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Characterization of the *gun* phenotype under photo-bleaching conditions

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Summary

Protein complexes involved in biochemical processes of organelles are composed of subunits encoded in the organelles and the nucleus. To guarantee energy-saving assembly and efficient functioning of such protein complexes, a proper regulatory network is required. The anterograde control of the nucleus over the organelles is extensive and the principal parameters are known. It is also accepted that organelles send information about their developmental and metabolic state to the nucleus ('retrograde signaling') in order to adapt nuclear gene expression. But, the nature of the molecules that relay information to the nucleus is still unclear. In a mutant screen, designed to find factors involved in retrograde signaling in *A. thaliana*, the *genomes uncoupled* (*gun*) mutants were identified more than 15 years ago. Under photo-bleaching conditions induced by norflurazon (NF), an inhibitor of carotenoid biosynthesis, the expression of the nuclear localized gene encoding photosystem II chlorophyll a/b-binding protein (*LHCB1.2*) is suppressed in wild-type plants. In the *gun* mutants, this suppression is less pronounced. Since four out of the five known *gun* mutants are affected in the tetrapyrrole biosynthesis pathway, it was suggested that tetrapyrroles are involved in retrograde signaling. However, recent studies have cast doubt on that theory. In this thesis the performance of photo-bleached *gun* mutants was characterized in more detail. A before unknown phenotype of NF-treated *gun* mutants is described which is not due to NF resistance. In comparison to NF-treated wild-type seedlings the *gun2-5* mutants affected in tetrapyrrole biosynthesis showed an enhanced growth capability, carotenoid enrichment, less anthocyanin accumulation and they retained plastome-encoded proteins. Replacement of NF by other inhibitors of carotenoid biosynthesis (such as amitrole) revealed that the growth and pigmentation phenotype is not coupled to the *LHCB1.2* mRNA accumulation phenotype. Furthermore, it is shown that no simple correlation between any single metabolite, pigment or reactive oxygen species and *LHCB1.2* expression exist. The observed heme accumulation caused by NF treatment is also not related to the *LHCB1.2* de-repression phenotype. Application of abscisic acid (ABA) to NF-treated wild-type plants was sufficient to increase *LHCB1.2* mRNA levels, but ABA is not involved in GUN-dependent signaling associated with tetrapyrrole biosynthesis. It is discussed that more natural conditions are necessary to uncover the regulatory network of GUN signaling.

Zusammenfassung

Plastidäre Proteinkomplexe bestehen aus verschiedenen Untereinheiten, welche entweder in den Plastiden oder in dem Zellkern kodiert werden. Exakte Zusammensetzung als auch Funktionsweise solcher Proteinkomplexe erfordern daher ein präzises regulatorisches Netzwerk. Mit Hilfe bestimmter Faktoren, von denen viele bekannt sind, übt der Zellkern starken Einfluss auf die Plastiden aus (‚anterograde control'). Plastiden senden andererseits Informationen über ihren aktuellen Zustand an den Zellkern (‚retrograde signaling'), um dessen Genexpression an deren Bedürfnisse anzupassen. Wie diese Informationen vermittelt werden ist noch unbekannt. Auf der Suche nach Faktoren, welche an dem plastidären Signal beteiligt sind, wurden vor mehr als 15 Jahren die sogenannten *genomes uncoupled* (*gun*) Mutanten identifiziert. Werden Pflanzen (*A. thaliana*) photooxidativen Stress ausgesetzt, z. B. durch die Applikation von Norflurazon (Inhibitor der Carotenoidbiosynthese), ist die Expression von dem im Kern lokalisierten Gen welches das Photosystem II Chlorophyll a/b Bindeprotein kodiert (*LHCB1.2*) stark vermindert. Diese Reaktion ist in den *gun* Mutanten gestört. Vier von fünf dieser Mutanten sind in der Tetrapyrrolbiosynthese beeinträchtigt. In aktuellen Studien konnte jedoch gezeigt werden, dass keine Verbindung zwischen der Anreicherung von Tetrapyrrolen und der *LHCB1.2* Expression besteht. In der vorliegenden Arbeit wurde das spezifische Verhalten photooxidativ geschädigter *gun* Mutanten genauer untersucht. Ein bisher unbekannter Phänotyp konnte identifiziert werden. Die *gun* Mutanten, welche bei der Tetrapyrrolbiosynthese beeinträchtigt sind, wachsen im Vergleich zu Wildtyppflanzen besser auf Norflurazon, synthetisieren einige Carotenoide, akkumulieren weniger Anthocyanine und enthalten mehr plastidär kodierte Proteine. Eine mögliche Norflurazonresistenz konnte ausgeschlossen werden. Die Verwendung von anderen Inhibitoren der Carotenoidbiosynthese (z.B. Amitrol) zeigt, dass der Wachstumsphänotyp nicht mit dem *LHCB1.2* Expressionsphänotyp korreliert. Darüberhinaus steht weder ein Metabolit, Pigment oder reaktives Sauerstoffmolekül noch die Anreicherung von Häm mit der *LHCB1.2* Expression in Verbindung. Zugabe von Abscisinsäure zu Norflurazon behandelten Wildtyppflanzen führt ebenfalls zu einer erhöhten Expression von *LHCB1.2*, allerdings unabhängig von dem GUN-spezifischen Effekt. Die Notwendigkeit natürlichere Bedingungen als Grundlage für die Erforschung des regulatorischen GUN-Netzwerkes zu verwenden wird diskutiert.

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Abbreviations

A. thaliana	*Arabidopsis thaliana*
ABA	abscisic acid
ABI4	ABA-insensitive 4
ADP	adenosine diphosphate
aKG	a-ketoglutarate
ALA	5-aminolevulinic acid
AMP	adenosine monophosphate
atABC1	ATP-binding-cassette protein
ATP	adenosine triphosphate
AtpB	β subunit of ATP synthase
BSA	bovine serum albumin
C. reinhardtii	*Chlamydomonas reinhardtii*
cDNA	complementary deoxyribonucleic acid
ChlD	D subunit of Mg-chelatase
ChlH	H subunit of Mg-chelatase
ChlI	I subunit of Mg-chelatase
chlm	*Mg-proto methyltransferase*
CPTA	2-(4-chlorophenylthio)triethylamine hydrochloride
crd1	*Mg-protoME cyclase subunit*
CTP	cytidine triphosphate
Cyt b_6/f	cytochrome b_6/f
D	dark
DGDG	digalactoglycerolipids
DNA	deoxyribonucleic acid
DP	dipyridyl
DTT	dithiothreitol
ECL	electrochemiluminescence
EDTA	ethylene diamine tetraacetic acid
EMS	ethyl methane sulfonate
Ex	executer

Fe-proto	iron protophorphyrin
FLU	negative regulator of chlorophyll biosynthesis (*flu* – *flu*orescent)
fwp	forward primer
F16P	fructose-1.6-bisphosphate
F6P	fructose-6-phosphate
GTP	guanosine triphosphate
gun	*genomes uncoupled*
GUS	β-glucuronidase
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
HCl	hydrochloric acid
HEMA	gene encodes glutamyl tRNA reductase
HPLC	high performance liquid chromatography
HSP70A	*heat-shock protein 70A*
HXK	hexokinase
hy1/2	phytochrome-deficient long hypocotyl mutants 1 and 2
H_2O	water
h * υ	light energy
Lhca3	photosystem I type III chlorophyll a/b-binding protein
Lhcb1	photosystem II type I chlorophyll a/b-binding protein
LHCB	encoding photosystem II chlorophyll a/b-binding protein
lin2	*lesion initiation 2*
LL	low-light
LP	left primer
Mg	magnesium
MGDG	monogalactoglycerolipids
Mg-proto	magnesium protoporphyrin
Mg-protoME	Mg-proto methyl ester
MOT	malate/oxaloacetate translocator
mRNA	messenger RNA
MS	Murashige and Skoog
M6P	mannose-6-phosphate

NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	disodium phosphate
NaH_2PO_4	sodium phosphate
NaOH	sodium hydroxide
NEP	nuclear encoded polymerase
NF	norflurazone
NGE	nuclear gene expression
NH_4OH	ammonium hydroxide
n.d.	not detected
OGE	organellar gene expression
PAGE	polyacrylamide gel electrophoresis
PBD	porphobilinogen deaminase
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PEP	plastid encoded polymerase
PetB	cytochrome b_6 subunit of cytochrome b_6/f
PORA	Protochlorophyllide reductase A
POR-OX	overexpressing PORA
PPR	pentatricopeptide repeat
PQ	plastoquinone pool
ProtoIX	protoporphyrin IX
PsaB	photosystem I reaction centre protein B
PsbD	photosystem II D2 protein
PSI	photosystem I
PSII	photosystem II
PVDF	polyvinylidene fluoride
RbcL	large subunit of RubisCO
RBCS	gene encodes small subunit of RubisCO
RNA	ribonucleic acid
ROS	reactive oxygen species

RP	right primer
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcription PCR
RubisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
rwp	reverse primer
SD	standard deviation
SDS	sodium dodecyl sulphate
SigE	sigma factor E
STN7	thylakoid-associated serine-threonine protein kinase
T-DNA	transferred DNA
Tetpy	tetrapyrrole
TPT	triosephosphate translocator
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer RNA
TTP	thymidine triphosphate
UDP	uridine diphosphate
UDP-Glc	UDP glucose
WT	wild-type
Xantho	xanthophylls
2CPA	encoding 2-Cys peroxiredoxin-A
3PG	3-phosphoglycerate
^{33}P-dCTP	^{33}P radioactive labelled dCTP

Units

°C	degree Celsius
g	gram
h	hour
m	meter
mg	milligram
min	minute
ml	millilitre
mM	millimolar
nM	nanomolar
nm	nanometer
nmol	nanomolar
pmol	picomolar
s	second
v	volume
w	weight
µg	microgram
µl	microlitre
µM	micromolar
µmol	micromolar

1. Introduction

1.1. Photosynthesis and the origin of plastids

Energy is essential for life on earth. Heterotrophic organisms get their energy from organic molecules which were generated by autotrophic organisms using either sun light as energy source (photosynthesis) or oxidizing inorganic molecules (chemo-synthesis). During photosynthesis the absorbed light energy is used to produce organic molecules out of carbon dioxide and water. Moreover, in this process oxygen is produced as a by-product, the second necessary compound for life of heterotrophic organisms, hence for human beings. Therefore, photosynthesis summarized in the following equation, is the most important process of life.

$$6\ CO_2 + 6\ H_2O + h * \upsilon \rightarrow C_6H_{12}O_6 + 6\ O_2 \qquad \Delta G^{0'} = +2872\ kJ$$

Photosynthesis takes place in the plastids of higher plants and algae or in unicellular free-living photosynthetic bacteria. It is generally accepted that plastids evolved from endosymbionts more than one billion years ago. An ancient eukaryotic cell absorbed a unicellular free-living bacterium that was able to perform photosynthesis (Mereschkowsky, 1905; Martin and Kowallik, 1999; Cavalier-Smith, 2000; Brinkman *et al.*, 2002; Martin *et al.*, 2002; Dyall *et al.*, 2004; Reyes-Prieto *et al.*, 2007). Like mitochondria, which are thought to derive from an alpha-protobacterial ancestor (Gray *et al.*, 2001; Dyall *et al.*, 2004), plastids are separated from the cytosol by a membrane system and contain their own genome. Therefore, plant cells possess three different compartments which contain genetic information. In the circular and polyploid genome of plastids (plastome) from higher plants 100 to 120 genes are present coding for subunits of complexes involved in photosynthesis or protein translation and for tRNAs and rRNAs (Sugiura, 1992). However, most proteins required in plastids are encoded in the nucleus of the cell. Thus, genes were transferred during evolution from the plastid to the nucleus (Martin and Herrmann, 1998; Martin *et al.*, 1998; Abdallah *et al.*, 2000; Timmis *et al.*, 2004). Proteins encoded by the nuclear genome contain a specific transit peptide to facilitate transport across the membranes surrounding the plastid (Jarvis and Soll, 2001; Leister, 2003; Reyes-Prieto *et al.*, 2007). Moreover, many protein complexes involved in the biochemical processes of plastids, especially in

photosynthesis, are composed of subunits encoded in both the plastids and the nucleus (Race *et al.*, 1999; Leister, 2003; Figure 1.1.). Thus proper assembly of protein complexes and function of photosynthesis requires a tight regulation of nuclear and plastid gene expression, which is dependent on a precise signaling network.

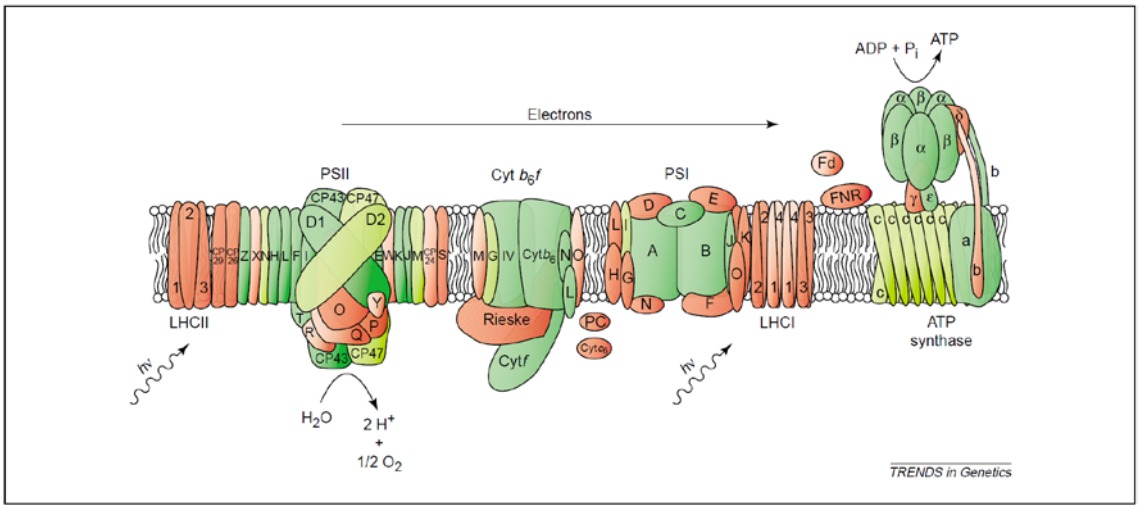

Figure 1.1: Composition of the photosynthetic apparatus in the thylakoid membrane (taken from Leister, 2003). The protein complexes photosystem II (PSII), cytochrome b_6/f (Cyt b_6/f), photosystem I (PSI) and the ATP synthase consist of nuclear encoded (marked red) and plastid encoded proteins (marked green).

1.2. The complex signaling network in plant cells

During evolution a complex interplay between the three genetically distinct compartments has been established consisting of three signaling routes (depicted in Figure 1.2.; reviewed inter alia in Leister, 2005; Bräutigam *et al.*, 2007; Pogson *et al.*, 2008; Woodson and Chory, 2008). Anterograde control mediates the control over the organelles by the nucleus. Information about the status of the organelles is transmitted by the so-called retrograde signal. The crosstalk between chloroplasts and mitochondria is made possible by complex metabolic exchange but can also affect nuclear gene expression (Pesaresi *et al.*, 2006). Last but not least all sources of signaling depend on the environmental conditions enabling fast adaptation processes in response to environmental changes.

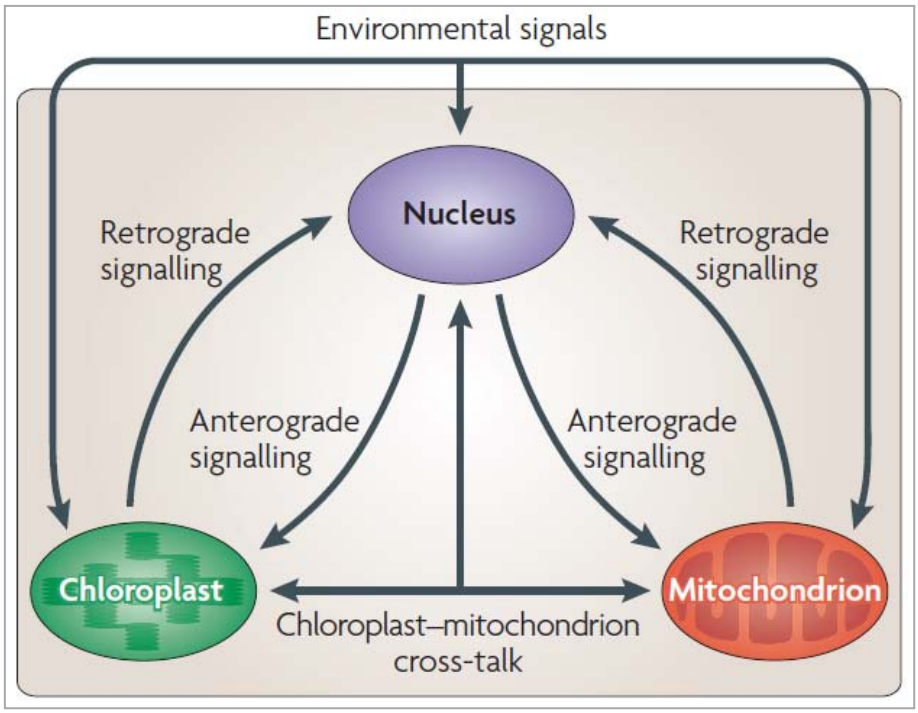

Figure 1.2: An overview of genome co-ordination between the nucleus and intracellular organelles (Woodson and Chory, 2008). The function of organelles is controlled by the nucleus (anterograde control or signaling), while the organelles send information to the nucleus in order to adapt nuclear gene expression to their current status (retrograde signaling). To optimize the complex metabolic interdependencies crosstalk between chloroplasts and mitochondria exists, which are also connected to the nucleus. Moreover, environmental conditions affect nuclear gene expression, as well as the functional activities of organelles, and thus affect both the anterograde and retrograde signaling.

Anterograde control is mainly due to the fact that the nucleus encodes almost all plastid proteins. Optimal performance of processes in plastids, like photosynthesis (see above), require appropriate expression of nuclear encoded components. Moreover, nuclear encoded proteins are involved in regulation of processes such as protein transport, assembly of multi protein complexes or biosynthesis of tetrapyrroles (summarized in Bräutigam *et al.*, 2007; Tanaka and Tanaka, 2007; Woodson and Chory, 2008). Furthermore, organellar gene expression is under control of nuclear encoded components. Transcription in plastids is mediated by two different polymerases. One of

them is encoded in the nucleus (NEP, nucleus encoded polymerase), while the genes for the core subunits of the second one are localized in plastids (PEP, plastid encoded polymerase). However, the transcription of PEP core subunits is performed by the NEP, and therefore also under nuclear control (Hess and Börner, 1999; Liere and Maliga, 2001). Additional nuclear encoded sigma factors are responsible for promoter specificity and transcript initiation of PEP (Isono *et al.*, 1997; Allison, 2000; Fujiwara *et al.*, 2000; Shiina *et al.*, 2005). In summary, nuclear control over the plastid is extensive and the principal functioning of anterograde control has been established.

The opposite is true for retrograde or plastid signaling. The concept that plastids are a source of specific signaling molecules that transmit information to the nucleus was developed 20 years ago (Taylor, 1989). But already 10 years earlier experiments with *albostrians* and *Saskatoon* mutant lines of *Hordeum vulgare* indicated the existence of such signals (Bradbeer *et al.*, 1979). These mutant lines developed in response to a recessive nuclear mutation either white or white-striped leaves which contained disrupted plastids. In the white parts of those leaves a reduction in the activity as well as in the expression of nuclear encoded plastid-localized proteins was observed that could be linked to the impairment of plastids (Bradbeer *et al.*, 1979; Hess *et al.*, 1994). Further experiments using mutations or inhibitors, such as norflurazon, chloramphenicol or lincomycin, specifically affecting pigment biosynthesis or protein synthesis in chloroplasts, could strengthen the hypothesis that specific metabolites or nucleic acids travel from plastids to the nucleus as signaling molecules (Mayfield and Taylor, 1984; Oelmuller *et al.*, 1986; Oelmuller and Mohr, 1986). After several years of research it seems to become clear that multiple retrograde signaling pathways exist (reviewed for example in Goldschmidt-Clermont, 1998; Brown *et al.*, 2001; Rodermel, 2001; Surpin *et al.*, 2002; Gray *et al.*, 2003; Leister, 2005; Nott *et al.*, 2006; Bräutigam *et al.*, 2007; Pogson *et al.*, 2008; Woodson and Chory, 2008). The signals have been assigned to four different sources: tetrapyrrole biosynthesis, protein synthesis in plastids, the redox state of plastids and the pool of reactive oxygen species (Beck, 2005; Bräutigam *et al.*, 2007; Kleine *et al.*, 2009). However, the exact nature of the proposed signaling pathways is still unknown.

The complexity in the coordination of gene expression between nucleus and organelles is not only due to the existence of the different compartments, but also the

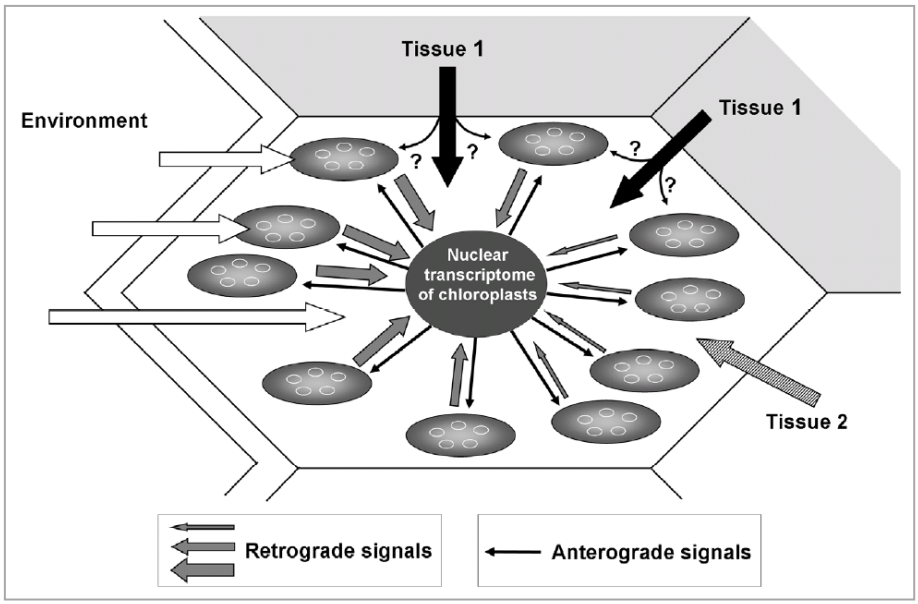

Figure 1.3: Complexity of coordination and communication between plastids and nucleus (Bräutigam *et al.*, 2007). Illustration of the 'gene copy number problem': nuclear localized genes are encoded by a single gene while plastid encoded genes are present in up to 10000 copies since cells contain many plastids and the plastome is highly polyploid. Moreover, special needs of plastids can differ among them dependent on their localization.

so-called 'gene copy number problem' (Figure 1.3.; Bräutigam *et al.*, 2007). While there is only one nucleus, normally containing one genome, cells harbor up to 100 plastids. Additionally the plastome is polyploid i.e. each plastid includes up to 100 identical plastomes and therefore several copies per gene, resulting theoretically in a 10000-fold higher gene copy number than in the nucleus. In order to maintain the stoichiometry of protein complexes consisting of nuclear and plastid encoded subunits, the transcription rate has to be adapted. Additionally, single plastids differ in their functional state dependent on their localization in the cell. Plastids in the neighborhood of different tissue cells have differential needs to those near to the environment resulting in diverse strength of retrograde signals or even unequal signals (Figure 1.3.). It is questionable if each signal is processed separately by the nucleus or if they are integrated into a coarse signal leading to a more general or averaged response (Bräutigam *et al.*, 2007). Following Occam's razor, i.e. simplest explanation tends to be

the best one, the second possibility seems to be more likely because it is the simpler one. The first one would assume that the nucleus can localize the origin of each signal and that the response is directed back to the source of the corresponding signal. No evidence for this kind of communication has yet been found.

1.3. Tetrapyrrole biosynthesis in higher plants

Tetrapyrroles are key-biomolecules that are cofactors of apoproteins involved in photosynthesis (chlorophyll), respiration and oxygen metabolism (heme), assimilation of nitrogen and sulphur (siroheme) as well as photoreception (phytochromobilin). All four compounds are synthesized by a common pathway starting with glutamyl tRNA (Figure 1.4.; Cornah *et al.*, 2003; Moulin and Smith, 2005). The enzymatic steps of this pathway are well known as are the important points of regulation (Von Wettstein *et al.*, 1995; Reinbothe and Reinbothe, 1996; Matsumoto *et al.*, 2004; Tanaka and Tanaka, 2007; Masuda, 2008). The need for a tight regulation is obvious since the four products have to be assembled with their corresponding apoproteins and the required amount differs strongly between them. Moreover, intermediates of the pathway are phototoxic, thus accumulation of them must be prevented (Moulin and Smith, 2005).

The first step of regulation is the synthesis of 5-aminolevulinic acid (ALA), which is regulated by hormones, the circadian clock, sugars and light via phytochrome and cryptochrome action (Reinbothe and Reinbothe, 1996; McCormac and Terry, 2002a; Moulin and Smith, 2005). Moreover, the glutamyl tRNA reductase, one enzyme of this step, is inhibited by heme (Vothknecht *et al.*, 1998). This is in accordance with the observation that mutants disturbed in phytochromobilin synthesis, with enhanced accumulation of heme, display reduced ALA synthesis, while plants affected in heme biosynthesis accumulate protoporphyrin IX (ProtoIX) indicating normal or even enhanced synthesis of ALA (Papenbrock *et al.*, 2001). Additionally, FLU, a negative feedback regulator of chlorophyll biosynthesis (mutants impaired in FLU accumulate protochlorophyllide when grown in the dark), has been shown to act as a regulator of ALA synthesis, independently of heme and able to bind to glutamyl tRNA reductase (Meskauskiene *et al.*, 2001; Goslings *et al.*, 2004). Altogether, feedback mechanisms of the two major branches (the chlorophyll- and the heme-branch) prevent accumulation of toxic intermediates.

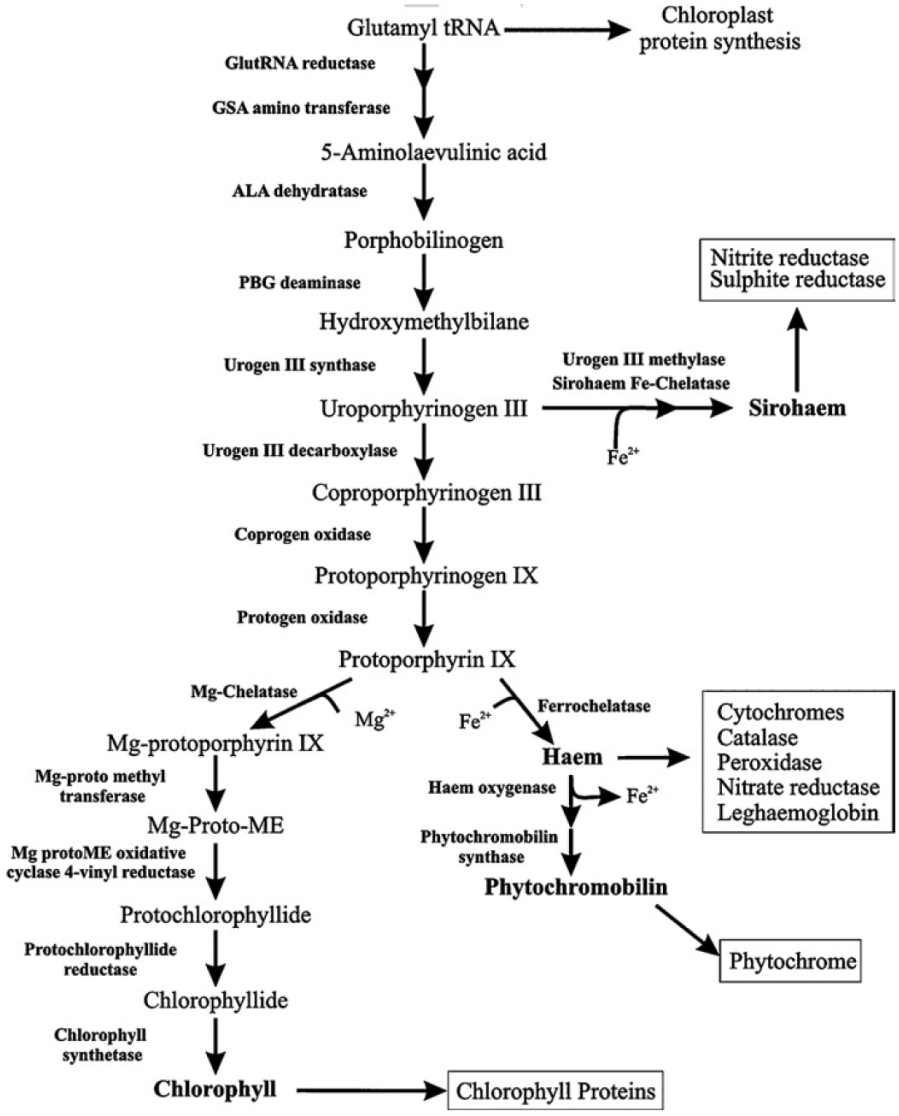

Figure 1.4: Tetrapyrrole biosynthesis pathway in higher plants (Moulin and Smith, 2005). Starting from glutamyl tRNA four end-products (chlorophyll, heme, phytochromobilin and siroheme) are synthesized which have to be assembled with their appropriate apoproteins (boxed). In addition to specific feedback mechanisms within tetrapyrrole biosynthesis it is suggested that intermediates are involved in retrograde signaling (for details see text).

Another important step of control is the branch point between the chlorophyll and the heme biosynthesis pathway (Figure 1.4.). The two competing chelatases incorporate either magnesium (Mg-chelatase; chlorophyll-branch) or iron (Fe-chelatase;

heme-branch) into ProtoIX. Although both reactions are similar, the two enzymes are quite different. While Mg-chelatase consists of three different subunits (ChlH, ChlI and ChlD), which are assembled into a hexameric ring structure, a process consuming ATP (Masuda, 2008), Fe-chelatase acts as a monomer or homodimer without any cofactor (Cornah *et al.*, 2003). Additionally, Fe-chelatase is inhibited by ATP, thus regulation between both enzymes is in part due to ATP availability. During the day the ATP level is higher thus Mg-chelatase will be more active while in the dark the opposite is the case (Walker *et al.*, 1997). This is just one part of the regulatory mechanism of tetrapyrrole biosynthesis evolved in higher plants.

Except for the steps of heme biosynthesis that were also found in mitochondria (Cornah *et al.*, 2003), tetrapyrrole biosynthesis takes exclusively place in plastids while the products are localized either in all cellular compartments (heme, siroheme), or in the cytosol and nucleus as it is the case for phytochromobilin, the chromophor of phytochrome. Only chlorophyll acts solely in the plastid. Accordingly, the final products or even intermediates (in the case of potential heme biosynthesis in mitochondria) have to be transported out of the plastid. Until now nothing is known about the nature of such transport processes. The only candidate protein suggested to export ProtoIX from the chloroplast (atABC1; Moller *et al.*, 2001) was re-evaluated and found to be involved in iron-sulfur cluster assembly and regulating iron homeostasis (Xu *et al.*, 2005).

1.4. Tetrapyrroles as signaling molecules

First evidence that tetrapyrrole intermediates could be involved in retrograde signaling was obtained from experiments with *Chlamydomonas reinhardi*. Blocking chlorophyll biosynthesis by feeding α,α-dipyridyl yielded in accumulation of magnesium-protoporphyrin IX methyl ester (Mg-protoME) followed by a reduction of *LHCB* (nuclear encoded gene for photosystem II chlorophyll a/b-binding protein) mRNA accumulation. The reduction of *LHCB* mRNA accumulation was not observed when inhibitors of chlorophyll biosynthesis prior to magnesium-protoporphyrin (Mg-proto) formation were used (Johanningmeier and Howell, 1984). In subsequent studies the expression of *HSP70* (encodes for heat-shock protein 70) was used as a reference as it is induced either by heat-shock or by light (Kropat *et al.*, 1995). In mutants defective

in Mg-proto formation the induction of this gene by light was disrupted while feeding of Mg-proto or Mg-protoME was sufficient for induction even in dark grown wild-type plants (Kropat *et al.*, 1997). Moreover, feeding of protoporphyrin IX (ProtoIX), protochlorophyllide and chlorophyllide failed to induce this light specific induction. More detailed analysis revealed that externally applied ProtoIX, contrary to Mg-proto, was transported to the plastids where it was converted into Mg-proto (Kropat *et al.*, 2000). When kept in the dark, the accumulated Mg-proto is retained in the plastids therefore it is not able to induce *HSP70* expression. It was concluded that light is necessary for Mg-proto release from the plastid for its proper function (Kropat *et al.*, 2000). Further research identified specific promoter elements inducible not only by light and Mg-proto but also by heme (von Gromoff *et al.*, 2006; von Gromoff *et al.*, 2008) and it was shown that the gene coding for glutamyl tRNA reductase (*HEMA*) responds to feeding of Mg-proto and heme (Vasileuskaya *et al.*, 2005). This is in accordance to known feedback mechanisms in tetrapyrrole biosynthesis. As most evidence was obtained by feeding experiments of chemical compounds, direct evidence for an *in vivo* signaling pathway is still missing.

The first genetic screen for mutants specifically affected in retrograde signaling was performed in *Arabidopsis thaliana* (Susek *et al.*, 1993). The screen was based on the fact that norflurazon (NF; an inhibitor of carotenoid biosynthesis that causes plastid disruption) inhibits the light-dependent induction of nuclear encoded photosynthesis genes like *LHCB* (Oelmuller and Mohr, 1986). Transgenic plants were generated harboring a construct containing a hygromycin resistance gene and a β-glucuronidase reporter gene (GUS), both under control of the full-length *LHCB1.2* promoter. Plants were mutagenized with ethyl methane sulfonate (EMS) and screened for hygromycin resistance and for GUS activity in photo-bleached conditions caused by NF. Five different mutant lines were selected in which the *LHCB1.2* promoter was still active. These were named *gun* (for *genomes uncoupled*) (Susek *et al.*, 1993). With the exception of *gun1*, which encodes a nucleic-acid-binding chloroplast protein (Koussevitzky *et al.*, 2007), all *gun* mutants were found to be affected in tetrapyrrole biosynthesis (Mochizuki *et al.*, 2001; Larkin *et al.*, 2003). Whereas *gun2* and *gun3* are allelic to the known photomorphogenic mutants *hy1* and *hy2* required for phytochromobilin synthesis (Parks and Quail, 1991; Mochizuki *et al.*, 2001), *gun5* is

affected in the gene encoding the H subunit of Mg-chelatase (Mochizuki *et al.*, 2001) and *gun4* in an before unknown gene. Biochemical analysis revealed that the protein GUN4 is able to bind ProtoIX and Mg-proto and thus is thought to be needed for regulation of Mg-chelatase activity (Larkin *et al.*, 2003; Davison *et al.*, 2005; Verdecia *et al.*, 2005; Adhikari *et al.*, 2009). Taken together, it is obvious that tetrapyrrole biosynthesis is connected to retrograde signaling in *A. thaliana*.

Early analysis of the *gun* mutants indicated that the H subunit of Mg-chelatase was responsible for the de-repressed expression of *LHCB1.2* after NF treatment because mutations in the I subunit of Mg-chelatase did not result in a *gun* phenotype although other parameters, like the content of tetrapyrroles, were comparable to those seen in *gun5* (Mochizuki *et al.*, 2001). ProtoIX and Mg-proto/Mg-protoME levels in untreated plants display no differences between wild-type and *gun5* mutant plants. Moreover, after NF-treatment it was not possible to detect ProtoIX or Mg-proto/Mg-protoME neither in wild-type nor in mutants. Hence, a possible involvement of tetrapyrrole intermediates as signaling molecules was ruled out (Mochizuki *et al.*, 2001). Recent experiments with *Synechocystis* sp. PCC 6803 support this initial idea (Osanai *et al.*, 2009). It could be shown that the H subunit of Mg-chelatase inhibited the transcription activity of sigma factor SigE by direct interaction thereby preventing SigE dependent gene expression. However, in *A. thaliana* another theory became dominant. Contrary to experiments described above, where Mg-proto was not detectable after NF treatment, it could be shown that Mg-proto accumulates more than 10-fold in NF-treated wild-type seedlings compared to untreated seedlings (Strand *et al.*, 2003). The increase was smaller in *gun2* and *gun5* mutants, suggesting that accumulation of Mg-proto correlates with the repression of *LHCB1.2*. Additionally, NF-grown *gun2* and *gun5* plants were treated for 8 h with dipyridyl (DP; Fe-chelatase inhibitor known to increase Mg-proto levels) in order to elevate the levels of Mg-proto and its methyl ester. Indeed, this was sufficient to prevent the de-repression phenotype suggesting that Mg-proto accumulation is responsible for the *LHCB1.2* repression (Strand *et al.*, 2003). To rule out possible toxic effects of DP an additional inhibitor was used (S23142; inhibitor of protoporphyrinogen oxidase), repressing the accumulation of Mg-proto. Indeed, the *gun* phenotype was again visible in *gun2* and *gun5* seedlings treated with NF, DP and S23142 but conflicting to this not in wild-type seedlings in which Mg-proto formation

was also inhibited by S23142. Altogether, the obtained results were inconsistent, hence a coherent explanation is missing. Even thought that Mg-proto could be visualized in the cytosol during stress conditions i.e. NF-treatment (Ankele *et al.*, 2007) it is still questionable how, and when it is transferred out of the plastid.

Compared to *gun* mutants affected in tetrapyrrole biosynthesis strong evidence exists that *gun1* is disturbed in another signaling pathway. The double mutant lines *gun1 gun4* and *gun1 gun5* showed a synergistic effect on *LHCB1.2* de-repression while their chlorophyll content was similar to the single mutants *gun4* and *gun5*, respectively (Mochizuki *et al.*, 2001). Cluster analysis of genes expressed differentially in NF-treated *gun* mutants compared to NF-treated wild-type plants (obtained by microarray analysis) pointed out that *gun1* showed a different gene expression profile than *gun2* and *gun5* which clustered together (Strand *et al.*, 2003). In addition to this, the expression of *HEMA1* is predominantly controlled by the *gun1* pathway, whereas *LHCB1.2* expression is affected by both pathways (McCormac and Terry, 2004) and repression of *LHCB1.2* by lincomycin, an inhibitor of plastid translation, is suppressed in *gun1* but not in the other *gun* mutants (Gray *et al.*, 2003; McCormac and Terry, 2004). On the other hand, recent analysis of global gene expression response to NF showed a similar pattern between a new *gun1* allele (*gun1-9*) and *gun5* (Koussevitzky *et al.*, 2007). This emphasizes that GUN1 could be involved in two or more proposed signaling pathways. Koussevitzky *et al.* (2007) proposed a model in which the tetrapyrrole dependent, the plastid translation dependent and the redox dependent pathway converge in the plastids upstream of GUN1 and that GUN1 is required for generating or transmitting a unknown signal to the nucleus which is common to all three pathways. To resolve the conflicts between the obtained results more data will be necessary.

Therefore, it is necessary to establish exact and reproducible methods to determine the amount of the most important tetrapyrrole biosynthesis intermediates in order to investigate if there is a direct link of endogenous tetrapyrrole accumulation to retrograde signaling. This is difficult to accomplish as the levels of intermediates are very low, owing to their phototoxic behavior, and high levels of chlorophyll interfere in currently used methods (Moulin and Smith, 2005). However, in the course of this thesis two groups independently solve these technical challenge. The obtained result clearly

showed that the accumulation of tetrapyrrole intermediates (except from the Fe-branch, which were not investigated) did not correlate with the expression of *LHCB1.2* in *A. thaliana* (Mochizuki *et al.*, 2008; Moulin *et al.*, 2008).

1.5. Aims of the thesis

The initial goal was to isolate mutants for all enzymes of the tetrapyrrole biosynthesis pathway in order to test them for their capability to exhibit the *gun* phenotype. The expression pattern of the reference gene *LHCB1.2* and the specific tetrapyrrole composition of the mutants determined after separation by reverse-phase HPLC should be correlated. Since the accumulation of ProtoIX, Mg-proto and Mg-protoME did not correlate to *LHCB1.2* mRNA accumulation, as it was published recently (Mochizuki *et al.*, 2008; Moulin *et al.*, 2008), we focused mainly on the characterization of norflurazon treated *gun* mutants in order to find an explanation for the *gun* specific de-repression of *LHCB1.2* mRNA accumulation.

It was intended to investigate whether heme accumulates after norflurazon treatment and if such an accumulation could be linked to the *LHCB1.2* de-repression phenotype.

The strong photo-oxidative damage caused by norflurazon treatment was planned to characterize in detail with the main focus on differences between wild-type plants and *gun* mutants. To this intent, protein, metabolite, lipid and pigment compositions were determined, as well as the expression pattern of marker genes specific for reactive oxygen species and the redox-state of plastids. Furthermore, phytoene levels were analyzed in norflurazon treated wild-type and *gun* seedlings to evaluate if the *gun* mutants are to some degree resistant to the inhibitor.

In a third approach we tested if different growth conditions would reveal information about the specific behavior of the *gun* mutants compared to wild-type plants. Thereby we also investigated if norflurazon can be replaced by other inhibitors of carotenoid biosynthesis such as amitrole, flurochloridone and CPTA.

2. Material & Methods

2. 1. Material

Standard chemicals (analytical grade) used in this work were purchased from Applichem (Darmstadt, Germany), Duchefa (Haarlem, Netherlands), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma-Aldrich (Steinheim, Germany). Radioactive labeled dCTP was obtained from Hartmann-Analytik (Braunschweig, Germany). For western analyses primary antibodies from Agrisera (Vänas, Sweden) and secondary antibodies from GE Healthcare (Munich, Germany) and Sigma-Aldrich (Steinheim, Germany) were used.

2. 2. Methods

2.2.1. Plant material, growth conditions and inhibitor treatments

The *gun1*, *gun2*, *gun4* and *gun5* mutants were obtained from Joanne Chory (Susek *et al.*, 1993; original designated *gun1-1*, *gun2-1*, *gun1-4* and *gun0-6*). The other lines, described in Table 2.1, were identified in the SIGnAL database (Alonso *et al.*, 2003). T-DNA insertions were confirmed by PCR using the primers listed in Table 2.2. After stratification (2 days at 4°C in the dark) seedlings were grown on Murashige and Skoog medium (MS medium; Murashige and Skoog, 1962; Sigma-Aldrich, Schnelldorf, Germany) containing 1.5% (w/v) sucrose and 0.3% (w/v) gelrite for 6, 10 or 12 days at 22°C under continuous light conditions (100 µmol m^{-2} sec^{-1}).

For photobleaching experiments, seedlings were grown on MS medium supplemented with 0.5 µM (or 5 µM) norflurazon (Sigma-Aldrich, Steinheim, Germany), 100 µM amitrol (Sigma-Aldrich, Steinheim, Germany), 100 µM flurochloridon (Sigma-Aldrich, Steinheim, Germany) or 15 µM (or 45 µM) CPTA (2-(4-chlorophenylthio)triethylamine hydrochloride; obtained from Ralph Bock), respectively. The effect of ABA on photo-bleached seedlings was tested by adding different concentrations to MS medium containing 0.5 µM norflurazon. For the ABA germination assay plants were grown on MS medium without sucrose containing different concentrations of ABA (Choy *et al.*, 2008).

Additionally different experiments with altered growth conditions were performed. To test the effect of light, plants were grown under low-light conditions (10

µmol m^{-2} sec^{-1}), as well as in the dark. A dark-light cycle of 12 h was used to analyze a potential influence of the circadian rhythm.

To obtain seeds plants were transferred to soil in the greenhouse after 12 days of growth on MS plates.

2.2.2. Nucleic acid extraction

For DNA isolation leaf tissue was homogenized in 400 µl extraction buffer containing 200 mM Tris/HCl (pH 7.5), 25 mM NaCl, 25 mM EDTA and 0.5% (w/v) SDS. After centrifugation for 3 minutes at 13.000 rpm, DNA was precipitated from the supernatant by the use of 300 µl isopropyl alcohol. After 5 minutes of centrifugation at 13.000 rpm the obtained pellet was washed with 70% (v/v) ethanol, centrifuged again (3 minutes at 13.00 rpm) and dried. The DNA was resolved in distilled water.

For total RNA isolation frozen plant tissue was grounded in liquid nitrogen and affiliated in 1 ml TRIZOL (Invitrogen, Carlsbad, California). After centrifugation for 10 minutes (13.000 rpm) and additional incubation at room temperature for 5 minutes 500 µl chloroform was added to the supernatant. Further 5 minutes of incubation on ice and centrifugation for 15 minutes at 13.000 rpm led to a clear phase separation. RNA was precipitated from the aqueous phase by adding 400 µl isopropyl alcohol, incubating 10 minutes at room temperature and centrifugation at 13.000 rpm for 10 minutes. The obtained pellet was washed with 70% (v/v) ethanol, centrifuged for further 5 minutes at 7.500 rpm and dried. RNA was resolved in RNase free water. Concentration and purity of RNA samples were determined spectroscopically using a GeneQuant *pro* RNA/DNA Calculator (GE Healthcare, Munich, Germany). Isolated RNA was stored at -80°C until further use.

2.2.3. Northern blot analysis

Northern blotting and hybridisation of probes were performed using standard procedures (Sambrook and Russel, 2001). 10 µg of total RNA were denatured for 5 minutes at 65°C and separated on an 1.2 % agarose gel, blotted on nylon membrane (Roche, Grenzach-Wyhlen, Germany) and hybridised for 16 hours at 62 °C (hybridisation buffer: 1.8 % (w/v) NaH_2PO_4 x H_2O; 6.6 % (w/v) Na_2HPO_4 x 2 H_2O; 7 % (w/v) SDS; 1 % (w/v) BSA; 1 mM EDTA) with cDNA probes labeled with ^{33}P-dCTP

using random hexamer primer and Klenow Fragment (New England Biolabs, Hertfordshire, United Kingdom). After washing three times (20 minutes at 62 °C; washing buffer: 1 % (v/v) SDS; 0.4 % (v/v) 0.5 M EDTA; 8 % (v/v) Na-P [74.4 % (v/v) 0.5 M Na_2HPO_4 x 2 H_2O and 25.6 % (v/v) 0.5 M NaH_2PO_4 x H_2O]) the filters were exposed to a phosphorimager screen and analysed by a Typhoon Variable Mode Imager (GE Healthcare, Munich, Germany) using ImageQuant version 5.2 (GE Healthcare, Munich, Germany).

Following primer combinations were used to generate specific cDNA probes: *Actin 1* (*At2g37620*), forward primer: 5´- TGCGACAATGGAACTGGAATG -3´, reverse primer: 5´- GGATAGCATGTGGAAGTGCATACC -3´; *LHCB1.2* (*AT1G29910*), forward primer: 5´- TCAGCTGATCCCGAGACATTC -3´, reverse primer: 5´- CTCTGGGTCGGTAGCAAGACC -3´

2.2.4. cDNA synthesis and real-time RT-PCR

cDNA was prepared from 1 µg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. cDNA was diluted 30-fold, and 2 µl of the diluted cDNA was used in a 20-µl iQ SYBR Green Supermix reaction (Bio-Rad, Munich, Germany). Reactions were performed in triplicate for expression analysis of genes affected by T-DNA insertions in corresponding mutant lines or in duplicate for ROS and metabolic marker genes. Thermal cycling consisted of an initial step at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C, 30 s at 55°C and 10 s at 72°C. Afterwards a melting curve was recorded. RT-PCR was monitored by using the iQ5 Real-time PCR Detection System (Bio-Rad, Munich, Germany). Baseline and threshold adjustments were performed according to the manufacturer's instructions. Investigated genes and corresponding primers are listed in Table 2.3. The relative abundance of transcripts was normalized to the constitutive expression levels of ubiquitin-protein ligase-like protein mRNA (*At4g36800*), actin1 mRNA (*At2g37620*) and 18S rRNA (*At3g41768*). The data were analyzed using LinRegPCR (Ramakers *et al.*, 2003) and according to Pfaffl (2001).

2.2.5. Protein isolation and Western blotting

Proteins from 6-day-old plants were homogenized in 2xSDS protein buffer (62.5 mM Tris/HCl (pH 6.8); 20% glycerin; 4% SDS; 100 mM DTT; 0.05% bromophenol blue), incubated for 7 minutes at 75°C and centrifuged for 15 minutes at 13.000 rpm. The amount of proteins in the supernatant was quantified by amidoblack staining (Schaffner and Weissman, 1973). For that purpose 5 µl of each protein extract were used. After addition of 195 µl water and 800 µl staining solution (10 % acetic acid, 90 % methanol and small amount amidoblack) solved material was centrifuged for 12 minutes at 13.000 rpm. The obtained pellet was washed in 1 ml washing solution (10 % acetic acid and 90 % methanol), mixed and centrifuged again for 12 minutes at 13.000 rpm. Air dried pellet was resolved in 1 ml 0.2 M NaOH. Extinction was measured at 615 nm and data were calibrated using a BSA calibration curve.

Proteins were then separated by vertical electrophoresis via 12% SDS-polyacrylamide gels and transferred to PVDF (polyvinylidene fluoride) membranes (Millipore, Billerica, USA) using a semi-dry blotter (Bio-Rad, Munich, Germany) according to Towbin *et al.* (1979). For protein transfer a current corresponding to 0.8 mA cm^{-2} was applied. Used cathode buffer contain 0.025 M Tris (pH 9.4), 40 mM glycine and 10 % methanol and the anode buffers contain 0.025 M Tris (pH 10.4) or 0.3 M Tris (pH 10.4), respectively, and 10 % methanol. Subsequently, filters were incubated with specific primary and corresponding secondary antibodies. Detection was done using Pierce Fast Western Blot Kit (Thermo Scientific, Rockford, USA).

2.2.6. Determination of metabolic levels

Phosphorylated intermediates of the central carbohydrate metabolism were determined from neutralized perchloric acid extracts of plants in a fluorescence microtiter plate reader as established by Haeusler *et al.* (2000). Carboxylic acid contents were determined from the same extracts according to Schneidereit *et al.* (2006).

For spectrophotometric determination of glucose-6-phosphate, fructose-6-phosphate and ATP an enzymatic assay was performed using in series glucose-6-phosphate dehydrogenase, phosphoglucose isomerase and, after addition of glucose, hexokinase (as described and referenced in Dietz and Heber (1986)). Metabolite amounts were calculated from changes in NADH-absorbtion at 340 nm.

2.2.7. Heme measurements

According to Thomas and Weinstein (1990), free heme was isolated from approximately 20 mg liquid nitrogen ground plant material by adding five times 1 ml acetone containing 10 mM NH_4OH. The heme content was determined using a chemiluminescent-based method described in Masuda and Takahashi (2006). 25 µl of isolated material was incubated for 30 minutes together with 75 µl of 100 mM Tris-HCl (pH 8.4) containing 25 nM horseradish peroxidase (Biozyme Laboratories, Blaenavon, South Wales, U.K.). After addition of luminol (50 µl ECL1) and hydrogen peroxide (50 µl ECL2) from Pierce Fast Western Blot Kit (Thermo Scientific, Rockford, USA) and further incubation for 30 minutes luminescence was measured (10 seconds initiation time) using a Tecan Safire2 microplate reader (Tecan Group, Männedorf, Switzerland).

2.2.8. Pigment analysis

Liquid nitrogen frozen plant material was homogenized in 1 ml acetone and centrifuged for five minutes at 12.000 rpm. Carotenoids and chlorophylls were monitored at 440 nm after separation by reverse-phase HPLC (LiChroCART 4-4 and LiChroCART 250-4, Merck, Darmstadt, Germany) according to Farber *et al.* (1997). Solvent A (acetonitril, methanol and 0.1 M Tris/NaOH (pH 8.0); 87:10:3) run for 9 minutes at a flow-rate of 2 ml per minute before a gradient to solvent B (methanol and hexane; 4:1) was set for 3.5 minutes. After five more minutes separation was done and all pigments determined at their specific retention time: neoxanthin (3.2 min), violaxanthin (4.2 min), antheraxanthin (6.3 min), lutein (9.3 min), zeaxanthin (10.3 min), chlorophyll b (13.3), chlorophyll b (13.9) and β-caroten (16.8 min). Specific conversion factors were used to determine the concentration of the pigments (Farber *et al.*, 1997).

For phytoene determination 30 mg liquid nitrogen ground plant material was homogenized in 2 ml methanol containing 6% potassium hydroxide and incubated for 20 minutes at 60°C. After addition of 1 ml 10% dieethyl ether in hexane and 1 ml saturated sodium chloride the upper phase was collected and the lower one was extracted a second time with 1 ml 10% dieethyl ether in hexane. Collected product was evaporated and dissolved in 120 µl acetone. Pigments were separated by reverse-phase HPLC on a GROM-SIL 120 ODS-5 ST C18 column (Alltech Grom, Rottenburg-

Hailfingen, Germany) by an isocratic flow in acetonitrile, methanol and isopropyl alcohol (85:10:5) at a flow-rate of 120µl per minute. Phytoene was monitored at 296 nm (absorption maxima at 275 nm, 285 nm and 297 nm; Herber *et al.* (1972); Giuliano *et al.* (1986)).

2.2.9. Lipid extraction and thin layer chromatography

Plants were ground in liquid nitrogen, solved in 1 ml dichlormethane and methanol (1:2) and centrifuged for 10 minutes at 13.000 rpm. After collecting the supernatant the pellet was extracted a second time in 300 µl dichlormethane and centrifuged again for 10 minutes at 13.000 rpm. Afterwards 450 µl 0.1 M sodium chloride were added to the combined extract and centrifuged for 5 minutes at 13.000 rpm. The resulting aqueous phase was collected, washed with 200 µl dichlormethane and centrifuged for further 5 minutes at 13.000 rpm. Both organic phases were combined, evaported with gaseous nitrogen and stored at -20°C until separation. Whenever possible, probes were kept dark during the process of isolation.

For thin layer chromatography samples were dissolved in 50 µl acetone loaded on an TLC Aluminum backed plate (Silicia Gel 60 F254, Merck, Darmstadt, Germany) and separated by a mixture of chloroform, methanol and water (65:25:4). According to De Santis-Maciossek *et al.* (1999) the chromatogram was sprayed with a ferrous sulfate/potassium permanganate solution and incubated for 20 minutes at 100°C.

2.2.10. Plant images

Images from plants were taken by a SteREO Lumar.V12 connected to an AxioCam MRc using AxioVision 4.7 software (Carl Zeiss, Munich, Germany).

Table 2.1: List of investigated T-DNA insertion lines selected from the SIGnAL database (Alonso *et al.*, 2003).

300-UTR3, 300 base-pair untranslated region from the 3' mRNA end to the last codon of translation; 300-UTR5, 300-UTR from the 5' end to the first codon of translation

Gene	Protein	Mutation	Mutant line	Insertion site[1]
AT1G58290	Glutamate tRNA reductase	*hema1-1*	SALK_053036.54.60.x	exon 3
		hema1-2	SALK_067622.41.75.x	300-UTR3
AT1G69740	ALA dehydratase	*alad1-1*	SALK_016544	intron 5
		alad1-2	SAIL_81_A04.v1	1000-promoter
AT5G14220	Protogen oxidase	*ppo1-1*	SALK_060822.50.90.x	exon 3
		ppo1-2	SALK_119885.49.55.x	exon 2
AT4G01690	Protogen oxidase	*ppo2-1*	SALK_017634	exon 9
		ppo2-2	SALK_143057.56.00.x	exon 7
AT5G13630	Mg-chelatase H-subunit	*chlh-1*	SALK_062726.54.50.x	exon 1
		chlh-2	AL754413	exon 3
AT3G59400	GUN4	*gun4-2*	SALK_026911.46.85.x	exon
		gun4-3	SALK_134687.40.35.x	300-UTR3
AT4G25080	Mg-proto methyl transferase	*chlm-1*	SALK_110265.54.80.x	300-UTR5
		chlm-2	BX546974	-
		chlm-3	FLAG_340C02	exon 2
AT3G56940	Mg-protoME oxidative	*crd1-1*	SALK_009052.47.55.x	300-UTR5
	cyclase 4-vinyl reductase	*crd1-2*	AL764967	exon 1
AT5G26030	Ferrochelatase	*fc1-1*	SALK_150001.42.45.x	300-UTR5
		fc1-2	AL756537	exon 3
AT3G09150	Phytochromobilin synthase	*gun3-2*	SALK_027641.19.40.n	1000-promoter
		gun3-3	SALK_104923.54.75.x	intron 1
		gun3-4	FLAG_255C02	1000-promoter
AT2G26670	Heme oxygenase	*gun2-2*	SALK_147500.54.50.x	300-UTR3
		gun2-3	AL763127	exon 1
AT5G63570	GSA amino transferase	*gsa1-1*	SALK_079035.23.50.x	exon 2
		gsa1-2	SALK_089320	exon 2
AT5G08280	PBG deaminase	*hemc-1*	SALK_040880	exon 5
		hemc-2	SALK_021519.56.00.x	300-UTR5
AT2G26540	Urogen III synthase	*hemd-1*	SALK_065522.48.85.x	exon 2
		hemd-2	SALK_147103.18.00.x	intron 4
AT2G40490	Urogen III decarboxylase	*heme2-1*	SAIL_574_B06	300-UTR5
		heme2-2	SAIL_355_D11	intron 3

AT3G14930	Urogen III decarboxylase	*heme1-1*	SALK_067370.19.95.x	exon 3
		heme1-2	SALK_107425.37.30.x	300-UTR5
AT1G03475	Coproporphyrinogen III	*cpo-1*	SALK_025589.49.40.x	exon 1
	oxidase	*cpo-2*	SALK_072155.55.75.x	intron 7
AT5G54190	Protochlorophyllide	*pora-1*	SALK_022639.28.45.x	exon 3 / 5
	reductase	*pora-2*	SALK_036137.56.00.x	1000-promoter
AT4G27440	Protochlorophyllide reductase[2]	*porb-1*	SALK_060191.43.55.x	exon 4
AT1G03630	Protochlorophyllide reductase[2]	*porc-1*	SALK_141477.22.00.n	300-UTR5
AT3G51820	Chlorophyll synthetase	*chlg-1*	SALK_112733.14.95.x	exon 6
		chlg-2	SALK_134433.51.80.x	exon 14
AT1G44446	Chlorophyll a oxygenase	*cao-1*	SALK_024295.33.50.x	exon 7 / 8
		cao-2	SALK_085897.49.80.x	exon 6

[1] Based on SIGnAL database (Alonso *et al.*, 2003); [2]TP39 & TP42 not available

Table 2.2: List of primer's used for confirmation of T-DNA insertions.
LP, gene specific left primer; RP, gene specific right primer

Mutant line	Primers (5' – 3')
hema1-1	LP – CTGAATGGCCAAGAGCTATTG
	RP – AACCTCTGGAGAAGCTTGAGG
hema1-2	LP – AATCTTAACCAAGTTTGATCAGCC
	RP – TATCAACAAGAAAACAACGAGAGC
alad1-1	LP – GAGACTTGTCCCTCAAAAGCC
	RP – AGAATTCAACAGGGGATGAGG
alad1-2	LP – GTACGGGATCTTGATTCCTCC
	RP – TAGTCGAAGGAAAGCTGCAAG
ppo1-1	LP – TCTACCTTAGATTGGGTAGAGAGC
	RP – ATTGTTGCAGATAGCAATGGC
ppo1-2	LP – GTTTGTGTTCGAACTTTAATGTGC
	RP – TGAAGATTCTAAAGCACGTTTGAC
ppo2-1	LP – AGCCAACAACTCCGTTTTAGC
	RP – CGCAAGGAGTTGAAACATTAGG
ppo2-2	LP – TGGTGGAAGCATAATAGGTGG
	RP – TGCGGAATCAAAACAAAAGAG
chlh-1	LP – TTCCCACTCTTGCGTAATGAC
	RP – AAGACGCCAACATCTTCATTG
chlh-2	LP – TCGATGTTCCTTACCTTGTGG
	RP – ATTGGGAATGTTCCCGATAAG
gun4-2	LP – TCAAACCTGAGCCATAAGTGAC
	RP – AAGGAGAAAGACATCAGAAGCTG
gun4-3	LP – CTCTGCTTCTTCCACCTCCTC
	RP – AGGTGCAGCTTTCTTGAACAG
chlm-1	LP – ATAATTGAATCGAAGAAGCTCACC
	RP – ACACACGCTCACATAAACATACAC
chlm-2	LP – TTGTTACTTGTATAATACTCT
	RP – GTACAGAAGGATATTCGACTC
chlm-3	LP – TGCATGCAGGGTATAGTAACAAAG
	RP – CTACTTCTCTAGGCTCATCGAAGC
crd1-1	LP – TCAAGCATAACGTAGACAAGGAAC
	RP – TACCAGAATGGTCTGGATATCACC
crd1-2	LP – CTGTTGTCGGAATTATTTTTCC
	RP – GAAGATCTCAGCCACAACAGG

fc1-1	LP – TGACTTTAATTAGTGAACGAAAACAAAG
	RP – GTCATAGACTCTCCGAAAGATCTCTC
fc1-2	LP – TCTGGGTTCAATCCTCTAACG
	RP – ACATAGCCTCGCCTTTGGTAC
gun3-2	LP – TGAAGATTTAAAAGCAAGCATTTG
	RP – TGGTAGAAAACAATTTTTCTATTTTAATG
gun3-3	LP – TGGATTCACCAGTCAATTTCC
	RP – AGAGAGTGTCCGAGGAAGGAG
gun3-4	LP – AGCAATCAGTTTGTACTGCTGAAG
	RP – AAATTCTTTTATTTTAGTTGGATAAACAC
gun2-2	LP – TGAGTTTGCTTATGTATTGGAAAAAG
	RP – GTTGATGCTTCATTGACTCTTCTTAG
gun2-3	LP – GCAAACTCAGTTTTTAATTACGAGC
	RP – TCAAACCATGGCGTATTTAGC
gsa1-1	LP – TTTCAGATCAAAGATGTCGGC
	RP – ATCACGTCTACCACCGTATGC
gsa1-2	LP – TTAAATTTCACACAAATTCCATGC
	RP – GTCTTTAGTAAGCTGGCGTAAACC
hemc-1	LP – TTTATTGCCTTTTGCAGGTTG
	RP – GAAGATCGGAGGAAATGAAGC
hemc-2	LP – CCAAATTCGCAAGAACAAAAC
	RP – TTCGGGTTTTCTGTTCAACAG
hemd-1	LP – TTGAAGATGCACAGATTGCAG
	RP – CTCTGAGCAAACCAGTTTTCG
hemd-2	LP – TTGTCTACTAAGTGTAACGACGGC
	RP – GGAATGTGTTGCTAGTGAGTTCAC
heme2-1	LP – AAATGTTGAAGGAACTAAAGCAGG
	RP – TATAGCTTCTGCAGAACAAACTCG
heme2-2	LP – CATTTTGATGAAGTTTTTGGTGAG
	RP – AGAAAGTGTAAATGGAGCTCCAAC
heme1-1	LP – AGCAGGAAGCGGAGAGAATAG
	RP – TCCAGTTTTGGGTCACTTCAC
heme1-2	LP – TTGATGAGAACGTTTAAGAGCAAG
	RP – CAATCTATTCCACAAGCTCACAAG
cpo-1	LP – CTGTGAAGCAGGGTTTTCTTG
	RP – GGGAGAGATTCATTACCGAGC
cpo-2	LP – TGGGCTGATCACTGTTTCTTC
	RP – ATTGGTGTTTACAGAATGCGC

pora-1	LP – TCATGTGCTCCGTAAAAGTCC
	RP – ATTGCAACGACTGGTTTGTTC
pora-2	LP – AACAACAAAACCGTCACCAAC
	RP – CATCTTTGCGGACAGAGAAAG
porb-1	LP – TCCAATGAACAAGATCAAGGC
	RP – TGATTGATGGAGGAGATTTCG
porc-1	LP – AATTGGGCTCAAAAAGGTTTC
	RP – TGGCTGTAACTGTCTGTGCAC
chlg-1	LP – AGGTTTGCCAAAAGAGTACCC
	RP – TACAAAACCAGTCACTTGGCC
chlg-2	LP – AAACTGCAAAATGGATATGCG
	RP – AACAAAAACATTCTGAAAGCAGAG
cao-1	LP – GCTGAAGAAACTTTCGATTGG
	RP – AGGTATGATCTGGATTTGGCC
cao-2	LP – TGTTTGCTCCGTTTAACATCC
	RP – TCACTGCAGATCTCAAGCATG

Table 2.3: List of primer's used for real-time PCR.

fwp, forward primer; rwp, reverse primer

Gene	Description	Primers (5' – 3')
	Loading control	
At4g36800	ubiquitin-protein ligase-like protein	fwp - CTGTTCACGGAACCCAATTC rwp - GGAAAAAGGTCTGACCGACA
At2g37620	actin 1	fwp - TTCACCACCACAGCAGAGC rwp - ACCTCAGGACAACGGAATCG
At3g41768	18S rRNA	fwp - TCAACTTTCGATGGTAGGATAGTG[1] rwp - CCGTGTCAGGATTGGGTAATTT[1]
	LHCB1.2	
At1g29910	light harvesting chlorophyll a/b binding protein 1.2	fwp - CCGTGAGCTAGAAGTTATCC rwp - GTTTCCCAAGTAATCGAGTCC
	ROS marker genes	
At2g43510	defensine-like protein	fwp - CTTAGTCATTTCCGATGTGCC rwp - GCATCTTCCACCTTTAGCTC
At1g10585	bHLH transcription factor	fwp - AAACTCAGTATTCGTTCACTGG rwp - GACATATGATAGCCTGGGCT
At1g17170	glutathione S-transferase	fwp - CGAGTACATAGACGAGACTTGG rwp - GTATTTCGATTAACTCCTTGGCTG
At1g26380	FAD-linked oxidoreductase	fwp - TGTCGCTAACAAATTCCCTG rwp - ATTATCTCCATCAGCTCATCGG
At5g13080	AtWRKY75	fwp - GTTCCCTAGGAGTTACTATAGGTG rwp - CATTTGAGTGAGAATATGCTCG
At4g34410	AP2 domain transcription factor	fwp - CGTCTTCAGTTTCATCTCCT rwp - TCATCATATTCATCCACTCCTC
AtCg01050	NADH dehydrogenase ND4	fwp - CTTCTTCTAACGACCTACGCT rwp - AAGTCCATCTATTCCCATTCTCC
At3g01140	AtMYB106	fwp - GCGCTTACAGAACCTAAACAG rwp - CATGGCGATATGATCATGATCAG
At4g23290	serine/threonine kinase - like protein	fwp - GGATACGCTGTTTCTAGGAG rwp - GATCATCCGTAGCATCATCTG
	Marker genes for different metabolic processes	
At1g77490	tAPX	fwp - AAACCTGAGACAAAGTACACGA rwp - CTCTGCATAGTTCTTGAATGAAGG

At5g52570	beta-carotene hydroxylase	fwp - AAATGAAGGGAGGTGAAGTG
		rwp - CTCTTGGTTTGTGATGTGAC
At5g17230	phytoene synthase	fwp - ATGACCTTGATGTGAAGAAACC
		rwp - TACACCAAACGTAGATTGCC
At5g67030	zeaxanthin epoxidase	fwp - CTATGTGACCGATAACGAAGGA
		rwp - GATTACTTTCACCCTAAACGCC
At1g08550	putative violaxanthin de-	fwp - TCAAGATGACTGGTATATCCTG
	epoxidase	rwp - GTTCTGGTATAATGCTATTGGG
At3g02730	thioredoxin f1	fwp - GTACACTCAATGGTGTGGTC
		rwp - CTTCCTTGACAACCTTGTTATCC
At3g15360	thioredoxin m4	fwp - CGTCGAAGTACCAAATCTGTC
		rwp - GAATTTGAACTTCCCTGCGA
At1g50320	thioredoxin x	fwp - AAGCCTTATCTCAGGAATATGG
		rwp - TGAAGAGAATGAAATGCGGT
At2g41680	putative thioredoxin	fwp - TCTTGTTGAATTTCACCAGCC
	reductase	rwp - TTTCTAAGAGCATACTGTCCCT
At3g26060	putative peroxiredoxin	fwp - ATCTTTGCCAAGGTTAACAAGG
		rwp - GAGTCTCTGAAAGCACAAGC
At2g25080	putative glutathione	fwp - TAATTCCTCAGCAACCTTTCTC
	peroxidase	rwp - CATCAATGTCCTTAACGGTG
At5g04140	ferredoxin-dependent	fwp - GAGTATCATTCAAACAACCCAGAG
	glutamate synthase	rwp - TCACGGAGGACATTAACAGG
At1g58290	glutamyl-tRNA reductase	fwp - GCAGCTGATCGATATACAAAGG
		rwp - CGGTTACAAGTACTAAGCACAG
At4g25080	mg-protoporphyrin IX	fwp - GTCTCTGCTTCCGATATTTCTG
	methyltransferase	rwp - ATTCCGTCTGCTTTGTTCTG
	ABA and GA_3 marker genes	
At5g52310	responsive to dessication	fwp – GGAGCTGAGCTGGAAAAAGAATTTGAT
	29a (RD29a)	CAGAAG[2]
		rwp – CCAATCTGAAGTTTCTCGGCAACCA
		TATCAG[2]
At1g20440	cold-regulated 47 (COR47)	fwp – GAAAAGACCGAGGAAGATGAGGAGAA
		CAAGCC[2]
		rwp - CCTCGGGATGGTCATGCTCCACCACAC
		TCTCCG[2]
At4g02780	ent-copalyl diphosphate	fwp - AATGACAGGAACATGAGATTGG
	synthetase 1 (GA1)	rwp - TGTGGGTGAAGAAGTTAGTTAGG

Genes affected by T-DNA insertion		
At1g58290	Glutamate tRNA reductase	fwp - CCTGTTGAGATGCGTGAGAA
		rwp - CAGCTGAGCTAACCGAAACC
At2g40490	Urogen III decarboxylase	fwp - TCTCATCTACCAAGTCATGCC
		rwp - TTGATAACTCTTCATGTACCTCCC
At3g14930	Urogen III decarboxylase	fwp - GTTACTCCTCGGGATTATCGT
		rwp - AAAGTGGATCGGAAGAGGAG
At5g14220	Protogen oxidase	fwp - CGCATAATGAAACGCAGAGA
		rwp - GACAGACACGGGTTCACCTT
At5g13630	Mg-chelatase H-subunit	fwp - TGTGGCAGTACCACTGGTGT
		rwp - CTCACGGACTCCCATTTTGT
At3g59400	GUN4	fwp - TCCCTCAAACAACCCACTTC
		rwp - GGCGTTTCATCGTTAAGCTC
At4g25080	Mg-proto methyl transferase	fwp - GTAACTCCACGGAGCAGAGC
		rwp - CAGAGACGATTGCTCCTTCC
At3g56940	Mg protoME oxidative	fwp - TCTGCCTCTCGGTTTATGTG
	cyclase 4-vinyl reductase	rwp - CAACCATTCTATCCAGTTTCCT
At5g54190	Protochlorophyllide	fwp - CCTTCAAGCTGCTTCTTTGG
	reductase A	rwp - TCTGTTCCCTCTTGCATCTC
At4g27440	Protochlorophyllide	fwp - TGCATTTAGACTTAGCCTCGT
	reductase B	rwp - AATGTATTCGTGTTCCCGGT
At1g03630	Protochlorophyllide	fwp - CCATCAAGGAACAGAGAAGAC
	reductase C	rwp - GCTAAACCTAAACCAGACGA
At5g26030	Ferrochelatase	fwp - TTTGCAAGCGAAGAACATTG
		rwp - GGCCAACACGACTCTGGTAT
At2g26670	Heme oxygenase	fwp - GGGTTTTGTGGAGGAGATGA
		rwp - CCACCAGCACTATGAGCAAA
At3g09150	Phytochromobilin synthase	fwp - TCTTTGACTTTGCGGGTTTC
		rwp - CTCCTTTGCCTTTGCTTCAC

[1] Taken from Ankele *et al.*, 2007; [2]Taken from Sanchez *et al.*, 2004

3. Results

3.1. Novel mutants of tetrapyrrole biosynthesis

Except for GUN1 (Koussevitzky *et al.*, 2007), all GUN proteins were found to be involved in different steps of tetrapyrrole biosynthesis (Mochizuki *et al.*, 2001; Larkin *et al.*, 2003). In order to investigate whether inactivation of further steps in the tetrapyrrole pathway also result in a *gun* phenotype, T-DNA insertion lines for all enzymes of the pathway were analyzed. For each enzyme, two or three independent lines were identified from the SIGnAL database (Alonso *et al.*, 2003), and seeds were obtained from public seed stocks. Genes and the corresponding mutant lines are listed in Table 2.1., together with the predicted location of the T-DNA insertion.

3.1.1. Screen for homozygous mutants

Lines that were supposed to harbor T-DNA insertions in the genes coding for ALA dehydratase, PBG deaminase and coprogen oxidase were not germinating in our hands (indicated by a X in Figure 3.1.), although mutants affected in PBG deaminase and coprogen oxidase were previously described (Ishikawa *et al.*, 2001; Strand *et al.*, 2003). This could be due to decreased seed vitality or embryo lethality caused by a total lack of the respective protein. Some other mutations in our screen could only be obtained in the heterozygous state (thin face in Figure 3.1.), either because homozygous seedlings were lethal or plants were not able to produce seeds. However, for some lines homozygous mutants could be obtained (underlined in Figure 3.1.). Only those lines were used for further analyses.

To confirm the effect of the inserted T-DNA in the mutant lines the expression level of the respective genes were monitored in 6-day-old plants by real-time PCR and compared to the level in wild-type plants (Table 3.1.). While in most mutant lines the expression of the corresponding gene was indeed reduced (*hema1-2*, *heme1-2*, *ppo1-1*, *chlm-1*, *chlm-3*, *crd1-1*, *porb-1*, *fc1-1* and *gun3-4*), the expression was not altered in one line (*gun2-2*) and even higher in four of them (*heme2-1*, *gun4-3*, *pora-2* and *porc-1*). Analyses of the original *gun* mutants reveal that the *gun4* mutant showed an even stronger expression, *gun2* exhibited a reduced transcript accumulation and the expression level was not altered in *gun5* (Table 3.1.). However, gene mutations in EMS (ethyl methane sulfonate) mutant lines (original *gun* mutants) is caused by base-pair and

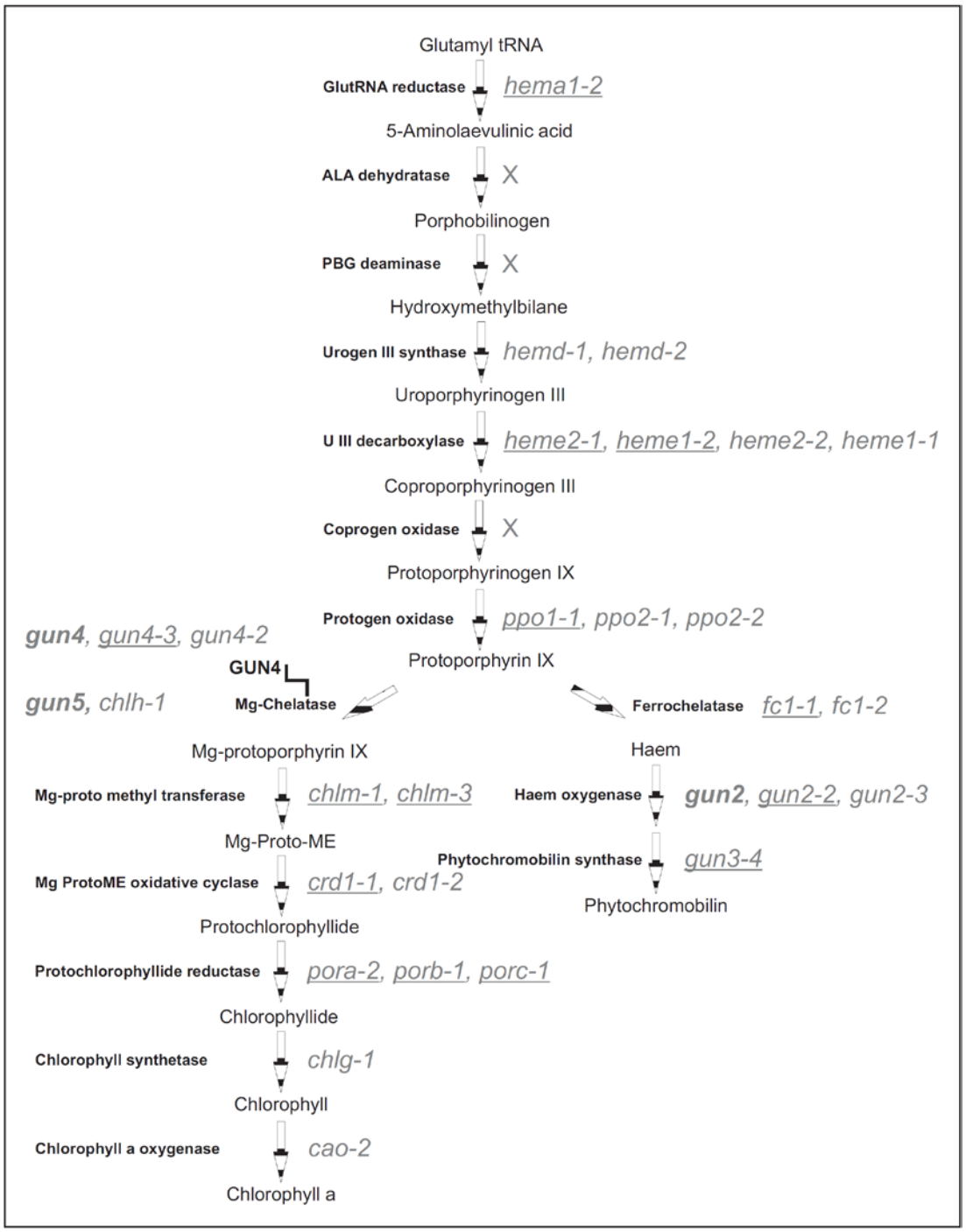

Figure 3.1: Schematic overview of the tetrapyrrole biosynthesis pathway in higher plants. For all enzymatic steps (indicated by arrows) two or more T-DNA insertion lines were analyzed. Some lines could only be isolated as heterozygous (thin face) and others were not viable (X). Only the homozygous lines (underlined) were used for further analysis. Also the known *gun* mutants used in this work are shown (bold face).

Table 3.1: Relative transcript abundance in mutant lines compared to wild-type plants. Plants were grown for 6 days under continuous light (100 µmol m^{-2} s^{-1}). Data for indicated genes were obtained in triplicate by real-time PCR analysis and normalized to the expression level of ubiquitin-protein ligase-like protein mRNA (*At4g36800*).

Mutant line	Gene	Ratio to WT level
hema1-2	*AT1G58290*	0.63
heme2-1	*AT2G40490*	2.2
heme1-2	*AT3G14930*	0.2
ppo1-1	*AT5G14220*	0.04
gun4	*AT3G59400*	3.01
gun4-3	*AT3G59400*	3.2
gun5	*AT5G13630*	1.07
chlm-1	*AT4G25080*	0.03
chlm-3	*AT4G25080*	0.25
crd1-1	*AT3G56940*	0.11
pora-2	*AT5G54190*	3930
porb-1	*AT4G27440*	0.46
porc-1	*AT1G03630*	1.95
fc1-1	*AT5G26030*	0.095
gun3-4	*AT3G09150*	0.04
gun2	*AT2G26670*	0.04
gun2-2	*AT2G26670*	1.08

thus aminoacid substitution and is not necessarily coupled to changes in the transcription rate. Moreover, T-DNA insertions (new tetrapyrrole biosynthesis mutants) can result in non-functional mRNA without changes in the expression of the gene.

3.1.2. Homozygous mutants have no *gun* phenotype

Insertion lines were grown in the presence of norflurazon (NF) for 6 days under continuous white light (100 µmol m^{-2} sec^{-1}) and real-time PCR analysis was performed to determine *LHCB1.2* expression levels (Figure 3.2.). After NF treatment the expression of the nuclear encoded gene *LHCB1.2* was strongly reduced in wild-type

plants compared to untreated seedlings (Figure 3.2.; Oelmuller *et al.*, 1986; Oelmuller and Mohr, 1986). This response is known to be affected in the *gun* mutants (Susek *et al.*, 1993; Mochizuki *et al.*, 2001), as it is visible for the controls *gun1* and *gun5*, as well as for a new *gun2* allele *gun2-2*. Those three mutants showed a higher expression of *LHCB1.2* after NF treatment than wild-type plants, but the expression was also reduced when compared to untreated wild-type plants (Figure 3.2.). The expression of *gun2* was not altered in *gun2-2* (Table 3.1.), therefore the observed *LHCB1.2* mRNA de-repression phenotype provide an indication that the detected *gun2* mRNA was affected by the inserted T-DNA and that the amount of functional GUN2 was decreased. However, none of the other novel mutant lines of the tetrapyrrole biosynthesis pathway exhibited this *LHCB1.2* de-repression phenotype. Among those, lines *gun4-3* and *gun3-4* were identified for which a de-repression was suggested, due to the proposed affection of the same alleles as in the known *gun* mutants. As the insertion site was either downstream (*gun4-3*) or upstream (*gun3-4*) of the gene (see Table 2.1.) it seems that the expressed mRNA were functional in both cases and that the reduced *gun3* expression in *gun3-4* (Table 3.1.) was still enough for proper development. It is noteworthy, that the

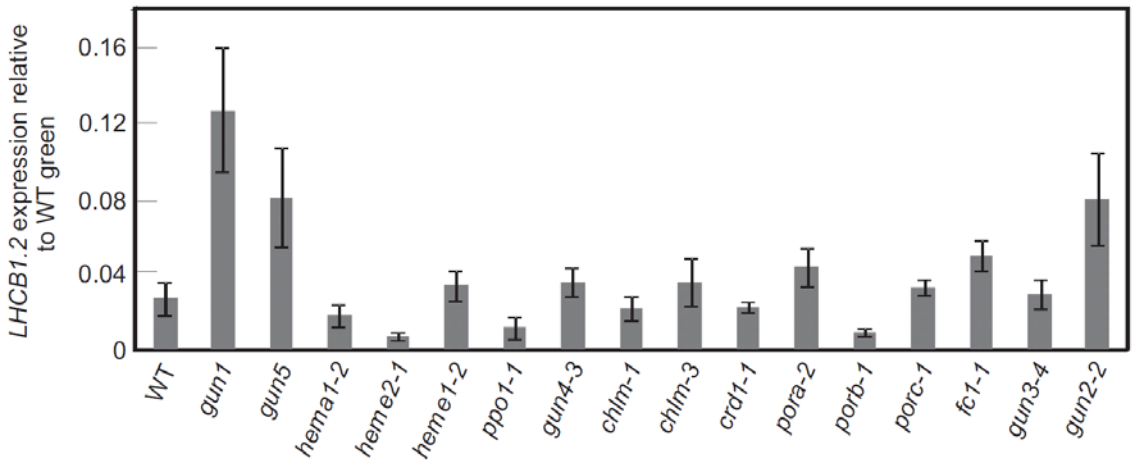

Figure 3.2: Real-time PCR analysis of *LHCB1.2* expression in seedlings grown in the presence of NF for 6 days under continuous light (100 µmol m^{-2} s^{-1}). The results were set relative to the expression level of wild-type grown without NF and normalized to the expression level of *ACTIN1*. Reactions were performed in triplicate. Bars indicate standard deviations.

T-DNA knockout lines for Mg-protoporphyrin methyltransferase (*chlm-1*) and a subunit of Mg-protoporphyrin methylester cyclase (*crd1-1*), did not show the de-repression phenotype, confirming previous data (Mochizuki *et al.*, 2008). To summarize, no novel *gun* mutants with a defect in tetrapyrrole biosynthesis could be identified by this strategy.

3.2. NF-grown *gun* seedlings exhibit a distinct growth phenotype

When grown under continuous light of 100 µmol m^{-2} s^{-1}, all *gun* mutant lines exhibited a pale green phenotype but with different severity (Figure 3.3; Mochizuki *et al.*, 2001). While g*un2* showed the strongest phenotype, the two mutant lines affected directly in chlorophyll biosynthesis (*gun4* and *gun5*) were less pale suggesting that the mutation had only a weak effect. Although the GUN1 protein is not involved in chlorophyll biosynthesis, the *gun1* mutant also displayed a light pale green phenotype. More surprisingly, the mutant lines also showed a different phenotype in the presence of NF (Figure 3.3.). This phenotype was not visible after two days but was clearly observable after 6 days and even more pronounced after 10 and 12 days. After 6 days anthocyanin accumulation was clearly visible in wild-type and *gun1* seedlings while in the other *gun* mutants this accumulation was largely decreased or not visible. Moreover, *gun2*, *gun4* and *gun5* plants seemed to grow better and showed a stronger leaf pigmentation phenotype. In *gun4* and *gun5* mutants this growth phenotype was more severe after 10 and 12 days while *gun2* stopped to grow after 6 to 8 days. Also wild-type seedlings showed less anthocyanin accumulation after 10 and 12 days while in *gun1* it was still visible. Taken together, the *gun* mutants which are affected in tetra-pyrrole biosynthesis seemed more vigorous than wild-type plants and exhibited a yellowish leaf coloration. In contrary the *gun1* seedlings seemed to be more fragile than wild-type plants and accumulated more anthocyanin indicated by the purple coloration of leaves. This suggests that the distinct growth and leaf coloration phenotypes of *gun2*, *gun4* and *gun5* are not strictly coupled to the de-repression of *LHCB1.2* transcription observable after NF treatment. Moreover, the T-DNA insertion lines affected at different stages of tetrapyrrole biosynthesis did not exhibit this growth phenotype as shown for 6-day-old plants in Figure 3.4. The seedlings of all investigated lines behaved similar to wild-type seedlings.

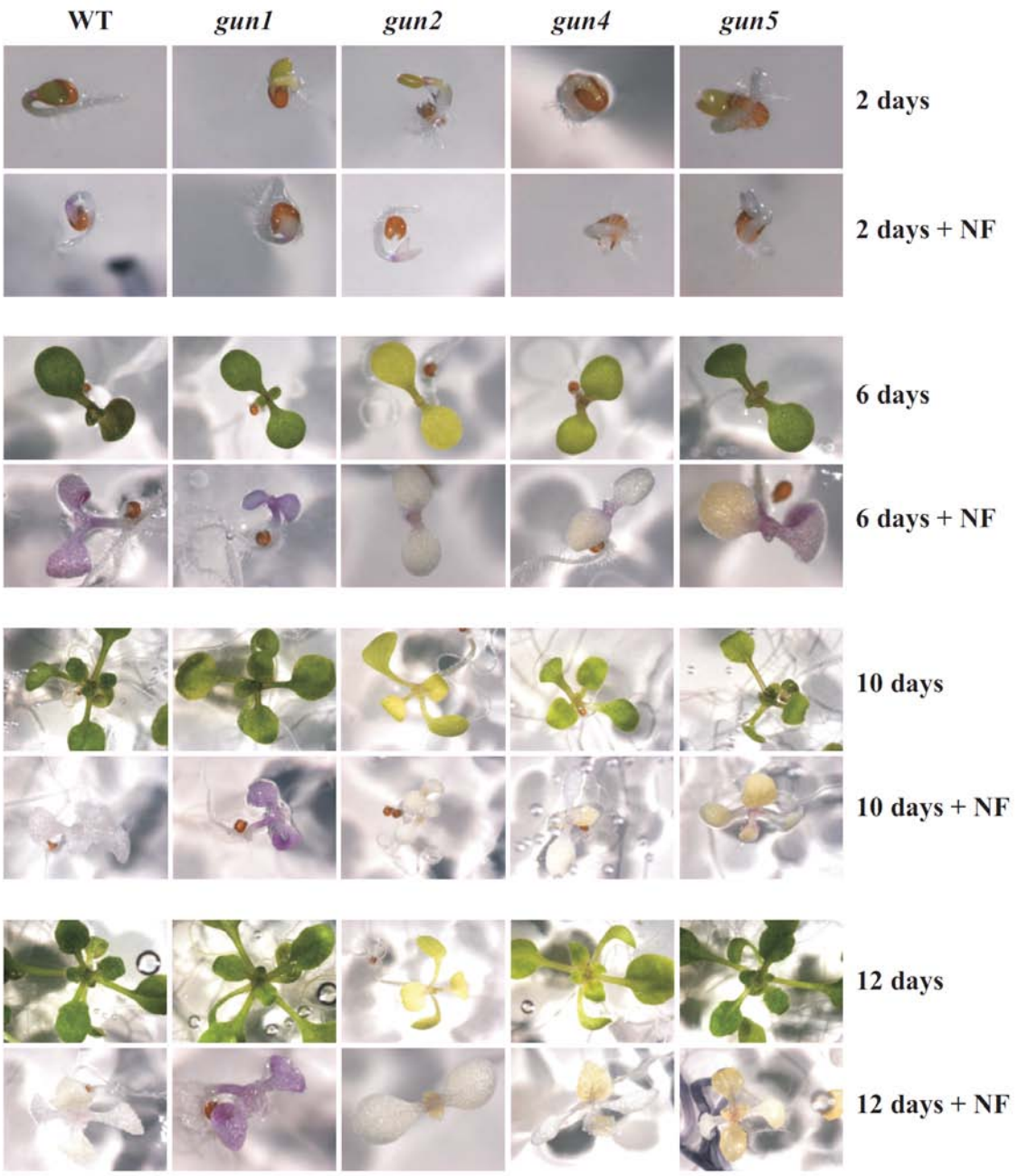

Figure 3.3: Images of plants grown in the absence or presence of NF (+ NF) for 2, 6, 10 or 12 days under continuous light (100 µmol m^{-2} s^{-1}).

3.3. Growth phenotype is not correlated with *LHCB1.2* de-repression

To investigate the relationship between the growth or pigmentation phenotype and *LHCB1.2* mRNA expression, we first tested if carotenoid biosynthesis inhibitors other than NF can mimic its function as a GUN-dependent repressor of *LHCB1.2*

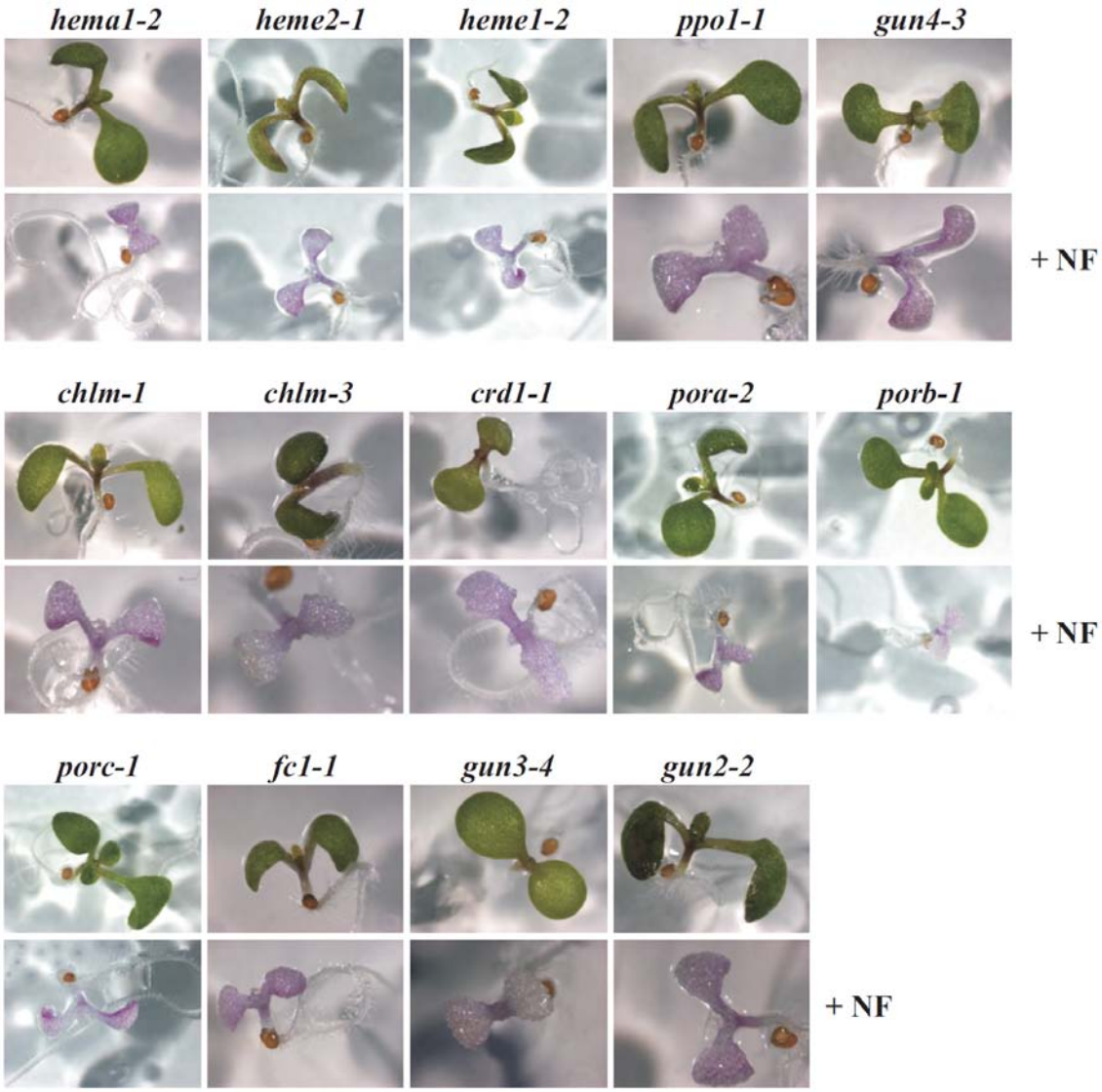

Figure 3.4: Images of T-DNA insertion lines affected in the tetrapyrrole biosynthesis pathway grown for 6 days under continuous light (100 µmol m^{-2} s^{-1}) in the absence or presence of NF (+ NF).

expression. For that, three different inhibitors were used (see also Figure 3.5): flurochloridone, amitrole and CPTA (2-(4-chlorophenylthio)triethylamine hydrochloride). Flurochloridone is a phytoene desaturase inhibitor (Pallett *et al.*, 1998), whereas amitrole inhibits lycopene cyclization (Agnolucci *et al.*, 1996) and thus acts downstream of NF by blocking the biosynthesis of δ- and γ-carotenes which are the precursors of α- and β-carotenes, respectively. CPTA also inhibits lycopene cyclization

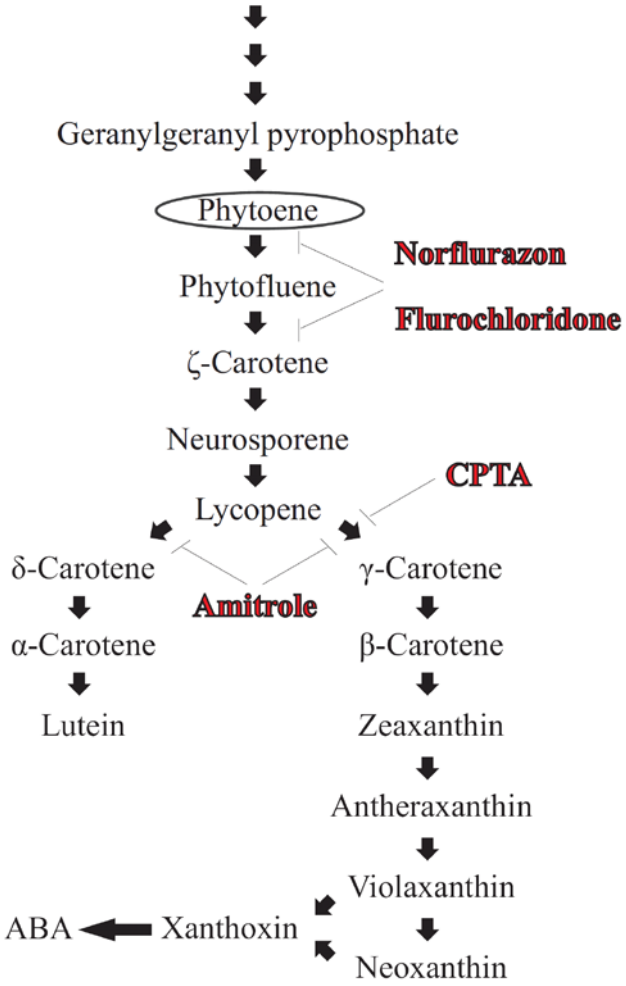

Figure 3.5: Schematic overview of the carotenoid biosynthesis pathway in plants. The different steps in chloroplast carotenoid biosynthesis are indicated by arrows. Note that in flowering plants the xanthophylls violaxanthin and neoxanthin are the precursors of xanthoxin, which is exported to the cytosol and there converted to ABA. The specific target sites of carotenoid biosynthesis inhibitors used in this work are indicated.

but in contrast to amitrole it affects only the biosynthesis of γ-carotenes (Al-Babili *et al.*, 1999). Additionally the concentration of NF was elevated to exclude the possibility that the amount was too low for complete inhibition.

When Northern blot analyses were performed on RNA isolated from wild-type and *gun* seedlings treated with either NF (0.5 µM or 5 µM), amitrole, fluorchloridone or CPTA (either 15 µM or 45 µM) and grown under continuous white light (100 µmol m^{-2} s^{-1}), similar differences between wild-type and *gun* seedlings in respect to *LHCB1.2*

mRNA levels were observed under all photo-bleaching conditions (Figure 3.6.). In contrast to non-photo-bleaching conditions where *LHCB1.2* mRNA was present in large amounts in all genotypes, the destruction of plastids using carotenoid biosynthesis

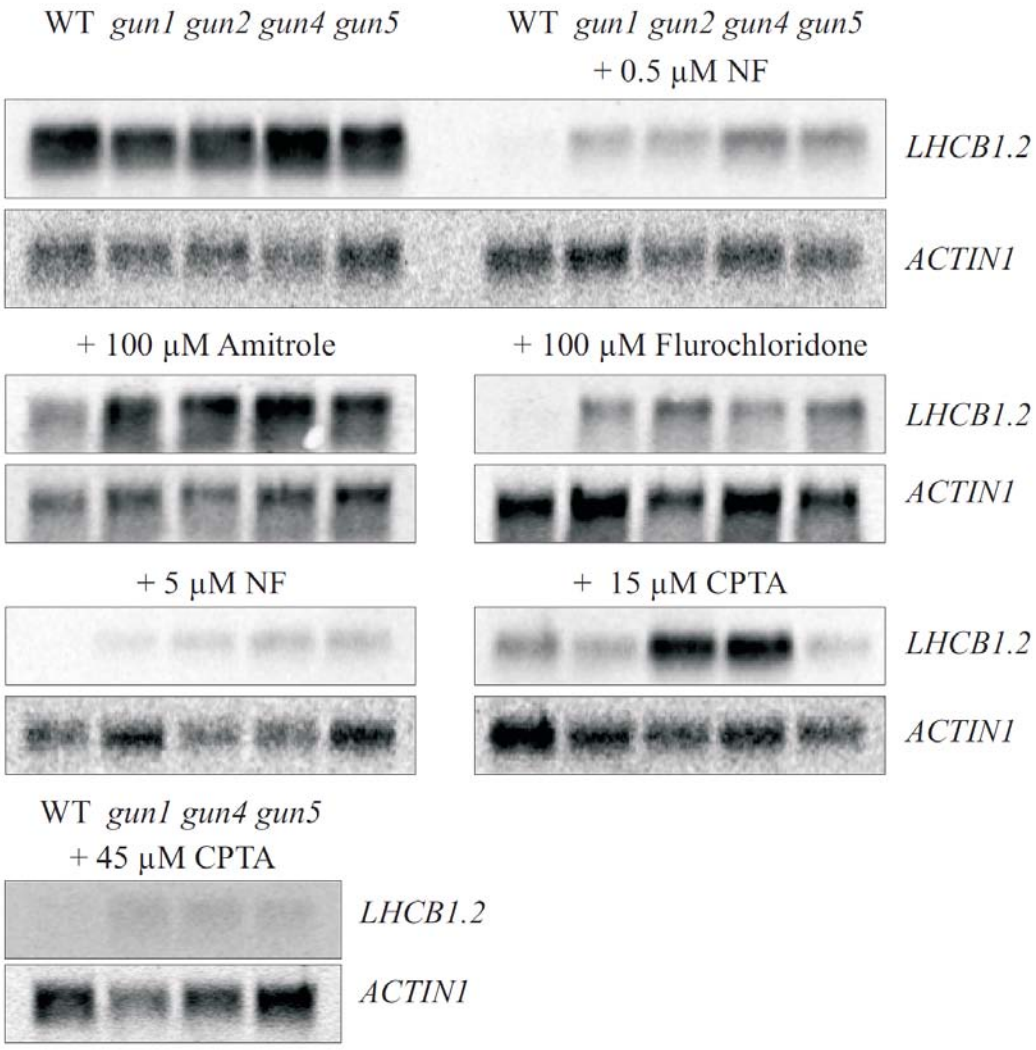

Figure 3.6: *LHCB1.2* mRNA levels in wild-type (WT) and *gun* seedlings, grown on MS plates without carotenoid biosynthesis inhibitors or supplemented with either 0.5 µM or 5 µM NF, 100 µM amitrole, 100 µM flurochloridone or 15 µM respectively 45 µM CPTA, were determined by Northern blot analyses. The *ACTIN1* mRNA level served as loading control. Seedlings were grown for 6 days under continuous light (100 µmol m^{-2} s^{-1}). Note that *gun2* was missing when experiments with 45 µM CPTA were performed.

inhibitors resulted in a strong decrease of *LHCB1.2* mRNA accumulation. This reaction on nuclear gene expression was somehow affected in *gun* mutants irrespective of which inhibitor was used. Therefore, *LHCB1.2* mRNA was more abundant after inhibition of carotenoid biosynthesis in *gun* mutants compared to wild-type seedlings but in all genotypes lower than in green plants.

We then asked if the characteristic NF-related growth or pigmentation phenotype observed in the three *gun* mutants with defects in the tetrapyrrole biosynthesis pathway (*gun2*, *gun4* and *gun5*) also appeared upon treatment with the other inhibitors of carotenoid biosynthesis. Indeed, amitrole and flurochloridone caused seedlings to display strong photo-oxidative damage while in the case of CPTA this was the case when supplemented with 45 µM CPTA but only to slight extent when 15 µM CPTA were given (Figure 3.7.). In the presence of amitrole, anthocyanin accumulation occurred in wild-type and all *gun* seedlings, whereas seedlings of all genotypes completely lacked pigmentation when grown in the presence of flurochloridone. Furthermore, seedlings grown on medium containing amitrole or flurochloridone were delayed in development. In contrast to seedlings grown on NF, they did not evolve first true leaves (Figures 3.3. and 3.7.). Contrary to amitrole and flurochloridone, CPTA failed to induce complete photo-oxidative damage, but only at a concentration of 15 µM (Figure 3.7.). Under this condition only the phenotypes of wild-type, *gun1* and *gun5* seedlings were affected which is in line with the reduced *LHCB1.2* mRNA levels seen in these three genotypes when compared to the transcript level of *gun2* and *gun4* seedlings (Figure 3.6.). It seemed that photo-destruction is less pronounced in *gun2* and *gun4* mutants resulting in a higher expression of *LHCB1.2* mRNA. However, higher concentrations of CPTA (45 µM CPTA) were sufficient to induce photo-oxidative damage in all genotypes (Figure 3.7.) resulting in *LHCB1.2* mRNA levels comparable to these seen when other inhibitors were used (Figure 3.6.).

Summarizing, it can be stated that the effects of NF on *LHCB1.2* mRNA expression in wild-type and *gun* seedlings can be mimicked by other carotenoid biosynthesis inhibitors. However, only after NF treatment the perturbation of tetrapyrrole biosynthesis in *gun2*, *gun4* and *gun5* result in a distinctive growth and leaf pigmentation phenotype.

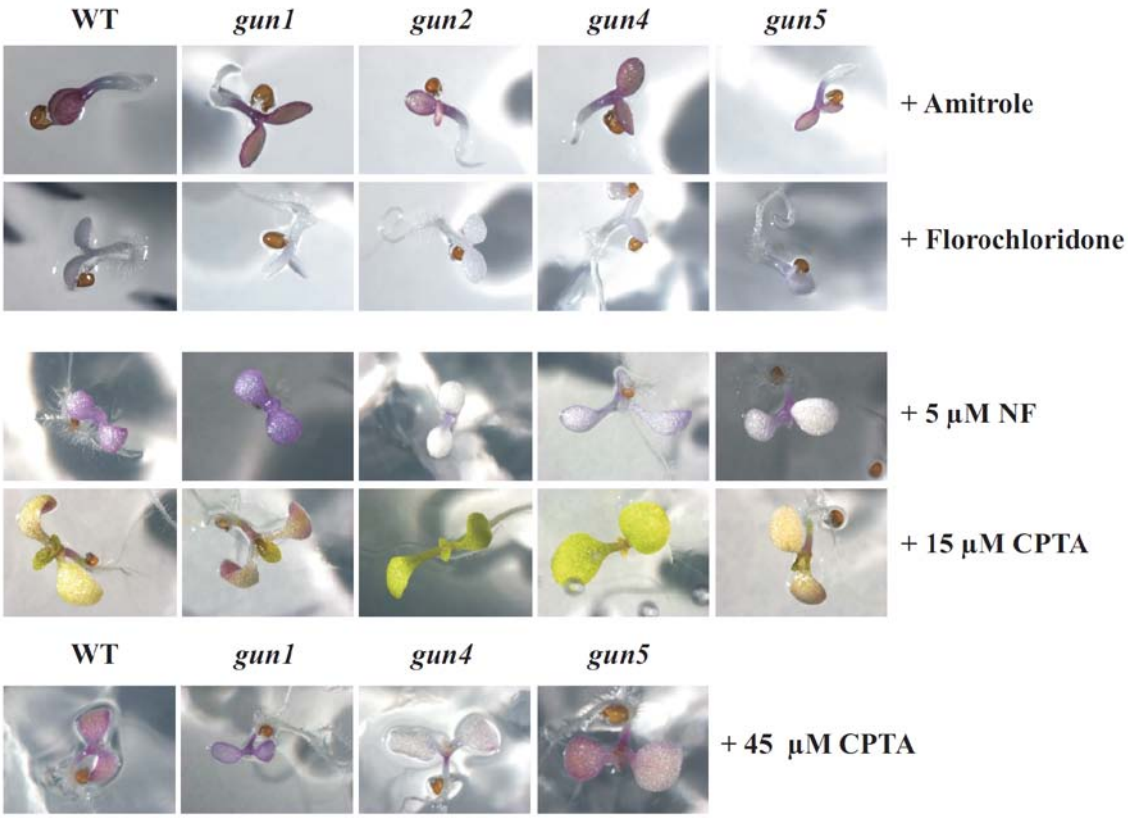

Figure 3.7: Images of seedlings grown on plates supplemented with different inhibitors of carotenoid biosynthesis (100 µM amitrole, 100 µM flurochloridone, 5 µM NF, 15 µM CPTA or 45 µM CPTA) for 6 days under continuous light of 100 µmol m^{-2} s^{-1}. Note that *gun2* was not available for experiments with 45 µM CPTA.

3.4. The *gun* mutants are not resistant to NF

Norflurazon is an inhibitor of phytoene desaturase, which has its target site at the desaturation step from phytoene to ζ-carotene (Figure 3.5.; Bartels and McCullou, 1972). In a screen for EMS (ethyl methane sulfonate)-mutagenized Arabidopsis plants with altered sensitivity to inducers of oxidative stress, seven strains were isolated that showed a certain degree of resistance to NF (Ezhova *et al.*, 2001). To investigate whether the higher *LHCB1.2* mRNA expression in the *gun* mutants or the growth phenotype of the mutants could be due to a resistance to NF, phytoene levels were determined in wild-type and *gun* plants. In the absence of NF, phytoene was not detectable in any of the genotypes (data not shown), while after NF treatment enhanced phytoene levels were detectable in WT plants, as well as in the *gun* mutant lines, as to

be expected in the case of a block in the reactions downstream of phytoene (Figure 3.8.). In *gun1* and *gun2*, the amount of phytoene was not as high as in wild-type plants (only 40% or 80%, respectively), whereas phytoene concentrations in *gun4* and *gun5* exceeded the wild-type values. These data suggested that the *gun* mutants are not generally resistant to NF. Furthermore, differences in the accumulation of phytoene caused by NF treatment could not be linked to any of the *gun* specific phenotypes.

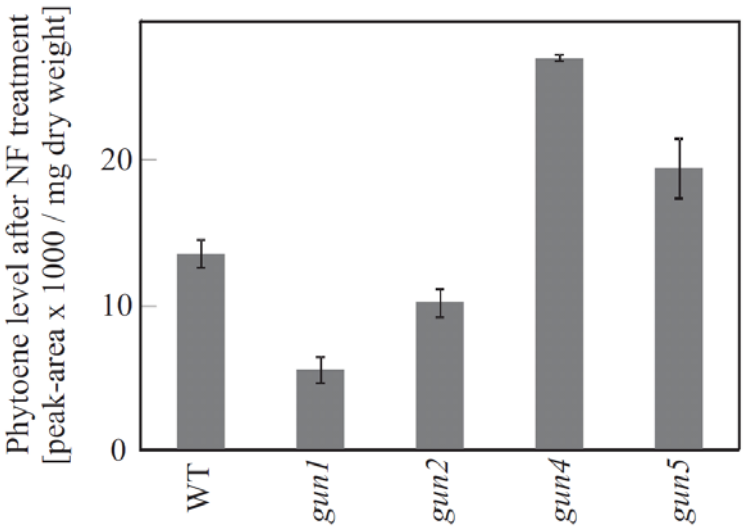

Figure 3.8: Phytoene level in NF-treated WT and *gun* seedlings grown for 6 days under continuous light of 100 µmol m^{-2} s^{-1}. Phytoene was separated by reverse-phase HPLC and monitored at 296 nm. Phytoene was not detectable in seedlings grown in the absence of NF. Bars indicate standard deviations from two replicates.

3.5. Nuclear-encoded photosynthetic proteins are not present in NF treated plants while some plastome-encoded proteins are retained in *gun* mutants

To test whether the effect of NF on *LHCB1.2* mRNA accumulation in the *gun* mutants also affected the abundance of the corresponding protein, Western analysis was performed. Also a putative effect of NF on the accumulation of other photosynthesis related proteins was studied. Total protein was isolated from WT and *gun* seedlings grown in the absence or presence of NF, and subjected to immunoblot analyses with

Figure 3.9: Western blot analysis of WT and *gun* seedlings. Proteins were extracted from seedlings grown in the absence (- NF) or presence of NF (+ NF) for 6 days under continuous light (100 µmol m^{-2} s^{-1}), fractionated by SDS-PAGE and blotted to a PVDF membrane (15 µg protein was loaded). Filters were probed with antibodies specific for the proteins indicated on the right side of the panels.
RbcL, large subunit of RubisCO; AtpB, β subunit of ATP synthase; PetB, cytochrome b_6; PsbD, photosystem II D2 protein; PsaB, photosystem I reaction centre protein B; Lhca3, photosystem I type III chlorophyll a/b-binding protein; Lhcb1, photosystem II type I chlorophyll a/b-binding protein

antibodies specific for representative proteins of the light-harvesting complexes (Lhca3 and Lhcb1), subunits of photosystem I (PSI) (PsaB), photosystem II (PSII) (PsbD) and the cytochrome b_6/f complex (PetB), as well as the β-subunit of ATPase (AtpB) and RbcL, the large subunit of RubisCO (Figure 3.9.). Although repression of *LHCB1.2* mRNA accumulation caused by photo-oxidative damage was attenuated in the *gun*

mutants, no Lhcb1 protein was detectable in wild-type or in the mutants. This suggests that there is no physiological relevance for the remaining *LHCB1.2* mRNA. Furthermore, the absence of most tested proteins in NF-treated WT and *gun* seedlings provided additional evidence for the severe photo-oxidative damage affecting plastids during NF treatment, and clearly showed that the remaining thylakoids (Susek *et al.*, 1993; McCormac and Terry, 2004) must be devoid of photosynthetic activity.

Interestingly, while AtpB and RbcL could be detected in NF-treated wild-type plants only in tiny amounts, a marked increase in their abundance was observed in NF-treated *gun* seedlings when compared to wild-type (to a less extent in *gun1*; Figure 3.9.). This indicates that translation of some plastome-encoded proteins continued in the *gun* mutants even after the photo-oxidative damage caused by NF treatment. The observation of an interplay between translation of plastome-encoded proteins and GUN signaling is compatible with the finding that the translation inhibitor lincomycin has the same effect as NF on *gun1* mutants (Gray *et al.*, 2003; Koussevitzky *et al.*, 2007), and might possibly explain how perturbations in tetrapyrrole metabolism (in *gun2*, *gun4* and *gun5*) and a defective nucleic acid binding protein (*gun1*) can similarly affect plastid signaling. Moreover, the differences of AtpB and RbcL amounts between the genotypes reflect the visible growth phenotype.

Under untreated conditions the levels of the tested proteins were not altered in *gun1*, *gun4* and *gun5* seedlings compared to wild-type, whereas in *gun2* mutants the core subunits of photsystem I and II, as well as PetB of the cytochrome b_6/f complex were present in higher amounts. The mutant line g*un2* showed the strongest phenotype (pale green) under normal growth conditions (Figure 3.3.), therefore the higher amounts of photosynthesis related proteins could be due to feedback regulation caused by affected capability to perform photosynthesis.

3.6. Alterations in heme levels do not explain the *gun* phenotype

Because the proteins GUN2–5 are all involved in tetrapyrrole biosynthesis (Mochizuki *et al.*, 2001; Larkin *et al.*, 2003), it was thought that intermediates of chlorophyll biosynthesis could function as signal molecules (Strand *et al.*, 2003; Ankele *et al.*, 2007). Testing this hypothesis revealed that in *A. thaliana* accumulation of tetrapyrroles of the Mg-branch did not correlate with *LHCB1.2* expression (Mochizuki

et al., 2008; Moulin *et al.*, 2008). Still it appeared possible that tetrapyrroles of the Fe-branch could be involved in signaling processes. Accordingly, heme was postulated to act as a plastid-derived regulator of nuclear gene expression in the green alga *C. reinhardtii* (von Gromoff *et al.*, 2008). In principle, *gun4* and *gun5* mutants should accumulate protoporphyrin IX (Proto IX), the precursor of both Mg-proto IX and Fe-proto IX i.e. heme (Figure 3.1.). Actually it could be shown that Proto IX accumulates in *gun5* mutants (Mochizuki *et al.*, 2008). This accumulation of Proto IX could, in turn, lead to an increase in biosynthesis of tetrapyrroles of the heme branch. Similarly, a block of the steps following heme biosynthesis, as it is the case for the *gun2* and *gun3* mutants, should also lead to a rise in heme levels.

To test if heme is somehow involved in retrograde signaling in *A. thaliana*, we determined heme levels in the *gun* mutants, as well as in wild-type seedlings, grown for 6 days on MS medium without or supplemented with NF (Figure 3.10.). Heme levels

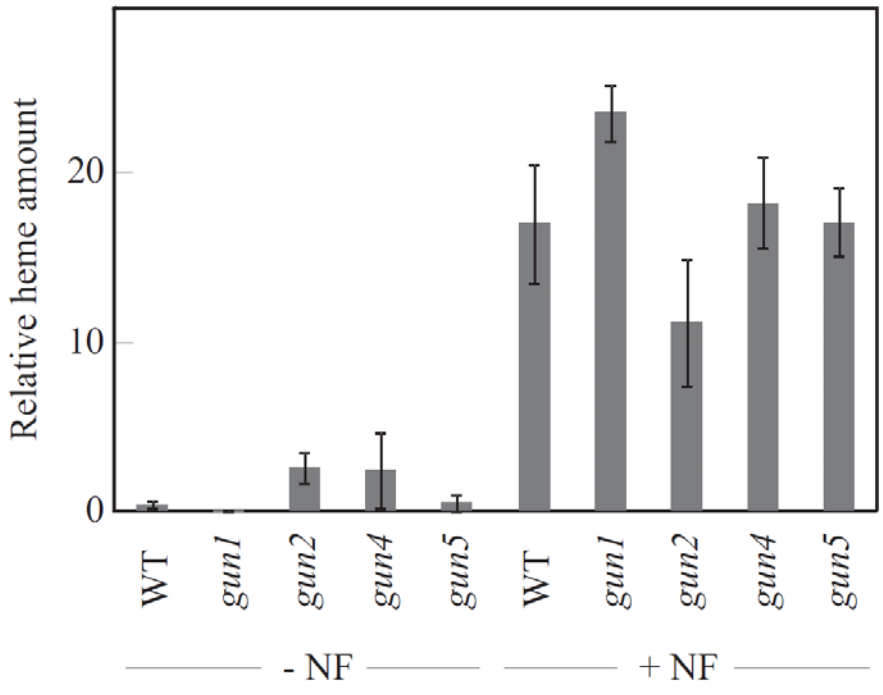

Figure 3.10: Relative heme amount in WT and *gun* seedlings grown for 6 days on MS plates in the absence (- NF) or presence of NF (+ NF) under continuous light (100 µmol m^{-2} s^{-1}). Heme was measured using a horseradish peroxidase-based assay. Data were obtained in triplicate. Bars indicate standard deviations.

were quantified by chemiluminescence, based on the incorporation of heme derived from the extracted plant material into externally supplied horseradish peroxidase apo-enzyme, with concomitant reconstitution of enzyme activity (Masuda and Takahashi, 2006). In the absence of NF, heme was barely detectable in seedlings of wild-type, *gun1* and *gun5* plants, while in *gun2* and *gun4* mutants very low levels were detectable (Figure 3.10.). However, in the presence of NF, heme levels were markedly increased in all seedlings. Heme levels were similar in wild-type, *gun4* and *gun5* seedlings, whereas its amount was slightly higher in *gun1* plants and lower in *gun2* mutants. Therefore, it is not possible to attribute the increased *LHCB1.2* mRNA levels in the *gun* mutants or their specific growth phenotype to an accumulation of heme.

3.7. *gun* specific phenotypes are not present when grown under low-light

The standard light intensities applied in this work caused strong photo-oxidative damage to the plants after NF treatment (Figure 3.3.). To elucidate the effect of NF on *LHCB1.2* mRNA accumulation and the different growth phenotypes under weak photo-oxidative or even non-photo-oxidative conditions, plants were exposed to low-light intensities (10 µmol m^{-2} s^{-1}) or were grown without light. No effect of NF was visible when plants were grown in the dark (Figure 3.11.), indicating that NF indeed acts specifically as an inhibitor of carotenoid biosynthesis. The fact that a phytoene desaturase mutant (*pds3*) showed the same effects as wild-type plants treated with NF (Qin *et al.*, 2007) and that plants existed which were resistant to NF (Chamovitz *et al.*, 1991; Ezhova *et al.*, 2001) underline the notion that the inhibitor acts specifically. Additionally, no differences could be observed between wild-type and *gun* seedlings. All plants showed a pale-green or yellowish coloration. Coloration was also visible in NF treated plants where carotenoid biosynthesis is known to be blocked. In this case the detectable coloration most likely came from pigments already stored in the seeds.

Exposure to continuous low-light was already sufficient to photo-bleach seedlings treated with NF (Figure 3.11.). In contrast to plants grown under higher light intensities, seedlings grown under low-light completely lacked pigmentation. Neither anthocyanin accumulation, explainable by reduced photo-oxidative damage, nor the yellowish pigmentation were visible. Moreover, the accumulation of *LHCB1.2* mRNA in seedlings grown under low-light conditions (10 µmol m^{-2} s^{-1}) varied from those seen

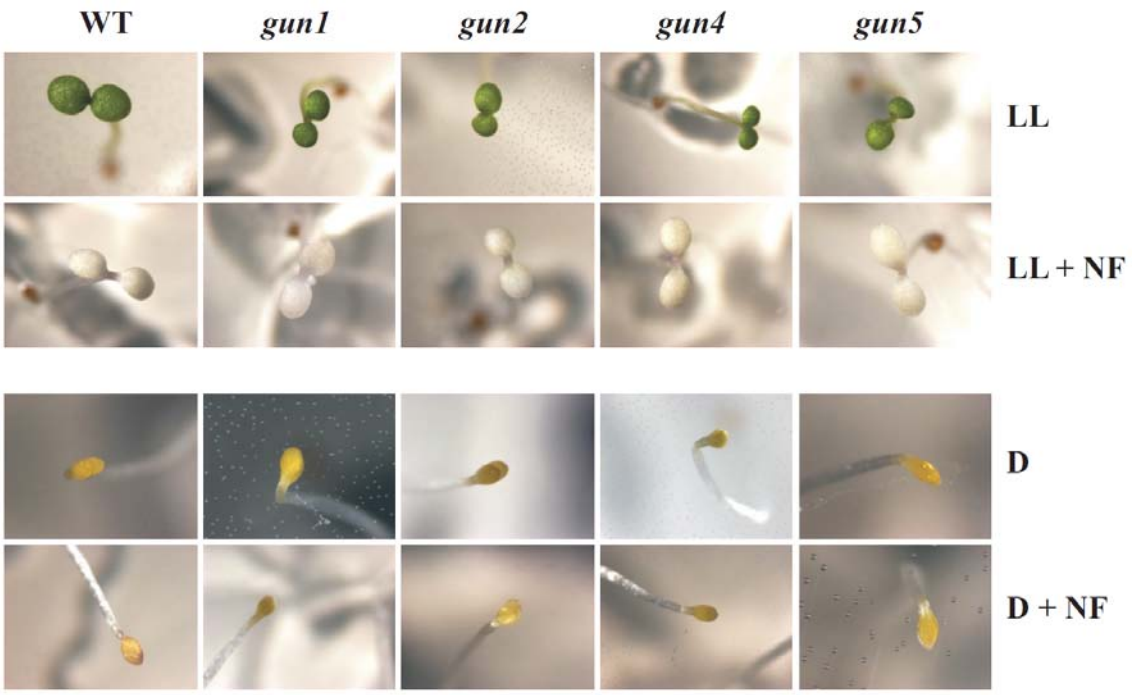

Figure 3.11: Images of wild-type and *gun* seedlings grown in the absence or presence of NF (+ NF) for 6 days either under low-light (LL; 10 µmol m^{-2} s^{-1}) or without light (D; dark).

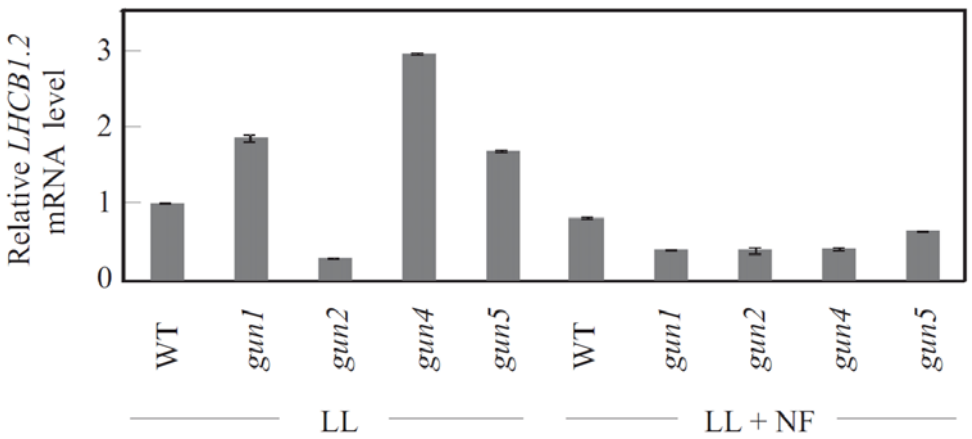

Figure 3.12: Real-time PCR analysis of *LHCB1.2* mRNA expression in WT and *gun* seedlings grown for 6 days under continuous low-light (LL) conditions (10 µmol m^{-2} s^{-1}) in the absence or presence of NF (+ NF). Reactions were performed in triplicate and results were normalized to the expression level of *At2g37620* coding for *ACTIN1*. Bars indicate standard deviations.

in seedlings grown under 100 µmol m^{-2} s^{-1} (Figure 3.12.). The typical de-repression of *LHCB1.2* mRNA accumulation caused by photo-oxidative damage was lost in wild-type and *gun* seedlings, whereas in *gun* mutants mRNA levels were slightly lower as in wild-type plants. This points out that the specific *gun* phenotypes are somehow linked to effects of strong photo-oxidative stress.

3.8. Altered accumulation of reactive oxygen species did not reveal an explanation

Photo-oxidative damage induced by inhibitors of carotenoid biosynthesis is caused by reactive oxygen species (ROS) produced as toxic by-products during photosynthesis. ROS are normally quenched by carotenoids (Frosch *et al.*, 1979; Apel and Hirt, 2004; Krieger-Liszkay, 2005). Beside this it has been proposed that ROS were somehow involved in retrograde signaling processes (Pesaresi *et al.*, 2007; Fernandez and Strand, 2008; Kleine *et al.*, 2009). Moreover, it was previously speculated that perturbations in tetrapyrrole biosynthesis might lead to localized ROS production (Moulin *et al.*, 2008). Our finding that the characteristic de-repression of *LHCB1.2* mRNA accumulation in NF-treated *gun* mutants does not occur in low-light conditions (Figure 3.12.) promoted us to investigate the expression of ROS marker genes. Thus, the steady-state transcript levels of genes, that have been described either as general oxidative stress response markers, or as being specifically regulated by hydrogen peroxide, superoxide or singlet oxygen (Gadjev *et al.*, 2006), in NF-treated wild-type and mutant plants were analyzed using real-time PCR and compared to data from untreated wild-type plants (Table 3.2.). As loading control, the expression of three different genes (*UBIQUITIN*, *ACTIN1* and *18S* rRNA) was used. In Table 3.2. only the values normalized to the expression of *UBIQUITIN* are shown.

In NF-treated wild-type seedlings, one of the five general oxidative stress response marker genes (bHLH transcription factor, *At1g10585*) was down-regulated, while the other four (defensine-like protein, *At2g43510*; glutathione S-transferase, *At1g17170*; FAD-linked oxidoreductase, *At1g26380* and AtWRKY75, *At5g13080*) were up-regulated (Table 3.2.). This is consistent with the induction of photo-oxidative stress by NF. Compared to wild-type, these marker genes exhibited very similar expression patterns in the four *gun* mutant lines. The overall up-regulation in NF-treated wild-type and *gun* seedlings was also visible for the marker gene specific for singlet oxygen (AP2

Table 3.2: The expression of ROS marker genes in NF-treated wild-type and *gun* seedlings grown for 6 days under continuous light (100 µmol m^{-2} s^{-1}) related to the expression in untreated wild-type seedlings. Data were obtained in duplicate using real-time PCR and normalized to the expression of *UBIQUITIN*. The three rows represent three independent experiments.

Gene	Description	WT	WT + NF	*gun1* + NF	*gun2* + NF	*gun4* + NF	*gun5* + NF
Specific for all ROS							
At2g43510	Defensine-like protein	1	1.39 1.24 2.38	3.58 2.13 2.84	1.34 1.66 3.47	2.20 1.62 2.31	0.84 1.09 1.59
At1g10585	bHLH transcription factor	1	0.48 0.38 0.48	1.08 0.79 0.60	1.25 1.11 1.17	0.96 1.34 0.46	1.10 0.17 1.15
At1g17170	Glutathione S-transferase	1	1.88 1.85 1.65	6.59 2.88 2.78	2.20 1.97 1.15	2.04 4.54 1.60	1.25 1.09 0.77
At1g26380	FAD-linked oxidoreductase	1	0.41 2.57 9.04	0.91 1.59 12.06	2.81 8.09 20.75	4.31 43.87 79.02	2.90 1.17 19.15
At5g13080	AtWRKY75	1	1.54 1.88 1.22	2.53 1.42 1.25	5.83 2.68 2.62	2.59 12.47 1.84	1.11 1.16 1.84
Specific for singlet oxygen							
At4g34410	AP2 domain transcription factor	1	12.59 1.69 16.97	20.08 10.34 10.24	10.43 5.45 21.68	4.28 178.8 20.48	1.47 15.03 7.40
Specific for superoxide							
AtCg01050	NADH dehydrogenase ND4	1	0.18 1.05 2.01	0.12 0.24 0.18	2.13 2.53 4.69	0.74 18.70 3.20	0.83 1.83 3.06
At3g01140	AtMYB106	1	0.77 1.55 13.20	2.40 1.89 12.89	3.30 2.34 20.07	0.63 11.97 6.96	1.19 1.37 11.55
Specific for hydrogen peroxide							
At4g23290	Serine/threonine kinase - like protein	1	0.17 0.20 0.47	0.04 0.04 0.06	0.19 0.10 0.21	0.18 0.04 0.42	0.32 0.10 0.39

domain transcription factor, *At4g34410*) and for one marker gene specific for superoxide (AtMYB106, *At3g01140*) (Table 3.2.). With respect to superoxide generation, these data were in accordance with the previous finding that superoxide generation was not altered in *gun* mutants relative to WT plants (Strand *et al.*, 2003). In contrast to that, the expression of the second marker gene specific for superoxide, which is encoded in the plastid (NADH dehydrogenase ND4, *AtCg01050*), was not markedly affected in NF-treated wild-type plants, but down-regulated in *gun1* mutants and up-regulated in *gun2*, *gun4* and *gun5* seedlings (Table 3.2.). This expression pattern reflects the visible phenotype. It suggests that in the tetrapyrrole biosynthesis mutants, plastids and their capability to perform transcription were less affected than in wild-type and *gun1* seedlings. Furthermore, transcripts of the marker gene specific for hydrogen peroxide stress (serine/threonine kinase like protein, *At4g23290*) were strongly down-regulated in all NF-treated genotypes (Table 3.2.). Taken together, the data do not support the idea that a general decrease or increase in a particular ROS molecule can be made responsible for differences between wild-type and *gun* seedlings with respect to *LHCB1.2* mRNA accumulation or the specific growth phenotype when grown on MS plates supplemented with NF.

3.9. Lipid composition is affected by NF treatment

Besides its photo-oxidative effect, NF does also affect the plastid lipid composition of plants (Abrous *et al.*, 1998; Di Baccio *et al.*, 2002). To test whether this reaction is influenced in the *gun* mutants, lipids were extracted from wild-type and *gun* seedlings grown in the absence or presence of NF and separated using thin layer chromatography (Figure 3.13.). As expected, in NF-treated plants some lipid bands were missing compared to untreated plants (Figure 3.13.; three blue bands above MGDG). However, this is the case for both wild-type and mutant plants. The two most prominent lipids in plastids, monogalactoglycerolipids (MGDG) and digalacto-glycerolipids (DGDG), were present in all genotypes, while the amount of MGDG was lowered in *gun1* grown on NF. This result did not correlate with the investigated phenotypes - *LHCB1.2* mRNA accumulation and the growth phenotype. In plants grown under non-photo-bleaching conditions some lipid bands were lowered in their intensity in *gun2* and were more prominent in *gun4* (Figure 3.13.).

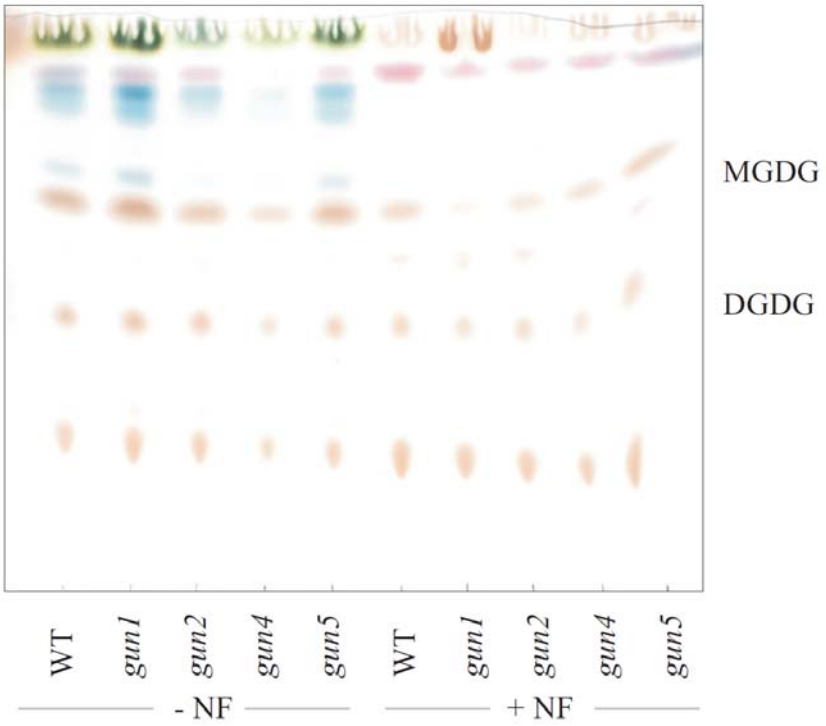

Figure 3.13: Total lipid extract from plants grown for 6 days under continuous light (100 µmol m^{-2} s^{-1}) on MS plates without (- NF) or supplemented with NF (+ NF). Lipids were separated by thin layer chromatography and visualized using ferrous sulfate/potassium permanganate solution incubated for 20 minutes at 100°C.
MGDG, monogalactoglycerolipids; DGDG, digalactoglycerolipids

3.10. Several metabolic processes are altered in NF-treated plants

Additionally to the proposed retrograde signaling pathways connected to plastid translation, ROS or tetrapyrrole biosynthesis, some indications exist that the organellar redox poise or alterations within the redox poise are involved in retrograde signaling. Also metabolic components produced in plastids could be responsible for signaling processes (for review see Baier and Dietz, 2005; Bräutigam *et al.*, 2007; Kleine *et al.*, 2009). In order to investigate whether changes in metabolic processes could be connected to the specific phenotypes seen in the *gun* mutants during photo-oxidative damage, we analyzed the expression levels of marker genes for a broad range of metabolic processes (Table 3.3.). We also measured the amount of several metabolic components in plants grown for 6 days in the absence or presence of NF (Table 3.4.).

Table 3.3: Real-time PCR analyses of the expression of marker genes for different metabolic processes in 6-day-old NF-treated wild-type and *gun* seedlings. Values were obtained as in Table 3.2. and presented in relation to untreated wild-type seedlings.

Gene	Description	WT	WT + NF	*gun1* + NF	*gun2* + NF	*gun4* + NF	*gun5* + NF
Marker genes for different metabolic processes							
At1g77490	tAPX	1	0.12 0.20 0.54	0.11 0.07 0.25	0.22 0.47 1.28	0.20 0.15 0.74	0.21 0.36 1.19
At5g52570	Beta-carotene hydroxylase	1	0.03 0.02 0.03	0.16 0.14 0.12	0.38 0.23 0.44	0.14 0.10 0.08	0.22 0.07 0.18
At5g17230	Phytoene synthase	1	0.12 0.11 0.72	0.34 0.17 0.79	0.44 0.22 0.92	0.20 0.08 0.45	0.35 0.12 0.89
At5g67030	Zeaxanthin epoxidase	1	0.37 0.24 0.88	0.63 0.36 0.49	1.48 0.77 1.44	0.73 0.58 0.66	0.94 0.47 0.96
At1g08550	Putative violaxanthin de-epoxidase	1	0.28 0.30 1.87	0.48 0.25 1.18	0.96 0.55 3.12	0.44 0.12 1.07	0.77 0.28 2.22
At3g02730	Thioredoxin f1	1	0.08 0.18 0.44	0.08 0.14 0.27	0.14 0.32 0.59	0.16 0.30 0.31	0.24 0.32 0.75
At3g15360	Thioredoxin m4	1	0.08 0.13 0.45	0.07 0.10 0.33	0.56 0.43 0.95	0.20 0.18 0.63	0.35 0.26 1.08
At1g50320	Thioredoxin x	1	0.21 0.19 0.54	0.28 0.29 0.48	0.47 0.55 1.04	0.30 0.35 0.52	0.45 0.40 0.88
At2g41680	Putative thioredoxin reductase	1	0.36 0.66 0.77	0.74 0.77 0.87	0,81 1.32 1.75	0.58 0.64 0.72	0.60 1.03 1.64
At3g26060	Putative peroxiredoxin	1	0.12 0.07 0.31	0.09 0.04 0.27	0.32 0.19 0.58	0.17 0.07 0.36	0.27 0.20 0.88
At2g25080	Putative glutathione peroxidase	1	0.49 0.22 0.50	0.42 0.22 0.36	2.36 0.90 1.36	0.77 0.19 0.50	1.32 0.26 1.10
At5g04140	Ferredoxin-dependent glutamate synthase	1	0.24 0.48 0.62	0.50 0.45 0.47	1.28 1.01 1.44	0.46 0.01 0.49	1.00 0.31 1.16
At1g58290	Glutamyl-tRNA reductase	1	0.16 0.19 0.71	0.30 0.23 0.78	1.18 0.75 2.49	0.31 0.05 0.51	0.59 0.23 0.90
At4g25080	Magnesium-protoporphyrin IX methyltransferase	1	0.21 0.17 0.29	0.21 0.19 0.24	0.98 0.82 1.31	0.35 0.33 0.36	0.54 0.32 0.51

Real-time PCR was performed to investigate the expression level for several marker genes in NF-treated wild-type and mutant seedlings compared to untreated wild-type plants. Again three different genes were used for loading control (*UBIQUITIN*, A*CTIN1* and *18S* rRNA).The overall picture for all genes tested was similar between wild-type and *gun* seedlings in the presence of NF: compared to non-photo-bleached wild-type plants the transcript levels were strongly down regulated (Table 3.3.; values normalized to *UBIQUITIN* are shown). This indicates that photo-oxidative damage of plastids by NF affects overall metabolic processes. This was the case for genes involved in processes either directly affected by NF (carotenoid biosynthesis: beta-carotene hydroxylase, *At5g52570*; phytoene synthase, *At5g17230*; zeaxanthin epoxidase, *At5g67030*; putative violaxanthin de-epoxidase, *At1g08550*) or indirectly caused by photo-oxidative stress (ROS detoxification and redox poise: tAPX, *At1g77490*; thioredoxin f1, *At3g02730*; thioredoxin m4, *At3g15360*; thioredoxin x, *At1g50320*; putative thioredoxin reductase, *At2g41680*; putative peroxiredoxin, *At3g26060*; putative glutathione peroxidase, *At2g25080*; ferredoxin-dependent glutamate synthase, *At5g04140*; or tetrapyrrole biosynthesis: glutamyl-tRNA reductase, *At1g58290*; Mg-proto IX methyltransferase, *At4g25080*).

Unexpectedly, measurement of metabolites like phosphorylated intermediates of the central carbohydrate metabolism, citric acid cycle intermediates and nucleotides revealed no marked differences between NF-treated and untreated plants irrespective of the genotype, although the amount of some metabolites like the investigated nucleotides were to slight extent lower in plants treated with NF (Table 3.4.). Furthermore, comparison between wild-type and *gun* mutants did not show for any of the measured metabolites a correlation with the *LHCB1.2* mRNA accumulation phenotype visible after NF treatment. Here three major observations have to be mentioned. First, many metabolites were less abundant in untreated *gun2* seedlings compared to the other untreated plants. This is in accordance with the strong pale green phenotype of the mutant line. However, when plants were treated with NF the differences between *gun2* and the other genotypes were less pronounced. Second, the comparison of NF-treated genotypes revealed that in *gun1* some metabolites, mainly citric acid cycle intermediates (e.g. a-ketoglutarate, malate and succinate) were more abundant than in the other seedlings, again suggesting that GUN1 protein is involved in other regulatory processes.

Table 3.4: Metabolites isolated from wild-type and *gun* seedlings grown for 6 days in the absence (- NF) or presence of NF (+ NF). Measurements were performed according to Haeusler *et al.* (2000) and Schneidereit *et al.* (2006) . Mean values (± SD) resulted from three independent experiments. Values in parentheses were measured by a different enzymatic assay according to Dietz and Heber (1986). Values correspond to nmol/g fresh weight.

n. d., not determined; aKG, a-ketoglutarate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; F6P, fructose-6-phosphate; F16P, fructose-1.6-bisphosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; M6P, mannose-6-phosphate; PEP, phosphoenolpyruvate; UDP, uridine diphosphate; UDP-Glc, UDP glucose; 3PG, 3-phosphoglycerate

	Wild-type	***gun1***	***gun2***	***gun4***	***gun5***
- NF					
	Phosphorylated intermediates				
G1P	7.1 ± 1.6	12.4 ± 7.7	13.3 ± 1.7	13.2 ± 5.5	17.1 ± 4.1
G6P	123.5 ± 18.6	156.5 ± 9.8	91.2 ± 11.7	112.9 ± 0.9	149.4 ± 13.3
(G6P)	151.1 ± 19.2	117.5 ± 34.9	117.2 ± 8.1	137.1 ± 8.4	117.0 ± 16.6
F6P	9.9 ± 1.4	20.9 ± 23.2	n. d.	15.5 ± 14.0	13.8 ± 2.6
(F6P)	39.9 ± 1.8	29.7 ± 2.8	29.4 ± 21.3	32.0 ± 4.8	33.2 ± 14.5
F16P	2.7 ± 0.9	5.3 ± 1.0	1.7 ± 0.9	4.4 ± 1.2	6.5 ± 0.2
M6P	12.5 ± 2.9	23.0 ± 5.2	18.0 ± 1.4	20.6 ± 4.2	28.3 ± 3.7
3PG	70.7 ± 5.3	96.3 ± 30.1	44.6 ± 6.7	73.9 ± 18.7	117.1 ± 9.1
UDP-Glc	58.2 ± 17.5	79.7 ± 19.1	46.5 ± 13.3	71.2 ± 16.2	101.7 ± 11.6
PEP	12.1 ± 1.1	13.1 ± 3.8	8.9 ± 1.3	11.9 ± 2.6	16.0 ± 2.3
Pyruvate	7.1 ± 4.5	20.5 ± 17.6	6.9 ± 3.2	13.8 ± 2.3	10.5 ± 10.0
	Citric acid cycle intermediates				
aKG	9.0 ± 2.9	14.2 ± 7.5	4.1 ± 2.6	12.9 ± 3.8	6.7 ± 5.8
Citrate	195.1 ± 48.6	362.5 ± 110.6	95.6 ± 22.5	219.0 ± 74.0	247.1 ± 34.2
Fumarate	88.4 ± 19.2	78.1 ± 22.2	35.0 ± 12.9	45.5 ± 4.0	41.2 ± 8.9
Malate	192.0 ± 53.0	211.2 ± 31.3	90.4 ± 8.2	142.4 ± 6.2	145.2 ± 21.9
Succinate	57.7 ± 15.6	53.1 ± 3.3	35.3 ± 7.7	32.3 ± 1.3	35.0 ± 8.9
	Nucleotides				
AMP	5.1 ± 1.2	7.2 ± 2.6	5.9 ± 1.3	7.0 ± 2.3	9.1 ± 1.2
ADP	19.6 ± 4.5	26.9 ± 5.9	17.8 ± 1.6	26.4 ± 5.5	32.0 ± 3.5
(ATP)	65.6 ± 13.0	48.3 ± 12.0	32.9 ± 9.6	80.2 ± 11.7	59.6 ± 25.7
UDP	5.9 ± 1.7	9.1 ± 2.8	5.6 ± 0.8	7.1 ± 1.9	11.6 ± 1.4

+ NF					
Phosphorylated intermediates					
G1P	13.2 ± 1.2	23.4 ± 3.1	14.6 ± 2.7	16.3 ± 4.4	16.1 ± 6.2
G6P	84.4 ± 15.8	200.0 ± 17.5	84.8 ± 13.1	101.6 ± 3.5	117.3 ± 32.8
(G6P)	126.8 ± 38.8	207.4 ± 67.2	82.6 ± 0.1	113.4 ± 14.7	70.3 ± 18.6
F6P	17.5 ± 9.2	32.4 ± 13.0	n. d.	21.3 ± 12.0	13.7 ± 9.0
(F6P)	26.7 ± 3.8	44.2 ± 4.6	23.8 ± 13.0	24.4 ± 17.3	30.4 ± 7.0
F16P	2.3 ± 1.5	12.5 ± 2.4	1.6 ± 0.5	3.9 ± 0.2	6.4 ± 1.9
M6P	10.7 ± 0.6	23.7 ± 2.0	16.1 ± 1.8	18.6 ± 3.5	19.1 ± 6.8
3PG	30.7 ± 8.4	49.7 ± 4.3	36.2 ± 11.7	49.0 ± 10.3	52.0 ± 19.0
UDP-Glc	70.5 ± 8.7	86.1 ± 5.1	53.0 ± 11.8	87.6 ± 12.2	94.1 ± 32.5
PEP	6.4 ± 0.7	6.8 ± 0.8	8.1 ± 1.8	9.2 ± 2.0	9.1 ± 3.7
Pyruvate	4.6 ± 2.0	9.7 ± 6.0	9.03 ± 4.1	7.6 ± 1.6	6.7 ± 3.9
Citric acid cycle intermediates					
aKG	4.8 ± 2.3	15.1 ± 12.1	4.6 ± 1.3	8.1 ± 0.9	5.8 ± 2.0
Citrate	102.6 ± 50.8	194.1 ± 26.4	67.6 ± 27.0	81.7 ± 11.3	203.4 ± 82.5
Fumarate	11.9 ± 1.9	14.4 ± 2.2	11.0 ± 13.2	9.7 ± 0.1	10.5 ± 2.5
Malate	69.0 ± 25.7	264.7 ± 40.8	56.4 ± 25.4	59.2 ± 12.1	81.9 ± 18.3
Succinate	47.4 ± 19.8	89.6 ± 11.2	40.0 ± 5.2	31.4 ± 12.5	40.0 ± 7.1
Nucleotides					
AMP	1.6 ± 0.1	2.5 ± 0.4	3.9 ± 2.5	3.7 ± 1.4	3.4 ± 1.7
ADP	8.2 ± 1.6	12.2 ± 2.1	16.8 ± 2.8	15.9 ± 2.6	19.8 ± 6.6
(ATP)	33.5 ± 8.1	21.4 ± 12.8	37.3 ± 14.0	59.6 ± 12.7	36.4 ± 3.8
UDP	4.8 ± 1.0	3.5 ± 3.4	1.9 ± 2.9	8.3 ± 1.8	9.4 ± 6.0

Third, AMP, ADP and, to a less extent, ATP accumulated more in NF-treated *gun2*, *gun4* and *gun5* seedlings than in wild-type and *gun1*, which is in correlation with the pigmentation or growth phenotype described above (3.2. and Figure 3.3.). Taken together, it can be stated that plants were able to use the supplemented sucrose to produce the metabolites needed to grow under photo-bleaching conditions.

3.11. The pigmentation phenotype is due to carotenoid enrichment

It was shown that several processes - synthesis of plastid-encoded proteins (Figure 3.9.), alteration of the expression of one plastid localized superoxide specific marker gene (i.e. NADH dehydrogenase ND4; Table 3.2.) and to a weaker extent the

accumulation of nucleotides (Table 3.4.) - were correlated with the pigmentation or growth phenotype observed in NF-treated *gun2*, *gun4* and *gun5* mutants. Those correlations could not explain the visible pigmentation phenotype. Therefore, we tried to measure chlorophyll fluorescence using pulse amplitude modulation technique and 77K fluorescence emission spectroscopy in NF-treated plants. However, it was not possible to measure any fluorescence in photo-bleached seedlings (data not shown). Hence, we determined the amount of several pigments. To this end, leaf pigments were isolated from 6-day-old NF-treated plants, separated by reverse-phase HPLC and monitored. In NF-grown wild-type and *gun* mutants, chlorophyll a and b, antheraxanthin, zeaxanthin and α-carotene were below the detection level while neoxanthin, violaxanthin and lutein were barely detectable in WT plants and not detectable at all in *gun1* seedlings. In contrast, these three pigments accumulated in the *gun2*, *gun4* and *gun5* seedlings to some extent, thus explaining the leaf coloration of these genotypes after NF treatment. As expected, this accumulation was much lower than in untreated plants (data not shown).

Table 3.5: Pigment levels in NF-treated WT and *gun* mutants grown for 6 days under continuous light of 100 µmol m^{-2} s^{-1}. Pigments were separated by reverse-phase HPLC and monitored at 440 nm. The results are reported in pmol/mg fresh weight. Mean values of at least three independent experiments ± SD are shown. Chlorophyll a, chlorophyll b, antheraxanthin, zeaxanthin and α-carotene could not be detected in seedlings grown in the presence of NF.

	Neoxanthin	**Violaxanthin**	**Lutein**
WT	0.04 ± 0.03	0.08 ± 0.07	0.07 ± 0.06
gun1	0 ± 0	0 ± 0	0 ± 0
gun2	0.20 ± 0.11	0.61 ± 0.23	0.86 ± 0.36
gun4	0.09 ± 0.03	0.30 ± 0.03	0.33 ± 0.09
gun5	0.14 ± 0.02	0.40 ± 0.08	0.43 ± 0.10

3.12. Involvement of abscisic acid in retrograde signaling

Since neoxanthin and violaxanthin are precursors of xanthoxin which is exported to the cytosol and there converted into the plant hormone abscisic acid (ABA) (see also Figure 3.5.), which has been previously associated with plastid signaling (Baier *et al.*, 2004; Baier and Dietz, 2005; Koussevitzky *et al.*, 2007; Kim *et al.*, 2009; Kleine *et al.*, 2009), we investigated the effect of ABA on nuclear gene expression under our conditions. Thus, we tested the impact of ABA on NF-induced *LHCB1.2* de-repression. We used very low ABA concentrations (0.125; 0.25; 0.5 and 1 µM) to test whether *LHCB1.2* expression in NF-treated wild-type seedlings could be rescued by ABA. Indeed, it seemed that in 6-day-old plants *LHCB1.2* mRNA expression could be gradually restored with increasing concentrations of ABA (Figure 3.14.). When compared with plants grown without ABA on NF, a concentration of 0.125 µM ABA revealed no effect, the *LHCB1.2* mRNA level was doubled at a concentration of 0.25 µM ABA and enhanced more than 5-fold when 1 µM ABA was feeded.

The influence of ABA on the growth capability of photo-bleached plants was different to the effect of ABA on nuclear gene expression. With rising concentrations of ABA, the plants grow worse than without ABA on MS plates containing NF (Figure 3.15.). The whole plants, and most prominent the roots, were smaller. When supplemented with even higher concentrations of ABA (starting with 2.5 µM), the germination rate was extremely reduced (data not shown). This again showed that effects on *LHCB1.2* mRNA accumulation could not be linked to a specific growth phenotype.

To investigate whether the *gun* mutants interfere in general, i.e. under non-photo-bleaching conditions, with regulatory processes linked to ABA we determined the germination rate of wild-type and *gun* mutant seeds at increasing ABA concentrations on MS medium without sucrose. The results (Figure 3.16.) indicated that the germination sensitivity of *gun4* and *gun5* seeds was indeed increased compared to wild-type, whereas *gun1* and *gun2* actually showed decreased sensitivity to ABA during the first days after germination. This implies that there is no simple link between the mode of action of ABA and *gun* signaling.

Moreover, we tested the expression level of two genes known to be regulated by ABA (*rd29* and *cor47*; Sanchez *et al.*, 2004) and of one gene involved in the synthesis

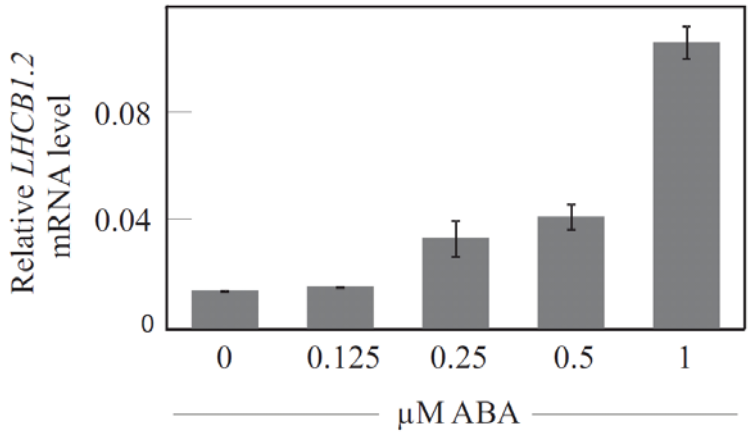

Figure 3.14: Changes in *LHCB1.2* mRNA accumulation in response to elevated concentrations (as indicated) of supplemented ABA in photo-bleached wild-type seedlings. Plants were grown for 6 days under continuous light (100 µmol m^{-2} s^{-1}) in the presence of 0.5 µM NF. Data were obtained in triplicate by real-time PCR, set relative to non-photo-bleached wild-type seedlings and normalized to the expression level of *ACTIN1*.

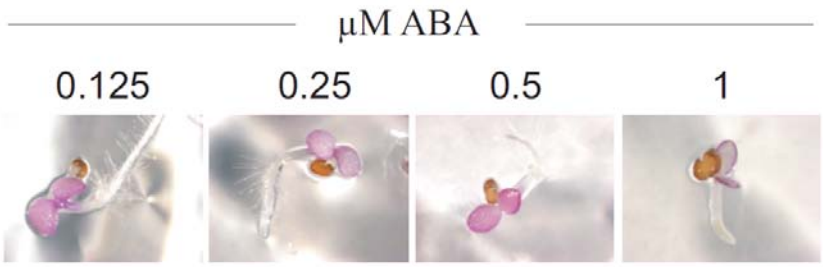

Figure 3.15: Pictures of NF-treated wild-type plants additionally supplemented with different concentrations of ABA (as indicated). Plants were grown on MS plates containing 0.5 µM NF.

of gibberellic acid, the antagonist of ABA, (*ga1*, Qin *et al.*, 2007) in wild-type and *gun* seedlings grown for 6 days in the absence or presence of NF. The expression pattern of these genes did not reveal any correlation with the investigated *LHCB1.2* mRNA de-repression and growth phenotypes (data not shown).

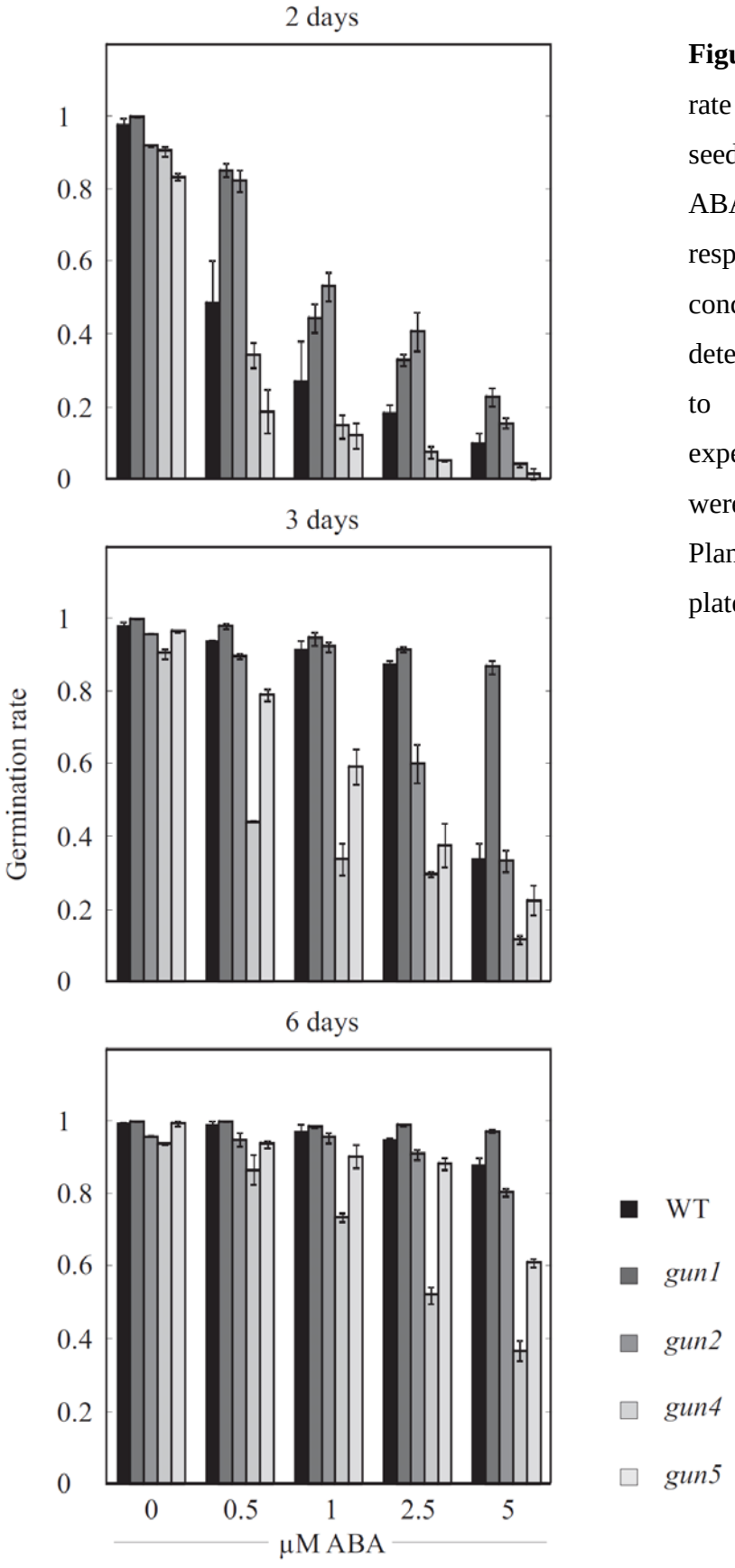

Figure 3.16: Germination rate of wild-type and *gun* seedlings in response to ABA. Germination rate in response to different concentrations of ABA was determined by counting 80 to 120 plants per experiment each day. Plants were stratified for two days. Plants were grown on MS plates without sucrose.

3.13. De-repression of *LHCB1.2* is age-dependent, not affected by day length and photo-bleached plants retain ability to green

Additional facts concerning the repression of *LHCB1.2* mRNA accumulation in photo-bleached seedlings: First, the differences between wild-type and *gun* seedlings in *LHCB1.2* mRNA repression were age dependent. When plants were grown for a longer time (e.g. 10 days) on MS plates containing NF, the levels of *LHCB1.2* mRNA were more similar between the genotypes than after 6 days (Figure 3.17.). During the work of this thesis plants were normally grown under continuous light, therefore it has to be noted that the specific down-regulated expression of *LHCB1.2* in response to photo-oxidative damaged plastids also occurred, irrespective of the day length, when plants were grown under dark-light cycle as it is shown for wild-type plants in Figure 3.18. Finally, it was possible to rescue photo-bleached seedlings of all genotypes by transferring them after 6 days on plates including NF to plates without NF (Figure 3.19.). This indicates that NF treatment did not result in a complete destruction of the plants.

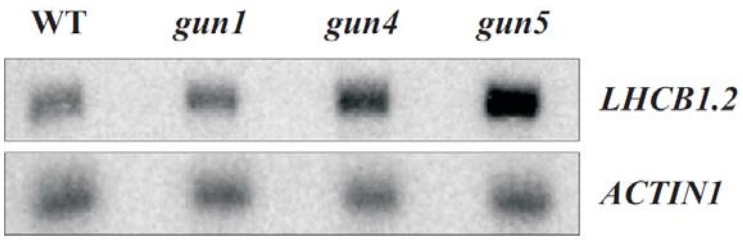

Figure 3.17: Northern blot analysis of *LHCB1.2* mRNA accumulation in NF-treated wild-type and *gun* seedlings grown for 10 days under continuous light of 100 µmol m^{-2} s^{-1}. The *ACTIN1* mRNA level was used as loading control.

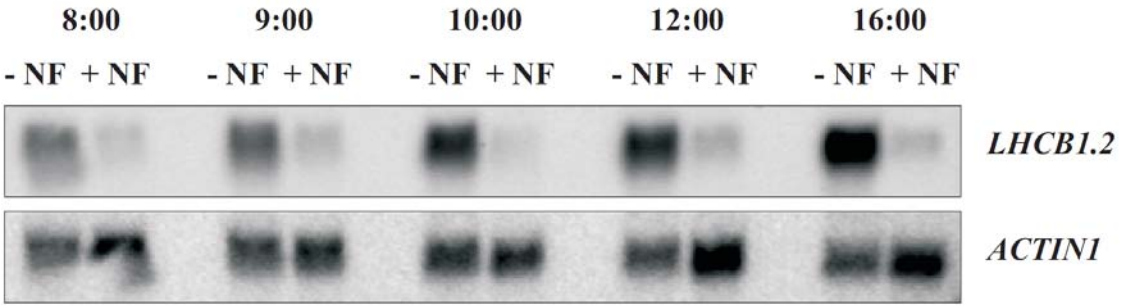

Figure 3.18: *LHCB1.2* mRNA level determined by Northern blot analysis from wild-type plants grown for 14 days under dark-light cycle (12 h / 12 h) in the absence (- NF) or presence of NF (+ NF). Plants were harvested at different time-points (as indicated) to test for an effect of the day length. The *ACTIN1* mRNA level served as a loading control.

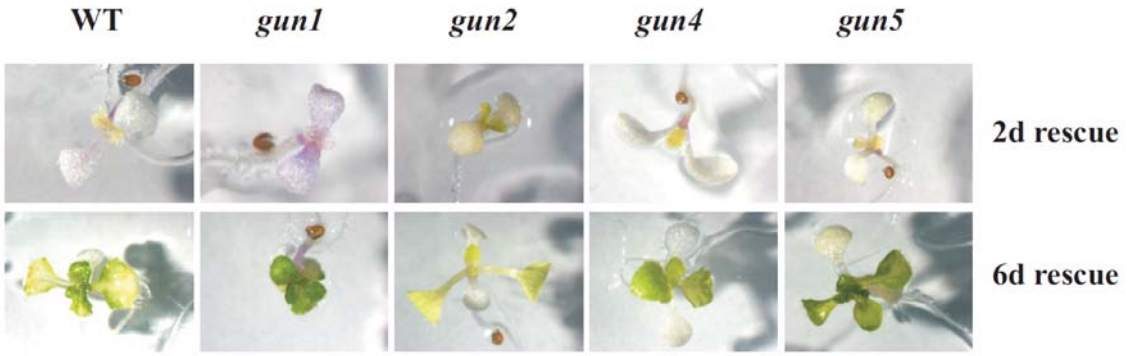

Figure 3.19: Rescue of photo-bleached seedlings. Wild-type and *gun* seedlings were grown for 6 days on MS plates containing NF and then transferred to plates without NF. Plants are shown 2 or 6 days after the transfer.

4. Discussion

4.1. No new *gun* mutants could be isolated

In order to correlate the composition of tetrapyrroles to the expression level of *LHCB1.2* when treated with norflurazon (NF), we wanted to isolate mutants for all enzymes of the tetrapyrrole biosynthesis pathway. An identical approach was undertaken by two independent groups, who could clearly show that neither magnesium-protoporphyrin IX (Mg-proto) nor any other precursor of chlorophyll is linked to the expression level of *LHCB1.2* (Mochizuki *et al.*, 2008; Moulin *et al.*, 2008).

Moreover, beside one new allele for *gun2* we could not isolate any new *gun* mutant line (3.1.). In most homozygous mutant lines analyzed the expression of the affected gene was down-regulated, while in some cases an over-expression could be observed (Table 3.1.). However, these expression studies yielded no information about the functionality of the detected transcripts. For example the *pora-2* mutant line showed a strong over-expression of the affected gene. The insertion was predicted to be in the last exon, while the used primers were designed to amplify a fragment at the 5'region of the gene. It is likely that the mRNA was truncated at the insertion site leading to a loss of PORA protein. The lack of the PORA protein could probably led to a feedback up-regulated expression of its gene. To answer this question, Northern blot analysis would be the method of choice, making it possible to resolve differences in transcript sizes. However, we stopped following these analyses as we failed to identify any novel *gun* mutants among the investigated T-DNA insertion lines affected in enzymes of tetrapyrrole biosynthesis. In this respect our results confirmed the assumption of Mochizuki *et al.* (2008), that a general disturbance in the accumulation of tetrapyrrole intermediates does not automatically lead to the *gun* phenotype. But we cannot exclude the possibility that beside the known *gun* mutant lines even the inhibition of additional steps in the tetrapyrrole pathway can trigger a *gun* phenotype under appropriate conditions, since not all tested mutant lines were knock-out lines and we only analyzed mutants with defects that were compatible with photoautotrophic growth. Moreover, in previous reports novel *gun* mutants were described which were either affected in genes for porphobilinogen deaminase (*PBD* knockout), coproporphyrinogen III oxidase (*lin2*) and D subunit of Mg-chelatase (*CHLD* knockout) or over-expressing protochlorophyllide oxidoreductase A (POR-OX) (McCormac and Terry, 2002b; Strand

et al., 2003; McCormac and Terry, 2004). Compatible with our results, the homozygous knockout lines for PBD are incapable of photoautotrophic growth (Strand *et al.*, 2003).

4.2. A newly observed pigmentation phenotype reveals insights into the complex interplay between biochemical processes in plastids

When *gun* mutants were grown on plates supplemented with NF, the typical repression of *LHCB1.2* mRNA accumulation, as known from wild-type plants, was attenuated. For more than 15 years the mutants were screened for this specific NF effect. A lot of studies, addressing this specific phenotype, were performed (e.g. Susek *et al.*, 1993; Mochizuki *et al.*, 1996; Vinti *et al.*, 2000; Mochizuki *et al.*, 2001; Larkin *et al.*, 2003; Strand *et al.*, 2003; McCormac and Terry, 2004; Ankele *et al.*, 2007; Koussevitzky *et al.*, 2007; Ruckle *et al.*, 2007; Larkin and Ruckle, 2008; Mochizuki *et al.*, 2008; Moulin *et al.*, 2008). Surprisingly, none of this publications, except Mochizuki *et al.* (2001), recognized that the *gun* mutants affected in tetrapyrrole biosynthesis exhibited an additional phenotype. In contrast to wild-type and *gun1* seedlings, those mutants grow slightly better on MS plates supplemented with NF and show a weak leaf pigmentation (3.2. and Figure 3.3.). By using different inhibitors of carotenoid biosynthesis (amitrole, flurochloridone and CPTA) we clearly demonstrated that the pigmentation phenotype is not coupled to the de-repression of *LHCB1.2* mRNA accumulation (3.3. and Figures 3.6. and 3.7.). While the differences in *LHCB1.2* expression between photo-bleached wild-type and *gun* seedlings were present independent of the used inhibitor, the pigmentation phenotype was visible only in NF-treated plants. Consequently, we tested whether the mutants were resistant to norflurazon by measuring the accumulation of phytoene. While undetectable in untreated plants, phytoene accumulated in all NF-treated seedlings with slightly reduced levels in *gun1* and *gun2* and even higher levels in *gun4* and *gun5* when compared to wild-type (Figure 3.8.). Hence it can be concluded that neither wild-type nor the *gun* mutants are resistance to NF.

Although the visible phenotype was not directly linked to retrograde signaling further analyses of it revealed new insight into the complex regulatory network of plastids. First of all, it could be shown that the weak pigmentation in the *gun* mutants was caused by enrichment of some carotenoids, namely neoxanthin, violaxanthin and

lutein which were hardly detectable in NF-treated wild-type plants, not present in *gun1* seedlings and elevated in *gun2*, *gun4* and *gun5* plants (3.11. and Table 3.5.). These results were difficult to explain since neoxanthin, violaxanthin and lutein are synthesized downstream of the NF-induced block in carotenoid biosynthesis. It could be speculated that the excess phytoene (relative to wild-type level) accumulated in *gun4* and *gun5* (see Figure 3.8.) is processed into carotenoids by an unknown route that bypasses the NF-induced block. For *gun2*, where a decrease (relative to wild-type level) in phytoene accumulation was associated with even higher levels (relative to *gun4* and *gun5*) of neoxanthin, violaxanthin and lutein, a less efficient block in carotenoid biosynthesis by NF appears plausible. Whatsoever, it seemed that there exists a regulatory link between tetrapyrrole and carotenoid biosynthesis, although no other mutant of tetrapyrrole biosynthesis investigated in this work exhibited this phenotype (Figure 3.4.). Recently it could be shown that a block in carotenoid biosynthesis resulted in inhibition of genes involved in many processes including chlorophyll biosynthesis (Qin *et al.*, 2007). Our data suggest that there exists a similar link from the tetrapyrrole biosynthesis to the carotenoid biosynthesis, because mutations in chlorophyll biosynthesis as in *gun4* and *gun5* trigger alterations in carotenoid biosynthesis when treated with NF. Moreover, in contrast to the known regulation of carotenoid biosynthesis e.g. via plastoquinone (Norris *et al.*, 1995), this link seems not to be based on the regulation of phytoene desaturase, but to bypass the blocked phytoene desaturase reaction. Interestingly, neoxanthin and violaxanthin are the precursors of abscisic acid which by itself is thought to be involved in retrograde signaling (see below).

A further observation was that the accumulation of anthocyanin in NF-treated seedlings differed among the genotypes. While anthocyanin accumulation was higher in *gun1* compared to wild-type plants, it was lower in the other three *gun* mutants (Figure 3.3.). Two explanations of this effect appear to be feasible. Anthocyanins are known to protect plastids from photo-oxidative stress and photoinhibition induced by high light flux (Vanderauwera *et al.*, 2005; Kleine *et al.*, 2007). Therefore, anthocyanins might be produced in response to photo-oxidative stress caused by NF to protect the seedlings. One interpretation of the lower anthocyanin accumulation in *gun2*, *gun4* and *gun5* mutants could be that these genotypes experience less plastid dysfunction during NF

treatment or that they are less susceptible to stress. Another possibility could be that the induction of anthocyanin biosynthesis in response to photo-oxidative stress depends on plastid signals affected in *gun2*, *gun4* and *gun5* but not in *gun1*. In contrast to recent suggestions (Koussevitzky *et al.*, 2007; Woodson and Chory, 2008), this would support the idea that *gun1* is impaired in a different pathway. The observation that anthocyanin accumulation depends on the presence of GUN1 when seedlings are grown under 50 µmol m^{-2} s^{-1} blue light on lincomycin (Ruckle *et al.*, 2007) is not contradictory to our results, since lincomycin and norflurazon have different effects on the chloroplasts. However, the finding that several processes were less affected in plastids of *gun2*, *gun4* and *gun5* mutants than in the wild-type and *gun1*, e.g. synthesis of plastid-encoded proteins (3.5. and below), alteration of the expression of the superoxide specific plastid localized marker gene (i.e. NADH dehydrogenase ND4; 3.8.) and to a weaker extent the accumulation of nucleotides (3.10.), are more compatible with the hypothesis that *gun2*, *gun4* and *gun5* were to some degree less affected by NF

After NF treatment neither Lhcb1, Lhca3, PsaB, PsbD nor PetB were detectable in wild-type or *gun* seedlings (3.5. and Figure 3.9.), indicating that residual thylakoids (Susek *et al.*, 1993; McCormac and Terry, 2004) were not functional anymore. Moreover, the lack of Lhcb1 in all genotypes argues against a physiological relevance of *LHCB1.2* mRNA de-repression in the *gun* mutants. This is in accordance with the lack of chlorophylls in NF-treated seedlings (see 3.11. and Table 3.5.), that normally stabilize the light-harvesting proteins. However, the plastome-encoded proteins RbcL and AtpB were detectable at low levels in NF-grown wild-type seedlings, which is consistent with the finding that RbcL is still present in photo-bleached wheat leaves (La Rocca *et al.*, 2004). Thus, the photo-oxidized plastids still maintained the ability to carry out protein synthesis. Surprisingly, RbcL and AtpB actually accumulate to a greater extent in the *gun* mutants, especially in *gun2*, *gun4*, and *gun5*. Again it is possible to link this observation to the ability of the *gun* mutants to grow better on NF than wild-type, or to a possible interplay between organellar gene expression (OGE) and *gun* signaling. Support for the latter possibility came from the observation that the repression of *LHCB1.2* expression in response to inhibition of OGE requires the GUN1 protein (Gray *et al.*, 2003; Koussevitzky *et al.*, 2007). GUN1 is a specific PPR protein which is associated with sites of active transcription on plastid DNA (Koussevitzky *et*

al., 2007). PPR proteins are known to be involved in all processes of gene expression i.e. RNA transcription, editing, splicing, processing, stability and translation (Andres *et al.*, 2007; Pogson *et al.*, 2008). Working out how the lack of such a protein and a disturbance in tetrapyrrole biosynthesis, as in *gun2*, *gun4* and *gun5*, can yield in enhanced capacity of gene expression in NF-treated *gun* plastids will be an important step to understand the molecular mechanisms that lead to *gun* signaling.

Taken together, the findings related to the specific growth and pigmentation phenotype seen in *gun* mutants affected in tetrapyrrole biosynthesis provide an indication of a complex interplay between biosynthesis of tetrapyrroles and carotenoids, the accumulation of anthocyanins, as well as organellar gene expression. Differences are mainly due to the fact that *gun2*, *gun4* and *gun5* were not that strongly affected by NF than wild-type plants. However, it is not possible to clarify what could be the reason for this specific growth phenotype.

4.3. Accumulation of tetrapyrroles does not correlate with *LHCB1.2* expression

After several years of conflicting results (reviewed in Pesaresi *et al.*, 2007; Kleine *et al.*, 2009), recent analyses using more precise and reproducible methods to measure tetrapyrroles demonstrated that accumulation of Mg-proto is not connected to the *LHCB1.2* mRNA accumulation. Thus, it is most likely that Mg-proto, or other chlorophyll precursors, are not involved in plastid-to-nucleus signaling in higher plants (Mochizuki *et al.*, 2008; Moulin *et al.*, 2008). Previously it was suggested that accumulation of Mg-proto is responsible for *LHCB1.2* repression (Strand *et al.*, 2003). To test this theory *in vivo*, two mutants of enzymes downstream of Mg-chelatase, *chlm* and *crd1*, were used as well as the double mutant *chlm crd1*. Actually, Mg-proto levels were elevated in those mutant lines but no effect on *LHCB1.2* transcript abundance could be observed. Furthermore, double mutants between *chlm* and *gun* mutants were generated in order to elevate Mg-proto levels in the *gun* background. Those mutants (*chlm gun1*, *chlm gun4* and *chlm gun5*) contained high levels of Mg-proto but the *LHCB1.2* de-repression phenotype was still present suggesting that the *gun* specific de-repression of *LHCB1.2* is not caused by less Mg-proto accumulation (Mochizuki *et al.*, 2008). In another study analyses of 16 different conditions in which seedlings were grown either under complete darkness or light/dark cycles, either untreated or treated

with NF, 5-aminolevulinic acid (ALA) or both revealed no correlation between *LHCB1.1* mRNA accumulation and the abundance of Mg-proto (Moulin *et al.*, 2008).

It was demonstrated that tetrapyrroles of the Mg-branch were not involved in signaling processes, but they had not tested tetrapyrroles of the Fe-branch although heme was suggested to be responsible for retrograde signaling in *C. reinhardtii* (von Gromoff *et al.*, 2008). Hence, we determined heme levels in wild-type seedlings and *gun* mutants grown on plates without or supplemented with NF. Interestingly, in the presence of NF, heme levels were higher than in untreated plants (3.6. and Figure 3.10.). This can be explained by an up regulated expression and thus activity of ferrochelatase I in response to oxidative stress (Singh *et al.*, 2002; Nagai *et al.*, 2007). A comparison between wild-type and *gun* seedlings revealed no correlation of heme levels with the de-repression of *LHCB1.2*, thus indicating that heme is also not involved in *gun* dependent signaling in *A. thaliana*. Additionally, the presence of heme indicates that tetrapyrrole biosynthesis still goes on in photo-damaged plastids.

While the assumption that tetrapyrroles were involved in retrograde signaling is not true for higher plants, evidence exists that it is the case in other photosynthetic organisms. For example in the green alga *C. reinhardtii* the expression of, amongst others, *HSP70* (encodes heat-shock protein 70) is known to be regulated by Mg-proto and heme (Kropat *et al.*, 1997; Kropat *et al.*, 2000; von Gromoff *et al.*, 2006; von Gromoff *et al.*, 2008). Furthermore, it was shown that tetrapyrroles can function as a cell-cycle coordinator in the red alga *Cyanidioschyzon merolae* (Kobayashi *et al.*, 2009). However, those results are mainly based on feeding experiments with chemical compounds. It has to be kept in mind that tetrapyrroles, namely Mg-proto, do not accumulate naturally in the cytosol. Moreover, accumulation of porphyrins is phototoxic thus generating radicals and singlet oxygen species (Keetman *et al.*, 2002; Moulin and Smith, 2005; Jung *et al.*, 2008), for which reason secondary effects cannot be ruled out. To discover the real nature of signaling processes, more natural conditions are required.

4.4. The *gun* mutants in the light of metabolic signaling

Beside, the finding that accumulation of tetrapyrroles does not correlate with *LHCB1.2* expression, new insights were obtained for other proposed retrograde

signaling pathways. For example the first component of a possible retrograde signaling pathway responsible for adjustment of photosystem stoichiometry in response to altered light quality was found (Bellafiore *et al.*, 2005; Bonardi *et al.*, 2005). When plants were shifted between light sources specifically absorbed by either photosystem I or photosystem II (Pfannschmidt *et al.*, 1999a; Pfannschmidt *et al.*, 1999b) mutants lacking STN7, a thylakoid localized protein kinase, were neither able to perform the short-term response i.e. state transition, nor the long-term response i.e. adjustment of the photosystem stoichiometry. Since the latter one requires regulatory changes in nuclear gene expression (NGE) and thus a signal from plastids to the nucleus, STN7 must be involved in such a retrograde signaling process. With respect to the proposed OGE-dependent signaling pathway it was suggested that GUN1 is responsible for signal transduction (Koussevitzky *et al.*, 2007). Actually, in contrast to wild-type as well as *gun2* to *gun5*, the expression of *LHCB* and *RBCS* genes was maintained in *gun1* seedlings when chloroplast protein translation was inhibited by lincomycin (Gray *et al.*, 2003). Additionally it could be shown that plastid signals that depend on GUN1 were involved in photomorphogenesis (Ruckle and Larkin, 2009).

Although these findings were in line with proposed specific retrograde signaling pathways, for no factor described so far the real nature of action is known. Furthermore, it was not possible to identify a specific protein or other agent that acts directly as a messenger by traversing the chloroplast membrane and passing through the cytosol in order to modify NGE. The theory that Mg-proto is exported from the chloroplast to the cytosol under stress conditions (Ankele *et al.*, 2007), became questionable in the light of recent findings (see above). Consequently, we presented alternative interpretations without the need of specific signaling molecules, based on the fact that plastids communicate their metabolic state to the cytosol by exchanging various metabolites (Figure 4.1.; Kleine *et al.*, 2009). Hence, the metabolic composition in NF-treated *gun* mutants and wild-type plants was analyzed in order to test if such metabolic signaling is responsible for *gun* specific phenotypes. Not surprisingly after NF treatment the plants were affected in all processes tested.

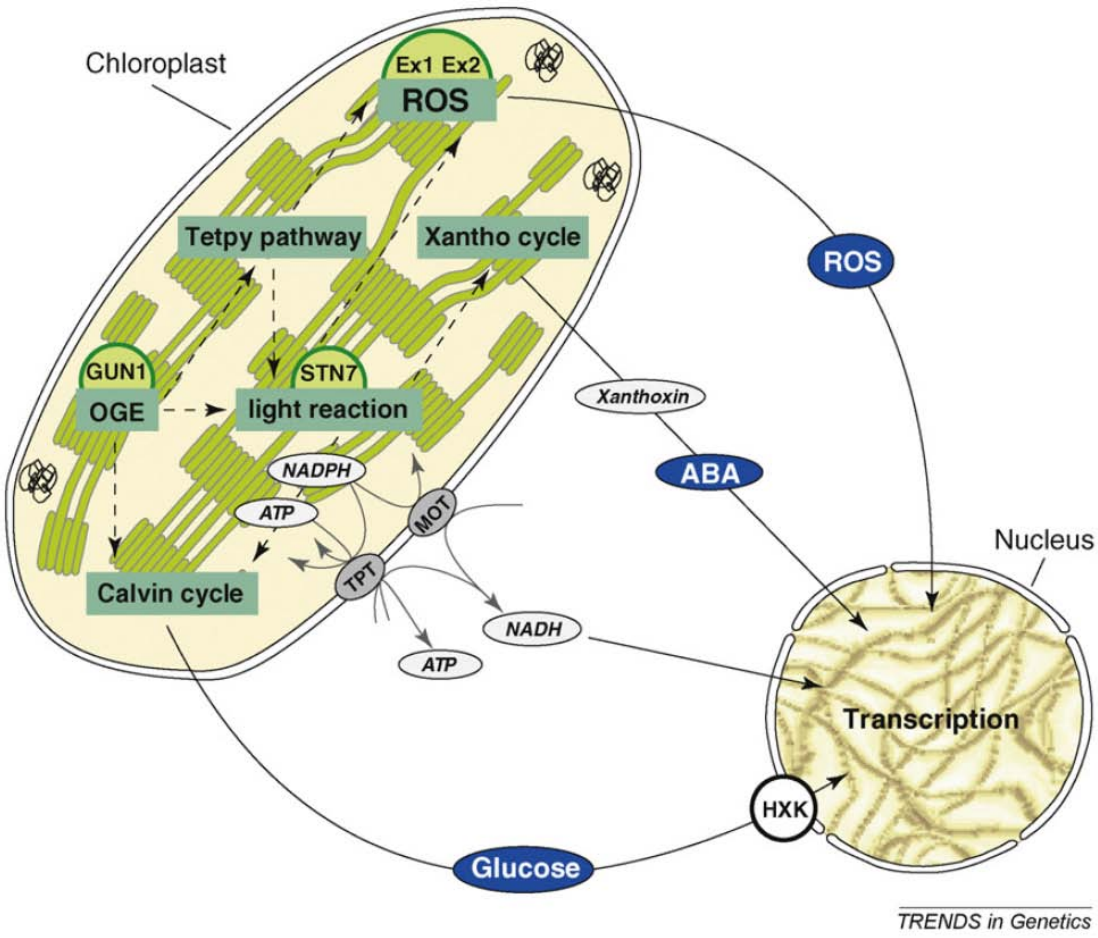

Figure 4.1: Overview of stimuli for and putative components of plastid signaling (taken from Kleine *et al.*, 2009). Chloroplast processes that stimulate plastid signaling are indicated in green and their interactions by dashed lines. Chloroplast proteins associated with plastid signaling (Ex1, Ex2, STN7, GUN1) are shown in light green, next to the corresponding chloroplast process. Signaling molecules thought to transit through the cytosol are shown in blue. For more details see the original publication.

ABA, abscisic acid; Ex1/2: Executer 1/2; HXK, hexokinase; MOT, malate/oxaloacetate translocator; OGE, organelle gene expression; ROS, reactive oxygen species; tetpy, tetrapyrrole; TPT, triosephosphate translocator; xantho, xanthophyll.

4.4.1. No correlation between expression of ROS marker genes and *gun* specific de-repression of *LHCB1.2* mRNA accumulation

Reactive oxygen species (ROS) are normally produced as toxic by-products during photosynthesis (Frosch *et al.*, 1979; Apel and Hirt, 2004; Krieger-Liszkay, 2005). For the understanding of signaling pathways dependent on the release of ROS the isolation of the *flu* mutant was a fruitful tool (Meskauskiene *et al.*, 2001). The corresponding protein (FLU) is a negative regulator of chlorophyll biosynthesis. Thus, mutants impaired in FLU accumulate protochlorophyllide when grown in the dark. When shifted from dark to the light the accumulated protochlorophyllide specifically generates singlet oxygen that acts as an activation signal for stress responses resulting in seedling lethality (op den Camp *et al.*, 2003). Indeed, it could be shown that this response has a genetic cause by isolating second-site mutations in the *flu* background. The singlet oxygen dependent stress response and the changes in NGE require two proteins named EXECUTER1 and EXECUTER2. This was deduced from the triple mutant *ex1*/*ex2*/*flu* where an up-regulation of almost all genes, can be observed in single *flu* mutant, are suppressed (Wagner *et al.*, 2004; Lee *et al.*, 2007). Moreover, it could be shown that after release of singlet oxygen a distinct set of genes was activated when compared to those induced by superoxide or hydrogen peroxide. Thus, providing evidence for different ROS dependent signaling pathways which in part antagonize each other (Laloi *et al.*, 2007). An additional screen for second-site mutants in the *flu* background was performed using a luciferase construct combined with the promoter of an singlet oxygen responsive gene. Eight mutants were found which either activate constitutively the reporter gene construct or abrogate the singlet oxygen specific up-regulation, indicating that multiple singlet oxygen dependent signaling pathways exist (Baruah *et al.*, 2009). Although it was not possible to show that ROS directly act as signaling molecules it is thought so for hydrogen peroxide (Mullineaux and Karpinski, 2002; Apel and Hirt, 2004; Mullineaux *et al.*, 2006). However, the polar nature of hydrogen peroxide has to be considered which might limit its capability to cross hydrophobic membranes. Moreover, hydrogen peroxide is not exclusively produced in plastids hence, it is difficult to explain how it can act specifically in plastid-to-nucleus signaling (Kleine *et al.*, 2009).

The expression pattern of chosen ROS marker genes was strongly altered in response to NF treatment (3.8. and Table 3.2.). It has to be mentioned that the results for some marker genes varied quite strongly between independent experiments. Technical problems could basically be ruled out as an explanation, because the same samples were also used for the detection of metabolic marker genes (see 3.9 and Table 3.3.) where no such strong variation occurred. Therefore, it has to be biological in such a way that plants were so strongly affected by photo-oxidative damage that many signaling and regulation pathways were concerned. Even slight differences during development would lead to noticeable changes in such disturbed conditions. This effect is also known from experiments where carotenoid biosynthesis was investigated in greater detail (Berry Pogson, personal communication). Most genes tested showed a strong up-regulation while the marker gene specific for hydrogen peroxide was down-regulated (3.8. and Table 3.2.). These data do not support the hypothesis that altered ROS levels are responsible for the *gun* phenotype, because wild-type and *gun* seedlings behaved similar in this respect. The expression of only one superoxide specific gene differed among the genotypes. Its specific expression pattern and the fact that this gene is encoded in the plastome fit with the described pigmentation or growth phenotype (4.2.). Although we could not correlate the expression of ROS marker genes with the *LHCB1.2* transcript abundance, it cannot be ruled out that transient changes in ROS levels might have occurred during NF treatment but remained undetected in our endpoint analyses. Detection of such transient changes has to be done in future analyses. For example seedlings can grow in the absence of NF before transferring them to plates supplemented with NF to get analyzed after defined periods. Another possibility could be to grow NF-treated plants under low-light conditions, where the *LHCB1.2* expression is not repressed (3.7. and Figure 3.12.), before inducing the repression by higher light intensities. Beyond this the concentration of NF can be diminished to reduce the photo-oxidative stress. The same effect could be initiated using mutant lines with inducible lesions in genes encoding enzymes of carotenoid biosynthesis in the *gun* background. Summing up, a reduced artificial influence to the plants will lead to more precise information.

4.4.2. Redox and metabolic state is not linked to *gun* specific phenotypes

Carbohydrates are synthesized by photosynthesis but needed for many processes outside the plastids e.g. respiration in mitochondria. Changes in production level could be sensed in the cytosol leading to adaptation processes via regulation of NGE. Furthermore, sugar metabolism is a very dynamic process regulated in response to environmental conditions, interacting with plant hormones and most interestingly with changes in photosynthesis (for review see; Rolland *et al.*, 2006). Recently it could be shown that a cytosolic localized hexokinase was actual involved in intracellular glucose dependent signaling processes. Interestingly those enzyme were also found in tiny amounts in the nucleus (Cho *et al.*, 2006). Thus hexokinase-dependent glucose signaling processes could be responsible for modulations in NGE in response to altered photosynthetic capacity. Next processes that could be involved in retrograde signaling are based on redox signals generated by the photosynthetic electron transport chain. Redox regulation is known to be involved in carbon fixation, starch metabolism, lipid synthesis as well as amino acid synthesis (Geigenberger *et al.*, 2005), thus providing a link between photosynthesis and the metabolic state of plastids. Moreover the redox state of plastids is mediated to the cytosol by carbohydrate shuttles such as the malate/oxalacetate and the triose-phosphate shuttle (Heineke *et al.*, 1991). In both cases a transfer of redox energy occurred between the chloroplastic NADPH and the cytosolic NADH system, hence can trigger information across cytosol to the nucleus.

The expression levels of all marker genes specific for metabolic processes tested were strongly down regulated in NF-treated plants, while no differences between wild-type and *gun* seedlings occurred (3.10. and Table 3.3.). The visualization of lipids resulted in the observation that NF had a strong effect on their composition, but again no genotype specific behavior to NF treatment was visible (3.9. and Figure 3.13.). Astonishingly the metabolites measured, like citric acid cycle intermediates, were not so strong affected suggesting that at least some processes were not disturbed (3.10. and Table 3.4.). These were in line with the observation that NF-treated plants can be rescued by transferring them to plates without inhibitor (Figure 3.19.). Furthermore, a possible effect of sucrose in the medium could be excluded by replacement via glucose or fructose (data not shown). Taken together, in our endpoint analyses no correlation with the *gun* specific phenotypes could be observed. To discover the involvement of

redox and metabolic processes in plastids to the *gun* specific signaling, more natural conditions are required.

4.4.3. Abscicic acid acts in retrograde signaling but independent of GUN

Another prominent candidate, that was suggested to be involved in plastid-to-nucleus signaling, the plant hormone abscisic acid (ABA), is produced from xanthophylls out of the plastids (see also 3.12. and Figure 3.5.). Production and availability of this hormone depends on the actual status of plastids. For example the xanthophyll-cycle, a redox reaction system between xanthophyll epoxidation and de-epoxidation, is activated under excess light in order to dissipate excess energy. For this reaction reduced and protonated ascorbic acid is required which by itself needs NADPH for regeneration. If this regeneration cannot compensate for ascorbate oxidation than the xanthophyll-cycle gets uncoupled, violaxanthin accumulates, thus yield in production of ABA (for further information and more detail see; Baier and Dietz, 2005). Indeed, it could be shown that ABA is able to regulate gene expression. Beside the ABA mediated redox regulation of *2CPA*, many ABA-responsive genes are known (Baier *et al.*, 2004; Sanchez *et al.*, 2004; Baier and Dietz, 2005). Moreover, a transcription factor involved in ABA signaling (ABI4) is thought to be part of the GUN1-dependent plastid signaling (Koussevitzky *et al.*, 2007) and it was demonstrated that the H-subunit of Mg-chelatase (also affected in *gun5*) is able to bind ABA and thus mediating seed germination, post-germination growth and stomatal movement (Shen *et al.*, 2006). Additionally, it was demonstrated that ABA supply can rescue impaired chloroplast development in *Arabidopsis thaliana ex1/ex2* (*executer*) mutant seedlings by readjustment of gene expression (Kim *et al.*, 2009).

In accordance to this, we have shown that application of low ABA concentrations to NF-treated wild-type plants resulted in a de-repression of *LHCB1.2* mRNA accumulation, as it is known from the *gun* seedlings (3.12. and Figure 3.14.). This points to a role for ABA as a positive regulator of photosynthetic related nuclear gene expression when present in small amounts (see also Kim *et al.*, 2009), contrary to the known function as a negative regulator when present in higher amounts (Penfield *et al.*, 2006; Koussevitzky *et al.*, 2007). However, two findings argue against an involvement of low ABA concentrations in GUN-dependent signaling associated with

tetrapyrrole biosynthesis. Firstly, the addition of low concentrations of ABA to NF-treated wild-type plants goes along with a stronger impact on growth capability (Figure 3.15.). This is in contrast to the observed growth and pigmentation phenotype seen in NF-treated *gun2*, *gun4* and *gun5* seedlings (see 4.2.). If the *LHCB1.2* de-repression phenotype seen in the *gun* mutants would be caused by higher ABA concentrations compared to wild-type, they should also have more problems to grow. Since they behave the other way around it is obvious that GUN-dependent signaling is not related to ABA. Moreover, it was not possible to link the germination rate dependent on sensitivity to ABA to the *gun* genotypes (Figure 3.16.). While *gun1* and *gun2* were less sensitive to ABA, *gun4* and *gun5* were more sensitive, indicating that no direct connection exists. To elucidate possible interactions between ABA and GUN-dependent signaling in more detail it is necessary to measure directly the amount of ABA in NF-treated plants in order to correlate it to the *LHCB1.2* transcription rate.

In summary, our data provide new insights into the physiological effect of NF in wild-type and *gun* seedlings as well as into the specific performance of photo-bleached *gun* mutants. Although we were not able to elucidate the signaling pathway responsible for de-repression of *LHCB1.2* mRNA accumulation, we could show that there is no simple correlation between any single metabolite, pigment or ROS molecule on the one hand and gene expression on the other. Our results were obtained with endpoint studies. To get a more precise picture and to elucidate the primary reactions, future analyses have to concentrate to more early events by using less invasive conditions. Thus, it would be possible to distinguish what is the cause and what the consequences of the observed data. Moreover, for this purpose the complex interplay between tetrapyrrole and carotenoid biosynthesis, organellar gene expression, ABA signaling, the metabolic state of plastids and the age dependency of GUN-related retrograde signaling (Figure 3.17.) have to be taken into account. According to this, it would be possible to elucidate the complex signaling network between plastids and the nucleus.

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Declaration / Ehrenwörtliche Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe. Der Autor hat zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder sich einer Doktorprüfung zu unterziehen. Die Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Ganzes vorgelegt.

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