A Plant Locus Essential for Phylloquinone (Vitamin K1) Biosynthesis Originated from a Fusion of Four Eubacterial Genes

A Plant Locus Essential for Phylloquinone (Vitamin K1) Biosynthesis Originated from a Fusion of Four Eubacterial Genes

Phylloquinone is a compound present in all photosynthetic plants serving as cofactor for Photosystem I-mediated electron transport. Newly identified seedling-lethal Arabidopsis thaliana mutants impaired in the biosynthesis of phylloquinone possess reduced Photosystem I activity. The affected gene, called PHYLLO, consists of a fusion of four previously individual eubacterial genes, menF, menD, menC, and menH, required for the biosynthesis of phylloquinone in photosynthetic cyanobacteria and the respiratory menaquinone in eubacteria. The fact that homologous men genes reside as polycistronic units in eubacterial chromosomes and in plastomes of red algae strongly suggests that PHYLLO derived from a plastid operon during endosymbiosis. The principle architecture of the fused PHYLLO locus is conserved in the nuclear genomes of plants, green algae, and the diatom alga Thalassiosira pseudonana. The latter arose from secondary endosymbiosises of a red algae and a eukaryotic host indicating selective driving forces for maintenance and/or independent generation of the composite gene cluster within the nuclear genomes. Besides, individual menF genes, encoding active isochorismate synthases (ICS), have been established followed by splitting of the essential 3’ region of the menF module of PHYLLO only in genomes of higher plants. This resulted in inactivation of the ICS activity encoded by PHYLLO and enabled a metabolic branch from the phylloquinone biosynthetic route to independently regulate the synthesis of salicylic acid required for plant defense. Therefore, gene fusion, duplication, and fission events adapted a eubacterial multienzymatic system to the metabolic requirements of plants.

PSI† (Photosystem I) is a highly conserved multisubunit complex in thylakoid membranes of cyanobacteria and photosynthetic eukaryotes (1). The light-driven plastocyanin-ferredoxin oxidoreductase activity of this enzyme is conducted by an intrinsic electron transfer chain of redox components consisting of six chlorophyll a molecules, two phylloquinones, and three [4Fe-4S] clusters (2). Particularly, the biosynthesis of the cofactor phylloquinone (PhQ), or 2-methyl-3-phytyl-1,4-naphthoquinone, has been subjected to a systematic reverse genetic analysis in the cyanobacteria strain Synechocystis sp. PCC 6803, providing evidence that PhQ biosynthesis involves almost the same eight enzymatic steps catalyzed by the Men proteins that are required for synthesis of menaquinone, a component of the respiratory chain in eubacteria (Fig. 1) (3–6). In Synechocystis PhQ-deficient mutants, the level of active PSI is reduced to about 50–60% of the wild type (WT), while Photosystem II (PSII) activity remains unaltered. These mutants grow photautotrophically under low light conditions because the missing quinone in PSI is functionally replaced by plastquinone (PQ) (3).

In plants, relatively little is known about PhQ biosynthesis, although biochemical analysis suggested a metabolic pathway similar to that of bacteria and that the principle reactions occur at the chloroplast envelope membrane (7). The menA homologous Arabidopsis thaliana gene, AtmenA, was recently demonstrated to be essentially required for PhQ biosynthesis and accumulation of PSI. However, conversely to the Synechocystis men mutants, the AtmenA knock-out plant was severely affected in the accumulation of PSII and PQ as well (8).

PhQ is also known as vitamin K1, a compound essential for human health involved in various physiological processes ranging from blood clot and bone metabolism to growth control and signal transduction (9). The major source of vitamin K in human nutrition is green vegetables (9), and circumstantial evidence suggests that also in plants PhQ could have additional functions apart from being a PSI cofactor. Some reports demonstrated the presence of PhQ in the plasma membrane, where it could act as a sensor/scavenger of reactive oxygen species and/or as an intermediate in cross-membrane electron transport chains (10, 11).

In the course of a genetic study aimed at PSI biogenesis using four allelic phylloquinone gbsence (pha) mutants in Arabidopsis, we identified an essential nuclear plant gene for PhQ biosynthesis, designated PHYLLO, which encodes a fusion of four formerly eubacterial genes of this pathway. We also demonstrate that both forms of isochorismate synthases, ICS1 and ICS2, encoded by two individual menF homologous genes in Arabidopsis contribute to the first step of vitamin K1 biosynthesis. An independent regulation of ICS1 secures an early branching point from the PhQ biosynthetic pathway to ensure induction of the salicylic acid (SA) metabolic route important under conditions of pathogenic attack (12). The findings suggest that the PHYLLO gene in plants has been shaped by subsequent gene fusion, duplication, and

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) DQ084385 (cDNA RAFL 09-32-C05 and DQ084386 (phyllo from Arabidopsis).

§ Received a Ph.D. scholarship from the CAPES Foundation, subordinated to the Brazilian Ministry of Education.

¶ Supported by Grants ME 1794/1 and SFB TR1 from the German Science Foundation. To whom correspondence should be addressed. Tel: 49-89-17861288; Fax: 49-89-1782274; E-mail: joerg.meurer@lrz.uni-muenchen.de.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Phytoquinone Biosynthesis in Plants

splitting events during evolution and that its product takes part in a multienzyme complex (metabolon) for PhQ biosynthesis. Furthermore, our data support the idea that PhQ could potentially exhibit functions other than connected with PSI electron transport in the plant cell.

EXPERIMENTAL PROCEDURES

Selection and Growth of Mutant Plants—The pha1 (accession Was-silewskija) and pha2 (Columbia) lines were selected from Arabidopsis mutant collections (13). Pha3, pha4, ics1, and ics2 mutants were obtained from the T-DNA insertion lines Salk_137597, Salk_039309, Salk_042603 and Salk_073287, respectively (Salk Institute, La Jolla, CA). Insertion sites and the genotypes of these lines were confirmed by PCR, nucleotide sequence, and segregation analyses. The mutants were grown on sucrose-supplemented Murashige and Skoog medium under a 14 h light/10 h dark period at a light intensity of 20 μmol photons m⁻² s⁻¹ (13). For feeding experiments hypocotyls of 3-week-old plants were cut off at their base, and plants were transferred and kept for 5 days either on Murashige and Skoog medium or on medium containing 1.0 mM NA (ABCR, Karlsruhe, Germany).

Immunology, Gene Expression, and Cellular Localization—Immunoblot analysis, isolation of poly(A)⁺ mRNA and total RNA, reverse transcription, as well as RNA gel blot analysis were carried out as described previously (13–16). The probes labeled for RNA gel blot analysis were amplified with primers specific for the PHYLLO locus. Quantitative two-step real-time reverse transcription-PCR for pha3 and WT plants was carried out essentially as described (16). A PCR product amplified from the cDNA clone RALF 09-32-C05 (17) using specific primers containing Sall restriction sites was cloned into the RFP expression vector P0L-DsRed (14). This produced a translational fusion of the MenF module of PHYLLO with the Discosoma sp. (DsRed) protein. The resulting fusion and the control DsRed were transiently expressed in tobacco protoplasts according to the standard polyethylene glycol protocol and images were captured using a fluorescence microscope equipped with a digital camera (Axioplan; Zeiss, Jena, Germany) (14).

Fluorimetry and Spectroscopy—Measurements of PSI and PSII activity were performed with the pulse amplitude modulated fluorimeter PAM101 connected with emitter detector subunits (Walz, Effeltrich, Germany) and equipped with a data acquisition system to record fast changes (13, 18).

Sequence Analysis, Map-based Cloning and Genetic Complementation—502 segregating F2 mutant plants from backcrosses of pha1 and pha2 with the WT (accession Landsberg erecta) were mapped using available and self-generated PCR-based molecular markers. Sequences of the RALF 09-32-C05 cDNA clone (form 1) (17), PHYLLO from Arabidopsis, ICS1, and ICS2 are available under the GenBank accession numbers DQ084385, DQ084386, AY056055, and NM_101744, respectively. The Arabidopsis homologues of the eubacterial MenB (At1g0550), MenA (At1g0600), MenG (At1g23360), and MenE (comprising a large gene family) were identified by BLAST searches (19). The genomic sequences of the PHYLLO locus from Chlamydomonas, Populus trichocarpa, and Thalassiosira pseudonana were retrieved from genome.jgi-psf.org/euk_cur1.html, and the rice PHYLLO homologue was found in the BAC clone GenBank accession number AP005800. These sequences were annotated by ab initio and comparative analyses as described previously (20). For complementation analysis, the RALF 09-32-C05 clone was cleaved with EcoRV and SpeI, and the resulting fragment was inserted into the plant binary transformation vector pS001 (14) within compatible SmaI and XbaI restriction sites, resulting in the clone binC05. To generate a full-length cDNA for complementation of pha mutants, the XbaI- and Bst1107I-cleaved fragment of binC05 was replaced by a PCR product modified by insertion of a guanine within the stop codon (form 4). Plants were transformed via Agrobacterium, and the offspring was selected with 10 mg/liter sulfadiazine (14). All primers used in this work are available upon request.
**RESULTS**

Characterization of pha Mutants—In an attempt to identify factors involved in PSI biogenesis in higher plants, the seedling lethal pha1 and pha2 mutants were selected based on their high chlorophyll fluorescence (hcf) phenotype, associated with photosynthetic lesions (13, 14). Further examination of these mutants by HPLC revealed that they completely lacked PhQ (Fig. 2A, Table 1). Despite of this absence, pha1 and pha2 exhibited 5–15% of WT PSI activity (Fig. 2B). The potential yield of PSII (minimal fluorescence yield/the maximum fluorescence yield (F/Fm)) was reduced to about 75% of WT (Fig. 2C) demonstrating that this photosystem is only moderately affected as in many other PSI mutants (14–16). Chlorophyll fluorescence analysis revealed that mutant plants failed to quench the fluorescence efficiently upon illumination, indicating that the limiting step of the linear electron transport lies behind PSII (Fig. 2C). Immunoblot analysis showed a reduced accumulation of the PSI core complex reaching 5–15% of the WT level. The other thylakoid membrane complexes, i.e. ATP synthase, cytochrome b6/f complex, NAD(P)H-dependent dehydrogenase, and PSII, however, accumulated at almost normal levels (Fig. 2D). As it was also evident from in vivo labeling experiments of thylakoid membrane proteins showing normal translation of PSI subunits in pha mutants (data not shown), the present data unequivocally indicate a specific reduction of PSI stability in pha mutants. Conversely, the lack of vitamin K1 in pha1 and pha2 is not a secondary effect related to the failure of plants to accumulate PSI, since other Arabidopsis mutants, like hcf101 and hcf145, with PSI amounts below 5% (15, 16), possess normal levels of PhQ (Table 1).

Identification of Four pha Mutations in the PHYLLO Locus—To identify possible PhQ biosynthetic defects, pha1 and pha2 mutants were grown in the presence of the metabolic precursor 1,4-dihydroxy-2-naphthoate (NA) (Fig. 1). Although the pha mutants accumulate only 41 ± 4% chlorophyll based on fresh weight, the NA-fed plants completely restore wild-type levels (Fig. 2E). NA feeding recovered also the PhQ content in pha mutants to about 0.5 μg/g fresh weight (Table 1) accompanied by a reestablishment of the accumulation and activity of PSI to about 50 and 70% of WT levels, respectively (Fig. 2, A–E), indicating a function of the mutated genes in the first steps of vitamin K1 biosynthesis (Fig. 1). Accordingly, both mutants were genetically mapped on the lower part of chromosome I (BAC clone T6L1) into a 9,788-bp interval (PHYLLO locus), surprisingly, revealing homology with four euubacterial men genes, menE, menD, menC, and menH. Two additional mutant lines, pha3 and pha4, which contain T-DNA insertions within this locus (Fig. 3A) also exhibited the pha phenotype as well as PhQ and photosynthetic recovery after NA supply (Fig. 2, A and B, Table 1), reinforcing the essential function of the PHYLLO locus in vitamin K1 biosynthesis.

**PHYLLO, a Tetramodular Fusion of Four men Genes**—Four lines of evidence demonstrate unequivocally that the observed regions in the PHYLLO locus homologous to four men genes represent a single, monocistronic gene. (i) All four pha mutants belong to the same complementation group (Figs. 2, A–C, and 4A). (ii) In the pha3 background (T-DNA insertion in the menE 5’ region, Fig. 3A) no accumulation of transcripts related to menD and menH regions could be detected by real-time RT-PCR analysis (data not shown). (iii) RNA gel blot analysis

**FIGURE 2.** The pha phenotype. A, HPLC showing retention peaks of PhQ (black triangles) and PQ (white triangles) in WT, pha1, and NA-supplemented plants. The PhQ amounts are expressed as percentage relative to WT. B, measurements of PSI activity. Shortly after the PSI pigment P700 was completely reduced (red) in the dark, far-red light (FR) was applied to fully oxidize (ox) P700. A saturating pulse (arrow) of heterochromatic light was given to follow re-reduction of P700. C, chlorophyll a fluorescence at room temperature reflects PSI activity. The Fm, the Fp, and the steady state fluorescence yield (F/Fm) was determined. NA-fed mutant plants showed fluorescence characteristics similar to those of WT indicating recovery of photosynthesis. Equivalent results shown for pha1 in A–C were obtained for all pha mutants, pha crosses, and the icl/cis2 double knock-out plants. D, immunoblot analysis of representative proteins of the major thylakoid membrane complexes, + NA, plants supplemented with NA; − NA, without NA. E, effects of NA feeding on the phenotype. WT and PSI mutant plants pha1, pha2, and hcf145 were grown for 3 weeks and then transferred to 1.0 mM NA-supplemented medium (+) or kept on normal Murashige and Skoog medium without NA (−). The pha1 and pha2 mutants greened upon the treatment and largely recovered photosynthetic activity (see C). No effect of NA on greening, PSI activity, and chlorophyll content could be detected for the PSI control mutant hcf145, which is not affected in PhQ biosynthesis.
Phyloquinone Biosynthesis in Plants

TABLE 1
PhQ and PQ content of wild type and Arabidopsis PSI mutant plants

| Lines          | PhQ | PQ |
|---------------|-----|----|
|               | −NA | +NA| −NA | +NA |
| Wild-type     | 3.04 | 0.52 | 5.15 | 0.96 |
| hcf415        | 3.21 | 0.42 | 5.36 | 0.36 |
| hcf101        | 3.22 | 0.35 | 5.63 | 0.06 |
| pha1          | 0.0  | 0.41 | 0.10 |
| pha2          | 0.0  | 0.59 | 0.08 |
| pha3          | 0.0  | 0.48 | 0.13 |
| pha4          | 0.0  | ND  | ND  |
| ics1/ics1; ics2 | ND  | ND  | ND  | ND  |
| ics1/ics1; ICS2/ics2 | 0.60 | 0.11 | ND  | ND  |

FIGURE 3. The PHYLLO locus. A, map of PHYLLO depicting exons (solid lines), introns (dashed lines), the transit peptide (TP), men-homologous regions (black bars), pha mutations positioned relative to the start codon, and the alternative splicing region (squared box). Pha1, insertion (Ins.) of a cytosine/thiamine (CT); pha2, transition of guanine (G) to adenine (A); pha3 and pha4, T-DNA insertions. B, the splicing forms 1–3, between exons 7 and 8, are presented in black bars containing the nucleotide sequences. Dashed lines indicate the guanine added to form 1 to generate form 4.

revealed a single band of 5.5 kb when probing 5’ central, and 3’ parts of the PHYLLO locus (Fig. 4B), indicating that a unique transcript covers the entire PHYLLO interval. (iv) The most convincing evidence is the existence of a functional full-length coding frame of the complete PHYLLO gene (see below).

Sequence analysis of a full-length cDNA (17) and overlapping RT-PCR products revealed that PHYLLO is composed of 28 exons encoding all four men-homologous regions (Fig. 3A). Additionally, three alternative splicing events (forms 1–3) were detected between exons 7 and 8 (Fig. 3B). Both form 1 and form 2 carry premature termination codons leading to the expression of a truncated menF homologous region. Differently, the form 3 contains shorter boundaries of exons 7 and 8, bypassing any stop codon, and encodes a fused reading frame of 5,347 bp comprising all four men-homologous modules (Fig. 3B). We conclude that only the form 3 corresponds to the functional full-length phylo transcript. Accordingly, a cDNA corresponding to the stop codon-containing form 1 transformed into the background of all pha mutants did not complement the mutations (data not shown). To obtain a full-length cDNA encoding a single tetra-modular reading frame of the entire PHYLLO locus, a guanine nucleotide was inserted into the context of the stop codon of form 1 (Fig. 3B). The resulting form 4 successfully complemented the pha3 and pha4 mutants (Fig. 4, C–H) allowing photoautotrophic growth, PhQ accumulation, and recovery of PSI activity (data not shown).

Conservation of the PHYLLO Locus—The PHYLLO product (form 3) consists of 1,715 deduced amino acid residues comprising the four Men modules preceded by a predicted transit peptide that directs the PHYLLO protein to the chloroplasts (Fig. 5). BLAST similarities (19), ranging from 40 to 50%, and the presence of highly conserved motifs confirmed the relationship of the four PHYLLO modules with known Men proteins in eubacteria. A cluster of all four men homologues is also present in the genomes of the plants P. trichocarpa and rice (Fig. 6A), implying conservation of PHYLLO in monocots and dicots. Furthermore, an entire PHYLLO locus is also present in the nuclear genomes of the green algae Ostroecoccus tauri strain OTTH0595 and Chlamydodesmos reinhardtii. Interestingly, the diatom alga Thalassiosira pseudonana, which arose from secondary endosymbiosis in the red algae lineage (21), also contains a PHYLLO locus, in which the menH module precedes the menC module (Fig. 6A). These results support a conservation of the fused PHYLLO locus within the green and red plant lineages.

Remarkably, men genes are clustered and partially imbedded in operons in some eubacterial genomes and in plastomes of the red algae Cyanidium caldarium and Cyanidioschyzon merolae (Fig. 6A). This reinforces the idea that PHYLLO derived from the structure of an eubacterial operon, which was presumably transferred during endosymbiosis from the chloroplast into the nucleus of the host genome (22). Once the operon has been transferred evolutionary driving forces could have established the gene cluster as a tetramodular fusion. The presence of PHYLLO in diatoms indicates that the fusion event happened either once before divergence of the green and red lineages but individual men genes have been maintained in plastomes of some red algae at the evolutionary base of the chlorophyll c-containing clade or the fusion occurred at least two times independently.

ICS1 and ICS2 Are Required for PhQ Biosynthesis in Arabidopsis—The evolutionary dynamics of the PHYLLO product becomes even more intriguing considering the fact that the C-terminal chorismate binding domain (12) is absent from the MenF module (isochorismate synthase) of PHYLLO in higher plants (Fig. 6A and B). Conversely, this module has maintained its structural integrity in the PHYLLO homologues of the green and red algae lineages (Fig. 6, A and B), suggesting a recent gene splitting event in the PHYLLO locus, which occurred only in higher plants. Therefore, we propose that the PHYLLO menF 5’ module,
whose product is only homologous to the N-terminal part of ICS, is not functional and that the ICS activity has been taken over by two additional genes (ICS1 and ICS2) present in *Arabidopsis*. To test this possibility, single and double knock-outs of these genes were analyzed (Fig. 7, A–E). Only the double knock-out plants showed the *pha* phenotype (Fig. 2, A–C, Table 1), indicating that, under normal conditions, ICS1 and ICS2 overlap in their function to synthesize PhQ (Fig. 1) and that the product of the *menF* 5' module of *PHYLLO* is unable to complement this deficiency.

*The Bulk of PhQ in Arabidopsis Is Not Associated with PSI*—Interestingly, offspring homozygous for *ICS* (ics1/ics1) and heterozygous for *ICS2* (ics2/ICS2) contains only about 18% of the WT PhQ content (Table 1) but 50–70% of WT PSI activity (data not shown). Similarly, 15% PhQ accumulation in the *pha* mutants after NA supply (Fig. 2A and Table 1) was sufficient to recover about 50% of PSI amount and up to 70% activity (Fig. 2, B–D). Three major conclusions can be drawn from this non-proportional numerical correlation: (i) PSI is a preferential metabolic sink for newly synthesized PhQ. (ii) An extrapolated amount of maximal 50% PhQ is sufficient for 100% PSI activity. (iii) Consequently, at least 15% of the PhQ is not associated to PSI and may be located elsewhere in plant membranes. The PhQ pool can be expanded even further from about 3.1 to 5.4 μg/g fresh weight when

---

**FIGURE 4.** *PHYLLO* represents a tetramodular monocistronic gene. A, scheme of allelism test crosses indicating the position of *pha* mutations and *men*-homologous regions (capital letters). B, Northern blot using probes amplified from the 5', middle, and 3' parts of *PHYLLO*. C, schematic representation of the *PHYLLO* locus indicating the T-DNA insertions of the *pha3* and *pha4* mutations and the positions of primers (arrows) used for screening of the transformed lines. D–H, genetic complementation of the *pha3* and *pha4* mutations with the full-length cDNA (form 4). D, the PCR product of 280 bp using primers 1 and 3 indicates the presence of the T-DNA in the complemented *pha3* lines (*pha3c*). E, the PCR product of 315 bp using primers 2 and 3 only amplifies in the WT plant, indicating homozygosity of the T-DNA insertion in *pha3c*. F, the PCR product of 518 bp using primers 1 and 5 indicates the presence of the T-DNA in complemented *pha4* lines (*pha4c*). G, the PCR product of 1,020 bp using primers 4 and 5 only amplifies in the WT, indicating homozygosity of the T-DNA insertion in *pha4c*. In all the PCR reactions positive controls generating products of 980 bp (D and E) and 315 bp (F and G) were used. H, the presence of the form 4 in all complemented mutant plants was confirmed by the BsaI cleavage of a cDNA-specific PCR product (P) of 271 generating fragments of 184 and 87 bp.

**FIGURE 5.** Subcellular localization of *PHYLLO*. Localization of *PHYLLO* in chloroplasts is revealed by merging the fluorescence induced by the *PHYLLO*::DsRed fusion and by the chlorophyll. The DsRed control is expectedly localized in the nucleus.
Phyloquinone Biosynthesis in Plants

A

Plant nuclear genomes

Arabidopsis

[menF* menD menC menH]

Oryza sativa and Populus trichocarpa

[menF* menD menC menH]

Green algae nuclear genomes

Ostreococcus tauri and Chlamydomonas

[menF menD]

Diatom algae nuclear genomes

Thalassiosira pseudonana

[menF menH]

Red algae plastid genomes

Cyanidium caldarium

[menD menE menA menB menF]

Cyanobioschyzon merolae

[menD menE menA menB menC]

Eubacterial genomes

Trichodesmium erythraeum

[menD menC menE menF]

E. coli

[menF menD menA menE]

B

Ec

MenF

MenD

MenA

MenE

IC5

MenH

At

MenF

MenD

MenA

MenC

MenH

PHYLLO algae

PHYLLO plants

MenF* module

DCH/HG1

FIGURE 6. Conservation of the PHYLLO locus. A, the PHYLLO genes found in plants and algae are indicated by open boxes. Black boxes represent men genes found in red algae plastomes and eubacterial genomes. The GenBank accession numbers of the genes are: Arabidopsis (DQ0843366, rice (AP008208), Populus (Scaffold LG X), Chlamydomonas (Scaffold 37), O. tauri (CR954217), T. pseudonana (Scaffold 48), C. caldarium (AF022186), C. merolae (AB002583), and the representative eubacterial genomes of the cyanobacterium T. erythraeum (AA040000003) and of E. coli (U00096). The tips of the black bars indicate the transcriptional orientation. The vertical displacements of black boxes in the red algal operons indicate overlapping of genes. B, schematic alignment of the proteins MenF in E. coli (Ec) (P38051); ICS, representing the Arabidopsis (At) ICS1 (AAAL17715) and ICS2 (NP_173321); PHYLLO of algae, representing PHYLLO homologues in Chlamydomonas, O. tauri, and T. pseudonana (where the MenH precedes MenC, as indicated by brackets); PHYLLO of plants, representing the PHYLLO homologues from Arabidopsis, rice, and P. trichocarpa. The fusions in the PHYLLO MenF module of plants result in the gene splitting event of the chlorisomat binding domain (pfam00425).

WT, hcf145, and hcf101 plants are fed with NA (Table 1), notably, without affecting PSI activity (data not shown). The fact that large amounts of vitamin K1 in Arabidopsis were not associated with PSI reinforces previous ideas suggesting that PhQ presumably exerts other functions in plant plasma membranes (10, 11).

DISCUSSION

Essential Role of Phyloquinone in Higher Plants—The analysis of pha and ics mutants showed that PhQ is essentially required for photoautotrophic growth in Arabidopsis. The deficiency at the level of PSI accumulation, which could be partially overcome by supplementation of the PhQ metabolic precursor NA, indicates that the lack of the cofactor was the primary cause for instability of PSI proteins. Nevertheless, 5–15% of the PSI core subunits and a corresponding activity of this complex could be detected in pha mutants and ics double knock-outs. This could be explained by a functional replacement of the missing PhQ by an alternative quinone, such as PQ, as has similarly been reported for Synechocystis men mutants (3). However, this replacement is not sufficient to allow normal accumulation of PSI and photoautotrophic growth suggesting that this quinone is not suited to fully replace PhQ for PSI assembly in Arabidopsis. The PQ content is reduced to about 55% based on fresh weight in pha mutants (Table 1). The same reduction could be observed in other PSI mutants, like hcf145 and hcf101, not primarily affected in PhQ biosynthesis. Therefore, the reduced PQ content is a secondary effect due to the deficiency of PSI. However, NA feeding increased the PQ content in pha mutants to more than 70% relative to wild type but expectedly not in hcf145 and hcf101. These data substantiate the potential of pha mutants to partially replace PhQ by PQ. In contrast to the pha mutants and the ics1/ics2 double knock-out, the recently described Arabidopsis menA mutant, designated abc4, which is also impaired in the PhQ biosynthesis, (i) completely failed to perform PSI activity, (ii) displayed a significant reduction of PSI polypeptides presumably related to a reduced PQ amount of 3% based on fresh weight, and (iii) showed a chlorophyll content that decreased with aging from 25 to 0% (8). We suggest that higher light intensities and continuous light used during growth could account for the severe deficiencies of the abc4 mutant affected in the same metabolic pathway like pha mutants. On the other hand, the differences could also be allele specific. For example, the Arabidopsis MenA homolog could exert a dual function for the transfer activity of phytyl and polypropenyl to generate phylloquinone and plastoquinone, respectively.

The Eubacterial Origin of PHYLLO—The PHYLLO locus constitutes a fusion of modules homologous to four eubacterial men genes, menF, menD, menC, and menH. Our data indicate that PHYLLO has either monophyletically or polyphyletically arisen from a eubacterially derived operon. This possibility is consistent with previous studies demonstrating that juxtaposed coding frames in operons are at the root of gene fusions, both in prokaryotes and eukaryotes (23, 24). This is further supported by the partial structural conservation of PHYLLO with men genes clustered in red algal plastomes (Fig. 6A) and in many eubacterial genomes, all containing a conserved co-localization of menF and menD genes (Fig. 6A, supplemental Table S1). Surprisingly, also the genome of the archaebacterium Halobacterium species NRC-1 contains a men cluster with adjacent menF and menD genes, presumably as the consequence of a lateral gene transfer (25), which emphasizes the tendency for this operon to be mobilized as a block. Although the men operons are generally quite conserved and men genes are still clustered in the cyanobacterium Trichodesmium erythraeum (Fig. 6A), in several eubacteria and other cyanobacteria, men genes were occasionally rearranged in their genomes leading to loss of men operon structures.

A Metabolon for PhQ Biosynthesis—PHYLLO genes have been conserved in the genomes of plants, green algae, and in the diatom alga T. pseudonana. Thus, a selective driving force may have been acting over a long evolutionary distance to maintain this cluster of men genes as a fusion, possibly corresponding to a need of a multifunctional association of proteins required to channel metabolic intermediates of PhQ biosynthesis (23, 26–32). This has been suggested similarly for the menaquinone pathway in the genomes of Escherichia coli and Bacillus subtilis, where co-localization of menF and menD genes in operons is essential for the proper channelling of isochorismate, the product of MenF, to the menaquinone pathway via MenD (27, 28). A tendency to co-localize the menF and menD genes is extended to several eubacterial
genomes, in line with the idea that conserved gene orders in operons of different bacteria often correspond to physical interactions of their gene products (29). These results suggest that the transcriptional linkage of men genes in an operon ensures a co-translational association of Men proteins promoting efficient assembly of a multienzymatic complex (27–29).

The nuclear gene PHYLLO also conserved co-localization of the menFD module. Therefore, it is conceivable that PHYLLO arose from the framework of a plastid operon as a result of selection forces adapting an analogous operon-directed association of enzymes in the euukaryotic genomes (23, 27–29). men genes are not fused in any of the eubacterial and plastid genomes sequenced so far. Therefore, it is feasible that the fusion event postdated gene transfer. The single gene fusion, which is exclusively present in euukaryotic genomes, expresses enzymes and multifunctional association of previously four enzymatic activities in one polypeptide. This is in accordance with several studies demonstrating the tendency of subunits of enzymatic complexes to be involved in gene fusion events (30–32). Furthermore, the additional presence of the MenH module in PHYLLO, corresponding to an enzymatic step non-successive to that of MenDC in the pathway (Fig. 1), could imply that PHYLLO takes part of a macromolecular enzymatic complex in association with the intermediate enzymes MenE and MenB, which could channel the MenDC products to MenH. A similar situation of substrate channeling and gene fusion has been reported for a bifunctional protein that does not catalyze consecutive reactions in the aspartate pathway (33). The absence of MenE and MenB modules from the multifunctional PHYLLO fusion in plants could reflect removal of the corresponding genes from the composite operon or they were present in a non-consecutive order within the operon as is the case in red algae plastomes (Fig. 6A) leading to exclusion from the fusion. On the other hand, sterical hindrances could have caused the failure to fuse the MenB and MenE modules to PHYLLO. Moreover, the NA-dependent increase of the PhQ pool was up to five times lower in mutants than in WT, Icsf101 and Icsf145 plants (Table 1). This uncovered deficiencies of pha plants for subsequent enzymatic steps after NA entry (Fig. 1), catalyzed by MenA and MenG, presumably due to destabilization of the multienzyme complex in absence of the interacting PHYLLO product. This strongly suggests that MenA and/or MenG contribute to the metabolon as well. Interestingly, the MenA- and MenB-encoding genes are also tightly clustered and separated by only 16.5 kb in the genome of Arabidopsis. Similarly, the MenB protein is encoded ~25 kb apart from PHYLLO in the genome of T. pseudonana. This unusual proximity of functionally related genes resembles the situation in yeast, where genes encoding subunits of stable complexes tend to be clustered within 10–30 kb (34).

A Metabolic Link between Plant Resistance and Photosynthesis—The gene fusion event that inactivated the MenF module of PHYLLO in higher plants must have postdated the establishment of a separate copy of the ICS gene. The low conserved N terminus of ICS1 and ICS2 reveals no significant similarity with eubacterial men genes present in the data base but shows a higher sequence similarity with the MenF 5’ module of PHYLLO in plants than with the corresponding region of PHYLLO in algae. Furthermore, ICS1 and ICS2 in Arabidopsis show higher sequence similarity with PHYLLO in algae than with any other putative chorismate-utilizing enzyme present in sequenced genomes of algae. These observations indicate that both ICS1 and ICS2 arose from a duplication of the menF module of PHYLLO. This may reflect a need to establish an individual ICS gene outside of the PHYLLO context, which is especially important under conditions of phytopathogenic attack to induce and to direct the metabolic flow from chorismate preferentially toward the synthesis of SA required for plant resistance (Fig. 1). This function has already been shown for the Arabidopsis ICS1 gene (12, 35). Here, we provide the genetic evidence that both ICS1 and ICS2 are also required for the synthesis of PhQ. Therefore, duplication and inactivation of the menF module of PHYLLO by a gene splitting event generated a branching point for PhQ and SA biosynthesis (Fig. 1). This has two possible consequent implications for the partitioning of the metabolic flow between the two routes when ICS1 is induced upon infection by pathogens. (i) The product of ICS1 is preferentially channelled to the synthesis of SA at the expense of channelling isochorismate for PhQ biosynthesis. Under this condition, it is conceivable that isochorismate for the PhQ pathway could be provided by the ICS2 gene product. Another intriguing possibility is that the switch of the metabolic flow between PhQ and SA biosynthesis could be fine-tuned by regulation of alternative splicing of exons 7 and 8 of PHYLLO, dictating the output rate of truncated MenF 5’ module/active PHYLLO full-length product. Ironically, alternative splicing takes place in the splitted region of PHYLLO in between the menF 5’ and the menD modules (Fig. 3, A and B). (ii) Conversely, the biosynthesis of both, PhQ and SA, could simultaneously increase following strong induction of ICS1 by phytopathogenic attack. This is in line with the dosis effect of ICS mRNA and the impact of NA feeding on PhQ levels (Table 1) and with a recent report
suggesting that PhQ or its intermediates could trigger cell death responses associated with plant defense against phytopathogens (35). In any case, a metabolic link between photosynthesis and plant resistance has now been established that provokes further investigations.

Acknowledgments—We are very grateful to Elli Gerick and Jens Hermann for excellent technical assistance.

REFERENCES
1. Ben-Shem, A., Frolow, F., and Nelson, N. (2003) Nature 426, 630–635
2. Golbeck, J. H. (2003) Annu. Rev. Biophys. Biomol. Struct. 32, 237–256
3. Johnson, T. W., Shen, G., Zybailov, B., Reategui, R., Beauparlant, S., Vassiliev, I. R., Bryant, D. A., Jones, A. D., Golbeck, J. H., and Chitnis, P. R. (2000) J. Biol. Chem. 275, 8523–8530
4. Johnson, T. W., Naithani, S., Stewart, C., Jr., Zybailov, B., Jones, A. D., Golbeck, J. H., and Chitnis, P. R. (2003) Biochim. Biophys. Acta 1557, 67–76
5. Sakuragi, Y., Zybailov, B., Shen, G., Jones, A. D., Chitnis, P. R., Van Der Est, A., Bittl, R., Zech, S., Stehlik, D., Golbeck, J. H., and Bryant, D. A. (2002) Biochemistry 41, 394–405
6. Meganathan, R. (2001) Vitam. Horm. 61, 173–218
7. Schultz, G., Ellerbrock, B. H., and Soll, J. (1981) Eur. J. Biochem. 117, 329–332
8. Shimada, H., Ohno, R., Shibata, M., Ikegami, I., Omai, K., Ohno, M. A., and Takamiya, K. (2003) Plant J. 41, 627–637
9. Saxena, S. P., Israels, E. D., and Israels, L. G. (2001) Apoptosis 6, 57–68
10. Lochner, K., Döring, O., and Böttger, M. (2003) Biofactors 18, 73–78
11. Bridge, A., Barr, R., and Morre, D. J. (2000) Biochim. Biophys. Acta 1463, 448–458
12. Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001) Nature 414, 562–565
13. Meurer, J., Meierhoff, K., and Westhoff, P. (1996) Planta 198, 385–396
14. Aman, K., Lezhneva, L., Wanner, G., Herrmann, R. G., and Meurer, J. (2004) Plant Cell 16, 3084–3097
15. Lezhneva, L., Amann, K., and Meurer, J. (2004) Plant J. 37, 174–185
16. Lezhneva, L., and Meurer, J. (2004) Plant J. 38, 740–753
17. Seki, M., Narusaka, M., Kamiya, A., Ishida, J., Satou, M., Sakurai, T., Nakajima, M., Enju, A., Akiyama, K., Oono, Y., Muramatsu, M., Hayashizaki, Y., Kawai, J., Carminci, P., Itoh, M., Ishii, Y., Arakawa, T., Shibata, K., Shinagawa, A., and Shinozaki, K. (2002) Science 296, 141–145
18. Klughammer, C., and Schreiber, U. (1994) Planta 192, 261–268
19. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
20. Gross, J., Stein, J. R., Fett-Neto, A. G., and Fett, J. P. (2003) Genet. Mol. Biol. 26, 477–497
21. Lopez, P. J., Descles, J., Allen, A. E., and Bowler, C. (2005) Curr. Opin. Biotechnol. 16, 180–186
22. Martin, W., Rujan, T., Richly, E., Hansen, A., Cornelsen, S., Lins, T., Leister, D., Stoebe, B., Hasegawa, M., and Penny, D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12246–12251
23. Yanai, I., Wolf, Y. I., and Koonin, E. V. (2002) Genome Biol. 3, 1–13
24. Anderson, J. O., and Roger, A. J. (2002) Eukaryot. Cell 1, 304–310
25. Kennedy, S. P., Ng, W. V., Salzberg, S. L., Hood, L., and DasSarma, S. (2001) Genome Res. 11, 1641–1650
26. Winkel, B. S. J. (2004) Annu. Rev. Plant Biol. 55, 85–107
27. Buss, K., Muller, R., Dahm, C., Gaitatzis, N., Skrzypczak-Pietraszek, E., Lohmann, S., Gassen, M. & Leistner, E. (2001) Biochim. Biophys. Acta 1522, 151–157
28. Rowland, B. M., and Taber, H. W. (1996) J. Bacteriol. 178, 854–861
29. Dandekar, T., Snel, B., Huynen, M., and Bork, P. (1998) Trends Biochem. Sci. 23, 324–328
30. Yanai, I., Derti, A., and DeLisi, C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7940–7945
31. Tsoka, S., and Ouzounis, C. A. (2000) Nat. Genet. 26, 141–142
32. Marcotte, E. M., Pellegrini, M., Ng, H. L., Rice, D. W., Yeates, T. O., and Eisenberg, D. (1999) Science 285, 751–753
33. James, C. L., and Viola, R. E. (2002) Biochemistry 41, 3726–3731
34. Teichmann, S. A., and Venia, R. A. (2004) Genetics 167, 2121–2125
35. Brodersen, P., Malinkovski, F. G., Hernaty, K., Newman, M. A., and Mundy, J. (2005) Plant Physiol. 138, 1037–1045