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

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A Dedicated Type II NADPH Dehydrogenase Performs the Penultimate Step in the Biosynthesis of Vitamin K₁ 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 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 demethylphylloquinone methyltransferase knockout. Chemical modeling and assay of purified demethylphylloquinone 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 demethylphylloquinone 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 phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone), also known as vitamin K₀, and menaquinones [2-methyl-3-(all-trans-polyprenyl)-1,4-naphthoquinones], also known as vitamin K₁. 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 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 PsaA/PsaB 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 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. Phylloquinone biosynthetic mutants in the cyanobacterium Synechocystis sp PCC 6803 and the green alga Chlamydomonas reinhardtii are still able to grow phototrophically, for these species recruit plastoquinone-9 in place of phylloquinone at the A₁ 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 demethylphylloquinone 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 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 demethylphylloquinone 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 phylloquinone biosynthesis and that loss of function of this gene blocks methylation of the demethylphylloquinone 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 triggers the accumulation of demethylphylloquinone (Xu et al., 2011). No biochemical work has been done on demethylphylloquinone methyltransferases beyond the demonstration that S-adenosyl-L-methionine (AdoMet) is the methyl donor in cell extracts (Kooping 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 a 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 Phylloquinone 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 phylloquinone and tocopherol biosynthetic genes via hierarchical clustering of coexpression indicated that NDC1 is more closely correlated with genes involved in phylloquinone 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 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 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 demethylphylloquinone 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 (ndbB) Blocks the Transmethylation of Demethylphylloquinone

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 (Hovitt 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 nonspecific 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 demethylphylloquinone precursor, just as is observed for the Synechocystis demethylphylloquinone 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 DndbB 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 Piller 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 (εPSII), 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 demethylnaphthoquinone 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, α-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; EC 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, phyloquinone, 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 phyloquinone 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), phyloquinone formation was markedly decreased if purified NDC1 was not preincubated with flavine adenine dinucleotide (FAD) (Figure 6A).

Figure 2. Gene Clustering of Type II NAD(P)H Dehydrogenase Homologs with Vitamin K Biosynthetic Genes in Bacterial Genomes. Homologs display matching color and number. 1, Type II NAD(P)H dehydrogenase (EC 1.6.99.3); 2, demethylnaphthoquinone methyltransferase (EC 2.1.1.163); 3, o-succinylbenzoate synthase (EC 4.2.1.113); 4, 1,4-dihydroxy-2-naphthyl-CoA synthase (EC 4.1.3.36); 5, 1,4-dihydroxy-2-naphthoate polypropenyl transferase (2.5.1.74); 6, polypropenyl diphosphate synthase (2.5.1.30). Black arrows indicate genes of hypothetical function or of function unrelated to naphthoquinone biosynthesis.

Figure 3. Synecochystis ndbB Is the Functional Homolog of NDC1. (A) Levels of phyloquinone (left panel) and of demethylphyloquinone (right panel) in wild-type Synecochystis, type II NAD(P)H dehydrogenase mutant strains ΔndbA, ΔndbB, ΔndbC, and ΔΔΔndbABC, and demethylnaphthoquinone methyltransferase ΔmenG knockout strain. Data are the means ± SE of three to four replicates. (B) Plastoquinol-9 (POH-9) and plastoquinone-9 (PO-9) content in wild-type and ΔndbB cells. Numbers above the bars indicate plastoquinol-9/plastoquinone-9 ratios. (C) α- and γ-tocopherol (TC) content in wild-type and ΔndbB cells. Data are the means of five to six replicates ± SE.
and the greater than a few micromolar, a second assay was developed in aqueous solution and could not be dispersed at concentrations ciency of 2.2

Phenotypes of Synechocystis

menadione were 2.4

flavin oxidoreductases (4.1 to 10.6

naphthoquinone oxidoreductases (Tie et al., 2011). The in-
markedly inhibited by dicumarol (Figure 6C), a competitive inhibitor of photosystem II in wild-type and

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Mutants.

Figure 4. Phenotypes of Synechocystis ΔndbB and Arabidopsis ndc1 Mutants.

(A) Growth of wild-type and ΔndbB Synechocystis cells under continuous low (30 μmol photons m⁻² s⁻¹) or high (800 μmol photons m⁻² s⁻¹) light intensities. Identical numbers of cells were plated on BG-11 medium without antibiotics and were incubated for 1 week at 30°C.

(B) Four-week-old wild-type and ndc1 Arabidopsis plants after 3 d of exposure to continuous high light (800 μmol photons m⁻² s⁻¹). Bar = 2 cm.

(C) Efficiency of photosystem II in wild-type and ndc1 Arabidopsis plants after 3 d of high light regime (800 μmol photons m⁻² s⁻¹). The open arrowhead indicates the end of actinic illumination.

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 nonrenpylaphthoquinone derivative, as a substrate and monitoring the oxidation of NADPH spectrophotometrically. NDC1 displayed typical Michaelis-Menten kinetics, and the K_m and K_cat 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 × 10³ 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_i) of NDC1 for dicumarol determined with menadione and NADPH as substrates was 5.5 μM, which was typical of the K_i values reported for naphthoquinone oxidoreductases (4.1 to 10.6 μM) assayed in similar conditions (Sánchez et al., 2001; Wrobé et al., 2002). Similar results were obtained with recombinant ndbB: Its K_m, K_cat, 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_i 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-succinylbenzoyl, is made from chro-minate in plastids (Figure 7). It is then exported to peroxisomes for its cyclization into 1,4-dihydroxyxaphthoate, 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; Kaiping et al., 1984). Our evidence that the plastoglobule-localized NDC1 mediates the mandatory reduction of the naphthoquinone ring to the phytlylation 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 |
|----------------|---------------------|---------------------|
| Level or Parameter | Wild Type | ndc1 |
| Chlorophyll a (nmoles g⁻¹ FW) | 1298 ± 38 | 1057 ± 36a |
| Chlorophyll b (nmoles g⁻¹ FW) | 830 ± 15 | 693 ± 53b |
| Fv/Fm | 0.726 ± 0.042 | 0.802 ± 0.038 |
| NPQ | 1.010 ± 0.214 | 1.909 ± 0.429 |
| ΔPSII | 0.321 ± 0.072 | 0.212 ± 0.079 |

Fluorescence parameters were calculated after 5 min of actinic illumination. Data are means of three (chlorophyll) or four (fluorescence parameters) biological replicates ± se. 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.
the ring methylation, the initial C3 of naphthoquinol becomes C2.

AdoMet and the Naphthalenoid Ring of Vitamin K.

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 phylloquinone and accumulate demethylphyloquinone 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 Physcomitrella patens 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, 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 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 phylloquinone biosynthesis. HPLC

methylation steps not only underscores the intricacy of the reactions of phylloquinone biosynthesis, but also points to a hitherto unsuspected constraint on the architecture of this pathway.

Figure 5. 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 demethylnaphthoquinone methyltransferase with demethylphyloquinone and AdoMet as substrates, in the presence or absence of DTT. Assays contained 1 μM demethylphyloquinone, 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.
analyses actually confirmed that the Arabidopsis to1 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 K 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 At1g23360 (MENG), At1g60550 (MENB), At1g30520 (MENE), At1g60600 (MENA), At5g08740 (NDC1), At4g32770 (VTE1), At2g18950 (VTE2), and At1g06570 (HPPD) and rice probe sets Os.490841. S1.at (NDC1), Os.6179.1.S1.at (MENG), Os.18701.1.S1.at (VTE1), Os.19161.1.S1.at (MENE), Os.52556.1.S1.at (MENA), Os.35677.1.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 phy tool. Degradation tracks were obtained using 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. (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 incubation 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) 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.
Steps are identified by their recommended Enzyme Commission numbers. Note that in plants, 2.2.1.9, 4.2.99.20, and 4.2.1.113 belong to a single multifunctional enzyme and that OSB-CoA ligase (6.2.1.26) appears to be dual targeted to chloroplasts and peroxisomes (van Oostende et al., 2011). There is also evidence suggesting that OSB-CoA doubles as an intermediate in the biosynthesis of anthraquinones (Heide et al., 1982). Dashed arrows and question marks indicate multiple and hypothetical steps, respectively. DHNA, 1,4-dihydroxynaphthoate; OSB, o-succinylbenzoate.

**Biological Material and Growth Conditions**

Seeds of Arabidopsis T-DNA insertion line GABI_565F06 (menG) and SALK_151963C (ito1) 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 (slr0851), ΔndbB (slr1743), ΔndbC (slr1484), and ΔΔΔndbABC were described by Howitt et al. (1999), and deletion strain ΔmenG (slr1653) 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'-CACCATGGTGATGAATTCACAAAGAAA-3' (forward) and 5'-TCAGATCCTCTACGATG-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'-CACCAGACAGAGATTCTGATAATGAAACAG-3' (forward) and 5'-TCAGAGACACAGAAACCTT-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'-GCCTAGAAATAATTTGGGTTATTTAGAAAGAGATACCTAG-3' (forward) and 5'-GGAAATTCTCAATGATGATGATGATGAGGCAAACCTT-3' (reverse), which contained the XbaI and EcoRI 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) pLysS (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-thiogalactopyranoside (500 μM) was added when
the OD₆₀₀ 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.
Enzymatic Assays

Prior to assays, NDC1 and NdbB preparations were thawed and incubated overnight at 4°C in the presence of up to 2-fold molar excess of FAD. For methyl transfer assays, methanolic solutions of demethylphylloquinone (44 to 130 pmol) 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 3.7 mM DTT, 0 to 25 µg 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 µM 0.05% (v/v) Tween 20, 100 µM methanol and centrifuged (18,000 g for 5 min). Supernatants (50 µL) were loaded onto Supelclean LC-18 SPE (Supelco) solid-phase extraction columns, and prenylated naphthoquinones were eluted with 2 mL 100% methanol. Eluates were evaporated to dryness, resuspended in 200 µL 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 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 0.09% (v/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. Preynlated 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 plastoquinone and tocochromanol 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 plastoehromanol-8) or diode array detection (plastoehromanol-9) as previously described (Block et al., 2013). Chlorophylls were quantiﬁed spectrophotometrically in acetone extracts of Arabidopsis leaves using the molar extinction coefﬁcients of 75.05 mM⁻¹ cm⁻¹ at 663 nm for chlorophyll a, and 47 mM⁻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 (Fₒ) during actinic illumination and dark relaxation (Fₘ) were determined using a 250-ms saturating light pulse (9600 µmol photons m⁻² s⁻¹), Fₘ (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: Fₘ/Fₐ = (Fₘ – Fₘ)/Fₐ, where Fₐ is the calculated variable ﬂuorescence; NPQ = (Fₘ – Fₐ)/Fₐ; αPSII = (Fₘ/Fₐ)²/Fₐ.

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), At4g32770 (VTE1), At2g18950 (VTE2), and At1g06570 (HPPP); rice CAE04724.1 (MENG), EAY75265 (MENB), EA205485 (MENE), NP_00104922 (MENA), NP_001057133 (NDC1), NP_001046545 (VTE1), B7FA90 (VTE2), and BAD26248 (HPPP); Synechocystis NP_441107 (NdbA), NP_441103 (NdbB), and NP_442910 (NdbC); and E. coli YP_026269 (MenG). 

Supplemental Data

Supplemental Figure 1. Quantiﬁcation of phylloquinone and of demethylphylloquinone 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

A.F., S.L., S.S., A.B., and G.J.B. designed and performed the research, analyzed the data, and wrote the article. P.H.D. designed the research and analyzed the data. W.F.J.V. contributed materials and analyzed the data. S.S.M. analyzed the data.

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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.