A Dedicated Type II NADPH Dehydrogenase Performs the Penultimate Step in the Biosynthesis of Vitamin K\textsubscript{1} in Synechocystis and Arabidopsis

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Mutation of Arabidopsis thaliana NAD(P)H DEHYDROGENASE C1 (NDC1; At5g08740) results in the accumulation of demethylphylloquinone, a late biosynthetic intermediate of vitamin K\textsubscript{1}. 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 demethylphylloquinone 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 $\gamma$-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 phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone), also known as vitamin K\textsubscript{1}, and menaquinones [2-methyl-3-(all-trans-polyprenyl)-1,4-naphthoquinones], also known as vitamin K\textsubscript{2}. The occurrence of phylloquinone 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). Phylloquinone from plant-based foods is the major source of vitamin K in the Western diet (Booth and Suttie, 1998). Vitamin K-synthesizing organisms use phylloquinone or menaquinone as redox cofactors in their electron transport chains. For instance, in plants, phylloquinone serves as a light-dependent one-electron carrier within photosystem I from chlorophyll a to the iron-sulfur cluster Fx of the heterodimeric PsA/PsbB 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 phylloquinone is similar to that of menaquinones in $\gamma$-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. Phylloquinone 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 phylloquinone at the A\textsubscript{1} site of photosystem I (Semenov et al., 2000; Lefebvre-Legendre et al., 2007). By contrast, loss of phylloquinone 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 phylloquinone biosynthesis in plastids (Lohmann et al., 2006). The cognate mutant is viable because it accumulates demethylphylloquinone, and the latter can replace phylloquinone in photosystem I, albeit with some loss of photosynthetic efficiency (Lohmann et al., 2006).

Kicking 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 demethylphyloquinone instead of phyloquinone 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 demethylnapthoquinone 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 (Shestopalov et al., 1997).

Another possibility is that NDC1 is not directly involved in phyloquinone biosynthesis and that loss of function of this gene blocks methylation of the demethylnapthoquinone 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 accumulation of plastid genome stability, also triggers the absorption of phyloquinone (Xu et al., 2011). No biochemical work has been done on demethylnapthoquinone methyltransferases beyond the demonstration that S-adenosyl-L-methionine (AdoMet) is the methyl donor in cell extracts (Kaiping et al., 1984; Koike-Takeshita et al., 1997). Yet, understanding the mechanism of transmethylation of the naphthalenoid ring could be significant, for the accumulation of demethylated naphthoquinone intermediates in ndc1 plants or in γ-proteobacteria suggests a missing step in vitamin K biosynthesis.

Therefore, we examined the connection between type II NAD(P)H dehydrogenases and vitamin K biosynthesis by means of coexpression analyses in Arabidopsis and rice (Oryza sativa), plant-prokaryote comparative genomics, and reverse genetics in selected cyanobacterial species. We then dissected the molecular mechanism of the transmethylation of the naphthalenoid precursor of vitamin K and established the crucial role of dedicated type II NAD(P)H dehydrogenases in this reaction.

RESULTS

Expression of NDC1 Correlates Strongly with Expression of Phyloquinone Biosynthetic Genes

Searching the GeneCAT coexpression database (Mutwil et al., 2008) with Arabidopsis MENG and NDC1 as queries showed that these two genes share over half of their top 500 coexpressors (Figure 1). Remarkably, such a correlation of expression is significantly higher than that found between NDC1 and tocopherol cyclase (VTE1), which are known functional partners in the recycling of tocopherol; NDC1 and VTE1 share only 30% of their top 500 coexpressors (Figure 1A). Similar results were obtained in rice, where the NDC1 ortholog shares 54% of its top 500 coexpressors with MENG and only 11% with VTE1 (Figure 1A). Expanding these analyses to other phyloquinone and tocopherol biosynthetic genes via hierarchical clustering of coexpression indicated that NDC1 is more closely correlated with genes involved in phyloquinone biosynthesis than with those linked to tocopherol biosynthesis (Figure 1B). All together, these in silico reconstructions indicate that there is a conserved and intimate functional connection between NDC1 and phyloquinone biosynthesis in plants and that the loss of phyloquinone and the accumulation of demethylphyloquinone in the Arabidopsis ndc1 knockout is unlikely to be the result of a nonspecific 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 demethylnapthoquinone 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 co-regulate the expression of the dehydrogenases with that of menaquinone biosynthetic genes. Such arrangements recapitulate the high level of correlation of expression between NDC1 and phyloquinone 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 (ndbB) 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 nongenotypic polar effect on naphthoquinone production. Phyloquinone 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 phyloquinone and instead accumulated its demethylnapthoquinone precursor, just as is observed for the Synechocystis demethylnapthoquinone 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 chro-
manol ring of tocopherols.

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

Whereas under low light intensity the growth of *Synechocystis* ΔnddB 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 Piller et al., 2011). To investigate this, Arabi-
dopsis 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 (εPSII), which is proportional to the rate of CO$_2$ fixation (Baker, 2008), than the wild type (Figure 4C, Table 1). Such a phenotype of increased photo-
sensitivity, loss of chlorophylls, and decrease of photosystem II efficiency in response to high light treatment is reminiscent of that described for the Arabidopsis demethylated naphthoquinone methyltransferase knockout (Lohmann et al., 2006).

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**Figure 1.** Correlation Analyses of the Expression of Arabidopsis NDC1 and Its Rice Homolog.

(A) Venn diagrams of the top 500 coexpressing genes of NDC1 versus the top 500 coexpressing genes of MENG (EC2.1.1.163) or of tocopherol cyclase (VTE1; EC5.5.1.24) in Arabidopsis and rice. The annotated and intersecting gene lists, as well as their correlation ranks, are provided in Supplemental Data Set 1.

(B) Hierarchical clustering reconstruction of the coexpression profile of NDC1 (red) with that of phylloquinone (green) and tocopherol (orange) biosynthetic genes in Arabidopsis and rice. Distance matrices of Pearson correlation coefficients were converted to Newick string using the unweighted pair group method with arithmetic mean analysis. Numbers indicate branch lengths. Arabidopsis gene numbers and rice probe sets used as inputs are provided in Methods. HPPD, 4-hydroxyphenylpyruvate dioxygenase (EC1.13.11.27); MENA, 1,4-dihydroxy-2-naphthoate phytyltransferase (EC2.5.1.74); MENB, naphthoate synthase (EC4.1.3.36); MENE, o-succinylbenzoic acid-CoA ligase (EC6.2.1.26); VTE2, homogentisate phytyltransferase (EC2.5.1.115).
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).
ometrically. NDC1 displayed typical Michaelis-Menten kinetics, using menadione, a nonprenylated naphthoquinone derivative, as a substrate and could not be dispersed at concentrations greater than a few micromolar, a second assay was developed in an aqueous solution and could not be dispersed. The efficiency of 2.2 s⁻¹ for menadione and NADPH as substrates was 5.5 s⁻¹, which was typical of the Ki values reported for naphthoquinone oxidoreductases (4.1 to 10.6 s⁻¹) reported for other eukaryotic oxidoreductases using NADH or NADPH as electron donors (Sánchez et al., 2001; Wrobel et al., 2002). Similar results were obtained with recombinant ndbB: Its Km, Vmax, and catalytic efficiency values for menadione were 2.4 μM, 3.2 s⁻¹, and 133.3 × 10³ M⁻¹ s⁻¹, respectively (Figure 6D). 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).

**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 phyloquinone rather than phyloquinone 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 demethyl/naphthoquinol 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 Phyloquinone Biosynthesis in Plastids**

It has been established that the biosynthesis of phyloquinone 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 phytosyntlated and methylated (Figure 7). Even within plastids, some phyloquinone biosynthetic intermediates are trafficked, for the demethylnaphthoquinone phyto/transferase (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; Kaiping et al., 1984). Our evidence that the plastoglobule-localized NDC1 mediates the mandatory reduction of the naphthoquinone ring between the phytosyntlation and

**Table 1. Chlorophyll Levels and Calculated Fluorescence Parameters of Wild-Type and ndc1 Plants Exposed to Continuous High Light Intensity (800 μmol Photons m⁻² s⁻¹) for 3 d**

<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&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chlorophyll b (nmol g⁻¹ FW)</td>
<td>830 ± 15</td>
<td>693 ± 53&lt;sup&gt;b&lt;/sup&gt;</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>(\phi_{PSII})</td>
<td>0.321 ± 0.072</td>
<td>0.212 ± 0.079</td>
</tr>
</tbody>
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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.
methylated, the initial C3 of naphthoquinol becomes C2. Note that due to the change in numbering priority after methyl group of AdoMet (green) is bonded to a positively charged sulfur (A) AdoHcy, S

Modeling of the Mechanism of Methyl Transfer between AdoMet and the Naphthalenoid Ring of Vitamin K.

(A) The naphthoquinol ring serves as the methyl group acceptor. The methyl group of AdoMet (green) is bonded to a positively charged sulfur atom and is a strong electrophile; C3 of the naphthoquinol ring (red dot) is the nucleophile. Note that due to the change in numbering priority after the ring methylation, the initial C3 of naphthoquinol becomes C2.

(B) The naphthoquinone ring serves as the acceptor. Ado, adenosine; AdoHcy, S-adenosyl-L-homocysteine; R, prenyl.

(C) Methyl transfer assays using *E. coli* demethylnaphthoquinine methyltransferase with demethylphylloquinone and AdoMet as substrates, in the presence or absence of DTT. Assays contained 1 μM demethylphylloquinone, 370 μM AdoMet, and 5 μg of MenG and were performed for 1 h at 30°C. The final concentration of DTT in the DTT-containing assay was 3.7 mM. Note that quinol forms are not detected in this assay as they readily reoxidize during solid-phase extraction prior to chromatographic analysis. HPLC traces have been offset for clarity. DPhQ, demethylphylloquinone; PhQ, phylloquinone.

(D) and (E) *E. coli* MenG (D) and time dependence (E) of phylloquinone formation.

Figure 5. Modeling of the Mechanism of Methyl Transfer between AdoMet and the Naphthalenoid Ring of Vitamin K.

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 oxygenic 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 demethylenaphthoquinol.

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 ablation of 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 phylloquinone and accumulate demethylphylloquinone rules out that other type II NADPH dehydrogenases contribute significantly to phylloquinone 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 demethylphylloquinone 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 thylakoid-localized oxidoreductase, and its cyanobacterial ortholog dsbB, 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 phylloquinone biosynthesis. HPLC...
Figure 6. Assays of NDC1 with Naphthoquinones as Electron Acceptors.

(A) Coupled assays with demethylnaphthoquinone methyltransferase. Assays contained 0.52 μM demethylnaphthoquinone, 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 0 μg 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) nDBB-catalyzed oxidation of NADPH as a function of menadione concentration.

(E) Inhibition of nDBB 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.

analyses actually confirmed that the Arabidopsis ion1 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 Demethylnaenuquinones 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 demethylnaenuquinone. This also suggests that were strategies developed to engineer vitamin K2 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

Demethylnaphthoquinone 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 Biomedicals and menaquinone-4 from Sigma-Aldrich. Unless mentioned otherwise, other reagents were from Fisher Scientific.
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 (sll0851), ΔndbB (sll1743), ΔndbC (sll1484), 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 −80°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’-CACCATGGTGGAATGTCACAGAAA-3’ (forward) and 5’-TCAGAATTATAACCCAGATC-3’ (reverse). The Arabidopsis NDC1 cDNA (At5g08740) lacking its predicted N-terminal presequence was amplified from total leaf RNA by RT-PCR using the primers 5’-CACCATGGAGAGATTCTGTGAATGACAG-3’ (forward) and 5’-TCAGAATTATAACCCAGATC-3’ (reverse); the italicized sequence indicates the initiation codon introduced to truncate the targeting presequence (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’-GGTCTAGAATTCTGTGAATGACAG-3’ (forward) and 5’-GGGAATCTCAGAGTGATGATGATGATGATG-3’ (reverse). 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the OD\textsubscript{600} reached \textasciitilde 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 NaH\textsubscript{2}PO\textsubscript{4} (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 KH\textsubscript{2}PO\textsubscript{4} (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_567283), NDB3 (NP_199880), NDB4 (NP_179763), At5g22140 (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 moellendorffii 1 (XP_002976807), S. moellendorffii 2 (XP_002978367), and S. moellendorffii 3 (XP_002977754); Physcomitrella patens 1 (XP_001768284), P. patens 2 (XP_001769969), P. patens 3 (XP_001757660), P. patens 4 (XP_001764062), P. patens 5 (XP_001766162), and P. patens 6 (XP_001759207); Chlamydomonas reinhardtii 1 (NA053723), C. reinhardtii 2 (NA074576), C. reinhardtii 3 (NA01), C. reinhardtii 4 (NA034), C. reinhardtii 5 (XP_001696489), C. reinhardtii 6 (NA02) (XP_001703643), and C. reinhardtii 7 (NA06) (XP_001703055); Cyanidioschyzon merolae 1 (XP_005537068), C. merolae 2 (XP_005538002), and C. merolae 3 (XP_005535723); Galdieria sulphuraria 1 (XP_005704866), and G. sulphuraria 2 (XP_005704866); 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 (WP_014407163), ndbA (NP_441107), slr0876 (WP_010872226), Klebsormidium flaccidum protein sequences were retrieved from the K. flaccidum genome project (http://www.plantmorphogenesis.bio.titech.ac.jp/~alga_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 NdbB 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, mannoionic solutions of demethylphylloquinone (44 to 130 pmoles) were evaporated to dryness in Pyrex screw-cap tubes. Assays (135 µL) contained demethylphylloquinone 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 2.5 µM of MenG, and 0 to 86 µg of NDC1. Reactions were initiated by the addition of AdoMet and were incubated for 15 to 180 min at 30°C and then stopped with 500 µL 100% methanol and centrifuged (18,000 g, 10 min) and analyzed by HPLC with 0.1-mm zirconia/silica beads, 550 nmol of 100% methanol, and 24 µM of methanol/dichloromethane (10:1) and analyzed by HPLC with fluorescence detection as previously described (Widholm 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 ndbB. Inhibition assays with dicumarol were performed at a concentration of menadione of 20 µM for NDC1 and to 24 µM for NdbB, 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 0.9% (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 µL of menaquinone-4 as an internal standard, and then ruptured by vortexing for 5 min. Prelyated naphthoquinones were phase-purified in 5 mL hexane and vigorously shaking. Hexane phases were combined and evaporated to dryness in Pyrex screw-cap tubes. Assays (135 µL) contained demethylphylloquinone in 50 mM KH₂PO₄ (pH 7.5), 0.2% (v/v) Tween 20, 0.37 mM AdoMet, 0.37 mM NADPH, 0 to 2.5 µM of MenG, and 0 to 86 µg of NDC1. Reactions were initiated by the addition of AdoMet and were incubated for 15 to 180 min at 30°C and then stopped with 500 µL of 100% methanol and centrifuged (18,000 g, 10 min) and analyzed by HPLC with 0.1-mm zirconia/silica beads, 550 nmol of 100% methanol, and 24 µM of methanol/dichloromethane (10:1) and analyzed by HPLC with fluorescence detection as previously described (Widholm et al., 2012). Phylloquinone was quantified according to the number of NDC1, the internal standard of menaquinone-4. For plastoquinone 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. 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 (Fₚ) was measured after a 250 ms saturating light pulse (4600 µmol photons m⁻² s⁻¹). Fₚ was determined using a 250-ms saturating light pulse (8600 µmol photons m⁻² s⁻¹). Fₙ (minimal fluorescence during actinic illumination and during dark relaxation) were measured with 10-µs weak detecting flashes. Fluorescence parameters were calculated as follows: F/Fₚ = (Fₙ – Fₚ)/Fₚ, where F is the calculated variable fluorescence; NPO = ([Fₙ – Fₚ'])/Fₚ' ; &Delta;PSII = ([Fₚ' – Fₚ])/Fₚ'.

**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 (MENE), At1g60600 (MENA), At1g08740 (NDC1), At1g32770 (VTE1), At2g18950 (VTE2), and At1g06570 (HPPD; rice CAE04724.1 (MENG), EAY75265 (MENB), EA205485 (MENA), NP_00104922 (MENA), NP_001057133 (NDC1), NP_001046545 (VTE1), B7FA90 (VTE2), and BAD26248 (HPPD); Synechocystis NP_441107 (NdbA), NP_441103 (NdbB), and NP_442910 (NdbC); and E. coli YP_026269 (MenG). Germplasm used is as follows: Arabidopsis demethylphylloquinone methyltransferase knockout line GABI_565F06; T-DNA insertion lines GABI_565F06 (meng; At1g23360) and SALK_151963C (ito1; At3g50820). Synechocystis sp PCC 6803 deletion strains ΔnbdA (ΔnbdA), ΔnbdB (ΔnbdB), ΔnbdC (ΔnbdC), and ΔΔΔΔnbdABC were those described by Howitt et al. (1999), and deletion strain ΔmenG (ΔmenG) was as described by Lohmann et al. (2006).


A Dedicated Type II NADPH Dehydrogenase Performs the Penultimate Step in the Biosynthesis of Vitamin $K_1$ in *Synechocystis* and Arabidopsis

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