Chloroplast lipid droplet type II NAD(P)H oxidoreductase, NDC1, is essential for vitamin E and K1 metabolism

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Submitted by

Lucia Eugeni Piller

Accepted on the recommendation by

Prof. Felix Kessler, thesis director

Dr. Dominique Rumeau

Prof. Jean-Marc Neuhaus

UNIVERSITÉ DE NEUCHÂTEL

Faculté des Sciences
Secrétariat-décanat de Faculté
Rue Emile-Argand 11
2000 Neuchâtel – Suisse
Tél : + 41 (0)32 718 21 00
E-mail : secretariat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Madame Lucia EUGENI PILLER

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"Chloroplast lipid droplet type II NAD(P)H oxidoreductase, NDC1, is essential for vitamin E and K1 metabolism"

sur le rapport des membres du jury composé comme suit:

- Prof. Felix Kessler, directeur de thèse, UniNE
- Prof. Jean-Marc Neuhaus, UniNE
- Dr Dominique Rumeau, CNRS CEA, Aix Marseille Université, France

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Le Doyen, Prof. R. Bshary

Abstract

Plant cells in different tissues contain specialized organelles belonging to the family of plastids. The chloroplast is the most prominent family member and responsible for photosynthesis in leaves. Most plastid types contain lipoprotein particles ("lipid droplets") termed plastoglobules. Little is known about plastoglobules that were long regarded as passive storage droplets. Indeed, the hydrophobic core of chloroplast plastoglobules contains neutral lipids such as the prenylquinones, carotenoids, triacylglycerols, phytyl esters and others unknown. Plastoquinone, plastochromanol-8, phylloquinone and tocopherol are prenylquinone molecules stored partly in the plastoglobule but functioning in the chloroplast thylakoids. Plastoglobules also carry proteins and some of these have been demonstrated to participate in metabolic reactions taking place at plastoglobules.

During my PhD work I demonstrated that NDC1 (NAD(P)H dehydrogenase C1 (At5g08740)), a candidate plastoglobule protein and predicted NAD(P)H-dependent quinone reductase is physically associated with the lipid droplets. A combined reverse genetic and in vitro approach demonstrated that NDC1 controls the overall REDOX state of the total plastoquinone reservoir. NDC1 does so by reducing the plastoquinone reservoir of plastoglobules. These findings provided evidence that plastoglobules are not simply a lipid storage site but have a role in energy metabolism. Besides its effects on the plastoquinone REDOX state NDC1 also facilitates plastochromanol accumulation and, surprisingly, is required for the last methylation step in phylloquinone (Vitamin K_1) biosynthesis. The *ndc1* mutant accumulates the non-methylated precursor, the 2-phythyl-1,4-naphtoquinone but up to now we have been unable to determine the precise mechanism. In conclusion, I have shown that NDC1 is a unique electron input device affecting the REDOX state of the overall plastoquinone pool. But more than that NDC1 is a key player at the intersection of a variety of

prenylquinone metabolic pathways. By mutant analysis, I identified that NDC1 is the second enzyme that is implicated in the tocopherol redox cycle. Presumably NDC1 plays a role as reducer of quinone intermediates foregoing the cyclization by VTE1. It has been also demonstrated that high light stress triggers far-ranging changes in prenylquinone composition studied in mutants and overexpressing lines of VTE1 and NDC1 enzymes. The discovery that NDC1 is a new component of phylloquinone biosynthesis pathway is the single most important result of my thesis.

Résumé

Les cellules végétales possèdent dans leurs différents tissus des organelles spécialisées appartenant à la famille des plastes. Le chloroplaste est le principal membre de cette famille et il est responsable de la photosynthèse dans les plantes. La majorité des plastes contiennent des particules lipoprotéiques ("gouttelettes lipidiques") appelées plastoglobules.

Peu est connu au sujet des plastoglobules qui ont été pendant une longue période imaginés comme des gouttelettes de stockage passif. En effet, le cœur hydrophobe des plastoglobules chloroplastiques contiennent des lipides neutres comme les prénylquinones (plastoquinone, plastochromanol-8, phylloquinone, tocophérol), les caroténoïdes, les triacylglycérols, les phytyl esters et d'autres lipides inconnus.

Les plastoglobules sont aussi composés de protéines et certaines d'entre-elles participent à des réactions métaboliques qui se déroulent dans les plastoglobules.

Pendant mon doctorat, j'ai démontré que NDC1 (NADP(H) déshydrogénase C1 (At5g08740), prédite comme une NAD(P)H-dépendante réductase de quinones, est physiquement associé aux gouttelettes lipidiques des chloroplastes..

Grâce à la génétique inverse et une approche *in vitro* il a été démontré que NDC1 contrôle l'état redox du réservoir de plastoquinone en injectant des électrons dans le plastoquinone à l'intérieur des plastoglobules. Cet effet sur l'état redox des plastoquinones facilitent l'accumulation du plastochromanol. Nous pouvons supposer que NDC1 puisse jouer un rôle en tant que réducteur des intermédiaires quinones qui précèdent la cyclisation par VTE1.

De manière surprenante, NDC1 est requis pour la dernière étape de méthylation lors de la biosynthèse de la phylloquinone (Vitamine K1). En effet, les mutants *ndc1* accumulent le

précurseur non-méthylé, le 2-phythyl-1,4-naphtoquinone ce qui montre que NDC1 est une enzyme indispensable de cette voie de biosynthèse.

L'ensemble des découvertes permettent d'affirmer que les plastoglobules ne sont pas un simple lieu de stockage de lipides mais ils possèdent un rôle dans les métabolismes biosynthétique et énergétique.

Keywords: Chloroplasts, plastoglobules, lipid bodies, sucrose gradient, prenylquinones, vitamin K_1, vitamin E, plastoquinone, plastochromanol, tocopherol, high light, lipidomics, redox cycle.

Mots-clés: Chloroplastes, plastoglobules, corps lipidiques, gradient de sucrose, prénylquinones. vitamine K_1, vitamine E, plastoquinone, plastochromanol, tocopherol, lumière forte, lipidomique, cycle rédox.

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List of abbreviations

ABA	abscissic acid
AAE14	acyl-CoA activating enzyme isoform 14
AOS	allene oxide synthase
Arabidopsis	*Arabidopsis thaliana,*
At	*Arabidopsis thaliana*
ATP	adenosine triphosphate
bp	base pairs
BSA	Bovine Serum Albumin
CAB	chlorophyll a/b binding protein
CaMV	cauliflower mosaic virus
CCD	carotenoid cleavage dioxygenase
cDNA	complementary DNA
Col	Columbia
DHNA-CoA thioesterase	1,4-dihydroxy-2-naphthoyl-CoA thioesterase
DMPBQ	2,3-dimethyl-6-phytyl-1,4-benzoquinone
DNA	desoxyribonucleic acid
dNTP	desoxy nucleotide triphosphate
DTT	1,4-dithio-DL-threitol
E. coli	*Escherichia coli*
ECHId	enoyl-CoA hydratase/isomerase
EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid
EST	expressed sequence tag
FBA	fructose-bis-phosphate aldolase
Fd	Ferredoxin
FITC	fluorescein isothiocyanate
FNR	ferredoxin $NADP^+$ reductase
FQR	ferredoxin-quinone reductase
gDNA	genomic DNA
GFP	green fluorescent protein
His6	hexahistidinyl-tag
HGA	homogentisic acid
HPPD	4-hydroxyphenylpyruvate dioxygenase

HPT	HGA phytyltransferase
HST	homogentisic acid solanesyl transferase
ICS 1/2	isochorismate Synthase 1 and 2
IPTG	isopropyl-β-D-1-thiogalactopyranoside
kDa	kilodalton
LB	Luria Bertani
LOO-	lipid peroxyl radical
Men	menaquinone synthesis
MPBQ	2-methyl-6-phytyl-1,4-benzoquinone
mRNA	messenger RNA
MS	Murashige and Skoog
MSBQ	2-methyl-6-solanesyl-1,4-benzoquinol
Ni-NTA	nickel-nitrilotriacetic acid
NPQ	non-photochemical quenching
NS	naphthoate synthase
PAP	plastid lipid associated protein
PBS	phosphate-buffered saline
PC-8	plastochromanol-8
PCR	polymerase chain reaction
PG	plastoglobule
PGL	plastoglobulin
-PP	pyrophosphate
PQ-9	plastoquinone-9
PQH_2	plastoquinol
PS	photosystem
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcription-PCR
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEPHCHC	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate
SHCHC	2-succinyl-6-hydroxy-2,4-cyclohaxadiee-1-carboxylate
TAG	triacylglycerol

TAT	tyrosine aminotransferase
TC	tocopherol cyclase
TEMED	N,N,N',N',-tetramethyl-ethylene diamine
TBS	tris-buffered saline
T-DNA	transfer DNA
UV	ultraviolet
VTE	vitamin E synthesis
v/v	volume per volume
WT	wild type
w/v	weight per volume
YFP	yellow fluorescent protein
Upper case, *italic*	gene (e.g. *NDC1*)
Lower case, *italic*	mutant allele (e.g. *ndc1*)
Regular case	protein (e.g. NDC1)

CHAPTER I.
GENERAL INTRODUCTION

1. INTRODUCTION

1.1. Evolution and organisation of plastids

Plastids (from Greek *plassein*, for "shape" or "to mould") are a group of highly versatile plant organelles resulting from an ancient endosymbiotic event (more than 1 billion years ago) where a primitive eukaryotic cell was invaded by a cyanobacterial ancestor (McFadden, 1999). Already in 1905, Mereschkowsky suggested the hypothesis that plastids have cyanobacterial origins acting as a "little workers, green slaves" within the cell (Martin and Kowallik, 1999). Comparisons of protein sequences or genes coding for chloroplast proteins with genes having cyanobacterial origin, confirm this endosymbiotic hypothesis and demonstrate a phylogenetic relation between plants and red algae (Sugita et al., 1997; Moreira et al., 2000; McFadden and van Dooren, 2004). Endosymbiosis allowed the transfer of the majority of the genetic material from the cyanobacterial chloroplast to the host cell nucleus (Sato, 2001; Timmis et al., 2004; Gould et al., 2008). Analysis of the nuclear genome of Arabidopsis demonstrates that about 18% of coding genes were acquired from chloroplast cyanobacterial ancestor (Martin et al., 2002). Cyanobacteria have approximately 3'000 genes compared to chloroplast genome that contain between 120 and 135 genes (Kaneko et al., 1996; Lopez-Juez and Pyke, 2005). Moreover, only 80 proteins are encoded by the chloroplast genome and are mainly components of photosynthetic complexes. During evolution, genes that were not essential for the endosymbiont disappeared while others were transferred to the plant nuclear genome (Martin et al., 1998; Timmis et al., 2004). The nuclear-encoded proteins to be imported into the organelle, acquired targeting sequences allowing the plant to produce highly specialized plastids.

In fact, different plastid types exist in higher plants that vary in size, shape, content and function (Thomson and Whatley, 1980). All plastids are derived from small undifferentiated

organelles, the meristematic proplastids, There are around 10-20 proplastids per cell ranging between 0.2 and 1 µm with limited internal membrane vesicles (Pyke and Leech, 1992; Waters et al., 2004). They develop into specific plastid types (chloroplasts, amyloplasts, etioplasts, elaioplasts and chromoplasts) (Lopez-Juez and Pyke, 2005) depending on tissue specificity as well as environmental stimuli. After differentiation, these colorless organelles can become either photosynthetic or non-photosynthetic (Bowsher and Tobin, 2001).

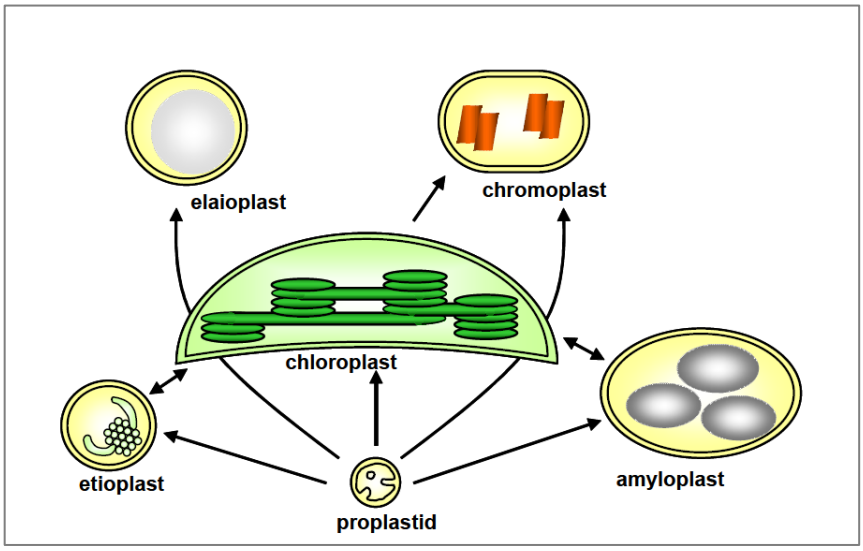

Figure 1: Diversity of plastid types and their interconversions. Chloroplasts occupy the center of the figure to signify their evolutionary roles as ancestors of all other plastid types, although during ontogeny all plastids derive from embryonic proplastids (Lopez-Juez and Pyke, 2005)

In the presence of light, proplastids become photosynthetic plastids in green tissues, the chloroplasts, producing chlorophyll. Conversely, a long darkness period allows the formation of etioplasts (Amrani et al., 1994). The main types of differentiated plastids in storage organ cells, like cotyledons, seed and tubers are amyloplasts and elaioplasts (Waters et al., 2004; Lopez-Juez and Pyke, 2005). The amyloplast is specialized in storing starch granules and has an active oxidative pentose pathway to assimilate nitrogen and generates energy (Neuhaus and Emes, 2000) while elaioplasts accumulate lipids. In mature petals and fruits, the plastids

that have the capacity to store pigments are called chromoplasts and derive from proplastids or chloroplasts (Weston and Pyke, 1999; Bramley, 2002).

1.2. Chloroplast organization and roles

In green tissues, chloroplasts represent the organelle performing photosynthesis and have a central role in plant metabolism. Their number is variable depending on the plant species from 10 to over 100 per cell (Waters et al., 2004). Their size is around 5-10 µm in diameter and 3-4 µm in thickness. Chloroplasts are delimited by an envelope consisting of an outer and inner membrane (Fig. 2). The envelope is the sites of lipid biogenesis and allows the exchange of solutes between the cytoplasm and the aqueous matrix of chloroplast, the stroma (Joyard et al., 1998). The envelope is also the site of preprotein import from the cytosol. The outer membrane is permeable to molecules up to 10 kDa, in contrast to the inner envelope membrane that is more selective and contains small transporters regulating influx and efflux of metabolites (Lopez-Juez and Pyke, 2005). Thylakoids are embedded in the stroma and form an extended membrane network arranged in stacks, the grana, that are interconnected by stromal lamellae. The photosynthetic apparatus is localized in this internal system. The stroma also contains multiple copies of plastid DNA, ribosomes, starch and protein lipid droplets termed plastoglobules (Greenwood et al., 1963; Lichtenthaler and Peveling, 1966; Thomson and Platt, 1973).

The four major protein complexes involved in the light reaction photosynthesis reside in the thylakoid membranes: the photosystems I and II (PSI and PSII), the cytochrome b_6f complex and the ATP synthase. Photosystems are physically separated in different domains of the thylakoid system. PSI is found exclusively in contact with stroma while PSII is limited to the grana membranes. The chloroplast thylakoid and envelope membranes are enriched in

galactolipids differing from the other plant membranes that are principally composed of phospholipids (Jarvis et al., 2000).

A B

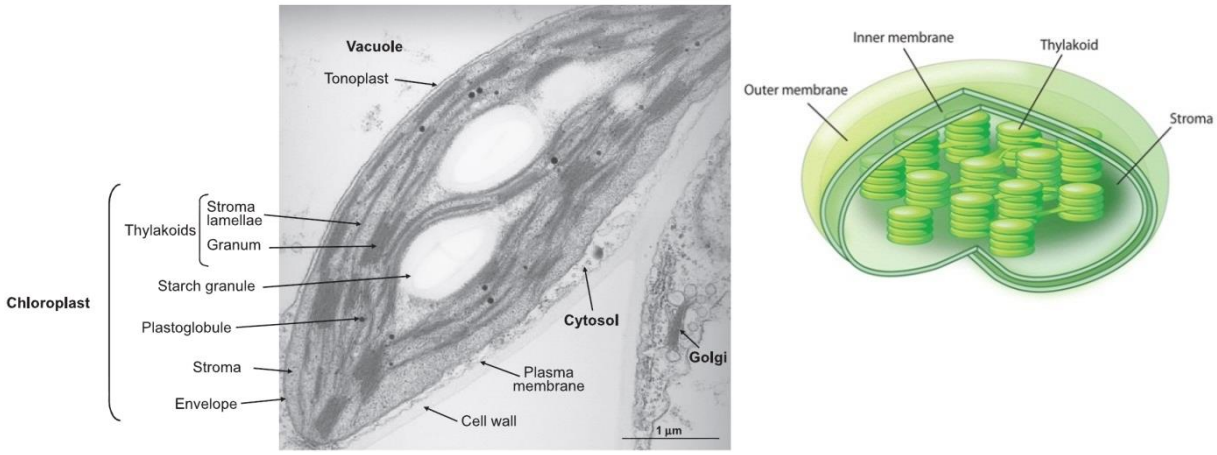

Figure 2: (A) Electron micrograph of an Arabidopsis cell. Chloroplasts are composed of the dual membrane envelope, thylakoids, stroma, starch granules and plastoglobules. Diverse compartments of the plant cell are indicated in bold lettering (Bréhélin and Kessler, 2008). **(B) Schematic representation of the chloroplast (http://www.nature.com)** showing the dual envelope membranes separated by an intermembrane space and enclosing the stroma containing many stacked thylakoids, so called grana.

The primary role of the chloroplast is photosynthesis initially leading to carbohydrates, many other metabolic pathways are also located in this organelle. They include nitrogen or sulphur assimilation starch synthesis, biosynthesis of fatty acids, phenolic groups, purine and pyrimidine bases, chlorophyll and other tetrapyrroles (Neuhaus and Emes, 2000).

1.3. Photosynthesis

Photosynthesis is a chemical process occurring in chloroplasts, using light energy to convert carbon dioxide (CO_2) into organic compounds. Photosynthesis comprises two distinct phases occurring within the chloroplast: the light-dependent reactions and the light- independent reactions (Waters and Langdale, 2009) (Fig.3).

$$6\ CO_2 + 12\ H_2O + \text{light energy} \longrightarrow C_6H_{12}O_6 + 6\ O_2 + 6H_2O$$

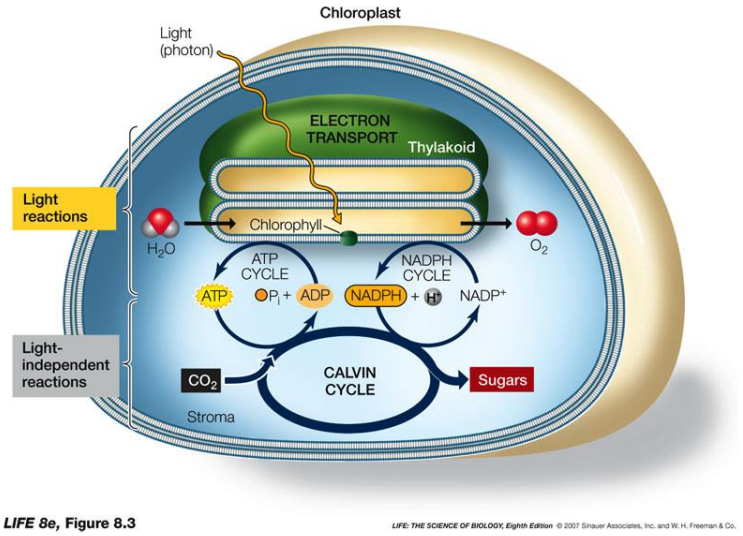

Figure 3: Schematic representation of the photosynthesis (Life: the science of biology, 8th edition). In chloroplasts, photosynthesis is separated in two different phases: the light reactions and the light-independent reactions that occur in thylakoid membranes and stroma, respectively.

The light-dependent reactions take place at the thylakoid membranes. The reactions of this phase result in electron transfer from water to $NADP^+$ resulting in NADPH via the protein complexes in thylakoid membranes: PSII, the cytochrome b_6f, PSI and ferredoxin $NADP^+$ reductase (FNR). The complexes are connected by electron carriers (plastoquinone PQ, plastocyanin and ferredoxin Fd). Along the way an electrochemical proton gradient is generated that is exploited by the ATP synthase to produce ATP. Both NADPH and ATP are

then used in the light-independent reactions occurring in the stroma and performing sugar production (Calvin-Benson cycle).

In summary, the classical pathway of photosynthesis, called linear electron transfer (LET) or "Z" scheme, allows the fixation of CO_2 by Rubisco to generate carbohydrates using NADPH and ATP (Fig. 4). Subsequently, sugar is exported to the cytosol or stored into the chloroplast as starch.

In addition to the LET, alternative electron transfer pathways have been proposed and are based on the cycling electron transfer (CET) (Fork and Herbert, 1993; Bukhov and Carpentier, 2004; Johnson, 2005) and chlororespiration (Bennoun, 1982; Rumeau et al., 2007). CET involves only PSI and the cyt b_6f complex to generate the electrochemical proton gradient (ΔpH) across the thylakoid membrane but without production of NADPH (Arnon et al., 1954; Munekage et al., 2003).

However, the mechanism of CET is not completely clear and at least two distinct pathways have been proposed (Joliot and Johnson, 2011):

- NDH-dependent pathway (NAD(P)H plastoquinone reductase complex): electrons are transferred to PQ via the complex NDH;
- Fd-dependent pathway (sometimes FQR): electron are transferred from Fd to PQ via a putative ferredoxin-quinone reductase (FQR);

The role and the contribution of CET into photosynthesis have been debated in recent years (Johnson, 2011; Joliot and Johnson, 2011). ATP synthesis and the control of light harvesting are presumed the two major functions for CET. Probably LET generates insufficient ATP to balance ATP:NADPH consumption of the Calvin cycle. The rapid light-induced production of ATP by CET could relieve this shortfall (Seelert et al., 2000; Allen, 2003). Moreover, a large proton gradient is generated by strong illumination to allow the formation of non-

photochemical quenching (NPQ) for the protection of PSII (Heber and Walker, 1992; Clarke and Johnson, 2001; Makino et al., 2002).

In higher plants, thylakoid membranes contain a large, multisubunit NDH complex resembling the bacterial complex I, which is involved in CET and chlororespiration (Peng et al., 2011). In this alternative route, originally discovered in cyanobacteria (Mi et al., 1995), electrons recycled from Fd are transferred to PQ and then to PSI through the cyt b_6f complex. However, the formation of ΔpH across thylakoid membranes is not sufficient for a substantial contribution to ATP synthesis (Okegawa et al., 2008) even though the chloroplast NDH complex is important to alleviate over-reduction of stroma under stress conditions (Munekage et al., 2004; Shikanai, 2007). Likewise, the NDH complex can interact with several units of PSI to form NDH-supercomplexes in thylakoid membranes (Peng et al., 2009).

Moreover, because chloroplast NDH is homologous to the mitochondrial complex I, it has been thought that it also could be involved in chlororespiration pathway that uses stromal NAD(P)H for the non-photochemical reduction of PQ to PQH_2. PQH_2 is then oxidized by a chloroplast targeted terminal oxidase (PTOX), homologous to mitochondrial alternative oxidase (AOX) using O_2 to produce H_2O (Cournac et al., 2000).

The second, Fd-dependent cyclic pathway is also associated with PSI. Until now, for this route, only two thylakoid proteins, PGR5/PGRL1 (Proton Gradient Regulation 5/PGR5-like 1) are known and play a regulatory rather than catalytic role. These two proteins help the operation of PSI cyclic electron transport (DalCorso et al., 2008) in which electrons are transferred from PSI via Fd to the PQ pool. Passage of electrons through the FQR enzyme remains hypothetical although some studies demonstrated the involvement of ferredoxin-$NADP^+$-oxidoreductase (FNR) protein in the FQR cycle. In fact, FNR has been found

attached to the cyt b_6f complex (Lehtimaki et al., 2010) and it has also been shown that PGRL1 physically interacts with FNR (DalCorso et al., 2008).

The cyclic electron flow allows the overall regulation of electron transport especially in response to environmental changes and metabolic cues (Foyer et al., 2012). In fact, CET supports the production of the proton motive force and allows ATP accumulation without net reduction of Fd or formation of NADPH. The possibility to switch between cyclic and non-cyclic pathway gives the possibility to plants to adjust the ratio of the two reductants (Fd and NADPH) to meet the needs of plant metabolism (Munekage et al., 2008; Livingston et al., 2010). Moreover, CET, by enhancing protonation of the lumen permits the protection of PSII limiting electron transport and triggering NPQ (Rumeau et al., 2007). The formation of ROS is also minimized by this regulating transport passing to the PSI and the cyt b_6f complex (Foyer et al., 2012).

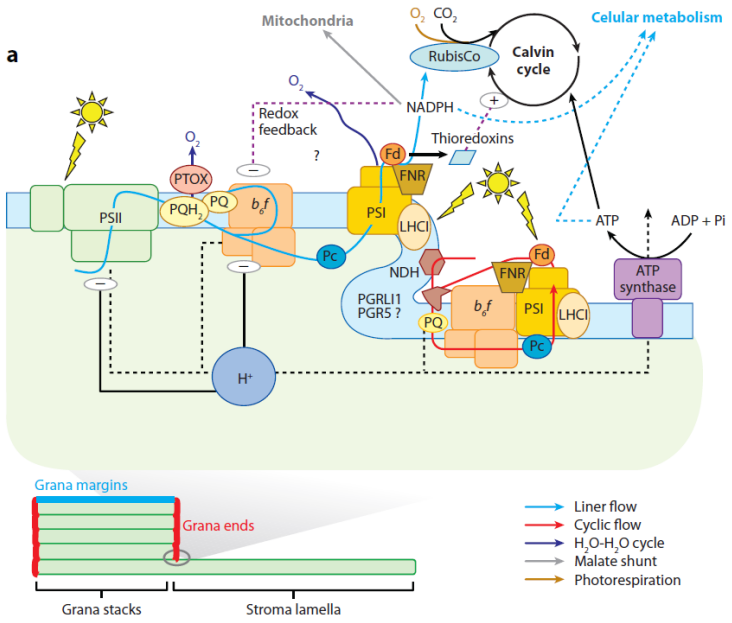

Figure 4: The complex picture of photosynthesis: branched pathways in photosynthetic electron flow, superimposed on highly regulated light excitation processes. Thylakoid membranes display a heterogeneous distribution of photosynthetic proteins, with PSII enriched in stacked membranes in the grana regions and PSI enriched in unstacked membranes of the stroma lamellae regions (lower panel).

Photosynthetic activity results from the balance between light absorption and its utilization for CO_2 assimilation. (a) Electron flow can be redirected to alternative pathways, which depends on changes in compartmentalization or in interaction between photosynthetic proteins. This modulates the efficiency of LEF (blue) and CEF (red). As shown here, compartmentalization may occur within supercomplexes, including all required electron carriers, or it may depend on a kinetic competition between the cyclic and linear pathway in a freely diffusing system. Red arrows point either to a direct reinjection of electrons from the PSI acceptor side to the plastoquinone pool or to an activity of the chlororespiratory enzyme NDH. Electron diversion at the PSI acceptor side also occurs via the water-water cycle catalyzed by the Mehler reaction (dark blue), or the export of reducing equivalents for a diversity of metabolic pathways (e.g., to the mitochondrion via the malate shuttle, gray). Electrons generated in PSII can be rerouted toward oxygen at the level of the plastoquinone pool, before reaching cyt b6f and PSI, via the catalytic activity of the chlororespiratory enzyme PTOX (dark blue). Gold line: photorespiratory activity of RuBisCo. Photosynthetic electron transfer generates an electrochemical proton gradient across the thylakoid membranes ($\Delta\mu H+$), which is used for ATP synthesis. Steady-state $\Delta\mu H+$ results from proton pumping by photosynthetic electron flow and proton consumption for ATP synthesis. The size of $\Delta\mu H+$ is modulated by the overall metabolic state through ATP/ADPxPi ratio. $\Delta\mu H+$ controls the rate of electron flow at the level of cyt b_6f and PSII complexes. The redox state of PSI electron acceptors, particularly the NADP/NADPH ratio, may also control cyt b6f complex activity (Adapted from Eberhard et al., 2008).

1.4. Type II NAD(P)H dehydrogenases family in *Arabidopsis thaliana*

Genes encoding hydrophobic subunits of NDH complex are absent in most microalgal species such as the green alga *Chlamydomonas reinhardtii* and in some gymnosperm species such as *Pinus thunbergii* (Ravenel et al., 1994; Peltier and Cournac, 2002; Mus et al., 2005). In *C. reinhardtii*, functions of the NDH complex in cyclic electron pathway and chlororespiration are mediated by a monomeric type II NAD(P)H:quinone oxidoreductase, NDA2 (Jans et al., 2008; Desplats et al., 2009). Type II NAD(P)H-dehydrogenases are non-proton pumping enzymes containing a flavin cofactor. In various organisms (bacteria, yeasts and plants) multiple sequences encoding putative NDH-2 have been found (Rasmusson et al., 1999; Melo et al., 2004; Michalecka et al., 2004).

In mitochondria of plants and fungi, the proton-pumping respiratory complexes I, II and IV work in parallel with type II NAD(P)H dehydrogenases and alternative oxidase (AOX). NADH and NADPH are oxidized by type II NAD(P)H dehydrogenase. In *Arabidopsis thaliana*, three nuclear gene families encode seven homologs of this protein class: two NDAs, four NDBs and one NDC (Michalecka et al., 2003) (Table 1). While the NDA and NDB sequence of plants have a common origin with fungal and protist sequences, phylogenetic analyses indicate that *NDC* gene in Arabidopsis and rice, have originated from cyanobacteria. The *NDC* gene most likely entered the plant cell via the chloroplast progenitor.

The seven proteins from all three families were shown to be mitochondrial (Elhafez et al., 2006). In that study, NDC1 (At508740) was protected in outer membrane-ruptured mitochondria indicating that it was imported to the matrix side of the inner mitochondrial membrane.

Table 1: summary of the data present in this study (Elhafez et al., 2006).

Gene	Locus	Location	Predicted	Actual	Mature	Tissue	Diurnal	Smith et al. (2004)	L–D
NDA1	At1g07180	Internal	57 kDa	60 kDa	56 kDa	C, L, B, F	A+ T+	+	+
NDA2	At2g29990	Internal	57 kDa	59 kDa	55 kDa	R, C, L, B, F	A– T-	–	–
NDC1	At5g08740	Internal	57 kDa	70 kDa	60 kDa	R, C, L, B, F	A– T–	–	+
NDB1	At4g28220	External	63 kDa	60 kDa	60 kDa	R, C, L, B, F	A– T–	–	–
NDB2	At4g05020	External	65 kDa	63 kDa	63 kDa	R, B, F	A– T+	+	–
NDB3	At4g21490	External	64 kDa	N/A	N/A	R, B, F	A– T–	–	–
NDB4	At2g20800	External	65 kDa	65 kDa	65 kDa	R, C, L, B, F	A– T–	–	–

The abbreviation, locus and intramitochondrial location are shown in the first three columns. The predicted mass, the apparent mass of precursor and mature proteins are indicated. The organs where expression is detected are indicated C = cotyledon, L = leaf, B = floral bud, F = flower, R = root. A summary of the diurnal expression data is given in the last three columns. The 'Diurnal' and 'L–D' columns refer to the data presented in Fig. 4A and B, respectively, with statistically significant differences ($P < 0.05$) indicated by +. 'A' and 'T' in the 'Diurnal' column refer to the ANOVA and *t*-tests performed on the quantitative RT–PCR data. An indication of which *NDs* were diurnally regulated based on data from Smith et al. (2004) is also given.

Previous studies using green fluorescent protein (GFP) fused to the unconserved N-terminal part of NDC1 (225 bp) demonstrated that this protein was targeted exclusively to mitochondria (Michalecka et al., 2003). Nevertheless, based on *in silico* predictions, it has been suggested that NDC1, NDA1 and NDA2 may be addressed to chloroplasts and have a

chlororespiratory function (Peltier and Cournac, 2002). Moreover, proteome analysis of Arabidopsis chloroplasts showed the presence of NDC1 in plastoglobules (Vidi et al., 2006; Ytterberg et al., 2006, Lundquist et al., 2012). Thus, NDC1 may be dually targeted to both mitochondria and chloroplasts, quite a widespread phenomenon in plants (Millar et al., 2006). The dual localization of NDC1 has been confirmed experimentally (Carrie et al., 2008). In this case, it appears that the mature protein sequence is necessary for dual localization and this behaviour is already known for other proteins (von Braun et al., 2007).

Eukaryotic cells are divided into membrane-enclosed compartments with varying functions, each containing its own set of proteins. After the endosymbiotic event, most mitochondrial or chloroplastic proteins were encoded by the nuclear genome, then translated on cytosolic ribosomes and directed to the appropriate organelle, following a specific pathway and guided by a targeting peptide. Studies of targeting sequences demonstrate that chloroplastic transit peptides (most of them have around 30–80 residues, the mean being 58) are longer and less structured than the plant mitochondrial pre-sequences (Zhang and Glaser, 2002).

Although targeting of proteins to mitochondria and chloroplasts is normally highly specific, recently, a third class of plant organellar proteins has been identified, corresponding to proteins targeted to both organelles (Duchêne et al., 2005; Pujol et al., 2007). As mitochondrial and chloroplastic proteins, they also have an N-terminal extension that in this case is called an “ambiguous” presequence, because it is recognized as an import signal to both mitochondria and chloroplasts (Peeters and Small, 2001).

1.5. Plastoglobules

Plastoglobules are low-density lipoproteins contained in all plastid types such as chloroplasts, chromoplasts and leucoplasts. They were discovered about 60 years ago by electron microscopy (Greenwood et al., 1963; Leggettbailey and Whyborn, 1963). Plastoglobules consist of a polar lipid monolayer contiguous with the outer leaflet of the thylakoid bilayer (Rey et al., 2000; Austin et al., 2006) (Fig. 5). These ubiquitous structures have long been regarded as passive lipid storage droplets. Their dimensions may vary from 30 nm to several µm (Lichtenthaler, 1968; Thomson and Platt, 1973). Their size and number increase as a function of environmental conditions and developmental stage. The former suggests an implication of plastoglobules in plant stress response, the latter a role in the transition of plastids from one type to another.

The hydrophobic core of lipid droplets contains neutral lipids such as prenylquinones, carotenoids, triacylglycerols and others (Kaup et al., 2002; Bréhélin and Kessler, 2008). PQ and tocopherol (vitamin E) are the principal components of plastoglobules (Lichtenthaler and Peveling, 1966; Tevini and Steinmuller, 1985; Austin et al., 2006; Vidi et al., 2006) whereas phylloquinone (vitamin K_1) was detected at lower concentrations (Lohmann et al., 2006). Developmental and stress-related variations of lipid composition suggest that plastoglobules are highly dynamic structures and that theirs functions evolve during plastid differentiation.

Plastoglobules have been shown to harbour structural proteins as well as enzymes. The protein composition allowed to hypothesize that plastoglobules do not simply store lipids but actively participate in diverse metabolic pathways.

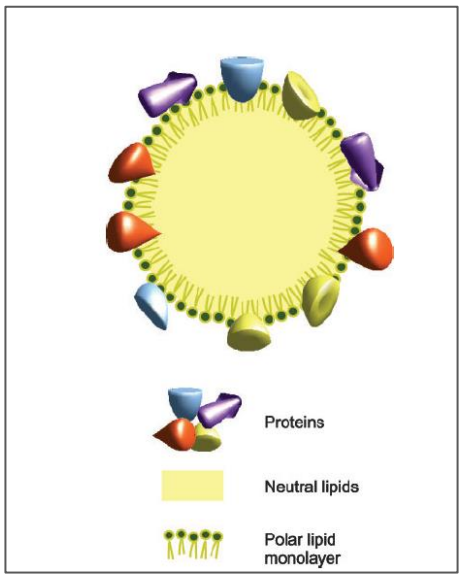

Figure 5: Model of plastoglobule structure. Plastoglobules are composed of neutral lipids surrounded by a polar lipid monolayer and coated with proteins (Bréhélin and Kessler, 2008).

In order to discover the composition and the functions of plastoglobules, two independent studies have determined the Arabidopsis plastoglobule proteome (Vidi et al., 2006; Ytterberg et al., 2006, Lundquist et al., 2012). Proteins are divided in three groups: Fibrillin/plastoglobulins, known metabolic enzymes and proteins of unknown function. The first group consists of presumed structural proteins, the fibrillin/plastoglobulins (i.e. PGL35, PGL40, PGL30.4) whereas six known enzymes originally composed the second group: three isoforms of fructose-biphosphate-aldolase (FBA1-3), the tocopherol cyclase VTE1, the allene oxide synthase (AOS) involved in jasmonate biosynthesis and the carotenoid cleavage dioxygenase (CCD4). However, FBA1-3a and AOS are more abundant in chloroplast compartments other than the plastoglobules and were therefore removed from the list (Lundquist et al. 2012)

The group of proteins with unknown function is composed principally of predicted enzymes presumably involved in lipid metabolism. For instance, two esterase/lipase/thioesterases,

PES1 and PSE2 (At1g54570 and At3g26840), have now been shown to be involved in thylakoid lipid metabolism. They have now been termed PES1 and -2 and esterify free fatty acids to phytol residues derived from chlorophyll catabolism resulting in fatty acid phytyl esters (FAPES) (Lippold et al. 2012). The ABC1-like kinases (At1g79600, At4g31390, At5g05200 and At5g71810) were predicted to regulate prenylquinone metabolism. It has now been shown that this indeed the case for At4g31390 (ABC1K1) and At1g79600 (ABC1K3) (Martinis et al. 2013, 2014, Lundquist et al. 2013). Moreover, NDC1 (At5g08740) belongs to this category of unclassified proteins and functional information predicts this enzyme to be a glutathion reductase dihydrolipoamide dehydrogenase, FAD-dependent pyridine nucleotide-disulphide oxidoreductase (Vidi et al., 2006).

During oxidative stress conditions such as high light, nitrogen deprivation, drought and others stresses (Nordby and Yelenosky, 1985; Locy et al., 1996) or during developmental stages, plants response by an enhanced synthesis of prenylquinones. Because these compounds are contained in plastoglobules, the number and the size of lipid droplets may increase under stress conditions. In parallel, thylakoid membranes are disassembled and the catabolism of the membrane lipids and chlorophyll leads to the accumulation of fatty acid phytyl esters (FAPEs) in plastoglobules (Lippold et al., 2012). The plant responses to oxidative stress include the enhanced synthesis of antioxidant molecules, especially tocopherol, phylloquinone and plastoquinol which are partly accumulated in plastoglobules (reviewed in following chapter published in Eugeni Piller et al. 2012).

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1.1. Goals and hypothesis of my PhD work

Plastoglobules (PG) are a major research topic in the Plant physiology laboratory at the University of Neuchâtel. A proteomics project lead to the identification of around thirty proteins that associate with PG. The laboratory systematically characterizes these proteins. In the case of my PhD thesis I decided to focus on NDC1, a NAD(P)H-dependent quinone reductase. We initially hypothesized that this protein may be involved in cyclic electron flow around PSI.

My first objectives were to carry out the initial characterization of NDC1 which included localization and analysis of enzymatic parameters. The results showed that NDC1 indeed localizes to PG and functions as a multifunctional quinone reductase.

In parallel *ndc1* mutant lines were obtained. These were to be used in bioenergetics studies to test the implication of NDC1 in cyclic electron flow in collaboration with Dr. Havaux's group at the CEA in Cadarache. The study demonstrated that NDC1 does not contribute to the major cyclic pathways but to a minor pathway directing electrons to the plastoquinone reservoir in PG.

The third objective was to characterize the (hypothetical) involvement of NDC1 in PG lipid metabolism. This part of my work demonstrated that NDC1 contributes to plastochromanol production and Vitamin E recycling after oxidation. In addition, we observed that NDC1 is required for Vitamin K1 (phylloquinone) biosynthesis. This was a big surprise and the single most important finding of my PhD thesis. Research by others has now confirmed this finding and demonstrated that NDC1 indeed catalyzes and indispensable step in Vitamin K1 synthesis in many different species.

CHAPTER II.
PLASTID LIPID DROPLETS AT THE CROSSROADS OF PRENYLQUINONE METABOLISM

2. PLASTID LIPID DROPLETS AT THE CROSSROAD OF PRENYLQUINONE METABOLISM

Lucia Eugeni Piller, Marion Abraham, Peter Dörmann, Felix Kessler and Céline Besagni

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2.1 General introduction

In this chapter, I present a review published in a special edition of the *"Journal of Experimental Botany"*. It concerns the role of plastoglobules in chloroplast lipid metabolism.

In chloroplasts, the hydrophobic core of plastoglobules is filled with neutral lipids such as the prenylquinones, triacylglycerols, carotenoids and others classes of lipids.

The major prenylquinone constituents of plastoglobules are tocopherols (vitamin E), plastoquinol (PQ) whereas plastochromanol-8 (PC-8) and phylloquinone (vitamin K1) are also present in varying but minor amounts. Specific proteins coat lipid droplets and three independent proteome studies divide these 34 proteins in three categories: PAP/fibrillins, chloroplast metabolic enzymes and unclassified proteins.

In this review, after a general introduction on plastoglobules, the implication of these lipid droplets in storage and biosynthesis of prenylquinones is discussed. Two of the plastoglobule proteins, the tocopherol cyclase VTE1 and the NAD(P)H quinone dehydrogenase C1, NDC1, are implicated in prenylquinone metabolism (see chapter 3 of the review for the NDC1 protein). In fact, the final steps of tocopherol synthesis require the activity of two proteins, VTE1 and VTE4. Several studies demonstrate that VTE1 is located in plastoglobules while VTE4 is localized to the inner envelope membrane. The differential location of these two

enzymes hints at the possibility of tocopherol metabolite trafficking inside the chloroplast. Furthermore, proteome analysis reveals the presence of NDC1 in plastoglobules. We demonstrate that this enzyme is important in PQ metabolism because it reduces PQ to PQH_2. Then, VTE1 catalyses the conversion of PQH_2 to PC8. NDC1 is implicated in the phylloquinone biosynthetic pathway and required for the AtMenG methylation step. The importance of these two enzymes, VTE1 and NDC1 in different steps of prenylquinone metabolic pathways suggests that plastoglobules play an essential role in prenylquinone metabolism in general and specifically in the production of vitamin K_1.

PLASTID LIPID DROPLETS AT THE CROSSROAD OF PRENYLQUINONE METABOLISM

Lucia Eugeni Piller, Marion Abraham[2], Peter Dörmann[1], Felix Kessler* and Céline Besagni

Laboratoire de Physiologie Végétale, Université de Neuchâtel, 2000 Neuchâtel, Switzerland

[1]Institute of Molecular Physiology and Biotechnology of Plants (IMBIO), University of Bonn, 53115 Bonn, Germany

[2] present address: Dongseo University, Division of Health Science, Jurye-2Dong, Sasang-gu, 617-764 Busan, South Korea

* to whom correspondence should be addressed

2.2 Abstract

Lipid droplets called plastoglobules (PG) exist in most plant tissues and plastid types. In chloroplasts, the polar lipid monolayer surrounding these low density lipoprotein particles is continuous with the outer lipid leaflet of the thylakoid membrane. Often small clusters of two or three PG, only one of them directly connected to thylakoids, are present. Structural proteins (known as PAP/fibrillins or plastoglobulins) together with lipid metabolic enzymes coat the PG. The hydrophobic core of PG contains a range of neutral lipids including the prenylquinones (tocopherols (vitamin E), phylloquinone (vitamin K_1) and plastoquinone (PQ-9). In this review we will discuss the function of PG and their associated enzymes in prenylquinone metabolism.

Key words: chloroplast prenylquinone metabolism, PG lipid droplets, plastoquinone; plastochromanol, tocopherol, phylloquinone.

2.3 Introduction

PG were discovered about 40 years ago as osmiophilic globules in electron microscopy of plant tissues (Greenwood et al., 1963; Leggettbailey and Whyborn, 1963). They are present in all tissues and plastid types such as chloroplasts, chromoplasts and leucoplasts. Easily isolated by flotation density centrifugation, PG were characterized as low density globules containing lipids and small amounts of protein (Greenwood et al., 1963; Leggettbailey and Whyborn, 1963; Lichtenthaler and Peveling, 1966; Lichtenthaler, 1968; Kessler and Vidi, 2007).

The first PG protein to be discovered was named fibrillin because it was identified in the carotenoid fibrils of red pepper chromoplasts. Technically, the fibrils are elongated lipid droplets (Deruère et al., 1994). Later, fibrillins were also discovered in association with PG in leaf tissue and termed plastid-lipid associated proteins (PAP) or plastoglobulins (Deruère et al., 1994; Pozueta-Romero et al., 1997; Kessler et al., 1999).

Until recently, PG were largely viewed as passive lipid storage droplets, their size and composition varying in function of the developmental stage or the type of plastids. However, recent proteome studies of the PG isolated from Arabidopsis chloroplasts and red pepper chromoplasts (Vidi et al., 2006a; Ytterberg et al., 2006b) revealed not only the presence of an entire family of plastoglobulin proteins but also enzymes. Many of these are predicted or known to participate in lipid metabolic pathways.

Electron tomographic experiments demonstrated that the PG constitutes a distinct structural and functional subcompartment of the thylakoids. This is underscored by the fact that its hydrophobic core is surrounded by a polar lipid monolayer contiguous with the thylakoid outer lipid leaflet (Austin et al., 2006b). PG dimensions range from 30 nm to 5 µm (Lichtenthaler, 1968; Thomson and Platt, 1973; Austin et al., 2006b). Several studies demonstrated that under biotic and abiotic stress conditions, size and number of the lipid

droplets increase. Moreover, the PG may connect resulting in grape-like clusters (Austin et al., 2006b). It has been suggested that PG clusters form by a two steps mechanism: a primary "blistering" event at the outer thylakoid lipid leaflet followed by a secondary blistering event at the surface of an existing PG. The resulting connections between PG and thylakoids as well as those between PG themselves provide the basis for a bidirectional metabolite conduit between PG and thylakoid membrane (Fig. 1).

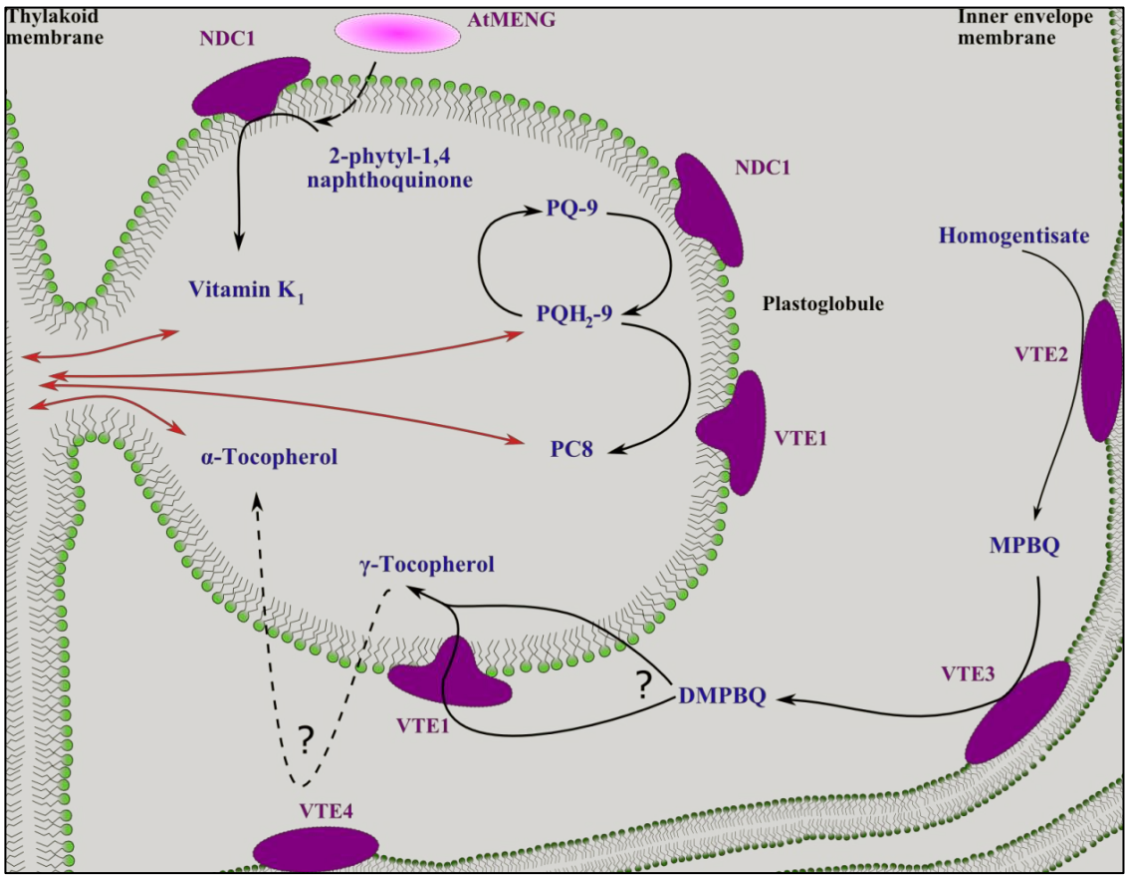

FIG. 1: Function of PG localized enzymes in prenylquinone metabolism. Prenylquinones are shown in blue, enzymes in violet. Bidirectional trafficking between the PG and the thylakoid membrane is represented by red arrows. PQ-9: plastoquinone; PQH_2-9: plastoquinol; PC8: plastochromanol-8; MPBQ: 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ: 2,3-dimethyl-5-phytyl-1,4-benzoquinol.

PG in various plant species differ with regard to size and number (Lichtenthaler, 2007). In older leaves of herbaceous plants such as spinach, the number of PG with a small diameter

(0.1-0.2 μm) increases to several hundred per chloroplast (Lichtenthaler, 1969). In older, sun-exposed leaves of beech and oak, PG are less numerous but significantly enlarged (Lichtenthaler, 1968). Moreover, in several-year-old Ficus leaves PG may reach diameters of 0.3-3.0 μm (Lichtenthaler and Weinert, 1970).

Studies of the PG core identified members of the neutral lipid class including prenylquinones, triacylglycerols (TAG), carotenoids and others. The prenylquinones, plastoquinol (PQH_2-9) and tocopherol (vitamin E), are among the major constituents of PG (Lichtenthaler and Peveling, 1966; Tevini and Steinmuller, 1985; Austin et al., 2006b; Vidi et al., 2006a) whereas phylloquinone (vitamin K_1) is present in minor amounts (Lohmann et al., 2006c) (Fig 1). However, no full lipidome of PG has been determined so far.

While traces of protein in PG were observed long ago (Leggettbailey and Whyborn, 1963), we now know that lipid droplets are coated with specific proteins. Two independent studies reported on the PG proteome, which consists of a total of about three dozen proteins. These belong to three categories: PAP/fibrillins, chloroplast metabolic enzymes and unclassified proteins (Vidi et al., 2006a; Ytterberg et al., 2006b).

The first group contains a total of eight of the thirteen-member Arabidopsis plastoglobulin/PAP/fibrillin family (Vidi et al., 2006a; Ytterberg et al., 2006b). Fractionation experiments demonstrated the enrichment and physical association of family members, PGL34 (At3g58010) and PGL35 (At4g04020), with PG (Vidi et al., 2006a; Vidi et al., 2007a). Based on their role in organizing red pepper carotenoid fibrils, the PAP/fibrillins are hypothesized to fulfil a structural role in PG too (Deruère et al., 1994). It is interesting to note that the cyanobacterial Synechocystis sp genome also contains two PAP/fibrillin homologs. Mutant analysis demonstrated that they serve, by an unknown process, to protect the organism from photooxidative damage (Cunningham et al., 2010).

The expression of plastoglobulins is regulated in response to abiotic and biotic stress as well as hormone treatment (Bréhélin and Kessler, 2008). Cold treatment induced the expression of a plastoglobulin in rice leaves (Lee et al., 2007). AtPGL30.4 (At3g23400) was identified as phosphorylated protein in the defence response to *Pseudomonas syringae* pv. Tomato DC3000 (Jones et al., 2006). In a comparative proteome study, four members of the Arabidopsis plastoglobulin family were found to accumulate under high light stress (Giacomelli et al., 2006). Abscisic acid also induced the expression of several plastoglobulins, AtPGL35 in particular (Gillet et al., 1998; Yang et al., 2006). Recently, it has been shown that plastoglobulins accumulate in response to light/cold stress-related jasmonate biosynthesis (Youssef et al., 2010).

The second group of proteins identified in the Arabidopsis PG proteome consists of known metabolic enzymes. This category includes the tocopherol cyclase VTE1 (vitamin E defective, At4g32770) involved in vitamin E synthesis, the carotenoid cleavage dioxygenase CCD4 (At4g19170) probably implicated in carotenoid metabolism, the three isoforms of fructose bisphosphate aldolase of the Calvin cycle (At2g21330, At4g38970 and At2g01140) and the allene oxide synthase AOS in jasmonate biosynthesis (At5g42650) (Kazan and Manners, 2011).

The third group of PG proteins consists of unclassified proteins. Some of them are predicted to be involved in lipid metabolism. For instance, ELT1 and 2 (Esterase/Lipase/Thioesterase, At1g54570 and At3g26840) may be involved in thylakoid lipid metabolism, while ABC1 (Activity of bc1 complex) - like kinases (At1g79600, At4g31390, At5g05200 and At5g71810) are predicted regulators of prenylquinone metabolism (Ytterberg et al., 2006b). Thus, the available data strongly suggests that PG, by the presence of enzymes, intervene in diverse aspects of thylakoid lipid metabolism.

2.3.1 PG response to stress

Under oxidative stress inducing conditions such as drought, high saline concentration, nitrogen deprivation, high light, viral infection, chilling and ozone (Nordby and Yelenosky, 1985; Locy et al., 1996; Rey et al., 2000; Oksanen et al., 2001; Gaude et al., 2007a; Lichtenthaler, 2007) as well as different developmental stages (senescence, fruit development) (Kaup et al., 2002), PG increase in size and number. This occurs in parallel to the disassembly of thylakoid membranes. Also, the lipid composition of PG will change dramatically due to the accumulation of fatty acid phytyl esters (FAPEs) from thylakoid catabolism (Gaude et al., 2007a; Bréhélin and Kessler, 2008).

In the thylakoid membrane, Reactive Oxygen Species (ROS) accumulate when the absorption of light by chlorophyll exceeds the capacity for energy utilization by the photosynthetic apparatus (Pospisil, 2011). The photosystems (PS) I and II are the major sites of free radical O_2^- generation. Plant responses against oxidative stress implicate different biochemical pathways including the enhanced synthesis of prenylquinones (Gruszka et al., 2008). Antioxidant action has been attributed to phylloquinone and plastoquinol although they are primarily known as electron carriers at PSI and PSII, respectively. Tocopherol does not play a role as electron carrier but is a key antioxidant lipid during high light stress (Munné-Bosch, 2005).

While prenylquinones partly accumulate in PG their true site of action is probably the thylakoid membrane where they scavenge ROS and protect the PS. Of the proteins in the PG proteome, the tocopherol cyclase VTE1 (Vidi et al., 2006a) and the NADPH quinone dehydrogenase C1, NDC1 (Eugeni Piller et al., 2011), are known players in prenylquinone metabolism and implicate PG as a metabolic compartment.

2.3.2 Implication of PG in storage and biosynthesis of tocopherols

Tocopherols belong to the amphipathic group of tocochromanols (vitamin E) that also includes tocotrienols (Falk and Munné-Bosch, 2010). The two types of tocochromanols differ in degree of saturation of their prenyl side chains. Synthesized only in photosynthetic organisms (plants, green algae and cyanobacteria), tocopherols are composed of a polar region derived from tyrosine and a hydrophobic polyprenyl side chain from the isoprenoid pathway (Fig. 2) (Valentin and Qi, 2005).

The group of tocopherols consists of four different forms, α-, β-, γ-, and δ-, which differ by the number and position of methyl groups on the chromanol ring (Mene-Saffrané and DellaPenna, 2010).

The presence of tocopherols is universal in higher plants albeit with differential tissue distribution of the various forms: α-tocopherol is predominant in leaves whereas in other organs such as seeds, flowers and roots, γ-tocopherol is the principle form (Horvath et al., 2006). In plastids, tocopherol inserts both in the envelope and thylakoid membranes whereby the polar chromanol group faces the hydrophilic surface (Dörmann, 2007). Around one third of the total plastid tocopherol is contained in the Arabidopsis PG core (Vidi et al., 2006a) (Fig 1). Under oxidative stress inducing conditions, such as high light, the production of tocopherols increases to protect membrane lipids from photooxidation and PSII from photoinactivation (DeLong and Steffen, 1997; Havaux et al., 2005c).

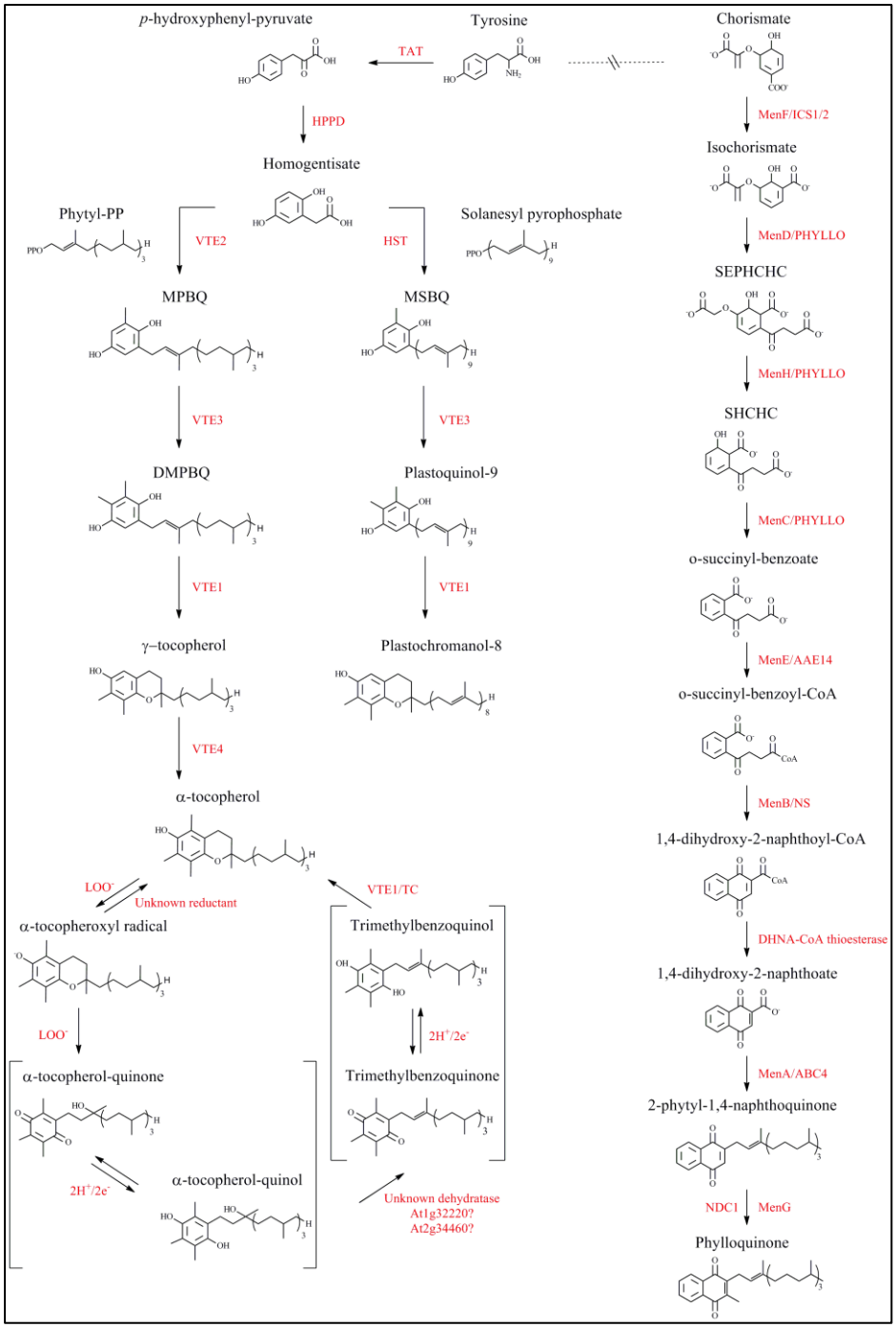

FIG. 2: The biosynthetic pathways of prenylquinones in Arabidopsis (adapted from Eugeni Piller et al., 2011). Summary of tocopherol, plastoquinol, plastochromanol and phylloquinone pathways in Arabidopsis. The enzyme abbreviations are shown in red. TAT: tyrosine aminotransferase; HPPD: p-hydroxyphenyl-pyruvate dioxygenase; HST: homogentisic acid solanesyl transferase; VTE: vitamin E synthesis; LOO-, lipid peroxy radical; TC: tocopherol cyclase; Men: menaquinone synthesis; ICS 1/2: isochorismate synthase 1 and 2; AAE14: acyl-CoA activating enzyme isoform 14; NS: naphtoate synthase; DHNA-CoA thioesterase: 1,4-dihydroxy-2-naphthoyl-CoA thioesterase; ECHId: enoyl-CoA hydratase/isomerase; -PP: pyrophosphate; MPBQ: 2-methyl-6-phytyl-1,4-benzoquinone; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinone; MSBQ: 2-methyl-6-

solanesyl-1,4-benzoquinol ; PQH_2, plastoquinol; SEPHCHC, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate; SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate. Adapted from (DellaPenna and Kobayashi, 2008; Eugeni Piller et al., 2011).

In older leaves, under high light stress, the level of α-tocopherol increases more than 4-fold (Szymanska and Kruk, 2010). The elimination of tocopherols drastically reduces the tolerance of photosynthetic organisms to high light stress (Maeda et al., 2005). The α-tocopherol accumulation correlates with an increase in size and number of PG in older leaves (Vidi et al., 2006a; Bréhélin et al., 2007a).

Three of the reactions of the tocopherol biosynthesis pathway, mediated by VTE2, VTE3 (Cheng et al., 2003) and VTE4, have been located at the chloroplast inner envelope (Soll et al., 1985). But surprisingly, the tocopherol cyclase VTE1 (Porfirova et al., 2002) was identified in the PG proteome (Vidi et al., 2006a; Ytterberg et al., 2006b). Its localization in PG was confirmed by physical fractionation, immunoelectron microscopy and expression of a fluorescent fusion protein (Vidi et al., 2006a). A serial immunoelectron tomography study revealed the penetration of VTE1 accros of the lipid PG monolayer. This may enable VTE1 to access substrates inside the PG and carry out the cyclase reaction (Austin et al., 2006b).

VTE1 catalyses the conversion of 2,3-dimethyl-5-phytyl-1,4-benzoquinol (DMPBQ) to γ-tocopherol and is required for the formation of the chromanol ring of all tocopherols (Soll et al., 1985) (Fig.2). If VTE1 were uniquely present in PG, DMPBQ would have to be moved from the inner envelope membrane to PG where the cyclase would convert it to γ-tocopherol. In support of this hypothesis, DMPBQ was indeed highly enriched in PG of the *vte1* mutant (Fig. 3).

The last step of α-tocopherol synthesis is carried out by the γ-tocopherol methyl transferase, VTE4, located at the chloroplast envelope (Zbierzak et al., 2010) (Fig. 1). Again, if VTE1 were exclusively located at PG, γ-tocopherol would have to be transported back to the inner envelope membrane to complete the synthesis of α-tocopherol (Zbierzak et al., 2010). Alternatively, it has been proposed that sufficient VTE1 for vitamin E synthesis may be still present at the envelope membranes and that at PG VTE1 serves other metabolic purposes such as the recycling of tocopherol oxidation products (DellaPenna and Kobayashi, 2008).

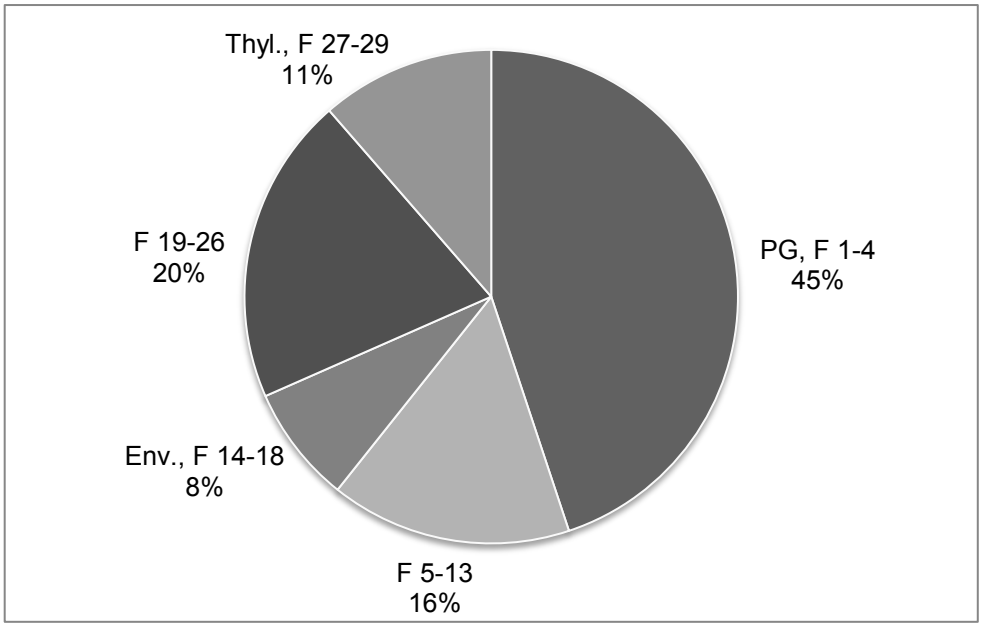

FIG. 3: Enrichment of DMPBQ in *vte1* PG. DMPBQ (2,3-dimethyl-5-phytyl-1,4-benzoquinol), the precursor of γ-tocopherol accumulates in the *vte1* mutants. Subplastidial chloroplast fractions were isolated from leaves of 8-week-old *vte1* mutant plants (Vidi et al., 2006a). Distribution of DMPBQ (in % of total) was measured in pooled chloroplast membrane fractions. Fractions F1-4 and F5-13 contained mostly PG, F14-18 envelopes (Env.), F19-26 some envelopes and thylakoids and F27-29 thylakoids (Thyl.). The highest amount of DMPBQ is present in the fractions enriched in PG (approx. 45%).

2.3.3 Role of PG in the tocopherol redox cycle

Tocopherol oxidation products form in response to high light stress (DellaPenna and Kobayashi, 2008). By *in vitro* chemical treatment twenty-three different oxidation products can be generated from α- and γ-tocopherol, but only two of these were detected *in vivo*: WT plants accumulated α-tocopherol-quinol (α-TQH_2) under high light conditions (DellaPenna and Kobayashi, 2008) and *vte4* mutant plants (containing only γ-tocopherol) accumulated γ-tocopherol-quinol. To determine whether α-TQH_2 was degraded or recycled to α-tocopherol, isolated WT and *vte1* chloroplasts were incubated with ^{14}C-labelled α-TQH_2 (DellaPenna and Kobayashi, 2008; Mene-Saffrané and DellaPenna, 2010). In WT chloroplasts, the incubation led to α-tocopherol accumulation, whereas in *vte1* mutants, a substrate of tocopherol cyclase, trimethylphytylbenzoquinone (TMPBQ), was detected (DellaPenna and Kobayashi, 2008). This is clear evidence that tocopherol oxidation products are recycled in higher plants. The α-tocopherol quinone (α-TQ) oxidation product was present in thylakoids, envelope membranes as well as plastoglobuli in chloroplast fractionation experiments (Kruk and Nowicka, 2010).

The proposed α-tocopherol redox cycle starts out by a two steps oxidation each characterized by the loss of a single electron (Mene-Saffrané and DellaPenna, 2010) (Fig. 2). In the first step, α-tocopherol is oxidized to the α-tocopherol radical by a lipid peroxy radical ($LOO^{\cdot}$). This product may be reduced back to α-tocopherol by an unknown reductant, possibly ascorbate, or be oxidized further by a second lipid peroxy radical resulting in the formation of α-TQH_2. To regenerate α-tocopherol, α-TQH_2 must undergo a dehydration step catalyzed by an as yet unidentified dehydratase. This will result in TMPBQ, which in turn will undergo the cyclase reaction catalyzed by VTE1 and results in the completion of the cycle. With regard to the potential role of PG in the cycle: not only are they enriched in tocopherol cyclase VTE1 (Austin et al., 2006b; Vidi et al., 2006a; Ytterberg et al., 2006b) but the PG proteome also

contains two predicted dehydratases (At2g34460 and At1g32220). In summary, the currently available evidence suggests that PG participate in the tocopherol recycling pathway.

2.3.4 Implication of VTE1 and NDC1 in plastoquinol metabolism

Plastoquinol (PQH_2-9) is well known as an electron and proton carrier in the photosynthetic transport chain between PSII and the cytochrome b_6f complex (Muh et al., 2011). But plastoquinol has also been shown to have a physiological antioxidant activity (Szymanska and Kruk, 2010). Structurally related to α-tocopherol, it exerts a photoprotective role on PSII during high light stress. It does so by scavenging singlet oxygen generated by chlorophyll at the reaction center (Kruk and Trebst, 2008) thereby inhibiting lipid peroxydation (Hundal et al., 1995). The plastoquinol head group as well as the isoprenoid chain are involved in the process which may confer additional antioxidant power over tocopherols (Gruszka et al., 2008).

PQ-9 is associated with Q_A and Q_B sites of PSII but also exists in a free form in thylakoid membranes. Together, these are considered the thylakoid or photoactive plastoquinone pool (Strzalka and Kruk, 1999). However, PQ-9 is also present in a separate pool contained in PG (Szymanska and Kruk, 2010; Zbierzak et al., 2010) (Fig.1). The PG PQ pool is not normally photoactive and does not directly participate in photosynthetic electron flow (Eugeni Piller et al., 2011). It might therefore serve both as a reservoir of antioxidant and to replenish the thylakoid pool (Zbierzak et al., 2010).

In Arabidopsis leaves, the level of plastoquinol dramatically increases under high light stress. The reduced form of PQ (PQH_2-9) increased 16- and 9- fold in old and young rosette leaves while the total amount of PQ (reduced plus oxidized) increased 8- and 11- fold, respectively

(Szymanska and Kruk, 2010). The majority of the plastoquinol under high light conditions is photosynthetically inactive and accumulates in PG. It is tempting to speculate that the plastoquinol that is irreversibly degraded by ROS in thylakoid membranes is replaced by plastoquinol from PG (Szymanska and Kruk, 2010). This may partially explain why plastoquinone accumulates to very high levels under high light conditions. NDC1, the NADPH-dependent quinone dehydrogenase C1 in PG functions to reduce the oxidized proportion of the non-photochemical pool of plastoquinol in PG. Indeed, the *ndc1* mutant had a significantly higher percentage of oxidized plastoquinone than the wild type (Eugeni Piller et al., 2011). Therefore, NDC1 constitutes a unique electron transport pathway separate from cyclic electron flow mediated by the NDH-complex or the PGR5 pathway (Shikanai, 2007; Peng et al., 2010). The NDC1-mediated electron pathway, however, is likely limited by the availability of plastoquinone inside PG that cannot rapidly be reoxidized as happens in NDH- and PGR5-dependent cyclic electron flow (Eugeni Piller et al., 2011).

Plastochromanol (PC8), derived from plastoquinol (PQH_2-9) by tocopherol cyclase activity, is present in leaves, seeds and other organs of Arabidopsis plants (Mene-Saffrané and DellaPenna, 2010; Szymanska and Kruk, 2010; Zbierzak et al., 2010). It constitutes 5-10% of the total tocochromanol, although this value may be higher in senescing leaves (Szymanska and Kruk, 2010). Around 50% of the PC8 is present in PG (Zbierzak et al., 2010). Together with γ-tocopherol, PC8 has been shown to be required for efficient germination after longer periods of seed quiescence (Mene-Saffrané and DellaPenna, 2010). The levels of the PC8 increase under high light stress as well as in aging leaves. Several studies have demonstrated that PC8 is an efficient singlet oxygen scavenger (Gruszka et al., 2008) and an inhibitor of lipid peroxidation (Olejnik et al., 1997). Its antioxidant activity is comparable to that of tocopherols (Olejnik et al., 1997). This is not surprising because plastochromanol has a chromanol group identical to that of γ-tocopherol differing only in the C40 polyunsaturated

solanesyl side chain instead of the phytol. Most likely, its source is the plastoquinol PQH_2-9 in the PG pool (Kumar et al., 2005; Kruk and Trebst, 2008) (Fig. 2) where the tocopherol cyclase is also present (Fig. 1).

In the *ndc1* mutant, PC8 was decreased (Eugeni Piller et al., 2011). Most likely, this is linked to the decrease of its direct precursor, PQH_2-9, the substrate of VTE1 (Grütter et al., 2006). In the *vte1* mutant, PC8 formation was entirely abolished but the overexpression of VTE1 induced a 2.4-fold increase of PC8 levels. This resulted in the proliferation of PG numbers and increased cluster formation (Kanwischer et al., 2005; Zbierzak et al., 2010).

2.3.5 Implication of PG in storage and biosynthesis of phylloquinone via NDC1

Phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone) or vitamin K_1 is an prenylquinone composed of a naphthoquinone ring and a prenyl side chain derived from phytyl-diphosphate (Fig. 2). It is synthesized in all organisms performing oxygenic photosynthesis. In higher plants, phylloquinone occurs in leaves where it serves as an electron carrier in the quinone/semiquinone turnover in PSI (Joyard et al., 2009). The overall stoichiometry of phylloquinone has been estimated at 3 mol of vitamin K_1 per 1 mol of PSI. However, only two molecules of phylloquinone are present for each PSI complex. This suggests that a separate pool exists that is not associated with PSI (Lohmann et al., 2006c; Bréhélin and Kessler, 2008). Interestingly, this is in good agreement with the around 30% of the total phylloquinone located in Arabidopsis PG (Lohmann et al., 2006c) (Fig.1).

The enzymatic reactions of phylloquinone biosynthesis take place at the inner membrane of the chloroplast envelope (Schultz et al., 1981) but recent evidence in Arabidopsis suggests that peroxisomes may also be implicated. In cyanobacteria and red algae, the pathway is catalysed by Men proteins including successively: MenF, MenD, MenH, MenC, MenE, MenB, MenA and MenG enzymes. The first step of phylloquinone biosynthesis (MenF) implicates the conversion of chorismate to isochorismate. In Arabidopsis, this reaction may be catalysed by two genes *ICS1* and *ICS2* showing homology with *MenF* gene (Ausubel et al., 2001; Gross et al., 2006; Garcion et al., 2008; Métraux et al., 2008).

The double homozygous *ics1 ics2* mutant was completely devoid of phylloquinone and plants remained smaller and had a pale green or yellowish phenotype compared to the WT or the single mutants. The two enzymes may form a complex in the chloroplast stroma to facilitate the efficient channelling of intermediates through the pathway (Ausubel et al., 2001; Métraux et al., 2008).

The conversion of isochorismate in *o*-succinyl-benzoate (OSB) implicates three distinct enzymes (Men D, Men H and Men C) in cyanobacteria. These functions are encoded by the composite gene PHYLLO in Arabidopsis (Gross et al., 2006). In cyanobacteria, the conversion of OSB to o-succinyl-benzoyl-coenzyme A is catalyzed by the ligase MenE. The presence of several MenE homologs in the Arabidopsis genome makes it difficult to assign the ligase function: it seems likely, however, that the OSB–CoA ligase corresponds to the Acyl-Activating Enzyme 14 (AAE14) (Browse et al., 2008). The *aae14* mutant is unable to grow on soil due to the lack of phylloquinone. Recently, it has been demonstrated that MenE/AAE14 is dually targeted to both chloroplasts and peroxisomes (Reumann et al., 2010). In the following step, OSB-CoA is converted to 1,4-dihydroxy-2-naphthoyl-coenzyme A by an enzyme orthologous to MenB, the naphthoate synthase (NS/ECHId) encoded by a single Arabidopsis gene (Gross et al., 2006; Browse et al., 2008; Babujee et al., 2010). Prior to these

studies, neither functional data nor subcellular localization had been reported for a MenB homologue in plants. Recently, NS/ECHId was localized to the Arabidopsis peroxisome. (Babujee et al., 2010; Reumann et al., 2010). It has also been proposed that the conversion of 1,4-dihydroxy-2-naphthoyl-coenzyme A to 1,4-dihydroxy-2-naphthoate (DHNA) is catalysed by the 1,4-dihydroxy-2-naphthoyl-CoA (DHNA-CoA) thioesterase and that this enzyme may also be a peroxisomal protein (Reumann, 2004; Reumann et al., 2010). These recent findings suggest that phylloquinone biosynthesis of higher plants is partially compartmentalized in peroxisomes.

The last steps of phylloquinone synthesis involve the attachment of the phytyl chain to DHNA by MenA/ABC4 (Shimada et al., 2005) and the methylation of 2-phytyl-1,4-naphthoquinone by MenG (Lohmann et al., 2006c). Genetic analysis of phylloquinone pathway in plants allowed the isolation of the *AtmenA* mutant of Arabidopsis, which lacks the DHNA phytyltransferase (Shimada et al., 2005). Total absence of vitamin K_1 in *AtmenA* mutant plants results in a drastic reduction in both growth and the accumulation of PSII and PQ-9.

In contrast, growth and photosynthesis was only slightly affected in *AtmenG* mutant plants raised under normal light conditions. This fact suggests that 2-phytyl-1,4-naphthoquinone can functionally replace phylloquinone as an electron carrier in PSI. Under high light stress, biochemical and physiological studies of the *AtmenG* mutant demonstrated a significant decrease in the level of PSI complexes caused by oxidative damage at the PSI reaction center. This lead to lowered PSII efficiency and negatively affected the performance of the entire photosynthetic electron transfer chain (Lohmann et al., 2006c).

Interestingly, a much higher proportion of the 2-phytyl-1,4-naphthoquinone in the *AtmenG* mutant than of phylloquinone in the wild type was present in PG. A non-targeted lipidomic

analysis of *ndc1* mutant plants lead to the unexpected discovery that, similar to AtmenG, phylloquinone was almost completely absent and that 2-phytyl-1,4-naphthoquinone accumulated instead (Eugeni Piller et al., 2011). Also, At*MenG* was normally expressed in *ndc1* plants. While this result is difficult to explain, it suggests the implication of NDC1 and PG in the AtMenG methylation step.

Interestingly, the multifunctional protein PHYLLO and AtMenG gave punctate fluorescence rather than the ring-like fluorescence typical of chloroplast envelope proteins when transiently expressed as GFP or YFP fusion proteins in Arabidopsis protoplasts. As the punctate fluorescence resembles that of NDC1 it may hint at a PG localization of PHYLLO and AtMenG (Gross et al., 2006; Lohmann et al., 2006c; Eugeni Piller et al., 2011). However, PHYLLO and AtMenG were not found in the PG proteome. Possibly they are only loosely or transiently associated with PG and lost during the purification procedure.

Together with the presence of phylloquinone and 2-phytyl-1,4-naphthoquinone, the new role for NDC1 suggests a role for PG in phylloquinone metabolism.

2.4 Conclusions

In the last few years several studies demonstrated the important role of PG in chloroplast lipid metabolism. The determination of the Arabidopsis PG proteome (Vidi et al., 2006a; Ytterberg et al., 2006b) paved the way to the discovery that PG do not only store lipids but actively participate in their synthesis.

The Arabidopsis PG proteome contains 34 proteins, assignable to three groups: plastoglobulins, metabolic enzymes and proteins of unknown function. While many of the unknown proteins are predicted to be enzymes, their function still remains to be discovered.

To discover the function of such candidate enzymes in lipid metabolism, a powerful non-targeted lipidomics analysis can be employed to correlate changes in metabolite profiles with enzyme function.

The final biosynthetic steps of tocopherol require VTE1 and VTE4. VTE1 was localized in PG while VTE4 was located at the inner envelope membrane. This hints at the interesting possibility of prenylquinone metabolite trafficking inside the chloroplast.

It is still not known whether PG are structurally required for proper chloroplast function but forward genetic screens have the potential to provide answers to this question. Proteome analyses of PG isolated from plants under various stress or developmental conditions may lead to the identification of yet new candidate proteins with important functions in lipid metabolism. But what we know for sure now is that PG are sitting right at the crossroads of the prenylquinone metabolic pathways.

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Chapter III.
Chloroplast Lipid Droplet Type II NAD(P)H Quinone Oxidoreductase Is Essential For Prenylquinone Metabolism And Vitamin K1 Accumulation

3. CHLOROPLAST LIPID DROPLET TYPE II NAD(P)H QUINONE OXIDOREDUCTASE IS ESSENTIAL FOR PRENYLQUINONE METABOLISM AND VITAMIN K_1 ACCUMULATION

Lucia Eugeni Piller[a], Céline Besagni[a], Brigitte Ksas[b,c,d], Dominique Rumeau[b,c,d], Claire Bréhélin[e], Gaétan Glauser[f], Felix Kessler[a,1] and Michel Havaux[b,c,d]

[a]Laboratoire de Physiologie Végétale, Université de Neuchâtel, 2000 Neuchâtel, Switzerland; [b]Laboratoire d'Ecophysiologie Moléculaire des Plantes, Institut de Biologie Environnementale et de Biotechnologie, Service de Biologie Végétale et de Microbiologie Environnementale, Commissariat à l'Energie Atomique, 13108 Saint-Paul-lez-Durance, France; [c]Unité Mixte de Recherche Biologie Végétale et Microbiologie Environnementales, Centre National de la Recherche Scientifique, 13108 Saint-Paul-lez-Durance, France; [d]Université Aix-Marseille, 13108 Saint-Paul-lez-Durance, France; [e]Laboratoire de Biogenèse Membranaire, Unité Mixte de Recherche 5200, Centre National de la Recherche Scientifique, Université Bordeaux 2, F-33076 Bordeaux, France; and [f]Chemical Analytical Service of the Swiss Plant Science Web, Université de Neuchâtel, 2000 Neuchâtel, Switzerland

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3.1 General introduction

In chapter III, I present the paper published in *"Proceedings of the National Academy of Sciences of the United States of America"* on the characterization of a chloroplast lipid droplet type II NAD(P)H quinone oxidoreductase, NDC1.

As explained in Chapter II, plastoglobules are a storage site of prenylquinones. Here we demonstrate that NAD(P)H dehydrogenase C1, NDC1, is associated with these low density lipoprotein particles by *in vivo* and *in vitro* experiments. In fact, transient expression of NDC1 fused to YFP resulted in a punctuate fluorescence inside the chloroplasts. *In vitro* import into isolated organelles supports a dual localization of this protein in chloroplasts and mitochondria.

Enzymatic assays demonstrate the capacity of recombinant NDC1 enzyme to use several synthetic substrates such as decyl-ubiquinone and even purified plastoglobules as quinone substrates. Moreover, using a non–targeted lipidomic approach, we demonstrate the

implication of this enzyme in the reduction of the PQ pool in chloroplast lipid droplets and the importance of NDC1 in the overall plastoquinone redox state. In *ndc1* mutant plants this reservoir is more oxidized than in WT. NDC1 also facilitates the normal accumulation of PC-8 by reducing PQ. In fact, it is preferentially PQH_2 that is converted into PC-8 by VTE1 that is also present in plastoglobules. Unexpectedly, lipidomic experiments also led to a completely unexpected discovery: NDC1 is required for the final step in phylloquinone biosynthesis, the AtMenG-dependent methylation step. In *ndc1* mutant plants vitamin K_1 is absent and replaced by its immediate precursor, the 2-phythyl-1,4-naphthoquinone. Moreover, the chemical precursor is accumulated to a concentration comparable to that of phylloquinone in wild type plants.

An implication of NDC1 in cyclic electron flow around PSI or chlororespiration, as we hypothesized previously, was not observed in the Arabidopsis system. Our results show that NDC1 does not have a similar function as NDH when several photosynthetic parameters were measured including F_v/F_m, $\Delta F/F_m'$, NPQ and others. Although no effect was detected on linear and cyclic electron transport, a unique afterglow thermoluminescence band (indicative of electron flow to PQ) appeared in the *ndh1* mutant background after high light treatment. This result indicates that NDC1 participates in a unique pathway of non-photochemical PQ reduction in chloroplasts parallel to cyclic and chlororespiratory electron flow.

In summary, this paper shows the presence and the importance of NDC1 in plastoglobules and the capacity of this enzyme to reduce different quinone substrates implicated in prenylquinone metabolism, most importantly the biosynthesis of phylloquinone.

Chloroplast lipid droplet type II NAD(P)H quinone oxidoreductase is essential for prenylquinone metabolism and vitamin K1 accumulation

Lucia Eugeni Piller[a], Céline Besagni[a], Brigitte Ksas[b,c,d], Dominique Rumeau[b,c,d], Claire Bréhélin[e], Gaétan Glauser[f], Felix Kessler[a,1] and Michel Havaux[b,c,d]

[a]Laboratoire de Physiologie Végétale, Université de Neuchâtel, 2000 Neuchâtel, Switzerland ; [b]CEA, IBEB, SBVME, Laboratoire d'Ecophysiologie Moléculaire des Plantes, 13108 Saint-Paul-lez-Durance, France ; [c]CNRS, UMR Biologie Végétale et Microbiologie Environnementales, 13108 Saint-Paul-lez-Durance, France ; [d]Université Aix-Marseille, 13108 Saint-Paul-lez-Durance, France ; [e]Laboratoire de Biogenèse Membranaire, UMR 5200 CNRS - Université Bordeaux 2, F-33076 Bordeaux, France; [f]Chemical Analytical Service of the Swiss Plant Science Web, Université de Neuchâtel, 2000 Neuchâtel, Switzerland

*to whom correspondence should be addressed

3.2 Abstract

Lipid droplets are ubiquitous cellular structures in eukaryotes and are required for lipid metabolism. Little is currently known about plant lipid droplets other than oil bodies. Here, we define dual roles for chloroplast lipid droplets (plastoglobules) in energy and prenylquinone metabolism. The prenylquinones, plastoquinone, plastochromanol-8, phylloquinone (vitamin K_1) and tocopherol (vitamin E) are partly stored in plastoglobules. This work shows that NDC1 (At5g08740), a type II NAD(P)H quinone oxidoreductase, associates with plastoglobules. It reduces a plastoquinone analogue *in vitro* and affects the overall redox state of the total plastoquinone pool *in vivo* by reducing the plastoquinone reservoir of plastoglobules. Finally, NDC1 is required for normal plastochromanol-8 accumulation and is essential for vitamin K_1 production.

3.3 Introduction

Energy production in plants largely relies on oxygenic photosynthesis implicating linear electron flow from water to NAD(P)H catalyzed by photosystems (PS) 1 and 2. For optimal energy production, however, plants and algae have additional pathways, such as chlororespiration and cyclic electron transport around PS1, allowing the electrons to be re-cycled into the intersystem electron transport chain (Cardol et al.; Johnson; Rumeau et al., 2007; Shikanai, 2007) (Fig. S1).

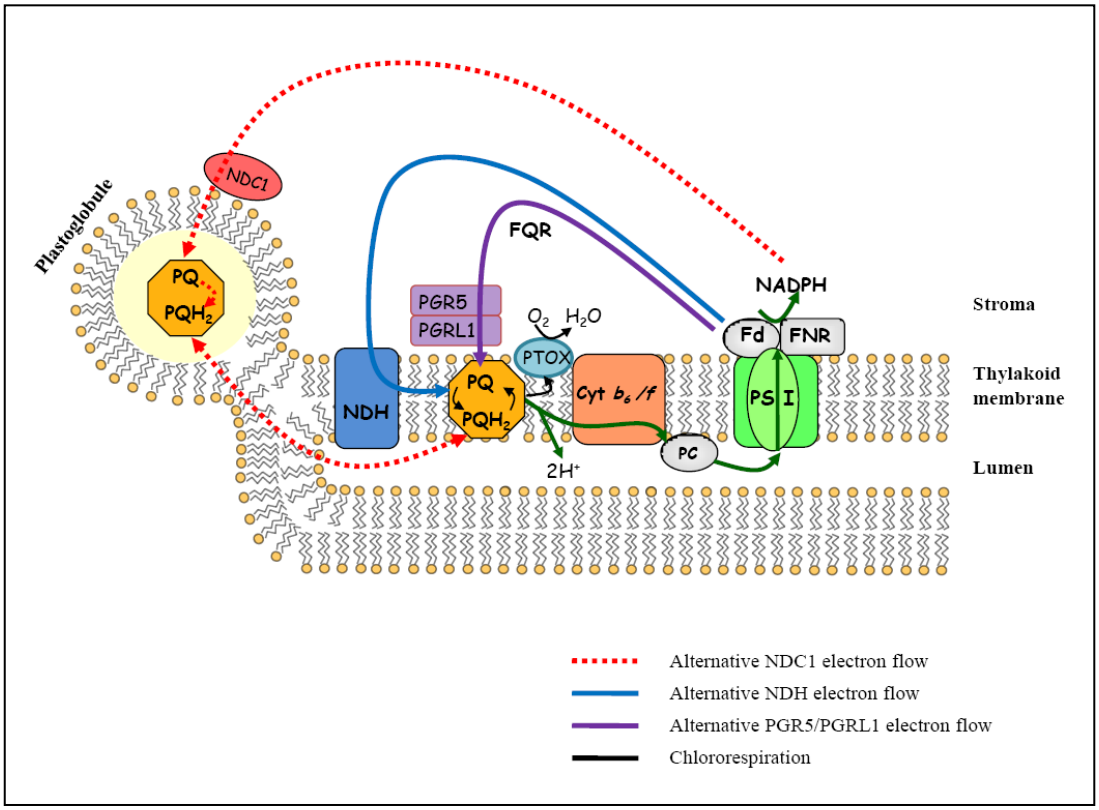

Fig. S1. Alternative electron flow pathways. 1) PGR5 (Proton Gradient Regulation) /PGRL1 (PGR5-like) pathways and 2) the NDH (NAD(P)H-dehydrogenase) complex transfer electrons from ferredoxin (Fd) back to the thylakoid plastoquinone pool (PQ/PQH_2). These two processes contribute to cyclic electron flow around Photosystem I. Chlororespiration involves transfer of electrons from PQH_2 directly to molecular oxygen via the plastid terminal oxidase (PTOX). NDC1 transfers electrons from NADPH to PQ present in the plastoglobules. The plastoquinone pools in the thylakoid and plastoglobules are linked by the outer lipid layer of the thylakoid which is contiguous with the plastoglobule polar lipid layer. FQR, ferredoxin plastoquinone reductase.

In angiosperms, those alternative electron flows are in part mediated by the PGR5 (Proton Gradient Regulation)/PGRL1 (Proton Gradient Regulation 5-like 1) pathway and in part by a multisubunit NDH (NAD(P)H:plastoquinone oxidoreductase) complex with similarity to bacterial and mitochondrial Complex I (Munekage et al., 2002; Shikanai, 2007). Both the PGR5/PGRL1 pathway and the NDH complex have the ability to reduce plastoquinone (PQ) using ferredoxin as the electron donor (Yamamoto et al.). A similar activity exists in the green alga *Chlamydomonas reinhardtii* but this organism does not encode the subunits of the NDH complex (Ravenel et al., 1994; Peltier and Cournac, 2002; Mus et al., 2005). Instead a monomeric type II NAD(P)H:quinone oxidoreductase, NDA2, takes over the function of the NDH complex in cyclic electron flow and chlororespiration in *C. reinhardtii* (Jans et al., 2008; Desplats et al., 2009). In *Arabidopsis thaliana* seven such type II NAD(P)H dehydrogenase homologs are encoded by three sub families (Michalecka et al., 2003): two NDAs, four NDBs and one NDC (termed NDC1). Their role, if any, in chloroplast electron pathways is not known. Interestingly, only *NDC1* appears to be of cyanobacterial origin (Michalecka et al., 2003). A study using GFP-fusions initially localized all seven homologs in mitochondria (Michalecka et al., 2003). Later, NDC1 was imported into isolated mitochondria as well as chloroplasts suggesting a dual localization (Elhafez et al., 2006; Carrie et al., 2008). Furthermore, NDC1 was identified in proteomics studies of chloroplast lipid droplets (plastoglobules) (Vidi et al., 2006b; Ytterberg et al., 2006a). The plastoglobules are a site of both prenylquinone metabolism and storage. Recently a large reservoir of PQ, probably not immediately involved in linear electron transport, was discovered in plastoglobules (Szymanska and Kruk; Zbierzak et al.; Bréhélin and Kessler, 2008). Tocopherol (vitamin E), phylloquinone (vitamin K_1) and plastochromanol-8 (PC-8), a chromanol-derivative of PQ, are also present in plastoglobules (Szymanska and Kruk; Zbierzak et al.; Lohmann et al., 2006a; Vidi et al., 2006b) (Fig. S2).

Here, we demonstrate that NDC1 functions in a new electron flow pathway to the plastoglobule PQ reservoir and thereby determines the overall PQ redox state. Unexpectedly, NDC1 also plays a key role in plastochromanol accumulation and promotes the production of phylloquinone beyond its non-methylated 2-phytyl-1,4-naphthoquinone precursor.

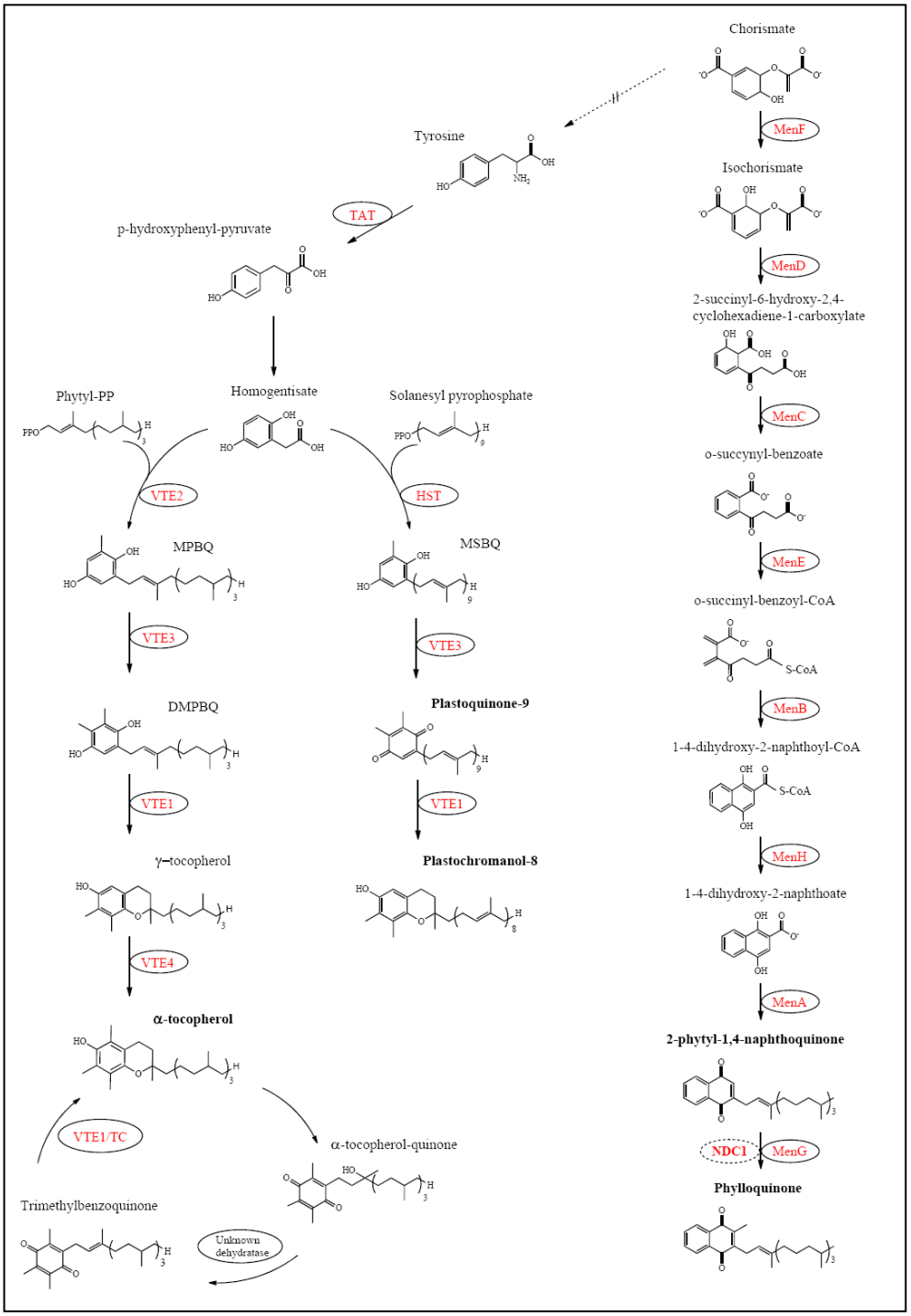

Fig. S2. Biosynthetic pathways of α-tocopherol, plastoquinone and phylloquinone. The enzyme abbreviations are shown in red. Note that NDC1 is required for the MenG-dependent methylation of 2-phytyl-1,4-naphtoquinone to phylloquinone. TAT: tyrosine aminotransferase; HST: homogentisic acid solanesyl transferase; TC: tocopherol cyclase; VTE: vitamin E synthesis; Men: menaquinone synthesis; -PP: pyrophosphate; MPBQ: 2-methyl-6-phytyl-1,4-benzoquinone; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinone; MSBQ: 2-methyl-6-solanesyl-1,4-benzoquinol.

3.4 Results

Chloroplast lipid droplet localization of NDC1. Earlier studies suggested either a mitochondrial or dual mitochondrial/chloroplast localization for NDC1 (Michalecka et al., 2003; Elhafez et al., 2006; Carrie et al., 2008). Two independent proteomics studies identified NDC1 as a chloroplast lipid droplet (plastoglobule) protein (Vidi et al., 2006b; Ytterberg et al., 2006a). Sucrose gradient flotation of total Arabidopsis chloroplast membranes followed by Western blotting demonstrated co-fractionation of NDC1 with the plastoglobule markers PGL35 and PGL40 in the low density fractions as well as the clear separation from the envelope marker Toc75 and the thylakoid marker LHCB2 (Fig. 1*A*). Transient expression of NDC1-YFP in *Nicotiana benthamiana* protoplasts resulted in punctate fluorescence mostly inside the chloroplasts (Fig. 1*B*). The punctate fluorescence in most cases colocalized with that of the neutral lipid dye Nile Red (Fig 1*D*). These results are consistent with the lipid droplet/plastoglobule localization of NDC1, indicated by the earlier proteomic studies and the fractionation experiment (Fig. 1*A*). Nevertheless, both isolated chloroplasts (Fig. 1*F*) and mitochondria (Fig. 1*G*) imported synthetic, full length [^{35}S]pre-NDC1 *in vitro* resulting in protease protected, mature forms in both cases.

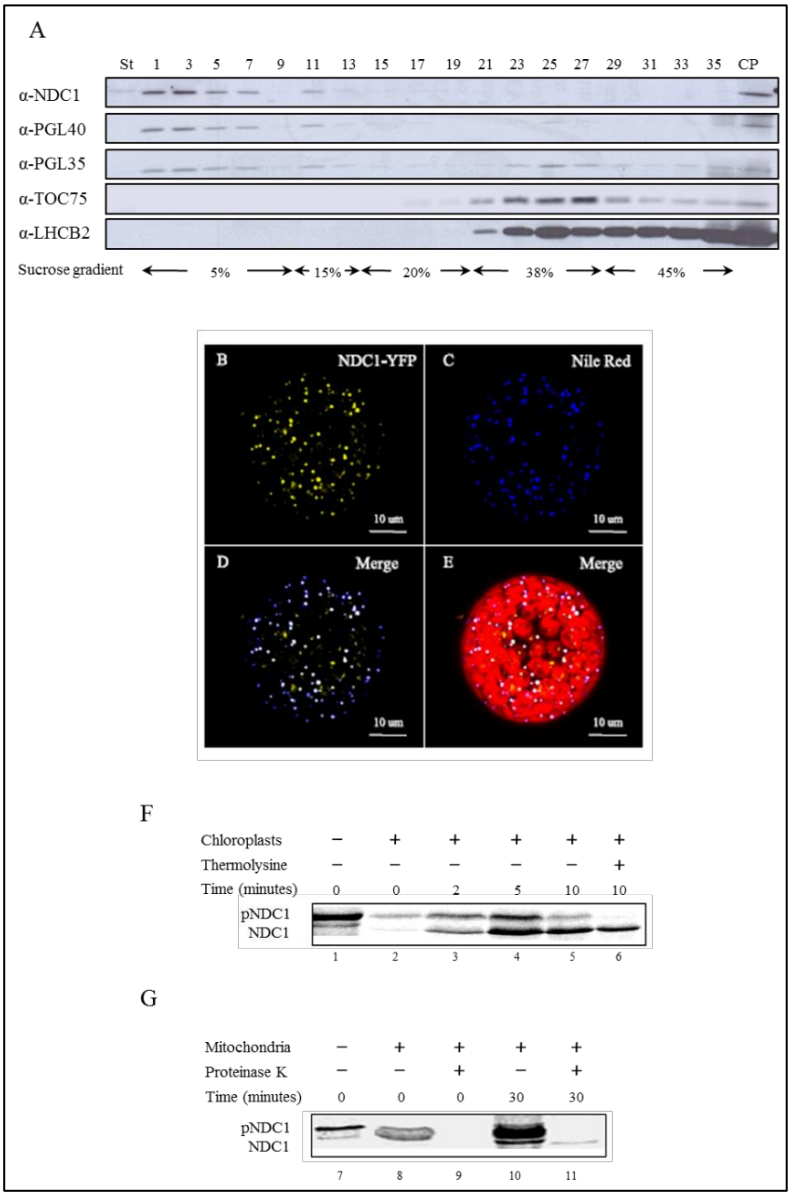

Fig. 1. NDC1 lipid droplet localization. (A) Western blotting of chloroplast membrane fractions separated by sucrose gradient flotation. Toc75 and LHCB2 are envelope and thylakoid markers, respectively. PGL35 and PGL40 are plastoglobule markers. CP: total chloroplast; St: stroma. (B) NDC1-YFP under the control of the CaMV 35S promoter was transiently expressed in tobacco protoplasts. Transformed protoplasts were analyzed by confocal laser microscopy. (C) The neutral lipid dye Nile Red reveals lipid droplets in chloroplasts (plastoglobules). (D) Merge 1 shows the superposition of NDC1-YFP and Nile Red in white and (E) Merge 2 shows the chlorophyll autofluorescence to visualize chloroplasts. (F) ^{35}S-labelled pre-NDC1 (lane 1) was incubated with isolated chloroplasts *in vitro* in a time course experiment (0, 2, 5 and 10 minutes (lane 2-5)). The imported, lower molecular mass NDC1 was resistant to exogenously added thermolysin protease (lane 6). (G) ^{35}S-labelled pre-NDC1 was incubated with isolated mitochondria *in vitro* (lane 7). The experiment was analyzed at the 0 and 30 minute time points (lane 8 and 10). The imported, lower molecular mass NDC1 was resistant to exogenously added proteinase K (lane 9 and 11).

Photosynthetic parameters of the *ndc1* mutant. We isolated two homozygous T-DNA insertion lines for the *NDC1* gene: Salk_024063 and GABI_614F03, named *ndc1-1* and *ndc1-3*, respectively. Immunoblotting using specific antibodies indicated the absence of the NDC1 protein and confirmed the homozygous knock-out nature of the two mutants (Fig. S3*A*). Neither of the lines had a visible phenotype (Fig. S3*B*).

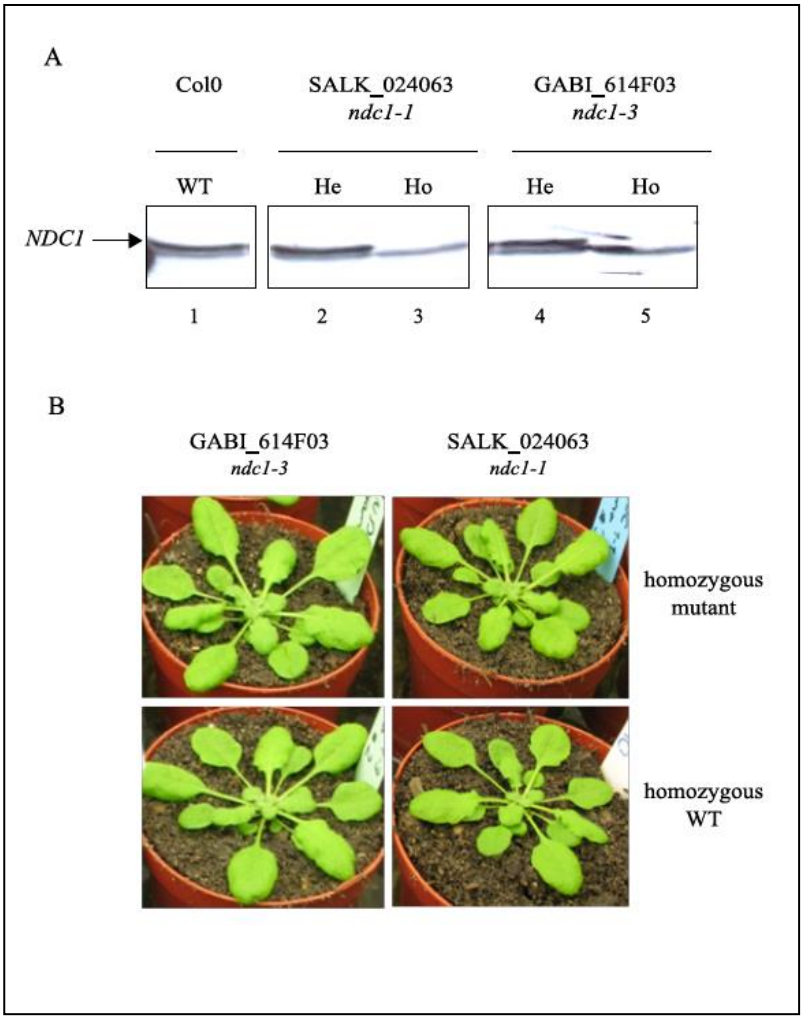

Fig. S3. Isolation of *ndc1* mutant lines. (A) Total extracts of two mutant lines *ndc1-1*, and *1-3* corresponding to 100 µg of protein each were analyzed by Western blotting. Homozygous (Ho) *ndc1-1* (Salk_024063) (lane 3) and *ndc1-3* (GABI_614F03) (lane 5) were null based on the absence of the upper band in the doublet. (B) Neither homozygous *ndc1-1* nor *ndc1-3* had a visible phenotype.

We measured several chlorophyll fluorescence parameters in WT and *ndc1-1* leaves (hereafter named *ndc1*) and also, for comparison purposes, in leaves of the *ndh*o mutant (hereafter named *ndh1*) lacking the NDH complex (Rumeau et al., 2005): the maximal quantum yield of PSII photochemistry (Fv/Fm) was determined in the dark while the actual PSII quantum yield (ΔF/Fm') and the non-photochemical quenching of chlorophyll fluorescence (NPQ) were measured at different photon flux densities (PFDs) (Fig. S4*A* and *B*). None of those parameters differed between WT and the *ndc1* mutant. We also analyzed alternative electron flows which donate electrons from the stroma to the PQ pool, using measurements of chlorophyll fluorescence (Fig. S4*C*), P700 redox state (Fig. S4*D*) and chlorophyll thermoluminescence (Fig. 2). The postillumination rise in chlorophyll fluorescence, which is indicative of PQ and Q_A reduction specifically mediated by the NDH complex (Rumeau et al., 2005), remained unchanged in *ndc1* when compared with WT (Fig. S4*C*). We also measured the postillumination reduction of oxidized P700, the PSI reaction center pigment (Maxwell and Biggins, 1976). While a slight increase in the half-time ($t_{1/2}$) of P700 reduction was observed in the *ndh1* mutant, indicating a slowdown of the electron donation to P700, the *ndc1* mutation had no effect on this parameter (Fig. S4*D*). Figure 2*A* shows the afterglow thermoluminescence band, induced by far-red pre-illumination, which corresponds to a heat-induced back electron transfer to the PQ pool in the dark (Miranda and Ducruet, 1995). In Arabidopsis, this pathway has been shown to reflect mainly the NDH activity (Havaux et al., 2005a; Lintala et al., 2009), as confirmed here by the strong reduction of this band in *ndh1*. Again, no difference was found between WT and *ndc1*. From those results, we can conclude that NDC1 and NDH do not fulfill a similar function and therefore NDC1 is probably not directly involved in cyclic or chlororespiratory electron flows (Fig. S1) under standard growth conditions.

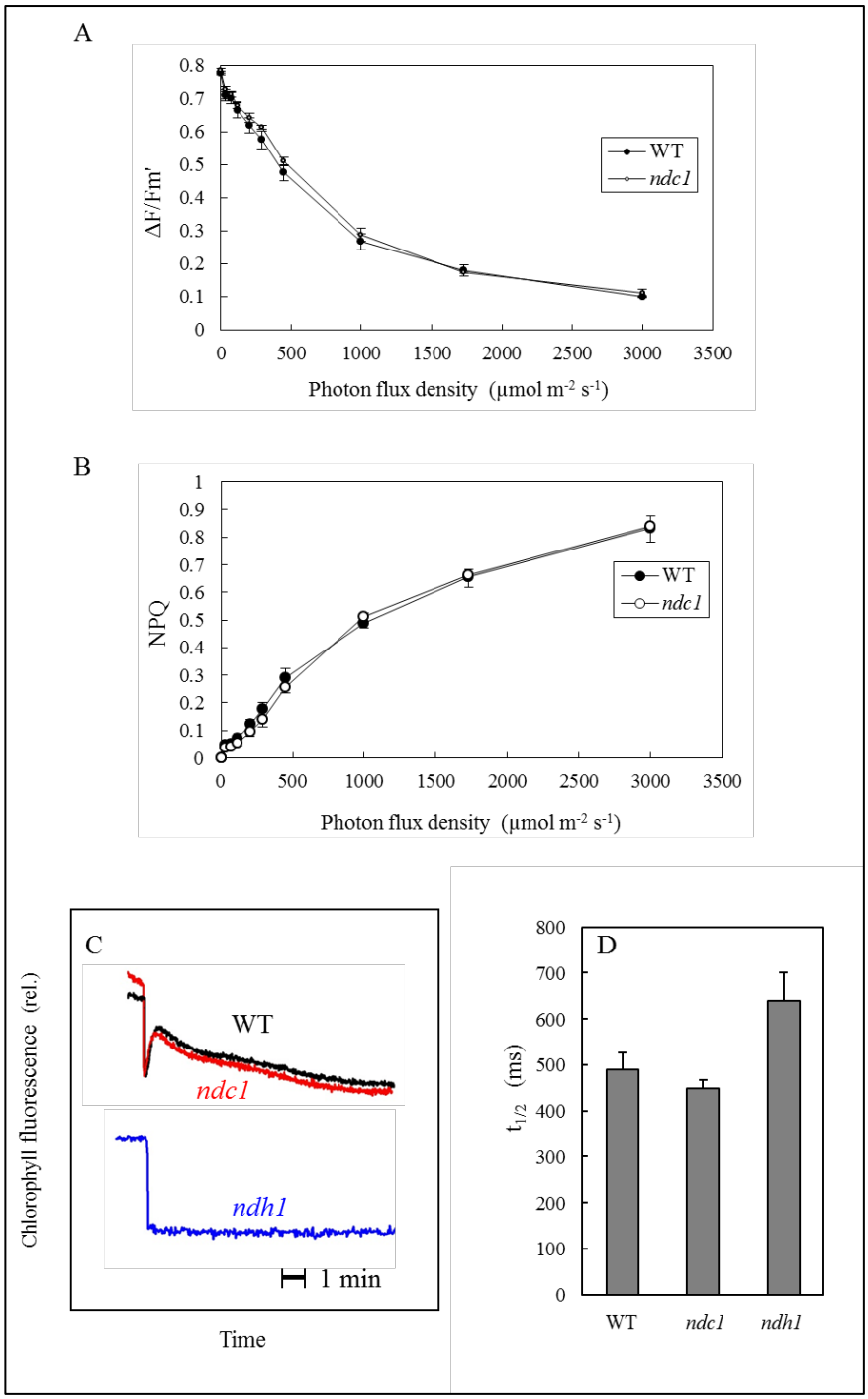

Fig. S4. Photosynthetic properties of WT and *ndc1* mutant leaves of Arabidopsis. A) Quantum yield of PSII photochemistry measured at different PFDs using the chlorophyll fluorescence parameter ΔF/Fm'. B) Non-photochemical energy quenching measured at different PFD using the NPQ parameter. Data are mean values of 3 separate experiments. C) Transient increase in chlorophyll fluorescence after a light to dark transition. For comparison purposes, the trace of the *ndh1* mutant is also shown. The data are representative traces of at least 5 separate experiments. D) Half-time $t_{1/2}$ of the postillumination re-reduction of oxidized P700 reduction. Data are mean values of 6 separate experiments.

We also analyzed plants previously exposed to high light conditions that induce accumulation of plastoglobules (Bréhélin et al., 2007b) and increase expression of the *NDC1* gene in the wild type (Fig. 2*B*) as well as the *ndh1* mutant (Fig. S5) (Yoshida and Noguchi, 2009). In WT, high light shifted the afterglow band towards lower temperatures compared to low light (Fig. 2*A* and *C*). This bandshift is indicative of an activation of the cyclic electron pathway: electrons are rapidly transferred to Q_B at room temperature, leading to charge recombination between Q_B- and the S states and to the merging of the afterglow band with the B band (Ducruet et al., 2005; Apostol et al., 2006). This activation was not observed in *ndh1* mutant leaves. Rather surprisingly, high light induced the appearance of a new band in this mutant, peaking at ca. 42°C (Fig. 2*C* and *D*). This band was insensitive to antimycin A (AA) (Fig. S6) but showed a high sensitivity to diphenyleneiodonium (DPI), a known inhibitor of type II NAD(P)H:quinone oxidoreductases (Mus et al., 2005) (Fig. 2*C*). Moreover, its amplitude was strongly reduced in the *ndc1 ndh1* double mutant (Fig. 2*D*), indicating the participation of NDC1 in this non-photochemical electron flow.

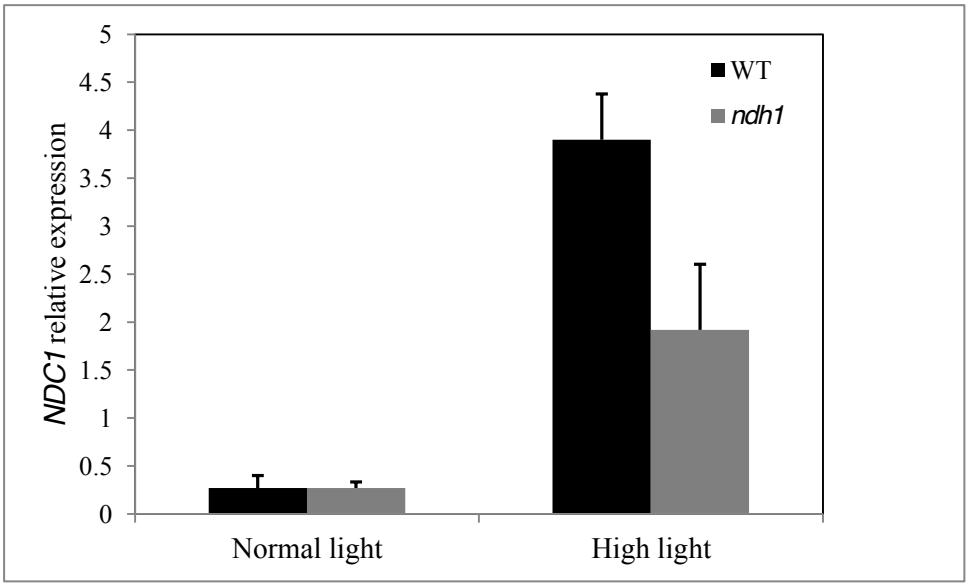

Fig. S5. Analyses of *NDC1* expression in normal and high light conditions. After extraction of RNA from WT and *ndh1* mutant plants, transcript levels were quantified by real-time reverse transcription (RT)-PCR with *ACTIN2* as the reference. Values are means of three replicates.

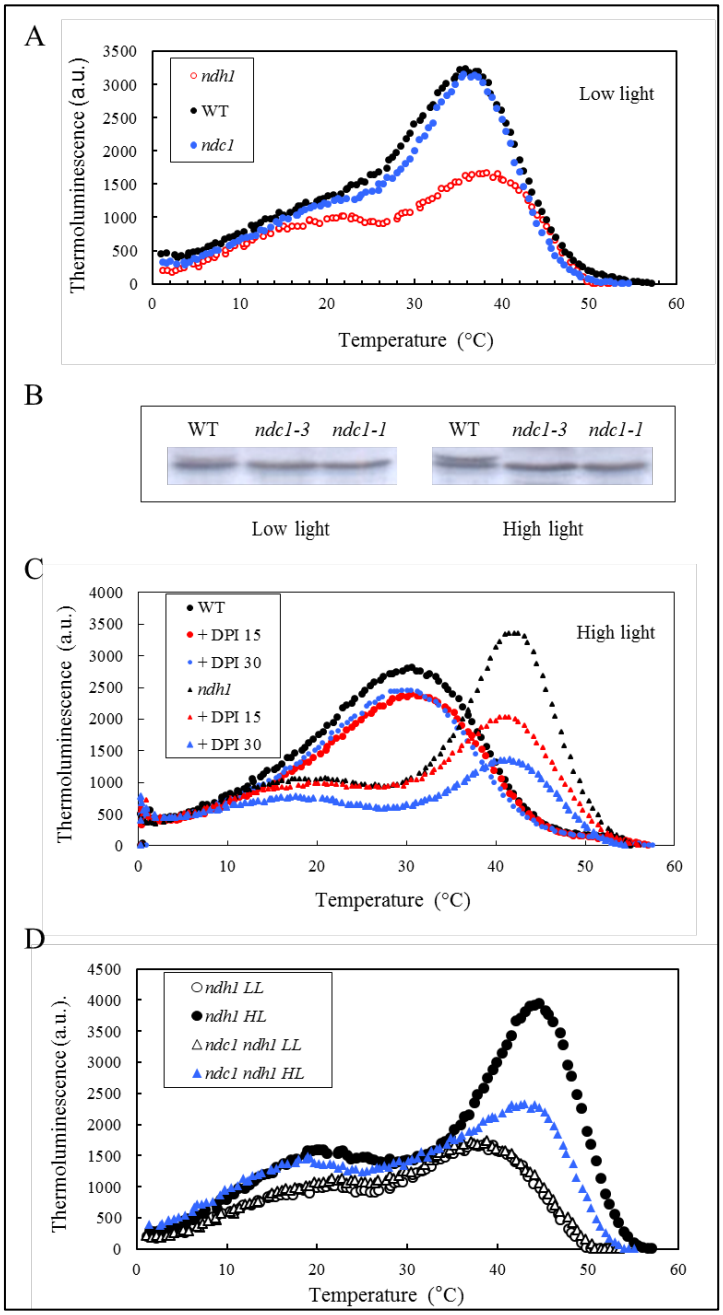

Fig. 2. Thermoluminescence measurements in WT, *ndc1* and *ndh1* mutant leaves of Arabidopsis. (A) Far-red light-induced thermoluminescence signal in WT Arabidopsis leaves and in mutant leaves (*ndc1, ndh1*) grown under standard conditions. The band peaking at temperature >35°C is the so-called afterglow band. The shoulder observed at lower temperature (ca. 20°C) is a B band corresponding to the $S_{2/3}$ Q_B- charge recombination. The data are representative traces of at least 6 separate experiments. (B) Immunodetection of NDC1 in total extracts of WT and two *ndc1* mutant lines (*ndc1-1* and *ndc1-3*) under normal and high light growth conditions. The upper band, weakly visible in the WT under low light

conditions and more strongly under high light conditions, corresponds to the NDC1 protein. The band is absent from the *ndc1-1* and *ndc1-3*. The lower band is a non-specific, crossreacting signal. (C) Effects of diphenyleneiodonium (DPI, 15 and 30 μM) on the afterglow thermoluminescence band of WT and *ndh1* mutant leaves acclimated for 7 days to high light. Data are representative traces of 3 separate experiments; (D) Afterglow thermoluminescence band measured in leaves of the *ndh1* mutant and the *ndc1 ndh1* mutant acclimated for 7 days to high light. Data are representative traces of at least 6 separate experiments.

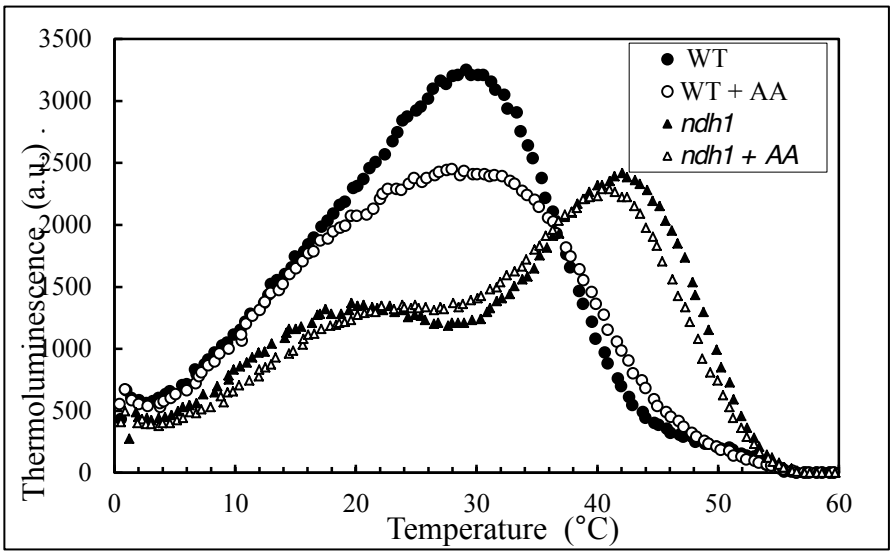

Fig. S6. Effects of 5 μM antimycin A (AA) on the afterglow thermoluminescence band of WT and *ndh1* mutant leaves acclimated for 7 d to high light. Data are representative traces of 3 separate experiments.

***In vitro* activity of NDC1.** NDC1 is a predicted NADH/NADPH:quinone reductase and the thermoluminescence experiments supported such a role *in vivo*. For *in vitro* experimentation, purified recombinant NDC1 protein was incubated with NADPH as the electron donor and decyl-PQ (Fig. 3*A*). The decyl derivative is more soluble due to the replacement of the highly hydrophobic isoprenoid chain. We tracked the reactions by recording the oxidation of NAD(P)H at 340 nm. For decyl-PQ we measured a K_m of 9 μM and V_{max} of 46 μmoles/ mg/

min (Fig. 3*A*). DPI inhibited decyl-PQ reduction in the presence of NAD(P)H with a half maximal inhibition at about 2.5 μM (Fig. 3*B*). Isolated plastoglobules (Fig. 3*C*) and decyl-ubiquinone (with NADH as the electron donor) (Fig. 3*D*) also functioned as NDC1 substrates *in vitro*.

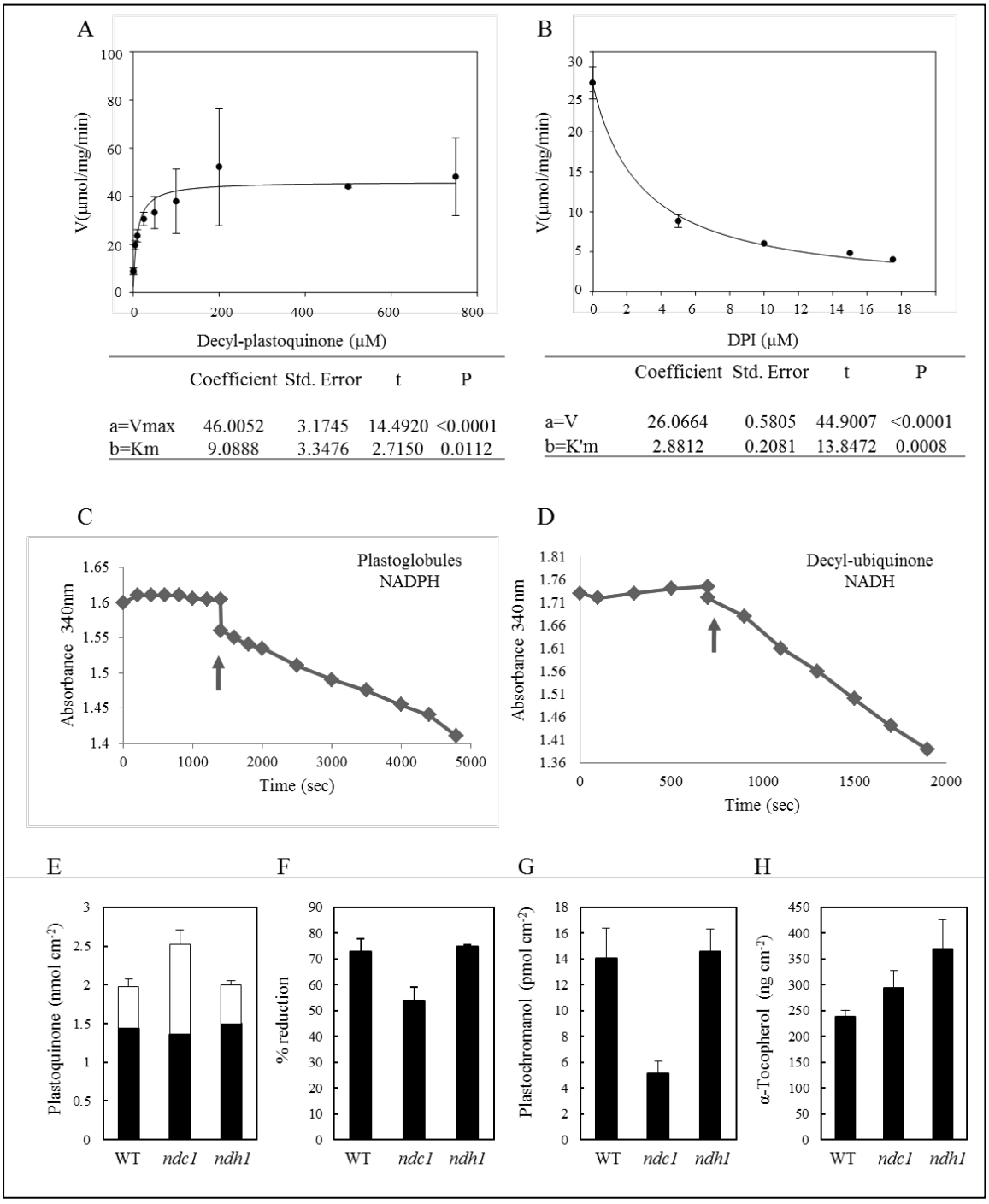

Fig. 3. NDC1 enzyme activity and prenylquinone quantification in the *ndc1* and *ndh1* mutants. (A) NDC1 activity was measured by monitoring NADPH oxidation in the presence of increasing concentrations of decyl-plastoquinone (a plastoquinone analog). (B) Increasing concentrations of the inhibitor diphenyleneiodonium (DPI) were added to the assay in the presence of 100 μM decyl-plastoquinone. The kinetic parameters were calculated using the SigmaPlot software. Each data point represents (B) two and (A) three experimental replicates. (C) NDC1 activity was measured in the presence of isolated plastoglobules and (D) in the presence of decyl-ubiquinone and NADH. The arrow indicates the time point of addition of purified NDC1. (E-H) Prenylquinones in leaves determined by HPLC. E) Total PQ, the white bar indicates the fraction of oxidized PQ, F) % reduction of the PQ pool, G) PC-8 and H) α-tocopherol in WT Arabidopsis leaves and *ndc1* or *ndh1* mutant leaves grown under standard conditions. Data are mean values of at least 3 separate experiments.

To determine how NDC1 affects prenyl-lipid levels in leaf extracts, we targeted PQ (Fig. 3*E* and *F*), its derivative PC-8 (Fig. 3*G*) as well as α-tocopherol (Fig. 3*H*) for quantification using HPLC coupled with UV and fluorescence detection systems. Neither tocopherol, nor PQ were diminished in *ndc1* relative to WT. Interestingly, the PQ pool was substantially more oxidized in *ndc1* (50% oxidation) compared to WT (30%) (Fig. 3*E* and *F*). Another striking result was the strong decrease of PC-8 in *ndc1* leaves (Fig. 3*G*). In contrast, the *ndh1* mutant did not show any significant change in the PQ redox state or in the PC-8 concentration.

Untargeted lipidomic analysis of the *ndc1* mutant. To determine the more general role of NDC1 in chloroplast metabolism we carried out untargeted lipidomics analyses (Fig. 4). Total lipid extracts of WT, *ndc1* (both *ndc1-1* and *-3*), *ndh1* single mutants and the *ndc1 ndh1* double mutant (*dk*) were prepared and injected into a ultra-high pressure liquid chromatography-quadrupole time of flight mass spectrometer (UHPLC-QTOFMS). The data obtained were subjected to multivariate analysis to determine differences in lipid content between samples (Fig. 4*A*). Using principal component analysis (PCA), two distinct groups

were observed (Fig. 4*A*): one containing WT and the *ndh1* mutant and the other containing the *ndc1* and *ndc1 ndh1* mutants. PCA loadings were then investigated and revealed that two variables were mostly contributive to the discrimination between both groups: *m/z* 450.3494 at 4.99 min for WT and *ndh1* and *m/z* 436.3336 at 4.64 min for *ndc*l and *ndc1 ndh1* (Fig. 4*B* and Table S1). The ion at *m/z* 450.3494 was associated to the molecular formula $C_{31}H_{46}O_2$ (error 0.9 ppm). A high resolution MS/MS experiment gave a main fragment at *m/z* 185.0604 corresponding to the raw formula $C_{12}H_9O_2$ (error 0.5 ppm) and a smaller fragment at *m/z* 225.0910 ($C_{15}H_{13}O_2$, error 2.7 ppm). These molecular and fragment ions were typical of phylloquinone which was further confirmed by the injection of a standard compound that yielded identical mass spectrum and eluted at identical retention time (Fig. S8).

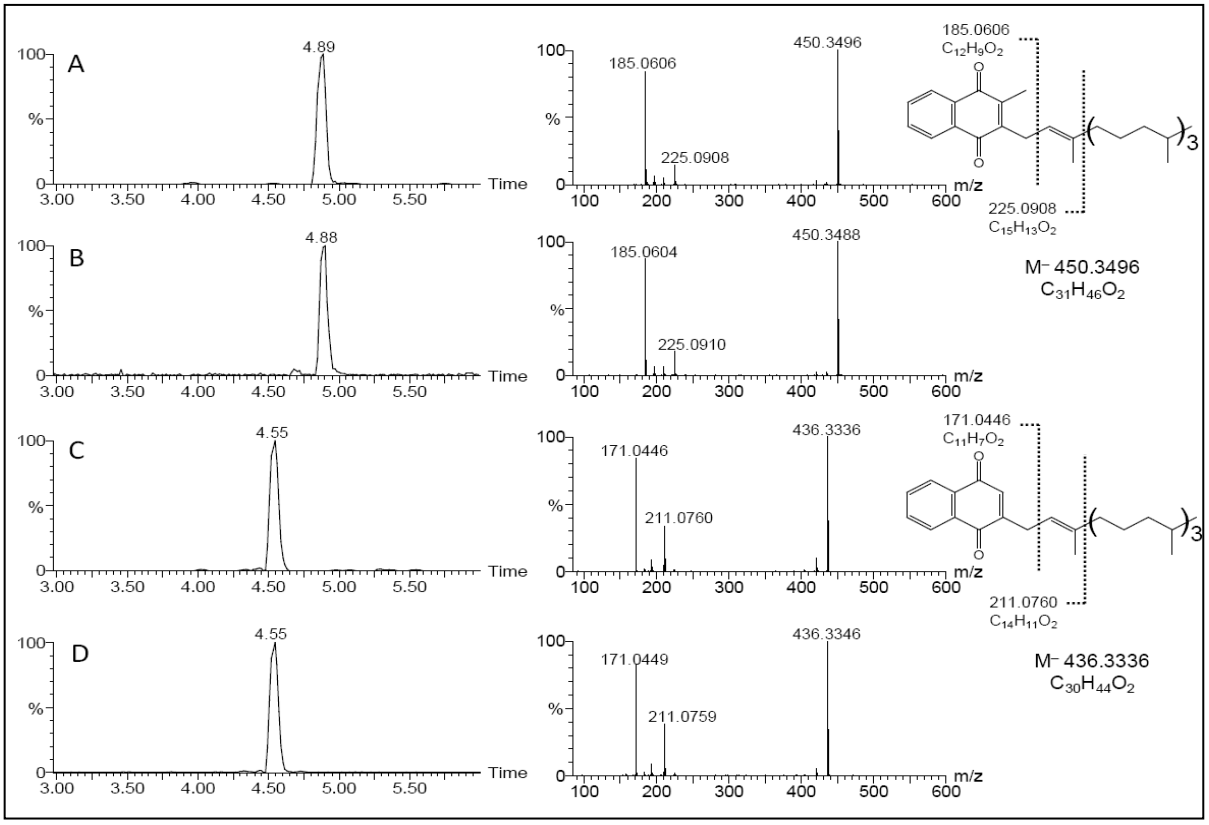

Fig. S8. Extracted ion chromatograms and product ion mass spectra of *m/z* 450.35 and *m/z* 436.33 for A) Phylloquinone standard. B) WT extract. C) *ndc1-1* extract. D) *AtmenG* extract.

The ion at *m/z* 436.3336 corresponded to the molecular formula $C_{30}H_{44}O_2$ (error 1.1 ppm) and could be due to the demethylated form of phylloquinone (2-phytyl-1,4-naphthoquinone). The loss of a methyl group was confirmed by MS/MS which generated a main fragment at *m/z* 171.0446 ($C_{11}H_7O_2$, error 0.0 ppm) together with a smaller fragment at *m/z* 211.0760 ($C_{14}H_{11}O_2$, error 0.5 ppm). No standard compound was available for this molecule and the ion at *m/z* 436.3336 was thus tentatively annotated as 2-phytyl-1,4-naphthoquinone. The latter was further confirmed using the *AtmenG* mutant known to accumulate 2-phytyl-1,4-naphthoquinone instead of phylloquinone (20): as in *ndc1*, the peak at *m/z* 436.3336 was present while phylloquinone was almost absent (Fig. S8). Phylloquinone levels in the whole plant were statistically similar in WT and *ndh1* mutant with 2.76 and 3.23 µg/g fresh weight (FW) respectively, while 2-phytyl-1,4-naphthoquinone was almost completely absent from both lines (0.07 and 0.06 µg/g FW in WT and *ndh1* respectively). Conversely in *ndc1* and *ndc1 ndh1* mutants, concentrations of phylloquinone were very low (0.02 and 0.05 µg/g FW respectively) and those of 2-phytyl-1,4-naphthoquinone were much higher with 2.82 and 2.83 µg/g FW respectively. Interestingly, the levels of 2-phytyl-1,4-naphthoquinone in *ndc1* and *ndc1 ndh1* mutants were similar to those of phylloquinone in WT and *ndh1* mutant (Fig. 4*C*).

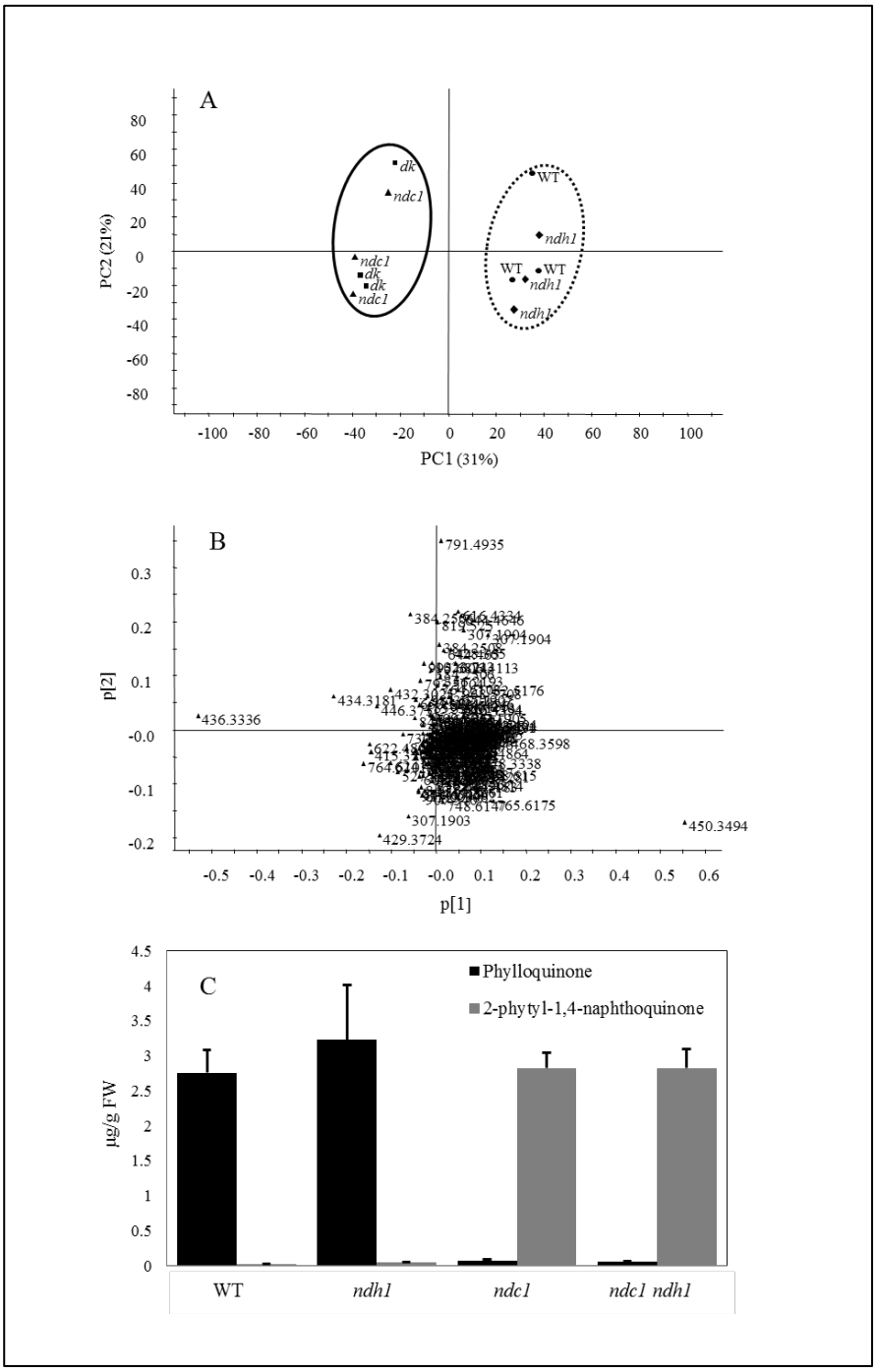

Fig. 4. Untargeted lipidomics of *ndc1* mutants. (A) Methanolic extracts from leaves of *ndc1*, *ndh1* and *ndc1 ndh1* (*dk*) mutants lines were analyzed using UHPLC-QTOFMS. (A) Principal component analysis score plots (PC1 x PC2) with their percentages of explained variance, based on normalized data from negative ion UHPLC-QTOFMS analyses. (B) Corresponding loading plot showing the most relevant variables responsible for the separation found in the score plot (436.3336 on far left corresponds to 2-phytyl-1,4-naphthoquinone; 450.3494 on far right corresponds to phylloquinone). (C) Quantification of phylloquinone and 2-phytyl-1,4-naphthoquinone in WT (Col0) and *ndc1*, *ndh1* and *ndc1 ndh1* mutant plants obtained from a standard solution of phylloquinone. Data represent the mean of two independent experiments (n=3 each).

A*tMENG* expression in the *ndc1* mutant. The absence of phylloquinone and its apparent replacement by the demethyl form in the *ndc1* mutant lines is surprising. It is known that AtMENG is required for the final methylation step of phylloquinone biosynthesis (Lohmann et al., 2006a). We used RT-PCR to determine whether *AtMENG* is still expressed in the *ndc1* and *ndc1 ndh1* mutants (Fig. S7). We detected no significant differences in the *AtMENG* expression levels between the WT and the *ndc1*, *ndh1* and *ndc1 ndh1* mutants. This indicates that *AtMENG* is normally expressed in the mutant background and strongly suggests that the active enzyme is also present.

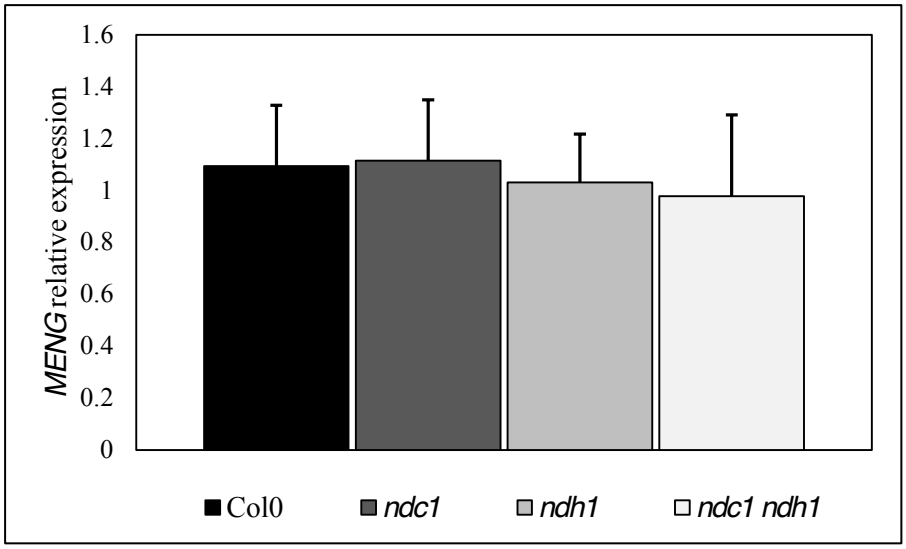

Fig. S7. Analyses of AtMENG expression in *ndc1* mutant lines. Transcript levels were quantified by real-time reverse transcription (RT)-PCR with *ACTIN2* as the reference. Values are means of three replicates.

3.5 Discussion

This study supports the function of NDC1 in chloroplasts. In chloroplast fractionation experiments NDC1 was almost exclusively detected in low density plastoglobule-containing fractions (Fig. 1*A*). Moreover, NDC1-YFP fluorescence co-localized with Nile Red staining (mostly in chloroplasts) emphasizing its association with lipid droplets (Fig.1*D*).

Nevertheless, this study also confirms *in vitro* import into both chloroplasts and mitochondria (Fig. 1*F* and *G*), and does not exclude the dual localization of NDC1. A possible explanation for these apparently disparate findings is that NDC1 accumulates predominantly in chloroplasts and only at lower levels in mitochondria. A potential function in mitochondria was supported by the ability of purified, recombinant NDC1 to use decyl-ubiquinone together with NADH as a substrate (Fig. 3*D*). Overall, our observations favor the function of NDC1 in chloroplasts: while no effect on linear or cyclic electron transfer in chloroplasts was observed (Fig. S4), a new afterglow thermoluminescence band (indicative of electron flow to PQ) appeared after highlight treatment of the *ndh1* mutant (Fig. 2): its characteristics (insensitivity to antimycin A, sensitivity to DPI, disappearance in the *ndc1 ndh1* double mutant) indicate that NDC1 functions in the chloroplast in a new pathway of non-photochemical PQ reduction parallel to cyclic and chlororespiratory electron flow (Fig. S1). Likely, this pathway corresponds to NDC1-catalyzed electron flow towards the PQ contained in the plastoglobules (Szymanska and Kruk; Bréhélin and Kessler, 2008) (Fig. S1). In support of this hypothesis, purified plastoglobules functioned as a quinone-containing substrate and accepted electrons from NADPH and the recombinant NDC1 enzyme *in vitro (*Fig. 3*C)*. In agreement with this data, the PQ pool was significantly more oxidized in the *ndc1* mutant than in the WT (Fig. 3*E* and *F*).

Decyl-PQ functioned as an *in vitro* substrate of recombinant NDC1 using NADPH as the electron donor (Fig. 3*A*). The ability of NDC1 to also use purified plastoglobules as a substrate was probably due to the PQ contained inside plastoglobules. This reaction may therefore reflect an *in vivo* scenario in which NDC1 binds to the plastoglobule surface thereby gaining access to and reducing the PQ substrate inside and resulting in a higher overall reduction of the PQ pool in leaves.

This interpretation is in good agreement with the results of Kruk and co-workers who reported a large PQ reservoir that is not immediately implicated in photosynthesis and may be located in plastoglobules (Szymanska and Kruk; Kruk and Karpinski, 2006). Moreover, they proposed that this PQ reservoir plays a role as a lipid antioxidant in the thylakoid membrane. In such a scenario NDC1 may function in the regeneration of reduced PQ upon oxidation. This would require the diffusion of PQ between plastoglobules and the thylakoid membrane, the compartment where PQ would likely function as an anti-oxidant in addition to its role as an electron carrier in photosynthesis. The diffusion of lipid compounds between plastoglobules and the thylakoid membranes has been proposed earlier and physical connections between the outer lipid leaflet of the thylakoid membrane and the plastoglobule polar lipid monolayer were observed (Szymanska and Kruk; Austin et al., 2006a; Bréhélin et al., 2007b; Bréhélin and Kessler, 2008).

Using HPLC as well as UHPLC-QTOFMS, we observed that the PC-8 concentration was strongly diminished in *ndc1* mutants (Fig. 3*G*). PC-8 is derived from PQ by the activity of VTE1, the tocopherol cyclase, present in the plastoglobule (Mene-Saffrané et al.; Szymanska and Kruk; Zbierzak et al.). For the cyclase to close the chromanol ring in dimethylphytylquinone (DMPQ, the precursor of γ-tocopherol) or in PQ, the quinol groups must preferentially be present in the reduced form (Grütter et al., 2006). Thus, the reduction of PC-8 levels may reflect the redox state of the plastoglobule PQ reservoir which is more oxidized than in WT. Interestingly, α-tocopherol was present at WT levels in the *ndc1* mutant indicating that NDC1 is not required for its accumulation.

The untargeted lipidomics experiments led to unexpected discoveries (Fig. 4). The most prominent differences between WT and the *ndc1* mutants using UHPLC-MS were the almost complete absence of phylloquinone and the appearance of an additional peak corresponding in mass to the 2-phytyl-1,4-naphthoquinone, precursor of phylloquinone, in *ndc1* mutants. It is

known that the complete absence of phylloquinone in Arabidopsis causes a lethal albino phenotype (Shimada et al., 2005). However, in the *AtmenG* mutant (like in *ndc1*) its immediate precursor, 2-phytyl-1,4-naphthoquinone still accumulates (Lohmann et al., 2006a). Surprisingly, the *AtmenG* mutant has no visible phenotype and it is therefore plausible that the 2-phytyl-1,4-naphthoquinone precursor functionally replaces phylloquinone. It is unclear why phylloquinone does not accumulate in the *ndc1* mutant. In RT-PCR experiments, *AtMENG* was expressed at the same level in both *ndc1* mutants and WT suggesting that the enzyme is still present. However, AtMENG activity may be downregulated at the enzyme level, for instance by the PQ redox state of the plastoglobules. Another possibility would be that in the absence of NDC1, AtMENG is no longer correctly recruited to the site of its activity that may implicate plastoglobules.

There are some indications that plastoglobules play a role in phylloquinone metabolism. A fraction of the total phylloquinone pool is present in the plastoglobules. In the *AtmenG* mutant the 2-phytyl-1,4-naphthoquinone precursor of phylloquinone accumulated to a large extent in plastoglobules (Lohmann et al., 2006a). Both the multiactivity protein PHYLLO and AtMENG gave punctate fluorescence when transiently expressed as GFP-fusion proteins in Arabidopsis protoplasts suggesting a plastoglobule localization (Gross et al., 2006; Lohmann et al., 2006a).

In conclusion, NDC1 represents a new electron input device for PQ. Unlike *C. reinhardtii* NDA2 (Jans et al., 2008; Desplats et al., 2009), it does not seem to be directly implicated in cyclic/chlororespiratory electron flows in thylakoids, but it rather plays a role in electron transfer to the plastoglobule PQ pool – a role that appears to be essential for prenylquinone metabolism.

3.6 Materials and Methods

Plant materials and growth conditions. Arabidopsis plants were grown on soil (Ricoter) or in vitro on 0.8% Phyto Agar (Duchefa) containing 0.5x Murashige and Skoog (MS) medium (Duchefa) under short day conditions (8h light, 16h dark, 200 µmol m^{-2} s^{-1}). For high light experiments plants aged 4 weeks were acclimated for 7 days by increasing photon flux density to 750 µmol m^{-2} s^{-1}. Pea (*Pisum sativum*) seeds were germinated on soil after one night in water and grown in a chamber for 2 weeks under a long day conditions (16h light, 8h dark). *Nicotiana tabacum* cv petit Havana plants used for protoplast isolation were grown for 4-5 weeks on 0.5x MS medium under long day conditions.

Chlorophyll fluorescence and thermoluminescence. Chlorophyll fluorescence emission from attached leaves was measured with a PAM-2000 fluorometer (Walz), as previously described (Rumeau et al., 2005; Havaux et al., 2009). Thermoluminescence measurements were performed on leaf discs (1 cm in diameter) with a custom-made apparatus that has been described previously (Havaux et al., 2005b). The sample was cooled at 1°C for 40 s, then it was illuminated for 30 s with far-red light (>715 nm, 3 W m^{-2}). Immediately after interrupting far-red illumination, temperature was increased from 1°C to 70°C at a rate of 0.5°C s^{-1}, and luminescence was measured during heating with a photomultiplier tube.

Prenyllipid quantification. Tocopherols, PQ (reduced and oxidized) and PC-8 were extracted, separated and quantified by HPLC using the method described in (Szymanska and Kruk; Kruk and Karpinski, 2006). The HPLC column was a Phenomenex C18 reverse-phase column (Kinetex, 2.6 µm, 100 x 4.6 mm).

P700 redox changes. The redox state of P700, the reaction center of PS1, was measured in attached leaves using a dual-wavelength emitter/detector ED-P700DW-E (Walz) connected to a PAM-101 unit, as previously described (Ravenel et al., 1994). P700 was oxidized by illuminating the leaf with far-red light (>715 nm, 25 W m^{-2}), and the rate of P700 re-reduction was measured in the dark after switching off the far-red light.

Chloroplast fractionation was carried out as described in (14).

Western Blot analysis was carried out as described in (14)**.** Anti-NDC1 serum was used at 1/1000 dilution on 5% milk-TBS.

***In vivo* targeting.** Transient transformation of protoplasts was done using the polyethylene glycol method (Greenspan et al., 1985; Koop et al., 1996) with reduced cellulase (1%) and macerozyme (0.25%) (Serva). 20 µg of pEarlyGate101-NDC1-YFP were used to transform 500,000 tobacco protoplasts. Fluorescence was monitored 48 to 80 h after transformation using a Leica TCS SP5 confocal microscope). Nile Red was used at 10 µg/ml (Greenspan et al., 1985).

***In vitro* import into isolated mitochondria and chloroplasts.** The radioactive full-length NDC1 protein was produced in a coupled transcription–translation system (TNT® T7 Quick Coupled Transcription/Translation System, Promega) using pET21-NDC1-H6 vector according to the manufacturer's instructions. Import of [^{35}S] labelled fusion protein into

purified pea mitochondria was performed according to (Moore et al., 1993). Import into purified Arabidopsis chloroplasts was performed according to (Agne et al.). All experiments were resolved by SDS-PAGE and analyzed by phosphorimaging.

Production of anti-AtNDC1 antibody. Full-length NDC1 protein was purified under denaturing conditions by nickel nitrilotriacetic acid affinity chromatography (Qiagen) according to the manufacturer's recommendations. Polyclonal antibodies were produced in rabbit (Eurogentec) and were affinity-purified against recombinant AtNDC1 coupled to Affi-Gel 10 (Biorad) according to the supplier's recommendations.

NDC1 enzyme assay. Activity was measured in 1 ml of 50 mM Hepes buffer (+KOH pH 7.2) containing 200 µM NADH and decyl-prenylquinone concentrations, from 0.5 and 750 µM. The reaction was started by the addition of 18 µg of nickel nitrilotriacetic acid affinity purified mature NDC1-H_6 and NADPH absorbance decay was measured at 340 nm.

Extraction and LC/MS analysis. 6-week-old plants (n=6) were ground to a fine powder under liquid nitrogen. Samples (100 mg) were extracted in 500 µL of MeOH for 2 min in a beadmill (Retsch MM 200, Arlesheim, Switzerland), centrifuged (4 min, 14'000 rpm), and the supernatant was recovered. The untargeted analysis of lipids was carried out using an Acquity UPLC system from Waters (Milford, USA) coupled to a Synapt G2 MS QTOF from Waters equipped with an APCI (atmospheric pressure chemical ionization) source. The separation was performed at 60 °C on an Acquity BEH C18 column (50x2.1 mm, 1.7 µm) at a flow rate of 500 µl/min under the following conditions: A = water; B = MeOH; 80-100% B in 6 min,

holding at 100% B for 3 min followed by re-equilibration at 80% for 2 min. The QTOF was operated at a resolution of 20000 (full half width maximum) in MS positive and negative ion modes over an m/z range of 225-1200 in centroid mode. Scan time was 0.5 s. The corona current was set to 12 μA and the cone voltage to 40 V. The source temperature was maintained at 120 °C, the APCI probe at 370 °C and desolvation and cone gas flows were set to 800 l/hr, respectively 20 l/hr. Accurate mass measurements (< 2 ppm) were obtained by infusing a solution of leucin-enkephalin at 400 ng/ml at a flow rate of 10 μl/min through the Lock Spray™ probe (internal calibration). MS/MS product ion spectra were obtained for phylloquinone and its demethylated form using precursor ions at m/z 450.3 and 436.3 respectively. The collision energy was set to 25 eV and argon was used as collision gas at a flow rate of 2.1 ml/min.

Absolute quantification of phylloquinone and 2-phytyl-1,4-naphthoquinone was performed using calibration curves obtained from standard solutions of phylloquinone at 100ng/ml, 250 ng/ml, 1000 ng/ml, and 2500 ng/ml.

Data preprocessing and multivariate analysis. Peak picking and data processing including multivariate analysis were performed using Markerlynx XS software (Waters), enabling the generation of a list of variables characterized by their m/z, retention time and intensity. Peak areas of the extracted variables were normalized by dividing them by the sum of the intensities of all detected variables in each sample. Normalized variables were then Pareto scaled and analyzed by principal component analysis (PCA).

Real-Time reverse transcription-PCR. Total RNA was extracted from Col0 control plants and mutant lines using the RNeasy Mini Kit (Qiagen). 1 μg of total DNase treated RNA was

reverse transcribed with the M-MLV reverse transcriptase (Promega). Real-time PCR was performed using SYBR Green (Thermo Scientific) on an iCycler with ACT2 as a reference. Specific primers to respective genes were as follows: *ACT2* (forward, 5'-TGGAATCCACGAGACAACCTA-3' and reverse, 5'-TTCTGTGAACG ATTCCTGGAC-3'); *AtMENG* (forward, 5'- ATTCGATGCGGTTACGATGG-3' and reverse, 5'-ACTCCTTTGCAAGATCATAAAC-3'); *NDC1* (forward, 5'-AGCTTGATTGGTGAAATGCC-3' and reverse, 5'-CTGCGGTTATGCAGGAGTAG-3').

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Chapter IV.
Preparation of Plastoglobules from Arabidopsis Plastids for Proteomic Analysis and Other Studies

4. PREPARATION OF PLASTOGLOBULES FROM ARABIDOPSIS PLASTIDS FOR PROTEOMIC ANALYSIS AND OTHER STUDIES

Celine Besagni, Lucia Eugeni Piller, Claire Bréhélin

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General introduction

Chapter IV describes experimental procedures used during my research and especially, it details the isolation of pure and intact plastoglobules from *Arabidopsis thaliana* leaves. The purification of plastoglobules depends on two critical steps. At the beginning, intact chloroplasts are isolated by a Percoll gradient centrifugation. The second step allows the separation of plastid membranes by a discontinuous sucrose gradient. For that, chloroplasts are first broken and then the stroma is separated from total membranes by an ultacentrifugation step. The membrane pellet is resuspended in 45% sucrose and disrupted by a supplemental homogenization step. The plastid membrane suspension is then loaded on a discontinuous sucrose gradient ranging from 45% (at the bottom of the tube) to 5% (upper part of the tube). Overnight ultracentrifugation of the sucrose gradient allows the separation of chloroplast membrane fractions in function of component density. In fact, by flotation, plastoglobules are well separated from envelope membranes at the top of the gradient due to the low density of these lipid droplets. In addition, the procedure described achieves a good separation from plastoglobules that stay attached to thylakoids. The purity of plastid membrane distribution along the sucrose gradient was verified by Western blotting using antibodies specific to plastoglobules, mixed envelope or thylakoid membranes. Finally,

fractions can be used for proteomic analysis, *in vitro* localisation by Western blotting, enzymatic assays and other experimentation.

Preparation of plastoglobules from Arabidopsis plastids for proteomic analysis and other studies

Celine Besagni[1,3] , Lucia Eugeni Piller[1,3], Claire Bréhélin[2]

[1] Laboratoire de Physiologie Végétale, Université de Neuchâtel, Neuchâtel, Switzerland.

[2] Laboratoire de Biogenèse Membranaire, UMR5200 CNRS-Université de Bordeaux 2, Bordeaux, France.

[3] These authors contributed equally to the article.

4.1 Abstract

Plastoglobules are particles specifically located inside different types of plastids. They mainly contain lipids and proteins and are physically attached to thylakoids. Proteomic studies have underlined the role of plastoglobules in diverse plastid metabolic pathways, such as those producing vitamin K, vitamin E, and carotenoids, and have implicated them in plant response to stress. This chapter describes the isolation of pure and intact plastoglobules from Arabidopsis leaves. The procedure starts with the isolation of intact chloroplasts by centrifugation on Percoll gradient. Plastoglobules are then separated from the plastid membranes by flotation on sucrose gradient. Finally, the purity of the plastoglobule fraction is verified by immunoblotting.

Key words

Plastoglobules, Sucrose gradient, Chloroplast preparation, Arabidopsis, Immunoblot, Percoll gradient, Flotation, Protein precipitation, Plastid membranes.

4.2 Introduction

Plastoglobules are lipoprotein particles present in different plastid types such as proplastids, chloroplasts, chromoplasts, and gerontoplasts. Plastoglobules are mainly composed of isoprenoids, neutral lipids and proteins **(Bréhélin and Kessler, 2008)**. They are physically attached to thylakoids via the outer half of the lipid bilayer of the thylakoid, which surrounds plastoglobules **(Austin et al., 2006b)**. Long viewed as passive lipid droplets, plastoglobules have been receiving increasing attention in the last half decade. Indeed, proteomic studies of plastoglobules **(Vidi et al., 2006b; Ytterberg et al., 2006a)** have demonstrated their active role in plastid biology. This idea is reinforced by the fact that plastoglobule size and number vary depending on plastid type and environmental conditions. Currently, a growing body of evidence suggests that plastoglobules are involved in various metabolic pathways such as those leading to vitamin K **(Lohmann et al., 2006b)**, tocopherol **(Vidi et al., 2006b; Zbierzak et al., 2009)**, and carotenoids **(Ytterberg et al., 2006a)**, and in plant response to stress by accumulating antioxidants (e.g. tocopherols) and sequestrating toxic molecules (e.g. fatty acid phytyl esters) **(Gaude et al., 2007b)**. In addition, studies of the plastid ultrastructure suggested that plastoglobule size and number are regulated in correlation with the fitness of the thylakoid membranes, putatively playing the role of a reservoir for thylakoid membrane lipids **(Kessler et al., 1999; Bréhélin and Kessler, 2008)**.

Besides their physiological roles, plastoglobules may present a biotechnological interest for molecular farming by providing a hydrophobic environment necessary for the production of certain proteins, associated with a simple purification procedure **(Vidi et al., 2006b)**.

The identification of components (proteins or lipids) specifically localized in plastoglobules relies on preparation of a plastoglobule fraction exempt of any contamination. Such a contamination could originate either from light particles present in other compartments of the

cell (e.g. oil bodies), or from plastid membranes. Thus, the preparation of pure plastoglobules depends on two critical steps: (i) isolation of intact plastids on Percoll gradients and (ii) accurate separation of plastid membranes by flotation on sucrose gradient. The range of sucrose concentration (5 - 45 %) that is used for the second step allows the separation of plastoglobules from envelope membranes, which is generally not achieved with standard protocols designed for plastid membrane preparation. In addition, because plastoglobules are physically attached to thylakoids, the procedure must include a step to achieve a good separation of plastoglobules from thylakoids. Finally, the purity of the plastoglobule fraction is verified by immunoblotting, and plastoglobules are then available for proteomic analysis or other studies.

4.3 Materials

4.3.1 *Arabidopsis* Culture

1. Universal soil (Ricoter, Switzerland).
2. Culture trays (30 x 50 cm) with propagator lids.
3. Solbac biological control agent (Andermatt Biocontrol, Switzerland), dilute 1/400 (v/v) in tap water.
4. Phytotron: Percival AR-66L (CLF Plant Climatics GmbH, Germany), 8 hours of light per day, 120 µmol photons/ m / s, 20°C, 60 % humidity.

4.3.2 *Preparation of Intact Chloroplasts and Purification of Plastoglobules*

1. HB buffer: 450 mM sorbitol, 20 mM Tricine-KOH pH 8.4, 10 mM ethylenediaminetetraacetic acid (EDTA) (*see* **Note** [i]), 10 mM $NaHCO_3$, 1 mM $MnCl_2$, 5 mM Na-ascorbate, 0.05% (w/v) bovine serum albumin (BSA) fraction V, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (*see* **Note** [ii] **and Note** [iii]). Prepare freshly (*see* **Note** [iv]).
2. RB buffer (8x) stock: 2.4 M Sorbitol, 160 mM Tricine-KOH pH 7.6, 20 mM EDTA (*see* **Note** [i]), and 40 mM $MgCl_2$. Working buffer is prepared by diluting 100 mL of RB buffer (8x) in 700 mL of water. Store at -20°C (*see* **Note** [v]).
3. 40 % (v/v) and 85 % (v/v) Percoll solutions: mix 40 or 85 mL of Percoll (pH 8.5-9.5; Sigma; MO, USA) with 12.5 mL of RB buffer (8X) and adjust volume to 100 mL with deionised water. These solutions can be kept several months at -20°C.
4. 80% (v/v) acetone diluted in water. This can be stored for several weeks at room temperature (*see* **Note** [vi]).
5. TrE buffer: prepare 10x stock solution with 50 mM Tricine-KOH, pH 7.5, 2 mM EDTA, and 2 mM dithiothreitol (DTT) (*see* **Note** [vii]). For use, 100 mL of

TrE (10x) is diluted in 900 ml of water. Buffer can be stored at -20°C for several months.

6. Sucrose (0.6 M) dissolved sucrose in TrE buffer (1x). The solution can be stored at -20°C for several months.
7. 45, 38, 20, 15 and 5% (w/v) sucrose solutions: sucrose is dissolved with TrE buffer (10x) and autoclaved deionised water to obtain desired sucrose concentration in TrE (1X). The solutions can be stored at -20°C for several months.
8. Centrifuges: superspeed refrigerated centrifuge (e.g., Sorvall RC-5B, Thermo Scientific, MA, USA), with fixed-angle rotor (e.g., Sorvall SLA1500) and corresponding plastic 250 mL bottles (Nalgene, NY, USA), or with swinging-bucket rotor (e.g., Sorvall HB-6) and corresponding open polycarbonate 50 mL tubes (Nalgene); refrigerated bench-top centrifuge (e.g., Eppendorf 5810R), with swinging-bucket rotor (e.g., Eppendorf A4-62) and capped polypropylene 50 mL tubes (Falcon, BD biosciences, CA, USA); ultracentrifuge (e.g., Beckman L7, Beckman Coulter Inc., CA, USA), with swinging-bucket rotor (e.g., Beckman SW 28) and UltraClear^{TM} SW28 tubes (25 X 89 mm, Beckman).
9. Waring blender homogeniser.
10. Miracloth and cheesecloth.

11. 15- and 50- mL Potter-Elvehjem tissue grinder with Teflon pestle.
12. Spectrophotometer.

4.3.3 *Proteins Precipitation and SDS-Polyacrylamide Gel Electrophoresis*

1. Sample buffer (SB 4x) stock: 200 mM Tris-HCl pH 6.8, 400 mM DTT, 8 % (w/v) sodium dodecyl sulphate (SDS) , 0.4 % (w/v) bromophenol-blue, and 50 % (w/v) glycerol. 100 µL of SB (4x) should be diluted in 300 µl of water and then used to resuspend dried proteins.
2. Stock solutions for running gel: 40% (w/v) acryl/bisacrylamide (37.5/1), 2 M Tris-HCl, pH 8.8; 20 % SDS, N,N,N′,N′-tetramethylethylenediamine (TEMED), 10 % (w/v) ammonium persulfate (APS) prepared in water and stored frozen in single-use aliquots (200 µL) at -20ºC (*see* **Note** [viii]).
3. Stock solutions for stacking gel: 40% (w/v) acryl/bisacrylamide (37.5/1), 0.5 M Tris-HCl, pH 6.8, 20% (w/v) SDS, Temed, and 10% (w/v) APS.
4. Running buffer: prepare (5x stock solution): 100 mM Tris, 1 M glycine, pH 8.3, and 0.5% (w/v) SDS. Store at

room temperature. For use, dilute 100 mL of 5x stock with 900 mL of water.

5. SDS-PAGE Molecular Weight Standards, Broad Range (BioRad, CA, USA), or similar.
6. Microcentrifuge.
7. Speed Vacuum Concentrator 5301 (Eppendorf, Germany), or similar.
8. Electrophoresis system for SDS-PAGE (e.g., PerfectBlue Dual Gel System Twin ExWS with glass plate (20 X 10 cm), a 20-teeth comb, and two 0.8-mm spacers; PeqLab Biotechnologies GmbH, Germany).
9. Power supply (e.g., Power PAC 300, BioRad).

4.3.4 *Verification of Plastid Membrane Fractions Purity by Immunoblotting*

1. Nitrocellulose membrane (e.g., Protran R, 0.45 μm pore size, Whatman, UK).
2. Blotting paper (e.g., 3 MM Whatman).
3. Transfer tank (e.g., Trans-Blot TM Cell, BioRad).
4. Rocking platform (e.g., Stuart SSM3 3D gyratory rocker, Bibby Scientific Limited, UK).
5. Transfer buffer: 15 mM NaH_2PO_4, 0.05% (v/v) SDS, 20% (v/v) ethanol. Prepare freshly.
6. AmidoBlack staining solution: 45% (v/v) ethanol, 10% (v/v) glacial acetic acid, and 0.1% (w/v) AmidoBlack

10B (Merck, NJ, USA). Store at room temperature (*see* **Note** [ix]).

7. Destaining buffer: 40% (v/v) ethanol, 10% (v/v) acetic acid. Store at room temperature.
8. PBS buffer (10x) stock: 1.4 M NaCl, 27 mM KCl, 0.1 M Na_2HPO_4, and 20 mM KH_2PO_4. Dilute 100 mL with 900 mL water to obtain 1x PBS for use.
9. TBS buffer (10x) stock: 10 mM Tris-HCl, 0.15 M NaCl, pH 7.5. Dilute 100 ml with 900 ml water to obtain 1x TBS for use.
10. Blocking buffer: 5% (w/v) nonfat dry milk in 1x PBS or 1x TBS.
11. Primary antibodies: (1) the anti-AtPGL35 serum (Agrisera, Sweden), specific to plastoglobules, has been raised against the *Arabidopsis thaliana* plastoglobulin AtPGL35. Use it diluted 1:3'000 in PBS with 5% (w/v) nonfat dry milk; (2) anti-LHCB2 serum (Agrisera) is a marker of thylakoid membranes and recognizes chlorophyll a/b-binding proteins of the light harvesting antenna complex II (LHCII). Use it diluted 1:3'000 in PBS, 5% (w/v) nonfat dry milk; (3) anti-Toc75 serum (Agrisera) is specific to the plastid envelope membrane and has been raised against the 75 kDa component of the translocon at the outer envelope membrane of chloroplasts (TOC). Use it diluted 1:3'000 in TBS, 5%

(w/v) nonfat dry milk. Sera should be stored at -20°C; however, an aliquot of around 50 µL can be kept at 4°C for daily use.

12. Secondary antibody: horseradish peroxidase-coupled goat anti-rabbit IgG (Bio-Rad) diluted 1:3'000 in PBS or TBS with 5% (w/v) nonfat dry milk.
13. Enhanced chemiluminescent (ECL) reagent: 0.1 M Tris-HCl, pH 8.5, 0.2 mM *p*-coumaric acid (Fluka, Switzerland), 1.25 mM 3-aminophthalhydrazide (Luminol, Fluka) (*see* **Note** [x]).
14. Chemiluminescent detection in dark room: Hyperfilm ECL 18 X 24 cm X-ray film (GE Healthcare, NJ, USA) placed in an X-ray film cassette (e.g., REGO Gollwitzer GmbH, Germany) and developed in a tabletop processor (e.g., SRX-101A, Konica Minolta, Japan). Alternatively, the luminescent signal may be monitored using a luminescence imaging device (e.g., ChemiDock system and the QuantityOne software, both from Bio-Rad).
15. Stripping buffer: 62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 100 mM β-mercapto ethanol (*see* **Note** [xi]).

4.4 Methods

4.4.1 *Arabidopsis culture*

1. Sow *Arabidopsis thaliana* seeds on six trays filled with universal soil (*see* **Note** [xii]). The soil is first watered with Solbac solution to prevent the growth of insect larvae. Trays are covered with transparent plastic propagator lids (or cling film) to ensure appropriate humidity for germination.
2. After 2 days at 4°C, place the trays in a phytotron for 6-8 weeks. Lids are removed after 2 weeks to avoid algae and / or fungi development.

4.4.2 *Preparation of Intact Chloroplasts and Purification of Plastoglobules*

We describe here the procedure we routinely follow to obtain pure Arabidopsis plastoglobules. Each step of the protocol below should be performed at 4°C to preserve the integrity of the chloroplasts, and to avoid protein degradation. When the starting material, and consequently, the amount of isolated chloroplasts, are limiting, smaller sucrose gradients can be prepared in appropriate tubes by proportionally reducing the volume of each sucrose solutions, as exemplified in Vidi et al. (Vidi et al., 2007b).

1. Prior to harvest, place plants in the dark for 24-48 hours to avoid starch accumulation (*see* **Note** [xiii]).
2. Harvest Arabidopsis leaves with scissors or scalpel, weigh them in a beaker (*see* **Note** [xiv]), and then maintain them in chilled water for 30 min (*see* **Note** [xv]).
3. Prepare six Percoll gradients as follows. Start by pouring 15 mL of 40 % Percoll solution into a 50-mL open tube. Then, with a glass Pasteur pipette, carefully introduce 5 mL of 85 % Percoll solution below the 40 % Percoll layer. Keep the gradients at 4°C.
4. Using a Waring blender homogeniser, grind the leaves three times in 500 mL of cold HB buffer (5 s at high strength and then two times 3 s at low strength) (*see* **Note** [xvi]).
5. Filter the homogenate immediately through two layers of cheesecloth and one layer of Miracloth placed in a funnel on top of an Erlenmeyer flask. Gently squeeze the homogenate inside the cheesecloth to extract most of the liquid.
6. Divide the filtrate between three or four 250-mL bottles and the centrifuge for 10 min at 1'075 x g in an SLA1500 fixed-angle rotor.
7. Gently resuspend each pellet with 1-2 mL of 1x RB buffer (*see* **Note** [xvii]) and pool the crude chloloroplast extracts in a 50-mL Falcon tubes. If necessary, add

additional RB buffer to resuspend any residual pellet material, and at the end, rinse the tube with small amount of additional RB. Gently mix the suspension by inverting the tube and then load 2-3 mL of crude chloroplast extract onto each Percoll gradient.

8. Centrifuge the gradients for 10 min at 13'600 x g in an HB-6 swinging-bucket rotor (*see* **Note** [xviii]). At the end of the centrifugation, intact chloroplasts are located at the interface between the 85 and 40 % Percoll phases. The green ring situated in the upper part of the gradient corresponds to broken chloroplasts and should be eliminated.
9. Aspirate most of the 40% Percoll layer with a vacuum aspirator, delicately collect the band of intact chloroplasts with a plastic pipette (with large opening) and transfer to a 50-mL Falcon tube.
10. Distribute chloroplasts from all gradients among two 50-mL Falcon tubes and dilute with 10 volumes of RB buffer (1x). Gently invert the tube to mix.
11. Centrifuge the suspension 2 min at 2'600 x g in a swinging-bucket rotor (A4-62).
12. Carefully decant the supernatant as the pellet is very loose.
13. Resuspend the pellet in 5 ml of TrE buffer (1x) and quantify the chlorophyll (*see* **Note** [xix]).

14. Adjust the sample volume to 50 mL with TrE buffer (1x) to wash the chloroplasts, and then centrifuge for 10 min at 2'600 x g in a swinging-bucket rotor (A4-62).
15. Carefully decant the supernatant and then resuspend (*see* **Note** [xx]) the chloroplast pellet with 0.6 M sucrose/TrE to a concentration of 2-3 mg / mL of chlorophyll. Add a cocktail of antiproteases to preserve protein integrity.
16. Incubate on ice for 10 min and then freeze at -80°C for at least 1 h (*see* **Note** [xxi]).
17. Thaw the chloroplast suspension and dilute with 2 volumes of TrE buffer (1X).
18. Homogenize chloroplast suspension for at least 20 strokes in a 50-mL Potter homogenizer and then transfer the homogenate into UltraClear SW28 tubes (*see* **Note** [xxii]).
19. Carefully balance the tubes and then perform ultra-centrifugation at 100'000 x g in a swinging-bucket rotor for 1 h (*see* **Note** [xxiii]).
20. Remove the supernatant, which corresponds to the stroma, and resuspend the pellet of total membrane (which contains plastoglobules) into 45% sucrose/TrE to reach a concentration of 2-6 mg of chlorophyll per mL (typically, ~ 10-20 mL 45% sucrose/TrE solution will be required) (*see* **Note** [xxiv] **and Note** [xxv]).

21. Homogenize the resuspended membranes by 20 strokes in a 15-mL Potter homogenizer (*see* **Note** [xxvi]).
22. Pour 5 mL aliquots of the membranes homogenate into the required number of UltraClear SW28 tubes, and carefully overlay each aliquot with the sucrose/TrE solutions in the following order: 6 mL of 38% sucrose, 6 mL of 20%, 4 mL of 15% and finally 8 mL of 5% sucrose to the top of the tube (*see* **Note** [xxvii]).

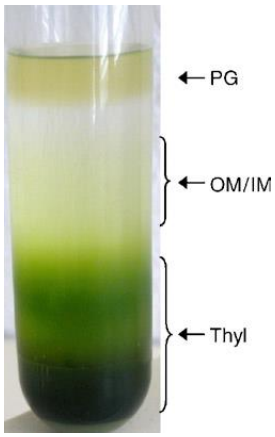

Fig. 1: Separation of total membranes from isolated Arabidopsis chloroplasts by flotation on a discontinuous sucrose gradient. Plastoglobules (PG) are visible as a yellowish layer at the top of the gradient. Outer and inner envelope membranes (OM/IM) are yellow; thylakoid membranes (Thyl) are green.

23. Carefully balance the tubes and ultracentrifuge them overnight at 100'000 x g (*see* **Notes** [xxviii] **and** [xxix]). An example of the gradient that it is obtained after the overnight centrifugation is given in **Fig. 1**.
24. For each gradient, collect 1-mL fractions with a micropipette, starting from the top of the gradient

(fraction 1) and ending at the bottom (approximately 32 fractions), and store them at -20°C. An illustration of the content of plastoglobules, envelopes and thylakoid membrane fractions observed with a confocal microscope is given **Fig. 2**. Typically, plastoglobules are contained in fractions 1-6, envelopes in fractions 14-18, and thylakoid membrane in fractions 25-32. However, the exact plastid membrane distribution varies from one experiment to another and has to be checked by immunoblotting as describe below.

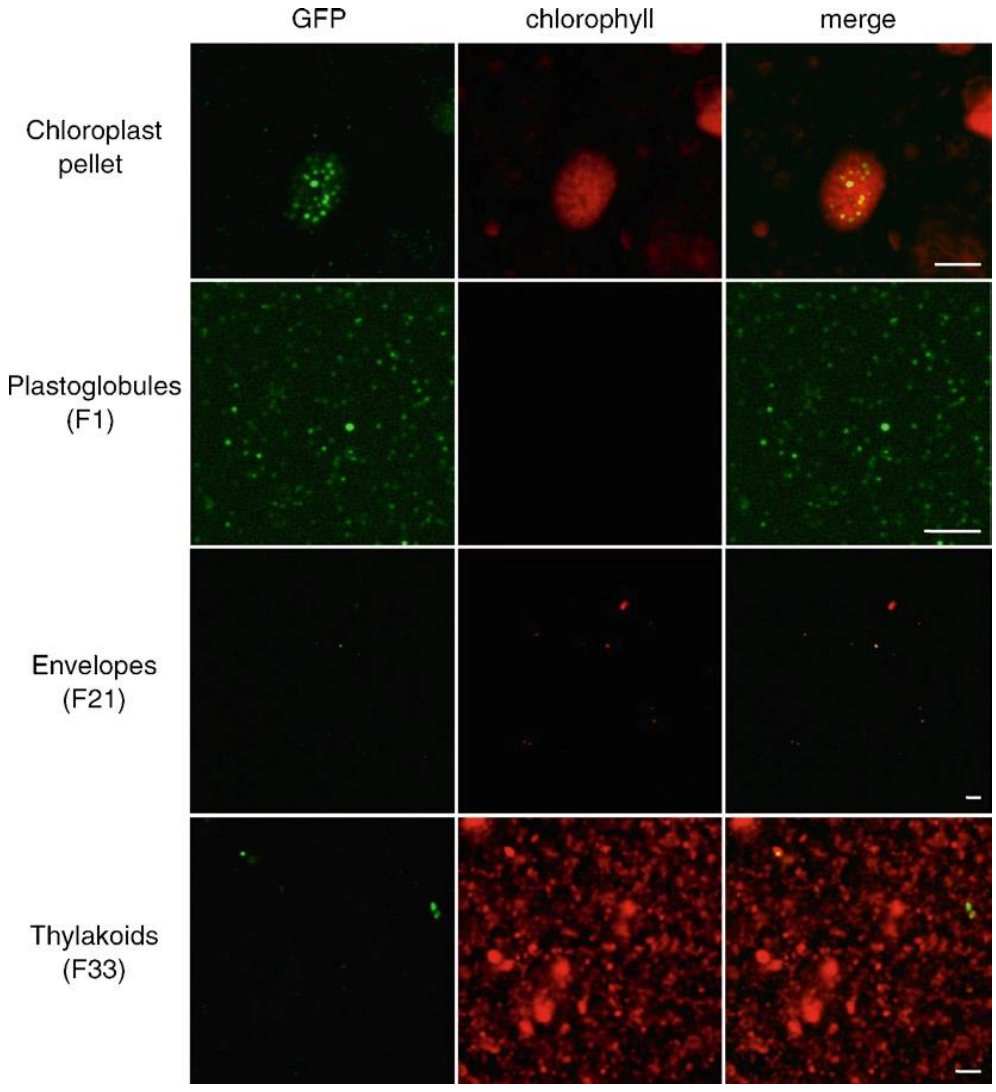

Fig. 2: Observation of the different plastid membrane fractions by confocal microscopy. Chloroplast membranes from Arabidopsis leaves expressing a plastoglobule marker (AtPGL35) fused to the green fluorescent protein (GFP) under the control of the constitutive 35S promoter were separated by flotation on a sucrose gradient. Fluorescence of fractions F1 (plastoglobules), F21 (envelopes), F33 (thylakoids) and of intact chloroplasts is visualised by confocal laser scanning microscopy. GFP and chlorophyll autofluorescence were monitored with a LEICA TCS SP5 microscope (LEICA Microsystems) using the 488 nm laser line and detection windows of 490–540 nm and 650-800 nm respectively. Bar scale: 5 µm.

4.4.3 *Proteins Precipitation and SDS-PAGE*

Here, we describe the procedure that we routinely follow to verify the plastid membrane distribution among the sucrose gradient fractions, and the purity of the plastoglobule fraction before subsequent analysis. However, the procedure needs to be modified depending on the goal of the experiment. For example, for mass spectrometry (MS) analysis, plastid membranes containing 25-30 mg of chlorophyll are loaded on one sucrose gradient and total proteins contained in 1 mL of the plastoglobule fraction are precipitated and separated on SDS-PAGE. Diverse staining methods (e.g., colloidal blue or silver staining) can then be used to visualize proteins before MS analysis.

4.4.3.1 Protein Precipitation

1. Different volumes of each even (or odd) fraction are transferred to microtubes for protein precipitation: 400 µL of fractions 1-18 (split in two tubes of 200 µL for

each fraction), and 200 µL of the upper fractions, stroma (St) and intact chloroplasts (*see* **Note** [xxx]).

2. To 200 µL of sample, add 160 µL of chloroform and 480 µL of methanol.
3. Vortex the tubes for 1 min and then add 640 µL of deionised H_2O.
4. Centrifuge the tubes for 1 min at maximum speed in a microcentrifuge.
5. Remove the upper phase, taking care not to disturb the protein band at the interphase (*see* **Note** [xxxi]).
6. Add 480 µL of methanol to the lower phase and vortex.
7. Centrifuge for 5 min at maximum speed in a microcentrifuge, remove the supernatant, and dry the protein pellet with a speed vacuum concentrator (around 10 min at 30°C) (*see* **Note** [xxxii]).
8. Resuspend the proteins in SB buffer: 20 µL each for fractions 1-18 (10 µL for both each tube of each fraction, which are then pooled together), 15 µL each for fractions 19-24, 30 µL for fractions 25-28 and stroma, and 60 µL for total chloroplasts and for each of the remaining fractions (*see* **Note** [xxxiii]).
9. Heat the protein samples for 10 min at 65°C. Proteins are now ready to be loaded on a SDS-PAGE gel.

4.4.3.2 Separation of Proteins by SDS-PAGE

The following steps describe the preparation and the use of a 12 % SDS-PAGE gel using the PerfectBlue Dual Gel Twin ExWS electrophoresis system (PeqLab).

1. Scrupulously clean with deionised water and technical ethanol the glass plates (20 x 10 cm) before assembling the electrophoresis system.
2. For one 12% acrylamide running gel, mix in a 50-mL Falcon tube: 5 mL of deionised water, 3 mL of 40% acryl/bisacrylamide (*see* **Note** [xxxiv]), 2 mL of 2 M Tris-HCl, pH 8.8, and 50 µL of 20% SDS. When everything is ready, add 5 µL of TEMED and 80 µL of 10 % APS (*see* **Note** [xxxv]) to the running gel solution, rapidly mix, and pour the gel leaving a space for the stacking gel (*see* **Note** [xxxvi]). Overlay with isopropanol. The gel should polymerize in less than 30 min at room temperature.
3. Remove the isopropanol with absorbing paper and prepare stacking gel by mixing in a 50-mL Falcon tube: 3.9 mL of water, 500 µL of 40% of acry/bisacrylamide, 600 µL of 0.5 M Tris-HCl, pH 6.8, and 25 µL of 20% of SDS. Finish by adding 5 µL of TEMED and 40 µL of 10% APS. Mix and pour stacking gel on the running gel until reaching the top of the plate and carefully insert the

comb. The stacking gel should polymerize within 20 minutes at room temperature.

4. Once polymerized, assemble the gel into the electrophoresis system and add the running buffer to the upper and lower chambers. Carefully remove the comb and wash the wells by expulsing running buffer into the wells with a micropipette.
5. Load different volumes of each sample in the wells: 20 µL for fractions 1-18, and 15 µL for the remaining samples. One well is loaded with 5 µL of the molecular weight marker.
6. Complete the assembly of the electrophoresis system and connect to a power supply. The gel can be run at 15 mA until the front migration penetrates the running gel and then at 30 mA until the dye blue front reaches the edge of the glass plates (around 45 minutes).

4.4.3.3 Verification of Plastoglobules Purity by Immunoblotting

1. Open a blotting transfer cassette in a tray filled with transfer buffer. Cut two pieces of 3 MM paper and a sheet of nitrocellulose membrane at the dimensions of the cassette. Soak the 3 MM papers, the nitrocellulose, and two sponges with transfer buffer.

2. Disassemble the SDS-PAGE electrophoresis system and remove the stacking part of the gel. Cut a small piece of the gel at one corner to allow future orientation of the gel. Soak the running gel in transfer buffer for 2-3 s.
3. Assemble the blotting "sandwich" in the cassette with soaked elements as follows. On the cathode side, avoiding any air bubble imprisonment, lay out the following: a sponge, a sheet of 3 MM paper, the running gel, the nitrocellulose membrane, a sheet of 3 MM paper, and a sponge (*see* **Note** [xxxvii]).
4. Close the cassette and introduce it into the transfer tank. It is crucial to pay attention to the orientation of the cassette in the tank with the gel on the cathode side and the membrane on the anode side. Fill the tank with transfer buffer. A magnetic stir bar is added in the tank and the tank is placed on top of a magnetic stirrer to ensure constant homogenization of the buffer. Transfer is performed at 4°C during 2 h at 250 mA (*see* **Note** [xxxviii]).
5. Disassemble the sandwich and stain the nitrocellulose membrane in a bath of AmidoBlack staining solution for approximately 10 min. Distain the membrane by three successive washes with distaining solution until a good contrast is obtained.

6. Rinse the membrane with deionised water and take a picture of it with a scanner or a camera. Mark the position of the standards with a pen.
7. To remove any trace of distaining solution, wash the membrane three times for 2 min each time on a rocking platform with PBS or TBS buffer, depending on the buffer to be used for the following steps.
8. Incubate the membrane in 20 mL of blocking buffer for 1h at room temperature on a rocking platform.
9. Incubate the membrane with the desired primary antibody at the adequate dilution for 2 h at room temperature or overnight at 4°C on a rocking platform.
10. Remove the primary antibody solution and wash the membrane three times for 10 min with PBS or TBS (*see* **Note** [xxxix]).
11. Incubate the membrane with secondary antibody solution for 30 min at room temperature on a rocking platform.
12. Wash the membrane in PBS or TBS three times 10 min each time on a rocking platform.
13. In a square Petri dish, mix 2 ml of ECL reagent and 6.6 µl of 3% H_2O_2 (*see* **Note** [xl]). Remove excess liquid from the membrane using absorbing paper and place it into the Petri dish. Incubate the membrane for 2 min with the ECL solution making sure the membrane is uniformly covered with the solution. Remove excess ECL solution

from the membrane using absorbing paper and place it into an X-ray film cassette. Cover the membrane with a clear plastic material such as wrapping film, and, once in a dark room, place a film on it. Expose the film for 30 s to 10 min. The X-ray film is developed in the dark room with a tabletop processor or by successive baths in standard developer and fixative solutions. Alternatively, the luminescent signal could be monitor using a luminescence imaging device. An example of the results that are obtained is given **Fig. 3**.

14. It may be necessary to remove the previous antibody before hybridizing a new one. For this, incubate the membrane 30 min in stripping buffer and then wash it with three successive baths of PBS or TBS for 10 min each. After stripping, the membrane must be blocked and the protocol restarts at step of Subheading 3.4.

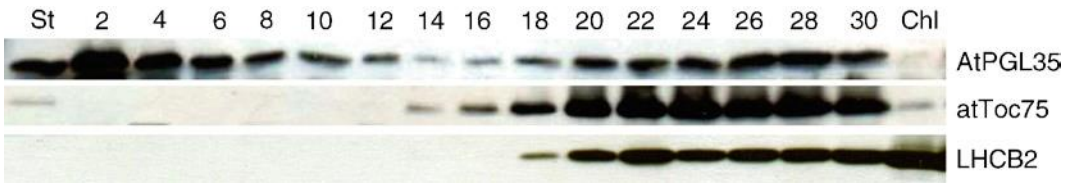

Fig. 3: Immunoblot analysis of chloroplast membrane fractions isolated from 6 week-old plants. Chloroplast membranes were separated by ultracentrifugation on a sucrose gradient. Fractions of 1 mL were collected from the top (fraction 1 in 5% sucrose) to the bottom (fraction 37 in 45% sucrose) of the sucrose gradient. Proteins from 400 µL of odd fractions 1-17, 200 µL of odd fractions 19-23, 100 µL of odd fractions 25, 27 and stroma (St), 50 µL of upper odd fractions and total chloroplasts (Chl)

were precipitated, separated by SDS/PAGE and transferred to a nitrocellulose membrane. The membrane is sequentially probed with antibodies raised against membrane marker proteins: AtTOC75 (outer envelope membrane protein), AtPGL35 (plastoglobule protein) and CAB (thylakoid protein).

4.5 Notes

i. EDTA is not soluble below pH 8, thus, NaOH should be added when preparing EDTA solution to help dissolution and after to reach the desired pH.

ii. HB buffer is an isotonic solution which will prevent osmotic shock and thus preserve intact chloroplasts. It is prepared with the following stock solutions that are autoclaved and can be stored several months at room temperature: 1 M Tricine-KOH, pH 8.4; 0.5 M EDTA; 0.5 M $NaHCO_3$; and 1 M $MnCl_2$.

iii. PMSF is a protease inhibitor. It is insoluble in water and must be dissolved at 0.2 M in solvent such as isopropanol. It is toxic: wear adequate protective clothes when handling stock solution.

iv. HB buffer can be prepared the day before and stored at 4°C. However, sodium ascorbate, BSA, and PMSF should be added just before use.

v. RB buffer is prepared with the following stock solutions that are autoclaved and can be stored several months at room temperature: 1 M Tricine-KOH, pH 7.6; 0.5 M EDTA, pH 8.5; 1M $MgCl_2$.

vi. Since acetone is highly volatile, the concentration of acetone in the bottle will decrease with time and the extraction of chlorophyll will then be less efficient. Prepare new 80% acetone solution periodically.

vii. TrE buffer can be prepared with the following stock solutions: 1M Tricine-KOH, pH 7.5; 0.5 M EDTA, pH 8; and 1M DTT (stored at -20°C).

viii. APS is unstable at 4°C. Thus, after 2-3 days at 4°C a new aliquot of APS should be open.

ix. AmidoBlack solution must be stored at room temperature in a bottle surrounded by aluminium paper to avoid light damages. It can be reused several times until loss of efficiency but it is recommended to filtrate it from time to time.

x. ECL solution is prepared in an opaque bottle to prevent light damages, and it may be conserved at 4°C for several weeks. 50 ml of this solution should be prepared by mixing 50 mL of water with the following stock aliquots stored at -20°C: 110 µL of 90 mM coumaric acid and 250 µL of 250 mM luminol.

xi. β-Mercaptoethanol is toxic. Work under the hood and wear adequate protective clothes.

xii. In order to ensure homogenous density, seeds are mixed with water in a saltshaker and then distributed on soil. Plant density is critical: high density is needed to obtain enough starting material; however, too high density will induce early flowering plants with very small leaves. For each 30 x 50 cm tray, an amount of Arabidopsis seeds corresponding to a volume of approximately 20 µL are used.

xiii. Starch accumulates as heavy granules in chloroplasts which might break chloroplasts during centrifugation. Therefore, it is critical to avoid starch formation before starting chloroplast preparation.

xiv. Leaves collected from six trays should weigh 150-300 g.

xv. Plants are immerged in chilled water to allow decantation of the soil that might have been collected with leaves.

xvi. Leaves are wrung to eliminate most of the tap water and then introduced in the homogeniser. Enough HB buffer is added to the leaves to allow good grinding. The total leaf amount is generally too important to be introduced all at the same time into the homogenizer; thus, only half or a third of the leaf volume is ground at a time. While the first-round homogenate is filtering, the next batch of leaves is ground with new HB buffer.

xvii. Resuspension of the pellet should be done as carefully as possible so as not to break the chloroplasts. Gently shake bottles on ice and use plastic Pasteur pipettes with large openings to transfer the solution into a new tube. The complete resuspension of the whole pellet is frequently not achieved with the first 1-2 mL of RB buffer. In such cases, add a supplemental 1-2 mL of RB buffer onto the remaining pellet and carefully scrape the pellet with a plastic Pasteur pipette to help its resuspension. Nevertheless, pay attention not to resuspend the pellet in an excessive total volume of RB, to allow loading the Percoll gradients with no more than 3 mL of crude extract. If necessary, prepare extra Percoll gradients.

xviii. Disconnect the break so as not to disturb the gradient during deceleration.

xix. Chlorophyll content is measured by diluting 5 µl of resuspended chloroplasts in 1 mL of 80 % acetone. Mix well by vortexing and spin for 2 min at maximum speed in microcentrifuge. Transfer the supernatant into a quartz cuvette (plastic cuvette can be used if the plastic is resistant to acetone) and measure the absorbance at 652 nm (A_{652}) against a blank of 80% acetone. Chlorophyll concentration is calculated as follows:

[chlorophyll] (mg/mL) = A_{652} x dilution factor / 36

xx. The pellet can be resuspended by vortexing, since at this step the integrity of chloroplast need not to be preserved.

xxi. The freezing and thawing cycle participates in the disruption of the chloroplasts.

xxii. Defrosting and mechanical grinding using a Potter homogenizer breaks the chloroplasts.

xxiii. Dilution of the chloroplast suspension followed by ultracentrifugation at 100'000 x g allows the separation of stroma from other chloroplast components (i.e., total chloroplast membranes, including plastoglobules). For protein analysis, an aliquot of the stroma fraction is taken from the supernatant and stored at -80°C.

xxiv. The volume of 45 % sucrose solution to be used for membrane resuspension is determined in order to (i) reach 2-6 mg / mL of chlorophyll concentration and (ii) allow the loading of 2-4 gradients (5 mL on each gradient). With this concentration, each gradient will be load with 10-30 mg of total membranes, which is the optimal chlorophyll amount needed for efficient plastoglobule preparation with a gradient of 30 mL.

xxv. At this step, the membrane solution can be stored at -80°C for later fractionation.

xxvi. Efficient homogenisation with 20 strokes of the Potter homogenizer is critical to detach plastoglobules from the thylakoid membranes. Possibly, the chloroplast suspension can be sonicated for 1-2 min to help dissociation of plastoglobules from the thylakoids.

xxvii. Use plastic Pasteur pipettes to gently load each sucrose solution on top of the preceding phase, taking care not to disturb the gradient.

xxviii. This ultracentrifugation allows the separation of the different plastid membranes (envelopes, thylakoids and plastoglobules) by flotation in the discontinuous sucrose gradient.

xxix. Disconnect the break so as not to disturb the gradient during deceleration.

xxx. The precipitated volume of the first fractions (1-18) is doubled because of the low protein abundance in these fractions.

xxxi. The proteins are present at the interface as a white band. However, in the first fractions, this white band is not visible due to low protein abundance. This does not prejudge the success of the experiment.

xxxii. The protein pellet can also be dried by leaving tubes open under a chemical hood for 1 h to overnight.

xxxiii. The abundance of proteins in the last fractions makes it difficult to resuspend the pellet. This is why a higher volume of SB is added to these fractions.

xxxiv. Non polymerized acrylamide is toxic; therefore, wear appropriated protective clothes.

xxxv. APS and TEMED catalyze the gel polymerization. High temperature results in faster polymerisation. It may be useful to pour gel in cold room during summer time.

xxxvi. The space needed for the stacking gel is approximately 1 cm below the comb.

xxxvii. To remove any air bubbles which may have formed between the different layers of the sandwich and which will interfere with the blotting process, use a plastic spatula and gently roll air bubbles out.

xxxviii. Alternatively, transfer could be done overnight at 100 mA, in the cold room.

xxxix. Primary antibody solution can be kept at -20°C and reused three to five times depending on the antibody.

xl. Once H_2O_2 is added to the ECL reagent, the stability of the solution is approximately 15 min. Thus the following steps need to be rapidly performed.

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CHAPTER V.
ROLE OF PLASTOGLOBULES IN METABOLITE REPAIR IN THE TOCOPHEROL REDOX CYCLE

5. ROLE OF PLASTOGLOBULES IN METABOLITE REPAIR IN THE TOCOPHEROL REDOX CYCLE

Lucia Eugeni Piller[1], Gaétan Glauser[2], Felix Kessler[1]* and Céline Besagni[1]

[1]Laboratoire de Physiologie Végétale, Institute of Biology, Université de Neuchâtel, Neuchâtel, Switzerland

[2]Chemical Analytical Service of the Swiss Plant Science Web, Neuchâtel Platform for Analytical Chemistry, Faculty of Sciences, Université de Neuchâtel, Neuchâtel, Switzerland

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5.1 General introduction

In Chapter V, I present the last paper of my thesis work, published in 2014 on « Frontiers in Plant Science », about the role of NDC1 in tocopherol redox cycle.

As mentioned before, to prevent damage of photosystems and thylakoid membranes, higher plants have developed various strategies. Among these adaptive responses, plants synthetize different lipid and water soluble antioxidants such as vitamin E and ascorbate. In PG, where storage and metabolism of tocopherol in part take place, two metabolic enzymes, VTE1 and NDC1 involved in prenylquinone pathways, and two kinases ABC1K3 and ABC1K1/PGR6 implicated in PC8 regulation, are located.

Tocopherol is converted into 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ) that is methylated by VTE3 to 2-3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ). VTE1 catalyzes the reaction leading the conversion of MPBQ and DMPBQ to δ- and γ- tocopherol, respectively. Then, another methyl transferase, VTE4, converts δ- and γ- tocopherol into β- and α-tocopherol.

Submitted to HL stress, the only tocopherol oxidation product currently known (α-tocopherol

quinol (α-TQH_2) is accumulated in plants. Interestingly, this compound has been reported to have specific roles such as dissipation of excess energy, protection of PSII against photoinhibition as well as strong antioxidant activity. Recent research demonstrated that the tocopherol redox cycle is a plastid-based mechanism.

In the present study, using a non-targeted lipidomics approach, we reported that NDC1 participates in the tocopherol recycling pathway. It was supposed that NDC1 reduces α-TQ to α-TQH_2 in a fashion analogous to the reduction of PQ to PQH_2 under HL stress. In fact, under HL, α-tocopherol is the compound that increases most among 500 others products analyzed in wild type plant. In these same conditions, *ndc1* mutant plants accumulate α-TQ. Unfortunately, our current methodology does not allow the detection of α-TQH_2 but I demonstrated earlier that NDC1 has a broad specificity for the reduction of quinolic substrates *in vitro*. We therefore hypothesize that NDC1 reduces α-TQ to α-TQH_2 prior to cyclization by VTE1 to give α-tocopherol. Another role played by NDC1 is plastoquinone reduction.

NDC1 overexpressing plants (35S:NDC1-YFP) showed a higher PQH_2/PQ ratio demonstrating the effect of this enzyme on the redox state of PQ reservoir. Moreover, the total PQ was increase in the overexpressing line. As shown in Chapter 3, the PQ pool present in PG is not directly implicated in photosynthesis. Thus, in 35S:NDC1-YFP plants, the increase of total PQ may be essential to preserve sufficient oxidized PQ to allow electron transport in the thylakoid membranes.

In summary, we showed that NDC1 is an important component of the tocopherol redox cycle, likely by reducing α-TQ to α-TQH_2. As this enzyme is located in PG and *ndc1* mutants had a higher concentration of α-TQ, PG appear to be a metabolic repair site in the tocopherol redox cycle.

ROLE OF PLASTOGLOBULES IN METABOLITE REPAIR IN THE TOCOPHEROL REDOX CYCLE

Lucia Eugeni Piller[1], Gaétan Glauser[2], Felix Kessler[1]* and Céline Besagni[1]

[1]Laboratoire de Physiologie Végétale, Institute of Biology, Université de Neuchâtel, Neuchâtel, Switzerland

[2]Chemical Analytical Service of the Swiss Plant Science Web, Neuchâtel Platform for Analytical Chemistry, Faculty of Sciences, Université de Neuchâtel, Neuchâtel, Switzerland

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5.2 Abstract

Plants are exposed to ever changing light environments and continuously forced to adapt. Excessive light intensity leads to the production of reactive oxygen species that can have deleterious effects on photosystems and thylakoid membranes. To limit damage, plants increase the production of membrane soluble antioxidants such as tocopherols. Here, untargeted lipidomics after high light treatments howed that among hundreds of lipid compounds alpha-tocopherol is the most strongly induced, underscoring its importance as an antioxidant. As part of the antioxidant mechanism, α-tocopherol undergoes a redox cycle involving oxidative opening of the chromanol ring. The only enzyme currently known to participate in the cycle is tocopherolcyclase (VTE1, At4g32770), that re-introduces the chromanol ring of α-tocopherol. By mutant analysis, we identified the NAD(P)H-dependent quinone oxidoreductase (NDC1,At5g08740) as a second enzyme implicated in this cycle. NDC1 presumably acts through the reduction of quinone intermediates preceding cyclization by VTE1. Exposure to highlight also triggered far-ranging changes in prenylquinone composition that we dissect herein using null mutants and lines overexpressing the VTE1 and

NDC1 enzymes.

Keywords: Arabidopsis, high light, lipidomics, NAD(P)H dehydrogenase C1, plastoglobule, prenylquinone, redox cycle

5.3 Introduction

In their natural environment, plants are exposed to many kinds of stress, such as heat, drought and high light (Suzuki et al., 2012) especially during summer or due to anthropogenic activities including herbicides, air pollutants and acid rain (Lichtenthaler, 1998). For plants growing under field conditions, occasional exposure to high or even excessive light intensities is normal but has the potential to damage the photosynthetic apparatus. Exposure to high light (HL) generates reactive oxygen species (ROS) in chloroplasts, principally singlet oxygen in PSII (Krieger- Liszkay, 2005) and superoxide in PSI (Asada, 1999). To protect membrane lipids from photooxidation and PSII from photoinhibition, higher plants have developed a variety of adaptive strategies. In addition to modifications of pigment composition (Lichtenthaler et al., 2007) and chloroplast ultrastructure (Austin et al., 2006; Bréhélin et al., 2007), plants synthetize various lipid and water soluble antioxidants such as tocopherol (vitamin E) and ascorbate, respectively (Delong and Steffen, 1997; Noctor and Foyer, 1998; Smirnoff, 2000; Smirnoff and Wheeler, 2000; Sattler et al., 2003; Havaux et al., 2005; Van Breusegem et al., 2008). Storage and metabolism of tocopherol but also phylloquinone (Vit K), plastoquinone (PQ) and its derivative plastochromanol (PC8) (Lohmann et al., 2006; Vidi et al., 2006; Szymanska and Kruk, 2010b; Zbierzak et al., 2010; Eugeni Piller et al., 2012) in part take place at chloroplast lipid droplets (plastoglobules; PG) implicating them in light stress responses. PG are attached to the thylakoid membrane by the shared outer lipid leaflet. This arrangement leads to a conduit that may allow the diffusion of lipid molecules between the two compartments (Austin et al., 2006). At least, two metabolic enzymes involved in

prenylquinone pathways are located at PG: the tocopherol cyclase VTE1 and NDC1 (Vidi et al., 2006; Ytterberg et al., 2006; Eugeni Piller et al., 2011; Lundquist et al., 2012). In addition, two kinases ABC1K3 and ABC1K1/PGR6 have been implicated in the regulation of PC8 production as well as α-tocopherol over accumulation under HL. They may function via phosphorylation of VTE1 (Lundquist et al., 2012; Martinis et al., 2013, 2014). Under HL stress, the synthesis of tocopherol is enhanced suggesting that this molecule exerts an essential role as lipid antioxidant (Munné-Bosch, 2005; Eugeni Piller et al., 2012). It has been shown that tocopherol is important in the maintenance of PSII function (Porfirova et al., 2002; Havaux et al., 2005). A large proportion of total plastid tocopherol is accumulated in the PG core which enlarges during oxidative stress (Vidi et al., 2006; Bréhélin et al., 2007). The tocopherol head group is derived from homogentisic acid that is converted to 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ) by the activity of homogentisate phytyltransferase VTE2 (Figure 1A) (CollakovaandDellapenna,2001). Then MPBQ is methylated by VTE3 to form2-3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ) (Shintani et al., 2002; Cheng et al., 2003). VTE1 introduces the chromanol ring in MPBQ and DMPBQ leading to δ- and γ-tocopherols, respectively (Porfirova et al., 2002; Sattler et al., 2003). The last methylation step of tocopherol biosynthesis is catalyzed by VTE4 converting the δ- and γ-tocopherol into β- and α-tocopherol, respectively (Shintani and Dellapenna, 1998; Cheng et al., 2003). It has been demonstrated that mutations in Arabidopsis affecting steps of the tocopherol pathway (*vte1* and *vte4* mutants), strongly reduces the tolerance of photosynthetic organisms to HL stress (Maeda et al., 2005; Dellapenna and Pogson, 2006).

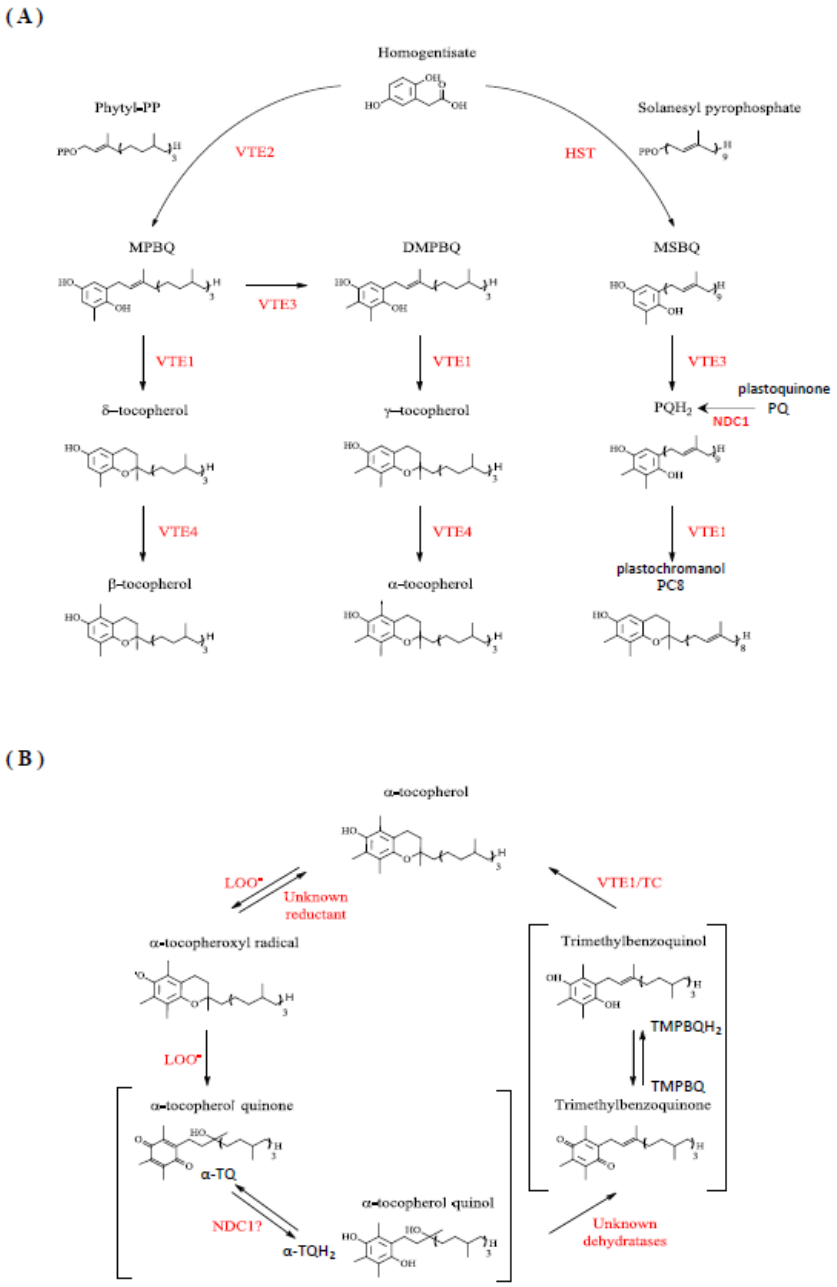


FIGURE 1. Tocopherols and tocochromanol biosynthesis and α-tocopherol biosynthesis oxidation pathway in Arabidopsis. (A) Summary of tocopherol, plastoquinol and plastochromanol pathways in Arabidospis. The enzyme abbreviations were shown in red. HST, homogentisic acid solanesyl transferase; VTE, vitamin E synthetis; -PP, pyrophosphate; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; DMPBQ, 2-3-dimethyl,6-phytyl-1,4-benzoquinone; MSBQ, 2-methyl-6-solanesyl-1,4-benzoquinol; PQH_2 plastoquinol; PC8, plastochromanol. Adapted from Dellapenna and Kobayashi (2008), Eugeni Piller et al., 2012. **(B)** α-tocopherol recycling pathway in plants. The enzyme abbreviations were shown in red. LOO-, lipid peroxy radical; TC, tocopherol cyclase. Adapted from Dellapenna and Kobayashi (2008), Mene-Saffrané and Dellapenna (2010), Eugeni Piller et al. (2012).

In response to HL stress, plants accumulate tocopherol oxidation products. In contrast to animal membranes, only one such product has been reported to accumulate in plants, namely α-tocopherolquinol (α-TQH2) (Figure 1B) (Dellapenna and Kobayashi, 2008; Mene-Saffrané and Dellapenna, 2010). A part from being a product of tocopherol oxidation, several functions for α-TQH2 have been proposed: dissipation of excess energy, protection of PSII against photoinhibition (Kruk et al., 2000, 2003; Munné-Bosch, 2005) as well as a strong antioxidant activity (Kruk and Trebst, 2008; Nowicka and Kruk, 2010). Recent studies demonstrate the existence of a plastid-based mechanism for a tocopherol redox cycle (Kobayashi and Dellapenna, 2008; Mene-Saffrané and Dellapenna, 2010; Eugeni Piller et al., 2012). In the first step of this recycling pathway, an α-tocopherol radical is formed by α-tocopherol oxidation via a lipid peroxyradical (LOO−). This compound is then oxidized by a second lipid peroxy radical to form α-tocopherol quinone (α-TQ) that is successively reduced to give α-TQH_2. A yet unidentified plastid-dehydratase activity converts α-TQH_2 to trimethylbenzoquinone (TMPBQ) (or $TMPBQH_2$), which is then cyclized by VTE1 leading to the regeneration of α-tocopherol and completion of the cycle. In the present study, we used ultra-high pressure liquid chromatography-mass spectrometry to analyze the composition of prenylquinones that play a fundamental role in light stress response in a variety of genetic backgrounds. We report that the PG-localized NAD(P)H-dependent quinone oxidoreductase NDC1 participates in the tocopherol redox cycle. NDC1 most likely functions by reducing α-TQ to α-TQH_2. This hypothesis is supported by the implication of NDC1 in the analogous reduction of PQ to PQH_2 in PG under HL stress (Eugeni Piller et al., 2011).

5.4 MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis thaliana wild-type plants (WT) refers to var Columbia-2 (Col2). In this work, the ndc1 mutant always corresponds to the T-DNA insertion line SALK_024063 from the Nottingham Arabidopsis Stock Center (http://arabidopsis.info; Alonso et al., 2003). The mutant line vte1, obtained by EMS mutagenesis (Porfirova et al., 2002), and the overexpressing 35S:VTE1-YFP plants (Kanwischer et al., 2005) area gift from Dr. P. Dörmann (Max Planck Institute, Golm, Germany). The 35S:NDC1-YFP plants were obtained as described below. Plants were grown on soil (Jiffy) under moderate light conditions (150 µmol m^{-2} s^{-1}, 22°C, 8/16h light/dark period) in a controlled environment room. For HL stress, 5 weeks old plants were exposed to 500 µmol m^{-2} s^{-1} (25°C, 8/16 h light/dark period).

OVEREXPRESSION OF NDC1 IN *A. THALIANA* LEAVES

Plants overexpressing NDC1-YFP under the 35S promoter were obtained using the Gateway recombination technology (Invitrogen). The NDC1 coding sequence was introduced into a donor vector pDONR™221, and subsequently transferred into an appropriate destination vector, the pEarlyGate101-YFP binary vector, resulting in pEarlyGate101-NDC1-YFP was transferred into Arabidopsis WT plants using the floral dip method (Clough and Bent, 1998). Transformed plants were selected for BASTA resistance and confirmed by segregation analysis.

WESTERN BLOT ANALYSIS

Total protein was isolated from Arabidopsis leaves according to Rensink et al. (1998) and concentrated by chloroform-methanol precipitation (Wessel and Flugge, 1984). Twenty µg of protein were separated by SDS-PAGE and blotted onto nitrocellulose membrane for

immunodetection. Immunodetection was carried out using anti-NDC1 serum at 1/1000dilution in 5% fat free milk powder /TBS (Eugeni Piller et al., 2011).

CONFOCAL MICROSCOPY

Protoplasts were released from plants overexpressing NDC1-YFP by overnight digestion with macerozyme (0.25%, Serva) and reduced cellulose (1%, Serva) in a solution containing 400mM Mannitol, 5mM MES and 8mM CaCl2. Protoplasts were filtered and loaded on a sucrose gradient (21 and 42%) and centrifuged for 10 min at 50×g. Intact protoplasts were resuspended and fluorescence was monitored with a Leica TCS SP5 confocal microscope using the appropriate parameters for YFP (514-nm laser lines, 520–588-nm detection windows).

PRENYLLIPID EXTRACTION FROM WHOLE PLANTS OR PG FRACTIONS AND LIPIDOMICS PROFILING

Prenylquinones were extracted from whole plants using an established method (Martinis et al., 2011). Leaves were ground in liquid nitrogen in a mortar. 100mg were re-suspended in 500 µl of tetrahydrofuran (THF, analytical grade, Normapur). Glass beads (1mm) were added and samples homogenized at 30Hz, 3min (Retsch MM300). After centrifugation, 200µl were transferred to a suitable HPLC vial. To measure prenylquinones contained in PG, intact chloroplasts were isolated by centrifugation on a Percoll gradient. Subsequently, PG were separated from thylakoid membranes by flotation on a sucrose gradient as described in Besagni et al. (2011). Four hundred µl of PG and thylakoid fractions were added to 600 µl of water and extracted three times with an equal volume of ethylacetate. Organic phases were pooled, evaporated and pellets were dissolved in 100µl of THF/water (85/15v/v) and the solution transferred to an appropriate HPLC vial (Kessler and Glauser, 2014). The quantification of prenyllipids was performed using reverse-phase ultra-high pressure liquid

chromatography (Acquity UPLC™, Waters) coupled to quadrupole-time-of-flight mass spectrometry (SynaptG2, Waters) (UHPLC-QTOFMS). Absolute concentrations of α-tocopherols, α-tocopherolquinone, plastochromanol and plastoquinone were calculated based on calibration curves obtained from pure standards. The method was also used for untargeted lipid profiling (Eugeni Piller et al., 2011; Martinis et al., 2011; Kessler and Glauser, 2014).

DATA PRE-PROCESSING AND STATISTICAL ANALYSIS

For comparison of metabolic profiles, raw spectrometric data were processed using MarkerlynxXS™ (Waters) which performs automatic peak detection and deconvolution in each chromatogram. The parameters were as follows: initial and final retention times 0.5–3.0 min, mass range m/z 300–1200 Da, mass tolerance 0.02 Da, retention time window 0.10 min, automatic peak with detection, automatic measurement of peak-to-peak baseline noise, intensity threshold 400 counts, no smoothing, noise elimination level disabled, deisotope filtering function applied. Peak areas for individual variables were normalized to the total integrated area per sample. Variables were Pareto-scaled before applying principal component analysis (PCA).

STATISTICAL TESTS

Multivariate statistical analysis of lipid profiles was performed using EZinfo (Umetrics). Univariate Analyses were performed using the software SigmaPlot version 12.0. Data were first analyzed using Shapiro-Wilk to determine whether data were normally distributed. When data passed the test, the Student's test (t-test) was applied to evaluate statistically significant difference between values ($p < 0.05$). For non-normally distributed data, the Mann-Whitney *U*-test was used.

RESULTS CHARACTERIZATION OF NDC1 OVEREXPRESSING PLANTS To improve our understanding of prenylquinone biosynthesis and metabolic regulation, we used Arabidopsis wild type (WT) plants, *vte1* and *ndc1* mutants as well as 35S:VTE1-YFP and 35S:NDC1-YFP overexpressing lines exposed to either moderate light conditions or HL stress in lipidomics studies. With the exception of 35S:NDC1-YFP the different lines were characterized previously, including two independent homozygous *ndc1* mutant lines SALK_024063 and GABI_614F03 (Eugeni Piller et al., 2011) that showed no differences in phenotype or prenylquinone composition. The transgenic plants overexpressing NDC1 fused to yellow fluorescent protein (YFP) under the 35S promoter (35S:NDC1-YFP) were engineered for this study. The expression of NDC1-YFP was verified by Western blotting using an antibody against NDC1 (Figure 2A). 35S:NDC1-YFP plants but neither wildtype nor *ndc1* gave a band at around 80kDa corresponding to the predicted mass of the fusion protein. Endogenous NDC1 protein (57 kDa) was not detected in WT and overexpressing plants due to the low amount of total protein (20 μg) loaded (100 μg are necessary; Eugeni Piller et al., 2011). The 35S:NDC1-YFP plants had no apparent phenotype. Protoplasts isolated from 35S:NDC1-YFP plants were analyzed by confocal microscopy and gave punctate fluorescence inside the chloroplasts which is in agreement with the PG localization of NDC1 (Vidi et al., 2006; Ytterberg et al., 2006; Eugeni Piller et al., 2011; Lundquist et al., 2012) (Figure 2B). Moreover, the 35S:NDC1-YFP construct was previously tested by transient expression in Arabidopsis WT protoplasts in the presence of the neutral lipid dye Nile Red which stains PG. The NDC1-YFP and Nile Red signals colocalized by confocal microscopy (Eugeni Piller et al., 2011).

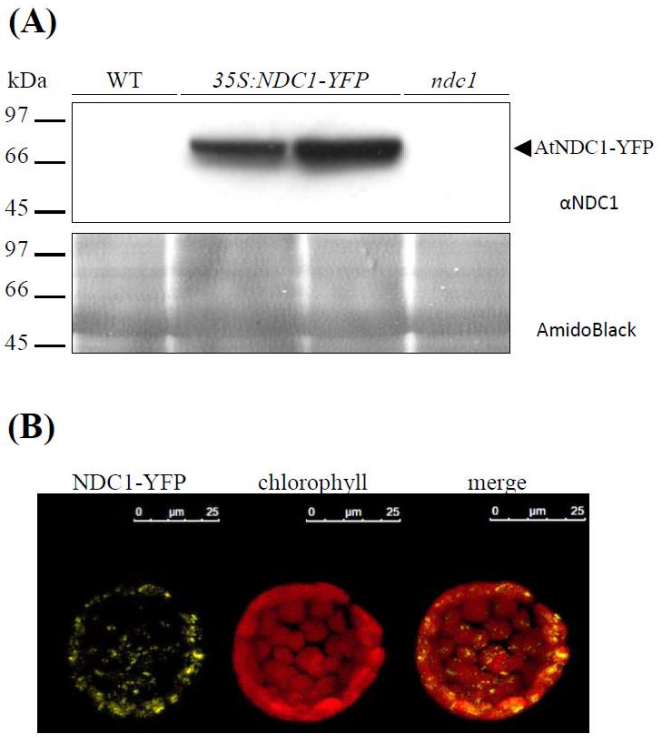


FIGURE 2. Characterization of NDC1 overexpressing plants. (A) Immunoblot with anti-NDC1. Twenty micrograms of total protein extract of WT, 35S:NDC1-YFP (two independent lines) and *ndc1* plants were separated by SDS-PAGE and transferred to nitrocellulose; A band corresponding to the NDC1-YFP fusion protein was detected using anti-NDC1 antibodies. The notrocellulose membrane stained by AmidoBlack is shown to control loadings. **(B)** Protoplasts isolated from Arabidopsis overexpressing 35S:NDC1-YFP were analyzed by confocal laser microscopy (left hand panel). Autofluorescence of chlorophyll in red identifies chloroplasts (center panel). The merge of NDC1-YFP and chlorophyll fluorescence is shown in the (right panel).

NDC1 AND VTE1, TWO PLASTOGLOBULE ENZYMES INVOLVED IN PRENYLQUINONE METABOLISM

To understand the dynamics of prenylquinone synthesis under changing light conditions, we first analyzed the global lipid composition in *ndc1*, *vte1*, 35S:NDC1-YFP, 35S:VTE1-YFP, and WT genetic backgrounds. The data obtained, using the UHPLC-QTOFMS-based method, were subjected to multivariate analysis. Using this method more than 500 different

compounds were detected, not all of which could be identified (Supplementary Table1). To investigate the difference in lipid contents, a principal component analysis (PCA) model was established from the datasets. PCA identifies and ranks major sources of variance and allows clustering of samples based on similarities and differences in the measured parameters. Under moderate light conditions, PCA showed the separation of five distinct groups characteristic for each of the genotypes tested in triplicate (Figure 3A). PCA loadings revealed that prenylquinones mostly contributed to the separation of these groups (Figure 3B). Most of the other lipids extracted were near the origin, suggesting that their contribution to metabolic difference was negligible. As expected, *vte1* plants accumulated DMPBQ lacking the chromanol ring of tocopherols. In the wildtype loadings, α-tocopherol appeared instead of DMPBQ. The35S:VTE1-YFP plants accumulated additional VTE1 products: PC8 and γ-tocopherol. The separation of *ndc1* from the other genotypes was mainly based on the presence of the 2-phytyl-1,4-naphthoquinone, the dimethyl precursor of phylloquinone (Eugeni Piller et al., 2011). Interestingly, an accumulation of PQ was also observed in *ndc1*.

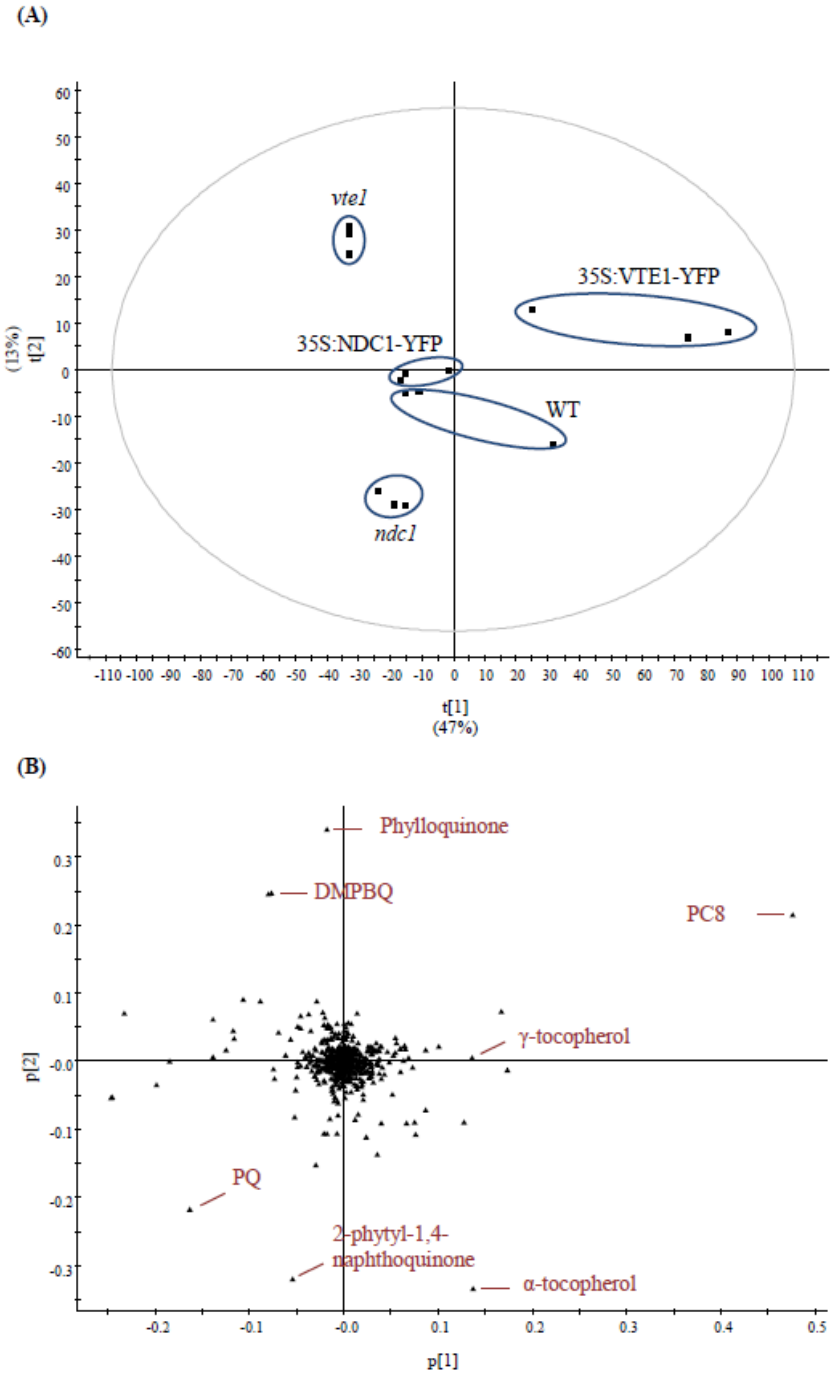


Figure 3: Untargeted lipidomics showing differences in lipid profiles between five different genotypes under moderate light conditions. (A) Principal component analysis. **(B)** Corresponding loadings plot. Lipids were extracted from WT, *ndc1*, *vte1*, 35S:NDC1-YFP and 35S:VTE1-YFP plants. Data are means of three experiments (n=3).

The PCA score plot indicated similar prenylquinone-lipid compositions for a representative 35S:NDC1-YFP line and the WT under moderate light conditions. This finding together with the absence of a visible phenotype suggests that the insertion of the 35S:NDC1-YFP encoding T-DNA construct was without positional effects. To investigate the implication of VTE1 and NDC1 plants in prenylquinone metabolism under HL stress, we compared the lipid profiles of wildtype plants with mutants after 4 and 8 days of HL exposure (Figure 4). Whereas *vte1* was blocked at the DMPBQ stage, WT plants strongly accumulated antioxidant lipids: γ-, α-tocopherol, PQ, PQH_2, and PC8 (Figures 4A, B). *ndc1* differed from WT by the presence of the phylloquinone precursor and PQ but interestingly also by the accumulation of α-TQ (Figures 4C, D).

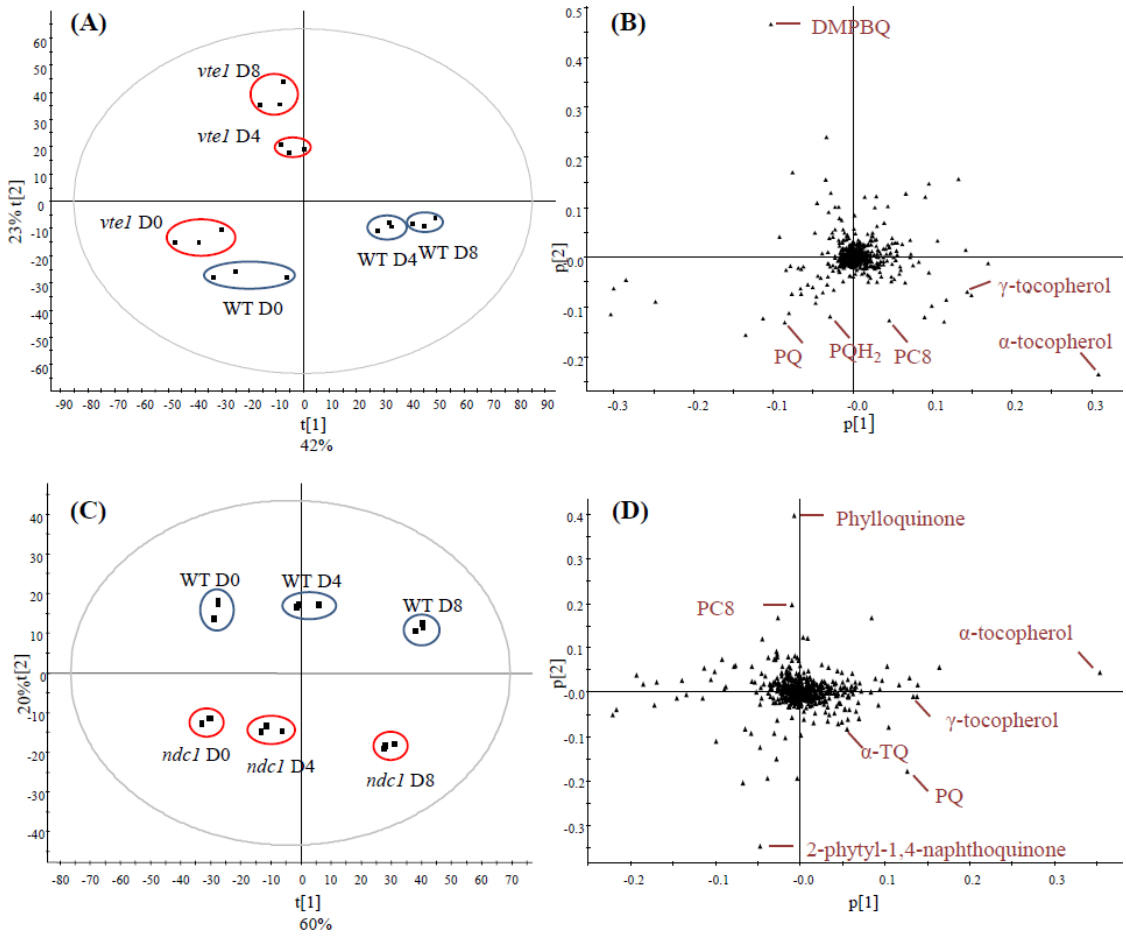


Figure 4: Comparison of the lipid profile between WT and *vte1* or *ndc1* mutant plants and after HL stress. (A) Principal component analysis showing difference between WT and *vte1* and **(B)**

corresponding loadings plot. **(C)** Principal component analysis showing difference between WT and *ndc1* and **(D)** corresponding loadings plot. Plants grown under moderate light conditions (D0) were exposed to continuous HL (500µE m^{-2} s^{-1}) for 4 (D4) and 8 days (D8). Data are means of three experiments (n=3).

α-TOCOPHEROL QUINONE, AN INTERMEDIATE OF THE TOCOPHEROL REDOX CYCLE ACCUMULATES UNDER HL STRESS IN *ndc1*

To assess the quantitative impact of NDC1 and VTE1 on prenylquinones after HL stress, we quantified, using pure standards, the principal compounds that were distinguished by PCA: α-tocopherol and α-TQ. As expected under HL conditions, the levels of α-tocopherol (Figure 5A) and oxidized α-TQ (Figure 5B) increased in WT, about 3 (Student's t-test $p_{D0\text{-}D8} = 0.0014$) to 6-fold ($p_{D0\text{-}D8} = 0.009$) respectively after 8 days. The level of α-tocopherol increased about 3-fold in *ndc1*, 35S:NDC1-YFP and 35S:VTE1-YFP after 8 days of HL, in the same manner as in WT (P_{D8}WT-*ndc1* = 0.7, WT-35S:NDC1-YFP = 0.063, WT-35S:VTE1-YFP = 0.105). With regard to the concentration of oxidized α-TQ, pronounced differences were observed between WT and mutant plants. In WT, the concentration of α-TQ increased under HL stress. However, *ndc1* accumulated at least 3 times as much α-TQ as WT under moderate and HL conditions. The finding for α-TQ was confirmed for two *ndc1* T-DNA insertion alleles (Supplementary Figure 1). In contrast, in 35S:NDC1-YFP and 35S:VTE1-YFP the level of α-TQ remained unchanged after HL stress and was about 8 times lower than in the WT at D8.

NDC1 IS IMPLICATED IN THE REGENERATION OF REDUCED PQ AND THE FORMATION OF PC8

The concentrations of total PQ including the proportion of the oxidized and reduced forms (Figure 6A) and of PC8 (Figures 6B, C) were measured. In WT plants, the concentration of total PQ was unchanged after 8 days of HL ($p_{D0\text{-}D8} = 0.471$) while a slight decrease of PC8 was observed ($p_{D0\text{-}D8} = 0.013$). In 35S:VTE1-YFP, the concentration of total PQ was generally

lower than in the WT and this difference increased under HL (p_{D0} = 0.068, p_{D4} = 0.03, p_{D8} = 0.012). Concomitantly, PC8 concentration in 35S:VTE1-YFP was 10 times higher than WT under moderate light conditions and 27 times higher after 8 days of HL. As expected, PC8 was not at all detectable in *vte1* (data not shown). *ndc1* and 35S:NDC1-YFP, accumulated the highest concentrations of total PQ under HL. In *ndc1*, the concentration of oxidized PQ was significantly higher than in the WT after 8 days of HL (PQox pD8*ndc1*-WT = 0.0078). In contrast, in 35S:NDC1-YFP, the accumulation of the reduced form PQH_2 made the difference (PQred p_{D8}35S:NDC1-YFP − WT = $8.50e^{-5}$). As expected, the concentration of PC8 detected in *ndc1* mutant plants was significantly lower than in the WT, but no difference was observed between WT and 35S:NDC1-YFP (p_{D8}WT-35S:NDC1-YFP = 0.1).

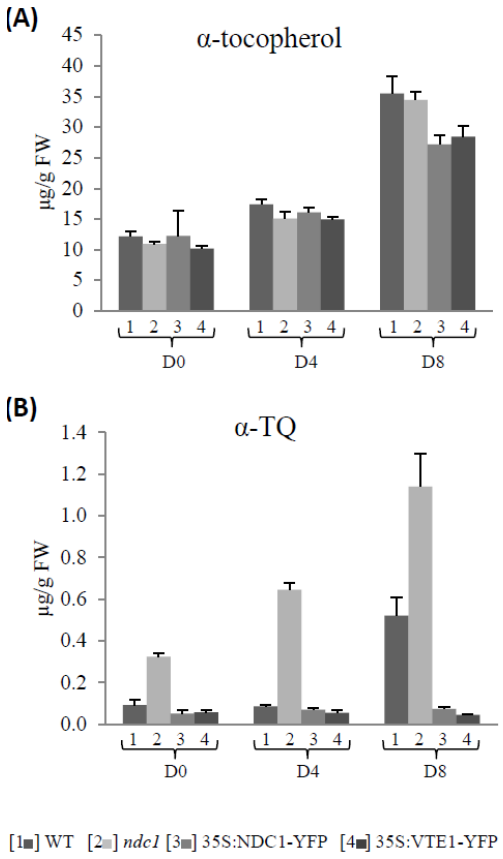


Figure 5: α-tocopherol and α-tocopherol quinone quantification in leaf and after HL treatment. **(A)** α-tocopherol. **(B)** α-tocopherol quinone. Lipids were extracted from [1] WT, [2] *ndc1*, [3] 35S:NDC1-YFP and [4] 35S:VTE1-YFP plants and quantified using purified standards. Plants grown under moderate light conditions (D0) were exposed to continuous HL (500µE m-2 s-1) for 4 (D4) and 8 days (D8). Data are means of three experiments (n=3).

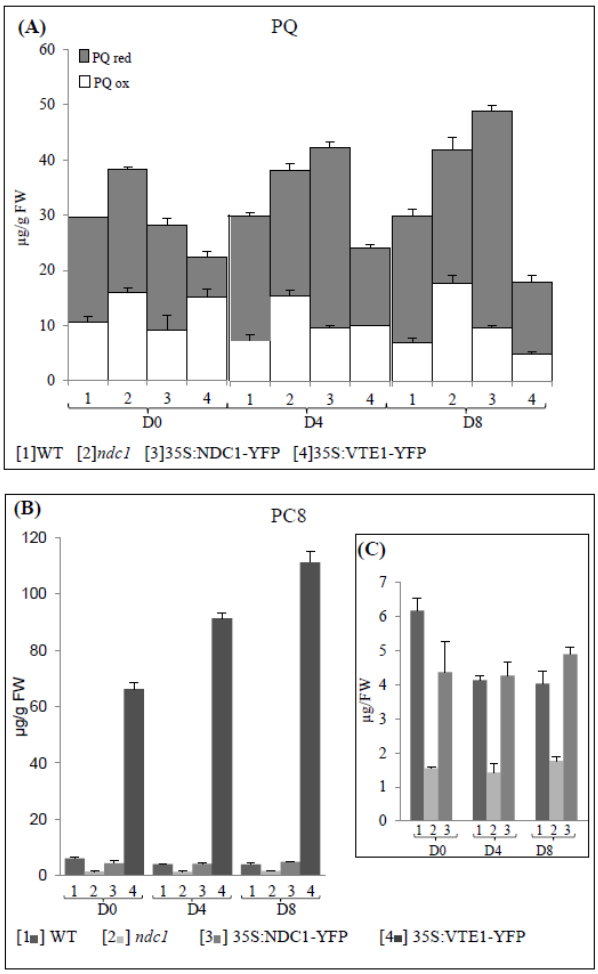
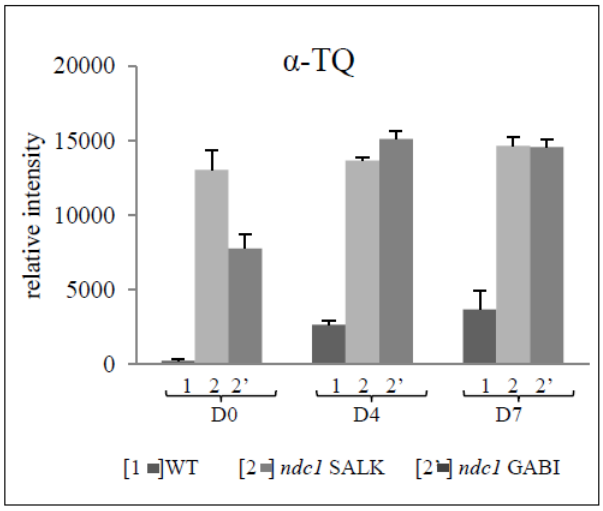


Figure 6: Plastoquinone and plastochromanol quantification in leaf and after HL treatment. Lipids were extracted from [1] WT, [2] *ndc1*, [3] 35S:NDC1-YFP and [4] 35S:VTE1-YFP grown under moderate light conditions (D0) and after 4 (D4) and 8 (D8) days of continuous HL exposition (500 µE m^{-2} s^{-1}). **(A)** Total PQ was quantified using purified PQ as a standard. The white and grey bars indicating respectively the fraction of oxidized (ox) and reduced (red) PQ. **(B)** and **(C)** Quantification of PC8 using purified PC8 as a standard. Histogram presented figure C was a magnification of panels [1, 2, 3] figure B. Data are means of three experiments (n=3).



Supplementary Figure 1: Amount of α-tocopherol quinone in leaf and after HL treatment. Lipids were extracted from [1] WT and two different *ndc1* mutant lines: [2] *ndc1* SALK_024063 and [2'] *ndc1* GABI_614F03. Plants grown under moderate light conditions (D0) were exposed to continuous HL (500 µE m^{-2} s^{-1}) for 4 (D4) and 7 days (D7). Data are means of three experiments (n=3).

PLASTOGLOBULES, A MAJOR COMPARTMENT OF PRENYLQUINONE METABOLISM AND REPAIR

To analyze the distribution of the prenylquinones, we isolated chloroplasts from leafs of WT and *ndc1* after 7 days of HL and separated the thylakoid membranes and the plastoglobules. We then carried out prenyllipid profiling on whole leafs, isolated chloroplast, thylakoids and plastoglobules (Figures 7A, B). The galactolipids, MG1; monogalactosyldiglyceride (18:3/16:3) and MG 2; monogalactosyldiglyceride (18:3/18:3), abundant chloroplast membrane lipids contributing to the thylakoids as well as the lipid monolayer of plastoglobules, were used as an internal reference to assess the enrichment of prenylquinones. Clearly, the prenylquinone compounds were enriched in plastoglobules, i.e., small peaks for MG1 and −2, large peaks for α-tocopherol and PQ/PQH2 when compared to the thylakoid membranes in both WT and *ndc1* (approximately 30- and 40-fold, respectively). Using γ-tocopherol as an internal reference (Figure 7C), increase of α-TQ and decrease of PC8 were

observed in *ndc1* PG. For α-TQ, the enrichment in PG compared to thylakoid membranes was 3-and5-fold, respectively in WT and *ndc1*. Note that α-TQ cannot be seen as peak in the chromatograms (Figures 7A, B) due to its relatively low abundance. As expected phylloquinone (Vit K) was detectable only in WT but not in *ndc1* PG.

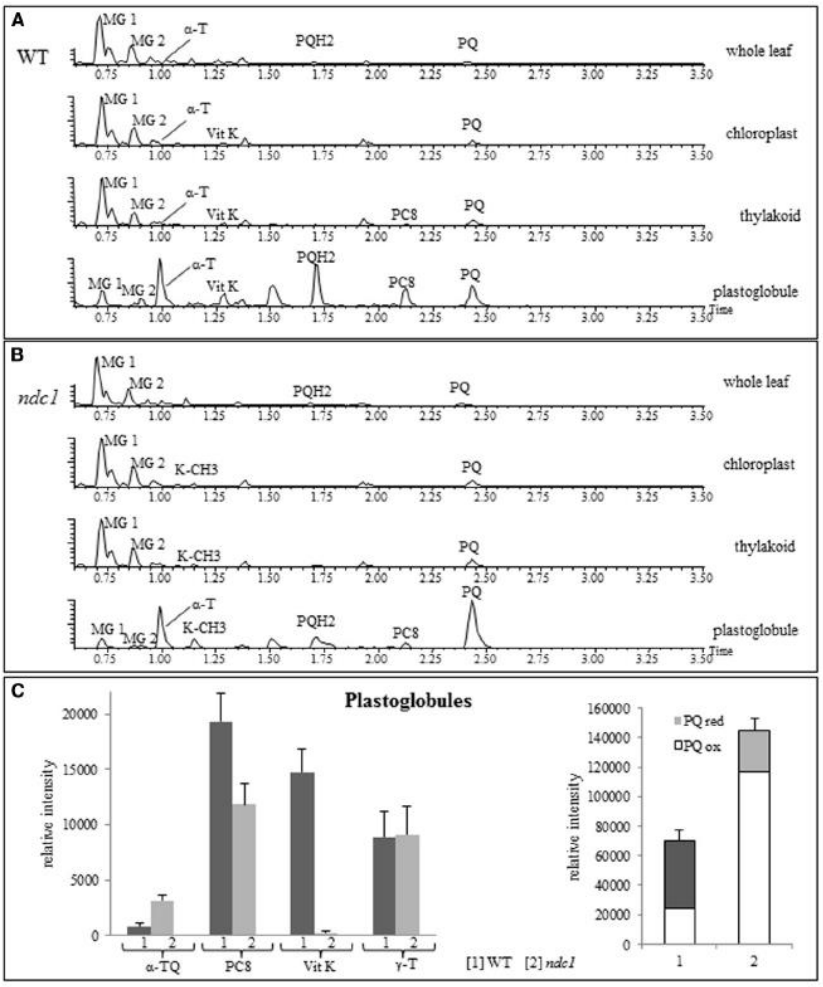


Figure 7: Amount of prenylquinones in chloroplast fractions isolated from WT and *ndc1* mutant plants under HL. UHPLC-QTOFMS chromatograms of chloroplast fractions showing prenylquinone enrichment in plastoglobules compared to other compartments. **(A)** in WT and **(B)** in *ndc1* mutants. MG 1, monogalactosyldiglyceride (18:3/16:3), MG 2, monogalactosyldiglyceride (18:3/18:3), α-T (alpha-tocopherol), Vit K (phylloquinone), PQH_2 (plastoquinol), PQ (plastoquinone), PC8 (plastochromanol), K-CH_3 (demethylphylloquinone) after 7 days of HL. Data are means of four experiments (n=4). **(C)** Quantity of α-TQ (α-tocopherol quinone), Vit K, PC8, PQ/PQH_2, γ-T (gamma-tocopherol) was estimated from [1] WT and [2] *ndc1* plastoglobule fractions. Data are means of four experiments (n=4).

DISCUSSION

In this study we analyzed the dynamics of prenyllipid metabolites during the change from moderate light to HL conditions using a non-targeted lipidomics approach. It is known that during acclimation to HL conditions, several prenylquinones accumulate in Arabidopsis leaves (Kobayashi and Dellapenna, 2008; Szymanska and Kruk, 2010b; Eugeni Piller et al., 2011). The four Arabidopsis genotypes used in these experiments resulted in distinct prenylquinone signatures. The most typical compounds that accumulated in each of the respective lines were: DMPBQ in *vte1*, 2-phytyl-1,4-naphthoquinone in *ndc1*, δ-, γ-tocopherol and PC8 in 35S:VTE1-YFP and finally PQH_2 in 35S:NDC1-YFP overexpressing plants (Figure 3). Overall, α-tocopherol was the prenylquinone that increased most during the course of light stress among the 500 compounds analyzed, except in the *vte1* mutant that lacks the tocopherol cyclase (Figure 4). This is testimony to the importance of α-tocopherol as a lipid antioxidant at the thylakoid membrane, which is subject to photooxidation and photoinhibition at PSII due to increased ROS production under HL stress (Kobayashi and Dellapenna, 2008). We previously showed that the α-tocopherol accumulation coincides with an increase in size and number of PG under HL stress (Martinis et al., 2014). This work demonstrates the implication of NDC1 in the tocopherol redox cycle. During HL stress, *ndc1* mutant plants accumulate the α-tocopherol oxidation product, α-TQ (Figure 4D). It has already been demonstrated that NDC1 is an enzyme with a wide specificity and able to reduce a range of quinolic substrates in vitro such as decyl-PQ, decyl-ubiquinone as well as in purified plastoglobules due to their contents of prenylquinones (Eugeni Piller et al., 2011). For α-tocopherol recycling to proceed efficiently it is likely that α-TQ must be present in the reduced form (α-TQH_2). However, our current methodology does not allow the detection of α-TQH_2. Nevertheless it is highly probable that NDC1 functions in the reduction of α-TQ to α-TQH_2 to regenerate α-tocopherol. An analogous reaction mechanism has been demonstrated

for the formation of γ-tocopherol, in which VTE1 closes the chromanol ring preferentially in the reduced form of DMPBQ (Grütter et al., 2006).

NDC1 is also implicated in the reduction of PQ to PQH_2 as 35S:NDC1-YFP plants exhibited higher PQH_2/PQ ratios (Figure 6A). Thus, NDC1 may directly influence the redox state of the PQ reservoir. Most likely, this increase in PQH_2 concerns primarily the proportion of the plastoquinone pool present in plastoglobules that is not directly implicated in photosynthesis (Eugeni Piller et al., 2011). The observed increase of total plastoquinone in 35S:NDC1-YFP plants (Figure 6A) may be necessary to maintain sufficient oxidized PQ to allow electron transport to proceed efficiently at the thylakoid membranes. 35S:VTE1-YFP plants showed a decrease of total PQ after HL stress (Figure 6A). It is important to note that VTE1 catalyzes the production of PC8 from PQH_2 (Zbierzak et al., 2010). Therefore, the decrease of total PQ is readily explained by the pronounced accumulation of PC8 in this genotype (Figure 6B). PQH_2 may continuously be siphoned off as a substrate of VTE1 to form PC8 explaining the decrease of PQ and the increase of PC8 in35S:VTE1-YFP. However, in agreement with Szymanska and Kruk (2010a), our results show that the production of PC8 is not influenced by HL in WT, *ndc1* and 35S:NDC1-YFP plants but an increase is apparent in 35S:VTE1-YFP plants. Potentially, this could be explained by an increased flux of PQH_2 to PG under HL in the presence of elevated concentrations of VTE1-YFP. In conclusion, we identify NDC1 as a novel enzyme that participates in the α-tocopherol redox cycle probably by reducing α-TQ to α-TQH_2. In this recycling pathway, VTE1 was already known to convert $TMPBQH_2$ to α-tocopherol. By hosting both NDC1 and VTE1, PG appear to play a role as metabolic repair site in the tocopherol redox cycle. This hypothesis is supported by the enrichment of α-TQ in the PG of *ndc1* compared to the WT (Figure 7C). Also, the overexpression of both NDC1-YFP and VTE1-YFP suppressed the increase of α-TQ observed in the wild type under HL (Figure 5B). This may be explained by higher levels of NDC1 or VTE1 activity that may

affect the reaction kinetics of the tocopherol redox cycle and accelerate the reduction of α-TQ. Beyond its role in tocopherol recycling and plastoquinone reduction, *ndc1* lacks phylloquinone and accumulates its dimethyl precursor instead (Figure 4D) (Eugeni Piller et al., 2011). This indicates that NDC1 has "moonlighting" role in the final methylation step of phylloquinone biosynthesis that is catalyzed by AtMENG. In the future, it will be of great interest to investigate the mechanisms of NDC1 in more detail and to determine the role of this unusual enzyme in other species.

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CHAPTER VI.
GENERAL CONCLUSIONS
&
PERSPECTIVES

6. GENERAL CONCLUSIONS AND PERSPECTIVES

6.1 Conclusion

Long viewed as passive lipid droplets, PG have been receiving increasing attention in the last few years. The determination of the Arabidopsis PG proteome in 2006, lead to the discovery that PG do not only store lipids but actively participate in their metabolism. This idea is supported by the observation that plastoglobule size and number vary depending on plastid type and environmental conditions. Furthermore, several recent studies indicate that plastoglobules are involved in various pathways such as those leading to the biosynthesis of vitamin K1 (phylloquinone), vitamin E (tocopherol), and carotenoids. Moreover, they are involved in plant responses to stress by lipid antioxidant accumulation (e.g. tocopherol, plastochromanol) and elimination of potentially toxic molecules (e.g. fatty acids and phytol that are converted to and stored as phytyl esters). This present thesis confirms the important role of PG in chloroplast lipid metabolism and, in addition introduces a new aspect of energy metabolism.

During my research, a powerful non-targeted lipidomics analysis was employed to discover the function of one candidate enzyme in lipid metabolism, the protein NDC1 (NADPH quinone oxidoreductase C1) and to correlate changes in metabolite profiles with NDC1 function.

First, we demonstrated that NDC1 is associated with PG. NDC1 does not seem to be directly implicated in cyclic or chlororespiratory electron flow in thylakoids but represents a new electron input device for PQ. In fact, a large, separate pool of non-photoactive plastoquinol exists in PG and this pool may function as an antioxidant reservoir for the thylakoid membrane. We showed that the regeneration of oxidized plastoquinol in PG is at least in part carried out by the PG-localized NDC1. In fact, recombinant, purified NDC1 was able to

reduce a plastoquinone analogue in vitro and plays a role in electron transfer to the plastoglobule PQ pool in vivo, which affects the overall redox state of the total plastoquinone pool. Surprisingly, NDC1 also appears to be essential for phylloquinone synthesis: *ndc1* mutant plants lacked vitamin K1 and instead accumulated its precursor, the 2-phytyl-1,4-naphthoquinone. Normally, the conversion to phylloquinone requires the methyltransferase AtMenG. As NDC1 is not predicted to function as methyltranferase its role in this pathway was unclear at the time of publication. However it has now been shown that NDC1 catalyzes the reduction of 2-phytyl-1,4-naphthoquinone and that this is an essential step in the biosynthetic pathway not only in Arabidopsis but also in cyanobacteria. Without reduction of the naphthoquinone intermediate AtMenG-dependent methylation cannot proceed.

NDC1 is also essential for normal plastochromanol-8 accumulation: A strong decrease of PC-8 levels is observed in the *ndc1* mutant plants. PC-8 is produced by VTE1from a fraction of the non-photochemical pool of reduced plastoquinol in PG. In the *ndc1* mutants, the reduced proportion of plastoquinone is diminished and therefore less efficiently converted to PC-8.

Finally, NDC1 is also involved in tocopherol recycling. Recycling of the tocopherol oxidation product α-tocoquinone requires VTE1 activity in the final step that re-introduces the chromanol ring. This reaction too occurs preferentially in the reduced state of the recycling intermediate.

In conclusion, NDC1 protein is located at the PG and is important for prenylquinone synthesis by allowing the regeneration of the plastoglobule PQ pool and playing a role in phylloquinone production, PC-8 accumulation and tocopherol recycling. The common denominator in all of these reactions is the dependency of a biochemical reaction on the reduced state of its substrate. It appears that PG provide a favourable chemical environment for these reactions to occur.

6.2 Perspectives

1. Characterization of new Arabidopsis proteins

   Proteome analyses of PG in 2006 yielded a list of known as well as unknown proteins (Vidi et al., 2006, Ytterberg et al., 2006). To discover new functions of plastoglobules, an obvious approach is to characterize the unknown proteins. In addition, the analysis of the proteome of plants growing under various stresses or at different developmental stages is of great interest. This approach may lead to the identification of yet new candidate proteins and implicate PG yet in other (lipid) metabolic pathways. For now the PG are firmly established at the crossroads of the prenylquinone metabolic pathways.

2. Plastoglobule proteins in crop species

   In plants, phylloquinone (vitamin K1) functions as an essential redox carrier in photosynthetic electron transport and tocopherol (vitamin E) as an important lipid anti-oxidant. Independently of their role in plants, vitamins E and K are indispensable for human and animal health. Vitamin K, for instance, is an essential factor in mammalian metabolism. It is required for blood coagulation and bone formation and like all vitamins must be supplied with the diet. Deficiency in vitamin K causes anemia and hemorrhage in new-borns.

   Vitamin K and E are principally found in green vegetables and some fruits. It will be of great interest to investigate NDC1 as well as factors regulating vitamin E in crop species such as tomato or pea. New ways to fortify vitamin contents in crop species may be identified. On a more general note, it will be of interest to determine PG proteomes and lipid profiles in various crops to understand the wider role of PG under different stress conditions such as drought, heat or high light in cultivated plants.

3. Molecular farming

Plants are well known as an alternative system for the production of recombinant proteins. Plant expression systems allow large-scale production of recombinant proteins and offer the possibility of lowering production costs (by a factor of 10 to 100) compared to traditional systems (Ma et al., 2003; Fischer et al., 2004; Ma et al., 2005a,b). Moreover, transplastomic plants (produced by introducing DNA into the chloroplast genome) enable high yields of recombinant proteins due to a high transgene copy number and limited epigenetic phenomena. Besides their physiological roles, plastoglobules may present a biotechnological interest for molecular farming. In fact, the plastoglobule core allows a hydrophobic environment necessary for the production of certain chemical compounds. Proteins of interest could be targeted to plastoglobules by fusion with a fibrillin/plastoglobulin (i.e. PGL35) (Shanmugabalaji et al., 2012, Vidi et al., 2006). Finally, due to the low density of PG, the proteins of interest could be easily co-purified using sucrose gradient flotation. The plant physiology laboratory has made important progress in this direction but much more remains to be discovered.

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