Phylloquinone (vitamin K$_1$) biosynthesis in plants: two peroxisomal thioesterases of lactobacillales origin hydrolyze 1,4-dihydroxy-2-naphthoyl-coa

Joshua R. Widhalm$^1$, Anne-Lise Ducluzeau$^1$, Nicole E. Buller$^2$, Christian G. Elowsky$^1$, Laura J. Olsen$^2$ and Gilles J. C. Basset$^{1,*}$

$^1$Center for Plant Science Innovation, University of Nebraska-Lincoln, Lincoln, NE 68588, USA, and $^2$Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA

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*For correspondence (e-mail gbasset2@unl.edu).

SUMMARY

It is not known how plants cleave the thioester bond of 1,4-dihydroxy-2-naphthoyl-CoA (DHNA-CoA), a necessary step to form the naphthoquinone ring of phylloquinone (vitamin K$_1$). In fact, only recently has the hydrolysis of DHNA-CoA been demonstrated to be enzyme driven in vivo, and the cognate thioesterase characterized in the cyanobacterium Synechocystis. With a few exceptions in certain prokaryotic (Sorangium and Opitutus) and eukaryotic (Cyanidium, Cyanidioschyzon and Paulinella) organisms, orthologs of DHNA-CoA thioesterase are missing outside of the cyanobacterial lineage. In this study, genomic approaches and functional complementation experiments identified two Arabidopsis genes encoding functional DHNA-CoA thioesterases. The deduced plant proteins display low percentages of identity with cyanobacterial DHNA-CoA thioesterases, and do not even share the same catalytic motif. GFP-fusion experiments demonstrated that the Arabidopsis proteins are targeted to peroxisomes, and subcellular fractionations of Arabidopsis leaves confirmed that DHNA-CoA thioesterase activity occurs in this organelle. In vitro assays with various aromatic and aliphatic acyl-CoA thioester substrates showed that the recombinant Arabidopsis enzymes preferentially hydrolyze DHNA-CoA. Cognate T-DNA knock-down lines display reduced DHNA-CoA thioesterase activity and phylloquinone content, establishing in vivo evidence that the Arabidopsis enzymes are involved in phylloquinone biosynthesis. Extraordinarily, structure-based phylogenies coupled to comparative genomics demonstrate that plant DHNA-CoA thioesterases originate from a horizontal gene transfer with a bacterial species of the Lactobacillales order.

Keywords: Arabidopsis, chloroplast, hotdog-fold, peroxisome, phylloquinone, Synechocystis.

INTRODUCTION

In plants and certain species of cyanobacteria, phylloquinone (2-methyl-3-phytyl-1,4-naphtho-quinone or vitamin K$_1$; Figure 1a) is a vital redox co-factor required for electron transfer in photosystem I and the formation of protein disulfide bonds (Brettel et al., 1986; Sigfridsson et al., 1995; Singh et al., 2008; Furt et al., 2010; Karamoko et al., 2011). A closely related form called menaquinone [2-methyl-3-(all-trans-poly-prenyl)-1,4-naphthoquinone or vitamin K$_2$] is synthesized by red algae, diatoms, and most archaeal and bacterial species (Collins and Jones, 1981; Yoshida et al., 2003; Ikeda et al., 2008). In vertebrates, vitamin K is needed for blood coagulation, bone and vascular metabolism, and signaling (Booth, 2009). For humans in particular, the phylloquinone of green leafy vegetables and vegetable oils, such as that of Glycine max (soybean), Helianthus annuus (sunflower), Olea europaea (olive), and Brassica sp. (canola), is the main contributor of dietary vitamin K (Booth and Suttie, 1998).

Despite the importance of phylloquinone in photosynthesis and human nutrition, the molecular architecture of its biosynthesis in plants has only recently been explored. The immediate precursor of the redox active naphthoquinone ring of phylloquinone is chorismate (Figure S1). It is first isomerized to serve as a substrate for an atypical multi-functional enzyme, termed PHYLLO, that catalyzes sequential steps of addition, elimination and aromatization, suggestive of a channeling mechanism (Gross et al., 2006). The product from PHYLLO, o-succinylbenzoate, is
then activated by ligation with CoA and cyclized, yielding the CoA thioester of 1,4-dihydroxy-2-naphthoate (DHNA). DHNA-CoA is subsequently hydrolyzed, and DHNA is prenylated and methylated (Shimada et al., 2005; Lohmann et al., 2006; Kim et al., 2008). In agreement with radiolabeling assays showing that the prenylation and methylation reactions are associated with plastidial membranes (Schultz et al., 1981; Gaudillière et al., 1984; Kaiping et al., 1984), several enzymes involved in the formation of the naphthoquinone ring and its subsequent conjugation to the phytol moiety have been shown to occur in the chloroplast (Shimada et al., 2005; Gross et al., 2006; Lohmann et al., 2006; Strawn et al., 2007; Garcia et al., 2008; Kim et al., 2008). This apparent all-plastidial localization of the phylloquinone biosynthetic pathway has nevertheless recently been challenged by proteomic studies that identified homologs of prokaryotic DHNA-CoA synthase in Arabidopsis. Searching the Pfam database of protein families (Finn et al., 2010), we show that these plant enzymes are not orthologous to cyanobacterial DHNA-CoA thioesterase orthologs but are classified in two separate phylogenetic subfamilies having distinct catalytic residues and quaternary structures (Benning et al., 1998; Thoden et al., 2002; Cantu et al., 2010).

In this study, we identified two Arabidopsis members of the 4HBT family encoding highly specific DHNA-CoA thioesterases that are targeted to peroxisomes and participate in phylloquinone biosynthesis. Using phylogenetic reconstructions, we show that these plant enzymes are not orthologous to cyanobacterial DHNA-CoA thioesterase, and probably originate from a lateral gene transfer from a bacterium of the Lactobacillales order.

**RESULTS**

**Arabidopsis genes At1g48320 and At5g48950 encode for members of the 4HBT family that fully complement synecochystis DHNA-CoA thioesterase knock-out**

Searching the Pfam database of protein families (Finn et al., 2010) for entries containing a predicted 4HBT domain identified 12 hotdog-fold proteins in Arabidopsis. Cognate full-length cDNAs were obtained for all of them, except the putative product of gene At1g68280 (see the Experimental procedures). Mining expressed sequence tag and microarray databases did not detect any hits for At1g68280 either. Analysis of the genomic context of At1g68280 showed that this gene actually occurs as a tandem repeat of gene At1g68280 (the corresponding deduced proteins being 83% identical), indicative of a recent event of gene duplication. Because such features typify At1g68280 as a pseudogene, it was not investigated further. The other 11 cDNAs were individually subcloned into expression vector pSynExp-2 under the control of the cyanobacterial psbA2 promoter (Sattler et al., 2003) and introduced into *Synechocystis* strain Δslr0204. This strain is a DHNA-CoA thioesterase.
knock-out that lacks phylloquinone and is photosensitive (Widhalm et al., 2009). Out of the 11 aforementioned cDNAs, two corresponding to genes At1g48320 and At5g48950 restored cell growth at high-light intensities, as did the reintroduction of gene Slr0204, providing initial genetic evidence for the existence of functional plant DHNA-CoA thioesterases (Figure 2a). The deduced At1g48320 and At5g48950 proteins share 63% identity and 78% similarity, indicating that they are likely paralogs. Remarkably, each of these Arabidopsis proteins shows low percentages of homology (approximately 15% identity/approximately 30% similarity) with Synechocystis Slr0204. Only cells expressing the At1g48320, At5g48950 and endogenous Slr0204 proteins displayed phylloquinone levels similar to that of the wild-type reference strain (Figure 2b). The At1g48320 and At5g48950 proteins, previously named small thioesterase type 1 and 2, respectively (Reumann et al., 2004a), and are de facto listed in the AraPerox database of putative proteins of Arabidopsis peroxisomes (http://www3.uis.no/araperoxv1; Reumann et al., 2004b). Similarly, a survey of the TAIR database (http://www.arabidopsis.org/index.jsp) confirmed that signature fragments of AtDHNAT1 have been identified through large-scale proteomic experiments in purified peroxisomes, and that the N-terminally eYFP-tagged protein is targeted to this organelle (Reumann et al., 2009). To verify the subcellular localization of AtDHNAT2, its full-length cDNA was fused to the C-terminal end of GFP. Co-expression of this fusion protein with an RFP-tagged peroxisomal marker resulted in a distinctive punctate pattern and co-localization of the green and red pseudocolors in peroxisomes (Figure 3a,b,d). To confirm these findings, chloroplasts, mitochondria and peroxisomes were isolated from Arabidopsis leaves, using assays of marker enzymes to monitor the integrity and enrichment of each organelle preparation (Table 1). Of the three purified organelles, DHNA-CoA thioesterase activity was detected only in peroxisomes (Table 1). Enrichment and recovery of DHNA-CoA thioesterase activity (approximately threefold and approximately 2%, respectively) were similar to those of catalase (approximately fivefold and approximately 2%, respectively). These data indicate that Arabidopsis DHNA-CoA thioesterase activity occurs in peroxisomes, but not in plastids and mitochondria, and coincides with the subcellular localization of AtDHNAT1 and AtDHNAT2.

AtDHNAT1 and AtDHNAT2 display marked substrate preference for DHNA-CoA in vitro

To study the substrate specificity of AtDHNAT1 and AtDHNAT2, 6xhis-tagged versions were expressed in Escherichia coli and purified by affinity chromatography (Figure S2). The DHNA-CoA thioesterase-specific activities measured in crude extracts of clones harboring AtDHNAT1 and AtDHNAT2 were 8- and 38-fold higher than that of the control cell extract, respectively (Table S1). The purified enzymes displayed comparable DHNA-CoA thioesterase specific activity to Synechocystis Slr0204 DHNA-CoA thioesterase assayed at a similar substrate concentration, i.e. 74–118 μmol h⁻¹ mg⁻¹ for AtDHNAT1 and AtDHNAT2 at 90 μM DHNA-CoA versus 102 μmol h⁻¹ mg⁻¹ for Slr0204 at 65 μM DHNA-CoA (Figure 4; Widhalm et al., 2009). AtDHNAT2 also displayed activity towards...
benzoyl-CoA, although the measured value was approximately an order of magnitude lower than that obtained with DHNA-CoA as the substrate (Figure 4). All the other activities measured with the aromatic acyl-CoA thioesters and the short-chain aliphatic acyl-CoA thioester succinyl-CoA were detected at trace levels (Figure 4). No activity was detected against the long-chain aliphatic acyl-CoA thioester palmityl-CoA (Figure 4). AtDHNAT1 and AtDHNAT2 thus appear to have marked substrate preference for DHNA-CoA, and in that regard resemble cyanobacterial DHNA-CoA thioesterase (Widhalm et al., 2009).

DHNA-CoA thioesterase and marker enzyme activities were assayed in crude extracts (CEs), stromal fraction of percoll-purified chloroplasts (CPs), matrix fraction of percoll-purified mitochondria (MT) and matrix fraction of percoll-purified peroxisomes (PXs) of Arabidopsis leaves. NADP-linked glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fumarase and catalase were used as marker enzymes for chloroplasts, mitochondria and peroxisomes, respectively. Data are means of three biological replicates ± SEs, except for the marker assays on peroxisomes, for which single measurements were performed.

<table>
<thead>
<tr>
<th>Substrate [90 μM]</th>
<th>AtDHNAT1</th>
<th>AtDHNAT2</th>
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<tbody>
<tr>
<td>DHNA-CoA</td>
<td>74 ± 21</td>
<td>118 ± 23</td>
</tr>
<tr>
<td>Benzoyl-CoA</td>
<td>1.2 ± 0.46</td>
<td>15.1 ± 4.7</td>
</tr>
<tr>
<td>Phenylacetyl-CoA</td>
<td>0.48 ± 0.2</td>
<td>0.53 ± 0.2</td>
</tr>
<tr>
<td>Succinyl-CoA</td>
<td>2.1 ± 0.82</td>
<td>2.3 ± 0.17</td>
</tr>
<tr>
<td>Palmitoyl-CoA</td>
<td>&lt; 0.001</td>
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AtDHNAT1 and AtDHNAT2 participate in phylloquinone biosynthesis in Arabidopsis

Two T-DNA lines from the SAIL collection (Sessions et al., 2002) corresponding to insertions located in the first exon of

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**Figure 3.** Subcellular localization of AtDHNAT2.
(a) green pseudocolor of GFP-tagged AtDHNAT2.
(b) red pseudocolor of peroxisomal marker RFP-tagged 3-keto-acyl-CoA thiolase 2 (KAT2; fragment 1–99).
(c) blue pseudocolor of plastid autofluorescence.
(d) overlay.

**Figure 4.** Substrate specificity of AtDHNAT1 and AtDHNAT2. Purified recombinant AtDHNAT1 and AtDHNAT2 (0.013–2.700 μg) were assayed with various aromatic and aliphatic acyl-CoA-thioester substrates, all at the concentration of 90 μM. DHNA-CoA thioesterase activity was monitored by direct quantification of DHNA using HPLC with diode array and fluorescence detection; the hydrolysis of the other substrates was measured spectrophotometrically by derivatization of free CoA-SH with the thiol-reagent DTNB. Data are means ± SEs of three biological replicates.
AtDHNAT1 (SAIL_1253_B02) and in the second exon of AtDHNAT2 (SAIL_315_C08) were identified, and confirmed by DNA genotyping (Figure 5a,b). Yet, RT-PCR experiments using primer pairs designed to amplify cDNA regions located after and before the T-DNA insertions of the atdh-nat1 and atdh-nat2 mutants, respectively (Figure 5a), demonstrated that none of these loci were null (Figure 5c). However, each insertion line displayed a marked reduction in DHNA-CoA thioesterase specific activity compared with wild-type controls, indicating that the atdh-nat1 and atdh-nat2 T-DNA loci are not fully functional (Figure 6a). The loss in DHNA-CoA thioesterase activity ranged from approximately 60% for atdh-nat1 AtDHNAT2 and atdh-nat1 atdh-nat2–22% for AtDHNAT1 atdh-nat2 (Figure 6a). The remaining DHNA-CoA thioesterase activity was found to still co-purify with peroxisomes in the double knock-down mutant (Table 2). Compared with wild-type plants, phylloquinone levels were reduced by 34 and 33% in atdh-nat1 AtDHNAT2 and atdh-nat1 atdh-nat2, respectively, and by 18% in AtDHNAT1 atdh-nat2 (Figure 6b). This reduction in phylloquinone content was
**DISCUSSION**

We have identified two Arabidopsis cDNAs specifying peroxisomal DHNA-CoA thioesterases involved in the biosynthesis of phylloquinone. It is noteworthy that neither of them correspond to the four plastid-targeted proteins that we had previously proposed as putative Arabidopsis DHNA-CoA thioesterases based on homology searches with the cyanobacterial enzyme (Slr0204; Widhalm et al., 2008). Of those, three (At1g68260, At1g35250 and At1g35290) fail to complement the Synechocystis DHNA-CoA thioesterase knock-out, and in fact are orthologous to recently characterized *Solanum lycopersicum* (tomato) methylketone synthases involved in the biosynthesis of 3-ketoacid volatiles (Yu et al., 2010). The fourth one, At1g68280, a paralog of At1g68260, probably corresponds to a pseudogene.

DHNA-CoA thioesterase activity occurs in peroxisomes, thus establishing definitive evidence for a split of the phylloquinone biosynthetic pathway between this organelle and plastids. This arrangement is apparently not specific to Arabidopsis, for DHNA-CoA thioesterase activity is not detectable in plastids isolated from *Pisum sativum* (pea) seedlings either, one of the best sources for obtaining intact and highly pure chloroplasts (Claine, 1986), whereas it is readily measured in the initial whole extract (Table S4). In addition, orthologs of AtDHNAT in monocots, gymnosperms, mosses and Lycopodiophytes all display canonical peroxisomal targeting signals of type 1 (Figure S4). A tacit conclusion is that yet-to-be identified transport steps between plastids and peroxisomes are involved in the biosynthesis of phylloquinone. (Specifically for DHNA, an a priori reason against passive diffusion is the redox-active

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**Table 2** DHNA-CoA thioesterase activity in double knock-out mutant *atdhnat1 atdhnat2*

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<td>GAPDH</td>
<td>0.53 ± 0.0</td>
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<tr>
<td>Fumarase</td>
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<td>40 ± 19</td>
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<td>Catalase</td>
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Extracts of the double mutant atdhnat1 atdhnat2 still contain about 40% of the wild-type DHNA-CoA thioesterase activity, but the atdhnat1 and atdhnat2 T-DNA loci are not null. A detailed inspection of the AtDHNAT1 sequence shows that an ATG codon (nucleotide position 49) located 19 nucleotides after the predicted T-DNA insertion of SAIL_1253_B02 could actually serve as an alternative start codon (Figure S5). Similarly, mining the NCBI cDNA database identifies an alternatively spliced version of the AtDHNAT2 transcript (NM_203182) containing an in-frame stop codon (nucleotide position 932) located 122 nucleotides before the predicted T-DNA insertion of SAIL_315_C08 (Figure S5). Notably, both of the atdhnat1 and atdhnat2 alternative mRNAs would encode intact catalytic motifs (Figure S5). We also investigated the possibility that the gene At5g48370, which partially complements the Synechocystis slr0204 knock-out mutant (Figure 2), encodes for an additional plant DHNA-CoA thioesterase. A cognate T-DNA knock-out line (SALK_122483) was isolated, genotyped and confirmed by RT-PCR, but it did not display any statistically significant differences in phylloquinone content compared with wild-type Arabidopsis (Figure S6).

Besides invalidating their classification as Paal enzymes, a structure-adjusted phylogenetic reconstruction established plant DHNA-CoA thioesterases as new functional members

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**Figure 7.** Land-plant DHNA-CoA thioesterases are not of cyanobacterial descent.

(a) Non-exhaustive reconstruction of the structure-based maximum likelihood phylogeny of Sir0204-type, Paal-type and 4HBT-II-type hotdog thioesterases using the MABL website (http://www.phylogeny.fr). PDB numbers of reference structures are given in brackets and alignments are provided in Figure S3. Full names and taxonomic origin of species, and protein accession numbers, are listed in Table S3. Red arrows point to enzymes for which there is experimental evidence of DHNA-CoA activity.

(b) Vitamin K biosynthetic gene clusters mined from the NCBI genomic database and SEED resources for comparative genomics (http://the-seed.uchicago.edu/FIG/index.cgi). Cyanobacterial, red algae and euglyphida gene clusters are modified from Widhalm et al. (2009). Matching colors and numbers indicate orthology. 1, DHNA-CoA thioesterase; 2, OSB-CoA ligase; 3, OSB synthase; 4, DHNA prenyltransferase; 5, isochorismate synthase; 6, SHCHC synthase; 7, DHNA-CoA synthase; 8, SEPHCHC synthase.
of the TE11/4HBT-II-type subfamily of hotdog-fold CoA thioesterases. That plant and cyanobacterial DHNA-CoA thioesterases display similar substrate specificity while having radically different catalytic sites exemplifies that, in the hotdog-fold superfamily, functions cannot be assigned on the basis of a mere comparison of primary sequences if the organisms are phylogenetically too distant. Most importantly, unlike their rhodophytes and euglyphidae counterparts, plant DHNA-CoA thioesterases are not of cyanobacterial ancestry. Instead, they are monophyletic with *Lactobacillales* orthologs that are encoded in clusters of vitamin K biosynthetic genes. It therefore seems very probable that the nuclear-encoded plant DHNA-CoA thioesterase originates from an event of horizontal gene transfer with a menaquinone-synthesizing bacterium of the *Lactobacillales* order. There is actually a similar precedent for this erase originates from an event of horizontal gene transfer via vitamin K biosynthetic genes. It therefore seems very likely that the organisms are phylogenetically too distant. Most importantly, unlike their rhodophytes and euglyphidae counterparts, plant DHNA-CoA thioesterases are not of cyanobacterial ancestry. Instead, they are monophyletic with *Lactobacillales* orthologs that are encoded in clusters of vitamin K biosynthetic genes. It therefore seems very probable that the nuclear-encoded plant DHNA-CoA thioesterase originates from an event of horizontal gene transfer with a menaquinone-synthesizing bacterium of the *Lactobacillales* order. There is actually a similar precedent for this.

**EXPERIMENTAL PROCEDURES**

**Chemicals and reagents**

DHNA-CoA was synthesized as described in Widhalm et al. (2009). DHNA, benzoyl-CoA, phenylacetyl-CoA and menaquinone-4 were obtained from Sigma-Aldrich (http://www.sigmaaldrich.com). Phylloquinone was obtained from MP Biomedicals (http://www.mpbio.com). Unless otherwise mentioned, all other reagents were from Fisher Scientific (http://www.fishersci.com).

**Functional complementation of synechocystis**

Arabidopsis cDNA clones G61320 (At1g48320), U89448 (At5g48950), G21545 (At3g61200), G12298 (At1g04290), G60738 (At2g29590), G13733 (At3g16175), G67253 (At5g48370), G67273 (At2g30720), U14083 (At1g86280), U84163 (At1g35250) and U14921 (At1g35290) were obtained from the Arabidopsis Biological Resource Center. No clone was available for At1g68280, and no corresponding cDNAs were obtained from Sigma-Aldrich (http://www.sigmaaldrich.com). Phylloquinone was obtained from MP Biomedicals (http://www.mpbio.com).Unless otherwise mentioned, all other reagents were from Fisher Scientific (http://www.fishersci.com).

**Plant material and growth conditions**

Arabidopsis T-DNA insertion lines SAIL_1253_B02 (At1g48320) and SAIL_315_C08 (At5g48950) were obtained from the Arabidopsis Biological Resource Center at the Ohio State University (http://abrc.osu.edu). Seeds were allowed to germinate on Murashige-Skoog solid medium and were transferred to potting mix in a growth chamber at 22 °C (100 µM m⁻² s⁻¹) with 16-h days for 6 weeks. The double knock-out mutant was obtained by crossing individual homozygous mutants. For the preparation of chloroplasts and mitochondria, Arabidopsis seedlings (Col-0) were grown at 22 °C (100 µM m⁻² s⁻¹) with 10-h days for 2 weeks. For the isolation of peroxisomes, Arabidopsis plants were grown with 16-h days for 4 weeks.

**Plant genotyping and semi-quantitative RT-PCR analyses**

Arabidopsis plants were genotyped using the following primers: LP1, 5'-ATCCATCTCTGAAACCTGC-3', RP1, 5'-GTGCTTACAG GAGTGCTCTCG-3' (SAIL_1253_B02); LP2, 5'-CATCCATCTGTTGATCCA CGTG-3', RP2, 5'-TGTGTGATGAAATCTGTTG-3' (SAIL_315_C08); LP3, 5'-GCTGGAATCTTGCAAGAAATCTG-3', RP3, 5'-TCTTCA CCAACCATGATTTC-3' (SALK_122483); and T-DNA specific primer LB2, 5'-GCTTCTATTATATCTTCCCCCAATTCACTA-3' (SAIL lines) or LB1, 5'-GCTTGGACCCTTTGCTGCAACT-3' (SALK line). Total RNA from Arabidopsis leaves were extracted using the SV Total RNA Isolation System (Promega, http://www.promega.com). PCR was performed on cDNAs prepared from 50 ng of total RNA using the following gene-specific primers: AtDHNA1, 5'-CCTTGGTTTCTCCGTC-3' (RTfwd) and 5'-TACAACCTTGTGCCAGCA TT-3' (RT1rva); AtDHNA2, 5'-CTGATCCAAATCTCCGCG-3' (RTfwd); and 5'-CGCGGAATCTTTTCCAGTCTC-3' (RT2rva); At5g48370, 5'-GAATCTCTTCTCGATCCTC-3' (RTfwd) and 5'-CCTTCTGTCACCTCTCCTC-3' (RTrva); actin control, 5'-CTAAAG TCTCAAGATCAAAGGC-3' (forward) and 5'-TTAACATTGCAAAG AGTTTCAAGG-3' (reverse).

**Purification of Arabidopsis organelles**

For the isolation of chloroplasts and mitochondria, 2-week-old Arabidopsis seedlings were de-starched for 18 h prior to tissue disruption. Chloroplasts were purified on a Percoll-gradient as described by Weigel and Glazebrook (2002), except that ascorbate and bovine serum albumin were omitted from the extraction and wash buffers. The procedure for the preparation of mitochondria was modified from Douce et al. (1987). For that, 13 g of leaves was homogenized in 75 ml of homogenization buffer (20 mM sodium pyrophosphate-HCl, pH 7.5, 1 mM EDTA, 300 mM mannitol) in a regular blender (three 5-s pulses). All steps were performed at 4 °C. The homogenate was filtered through one layer of miracloth, and the remaining solid material was re-extracted with 25 ml of homogenization buffer. The filtrate was centrifuged (1500 g for 10 min), and the supernatant was collected to be re-centrifuged (10 000 g for 20 min). The resulting pellet was recovered and resuspended in 2 ml of sample buffer (10 mM HEPES-KOH, pH 7.2, 1 mM EDTA, 300 mM mannitol). The sample (2 x 1 ml) was then layered over a discontinuous density gradient consisting of 60% (1.5 ml) and 28% (6 ml) of percoll prepared in sample buffer. After centrifugation (41 000 g for 40 min), the mitochondrial fractions (approximately 2 x 1.5 ml) were collected at the interface of the percoll layers. Peroxisomes from wild-type Arabidopsis plants were prepared as described in Reumann et al. (2008), whereas those from the double *athnat1 dhnat2* knock-out mutant were prepared as described in Harrison-Lowe and Olsen (2006). Marker enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fumarase...
and catalase were assayed as described by Oostende et al. (2008). For recovery and enrichment calculations, DHNA-CoA thioesterase activity was measured in the desalted crude extracts from the chloroplast preparation.

Subcellular localization

Full-length At5g48950 cDNA was subcloned into pK7WG2 (Karimi et al., 2002) using Gateway™ technology, resulting in an in-frame fusion with the C-terminal end of GFP. A Kat2-eqFP611 peroxisomal marker cassette, encoding for a C-terminal fusion of Arabidopsis 3-ketoacyl-CoA thiolase 2 (residues 1–99) with GFP under the control of the 35S promoter (Forner and Binder, 2007), was subcloned into the EcoRI and Pst sites of binary vector pZP212 (Hajdukiewicz et al., 1994). 35S::GFP-AtDHNAT2 and 35S::Kat2-eqFP611 constructs were individually electroporated into Agrobacterium tumefaciens CS8C1, for subsequent co-infiltration into the leaves of Nicotiana benthamiana. Epidermal cells were imaged by confocal microscopy 2 days later.

Preparation of Arabidopsis crude protein extracts

Arabidopsis leaves (0.75 g) were flash-frozen in liquid nitrogen and ground to a fine powder with pestle and mortar. The powder was transferred to a 40-ml screw-cap tube and thawed with 2.25 ml of 50 mM freshly prepared DTT. Samples were centrifuged (10 000 g, 30 min at 4°C) to pellet debris. Supernatants were recovered and desalted on a PD-10 column equilibrated in 100 mM KH2PO4 (pH 7.0), 5 mM DTT and 10% glycerol (vol/vol).

Expression and purification of recombinant enzymes

At1g48320 and At5g48950 cDNAs were subcloned minus their stop codons using Gateway™ technology in expression vector pET-DEST42 (Invitrogen, http://www.invitrogen.com) for C-terminal fusion with a 6xHis tag. Constructs were introduced into E. coli BL21 CodonPlus (DE3)-RIL cells (Agilent, http://www.home.agilent.com). Starter cultures containing ampicillin were used to inoculate 500 ml of pre-warmed Luria–Bertani (LB) medium without antibiotic. When A600 reached approximately 0.9, isopropyl-1-thio-β-D-galactopyranoside (500 μM) was added, and incubation was continued for 2 h at 30°C. Subsequent operations were at 4°C. Cells were harvested by centrifugation, resuspended in 8 ml of extraction buffer [50 mM NaH2PO4 (pH 8.0), 300 mM NaCl, 10% glycerol (vol/vol) and 10 mM imidazole], and disrupted with 0.1-mm zirconia/silica beads in a MiniBeadbeater (Biospec Products Inc., http://www.biospec.com) at 5000 rpm for 20 s, five times. The extracts were centrifuged (at 14 000 g for 10 min) and the recombinant proteins were purified under native conditions with Ni-NTA His-Bind resin (Novagen, http://www.emdchemicals.com), following the manufacturer’s recommendations. Isolated proteins were immediately desalted on a PD-10 column (GE Healthcare, http://www.gelifesciences.com) equilibrated in 100 mM KH2PO4 (pH 7.0), 10% glycerol (vol/vol). Desalted fractions were frozen in liquid N2 and stored at –80°C.

Enzyme assays

The DHNA-CoA thioesterase assays (50 μl) contained 100 mM KH2PO4 (pH 7.0), 5 mM DTT, 35–90 μM DHNA-CoA and 0–49 μg of proteins, and were incubated in the dark for 10–20 min at 30°C. Negative controls containing boiled proteins and external standards of DHNA were incubated in parallel. Assays were terminated with the addition of 150 μl of ice-cold 95% ethanol (vol/vol). Samples were then centrifuged (at 16 000 g for 5 min at 8°C) and immediately analyzed by HPLC with fluorescence and diode array detection modules, as previously described (Widholm et al., 2009). The hydrolysis of the benzoyl-CoA, phenylacetyl-CoA, palmitoyl-CoA and succinyl-CoA substrates was measured spectrophotometrically using the DTNB-derivation method. Assays (375 μl) contained 100 mM KH2PO4 (pH 7.0), 90 μM substrates and 0.65–2.7 μg of recombinant AtDHNAT1 and 2. In addition, assays with palmitoyl-CoA contained 3 μM of BSA. Blank samples containing no enzyme or no substrate were included. Reactions were incubated for 60–180 min at 30°C, and were then mixed with 375 μl of an aqueous solution of 400 μM DTNB. Changes in A412 compared with blank samples were read after a 5-minute incubation.

Phytoquinone analyses

All steps were carried out in dimmed light to avoid the photodestruction of naphthoquinone species. Synechocystis cells (1.8 ml) were harvested by centrifugation, and resuspended in 225 μl of BG-11 medium (Williams, 1988). Cells were quantified by absorbance at 730 nm using the formula 0.25 unit A490 = 104 cells. A 150-μl aliquot was then added to 700 μl of 95% (vol/vol) ethanol and 220 μl of water into a pyrex screw-cap tube, spiked with 75 pmoles of menaquinone-4 as an internal standard. Arabidopsis leaves (8–21 mg fresh weight) were spiked with 150 pmoles of menaquinone-4 as an internal standard, and were homogenized in 1 ml of 100% (vol/vol) methanol using a pyrex tissue grinder. The extract was then transferred to a pyrex tube containing 0.6 ml of water. Synechocystis and Arabidopsis extracts were then partitioned with 5 ml of hexane. Upper phases were transferred into a new tube, and then evaporated to dryness under a gentle stream of gaseous N2. The residue was redisolved into 200 μl of ethanol. The samples (100 μl) were analyzed by HPLC on a 5 μm Supelco Discovery C-18 column (250 × 4.6 mm, Sigma-Aldrich), thermostated at 30°C and eluted in isocratic mode at a flow rate of 1 ml min−1 with methanol : ethanol (80 : 20 vol/vol), containing 1 mM sodium acetate, 2 mM acetic acid and 2 mM ZnCl2. Naphthoquinone species were detected fluorometrically (at 238 and 426 nm for excitation and emission, respectively) after reduction into a post-column chemical reactor (70 × 1.5 mm) packed with ~100 mesh zinc powder (Sigma-Aldrich). Phyloquinone and menaquinone-4 were quantified according to external calibration standards, and data were corrected for the recovery of the internal standard.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The biosynthetic pathway of phyloquinone.

Figure S2. Expression and purification of recombinant AtDHNAT1 and AtDHNAT2.

Figure S3. Phenotypes of wild-type, atdhnat1 AtDHNAT2, AtDHNAT1 atdhnat2 and atdhnat1 atdhnat2 Arabidopsis plants.

Figure S4. Sequence alignment of prokaryotic and eukaryotic DHNA-CoA thioesterases and related proteins.

Figure S5. Schemes of the atdhnat1 and atdhnat2 loci.
Figure S6. Molecular characterization of AtSG48370 T-DNA insertion mutant (SALK_122483) and phylloquinone analyses.

Table S1. DHNA-CoA thioesterase activity of Escherichia coli crude extracts.

Table S2. Primer sets used for the subcloning of Arabidopsis cDNA clones in expression vector pSynExp-2.

Table S3. Accession numbers and taxonomic origin of proteins used for phylogenetic reconstruction.

Table S4. DHNA-CoA thioesterase activity in pea seedling crude extracts and percoll-purified chloroplasts.

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REFERENCES


