (Hess et al., 1991; Hess et al., 1994). Subsequent reports established the role of plastid signals to ensure a complete differentiation to
chloroplasts (Mayfield and Taylor, 1984; Oelmüller et al., 1986; Oelmüller and Mohr, 1986). Although plastid-to-nucleus signalling have attracted much interest in recent years and progress has been made very little is known about the mechanisms involved (Nott et al., 2006; Bräutigam et al., 2007; Fernández and Strand, 2008; Kleine et al., 2009).

**What triggers retrograde signalling?**
In short, retrograde signals are triggered when the plastid experiences shifts in the surrounding environment. In young seedlings, chloroplasts send signals to the nucleus for correct development and in adult plants, retrograde signals may be responsible for inducing stress responses, fine tuning of nuclear gene expression, both in response to chlorophyll synthesis- and photosynthetic flux, and recovery from damage (Nott et al., 2006). Considering the fluctuating environment that the plants live in, retrograde signalling is a constant on-going process. However, the first signals that plastid send in response to light are to start developing photosynthetic chloroplasts. These signals are investigated with de-etiolated seedlings, mutants containing abnormal chloroplasts or chemical inhibitors of chloroplast development.

Norflurazon (NF), an inhibitor of the carotenoid biosynthesis, has been used to screen for retrograde signalling mutants (Susek et al., 1993). Seedlings that are grown on NF experience photobleaching since carotenoids protect the plant from oxidative stress (Sandmann and Böger, 1997). As a consequence, NF-grown seedlings contain chloroplasts without thylakoid membranes, photosynthetic pigments and several photosynthetic proteins (Ankele et al., 2007).

Another option to block chloroplast biogenesis is to inhibit plastid translation or transcription. The inhibition is accomplished by adding antibiotics to developing seedlings. Certain antibiotics inhibit bacterial translation and, since chloroplasts have bacterial ancestry and bacterial translational machinery, this will only affect the translation in the chloroplast and the mitochondria. Chloramphenicol, erythromycin and lincomycin, inhibitors of plastid translation, have been used to elucidate plastid signals (Oelmüller et al., 1986; Sullivan and Gray, 1999; Koussevitzky et al., 2007; Ruckle et al., 2007). Chloramphenicol inhibits peptidyl transferase activity and prevents the formation of the peptide bond in bacterial protein translation (Jardetzky, 1963). Erythromycin and lincomycin with similar mode of action as chloramphenicol, inhibit translation in plastids as well (Sullivan and Gray, 1999; Mulo et al., 2003).
To study retrograde signalling in mature chloroplasts, different stress conditions that affect the processes in the chloroplast are used. Several studies using different light qualities (Pfannschmidt et al., 1999; Ruckle et al., 2007; Brautigam et al., 2009; Ruckle and Larkin, 2009) or intensities (Escoubas et al., 1995; Karpinski et al., 1999) have demonstrated the importance of retrograde signalling. Plastid-to-nucleus signalling has also been found to be induced during cold stress (Wilson et al., 2003; Svensson et al., 2006; Nakayama et al., 2007). Oxidative stress, caused by excess light or cold, can also be induced by treating plants with the chemical compound, methyl viologen (MV). MV accelerates production of reactive oxygen species in chloroplasts by photoreduction of O₂ at PSI, resulting in higher levels of singlet oxygen and H₂O₂ (Mano et al., 2001; Yabuta et al., 2004).

**Figure 5.** Suggested plastid components of retrograde signalling. (1) Chlorophyll intermediates. (2) Plastid gene expression. (3) Plastid protein import machinery. Reactive oxygen species: H₂O₂ (4) and ¹O₂ (5). (6) Redox state of the PQ-pool. (7) The electron acceptor NADPH. (8) Photosynthetic metabolites.
Although the retrograde signalling is a complex network of different signals, certain plastid key players have been identified in plants (Figure 5). These players can be divided into four main signalling pathways: (1) chlorophyll intermediates (Fig 5, signal 1), (2) plastid gene expression (Fig 5, signal 2), (3) plastid protein import (Fig 5, signal 3), and (4) photosynthesis related signals (Fig 5, signal 4-8). These signals and their interacting partners in the cytosol in addition to the nuclear elements will be discussed below in greater detail.

**Chlorophyll intermediates**

The first evidence that suggested tetrapyrroles might act as signalling components came from work in the photosynthetic unicellular green alga *Chlamydomonas reinhardtii*. Using inhibitors in different steps of the chlorophyll biosynthesis pathway, accumulating tetrapyrroles could be identified as possible signalling molecules to regulate *LHCB* genes (Johanningmeier and Howell, 1984; Johanningmeier, 1988; Jasper *et al.*, 1991). The results pointed at the porphyrins between ProtoIX and Mg-ProtoIX-ME to be responsible for repressing *LHCB* (Figure 6).

![Figure 6](image_url)  
*Figure 6.* Molecular structure of intermediates in the tetrapyrrole biosynthesis with an implied role in retrograde signalling. Enzymes and mutants discussed in the text are highlighted.
In addition to a repressing role, Mg-ProtoIX and Mg-ProtoIX-ME are also required for the induction of HEAT SHOCK PROTEIN 70A/B (HSP70A and HSP70B) in *C. reinhardtii*. Light plays a role together with cytosolic Mg-ProtoIX to induce the expression of HSP70 (Kropat *et al.*, 1997; Kropat *et al.*, 2000). In *C. reinhardtii*, Mg-ProtoIX was also found to induce HEMA, encoding glutamyl-tRNA reductase, the first enzyme in the tetrapyrrole pathway (Vasileuskaya *et al.*, 2004). The cytosolic localization of Mg-ProtoIX rises the question how the intermediate exits the plastid. The transport of Mg-ProtoIX will be discussed later in the results and discussion chapter.

Evidence of tetrapyrrole signalling also comes from higher plants. Studies from etiolated seedlings of barley and cress confirm the involvement of Mg-ProtoIX and Mg-ProtoIX-ME in the light regulation of *LHCB* expression (Kittsteiner *et al.*, 1991; Oster *et al.*, 1996; La Rocca *et al.*, 2001). In *Arabidopsis*, tetrapyrrole involvement in retrograde signalling was confirmed analyzing the genome uncoupled (*gun*) mutants (Strand *et al.*, 2003). Seeds containing a screenable marker fused to a *LHCB* promoter were mutagenized with ethyl methane sulfonate (EMS) and screened. Seedlings that still displayed a high *LHCB* expression on NF were isolated (Susek *et al.*, 1993).

Of the mutants isolated, *gun2-5* were all found to have perturbations in defined steps of the tetrapyrrole biosynthesis (Figure 6) and show a pale phenotype. The *gun2* and *gun3* mutants have mutations in enzymes involved in the heme branch of tetrapyrrole biosynthesis and they are allelic to LONG HYPOCOTYL 1 and 2 (*hy1* and *hy2*) (Figure 6) and, as a consequence, plastids in *gun2* and *gun3* accumulate heme (Mochizuki *et al.*, 2001). Accumulating heme will negatively regulate the first step of tetrapyrrole biosynthesis (Pontoppidan and Kannangara, 1994; Terry and Kendrick, 1999). *GUN4* encodes a tetrapyrrole binding protein proposed to stimulate Mg-chelatase activity (Larkin *et al.*, 2003). However, the exact mechanism of *GUN4* is still unknown. Lastly, the *gun5* mutant has a point mutation in the porphyrin binding H-subunit of Mg-chelatase (Mochizuki *et al.*, 2001). The *gun2/3/4/5* mutations all result in decreased accumulation of Mg-ProtoIX and Mg-ProtoIX-ME (Figure 6). The inability of the *gun* mutants to accumulate Mg-ProtoIX was also suggested to be the reason for their phenotype and that accumulating Mg-ProtoIX is the trigger needed to repress *LHCB* on NF (Strand *et al.*, 2003).

Other mutations in enzymes involved in the tetrapyrrole biosynthesis strengthen the role of Mg-ProtoIX as a signalling molecule. Impaired retrograde signalling can be seen in mutants of both porphobilinogen deaminase and coproporphyrinogen
oxidase (Figure 4, (Strand et al., 2003). In transgenic Tobacco, the increased or decreased pool of Mg-ProtoIX could be correlated to the expression of PhANGs in plants over- or under expressing CHLM, encoding Mg-ProtoIX methyl transferase (Figure 6, (Alawady and Grimm, 2005). In Arabidopsis, knocking out the CHLM gene results in accumulation of Mg-ProtoIX and repression of LHCb (Pontier et al., 2007; Mochizuki et al., 2008).

Mutations in the D-subunit of Mg-chelatase also show a gun phenotype (Strand et al., 2003). To further establish the role for Mg-ProtoIX in signalling, mutations in the CRD subunit of the cyclase (CHL27) that catalyses the conversion from Mg-ProtoIX-ME to divinyl protochlorophyllide, does not show a gun phenotype, although chloroplast development is severely impaired (Ankele et al., 2007; Bang et al., 2008). The crd mutant accumulates more Mg-ProtoIX and Mg-ProtoIX-ME than wild type (Tottey et al., 2003), again suggesting that the chlorophyll intermediates upstream of protochlorophyllide are involved in signalling. The cyclase complex is highly regulated by redox and is inhibited by conditions that trigger plastid-to-nucleus signalling (Stenbaek et al., 2008), further suggesting that Mg-ProtoIX and Mg-ProtoIX-ME could accumulate during oxidative stress. In addition, feeding of Mg-ProtoIX to protoplasts was able to repress LHCb expression, whereas addition of ProtoIX, heme or porphobilinogen did not repress the luciferase gene driven by the LHCb promoter (Strand et al., 2003).

Though there is clear genetic and biochemical evidence of Mg-ProtoIX signalling, there are results that do not fit into the model. The chlm and crd mutants have higher levels of Mg-ProtoIX in control conditions, but show normal expression of LHCb, suggesting that the Mg-ProtoIX mediated signal is only associated with stress conditions (Mochizuki et al., 2008). Mutations in the last subunit of Mg-chelatase (CHLI, cs and ch42 mutants) do not show the expected gun phenotype (Mochizuki et al., 2001). Both cs and ch42 have less Mg-chelatase activity than the gun5 mutant, but show wild type repression of LHCb on NF (Mochizuki et al., 2001).

However, there are two CHLI genes in Arabidopsis, CHLI1 and CHLI2, and recent studies have shown that knocking out both genes can give a gun phenotype on NF (Huang and Li, 2009), explaining the phenotype in the single mutants. The role of Mg-ProtoIX as a plastid signal was recently questioned (Mochizuki et al., 2008; Moulin et al., 2008). These reports showed that the steady state level of Mg-ProtoIX could not be responsible for repression of LHCb on NF. Using similar setup as Strand et al. (2003), it was shown that seedlings on NF contain less Mg-ProtoIX than seedlings grown in control conditions. Even though there is
overwhelming evidence that points at Mg-ProtoIX as a mediator in signalling, it is still an open question if the molecule itself or a Mg-ProtoIX derived molecule is responsible for the signalling.

**Plastid gene expression**
A second plastid signal was identified using the plastid translation inhibitors chloramphenicol and lincomycin. Applying chloramphenicol to young seedlings results in repression of PhANGs. However, the effects of antibiotics can only be seen in seedlings treated early in development, suggesting that an early translated plastid protein is the origin of the signal or that the plastid never reach the developmental stage to send a signal (Sullivan and Gray, 1999). Using inhibitors of PEP transcription at this stage showed similar results (Mathews and Durbin, 1990; Pfannschmidt and Link, 1997). Studies of the constitutively photomorphogenetic *Arabidopsis cop1-4* and pea *lip1* mutants showed that their over-accumulation of *LHCb* transcripts in the dark could be reversed by applying lincomycin (Sullivan and Gray, 1999). The danger in working with translation inhibitors is that they give pleiotropic effects (Mulo et al., 2003). Since the mitochondria also have a bacterial ancestry and a bacterial translation system, it is possible that effects could reach the mitochondria as well. Certain antibiotics will even affect the cytosolic translation (Mulo et al., 2003).

Studying mutants with impairments in components of the plastid and mitochondria translation system showed that it is necessary to mutate both translation systems to see the effect of nuclear gene expression (Pesaresi et al., 2006). So far, very little is known about how plastid gene expression mediated signalling occurs. The best characterized candidate in the pathway is the last of the *gun* mutants, *gun1*. *GUN1* encodes a chloroplast localized pentatricopeptide repeat (PPR) protein (Koussevitzky et al., 2007).

Although the exact function of GUN1 is not known, it has been suggested to be the proposed (Richly et al., 2003) master switch in plastid- to nucleus signalling during chloroplast development incorporating many signals to a common nuclear targeted signal (Koussevitzky et al., 2007). The *gun1* mutant displayed a *gun* phenotype both on NF and lincomycin contrary to *gun2/3/4/5* that only showed the phenotype on NF (Koussevitzky et al., 2007), suggesting that GUN1 is part of the signalling pathway that is affected by the inhibition of plastid protein synthesis. Seedlings with a mutated GUN1 protein showed a slower chloroplast development in response to light and affected hypocotyl elongation, anthocyanin biosynthesis and cotyledon opening (Ruckle and Larkin, 2009).
**Plastid protein import**

The third plastid signal that has been proposed derives from the plastid protein import machinery. Using the _plastid protein import_ 2 (ppi2) mutant in _Arabidopsis_, that lacks the most abundant protein of the import receptor TOC159 family, Kakizaki and co-workers could show that the rate of protein import into the chloroplast is tightly coordinated with nuclear gene expression (Kakizaki _et al._, 2009). The ppi2 mutant is albino and does not survive beyond the seedling stage of development, because of its inability to accumulate photosynthesis related proteins (Bauer _et al._, 2000). The effect in the ppi2 mutant seems to be specific to photosynthesis related proteins since both the expression and accumulation of non-photosynthetic genes and proteins, respectively, are normal (Bauer _et al._, 2000). The TOC159 mutated in the ppi2 mutant, is one of four family members in _Arabidopsis_ and it recognizes photosynthesis related proteins, but not non-photosynthetic proteins, partly explaining the phenotype of the ppi2 mutant (Smith _et al._, 2004).

The plastid import mediated signal could not be correlated to tetrapyrrole mediated signalling, but intriguingly, seems to be in the same pathway as GUN1 (Kakizaki _et al._, 2009). These results further strengthen the ‘master switch’ role of GUN1 in developing seedlings. Other mutants of plastid protein import show a similar phenotype, suggesting that the signal is triggered by a general impairment of plastid protein import (Kakizaki _et al._, 2009), and not only by a defect in importing photosynthesis related proteins. It is not yet known what signal molecule that is responsible for the signalling in response to an impaired plastid protein import but suggestions have proposed a feedback regulation from accumulating precursors in the cytosol (Kakizaki _et al._, 2009). Another possibility is an interaction with the last group of signals originating in the plastid, the redox signals.

**Photosynthesis related signals**

The fourth type of plastid signal to control nuclear gene expression is a group of signals all related to the photosynthetic machinery in the chloroplast. Three different groups of signals have been proposed; (1) Reactive oxygen species (ROS) generated at the photosystems, (2) Redox state of components in or coupled to the photosynthetic electron transport chain (PET), and (3) metabolite exchange between the chloroplast and the cytosol (Nott _et al._, 2006; Fernández and Strand, 2008; Pogson _et al._, 2008; Kleine _et al._, 2009). In linear PET, explained earlier, electrons are delivered from PSII to the plastoquinone (PQ) pool, and via the cytochrome b6f complex and plastocyanin (PC) they end up at PSI. At the acceptor side of PSI thioredoxin, a redox active protein is photosynthetically reduced by...
ferrodoxin, which in turn acquires its electrons from the end electron acceptor in PET, NADPH. Reduced thioredoxin is used to activate many of the enzymes associated with photosynthesis (Schürmann and Buchanan, 2008). If photon fluence exceeds the utilization rate of photosynthesis, excited chlorophyll molecules transfer their energy to other molecules, creating toxic compounds, ROS.

ROS is continuously produced in plants at all light intensities, but accumulate during photooxidative stress conditions (i.e. high light, cold). There are three major sites for ROS production coupled to photosynthesis in plants. At the light harvesting complexes associated with PSII and the PSII reaction center, excited chlorophyll molecules can react with oxygen, creating singlet oxygen $^{1}\text{O}_2$ (Niyogi, 1999). Singlet oxygen is normally quenched by xanthophylls associated with PSII (Kühlbrandt et al., 1994; Niyogi, 1999). At the acceptor side of PSI, oxygen can acquire electrons, creating super oxide $\text{O}_2^-$ (Niyogi, 1999). Super oxide is rapidly converted to $\text{H}_2\text{O}_2$ spontaneously or by enzymatic dismutation (Apel and Hirt, 2004). $\text{H}_2\text{O}_2$ can then be detoxified by chloroplastic ascorbate peroxidises (APXs) to $\text{H}_2\text{O}$. Mutants that lack cytosolic or chloroplastic scavenger enzymes trigger different signalling pathways, indicating that the compartmental source of the produced ROS is important (Miller et al., 2007). Singlet oxygen and $\text{H}_2\text{O}_2$ activate distinct sets of nuclear genes (Laloi et al., 2007) even though they cause similar damage (Foyer and Noctor, 2005). Though they regulate different genes, there is a cross talk between the $\text{H}_2\text{O}_2$ and singlet oxygen mediated pathways, thought to contribute to robustness when plants are exposed to stress conditions (Laloi et al., 2007).

If the chloroplast contain an excess of $\text{H}_2\text{O}_2$, it is possible that it traverses the envelope membrane to start a signal further transduced to the nucleus (Mullineaux and Karpinski, 2002). The $\text{H}_2\text{O}_2$ transport may be mediated by pores in the envelope, called aquaporins (Bienert et al., 2007; Dynowski et al., 2008). $\text{H}_2\text{O}_2$ could therefore function as a high light signal in plants. Addition of external $\text{H}_2\text{O}_2$ supports this hypothesis (Karpinski et al., 1999). Genes that are induced by $\text{H}_2\text{O}_2$ include $\text{APX2}$ and the genes encoding the zinc finger transcription factors $\text{ZAT10}$ and $\text{ZAT12}$. $\text{ZAT10}$ and $\text{ZAT12}$ are responsible of inducing many of the genes up regulated by high light (Davletova et al., 2005b; Rossel et al., 2007).

Recent studies suggest that $\text{H}_2\text{O}_2$ oxidizes the electron acceptor $\text{Q}_A$, responsible of moving electrons to PQ (Karpinska et al., 2000; Laloi et al., 2007), indicating a cross talk between ROS and components in PET. $\text{H}_2\text{O}_2$ oxidification of $\text{Q}_A$ would result in an increase in electron transport and, as a consequence, the likelihood for production of ROS would be lower. Thus, $\text{H}_2\text{O}_2$ could regulate the amount of
singlet oxygen (\(1^\text{O}_2\)) produced at PSII. \(1^\text{O}_2\) itself has also been suggested to function as a signal (op den Camp et al., 2003). The isolation of the fluorescent (flu) mutant (Meskauskiene et al., 2001) showed that \(1^\text{O}_2\), thought to remain in the chloroplast, was responsible of regulating nuclear genes that differ from \(\text{O}_2^-/\text{H}_2\text{O}_2\) induced genes (op den Camp et al., 2003). Genes activated by \(1^\text{O}_2\) are primarily involved in cell death (Danon et al., 2005). The flu mutant accumulates the chlorophyll precursor protochlorophyllide and consequently generates \(1^\text{O}_2\) upon illumination. The short half life of \(1^\text{O}_2\) (200 ns) (Gorman and Rodgers, 1992), suggests that the signalling pathways need adjacent components to relay the signal.

Isolated in a suppressor screen in the flu background, executor1-2 (ex1 and ex2), was found to be part of the \(1^\text{O}_2\) signalling pathway (Wagner et al., 2004; Lee et al., 2007b). EX1 and EX2 are two chloroplast-localized proteins that are thought to be associated with the thylakoid membrane. Studies with double and triple mutants of ex1/2 and flu show that mutations in both EX genes are required to suppress the \(1^\text{O}_2\) induced genes (Lee et al., 2007b). EX1 and EX2 potentially could work as sensors or mediators of \(1^\text{O}_2\) accumulation in chloroplasts but the exact function is still not known. Bioinformatic studies show that the EX proteins are conserved between higher plants, but unrelated to any proteins with known function (Lee et al., 2007b).

In addition to the signalling role of ROS, components in PET have also been implicated to relay information to the nucleus. The redox state of the PQ pool was early proposed as an origin for plastid signals. Using inhibitors on both sides of the PQ pool and different light qualities, leaving the PQ pool fully reduced or oxidized, it was found that the redox state was responsible for regulating a number of photosynthesis genes (Escoubas et al., 1995; Pfannschmidt et al., 1999; Pfannschmidt et al., 2001). More recent studies questioned the importance of the PQ pool as a plastid- to nucleus signalling, suggesting that reducing molecules at the acceptor side of PSI, like thioredoxin, was of greater importance (Piippo et al., 2006).

Still, the role of the PQ pool can not be neglected and several studies suggest that the PQ pool and thioredoxin produce parallel signals communicated to the nucleus (Fey et al., 2005; Piippo et al., 2006; Adamiec et al., 2008; Brautigam et al., 2009). A key regulator in this pathway is THYLAKIOD ASSOCIATED KINASE STATE TRANSITION7 (STN7). STN7 is responsible for acclimation of photosynthesis to fluctuating conditions (Bellafiore et al., 2005; Bonardi et al.,
The acclimation is activated by a reduced PQ pool and inactivated by reduced thioredoxin (Bellafiore et al., 2005).

A gene that has been found to be regulated by the acceptor side of PSI but not by the redox state of the PQ pool is 2-CYSTEINE PEROXIREDOXIN A (2CPA) (Baier et al., 2004). 2CPA is a chloroplastic peroxiredoxin involved in protecting the plant against redox damage (Baier and Dietz, 1997). In an attempt to isolate redox imbalance (rimb) mutants, the promoter of 2CPA was fused to a reporter gene and screened for low 2CPA expression (Heiber et al., 2007). Low expression of 2CPA should be a result of impaired signalling from the chloroplast. Five mutants were isolated and they showed reduced expression of other antioxidant enzymes in addition to increased sensitivity to H$_2$O$_2$ and low CO$_2$ (Heiber et al., 2007). The mutated genes in the rimb mutants are still unknown.

The last proposed signal coupled to photosynthesis is photosynthetic metabolites that can cross the envelope to the cytosol. The end products of photosynthesis, carbohydrates in the form of glucose or sucrose, have been known to repress PhANG expression (Rolland et al., 2006). In addition, the rate of CO$_2$ fixation, consequently resulting in higher levels of carbohydrates, was shown to be a component in retrograde signalling (Piippo et al., 2006). The flux of carbohydrates from chloroplast to cytosol could also be coupled to signalling. Both the malate/oxaloacetate and triose-phosphate shuttles use chloroplastic NADPH, reducing cytosolic NADH, to accomplish transport over the membrane (Heineke et al., 1991).

**Cytosolic components**

Cytosolic components are the ‘big black box’ in retrograde signalling. Although many components in plastid to nucleus signalling have been found in the plastid and nucleus, the players in the cytosol -the relay stations- have remained elusive. Many of the proposed plastid signals have properties that make them unsuitable as long distance signals (Kleine et al., 2009). At some level, plastid signals are presumed to interact with other cytosolic signalling pathways to regulate nuclear gene expression. There is evidence suggesting that a phosphorylation cascade might be involved. The best model for cytosolic components functions in the ROS pathway. H$_2$O$_2$ activates cytosolic mitogen-activated protein kinases (MAPK) and regulates gene expression. In many eukaryotes, signals are transduced via phosphorylation involving MAPKs and their downstream components MAPK kinases (MAPKK) and MAPKK kinases (MAPKKK) (Apel and Hirt, 2004). In *Arabidopsis*, H$_2$O$_2$ activates MAPK3, MAPK4 and MAPK6 via the MAPKKKs,
ANP1 and MEKK1 (Kovtun et al., 2000; Nakagami et al., 2006). The activated MAPKs can then activate transcription factors to regulate gene expression.

Since many genes involved in photosynthesis are regulated by light and plastid signals, it comes as no surprise that photoreceptors have been implicated in retrograde signalling. In plants, there are the blue light receptors, cryptochromes and phototropins, in addition to phytochromes, the red and far red light receptors (Jiao et al., 2007). Photoreceptors are involved in many processes in plants including germination, photomorphogenesis, shade avoidance, input for the circadian clock and flowering time. In respect to plastid-to-nucleus signalling, Phytochromes (PHY) have been closely linked to \textit{LHCB} expression (Lopez-Juez et al., 1998). Studies of \textit{cab under expressed} (\textit{cue}) mutants in \textit{Arabidopsis} revealed that plastid signals are needed for PHY control of nuclear gene expression (Vinti et al., 2005). Of the photoreceptors, only mutants of CRYPTOCHROME1 (CRY1) show a \textit{gun} phenotype when grown on lincomycin (Ruckle et al., 2007). Genetic experiments with other mutants involved in regulating \textit{LHCB} expression combined with different light qualities suggested that a plastid signal could turn CRY1 from a positive- to a negative regulator of \textit{LHCB} (Ruckle et al., 2007). CRY1 have also been found to be involved in the cell death response mediated by the EX proteins (Danon et al., 2006), again suggesting a cross talk between different plastid signals.

The plant hormone abscisic acid (ABA) has been repeatedly associated with retrograde signalling. It does not fit into the model as a plastid signal since it is synthesised in the cytosol, not in the chloroplast, but many of its precursors are synthesized in the plastid (Seo and Koshiba, 2002). ABA derives from xanthophylls that are produced in the chloroplast and transported to the cytosol as xanthoxin. Xanthoxin is converted in the cytosol to ABA that triggers signals controlling nuclear gene expression (Finkelstein and Rock, 2002). ABA is involved in embryo and seed development, germination and seedling development, in addition to a role in different stress responses (Cutler et al., 2010). The chloroplastic part of ABA biosynthesis is thought to be highly dependent on the photosynthetic reactions. For example, NADPH is required for synthesizing xanthophylls (Baier and Dietz, 2005). The action of ABA can therefore be connected to retrograde signalling during oxidative stress. Both \textit{APX2} and \textit{2CPA} expression is regulated by ABA (Baier et al., 2004; Chang et al., 2004).

Many other photosynthetic genes have ABA responsive elements in their promoters. Furthermore, there is a possible crosstalk between ABA and the \textit{gun} signalling pathway. GUN5, the H-subunit of Mg-chelatase, has been found to bind to ABA and could possibly function as an ABA receptor (Shen et al., 2006).
Exogenously applied ABA increased the amount of Mg-ProtoIX, but decreased the amounts of chlorophyll (Shen et al., 2006). The possible crosstalk between ABA and gun signalling could also be part of the NF response since NF inhibits carotenoid synthesis, a precursor in ABA biosynthesis (Kleine et al., 2009). An actual biological role for GUN5 as an ABA receptor is still under debate (McCourt and Creelman, 2008), but recent findings suggests that GUN5 spans the envelope membrane of the chloroplast and function as a ABA receptor (Wu et al., 2009; Shang et al., 2010).

An example from yeast could give some more hints about cytosolic partners for retrograde signalling in plants. In yeast, HEAT SHOCK PROTEINS (HSPs) build up a chaperone complex together with the transcription factor HEME Responsive Transcriptional Activator 1 (HAP1). Different members of the HSP90 and HSP70 family of heat shock proteins are involved in the complex. The tetrapyrrole heme and the HSP complex interact with HAP1 to regulate its activity (Zhang and Hach, 1999; Lan et al., 2004). The yeast complex regulates genes encoding proteins involved in respiration and protection against oxidative damage (Zhang and Hach, 1999). The complex is conserved between species and appears to have an important role in plants as well. Identified members of the plant complex include HSP90 and HSP70 (Owens-Grillo et al., 1996; Stancato et al., 1996). No plant homolog to HAP1 has been identified, however. In plants, there are seven members of the HSP90 family (Krishna and Gloor, 2001). The HSP90 family of proteins is divided into four cytosolic (HSP90.1-4), one chloroplastic (HSP90.5), one mitochondrial (HSP90.6) and one protein targeted to the endoplasmic reticulum (HSP90.7). Although homologs of the components in the yeast complex have been found in plants, the mechanisms and the biological role of the plant complex are so far unknown.

**Nuclear targets**

The regulation of PhANGs involves a complex network of different signals. Common to most promoters of PhANGs is that they have elements that respond to both plastid and light signals (Nott et al., 2006). In all cases so far studied, the cis-elements responsible for plastid- and light signals have been inseparable (Simpson et al., 1986; Vorst et al., 1993; Bolle et al., 1996; Strand et al., 2003; Brown et al., 2005; von Gromoff et al., 2006; Koussevitzky et al., 2007). As mentioned before, in Chlamydomonas reinhardtii, both Mg-ProtoIX and light are needed for inducing HSP70A expression (Kropat et al., 2000). The cis-element responsible for light/Mg-ProtoIX induction is separate from the heat induced element (von Gromoff et al., 2006). The cis-element found in C. reinhardtii:
(G/C)CGA(C/T)N (A/G)N₁₅ (T/C/A) (A/T/G) (von Gromoff et al., 2006) is clearly distinct from the elements found in higher plants.

The best characterized cis-element in higher plants responding to plastid signals is the G-box (CACGTG) or the closely related CUF1 element (CACGTA). It was demonstrated that 42 of the 70 genes affected by the NF induced accumulation of Mg-ProtoIX, showed these elements in their promoters (Strand et al., 2003). The genes identified in the experiment all encoded predicted plastid localized proteins involved in tetrapyrrole biosynthesis, light harvesting and other components of photosynthesis (Strand et al., 2003). The 5’-end of the CUF1 element (CCAC) resembles an S-box, a cis-element responsive to sugar and ABA signalling (Acevedo-Hernández et al., 2005). The CACC element is the core element required for binding to a transcription factor, ABA INSENSITIVE 4 (ABI4), found to be important in plastid- to nucleus signalling (Koussevitzky et al., 2007).

To be able to regulate nuclear gene expression, plastid signal pathways have to involve transcription factors in the nucleus. ABI4 has been suggested to be one of those. Downstream of GUN1, ABI4 is thought to repress LHCB expression predominantly during early seedling development (Koussevitzky et al., 2007). The abi4 mutant shows a gun phenotype when grown on lincomycin and over expressing ABI4 in the gun1 background suppresses the gun phenotype (Koussevitzky et al., 2007). In addition to a repressing role, abi4 and gun1 showed decreased induction of ZAT10 and ZAT12 in response to high light, indicating that ABI4 is involved in several signalling pathways (Koussevitzky et al., 2007). ABI4 was originally isolated in a screen for ABA insensitive mutants (Finkelstein et al., 2002), indicating that there is a cross talk between plastid- and ABA signalling pathways.

The cross talk between ABA and other plastid signals became more evident when GUN5 was proposed as an ABA receptor (Shen et al., 2006). The location of GUN5 inside the chloroplast made it improbable as a functional receptor but a recent finding shows that GUN5 actually is able to span the envelope membrane (Shang et al., 2010). The envelope location of GUN5 is very interesting since exogenously applied ABA was found to increase the level of Mg-ProtoIX (Shen et al., 2006). Moreover, GUN5 in itself can directly interact with transcription factors that belong to the WRKY superfamily (Shang et al., 2010). Three transcription factors were found to interact with GUN5; WRKY40, WRKY18 and WRKY60 (Shang et al., 2010). The transcription factors are later transported to the nucleus to regulate gene expression of ABA responsive genes.
Figure 7. Current model of identified transcription factors (TFs) and their role in plastid-to-nucleus signalling. Tetrapyrrole signalling, triggered by accumulating Mg-ProtoIX operates with so far unknown TFs. Different conditions (NF, PPI and LIN) triggers a GUN1 mediated pathway. On NF and LIN, GUN1 repress PhANG expression via ABI4. Impairment in the plastid import machinery triggers a signal via GUN1 and GLK1, independent of ABI4. GLK1 interacts with GBF to positively regulate PhANG expression. On lincomycin, HY5 functions in a GUN1 independent pathway to negatively regulate PhANG expression.

Another transcription factor that demonstrates a *gun* phenotype on lincomycin is mutants of LONG HYPOCYTOL 5 (HY5). HY5 plays an important role and has been extensively investigated during photomorphogenesis (Holm *et al.*, 2002). Genetic studies showed that HY5 is involved in a signalling pathway distinct from GUN1, and that this pathway includes the photoreceptor CRY1 to repress LHCB expression (Ruckle *et al.*, 2007). The *hy5* and *cry1* mutants exhibit a long hypocotyl, demonstrating their important role during photomorphogenesis. Both HY5 and CRY1 have earlier been shown to be positive regulators of PhANG expression (Jiao *et al.*, 2007; Lee *et al.*, 2007a). Ruckle and co-workers (2007) suggested that an unknown plastid signal could convert CRY1 and thereby the downstream component HY5 from positive to negative regulators of LHCB expression.

Lastly, *AtGLK1*, was recently suggested as a positive regulator of plastid protein import and PhANG expression and to function in a GUN1 dependent pathway (Kakizaki *et al.*, 2009). Although GUN1 is involved, ABI4 is suggested not to take part in this pathway. Studies with the *plastid protein import 2* (*ppi2*) mutant revealed that a non functional plastid import system generates a signal, via GUN1, that represses PhANG expression through the suppression of *AtGLK1* (Kakizaki *et al.*, 2009). *AtGLK1* interacts with G-box binding factors (GBFs) to further
regulate the gene expression (Tamai et al., 2002). Double mutants of AtGLK1 and its homolog, AtGLK2, show a weak gun phenotype when grown on both NF and lincomycin, suggesting that they are part of the plastid-to-nucleus signalling network (Waters et al., 2009). The current model (Figure 7) for plastid signal responsive transcription factors is that the negative regulators (ABI4 and HY5) block the G-box from the positive regulators (AtGLK1 and GBFs) of PhANG expression (Koussevitzky et al., 2007; Kakizaki et al., 2009).

**AIM**

The overall aim for this thesis was to obtain a better understanding of plastid-to-nucleus signalling pathways. Two main questions permeate this thesis:

1. What components interact and relay Mg-ProtoIX mediated signalling?
2. What novel proteins are involved in redox sensing?

All work presented in this thesis is from the model plant *Arabidopsis thaliana.*
RESULTS AND DISCUSSION

The scope of this thesis is extensive but I will try to break down the results from the papers into parts and build them up as a consecutive story. The importance of plastid to nucleus signals is only starting to emerge, and this discussion will try to incorporate my findings with other recent results to paint a picture of retrograde signalling as it stands today. Some parts will only be discussed briefly while others, such as tetrapyrrole and redox signalling will be discussed more extensively. This discussion will take you from the novel technique of visualization of tetrapyrroles in plant cells (Paper I) to the identification of putative cytosolic interaction partners, confirming the biological role of accumulating Mg-ProtoIX (Paper II), to an example of how Mg-ProtoIX mediated signalling regulates transcription factors and gene expression in the nucleus; a process that is dependent on the photoreceptors (Paper III). Photoreceptors will bring us to high light stress and how genes are regulated during this condition (Paper IV), and finally to the isolation of a mutant of a novel chloroplastic protein that is involved in transducing the high light signal to regulate nuclear gene expression (Paper V).

Mg-ProtoIX MEDIATED SIGNALLING
– ACCUMULATION AND TRANSPORT TO THE CYTOSOL

Mg-ProtoIX accumulates during stress conditions
The role of chlorophyll intermediates in retrograde signalling derives from overwhelming genetic and biochemical evidence supporting the regulating role of tetrapyrroles in nuclear gene expression. However, the actual mechanism of tetrapyrrole signalling remains elusive. Is it the molecule itself or is it a tetrapyrrole derived molecule that is the signal? Here, we show that Mg-ProtoIX accumulates after a number of stress conditions: norflurazon (NF) (Paper I, Fig 4; Paper II, Fig 3), methyl vinylugen (MV) (Paper II, Fig 3), cold (Paper III, Fig 2), and during photoperiodic control conditions (Paper III, Fig 4). These findings align well with earlier reports (Papenbrock et al., 1999; Strand et al., 2003; Wilson et al., 2003; Aarti et al., 2006). However, the findings by Strand et al. (2003) are contradictory to more recent findings (Mochizuki et al., 2008; Moulin et al., 2008). The later studies found no accumulation of tetrapyrroles in seedlings grown on NF. The steady state level of tetrapyrroles could therefore not be directly linked to changes in gene expression. The contradiction resulted in scepticism about Mg-ProtoIX as a signalling molecule (Kleine et al., 2009; Pfannschmidt, 2010). The results presented in this thesis once more points at Mg-ProtoIX as a signalling molecule.
To fully understand tetrapyrrole signalling, more detailed studies must be conducted. What is clear is that the conditions used must be carefully chosen together with the time frame of the experiment. The difference in observed accumulation of tetrapyrroles presented by Strand et al. and by Mochizuki, Moulin and co-workers could in part be explained by the above mentioned parameters. Firstly, photoperiodic conditions resulted in transient higher levels of tetrapyrroles compared to continuous conditions (Paper III, Fig 4, (Papenbrock et al., 1999). Secondly, NF is an artificial condition that gives pleiotropic effects that eventually kills the plant (Sandmann and Böger, 1997). Possibly Mochizuki, Moulin and co-workers could have failed to detect an earlier transient peak of Mg-ProtoIX accumulation because of the nature of inhibition and concentration of NF together with the measurements of tetrapyrroles after 4-6 days of treatment (Mochizuki et al., 2008; Moulin et al., 2008).

In both Arabidopsis and cucumber, Mg-ProtoIX and Mg-ProtoIX-ME have been found to accumulate following exposure to oxidative stress (Aarti et al., 2006; Stenbaek et al., 2008). The reason for the accumulation is believed to be an inactivation of the aerobic cyclase, the enzyme that converts Mg-ProtoIX-ME to protochlorophyllide. To confirm an inactivation of enzymes downstream of Mg-ProtoIX after oxidative stress, we used 7 days old photoperiodic grown seedlings on media without NF. The seedlings were then treated with NF and exogenously applied ALA to examine the effect in developed chloroplasts (Paper II, Fig 3). Indeed, Mg-ProtoIX accumulated in response to NF. Similar results were observed following exposure to MV (Paper II, Fig 3). After MV treatment, Mg-ProtoIX and Mg-ProtoIX-ME accumulated, but declined after 4 h of treatment towards control levels. Paper II clearly show that accumulated Mg-ProtoIX following exposure to artificial conditions resulting in oxidative stress is transient. More importantly, results in Paper III show that under conditions experienced by plants in their natural environment (photoperiodicity and cold); Mg-ProtoIX demonstrates a similar transient accumulation (Paper III, Fig 2, 4).

An interesting effect in the Mg-ProtoIX under accumulating mutants, gun4 and gun5, is that they showed a different response to cold treatment (Paper III, Fig 1). While the gun4 mutant recovered from the pale phenotype, the gun5 mutant exhibited an even more severe, paler, phenotype after cold treatment compared to their warm grown control and wild type. Long term exposure to cold resulted in dysfunctional chloroplasts in the gun5 mutant. The gun4 mutant, on the other hand, adapted to cold and exhibited wild type chloroplasts following cold exposure (Paper III, Fig 1). The main reason for this difference is the mutants’ ability to synthesise tetrapyrroles. By measuring the chlorophyll intermediates Mg-ProtoIX
and Mg-ProtoIX-ME, we showed that the gun4 mutant were able to synthesize
tetrapyrroles close to wild type levels after cold treatment whereas the gun5 mutant
had a constant low level of tetrapyrroles (Paper III, Fig 2).

These results gave indications about GUN4’s mode-of-action. GUN4 was
suggested to be involved in the release of Mg-ProtoIX from the chelatase enzyme
(Peter and Grimm, 2009), but also implied in substrate channelling and retrograde
signalling (Larkin et al., 2003). Arabidopsis GUN4, and the Synechosystis homolog,
showed higher affinity for Mg-ProtoIX, compared to ProtoIX and Mg-ProtoIX-
ME (Davison et al., 2005; Peter and Grimm, 2009). The direct interaction of
CHLH and the next downstream enzyme in tetrapyrrole biosynthesis, CHLM
(Alawady et al., 2005), suggested that GUN4 is not directly involved in metabolic
substrate channelling between CHLH and CHLM, but rather binds excessive
amounts of Mg-ProtoIX (Peter and Grimm, 2009). Our results further supported
this model. During cold temperatures, the rate of chlorophyll synthesis slows down
and thus less excessive amounts of Mg-ProtoIX are produced, explaining the
recovery of the gun4 mutant in cold. Mutations in CHLH (gun5), on the other
hand, will result in low levels of Mg-ProtoIX and Mg-ProtoIX-ME after cold
temperatures since the impaired production of Mg-ProtoIX is still present.

In summary, we show that Mg-ProtoIX accumulates when plants are exposed to
oxidative stress. The accumulation of Mg-ProtoIX is partly due to the redox
regulated inactivation of the cyclase reaction (Stenbaek et al., 2008). The observed
decline of Mg-ProtoIX levels after accumulation indicates that the accumulation is
transient. The transient accumulation of tetrapyrroles in different environmental
fluctuations is unquestionable and supports a signalling role, but how is the signal
transduced?

**Cytosolic activity of Mg-ProtoIX**

There are a number of experiments that suggest a cytosolic activity for Mg-
ProtoIX. The first line of evidence comes from feeding experiments. Feeding of
Mg-ProtoIX directly to: plant roots (Paper III, Fig 3), protoplasts (Strand et al.,
2003) and to C. reinhardtii (Kropat et al., 1997), was enough to regulate nuclear
gene expression. The important finding that the downstream intermediate, Mg-
ProtoIX-ME, did not accumulate strongly suggested that exogenously applied Mg-
ProtoIX stayed in the cytosol (Paper III, Fig 3; (Kropat et al., 1997; Strand et al.,
2003). If Mg-ProtoIX entered the chloroplast, it would be converted to Mg-
ProtoIX-ME. Exogenously applied heme, protoporphyrinIX or porphobilinogen
had no effect on nuclear gene expression in protoplasts (Strand et al., 2003) or in
C. reinhardtii (Kropat et al., 1997). In a novel experiment to visualize tetrapyrroles
in vivo, we took advantage of the photoreactive properties of tetrapyrroles in combination with confocal laser scanning microscopy (Paper I). NF grown seedlings were fed with ALA, the precursor of tetrapyrroles, to boost the biosynthesis of chlorophyll intermediates. Mg-ProtoIX and ProtoIX could not only be visualized in the plastids but also in the cytosol (Paper I, Fig 5), indicating that, during stress conditions, Mg-ProtoIX can be exported from the plastid to the cytosol. The use of NF gives, as mentioned before, a variety of effects on plant cells. The effects of NF could potentially cause an effect on the envelope membrane of the chloroplast, but TEM images demonstrated a functional barrier (Paper I, Fig 5, 6). The visualization and feeding experiments all demonstrates that a cytosolic localization of Mg-ProtoIX is possible.

Transport of Mg-ProtoIX to the cytosol

The cytosolic action of Mg-ProtoIX requires transport from the chloroplast to the cytosol. How is this accomplished? Transmembrane movement of porphyrins like heme and Mg-ProtoIX is problematic because of the unfavourable energetics of moving the carboxylate side of porphyrins over the lipid double layer (Krishnamurthy et al., 2007). The unsuitable properties make a simple passive diffusion of porphyrins improbable. Nevertheless, numerous porphyrins have been found outside of the chloroplast. The dual synthesis of heme in chloroplast and mitochondria requires that an intermediate is transported over the chloroplast envelope (Tanaka and Tanaka, 2007). How this is done and what intermediate(s) that is transported is unknown.

In yeast, ATP-BINDING CASSETTE B6 (ABCB6) mediates transport of heme and porphyrins from the cytosol into mitochondria (Krishnamurthy et al., 2006). No plant homolog to ABCB6 has been found so far. The energy dependency of ABCB6 suggests that there should be an active transport of tetrapyrroles in the other direction as well (mitochondria to cytosol), but no transporter have been found to date. A chloroplastic ABC transporter was suggested to be involved in transporting and distributing of ProtoIX (Moeller et al., 2001), but the actual role of the transporter in plastid-to-nucleus signalling remains to be determined. There is a number of reports demonstrating that other porphyrins can be found in the cytosol during different conditions (Paper I, Fig 5; (Jacobs and Jacobs, 1993; Nott et al., 2006), but no clear evidence of an active transport. In fact, recent findings challenge the need of an active transporter of tetrapyrroles in plants.

To elucidate how tetrapyrroles are transported from the chloroplast it is important to know where the enzymes in the tetrapyrrole biosynthesis are localized. The first steps of tetrapyrrole biosynthesis is situated in the stroma (Eckhardt et al., 2004),
but later steps—from Mg-Chl to NADPH-protochlorophyllide oxireductase (POR)- are found in both the thylakoid and envelope membrane (Eckhardt et al., 2004; Shang et al., 2010). Recent findings even suggest that CHLH/GUN5 can traverse the envelope membrane, including a cytosolic localization of the C- and N-terminus, where CHLH functions as an ABA receptor (Shang et al., 2010). The envelope localization of CHLH indicates that Mg-ProtoIX could be transported over the membrane through the chelatase subunit, eliminating the necessity of a classical transport protein. Furthermore, higher levels of tetrpyrroles, as seen in stress conditions, have been found to promote interaction between CHLH and chloroplast membranes (Adhikari et al., 2009).

Altogether, these results suggest that during conditions with increased tetrpyrrole levels, CHLH associates more strongly to the envelope membrane, a process that requires GUN4 (Adhikari et al., 2009), to transport Mg-ProtoIX over the envelope membrane. Cytosolic ABA could also promote Mg-ProtoIX production as proposed in an earlier study (Shen et al., 2006). The localization of CHLM, the enzyme that adds the methyl group on Mg-ProtoIX to produce Mg-ProtoIX-ME has been shown to be both in the thylakoid and envelope membrane (Block et al., 2002). The exact localization (inner or outer envelope membrane) is still unknown, but it has been found to bind physically to CHLH (Alawady et al., 2005). Interestingly, CRD1 that is part of the cyclase system to convert Mg-ProtoIX-ME to protochlorophyllide localizes to the thylakoid and inner envelope membrane (Tottey et al., 2003), making a cytosolic transport of downstream intermediates of Mg-ProtoIX-ME improbable.

Taken together, we show that Mg-ProtoIX and Mg-ProtoIX-ME accumulate after various conditions that affect photosynthetic activity, especially conditions that generate ROS and result in oxidative stress. The accumulation is transient and results in accumulation of tetrpyrroles both in the plastid and the cytosol. The transport over the plastid membrane might not involve an active transporter but rather the CHLH enzyme itself that spans the envelope membrane. The results presented here and other published results strongly suggest that Mg-ProtoIX is a plastid signal and that it is able to be transported over the envelope membrane. The question now is what the signal actually is affecting.
**Mg-ProtoIX MEDITATED SIGNALLING**

– CYTOSOLIC INTERACTION PARTNERS TO Mg-ProtoIX

**Isolation of putative Mg-ProtoIX interacting partners**

In order to identify cytosolic components in Mg-ProtoIX mediated signalling, we used a novel column-based technique (Paper II). Mg-ProtoIX was covalently linked to an Affi-gel matrix, and proteins from *Arabidopsis* were allowed to pass through. The proteins trapped within the column were eluted and identified with mass spectrometry. To test the specificity of the column, a number of controls were run. Firstly, antibodies from a known Mg-ProtoIX binding protein, CHLH, and a protein that was not supposed to bind, cytosolic fructose-1,6-bisphosphate, were tested in sample and eluate. Secondly, protein samples were incubated with Mg-ProtoIX before being applied on the column. Thirdly, different concentrations of NaCl were used as eluent to confirm a strong interaction between protein and column. All tests showed that the column specificity was high (summarized in Paper II, Fig S1).

We identified proteins from various functional categories (Paper II, Fig 1, Table S1). Expression profiles of the identified proteins suggested that a majority of them are regulated by stress (Paper II, Fig 1). Cold, salt, ABA and NF treatment all showed a higher representation of the genes encoding the proteins found in our study, compared to a random set of genes. The list of proteins could prove to be an important tool to elucidate Mg-ProtoIX mediated signalling.

Using bioinformatic tools, we investigated if there was any common amino acid motif responsible for tetrapyrrole binding. In addition, we used known Mg-ProtoIX binding proteins to find a common motif. We could not find any common motif among our proteins or among the known binding proteins (Paper II, Table S2, S3); suggesting that binding of Mg-ProtoIX is not due to an amino acid motif but rather a protein fold. This is in accordance to previous studies, where porphyrins were found to bind GUN4 in a “cupped hand” fold (Verdecia et al., 2005). Due to the phototoxicity of Mg-ProtoIX and the absence of transcription factors in our study, a direct nuclear effect of Mg-ProtoIX is improbable. The following parts will discuss a few examples of how the Mg-ProtoIX signal could be relayed from the cytosol to regulate nuclear gene expression.
**HSP90 binds to Mg-ProtoIX and suppresses the gun phenotype**

An interesting group of proteins found in the list of putative Mg-ProtoIX interacting proteins is the HEAT SHOCK PROTEIN90 (HSP90) family. HSP90 proteins are important in many developmental processes in the plant cell. For example, hypocotyl and root elongation as well as greening upon dark-to-light transfer and gravity responses are all dependent on HSP90 chaperones (Sangster and Queitsch, 2005). HSP90 proteins function in a molecular chaperone complex and are responsible of assisting other polypeptides to fold correctly and prevent unproductive interactions (Mayer and Bukau, 1999). The HSP90 proteins in *Arabidopsis* are divided into four cytosolic (HSP81.1-4), one chloroplastic (HSP88.1), one mitochondrial (HSP89.1) and one protein targeted to the endoplasmic reticulum (HSP90.7) (Krishna and Gloor, 2001). We identified 5 of the 7 HSP90 members in our proteomic study; HSP81.1, HSP81.2, HSP81.3, HSP88.1 and HSP90.7 (Paper II, Table S1). The direct interaction between Mg-ProtoIX and one of the cytosolic members, in addition to the plastid localized member, could be confirmed *in vitro*, and implies that HSP90 has a role in Mg-ProtoIX mediated signalling (Paper II, Fig 2).

To further investigate the biological role of the interaction between HSP90 and Mg-ProtoIX, we constructed RNA interference (RNAi) lines that down-regulate all four cytosolic members (HSP81.1-4) in both wild type and the *gun5* background. The repression in the selected lines was 40-60% of wild type expression (Paper II, Fig 5). The *gun* phenotype seen in the *gun5* mutant grown on NF was suppressed in the *gun5*-HSP81 RNAi lines, whereas wild type HSP81 RNAi lines showed wild type repression (Paper II, Fig 6), suggesting that HSP81 is part of the Mg-ProtoIX mediated signalling pathway regulating *LHCB2.4* expression. The biological role of the interaction between Mg-ProtoIX and HSP81 could be seen in the *gun5*-HSP81 RNAi lines after methyl viologen (MV) treatment as well as on NF (Paper II, Fig 6). The *gun5*-HSP81 RNAi lines showed a suppression of the uncoupled *LHCB* response after treatment with MV compared to the single mutant. Down regulating the different *HSP81* genes could possibly have an indirect effect on tetrapyrrole levels, thereby affecting *LHCB2.4* expression. However, the levels of Mg-ProtoIX in the *gun5*-HSP81 RNAi lines were unaffected compared to the *gun5* mutant, suggesting that HSP81 functions downstream of accumulating Mg-ProtoIX (Paper II, Table 1).

There are two plausible scenarios for how Mg-ProtoIX together with HSP81 regulates *LHCB* expression during stress conditions. The first scenario involves a complex similar to the regulatory complex that has been extensively studied in yeast. In yeast, HSP90 is part of a protein complex with HSP70 and the
transcriptional activator HAP1. HAP1 binds the tetrapyrrole heme to activate expression of genes encoding proteins involved in respiration and protection against oxidative damage (Zhang and Hach, 1999). Could Mg-ProtoIX have a similar role in plants to control expression of photosynthetic genes? The results in Paper II suggest that the HSP90 complex is inactivated by the interaction with Mg-ProtoIX and therefore LHCB2.4 transcription is impaired. The results further suggest that the signal is only active during stress conditions, since both gun5 and the HSP81 RNAi lines show wild type expression in control conditions. However, we could not find any transcriptional activator in our proteomic study and no homolog of HAP1 has so far been identified in the Arabidopsis genome. The direct interaction between Mg-ProtoIX and HSP81.2 suggests that the plant complex is different from that in yeast. While heme interacts with the transcriptional activator in yeast, tetrapyrroles interact directly with the heat shock proteins in plants (Paper II, Fig 2).

The second scenario that could explain the LHCB regulation involves the folding and guiding function of the HSP90 complex. HSP90 members have been found to deliver precursor proteins to the chloroplastic import machinery (Qbadou et al., 2006). The import machinery itself is able to send signals to regulate nuclear gene expression shown in mutants with impaired TOC/TIC complexes (Kakizaki et al., 2009). If the import machinery is impaired that would result in an accumulation of photosynthetic precursors in the cytosol causing a feed-back regulation of gene expression. This type of feed-back regulation is important in various processes in both animals and plants (Pfannschmidt, 2010). Mg-ProtoIX bound to HSP90 might have a role in the import of polypeptides to the chloroplast. If Mg-ProtoIX prevents photosynthetic precursors to be imported to the chloroplast or their stability, this could possibly trigger a signal to repress the expression of photosynthesis related genes.

Are there any results that support this second scenario? The idea that Mg-ProtoIX is involved in chloroplast protein import or protein stability during import is plausible, since other tetrapyrroles have been found to be highly involved in this process. Protochlorophyllide, the downstream product of Mg-ProtoIX-ME and atomic oxygen have been shown to be required for import of the protein PORA in Arabidopsis cotyledons (Kim and Apel, 2004). In addition, chlorophyllide a has been suggested to be important in translocating LHC proteins to the chloroplast (Kuttkat et al., 1997; Hoober and Eggink, 2001; Reinbothe et al., 2006). Previous studies also implied that Mg-ProtoIX could be important in the translocation and/or the stability of plastid proteins involved in tetrapyrrole biosynthesis. Albino chlm mutants, that over-accumulate Mg-ProtoIX, did not show a reduction of
POR and CHL27 proteins, even though the mutant was unable to synthesise protochlorophyllide (Pontier et al., 2007). Also, knock-out mutants of GUN4, which had low levels of Mg-ProtoIX, demonstrated wild type level of CHLM transcription but a decreased level of CHLM protein (Peter and Grimm, 2009). Both results support the notion that Mg-ProtoIX could be involved in the import or stability of these proteins. Intriguingly, our proteomic study (Paper II) identified a number of other heat shock proteins involved in chloroplast protein import in addition to the HSP90 proteins (Paper II, Table S1). Four members of the HSP70 family, including a stromal member that has been found to be crucial for plastid protein import (Su and Li, 2010); HSP93-V, a stromal protein involved in translocation (Kovacheva et al., 2005); two Chaperonin 60 (CPN60) proteins, involved in folding newly imported polypeptides but also part of the translocation process (Jarvis, 2008).

**Figure 8.** Working model for the repressing role of Mg-ProtoIX interacting with HSP90. After NF or MV treatment, Mg-ProtoIX accumulates in the cytosol and interacts with cytosolic HSP90. Two different pathways are plausible. Either a direct pathway (1) that involves a transcriptional complex similar to that found in yeast (Zhang and Hach, 1999); (2) a more indirect pathway involves the TIC/TOC machinery, or (3) interaction between Mg-ProtoIX and HSP90 impairs the plastid protein machinery that, in turn, sends a signal to the nucleus.
The HSP90-Mg-ProtoIX interaction can be summarized in a working model (Figure 8). After treatment with NF or MV, Mg-ProtoIX accumulates and exits the chloroplast via the envelope spanning CHLH protein. Cytosolic Mg-ProtoIX directly interacts with HSP90 and inactivates the complex. The first possible mode-of-action (Figure 8, arrow 1) is a direct effect of LHCB expression that involves in a complex similar to that described in yeast. The complex possibly incorporates unknown transcription regulators in the nucleus to control gene expression. An indirect pathway involves the chloroplast import machinery and/or the stability of proteins that will be imported (Figure 8, arrow 2). The interaction between Mg-ProtoIX and HSP90 prevents an efficient import of photosynthesis proteins or adjust their stability, thus triggering a signal that represses LHCB expression (Figure 8, arrow 3). Future experiments will hopefully elucidate the correct pathway.

**Mg-ProtoIX involvement in the activated methyl cycle**

In the table of putative interaction partners to Mg-ProtoIX, one group of proteins stands out and is of special interest. All these proteins are involved in Methionine (Met) metabolism and are important since they indirectly determines the rate of tetrapyrrole synthesis. S-Adenosyl-Methionine (SAM) is synthesised from methionine and is, in turn, used to donate a methyl group to Mg-ProtoIX in the formation of Mg-ProtoIX-ME (Bouvier et al., 2006). Met and SAM are involved in a wide range of processes in plants. For example, as an amino acid, Met is important in protein synthesis; SAM has an important regulatory function as a methyl donor in many pathways, including chlorophyll synthesis (Hesse et al., 2004). The biosynthesis of Met is predominantly localized in the plastid, but the conversion from Met to SAM is localized in the cytosol (Ravanel et al., 2004). The key regulatory point in the biosynthesis is the Cystathionine \( \gamma \)-Synthase (CGS) protein (Hesse et al., 2004). CGS is a plastid localized enzyme that catalyses the formation of cystathionine, an intermediate in the Met biosynthesis (Ravanel et al., 2004).

Our proteomic approach identified several proteins involved in SAM biosynthesis as putative Mg-ProtoIX interacting partners (**Paper II, Table S1**). The conversion of Homocysteine (Hcy) to Met is catalysed by Methionine Synthase (MS), and there are three family members in *Arabidopsis*; two cytosolic and one chloroplastic (Ravanel et al., 2004). Both cytosolic enzymes were identified in our study. The consecutive step, where SAM is formed from Met and an adenosyl group, is catalysed by Metionine Adenosyl Transferase (MAT). Four isoforms exist in *Arabidopsis*, all localized in the cytosol (Hesse et al., 2004). Three of the isoforms were found in our study (MAT1, MAT 2 and MAT4). In addition, we identified
one isoenzyme of S-Adenosyl Homocysteine Hydrolase (SAHH), responsible for converting downstream products of SAM back to Hcy (Hesse et al., 2004). In summary, we could identify a majority of the enzymes in a process called “activated methyl cycle” (AMC) (Ravanel et al., 2004) as potential interaction partners to Mg-ProtoIX. AMC consists of the above mentioned enzymes and a SAM transporter, SAMT1, localized in the envelope membrane (Bouvier et al., 2006). AMC and SAMT1 forms a cycle since the waste product from SAM methylation reactions, S-Adenosyl Homocysteine (SAHC), is transported back to the cytosol through SAMT1 (Bouvier et al., 2006), and converted back to Hcy by SAHH. However, the in vivo interaction and the biological role for Mg-ProtoIX and the enzymes in the activated methyl cycle remain unknown.

Mg-ProtoIX is known to be a negative regulator of PhANGs (Paper I) and other nuclear genes encoding plastid proteins, for example COR15a (Paper III). To further investigate the involvement of Mg-ProtoIX in SAM biosynthesis, we checked the mRNA level of CGS after stress conditions where Mg-ProtoIX accumulates (Figure 9). Using the same feeding experiment as presented in Paper III, where roots from Arabidopsis were incubated with Mg-ProtoIX solution, we could see that a higher level of cytosolic Mg-ProtoIX repressed CGS expression (Figure 9A-B). CGS regulation was also impaired in gun5 plants grown under photoperiodic conditions after cold treatment (Figure 9C). In wild type, CGS expression was repressed after 96 h of cold treatment. The gun5 mutant showed a lower repression compared to wild type, and this correlated with the accumulation of Mg-ProtoIX (Paper III, Fig 2). These results indicate that Mg-ProtoIX mediated signalling is involved in regulating CGS expression during stress conditions.

**Figure 9.** Mg-ProtoIX is potentially involved in regulating CGS expression during stress conditions. (A) Uptake of Mg-ProtoIX in leaves through roots of plants incubated in Mg-ProtoIX solution compared to plants incubated with MS. (B) CGS expression in corresponding plants as in (A). (C) CGS mRNA levels in wild type and gun5 plants after 96 h of cold treatment compared to warm grown plants. All experiments are done on 3 week old plants grown in cont. light (A-B) or photoperiodic conditions (C). Expression data are presented as mean ± 95% CI of at least three biological replicates. Significant differences compared to Col were determined using One-way ANOVA (GraphPad).
A working model for the potential role of Mg-ProtoIX mediated regulation of Met metabolism is presented in figure 10. The results presented here suggest that Mg-ProtoIX regulate the Met metabolism via two mechanisms. (1) Expression of CGS is regulated by accumulating Mg-ProtoIX. Intriguingly, the CGS promoter consists of a CUF-1 element, found to be important in Mg-ProtoIX mediated signalling (Strand et al., 2003). (2) Mg-ProtoIX regulates proteins in the activated methyl cycle. The interaction with the indicated enzymes in figure 10 might have an important role in acclimating plants to stress conditions. A possible fate for enzymes that bind Mg-ProtoIX is to be targeted for proteolysis. Previous studies suggested that tetrapyrrole binding is important for protein stability for numerous proteins involved in tetrapyrrole synthesis (Pontier et al., 2007; Tanaka and Tanaka, 2007; Peter and Grimm, 2009) and photosynthesis (Eichacker et al., 1996). Another option is that tetrapyrrole binding inhibits enzymatic activity, as...
shown for GlutRNA reductase (Vothknecht et al., 1998) and glutathione S-transferases (Lederer and Böger, 2003). The inactivation of the AMC would most probably increase the level of Met in the cell, since conversion to SAM is inactivated. Met transported from the chloroplast would accumulate, something that is important for acclimation of plants to stress conditions (Joshi et al., 2010). The AMC could potentially be an important feed-back regulation point following exposure to stress conditions that result in accumulation of Mg-ProtoIX and Mg-ProtoIX-ME. Accumulating tetrapyrroles would inactivate the biosynthesis of SAM, resulting in lower synthesis of Mg-ProtoIX-ME. More detailed experiments will hopefully elucidate the impact Mg-ProtoIX mediated signalling has on Met metabolism and its consequences for plant stress adaptation and development.

Transport and degradation of Mg-ProtoIX
The overwhelming published results, in addition to the results in this thesis, show that Mg-ProtoIX triggers a signal to control various processes coupled to the chloroplast during different growth conditions. However, the major problem with Mg-ProtoIX as a signalling molecule is its photoreactivity. Accumulated Mg-ProtoIX could pose a threat to plant survival. Our finding that the accumulation of Mg-ProtoIX is transient implies that an efficient degradation system exists. What also could be important is a cytosolic carrier system that protects tetrapyrroles from oxidation.

The carrier system consists, most likely, of a group of proteins called Glutathione S-Transferases (GSTs). GSTs are an abundant and diverse group of proteins involved in normal cellular metabolism as well as detoxification and transport of harmful compounds (Dixon et al., 2010). They are present in most cellular compartments, including the plastid and nucleus (Dixon et al., 2009). GSTs have long been associated with responses to stress conditions. For example, several of the plant GSTs are up-regulated during oxidative stress (Dixon et al., 2010). Previous studies reported that different isoforms of GSTs in Zea mays can interact with tetrapyrroles, including Mg-ProtoIX (Lederer and Böger, 2003; Dixon et al., 2008). The tested GST isoforms in Zea mays bound tetrapyrroles non-covalently and apparently did not degrade their bound ligand (Lederer and Böger, 2003). We identified three different GST isoforms in our proteomic study (Paper II, Table S1) that could potentially be involved in Mg-ProtoIX protection and transport in the cytosol.

The degradation of toxic tetrapyrroles might involve Peroxidases (PRXs). PRXs are a superfamily of proteins and their main function is to catalyse the reduction of H₂O₂ (Cosio and Dunand, 2009). PRXs are also known to bind tetrapyrroles and
detoxifying them into non-toxic compounds (Dayan et al., 1998; Dayan et al., 1999). Three peroxidases that belong to the class III family were identified in our proteomic study (Paper II, Table S1). Class III PRXs incorporates the tetrapyrrole heme to be active and PRX expression is often up-regulated in response to oxidative stress (Cosio and Dunand, 2009). We also identified Ascorbate Peroxidase 1 (APX1) in our study. APX1 have a central role in protecting plants during oxidative stress, through its role in H$_2$O$_2$ scavenging (Davletova et al., 2005a).

The biological role of the Mg-ProtoIX-GST and Mg-ProtoIX-PRX interactions remains to be solved. However, the identification of members from these protein families as putative Mg-ProtoIX interactors suggests that they are important in Mg-ProtoIX signalling and the involvement of APX1 in particular could hint of a cross talk between Mg-ProtoIX mediated signalling and ROS signalling. Our identification of putative interaction partners to Mg-ProtoIX could prove an important tool in how this signal is mediated and transduced in the cytosol. However, there is still a great challenge for the future to elucidate the biological role for these potential interactors to Mg-ProtoIX. On one hand, the identification and role of cytosolic components in plastid-to-nucleus signalling is still a very open question. On the other hand, nuclear components and the nuclear genes affected by plastid signals are something that is understood better. The results presented in this thesis will further shed light on the targets for plastid-to-nucleus signalling.

**Mg-ProtoIX MEDIATED SIGNALLING – NUCLEAR GENE REGULATION**

**Expression of photosynthetic associated nuclear genes**

The traditional genes investigated in plastid-to-nucleus signalling are photosynthetic associated nuclear genes (PhANGs). $LHCB$ and $RBCS$ respond to both light and plastid signals (Nott et al., 2006) and have been extensively investigated. Our results further strengthen the proposed role of Mg-ProtoIX in regulating nuclear gene expression. We used the Mg-ProtoIX under- and over-accumulating gun5 and crd, respectively, and showed that crd did not exhibit a genome uncoupled lack of repression of $LHCB1.1$ and $RBCS$ on norflurazon (NF) (Paper I, Fig 7). The gun5 mutant showed a constant low level of Mg-ProtoIX and Mg-ProtoIX-ME under photoperiodic conditions (Paper III, Fig 4), cold exposure (Paper III, Fig 2), and MV treatment (Paper II, Table 1).

The crd mutant displayed a massive accumulation of Mg-ProtoIX-ME and slightly higher levels of Mg-ProtoIX compared to wild type under photoperiodic
conditions (Paper III, Fig 4). The non-gun phenotype of crd is consistent with findings in barley (Hordeum vulgare) (Gadjieva et al., 2005), and further strengthens the hypothesis that Mg-ProtoIX and/or Mg-ProtoIX-ME is responsible for triggering a signal. In addition to have an impaired PhANG expression on NF, the gun5 mutant exhibited a mis-regulation of other nuclear encoded genes. All six sigma factors, required for plastid gene expression by PEP, were not repressed after NF treatment as they were in wild type and the crd mutant (Paper I, Fig 10). The mis-regulation of the sigma factors in the gun5 mutant resulted on one hand in mis-regulation of PEP transcribed plastid genes (Paper I, Fig 8). On the other hand, RPOPT, encoding the NEP enzyme, displayed wild type like expression (Paper I, Fig 10). NEP transcribed plastid genes consequently showed wild type expression in the gun5 mutant (Paper I, Fig 9). Taken together, these results establish that the Mg-ProtoIX triggered signal is responsible for regulating nuclear photosynthesis related genes, in addition to a role of regulating the plastid photosynthesis gene expression through the sigma factors.

Regulation of COR15a

Many nuclear photosynthesis genes have light regulated elements in their promoters, including genes that are not involved in photosynthesis. Non-photosynthetic genes with light responsive elements in their promoter include the Cold Responsive 15a (COR15a) gene. COR15a has been extensively investigated for its involvement in cold stress. COR15a encodes a stromal protein with cryoprotective properties (Nakayama et al., 2007), and COR15a expression responded quickly to cold temperatures (Paper III, Fig 2). In addition to the cold regulation, COR15a was regulated by light intensity (Paper IV, Table S1). We showed here that the expression of COR15a strongly correlated with the levels of Mg-ProtoIX and Mg-ProtoIX-ME in different conditions: cold exposure (Paper III, Fig 2), exogenously applied Mg-ProtoIX (Paper III, Fig 3) and photoperiodicity (Paper III, Fig 4).

The regulation of COR15a, performed by the Mg-ProtoIX triggered signal, seems to only have a repressing role. Induction of COR15a after cold exposure was similar in the under accumulating gun5 mutant compared to wild type and the gun4 mutant (Paper III, Fig 2), suggesting that the activators of COR expression, the C/DRE Binding Factors (CBFs) (Guo et al., 2002), functioned normally in the gun4 and gun5 mutants. However, the repression of COR15a expression that occurs after long-term cold exposure was impaired in gun5 (Paper III, Fig 2). Similar results could be seen during photoperiodic conditions where gun5 superinduce COR15a expression during the day (Paper III, Fig 4). The gun4 mutant had impaired COR15a expression only during photoperiodic conditions.
and not after cold exposure, correlating strongly with the recovered levels of tetrapyrroles in the gun4 mutant (Paper III, Fig 2, 4).

The expression patterns in the two gun mutants also reflected in their visible phenotype after cold exposure, where the gun4 mutant recovered from its warm grown pale phenotype whereas the gun5 mutant had a more severe phenotype (Paper III, Fig 1). The crd mutant that over-accumulated Mg-ProtoIX and Mg-ProtoIX-ME exhibited a strong repression of COR15a during photoperiodic light conditions, again showing a tight correlation between COR15a expression and tetrapyrrole levels (Paper III, Fig 4). The regulatory role of Mg-ProtoIX might have an additional role in COR15a regulation. In our study to isolate cytosolic interactors of Mg-ProtoIX (Paper II), discussed in greater detail above, we identified Low expression of O3motically responsive genes 1 (LOS1) (Paper II, Table S1).

LOS1 encodes a translation elongation factor 2 like protein, important for new protein synthesis, especially after cold exposure (Guo et al., 2002). LOS1 was reported to be important in the translation of the CBF proteins and might also have a role in translating COR15a (Guo et al., 2002). The interaction between LOS1 and Mg-ProtoIX could be confirmed in vitro (Paper II, Fig 2). To determine the biological role for the interaction between LOS1 and Mg-ProtoIX requires a more detailed analysis.

In conclusion, our results strongly suggest that accumulation of Mg-ProtoIX and/or its methylester is responsible for regulation of COR15a in addition to PhANGs. Furthermore, our results show that accumulating Mg-ProtoIX is a negative regulator of COR15a expression. To regulate nuclear gene expression, Mg-ProtoIX mediated signalling needs to incorporate transcription factors.

Mg-ProtoIX regulates COR15a via HY5

To further elucidate the Mg-ProtoIX mediated regulation of COR15a, we investigated transcription factors known to be influenced by plastid signals. Most transcription factors involved in retrograde signalling are part of the GUN1 signalling pathway. This includes ABI4 (Koussevitzky et al., 2007) and AtGLK1 (Kakizaki et al., 2009). However, gun1 mutants did not show impaired COR15a expression (Paper III, Fig 5), indicating that the plastid derived signal regulating COR15a expression is independent of GUN1. A transcription factor that has been shown to function in a GUN1 independent pathway is HY5 (Ruckle et al., 2007). HY5 has been shown to bind to elements in promoters called G-boxes (Chattopadhyay et al., 1998), and was also found to bind the to COR15a promoter.
that includes at least two G-boxes (Paper III, Fig 5). G-boxes are known to be the binding site for transcription factors involved in light and plastid signalling (Strand et al., 2003; Lee et al., 2007a). Expression in the hy5 mutant implied that HY5 is a negative regulator of COR15a in photoperiodic conditions (Paper III, Fig 5). The expression of COR15a in the hy5 mutant at the end of the day was higher than wild type, similar to the expression in gun5 and gun4.

To investigate if HY5 is part of the Mg-ProtoIX mediated signalling pathway, we created double mutants of gun4 and hy5 as well as crd and hy5. Expression analysis of the double mutants revealed that HY5 and Mg-ProtoIX is part of the same pathway (Paper III, Fig 5). It also revealed that the pathway is more complex with more players involved. The double mutant gun4hy5 showed an additive effect compared to the single mutants, while crdhy5 showed expression similar to that of the hy5 mutant. Both the gun4 mutant (Larkin et al., 2003) and the hy5 mutant (Kleine et al., 2007) used in this experiment are leaky mutations, which could partly explain the additive effect seen in the double mutant. The results obtained implied that HY5 works downstream of Mg-ProtoIX and that more unknown factors regulate COR15a expression. A putative component could be that the HSP90 complex discussed above and in Paper II is involved in the regulation of COR15a expression.

In yeast, HSP90 functions in a complex that regulates gene expression (Lan et al., 2004). HSP90 binds the transcriptional regulator HAP1 and protects it from degradation, thereby activating transcription (Lan et al., 2004). The tetrapyrrole heme promotes the interaction between HSP90 and HAP1. In plants, the chloroplastic member of the HSP90 family has been found to bind Mg-ProtoIX (Paper II, Fig 2) and to be in separate yet interacting pathways with HY5 to control photomorphogenesis (Cao et al., 2000). In addition to the interaction between Mg-ProtoIX and the plastid HSP90, we showed that one of the cytosolic members of the HSP90 family bound to Mg-ProtoIX as well, suggesting that cytosolic HSP90 could be involved in the Mg-ProtoIX-HY5 pathway.

The additive effect observed in the gun4hy5 double mutants suggested that not only HY5 responds to accumulation of Mg-ProtoIX. The Golden 2-Like 1 and 2 (GLK1 and GLK 2) proteins have been found to be part of the COR15a regulation network and been suggested to function in a HY5 dependent pathway (Waters et al., 2009). Of the two GLK proteins, GLK1 has been shown to be part of retrograde signalling (Kakizaki et al., 2009). Similar to HY5, the GLK proteins are involved in light signalling and utilize the same promoter elements (Waters et al., 2009).
In summary, our results show that Mg-ProtoIX mediated signalling uses the transcription factor HY5 to repress the expression of \textit{COR15a}. However, our analysis showed that the regulation of \textit{COR15a} expression requires more unknown components. Previous studies together with results presented earlier in this thesis suggest that HSP90 and the GLK proteins could be involved in Mg-ProtoIX mediated regulation of \textit{COR15a} expression. The finding that HY5 is involved in \textit{COR15a} regulation further suggests that HY5 gets information from the plastid as well as the photoreceptors to fine-tune the expression of \textit{COR15a}.

**Photoreceptors**

Photoreceptors form a group of proteins that were not found in the biochemical approach in Paper 2. Nevertheless, photoreceptors have been implied in plastid-to-nucleus signalling in earlier studies (Lopez-Juez \textit{et al}., 1998; Nott \textit{et al}., 2006; Ruckle \textit{et al}., 2007). The involvement of HY5 in \textit{COR15a} regulation further suggested that photoreceptors are parts of the puzzle, since HY5 has been shown to be regulated by both cryptochromes and phytochromes (Jiao \textit{et al}., 2007). CRY1 was especially interesting to us because of its ability to convert HY5 from a positive to a negative regulator of gene expression (Ruckle \textit{et al}., 2007). It was even suggested that the conversion of HY5 via CRY1 was dependent on a plastid signal (Ruckle \textit{et al}., 2007).

![Figure 10. Expression analysis of \textit{COR15a} and tetrapyrrole levels in mutants involved in Mg-ProtoIX biosynthesis and light perception. All experiments were done on 3 week old plants grown in photoperiodic conditions. Samples for expression analysis were taken in the end of the light period. Tetrapyrrole samples were obtained in the middle of the light period. Expression data are presented as mean ± 95% CI of at least three biological replicates. Significant differences compared to Col were determined using One-way ANOVA (GraphPad). Tetrapyrrole levels (mean ± SD) were determined with HPLC (Paper III) including at least four biological replicates.](image)

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<tr>
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<th>Mg-ProtoIX (pmol/g FW)</th>
<th>Mg-ProtoIX-ME (pmol/g FW)</th>
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<tbody>
<tr>
<td>Col</td>
<td>109.4 ± 21.7</td>
<td>94.0 ± 16.1</td>
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<tr>
<td>gun5</td>
<td>47.6 ± 23.7</td>
<td>25.9 ± 6.5</td>
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<tr>
<td>cry1</td>
<td>112.5 ± 12.2</td>
<td>38.4 ± 6.5</td>
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<td>24.9 ± 3.2</td>
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<tr>
<td>phyA</td>
<td>91.3 ± 11.8</td>
<td>54.9 ± 31.2</td>
</tr>
<tr>
<td>phyAgun5</td>
<td>70.9 ± 15.0</td>
<td>18.1 ± 6.0</td>
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\textbf{Table 1.} Tetrapyrrole levels in different genotypes.
The conversion of HY5 from a positive to a negative regulator is interesting because in yeast, HAP1 functions as a repressor when the tetrapyrrole heme is not present but as an activator in the presence of heme (Hickman and Winston, 2007). The accumulation of Mg-ProtoIX during the day could therefore be of importance for COR15a expression in a similar way in plants. However, our COR15a expression analysis revealed that CRY1 and the Mg-ProtoIX triggered signal were independent of each other (Figure 10). The double mutant, gun5cry1, exhibited a COR15a expression level that was between either single mutant. The analysis further showed that CRY1 is required to positively regulate COR15a. The response in the cry1 mutants was not caused by changes in the tetrapyrrole levels (Table 1).

Further analysis of one of the red light receptors, PHYA, revealed that the photoreceptor is part of the same pathway as Mg-ProtoIX (Figure 10). PHYA senses pre-dominantly far-red light and is stable in dark and far-red conditions but quickly depleted in blue and red light (Parks and Spalding, 1999; Sharrock and Clack, 2002). That PHYA is involved retrograde signalling coincide with previous studies demonstrating that plastid derived signals are indispensable for phytochrome action on nuclear encoded plastid genes (Vinti et al., 2005). Mutants of PHYA showed a de-repressed expression of COR15a, similar to the gun5 mutant (Figure 10). We also checked the expression levels in the double mutant, phyAgun5. The phyAgun5 mutant showed a similar expression as either single mutant, indicating that they are in the same pathway. PHYA is part of the regulatory network of genes encoding tetrapyrrole biosynthesis (Tanaka and Tanaka, 2007). Tetrapyrrole levels could therefore be affected in the phyA mutants. We could not detect any difference in tetrapyrrole levels either in phyA or phyAgun5 compared to wild type and the gun5 mutant respectively (Table 1). This suggests that the effect on COR15a expression in the phyA mutant is independent of tetrapyrrole levels.

Previous studies have shown that phytochromes are important in COR15a regulation. However, studies showed that PHY function did not primarily utilize G-boxes but promoter motifs called CRT/DRE motifs (Kim et al., 2002), recognized by the CBF transcription factors. CBFs are transcription factors strongly induced after cold exposure and responsible for induction of COR15a expression (Guo et al., 2002). PHYs have been implicated to be repressors of the CBFs, thereby indirectly regulating COR15a expression (Kim et al., 2002; Franklin and Whitelam, 2007). The regulation of COR15a and CBF expression via the phytochromes is mostly prominent in moderate low and cold temperatures (Franklin and Whitelam, 2007). In conditions used in our study (23°C), CBFs are transcribed at low rates compared to low temperatures (Fowler et al., 2005). Thus,
the demonstrated fluctuations in \textit{COR15a} expression seen in our photoperiodic study are most likely in-dependent of CBF stimulation. The PHY action on cold-dependent \textit{CBF} expression seems to be restricted to PHYB and PHYD (Kim \textit{et al.}, 2002; Franklin and Whitelam, 2007). PHYA, on the other hand, does not have an obvious role in cold regulation of \textit{CBF} and \textit{COR15a} (Kim \textit{et al.}, 2002; Franklin and Whitelam, 2007). Our results suggest that PHYA could have a role in 23°C rather then in cold temperatures. If PHYB and PHYD also have a role in warmer temperatures remains to be shown.

The results from the different \textit{phyA} mutants allow us to speculate how the plant fine-tunes the \textit{COR15a} expression in warm, photoperiodically grown plants. Our results suggest that the plant uses plastid signals (Mg-ProtoIX) and signals from photoreceptors (PHYA) and integrate these signals to a response in gene expression. The involvement of HSP90, HY5 and PHYA in Mg-ProtoIX mediated signalling (\textit{Paper II}) requires more components, however. We identified a possible link between PHYA, HY5 and HSP90 in our proteomic study (\textit{Paper II, Table S1}). Protein Phosphatase 5 (PAPP5) is a protein that has been found to interact with both PHYA (Ryu \textit{et al.}, 2005) and HSP90 (De La Fuente Bentem \textit{van et al.}, 2005). In plants, PAPP5 functions as a co-chaperone to HSP90 in the folding and function of disease resistance proteins. Furthermore, in mammals; HSP90, PAPP5 and photoreceptors form complexes to regulate different processes (Chinkers, 2001). No direct interaction has been shown for PAPP5 and HY5, but they co-localize in nuclear bodies (Chen, 2008), suggesting that they could be involved in a complex.

In summary, our results indicate that plastid and light signals are both required for a precise fine-tuning of gene expression during photoperiodic conditions. A cross-talk between Mg-ProtoIX mediated signalling from the plastid and light signals from PHYA determines the \textit{COR15a} expression. The incorporation of the different signals could occur in a multi-protein complex that consists of HSP90 and PAPP5. The input from the chloroplast together with light intensity and quality determines how the complex controls transcriptional regulators like HY5.

\textbf{Model of Mg-ProtoIX mediated \textit{COR15a} regulation}

The results from the different experiments in Paper III and the plausible involvement of PHYA, PAPP5 and HSP90 can be summarized in a model illustrating how Mg-ProtoIX mediates regulation of \textit{COR15a} expression (Figure 11). A Mg-ProtoIX triggered signal turns HY5 to a negative regulator of \textit{COR15a} expression. The Mg-ProtoIX mediated signal is independent of CRY1 but dependent on PHYA. CRY1 is a positive regulator and PHYA a negative regulator.
of $\text{COR15a}$. CRY1 could function as a switch for HY5 that converts the transcription factor from positive to negative regulator, something that has been reported in HY5 mediated $LHCB$ regulation (Ruckle et al., 2007). If CRY1 mediates a signal through HY5 to regulate $\text{COR15a}$ expression is still unclear. We could not find a direct interaction between HY5 and Mg-ProtoIX, suggesting that more components are required. HSP90 and PAPP5 could be two components that link the plastid signal with the light signal. HY5 could then function as the transcriptional activator/inactivator in the complex, similar to the role of HAP1 in yeast (Zhang and Hach, 1999).

**Figure 11.** Model of Mg-ProtoIX mediated regulation of nuclear gene expression. In this model, Mg-ProtoIX regulates $\text{COR15a}$ expression via HY5 and PHYA. The regulation of $\text{COR15a}$ expression is independent of CRY1 mediated regulation of HY5. Two possible interaction partners in this regulatory complex are HSP90 and PAPP5.

**Summary: Mg-ProtoIX mediated signalling**

This thesis establishes tetapyrrole signalling as an important part of plant stress response. We show that the level of tetapyrroles strongly correlates with expression of genes encoding proteins required in the plastid. The genes regulated by the Mg-ProtoIX mediated signal include photosynthesis genes but also non-photosynthetic genes. The results presented here suggest that both $\text{COR15a}$ and $\text{CGS}$ expression are regulated by the Mg-ProtoIX mediated signal. The light responsive elements in their promoters seem to be common to all genes regulated by the Mg-ProtoIX mediated signal. We also postulate that Mg-ProtoIX interacts with different components in the cytosol to transduce the signal. Exemplified by the HSP90 family and AMC involved proteins, Mg-ProtoIX regulates various processes in the cell during stress. Mg-ProtoIX seems to be a general stress signal that controls many nuclear encoded plastid proteins. The emerging picture of light- and plastid-
signalling cross talk made photoreceptors a promising path to investigate. We could find that PHYA is part of the signalling network involved in Mg-ProtoIX mediated signalling. Thus, photoreceptors are an important part of retrograde signalling. However, CRY1 proved to be a positive regulator of *COR15a* but in an independent pathway distinct from Mg-ProtoIX signalling. In an attempt to understand how the photoreceptors and HY5 control nuclear gene expression, we investigated their impact in the high light response (Paper IV).

**HIGH LIGHT REGULATION OF GENES**

High irradiances are experienced by plants throughout their life cycle. For instance, the sun can hide behind clouds one moment, and direct sun light can hit the plant the next. These fluctuations in light intensity must be sensed by the plant, and appropriate processes must be activated to ensure plant survival. An excess of light will create reactive compounds that gives rise to damage within the plant. The obvious place for a plant to sense light intensity is the photosynthetic machinery. Not surprisingly, components involved in photosynthesis have been reported to trigger a signal (see Figure 5). These components include the PQ pool, ROS and metabolite production (Nott *et al.*, 2006; Fernández and Strand, 2008; Pogson *et al.*, 2008). To investigate if the photoreceptors are pieces in this network of signals, we tested if mutants of phytochromes (PHYA and PHYB) and cryptochromes (CRY1 and CRY2) in addition to their downstream targets (HY5 and HYH) had any impairment in the high light stress response (Paper IV).

**Transcriptome changes in response to high light**

To understand the impact of our high light treatment, we performed microarray analysis of treated versus untreated wild type seedlings. We used 7-day old seedlings grown in 100 μmol photons m⁻² s⁻¹ continuous light as control and seedlings grown under the same conditions but exposed to 1000 μmol photons m⁻² s⁻¹ for 3 h (HL) as high light sample. The analysis revealed that 992 genes changed their expression at least 2-fold in response to our high light treatment. 660 of these genes were up-regulated and 332 down-regulated (Paper IV, Table S1). The 992 genes represented about 4% of the genes on the chip used for analysis. Among the differentially expressed genes, we wanted to find marker genes that could be used to investigate the involvement of HY5, HYH and the photoreceptors. Two strongly induced genes after HL exposure were *Early Light Inducible Protein 1* and 2 (*ELIP1/2*). *ELIP1* (100-fold up-regulated) and *ELIP2* (88-fold up-regulated) were reported as high light inducible in a similar manner in a previous study (Heddad *et al.*, 2006). In addition to the *ELIP* genes, we chose *Ascorbate Peroxidase 2* (*APX2*) (6-fold up-regulated) and *Light-Harvesting Chlorophyll A/B-binding protein 2.4* (*LHCB2.4*) (4-fold down regulated) as marker genes.
**cry1 and hy5 mutants show impaired high light response**

Mutants of the respective photoreceptor (phyA, phyB, cry1 and cry2), in addition to the hyb and hy5 mutants, were exposed to 3 h of HL and checked for expression levels of the selected marker genes (Paper IV, Fig 1). A clear impairment of ELIP regulation could be seen in the cry1 and hy5 mutant. Both APX2 and LHC2.4 were regulated in wild type manner in these mutants, suggesting that the different genes are controlled by distinct signalling pathways. No clear mis-regulation could be seen in the other mutants investigated. The analysis revealed that CRY1 and HY5 mediate a significant part of the high light induction of the ELIP genes.

Furthermore, checking the ELIP induction in increasing blue light intensities showed that CRY1 functions as a sensor of increasing light (Paper IV, Fig 2). Blue light intensities from 10 μmol photons m⁻² s⁻¹ (corresponding to control light) to 200 μmol photons m⁻² s⁻¹ were used to check ELIP induction. The analysis showed that ELIP expression was increasing with increasing blue light intensities and that this induction was abolished in the cry1 mutant. These results identify CRY1 as a cytosolic/nuclear mediator of high light, able to regulate nuclear gene expression independent of direct input signals from the chloroplast.

**Impact of CRY1 and HY5 after high light exposure**

In order to understand the impact of the CRY1 and HY5 part of HL signalling, we used microarray analysis to identify those genes that were mis-regulated in cry1 and hy5 mutants after HL exposure (Paper IV, Fig 3). Genes that were mis-regulated compared to wild type already under control conditions were not included in the final list of genes. The cry1 mutant showed mis-regulation of 77 of the 992 genes identified in wild type response to HL (Paper IV, Table S5). 65 of the 992 genes were mis-regulated in the hy5 background (Paper IV, Table S6). Combining these gene lists left 26 genes that were CRY1- and HY5-dependent.

To understand more about the CRY1 regulon, we analyzed arrays on wild type seedlings exposed to high intensity blue light (BL) (Paper IV, Table S2). Comparison of the BL and HL list revealed that 49 of the 77 genes mis-regulated in the cry1 mutant, also were regulated in BL (Paper IV, Table 2). 38 of the 65 genes in the hy5 mutant were mis-regulated in HL and regulated in BL (Paper IV, Table 2). Expression of 23 of the 26 genes found mis-regulated in HL in both cry1 and hy5 were also regulated in BL. Thus, it is clear that CRY1 and HY5 are required for the regulation of a significant group of genes in response to high irradiances. Analysis of the promoters (500 bp spaces) (Benedict et al., 2006; Geisler et al., 2006) of the mis-regulated genes in cry1 and hy5 revealed that G-boxes were significantly over-represented, confirming the importance for this
element in CRY1-HY5 mediated signalling. In addition to the G-box, we could also identify novel cis-elements for CRY1 mediated signalling, CryR1 and CryR2 (Paper IV, Table 3). Bioactivity of these elements was suggested by their significant over-representation in the genes mis-regulated in the cry1 background (Paper IV, Table 3). The biological role for these elements is currently under investigation.

**CRY1, an important component in high light adaptation.**

CRY1 and HY5 have been extensively investigated for their role in photomorphogenesis and transitions from dark to light (Jiao et al., 2007), but does the finding that CRY1 is a high light sensor have any importance in adult plants? We measured maximum quantum efficiency of PSII (Fv/Fm) after high light exposure in adult plants of cry1 and hy5. Compared to wild type, only the cry1 mutant showed a drop in Fv/Fm after HL stress (Paper IV, Table 1). Visible phenotype of the cry1 mutant confirmed that these plants have an impaired HL response (Paper IV, Fig 4). Exposure to HL decreased the levels of chlorophyll in the cry1 mutant compared to wild type (Paper IV, Fig 4). The mis-regulation of genes involved in the phenylpropanoid pathway in both the cry1 and hy5 mutant (Paper IV, Fig S1) also resulted in an impaired anthocyanin accumulation after HL exposure (Paper IV, Fig S2). The results point to a key role for CRY1 and HY5 in the anthocyanin accumulation following HL exposure. However, the lesser amounts of anthocyanins and the mis-regulation of genes in the hy5 mutants did not result in short term HL sensitivity (Paper IV, Table 1). Since CRY1 is the mediator of high irradiances and HY5 is a downstream target, CRY1 controls a larger set of genes than HY5, partly explaining the differences in HL sensitivity between the cry1 and hy5 mutants.

We could show that both HY5 and CRY1 have important roles to play in adapting plants to fluctuations in light intensity. CRY1 in itself works as a cytosolic/nuclear high light sensor that mediates signals to regulate nuclear gene expression. The mechanism of CRY1 and HY5 mediated signalling in high light is so far unknown, but knowledge from photomorphogenesis allows us to speculate. In photomorphogenesis, CRY1 has been proposed to inhibit COP1 when plants are exposed to light (Yang et al., 2001). In the dark, COP1 binds to a number of transcription factors, including HY5, responsible for light driven transcription (Holm et al., 2002; Jiao et al., 2007). COP1 binding targets the transcription factors for degradation. In the light, when CRY1 inhibits COP1, HY5 is no longer degraded and can induce transcription.
All three proteins have been reported to have important roles in adult plants as well as in seedlings (Paper IV) (Yu et al., 2008). If the CRY1-COP1-HY5 signalling system is active during high light exposure is still an open question. HY5 in itself was found to be up-regulated in CRY1 dependent manner (Paper IV, Table 3) and the protein level increased after HL exposure (Paper IV, Fig S5), suggesting a role in the HL response. It further suggests that HY5 is not degraded via COP1 during HL conditions. The role of CRY1 as a high light sensor could potentially inhibit COP1 further in high irradiances allowing for a more stable HY5 protein. Another possibility is that HY5 is phosphorylated during HL exposure, something that results in higher stability for the HY5 protein (Hardtke et al., 2000). In addition to its putative role in HL exposure, COP1 might also be involved in the photoperiodic regulation of COR15a (Paper III). COP1 has been shown to have an important role in determination of flowering time (Yu et al., 2008) and might be involved in other regulatory processes under photoperiodic conditions.

Our findings show that both CRY1 and HY5 are important in high light response and that CRY1 in itself is a sensor of high light irradiances. Thus, CRY1 can transduce high irradiance signals independent of the chloroplast. Elucidating the retrograde signals that are active during high light exposure is a challenge. Most signals that have been identified have over-lapping functions and cross-talk between the signals is common (Pfannschmidt, 2010). In order to find novel components in plastid signalling, we set up a screen to isolate mutants that had an impaired regulation of LHCb expression after high light exposure (Paper V).

**ISOLATION OF REDOX INSENSITIVE MUTANTS**

When plants are exposed to high light, they need to adapt to the new condition. High light results in an excess of photons; the plant absorbs more photons than it can utilize in its photosystems. The excited molecules inevitably lead to ROS production and potential damage for the cell. A defence mechanism that is employed during high light is to reduce the size of the antenna complexes (Dietzel et al., 2008). The major proteins in the antenna complexes are the LHC proteins, responsible for chlorophyll binding. Therefore, exposure to high irradiances will result in down-regulation of LHC expression (Dietzel et al., 2008). The repression of LHC expression requires plastid signals (Bräutigam et al., 2007). We could isolate redox insensitive (rin) mutants that had impaired repression of LHCb1.1 after high light exposure using an Ethyl Methane Sulphate (EMS) mutagenised line, incorporated with a construct, consisting of the regulatory part of the LHCb1.1 gene fused to a reporter gene (Luciferase, LUC).
The isolation and positional cloning of rin2

Our screen was divided into three steps. First, mutated seedlings with high LUC activity after exposure to high light (3 h, 1000 μmol photons m⁻² s⁻¹) were identified and allowed to set seeds. Second, the seeds from the putative rin mutants were screened once again and checked for LUC activity. This time, HL treated seedlings were compared with wild type and control (seedlings that remained in 100 μmol photons m⁻² s⁻¹). Mutants that exhibited no or low repression of LUC activity after HL compared to its control were chosen. Third, endogenous LHCB1.1 expression was checked with semi-quantitative real time Polymerase Chain Reaction (rtPCR). Using this screen we could isolate the rin2 (rin2-1) mutant as a true redox insensitive mutant.

A mutated RIN2 gene results in a striking visible phenotype (Paper V, Fig 1). Seedlings of the EMS mutation, rin2-1, display pale/albino cotyledons and the true leaves are slightly paler than wild type. Using traditional map based cloning together with modern genome sequencing, we could identify the mutated gene in rin2-1 on top of chromosome 1 (Paper V, Fig 1). Complementation with the wild type version of RIN2 rescued the rin2-1 phenotype. Furthermore, a T-DNA insertion line (GABI_772D02, rin2-2) with a disrupted RIN2 gene demonstrated a similar, although more severe phenotype (Paper V, Fig 1). The mutation in rin2-1 creates a pre-mature stop codon, resulting in a protein that is 6 amino acids shorter than the wild type version of RIN2 (Paper V, Fig 1). The severe phenotype in the rin2-1 mutant suggests that the missing part of the C-terminus is required for correct function of RIN2. The rin2-2 mutant showed a clear reduction in growth compared to wild type. In addition, seedlings of both rin2-1 and rin2-2 had significantly lower levels of tetrapyrroles compared to wild type (Paper V, Table 1). The phenotypes in the different rin2 mutants suggest that RIN2 is an important factor in plant growth and development and that the C-terminus of the protein is essential for its function.

Localization of RIN2

The RIN2 protein has no described function in Arabidopsis. The sequence of RIN2 revealed that it is a plant specific protein that has no homology with other proteins with known function (Paper V, Fig 2). Predictions with available localization software placed RIN2 in the chloroplast. The predicted transit peptide (TP) seems to vary in sequence between different plant species, but the predicted mature form of the protein is highly homologous. These results are in line with the hypothesis that the TP sequence is not conserved between plastid localized proteins, but that it is rather the secondary fold that is important (Jarvis, 2008). We constructed a fusion protein (RIN2:YFP) to visualize and confirm the localization in vivo (Paper...
V, Fig 2). The YFP-signal clearly overlapped with the auto-fluorescence from chlorophyll, confirming the in silico prediction of a plastid localization. Furthermore, the YFP signal co-localized with a PEND:CFP construct (Arsova et al., 2010) that binds to DNA in the chloroplast, indicating that RIN2 might be localized in the complex that control plastid transcription/translation (Pfalz et al., 2006).

The lack of RIN2 homologs in algae and cyanobacteria suggests that RIN2 evolved late during evolution. A feature for proteins that evolved late is that they often are involved in regulatory functions that coordinate the different processes in the complex plant cell (Pfannschmidt and Liere, 2005). When we did a similarity search based on known motifs and domains, we found two stretches that resembled thiol oxidoreductase proteins. Thiol oxidoreductases are enzymes containing a catalytic redox-active disulfide bridge. They have been found to protect against oxidative stress but also to directly regulate proteins (Buchanan and Balmer, 2005).

Without a functional RIN2 protein, plants show impairment in chloroplast development. Transmission electron microscopy (TEM) images of chloroplast from the rin2 mutants grown in 150 μmol photons m^{-2} s^{-1} (hereafter abbreviated CL) showed that they had non-functional chloroplasts (Paper V, Fig 5). The rin2-1 mutant showed a clear reduction and the rin2-2 showed almost no internal membranes. Neither mutant was able to grow on media without added external carbon source (i.e. sucrose), further implying that the rin2 mutants are not able to assemble a functional photosynthetic machinery.

**Plastid expression profile in rin2 implies involvement in regulating of PEP activity**

The localization analysis indicated that RIN2 might be localized in a transcriptional complex in the chloroplast. Thus, we checked the expression of plastid encoded genes in rin2 and wild type seedlings (Paper V, Fig 4). The lack of a functional RIN2 protein results in a strong repression of genes associated with photosynthesis. Mutated RIN2 further leads to an increased expression of plastid house-keeping genes and the genes encoding the subunits for the Plastid Encoded Polymerase (PEP). In plastids, the Nuclear Encoded Polymerase (NEP) is responsible for transcription of non-photosynthetic genes, including the PEP subunits, while PEP transcribes the photosynthetic associated genes (Maliga, 1998). These results indicate that PEP activity is decreased in the rin2 mutants. Rosette plants from both rin2 mutants showed a decreased expression of PSBA and PSAA compared to wild type under control conditions, similar to the expression pattern seen in seedlings (Paper V, Fig. 6). Other mutants with impaired PEP
activity like the plastid transcriptionally active chromosome 2 (ptac2) and the chloroplast biogenesis 19 (clb19) mutants show highly similar expression profiles (Pfalz et al., 2006; Chateigner-Boutin et al., 2008).

Like RIN2, PTAC2 and CLB19 are newly evolved proteins only found in higher plants. Both PTAC2 and CLB19 are PentatricoPeptide Repeat (PPR) proteins. PPR proteins are involved in RNA editing, splicing and translation (Pogson et al., 2008). RIN2, however, does not contain any PPR motif in its sequence. PTAC2 was found to be localized in a multi-subunit complex in the plastid that is involved in plastid gene expression (Pfalz et al., 2006). CLB19 was shown to be involved in RPOA editing (Chateigner-Boutin et al., 2008). Both mutations result in an impaired PEP activity. Both ptac2 and clb19 mutants have a visible phenotype that resembles the rin2 mutants with albino/pale cotyledons (Pfalz et al., 2006; Chateigner-Boutin et al., 2008). Plastid gene expression reveals that PEP activity is impaired in the rin2 mutants.

**Light irradiance sensitivity in rin2 seedlings**

When grown in low light (10 μmol photons m⁻² s⁻¹, LL) conditions, the rin2 mutants partly recover their pale phenotype (Paper V, Fig 5). TEM images of rin2 chloroplasts from seedlings grown under the LL condition showed an almost normal thylakoid membrane structure in the rin2-1 mutant. The rin2-2 mutant still displayed a decreased internal membrane system compared to wild type, but a clear recovery compared to rin2-2 chloroplast grown in CL was observed. This difference indicated that the pale phenotype in rin2 could be partly explained by photooxidative stress caused by the exposure to higher light intensities. The plastid gene expression was nevertheless still mis-regulated in LL (Paper V, Fig 5). The rin2-1 mutant showed almost wild type expression for the checked plastid genes, while the rin2-2 mutant still had a significant decrease in genes associated with photosynthesis compared to wild type. Both mutants still had a mis-regulated RPOB gene, encoding one of the subunits in the PEP enzyme.

In contrast, nuclear gene expression in the rin2 mutants showed clear differences between CL and LL (Paper V, Fig 5). LHCB1.1, LHCB2.4 and RBCS showed a decreased expression level in the rin2 mutants compared to wild type when seedlings were grown in CL, whereas in LL, the nuclear gene expression was indistinguishable from wild type in both rin2 mutants. A functional PEP enzyme requires nuclear encoded sigma factors. The sigma factors are believed to determine promoter specificity for PEP (Lysenko, 2007). Both SIG2 and SIG6 have been reported to be important during chloroplast development (Lysenko, 2007). However, neither SIG2 nor SIG6 were mis-regulated in the rin2 mutants (Paper
V, Fig 5). The other sigma factors showed a slight increase in expression in the rin2 mutants grown in CL, except the SIG1 gene that showed a decrease compared to wild type (Paper V, Fig 5). In LL conditions, expression of SIG1 was the same in wild type and the rin2 mutants. Thus, the expression of the sigma factors cannot explain the impaired PEP activity in the rin2 mutants.

Furthermore, the rin2 mutations have a general effect on expression of photosynthesis genes, both in the nucleus and the plastid. The effect on nuclear gene expression in the rin2 mutants seems to be restricted to higher light intensities and could be a secondary effect caused by the oxidative stress that the mutants experience under those conditions. Hence, RIN2 might be involved in the protection of the plant from photooxidative damage and/or redox signalling.

RIN2, a mediator of redox signals?
The RIN2 gene is primarily expressed in green tissue (Paper V, Fig S1). Seedlings and mature leaves show higher expression than flowers and roots. The expression pattern suggests that RIN2 is expressed in tissues with mature chloroplasts, but also in seedlings during chloroplast development given the phenotype. Furthermore, it suggests that RIN2 is light regulated because of the low expression in roots. The presence of light responsive elements (G- and GATA-boxes) in the RIN2 promoter is consistent with the expression profile of RIN2 in photoperiodic conditions (Paper V, Fig S1). The RIN2 expression is also slightly up-regulated after high light (HL) exposure (Paper V, Fig S1), indicating that RIN2 could have a role in the HL response.

To further elucidate the function of RIN2 and investigate the light sensitivity of the rin2 mutants, we used HL exposure (3 h, 1000 μmol photons m⁻² s⁻¹). The rin2-1 mutant was isolated, as described above, because of its impairment of repressing LHCBI.1 expression after HL exposure. When the rin2-2 mutant was included in our analysis, it demonstrated similar response to HL exposure as the rin2-1 mutant (Paper V, Fig 3). Comparing the level of expression between the wild type and the rin2 mutants revealed that, even if the rin2 mutants did not down-regulate LHCBI.1 expression after high light exposure, they still did not have higher expression than wild type because of their lower expression in CL.

Interestingly, rosette plants of the rin2 mutants showed the same mis-regulation of LHCBI.1 expression after HL as the seedlings (Paper V, Fig 6). Another nuclear encoded photosynthesis gene, LHCBI.4, also showed mis-regulation after HL exposure in the rin2 mutants (Paper V, Fig 6). We also checked the expression in rosette plants of the plastid encoded PSBA and PSAA genes, encoding reaction
centre proteins in PSII and PSI, respectively (Paper V, Fig 6). Both genes showed an impaired regulation in response to HL compared to wild type where the expression was clearly repressed following exposure to HL.

The mis-regulation of both nuclear and plastid genes involved in photosynthesis gave hints about an impaired in response to high light in the rin2 mutants. Measuring the photosynthetic activity in adult rin2 plants confirmed the impairment (Paper V, Fig 7). The electron transport rate was lower and the non-photochemical quenching higher in both rin2 mutants compared to wild type. The effect was greater in the T-DNA line, rin2-2. The levels of ROS were also checked using staining compounds (Paper V, Fig S2). Levels of H$_2$O$_2$ were slightly higher than in wild type while levels of super oxide were lower.

Similar to the results obtained from seedlings, rosette plants of the rin2 mutants showed an impaired retrograde signalling in response to high light exposure. In addition, the rin2 mutants have decreased expression of plastid encoded photosynthesis genes in control conditions and mis-regulate these genes after high light exposure. The effect on gene expression results in disturbed photosynthetic acclimation to high light intensities. The results from the HL exposure imply that RIN2 has a function in both developing and mature chloroplast during moderate and high light intensities.

**Redox regulation of PEP**

Redox regulation of the PEP enzyme has been shown in previous studies with different combinations of light intensities and light qualities (Pfannschmidt and Liere, 2005). In low and moderate light, it is thought that PEP is regulated by the redox state of the PET components (Pfannschmidt and Liere, 2005). In higher light intensities other components add their input to regulate PEP. For instance, different phosphorylation events have been shown to be important. Phosphorylation of sigma factors as well as phosphorylation of PEP itself regulates plastid gene expression (Baginsky et al., 1997; Shimizu et al., 2010). In mustard, a kinase, PTK, has been found to phosphorylate both sigma factors and components of PEP (Pfannschmidt and Liere, 2005).

PTK phosphorylates PEP under different light regimes (Pfannschmidt and Liere, 2005; Steiner et al., 2009). The phosphorylation of PEP results in lower transcription rate under moderate light conditions while a non-phosphorylated enzyme is more active during short high light exposure (Pfannschmidt and Liere, 2005). Additional identified components in the PEP complex support the regulation by phosphorylation. A recent study identified a thioredoxin (TRX z)
involved in the regulation of PEP (Arsova et al., 2010). The redox state of TRX z regulates kinases that, in turn, regulate PEP activity during dark-to-light transitions (Arsova et al., 2010). The involvement of TRXs and kinases implicate that phosphorylation work synergistically with thiol modifications to regulate PEP activity (Steiner et al., 2009).

The similarity between the rin2 and trx z mutants (Arsova et al., 2010) in respect to phenotype (Paper V, Fig 1), gene expression (Paper V, Fig 4), and the putative thiol oxireductase stretches indicate that RIN2 and TRX z could have similar roles in regulating PEP activity. In addition, RIN2 co-localize with DNA in chloroplast (Paper V, Fig 2), further suggesting that RIN2 somehow is involved in the complex that regulates transcription/translation of plastid gene expression. Identifying interaction partners to RIN2 could clarify this issue. It would also be interesting to investigate the high light response in the trx z mutant and compare it to the rin2 mutants to see if they share the impaired high light repression of PhANGs.

**RIN2 and retrograde signalling**

Given our results indicating that RIN2 somehow is involved in retrograde signalling during high light exposure, we wanted to know if the rin2 mutants demonstrated a classical gun phenotype (Susek et al., 1993). Indeed, the rin2 mutants showed a gun phenotype when grown on NF (Paper V, Fig 8). The gun phenotype in the rin2 mutants raises the possibility that RIN2 functions in the same pathway as the gun1 and gun5 mutants. Since the rin2 mutants both showed a lower level of tetrapyrroles in control conditions, the gun phenotype in rin2-1 and rin2-2 could be an effect of impaired tetrapyrrole accumulation on NF. Indeed, when the rin2 mutants were exposed to the plastid translation inhibitor, chloramphenicol (chl), they did not show a gun phenotype (Paper V, Fig 8). Only gun1 demonstrated the gun phenotype on chl. Thus, the gun phenotype of the rin2 mutants on norflurazon is most likely explained by reduced tetrapyrrole accumulation in the rin2 mutants. However, double mutants of gun1 and the different rin2 mutants showed a more severe phenotype then the rin2-1 or rin2-2 single mutants (Paper V, Fig 9). The phenotype of the gun1rin2 mutants could suggest that GUN1 somehow is involved in RIN2 mediated response. A more detailed analysis will hopefully elucidate the cross talk between the gun and rin2 mutants.
Summary: rin2
The rin2 mutants are, so far, the first mapped and characterized mutants with impaired plastid-to-nucleus signalling in response to high light. RIN2 encodes a novel plastid localized protein. The rin2 mutants have impaired development of chloroplasts in cotyledons and effects on gene expression both in seedlings and adult plants. Our results so far suggest that RIN2 is involved in PEP regulation in a redox state-manner, and that this regulation occurs both in seedlings and rosette plants. However, the actual mode-of-action for RIN2 remains to be solved. It is tempting to speculate that RIN2 functions in a phosphorylation network, but no such evidence exist so far. Clearly, the direct or indirect effect of RIN2 on PEP activity is also responsible for sending a retrograde signal to the nucleus after high light exposure. The involvement of plastid gene expression (PGE) in plastid-to-nucleus signalling has been shown in young seedlings but never in rosette plants (Nott et al., 2006). The exact mechanism of PGE mediated signalling is under debate and it is unclear if the plastid actually produces a direct product (mRNA, protein) in this signalling pathway (Kleine et al., 2009). RIN2 could potentially shed some light into this question since the signal seems to be important in both seedlings and rosette plants. The isolation of rin2 opens up the retrograde signalling field in respect to high light sensing and signalling. What proteins interact with RIN2? Is RIN2 a specific high light signalling component or do other mutants with impaired PEP activity share this high light phenotype? What part does PGE signalling play in rosettes plants? Important and intriguing questions that hopefully can be answered with the help of the rin2 mutants.

CONCLUSIONS AND FUTURE PERSPECTIVES

The work presented in this thesis is an important step for better understanding of plastid-to-nucleus signalling in higher plants. Traditionally, retrograde signalling is investigated in conditions that are artificial and unnatural to plants. Here, we complement the traditional conditions with physiologically important conditions. We show that the natural conditions, conditions that the plant faces in its development and growth also can be used in studying retrograde signalling.

This thesis establishes the role of Mg-ProtoIX as a signalling molecule with plastidic origin that can exit the chloroplast under specific conditions. Furthermore, Mg-ProtoIX mediated signalling must be studied with carefully chosen time points. As with many other signal molecules, Mg-ProtoIX accumulation is transient. The HSP90 proteins are the first cytosolic component identified in tetrapyrrole signalling. The HSP90 complex, where Mg-ProtoIX
seems to be an important player, has to be further investigated. One interesting question is if the HSP90-Mg-ProtoIX complex involves the photoreceptors to fine-tune nuclear gene expression. This is an important question in respect to the utilization of light regulatory element in both plastid and light signalling. The utilization of common promoter elements requires a common factor, and the HSP90 complex could be one of them. The list of other putative interacting partners with Mg-ProtoIX could prove to be an important tool in elucidating how tetrapyrrole mediated signalling occurs.

One of the big challenges in retrograde signalling is to find marker genes better suited then the traditional *LHCB* and *RBCS* genes. *LHCB* and *RBCS* are regulated by a multitude of stresses, circadian rhythms and hormones (Pfannschmidt, 2010) making them unsuitable for more detailed investigations. We have to find marker genes that can be investigated during natural conditions. We have used a new marker gene, *COR15a*, which represents a non-PhANG gene that clearly is regulated by plastidic signals. New, carefully designed mutant screens using *COR15a* could prove fruitful in elucidating retrograde signalling. Taking advantage of the results from this thesis, one could use mutagenised lines of different mutant backgrounds in combination with various light quality and intensity to clarify the cross-talk between light and tetrapyrrole signalling. For the future though, new marker genes have to be identified in other plastid signal pathways, for example in ROS signalling and PGE signalling.

To be able to understand the plastid-to-nucleus signals, we have to make more detailed analysis of the transcription factors involved. Here, we identified HY5 as part of the tetrapyrrole mediated signalling pathway to regulate *COR15a* expression. All identified transcription factors so far bind to or adjacent to the G-box *cis*-element. This includes ABI4, GLK1-2, HY5 and GBFs. To separate plastid from light signals could therefore prove impossible since they seem to be dependent on each other. What could be done is a more extensive analysis of the known transcription factors and their relationship to each other. The difficulty with working with the transcription factor mutants as well as chloroplast development mutants is their strong phenotype. The phenotype often gives rise to pleiotropic effects, making it hard to discriminate between primary and secondary effects. For the future, inducible systems, creating phenotypes from earlier wild type looking plants would be very useful. If experiments are designed correct, this might give us the tools they need to find the primary effects of plastidic signalling.

Another problem in retrograde signalling that has to be resolved in the future is how the specificity of the different plastid signals is determined. For instance, ROS
signals are produced in the mitochondria, peroxisome and chloroplast. How does the nucleus know which genes to regulate and where to send the product, the proteins? The ROS signals often have short half life and they are required to interact with other components in the signalling pathway to transduce the signal. A possibility is that ROS signals react with peptides and oxidize them (Møller and Sweetlove, 2010). The oxidized peptides could act as more specific plastid signals. Our finding that HSP proteins are involved in retrograde signalling raises the possibility that unfolded or mis-folded proteins could act as signalling components creating a feed-back regulation of nuclear genes. Using proteomic and spectroscopy approaches of samples from different stress conditions could answer some of these questions.

Our understanding of retrograde signalling is still rudimentary. The identified proteins in plastidic signalling often have an unknown mode-of-action. Finding the mechanisms for GUN1, STN7, EX1 and EX2 is therefore of utmost importance. We identified a new component in plastid-to-nucleus signalling, RIN2. This is the first reported protein that has an impaired high light regulation of nuclear encoded photosynthesis genes. Unfortunately, RIN2 is another protein with unknown function. Our results indicate that RIN2 is involved in regulation of PEP activity. The impaired nuclear and plastid gene expression after high light exposure suggest that there is a dependency for a functional PEP enzyme to also regulate nuclear gene expression. The results presented here are the first that show that there might be PGE mediated signalling in both seedlings and rosette plants. The rin2 mutants together with other PEP deficient mutants (ptac2, clb19), gun mutants and other mutants (trx z) might explain more about how the PGE is involved in regulating nuclear gene expression. Finding interaction partners to RIN2 as well as GUN1 will also help in unfolding the mysteries concerning these proteins.

The work in this thesis presents new findings in plastid-to-nucleus signalling, but more importantly, it opens up the field with many new threads of thinking and possible experiments for the future. Our understanding of the signals that originate in the chloroplast is still basic and we need to focus our efforts in breaking the bottlenecks that exists today. The chloroplast talks, we just need to figure out the language.
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