A Dedicated Type II NADPH Dehydrogenase Performs the Penultimate Step in the Biosynthesis of Vitamin K$_1$ in *Synechocystis* and Arabidopsis

Abdelhak Fatihi  
*University of Nebraska-Lincoln, abdou.fatihi@unl.edu*

Scott Latimer  
*University of Nebraska-Lincoln*

Stefan Schmollinger  
*University of California, Los Angeles*

Anna Block  
*University of Nebraska-Lincoln*

Patrick Dussault  
*University of Nebraska-Lincoln, pdussault1@unl.edu*

See next page for additional authors

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A Dedicated Type II NADPH Dehydrogenase Performs the Penultimate Step in the Biosynthesis of Vitamin K₁ in *Synechocystis* and Arabidopsis

Abdelhak Fatihi, Scott Latimer, Stefan Schmollinger, Anna Block, Patrick H. Dussault, Wim F.J. Vermaas, Sabeeha S. Merchant, and Gilles J. Basset

Department of Agronomy and Horticulture, and Center for Plant Science Innovation, University of Nebraska, Lincoln, Nebraska 68588

Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095

Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588

School of Life Sciences and Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, Arizona 85287

Institute for Genomics and Proteomics, University of California, Los Angeles, California 90095

Mutation of *Arabidopsis thaliana* NAD(P)H DEHYDROGENASE C1 (NDC1; At5g08740) results in the accumulation of demethylphyloquinone, a late biosynthetic intermediate of vitamin K₁. Gene coexpression and phylogenomics analyses showed that conserved functional associations occur between vitamin K biosynthesis and NDC1 homologs throughout the prokaryotic and eukaryotic lineages. Deletion of *Synechocystis ndbB*, which encodes for one such homolog, resulted in the same defects as those observed in the cyanobacterial demethylnaphthoquinone methyltransferase knockout. Chemical modeling and assay of purified demethylnaphthoquinone methyltransferase demonstrated that, by virtue of the strong electrophilic nature of S-adenosyl-L-methionine, the transmethylation of the demethylated precursor of vitamin K is strictly dependent on the reduced form of its naphthoquinone ring. NDC1 was shown to catalyze such a prerequisite reduction by using NADPH and demethylphyloquinone as substrates and flavine adenine dinucleotide as a cofactor. NDC1 displayed Michaelis-Menten kinetics and was markedly inhibited by dicumarol, a competitive inhibitor of naphthoquinone oxidoreductases. These data demonstrate that the reduction of the demethylnaphthoquinone ring represents an authentic step in the biosynthetic pathway of vitamin K, that this reaction is enzymatically driven, and that a selection pressure is operating to retain type II NAD(P)H dehydrogenases in this process.

INTRODUCTION

Vitamin K is a generic term used to designate a class of naphthoquinone derivatives that display some biological activity as cofactors for γ-glutamyl-carboxylases involved in blood coagulation, bone and vascular metabolism, and cell growth regulation (Booth, 2009; Beulens et al., 2013). Natural forms of the vitamin include phyloquinone (2-methyl-3-phytyl-1,4-naphthoquinone), also known as vitamin K₁, and menaquinones (2-methyl-3-(all-trans-polyprenyl)-1,4-naphthoquinones), also known as vitamin K₂.

The occurrence of phyloquinone appears to be restricted to green plants and some species of cyanobacteria, while menaquinones are widespread among archaea, bacteria, red algae, and diatoms (Collins and Jones, 1981; van Oostende et al., 2011). Phyloquinone from plant-based foods is the major source of vitamin K in the Western diet (Booth and Suttie, 1998). Vitamin K-synthesizing organisms use phyloquinone or menaquinone as redox cofactors in their electron transport chains. For instance, in plants, phyloquinone serves as a light-dependent one-electron carrier within photosystem I from chlorophyll a to the iron-sulfur cluster Fx of the heterodimeric PsA/PsB reaction center (Brettel, 1997). It also doubles as an electron acceptor for the formation of protein disulfide bonds in the lumen of chloroplasts (Furt et al., 2010; Karamoko et al., 2011).

The biosynthetic pathway of phyloquinone is similar to that of menaquinones in γ-proteobacteria (van Oostende et al., 2011). Chorismate and 2-oxoglutarate serve as precursors for the formation of the naphthalenoid ring, which is prenylated and then methylated. Phyloquinone biosynthetic mutants in the cyanobacterium *Synechocystis* sp PCC 6803 and the green alga *Chlamydomonas reinhardtii* are still able to grow photoautotrophically, for these species recruit plastoquinone-9 in place of phyloquinone at the A₁ site of photosystem I (Semenov et al., 2000; Lefebvre-Legendre et al., 2007). By contrast, loss of phyloquinone biosynthesis in *Arabidopsis thaliana* results in seedling lethality (van Oostende et al., 2011). However, one exception is the Arabidopsis mutant corresponding to demethylnaphthoquinone methyltransferase (MENG; At1g23360; EC 2.1.1.163), which catalyzes the last step of phyloquinone biosynthesis in plastids (Lohmann et al., 2006). The cognate mutant is viable because it accumulates demethylphyloquinone, and the latter can replace phyloquinone in photosystem I, albeit with some loss of photosynthetic efficiency (Lohmann et al., 2006).

Knocking out Arabidopsis NAD(P)H DEHYDROGENASE C1 (NDC1; At5g08740), which encodes a member of the type II NAD(P)H dehydrogenase family, also blocks the methylation of
the naphthalenoid ring (Eugeni Piller et al., 2011). NDC1 also affects the redox state of plastoquinone as well as the recycling of α-tocopherol in thylakoid-associated lipoprotein particles called plastoglobules (Eugeni Piller et al., 2011, 2014). However, the reason why the ndc1 knockout accumulates demethylphylloquinone instead of phylloquinone is unknown (Eugeni Piller et al., 2011; Besagni and Kessler, 2013). Because changes in the redox state of plastoquinone impact the expression of photosynthetic genes (Rochaix, 2013), it might seem that the loss of NDC1 simply triggers the downregulation of the transcription of demethylnaphthoquinone methyltransferase. This not the case, however, because the transcript level of this enzyme is unaffected in ndc1 knockout plants (Eugeni Piller et al., 2011). Such a situation is actually reminiscent of that observed in γ-proteobacteria, which can alternate between the accumulation of demethylmenaquinone or menaquinone depending on the growth conditions, but again without apparent changes in the transcription of the cognate methyltransferase (Shetepolav et al., 1997).

Another possibility is that NDC1 is not directly involved in phylloquinone biosynthesis and that loss of function of this gene blocks methylation of the demethylnaphthoquinone intermediate via a general alteration of plastid metabolism. There is a precedent for this: Arabidopsis knockout plants corresponding to gene MUTS-HOMOLOG1, which encodes an endonuclease involved in the maintenance of plastid genome stability, also triggered for this: Arabidopsis knockout plants corresponding to gene MUTS-HOMOLOG1, which encodes an endonuclease involved in the accumulation of demethylmenaquinone or menaquinone depending on the growth conditions, but again without apparent changes in the transcription of the cognate methyltransferase (Shetepolav et al., 1997).

Because in both situations, the ndc1 knockout accumulates demethylphylloquinone, NDC1 would then be required to coregulate the expression of dehydrogenases with that of phylloquinone biosynthesis in plants and that the loss of phylloquinone and the accumulation of demethylphylloquinone in the Arabidopsis ndc1 knockout is unlikely to be the result of a non-specific perturbation of plastid metabolism.

**Comparative Genomics Identify Conserved Physical Associations between Type II NAD(P)H Dehydrogenase Homologs and Vitamin K Biosynthetic Genes in Bacteria**

Searches of the STRING (Franceschini et al., 2013) and SEED (http://theseed.uchicago.edu/Fig/index.cgi) databases using Arabidopsis NDC1 as a query identified bacterial type II NAD(P)H dehydrogenases in the neighborhood of naphthoquinone biosynthetic genes (Figure 2). In three notable instances (Chloroflexi, Proteobacteria, and Actinobacteria), the association was detected with demethylnaphthoquinone methyltransferase itself (Figure 2). Furthermore, in Chloroflexi, Proteobacteria, Actinobacteria, and Firmicutes, these gene clusters display operon-like structures (Figure 2), pointing to the existence of a selective pressure to coregulate the expression of the dehydrogenases with that of menaquinone biosynthetic genes. Such arrangements recapitulate the high level of correlation of expression between NDC1 and phylloquinone biosynthetic genes in Arabidopsis and rice and indicate that the functional coupling between type II NAD(P)H dehydrogenases and vitamin K biosynthesis is not restricted to flowering plants.

**Deletion of Synechocystis slr1743 (ndb) Blocks the Transmethylation of Demethylphyloquinone**

To determine whether prokaryotic type II NAD(P)H dehydrogenases are directly involved in vitamin K biosynthesis, prenylated naphthoquinones were quantified in corresponding knockout strains of the cyanobacterium Synechocystis sp PCC 6803. The genome of this species encodes three type II NAD(P)H dehydrogenases, ndbA, ndbB, and ndbC, which are the gene products of slr0851, slr1743, and slr1484, respectively (Howitt et al., 1999). Because in Synechocystis none of these NDC1 homologs occurs in a cluster with quinone biosynthetic genes, these three genes could be deleted, either singly or in combination, without creating a non-specific polar effect on naphthoquinone production. Phylloquinone represented most of the pool of prenylated naphthoquinones in the ΔndbA and ΔndbC knockout strains, as is typically observed in wild-type Synechocystis (Figure 3). By contrast, the ΔndbB and ΔΔndbABC knockout strains contained only traces of phylloquinone and instead accumulated its demethylnaphthoquinone precursor, just as is observed for the Synechocystis demethylnaphthoquinone methyltransferase mutant (Figure 3A). Synechocystis ΔndbB cells also displayed a lower plastoquinol-9/plastoquinone-9 ratio and a lower tocopherol content than did the wild-type control (Figures 3B and 3C). This suggests that, like its plant homolog NDC1, ndbB contributes to
the reduction of plastoquinone-9 and the salvaging of the chromanol ring of tocopherols.

**Synechocystis** ΔndbB and Arabidopsis ndc1 Mutants Display Increased Photosensitivity to High Light

Whereas under low light intensity the growth of **Synechocystis** ΔndbB cells was indistinguishable from growth of wild-type cells, this mutant displayed marked growth retardation compared with the wild type when cultured at high light intensity (Figure 4). This result was surprising, for it had been reported that the Arabidopsis ndc1 mutant was not phenotypically different from its wild-type counterpart (Eugeni Pillier et al., 2011). To investigate this, Arabidopsis ndc1 knockout and wild-type plants that had been grown under normal light conditions and with day/night cycles were exposed to continuous high light. After 3 d of exposure to high light, ndc1 plants appeared smaller and paler than the wild type (Figure 4B). Chlorophyll quantification confirmed that chlorophyll a and chlorophyll b levels in the leaves of the ndc1 mutant were 19 and 16% lower than those of the wild type, respectively (Table 1). Measurements of chlorophyll a fluorescence in response to actinic illumination did not indicate that maximum quantum efficiency of photosystem II (Fv/Fm) or nonphotochemical quenching (NPQ) was decreased in ndc1 plants compared with the wild type (Table 1). However, high light acclimated ndc1 plants displayed lower photosystem II operating efficiency (ΦPSII), which is proportional to the rate of CO2 fixation (Baker, 2008), than the wild type (Figure 4C, Table 1). Such a phenotype of increased photosensitivity, loss of chlorophylls, and decrease of photosystem II efficiency in response to high light treatment is reminiscent of that described for the Arabidopsis demethylpentahydroquinone methyltransferase knockout (Lohmann et al., 2006).
The Reduction of the Demethylnaphthoquinone Ring Is a Prerequisite to Its Transmethylation

Having shown that the connection between type II NAD(P)H dehydrogenases and naphthoquinone metabolism is not solely specific but widespread throughout the prokaryotic and eukaryotic lineages of vitamin K-synthesizing organisms, we sought to elucidate the molecular mechanism that underlies the apparent epistasis between the loss of function of NDC1/ndbB and that of demethylnaphthoquinone methyltransferase. For that, we first modeled the mechanism of methyl transfer between AdoMet and the demethylated naphthoquinone precursor of vitamin K. The resulting model predicted that the transmethylation reaction strictly requires the reduced form (quinol) of the naphthalenoid ring, where C3 of the demethylnaphthoquinol moiety would act as the attacking nucleophile on the electrophilic methyl group of AdoMet, followed by proton exchanges to reform the C1-C2 double bond and the C1 phenol (Figure 5). By contrast, the alternative scenario starting from the oxidized form (quinone) of the naphthalenoid ring appears improbable because it would require the formation of an unstable cation intermediate (Figure 5B).

To test the proposal that the transmethylation reaction in vitamin K biosynthesis indeed depends upon the redox state of the naphthoquinone ring, demethylphyloquinone was purified from the leaves of Arabidopsis demethylnaphthoquinone methyltransferase knockout plants and used as a methyl acceptor in assays of purified demethylnaphthoquinone methyltransferase (MenG; 2.1.1.163), with AdoMet as methyl donor and in the presence or absence of DTT as a reductant. Demethylphyloquinone and its methylated product, phylloquinone, were then extracted on solid-phase cartridges and quantified by HPLC with fluorescence detection. Phylloquinone formation was readily detected in the DTT-containing assay (Figure 5C), and the corresponding methyl transferase activity was linear with both the amount of demethylnaphthoquinone methyltransferase and time (Figures 5D and 5E). By contrast, no phylloquinone was detected when DTT was omitted from the assay (Figure 5C).

NDC1 and ndbB Catalyze the Reduction of the Demethylnaphthoquinone Ring

Since the demethylnaphthoquinone methyltransferase-catalyzed reaction strictly requires the quinol form of the methyl-accepting substrate, we examined the possibility that the demethylnaphthoquinone ring could be reduced by NDC1. To do this, purified NDC1 was assayed with demethylphyloquinone and NADPH in a coupled assay with demethylnaphthoquinone methyltransferase and AdoMet. NDC1-dependent demethylnaphthoquinone methyltransferase activity was readily detected, demonstrating that NDC1 and NADPH can effectively replace DTT (Figure 6). Furthermore, consistent with the occurrence of a noncovalently bound flavin cofactor in type II NAD(P)H dehydrogenases (Melo et al., 2004), phylloquinone formation was markedly decreased if purified NDC1 was not preincubated with flavine adenine dinucleotide (FAD) (Figure 6A).
and the metrically. NDC1 displayed typical Michaelis-Menten kinetics, a substrate and monitoring the oxidation of NADPH spectrophotometrically. An aqueous solution and could not be dispersed at concentrations of 2.2

Typical of the Ki values reported for naphthoquinone oxidoreductases (Tie et al., 2011). The inactivation of the naphthoquinone oxidoreductase activity of ndbB was also markedly inhibited by dicumarol; the enzyme had a Ki value of 1.3 μM for this inhibitor with menadione and NADPH as substrates (Figure 6E).

Because demethylphylloquinone has a very low solubility in aqueous solution and could not be dispersed at concentrations greater than a few micromolar, a second assay was developed using menadione, a nonprenylated naphthoquinone derivative, as a substrate and monitoring the oxidation of NADPH spectrophotometrically. NDC1 displayed typical Michaelis-Menten kinetics, and the Km and Kcat of the enzyme for menadione were 20 μM and 0.44 s⁻¹, respectively (Figure 6B). This resulted in a catalytic efficiency of 2.2 × 10⁴ M⁻¹ s⁻¹, which was within the range of values (0.3 × 10⁴ to 145 × 10⁶ M⁻¹ s⁻¹) reported for other eukaryotic flavin oxidoreductases using NADH or NADPH as electron donors and menadione as an electron acceptor (Sánchez et al., 2001; Endo et al., 2008; Dong et al., 2009). Furthermore, the enzyme was markedly inhibited by dicumarol (Figure 6C), a competitive inhibitor of naphthoquinone oxidoreductases (Tie et al., 2011). The inhibition constant (K) of NDC1 for dicumarol determined with menadione and NADPH as substrates was 5.5 μM, which was typical of the K values reported for naphthoquinone oxidoreductases (4.1 to 10.6 μM) assayed in similar conditions (Sánchez et al., 2001; Wrobel et al., 2002). Similar results were obtained with recombinant ndbB. Its Km, Kcat, and catalytic efficiency values for menadione were 2.4 μM, 3.2 s⁻¹, and 133.3 × 10⁴ M⁻¹ s⁻¹, respectively (Figure 6D). The naphthoquinone oxidoreductase activity of ndbB was also markedly inhibited by dicumarol; the enzyme had a K value of 1.3 μM for this inhibitor with menadione and NADPH as substrates (Figure 6E).

DISCUSSION

A Missing Step in Vitamin K Biosynthesis

We establish here that the biosynthesis of vitamin K entails an additional bona fide step that is the enzymatic reduction of the demethylnaphthoquinone ring prior to its AdoMet-dependent transmethylation (Figure 7). Our data explain why the loss of function of NDC1/ndbB is epistatic with that of MENG and why the Arabidopsis ndc1 mutant accumulates the demethylated precursor of phylloquinone rather than phylloquinone itself. It is crucial to the interpretation of our results that AdoMet is strongly electrophilic and, therefore, that the transfer of its methyl group depends upon an electron pair originating from the methyl-accepting naphthalenoid ring. Chemical modeling and in vitro assays demonstrate that such an electron donation occurs exclusively with the quinol, i.e., reduced, form of the naphthalenoid ring. In plants and cyanobacteria, this prerequisite formation of demethylnaphthoquinol is catalyzed by a type II NADPH dehydrogenase, and comparative genomic data indicate that this is most probably also the case for the biosynthesis of menaquinone in Chloroflexi, Proteobacteria, Actinobacteria, and Firmicutes.

The Redox Properties of the Demethylated Naphthoquinone Ring Impact the Architecture of Phylloquinone Biosynthesis in Plastids

It has been established that the biosynthesis of phylloquinone in plants requires the coordinated synthesis and transport of metabolic intermediates between two subcellular compartments, plastids and peroxisomes (van Oostende et al., 2011; Reumann, 2013). The initial benzenoid intermediate, o-succinylbenzoate, is made from chorismate in plastids (Figure 7). It is then exported to peroxisomes for its cyclization into 1,4-dihydroxynaphthoate, which is transported back into plastids where it is phytlated and methylated (Figure 7). Even within plastids, some phylloquinone biosynthetic intermediates are trafficked, for the demethylnaphthoquinone phytyltransferase (2.5.1.74) and methyltransferase (2.1.1.163) activities are localized in the chloroplast envelope and thylakoid membranes, respectively (Figure 7; Schultz et al., 1981; Kaipeng et al., 1984). Our evidence that the plastoglobule-localized NDC1 mediates the mandatory reduction of the naphthoquinone ring between the phytlation and

<table>
<thead>
<tr>
<th>Level or Parameter</th>
<th>Wild Type</th>
<th>ndc1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a (nmol g⁻¹ FW)</td>
<td>1298 ± 38</td>
<td>1057 ± 36‡</td>
</tr>
<tr>
<td>Chlorophyll b (nmol g⁻¹ FW)</td>
<td>830 ± 15</td>
<td>693 ± 53‡</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>0.726 ± 0.042</td>
<td>0.802 ± 0.038</td>
</tr>
<tr>
<td>NPQ</td>
<td>1.010 ± 0.214</td>
<td>1.909 ± 0.429</td>
</tr>
<tr>
<td>δPSII</td>
<td>0.321 ± 0.072</td>
<td>0.212 ± 0.079</td>
</tr>
</tbody>
</table>

Fluorescence parameters were calculated after 5 min of actinic illumination. Data are means of three (chlorophyll) to four (fluorescence parameters) biological replicates ± so. Different superscript letters indicate significant differences from the wild type as determined by Fisher’s test (P < α = 0.05) from an analysis of variance. FW, fresh weight.
Indeed, having low midpoint redox potentials, naphthoquinols are prone to oxidation in vivo (Schoepp-Cothenet et al., 2009). The calculated rate of spontaneous reoxidation for the dihydroxynaphthoate ring gives a half-life of ~1.5 h at atmospheric O₂ concentration and standard ambient temperature and pressure. This is only a minimum estimate; the half-life of demethylnaphthoquinols is most probably much shorter in chloroplasts owing to the oxidative nature of photosynthesis and to the occurrence of reactive oxygen species. The proximity of demethylnaphthoquinone methyltransferase in thylakoids and of NDC1 in plastoglobules, which are permanently attached to thylakoids (Austin et al., 2006), is therefore not coincidental and is in fact dictated by the need for plants to promptly methylate demethylphyloquinol.

**Plants and Cyanobacteria Have Unique Demethylnaphthoquinone Oxidoreductases**

It should be emphasized that our results do not contradict previous reports indicating that NDC1 participates in the redox metabolism of two prenyl benzoquinones, plastoquinone-9 and the tocopherol recycling-intermediate α-tocopherol quinone (Eugeni Piller et al., 2011, 2014). In fact, our data show that ablating ndbB, the Synechocystis ortholog of NDC1, also results in lower plastoquinol-9/plastoquinone-9 ratio and lower α-tocopherol content compared with wild-type cells. It is therefore clear that NDC1 and ndbB are bifunctional oxidoreductases that are able to act both on prenyl naphthoquinones and on prenyl benzoquinones in vitro and in vivo. However, that the ndc1 and ndbB mutants contain only traces of phyloquinone and accumulate demethylphyloquinone rules out that other type II NADPH dehydrogenases contribute significantly to phyloquinone biosynthesis. In Arabidopsis, such an absence of functional redundancy is not wholly unexpected because out of seven type II NADPH dehydrogenases, only NDC1 is found in plastids (Carrie et al., 2008; Xu et al., 2013). A similar scenario applies to rice, where the NDC1 ortholog is the sole plastid-targeted type II NADPH dehydrogenase (Xu et al., 2013). By contrast, green fluorescent protein fusion experiments showed that the moss Physcomitrella patens possesses three plastid-targeted type II NADPH dehydrogenases (Xu et al., 2013), whereas two have been identified in the plastid of the green alga Chlamydomonas (Jans et al., 2008; Terashima et al., 2010). However, it would be premature to infer that mosses and green algae have multiple enzymes to reduce the demethylated naphthoquinone intermediate. First, as the case of Synechocystis illustrates, the capacity to reduce demethylphyloquinone is not common to all members of the type II NADPH dehydrogenase family. Second, phylogenetic reconstructions show that species within the Embryophytes, Charophytes, Chlorophytes, Rhodophytes, and Stramenopiles lineages each have a single type II NADPH dehydrogenase that is monophyletic with NDC1 and ndbB (Figure 8). Notably, the P. patens protein is one of the three type II NADPH dehydrogenases that is targeted to plastids (Xu et al., 2013). Similarly, one can exclude that Arabidopsis LUMEN THIOL OXIDASE1 (LTO1), a thylakloid-localized oxidoreductase, and its cyanobacterial ortholog dsdB, which both couple the formation of disulfide bonds in proteins to the reduction of prenyl naphthoquinones (Bader et al., 1999; Singh et al., 2008; Karamoko et al., 2011) contribute to phyloquinone biosynthesis. HPLC
analyses actually confirmed that the Arabidopsis *ito1* mutant does not display any significant differences in methylation state and total content of phylloquinone compared with wild-type plants (Supplemental Figure 1).

A Redox Switch Regulates the Output of Demethylmenaquinones versus Menaquinones

Finally, our results call for revisiting the question of how γ-proteobacteria are able to modify their naphthoquinone/demethylnaphthoquinone ratio in response to changes in growth conditions and independently of the transcription of the cognate methyltransferase (Shestopalov et al., 1997). One of the most striking instances of such a case is in *Escherichia coli*, the menaquinone pool of which switches from mostly demethylated to mostly methylated in a matter of seconds during the aerobic to anaerobic transition (Bekker et al., 2007). We propose that γ-proteobacteria have evolved the ability to make such rapid adjustments in the biosynthetic fluxes of their prenyl naphthoquinones via changes in the redox state of demethylmenaquinone. This also suggests that were strategies developed to engineer vitamin K₁ levels in γ-proteobacteria, these cells might be able to defeat attempts to increase the cognate biosynthetic fluxes simply by modifying the redox state of their pool of naphthoquinone intermediates.

METHODS

Chemicals and Reagents

Demethylphylloquinone was purified by HPLC from leaf extracts of the *Arabidopsis thaliana* demethylnaphthoquinone methyltransferase knockout line GABI_565F06 (Lohmann et al., 2006). Phylloquinone was from MP Biomedical and menaquinone-4 from Sigma-Aldrich. Unless mentioned otherwise, other reagents were from Fisher Scientific.

Bioinformatics

Microarray rice (*Oryza sativa*) probe sets were retrieved using the tBLASTn search mode of PLEXdb (http://www.plexdb.org/modules/tools/plexdb_blast.php) and the cognate rice proteins as queries. Coexpressing gene lists were identified in the GeneCAT database (http://genecat.mpg.de; Mutwil et al., 2008) using Arabidopsis genes At1g23380 (MENG), At1g60550 (MENB), At1g30520 (MENE), At1g60600 (MENA), At5g08740 (NDC1), At4g32770 (VTE1), At4g18950 (VTE2), and At1g06570 (HPPD) and rice probe sets Os.49084.1.S1_at (NDC1), Os.6179.1.S1_at (MENG), Os.18701.1.S1_at (VTE1), Os.19161.1.S1_at (MENE), Os.52566.1.S1_at (MENA), Os.35677.1.S1_at (MENG), Os.19292.1.S1_at (VTE2), and Os.11995.1.S1_at (HPPD) as data entries. For Venn analyses of the coexpressing genes of NDC1, MENB, and VTE1, the first 500 coexpressors (top 2.2% of the 22,810 probe sets for Arabidopsis and top 0.9% of the 57,342 probe sets for rice) of each list were aggregated using GeneVenn (http://genevenn.sourceforge.net; Pirooznia et al., 2007). Hierarchical clustering analyses were performed using the expression tree analysis tool of GeneCAT and then visualized with PhyFi (http://cgi-www.daimi.au.dk/cgi-chili/phyfi/go; Fredslund, 2006). Comparative genomics searches were performed with the STRING (http://string.embl.de) and SEED (http://theseed.uchicago.edu/FIG/index.cgi) databases using their associated tools and Arabidopsis NDC1 as an initial query.

![Figure 6. Assays of NDC1 with Naphthoquinones as Electron Acceptors.](http://example.com/figure6.png)

(A) Coupled assays with demethylnaphthoquinone methyltransferase. Assays contained 0.52 μM demethylphylloquinone, 370 μM NADPH, 370 μM AdoMet, 89 μg of NDC1, and 10 μg of MenG and were performed for 3 h at 30°C. Overnight preincubation of NDC1 with FAD prior to the assay stimulated phylloquinone formation −25-fold (upper trace) compared with no preincubation (lower trace). Inset shows the formation of phylloquinone as a function of the concentration of NDC1 preincubated with FAD. Note that the point at 0μg NDC1 also contained FAD. HPLC traces have been offset for clarity. DPhQ, demethylphylloquinone; PhQ, phylloquinone.

(B) NDC1-catalyzed oxidation of NADPH as a function of menadione concentration.

(C) Inhibition of NDC1 by dicumarol. Menadione concentration was 20 μM.

(D) nddB-catalyzed oxidation of NADPH as a function of menadione concentration.

(E) Inhibition of nddB by dicumarol.

The menadione concentration was 2.5 μM. The concentration of NADPH was 100 μM, and that of the FAD cofactor was 0.6 μM in (B) and (C) and 1.12 μM in (D) and (E). Data are means ± se of three replicates. Error bars smaller than the points are not visible.
**Biological Material and Growth Conditions**

Seeds of Arabidopsis T-DNA insertion line GABI_565F06 (meng) and SALK_151963C (lto1) were germinated on Murashige and Skoog solid medium. Two-week-old seedlings were then transferred to soil and grown at 22°C in 16-h days (110 μmol photons m\(^{-2}\) s\(^{-1}\)) for 4 weeks. For high-light treatment, plants were grown at normal light intensity (110 μmol photons m\(^{-2}\) s\(^{-1}\); 16-h days) for 4 weeks and then exposed for 3 d to continuous high light (800 μmol photons m\(^{-2}\) s\(^{-1}\)). Synechocystis sp PCC 6803 deletion strains ΔndbA (sll0861), ΔndbB (sll1743), ΔndbC (sll1864), and ΔΔΔndbABC were those described by Howitt et al. (1999), and deletion strain ΔmenG (sll1653) was described by Lohmann et al. (2006). Because the lack of tocopherols impacts macronutrient homeostasis in photomixotrophic conditions (Sakuragi et al., 2006), all Synechocystis mutants were isolated and propagated photoautotrophically at a light intensity of 50 to 65 μmol photons m\(^{-2}\) s\(^{-1}\) on solid BG-11 medium (Rippka et al., 1979). Selection and propagation plates contained 25 μg mL\(^{-1}\) erythromycin (ΔndbA), 25 μg mL\(^{-1}\) spectinomycin (ΔndbB and ΔmenG), 15 μg mL\(^{-1}\) zeocin (ΔndbC), or all three antibiotics (ΔΔΔndbABC). Homozygosity for the mutant loci was verified by PCR and the strains were stored at –80°C. For metabolite quantification, wild-type and mutant Synechocystis strains were streaked from their respective original 4°C stocks and grown photoautotrophically at a light intensity of 65 μmol photons m\(^{-2}\) s\(^{-1}\) at 30°C on solid BG-11 medium containing the appropriate antibiotics. For growth assays in normal and high light intensities, cells were scraped from propagation plates, resuspended in liquid BG-11 medium (Rippka et al., 1979), and quantified spectrophotometrically at 730 nm. Identical numbers of wild-type and mutant cells were then spotted on solid BG-11 medium without antibiotics, and the plates were incubated at 30°C under a continuous illumination of 30 or 800 μmol photons m\(^{-2}\) s\(^{-1}\).

**Expression, Purification, and Refolding of Recombinant Enzymes**

The Escherichia coli menG gene was amplified from strain K12 genomic DNA using the primers 5′-CACCAGTGAGTGAATCCAAAGAAA-3′ (forward) and 5′-TCAGACCTATACCCAGCATG-3′ (reverse). The Arabidopsis NDC1 cDNA (At5g08740) lacking its predicted N-terminal pre-sequence was amplified from total leaf RNA by RT-PCR using the primers 5′-CAGGATAGAGGAGATATACCATG-9′ (forward) and 5′-TCAGAACCAGACAAACC-3′ (reverse); the italicized sequence indicates the initiation codon introduced to truncate the targeting pre-sequence (T60–M). The PCR products were transferred into pDEST 17 (Invitrogen) using Gateway technology. The Synechocystis ndbB gene was amplified from strain PCC 6803 genomic DNA using the primers 5′-GGCATGGGTCGATGTTTCTGTTAATGAAGAACAG-3′ (forward) and 5′-TCAGAACCAGACAAACC-3′ (reverse), which contained the XbaI and EcoRl restriction sites (italicized), respectively. The digested PCR fragment was then cloned into the corresponding sites of pET-28b (Novagen). The meng and ndbB constructs were introduced into E. coli BL21 Star (DE3) pLYS5 (Invitrogen), while the NDC1 construct was introduced into BL21-CodonPlus (DE3)-RII (Agilent Technologies). Overnight starter cultures (1 mL) were used to inoculate 100 mL of Luria-Bertani medium (Luria and Burrous, 1957) prewarmed at 37°C. Isopropyl β-D-1-thiogalactopyranoside (500 μM) was added when
the OD_{600} reached ~0.7, and incubation was continued for 4 h. Subsequent operations were at 4°C. Cells were harvested by centrifugation (18,000 g for 5 min), resuspended in 2 mL of 50 mM NaH2PO4 (pH 8.0) and 300 mM NaCl, and disrupted with 0.1-mm zirconia/silica beads in a MiniBeadbeater (BioSpec Products). The extract from cells expressing C-terminally 6xHis-tagged ndbB was cleared by centrifugation (18,000 g for 15 min), and the recombinant enzyme was purified under native conditions using Ni-NTA His-Bind resin (Novagen), according to the manufacturer’s instructions. Extracts of cells harboring the menG or NDC1 constructs were centrifuged (18,000 g for 15 min), and the pellets containing the inclusion bodies were washed twice with 2 mL of 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 1% (v/v) Triton X-100. Inclusion bodies were then solubilized for 1 h in 2 mL of 50 mM HEPES (pH 7.5), 6 M guanidine-HCl, and 25 mM DTT. After removal of insoluble material (18,000 g for 15 min), solubilized proteins were renatured by briskly diluting supernatants 10-fold in 50 mM HEPES (pH 7.5), 0.2 M NaCl, 1 mM DTT, and 1 M 3-(1-pyridino)-1-propane sulfonate (Sigma-Aldrich). Purified NdbB, MenG, and NDC1 were desalted on a PD-10 column (GE Healthcare) equilibrated in 50 mM KH2PO4 (pH 7.5), 150 mM KCl, and 0.2% (v/v) Tween 20. Proteins were quantified using the Bradford method.

Figure 8. Maximum Likelihood Reconstruction of the Phylogenetic Relationships of Type II NAD(P)H Dehydrogenases within the Plant, Rhodophyte, Stramenopile, and Cyanobacterial Lineages. Arabidopsis NDC1 closest homologs were identified from fully sequenced prokaryotic and eukaryotic genomes using BLASTp searches. Protein sequences were aligned with MUSCLE (Edgar, 2004), and misalignments and divergent regions were curated with Gblocks (Castresana, 2000). The tree was constructed with PhyML (Guindon et al., 2010) and visualized with TreeDyn (Chevenet et al., 2006). Synechocystis slr0876 was used as an outgroup to root the tree. Numbers next to each branching indicate approximate likelihood ratio test. The NDC1/ndbB clade is displayed in green; green dots indicate proteins for which exists experimental confirmation of subcellular localization in plastids. Arabidopsis NDC1 (AED91343), NDA2 (NP_180560), NDA1 (NP_563783), NDB1 (NP_567801), NDB2 (NP_567829), NDB3 (NP_193880), NDB4 (NP_179673), At5g23140 (BAC42443), and AT3G44190 (NP_190005); rice Os06g0214900 (NP_001057133), Os07g0564500 (NP_001060003), Os01g0830100 (NP_001044694), Os05g0312000 (NP_001055220), Os06g0684000 (NP_001058394), Os11g0158200 (NP_001065100), Selaginella moellendorfii 1 (XP_002976807), S. moellendorfii 2 (XP_002978367), and S. moellendorfii 3 (XP_002977754); Physcomitrella patens 1 (XP_001768284), P. patens 2 (XP_001769669), P. patens 3 (XP_001757660), P. patens 4 (XP_001764062), P. patens 5 (XP_001766162), and P. patens 6 (XP_001759207); Chlamydomonas reinhardtii 1 (AD91343), C. reinhardtii 2 (NDA1) (XP_001703056), C. reinhardtii 3 (NDA2) (XP_001703643), C. reinhardtii 4 (NDA3) (XP_001702277), C. reinhardtii 5 (XP_001696489), C. reinhardtii 6 (NDA4) (XP_001703055), C. reinhardtii 7 (NDA5) (XP_001703055); Cyanidioschyzon merolae 1 (XP_005537068), C. merolae 2 (XP_005538302), and C. merolae 3 (XP_005535723); Galdieria sulphuraria 1 (XP_005704866), and G. sulphuraria 3 (XP_005705674); Ectocarpus siliculosus 1 (CBN78924), E. siliculosus 2 (CBN78752), E. siliculosus 3 (CBN76274), E. siliculosus 4 (CBJ25466), and E. siliculosus 5 (CBN8336); Synechocystis sp PCC 6803 ndbB (NP_441103), ndbC (NP_441407163), ndbA (NP_441107), slr0890 (NP_001073573), and slr0876 (NP_001087226). Klebsormidium flaccidum protein sequences were retrieved from the K. flaccidum genome project (http://www.plantmorphogenesis.bio.titech.ac.jp/-algae_genome_project/Klebsormidium/): K. flaccidum 1 (kfl00025_0340), K. flaccidum 2 (kfl00064_0190), K. flaccidum 3 (kfl00811_0040), K. flaccidum 4 (kfl00553_0030), and K. flaccidum 5 (kfl00123_0080).
(Bradford, 1976) using BSA as a standard. Aliquots of desalted proteins were flash frozen in liquid N₂ and stored at ~80°C.

**Enzymatic Assays**

Prior to assays, NDC1 and NdBΔ preparations were thawed and incubated overnight at 4°C in the presence of up to a 2-fold molar excess of FAD. For methyl transfer assays, methanolic solutions of demethylphyloquinone (44 to 130 pmoles) were evaporated to dryness in Pyrex screw-cap tubes. Assays (135 µL) contained demethylphyloquinone in 50 mM KH₂PO₄ (pH 7.5), 150 mM KCl, 0.2% (v/v) Tween 20, 0.37 mM AdoMet, 0.37 mM NADPH, 0 to 3.7 mM DTT, 0 to 250 mM FAD. For methyl transfer assays (1 mL) contained 50 mM HEPES/KOH (pH 7.5), methanol/dichloromethane (10:1) and analyzed by HPLC with 100% methanol and centrifuged (18,000 g). Eluates were evaporated to dryness, resuspended in 200 µL methanol, and phase-partitioned twice with 5 mL of methanol/dichloromethane (10:1) and analyzed by HPLC with fluorescence detection as previously described (Widhalm et al., 2012). Spectrophotometric assays (1 mL) contained 50 mM HEPES/KOH (pH 7.5), 0.05% (v/v) Tween 20, 100 µM NADPH, 0 to 500 µM of menadione, and 0 to 31 µg of NDC1 or ndBΔ. Inhibition assays with dicumarol were performed at a concentration of menadione of 20 µM for NDC1 and to 24 µM for NdBΔ, so that [dicumarol] = 2K for 1/NADPH oxidation = 0. Reactions were performed for 15 min at 30°C, and the oxidation of NADPH was monitored spectrophotometrically at 340 nm.

**Metabolite Quantification**

Synechocystis cells were directly harvested from BG-11 plates, resuspended in 200 µL of 0.09% (w/v) NaCl, and quantified by measuring the optical density at 730 nm. For naphthoquinone analysis, resuspended cells were transferred to Pyrex screw-cap tubes containing 1 mL of 0.1-mm zirconia/silica beads. 900 µL of 95% (v/v) ethanol, 400 µL of water, and 390 pmoles of menaquinone-4 as an internal standard, and then vortexed for 5 min. Prenylated naphthoquinones were phase-partitioned twice with 5 mL hexane and vigorous shaking. Hexane phases were combined and evaporated to dryness under nitrogen. Samples were resuspended in 300 µL of methanol/dichloromethane (10:1) and analyzed by HPLC with fluorescence detection as previously described (Widhalm et al., 2012). Phylloquinone was quantified according to external standards, and data were corrected for recovery of the internal standard of menaquinone-4. For plastocyanin and tocopherol analysis, 50 µL of resuspended cells were transferred to Pyrex screw-cap tubes containing 400 µL of 0.1-mm zirconia/silica beads, 550 µL of 100% (v/v) ethanol, and then vortexed for 5 min. Extracts were cleared by centrifugation (18,000g for 10 min) and analyzed by HPLC with fluorescence detection (plastoquinol-9, α- and γ-tocopherol, and phylloquinone-9) as previously described (Block et al., 2013). Chlophylls were quantified spectrophotometrically in acetone extracts of Arabidopsis leaves using the molar extinction coefficients of 75.05 m²·mol⁻¹·cm⁻¹ at 663 nm for chlorophyll a, and 47 m²·mol⁻¹·cm⁻¹ at 645 nm for chlorophyll b.

**Measurements of Photosynthetic Parameters**

Chlorophyll fluorescence was measured on detached and dark-adapted (30 min) leaves using a JTS-10 LED spectrometer (Bio-Logic Scientific Instruments). Actinic illumination (520 µmol photons m⁻² s⁻¹) was provided from green LEDs (520 nm; Bio-Logic Scientific Instruments) for 6 min to the adaxial surfaces of the leaves. The maximal fluorescence in the dark-adapted state (F₀) during actinic illumination and dark relaxation (Fₘ) were determined using a 250-ms saturating light pulse (9600 µmol photons m⁻² s⁻¹). Fₗ (initial fluorescence of dark-adapted leaves) and Fₘ (minimal fluorescence during actinic illumination and during dark relaxation) were measured with 10-µs weak detecting flashes. Fluorescence parameters were calculated as follows: F/Fₘ = (Fₗ - Fₘ)/Fₘ, where Fₘ is the calculated variable fluorescence; N/PQ = (Fₘ - Fₗ)/Fₘ; ΔPSII = (Fₗ - Fₘ)/Fₘ.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: Arabidopsis At1g23360 (MENG), At1g60550 (MENB), At1g30520 (MENA), At1g60600 (MENA), At1g908740 (NDC1), At1g32770 (VTE1), At2g18950 (VTE2), and At1g60570 (HPPD); rice CAE04724.1 (MENG), EAY75265 (MENA), EA205485 (MENA), NP_00104922 (MENA), NP_001057133 (NDC1), NP_001046545 (VTE1), B7FA90 (VTE2), and BAD226318 (HPPD); Synechocystis NP_441107 (NdbA), NP_441100 (NdbB), and NP_442910 (NdbC); and E. coli YP_026269 (MenG). Germlapsis used as follows: Arabidopsis demethylphyloquinone methytransferase knockout line GABI_565F06; T-DNA insertion lines GABI_565F06 (menG; At1g23360) and SALK_151963C (ito1; At3g50820). Synechocystis sp PCC 6803 deletion strains ΔndbA (str8051), ΔndbB (str8174), ΔndbC (str8148), and ΔΔΔndbABC were those described by Howitt et al. (1999), and deletion strain A. n. G. (str1653) was that described by Lohmann et al. (2006).

**Supplemental Data**

**Supplemental Figure 1.** Quantification of phylloquinone and of demethylphyloquinone in the leaves of wild-type and ito1 knockout plants.

**Supplemental Data Set 1.** Gene lists, including correlation ranks and functional annotations, of the top 500 coexpressors of MENG, NDC1, and VTE1 in Arabidopsis and rice.

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**AUTHOR CONTRIBUTIONS**


**REFERENCES**


A Dedicated Type II NADPH Dehydrogenase Performs the Penultimate Step in the Biosynthesis of Vitamin K₁ in Synechocystis and Arabidopsis
Abdelhak Fatihi, Scott Latimer, Stefan Schmollinger, Anna Block, Patrick H. Dussault, Wim F.J. Vermaas, Sabeeha S. Merchant and Gilles J. Basset

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**Supplemental Fig.1.** Quantification of phyloquinone and of demethylphyloquinone in the leaves of wild-type and *lto1* knockout plants. Same letter annotations above the error bars indicate that the corresponding values are not significantly different as determined by Fisher's least significant difference test from ANOVA (*p* > 0.1). Data are the means of 4 replicates ± SE.