Deficiency in Phylloquinone (Vitamin K$_1$) Methylation Affects Prenyl Quinone Distribution, Photosystem I Abundance, and Anthocyanin Accumulation in the Arabidopsis AtmenG Mutant

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Phylloquinone (vitamin K$_1$) is synthesized in cyanobacteria and in chloroplasts of plants, where it serves as electron carrier of photosystem I. The last step of phylloquinone synthesis in cyanobacteria is the methylation of 2-phytyl-1,4-naphthoquinone by the menG gene product. Here, we report that the uncharacterized Arabidopsis gene At1g23360, which shows sequence similarity to menG, functionally complements the Synechocystis menG mutant. An Arabidopsis mutant, AtmenG, carrying a T-DNA insertion in the gene At1g23360 is devoid of phylloquinone, but contains an increased amount of 2-phytyl-1,4-naphthoquinone. Phylloquinone and 2-phytyl-1,4-naphthoquinone in thylakoid membranes of wild type and AtmenG, respectively, predominantly localize to photosystem I, whereas excess amounts of prenyl quinones are stored in plastoglobules. Photosystem I reaction centers are decreased in AtmenG plants under high light, as revealed by immunoblot and spectroscopic measurements. Anthocyanin accumulation and chalcone synthase (CHS1) transcription are affected during high light exposure, indicating that alterations in photosynthesis in AtmenG affect gene expression in the nucleus. Photosystem II quantum yield is decreased under high light. Therefore, the loss of phylloquinone methylation affects photosystem I stability or turnover, and the limitation in functional photosystem I complexes results in overreduction of photosystem II under high light.

In plants and cyanobacteria, photosynthetic conversion of light into chemical energy is mediated via two photosystems, photosystem I (PSI) and photosystem II (PSII). PSII transfers electrons from water onto plastoquinone, an abundant prenyl quinone in thylakoids. PSI accepts electrons from plastocyanin, and they are subsequently transferred onto ferredoxin or flavodoxin. PSI forms dimers in thylakoids, with each PSI monomer harboring a special chlorophyll $a$ pair (reaction center P$_{700}$) for charge separation. The electron transfer chain through one PSI monomer encompasses two branches, each containing two further chlorophyll $a$ molecules (A$_{0}$ and A$_{1}$) and one phylloquinone (A$_{1}$). Electron flux merges at the iron-sulfur center F$_{X}$, which passes electrons on to the iron-sulfur centers F$_{B}$ and F$_{A}$ (1–3). The two branches of the PSI electron transfer chain are not structurally equivalent and differ in their lipid association: Whereas phosphatidylglycerol is found in close proximity to one of the branches, monogalactosyldiacylglycerol is associated with the other branch (2). It is presently unclear whether these branches are active to similar extents (4, 5).

Phylloquinone (vitamin K$_1$) is an essential component of the human diet, because it serves as a cofactor for $\gamma$-carboxylation of glutamyl residues in different proteins, such as blood coagulation factors, but cannot be synthesized in animals and humans (6). The phylloquinone molecule is composed of a naphthoquinone ring, which can exist in the oxidized quinone or in the reduced hydroquinone form, and a prenyl side chain derived from phytol-diphosphate. The biosynthesis of menaquinone, a bacterial vitamin K analog carrying an unsaturated side chain, and of phylloquinone has been studied in Escherichia coli and in cyanobacteria, respectively (7–9). The naphthoquinone ring is derived from chorismate, which is converted into 1,4-dihydroxy-2-naphthoate (DHNA) by six consecutive reactions (Fig. 1). Subsequently, DHNA is converted to 2-phytyl-1,4-naphthoquinone (PNQ) by DHNA phytlytransferase (MenA). The last step of phylloquinone synthesis in cyanobacteria involves the methylation at the C3-position of the naphthoquinone moiety by the menG gene product, thereby converting the unmethylated form, 2-phytyl-1,4-naphthoquinone, into phylloquinone.

The role of phylloquinone in photosynthesis has been studied by employing loss-of-function mutants of cyanobacteria.
(menA, menB, menD, menE, menG) (7, 9, 10). A block in biosynthesis resulting in total loss of phylloquinone has only a minor impact on photosynthesis or growth of Synechocystis under low light conditions, presumably, because other hydroquinones, such as plastoquinone, can largely substitute for phylloquinone deficiency in PSI (7). However, under high light conditions, photoautotrophic growth of Synechocystis menA and menB mutant strains is severely compromised (7).

Phylloquinone synthesis in plants has been studied by feeding biosynthetic precursors and by enzyme assays in spinach, Capsicum annuum, Euglena gracilis, and Gallium cell cultures (11–14). The first genetic study of phylloquinone synthesis in plants was presented by the isolation of the AtmenA mutant of Arabidopsis carrying a block in DHNA phytoltransferase. AtmenA plants are totally devoid of phylloquinone, contain reduced amounts of chlorophyll and PSI and show a severe reduction in photosynthetic efficiency (15). As a consequence, mutant plants are unable to survive in soil, providing strong evidence for the importance of phylloquinone as essential electron carrier of PSI (15). Furthermore, the gene and corresponding mutant of a multifunctional protein carrying four enzymatic domains putatively involved in phylloquinone synthesis (MenF, MenD, MenC, lipase) were recently isolated from Arabidopsis (16).

Disruption of the menG gene encoding PNQ methyltransferase resulted in phylloquinone deficiency in Synechocystis (10). In their PSI particles, mutant cells contained two molecules of 2-phytyl-1,4-naphthoquinone, the substrate of the MenG reaction, per PSI monomer. The gene encoding PNQ methyltransferase has not been isolated from higher plants, and the role of the final methylation reaction in phylloquinone synthesis for functional integrity of PSI in plants has not been studied. For this reason, the cDNA for an open reading frame identified as potentially homologous to menG and found in the fully sequenced genome of Arabidopsis, At1g23360, was isolated and its gene product tested for PNQ methyltransferase activity by expression in a heterologous system. The plastidial distribution of phylloquinone and of PNQ was measured in Arabidopsis wild type and in an AtmenG knock-out mutant. Furthermore, biochemical and physiological characterization of the AtmenG mutant demonstrated that the 3-methyl group of phylloquinone is required to sustain maximal efficiency of photosynthetic electron transport, particularly at high light conditions.

MATERIALS AND METHODS

Isolation of the At1g23360 cDNA and Complementation of the Synechocystis menG Mutant—A cDNA corresponding to the Arabidopsis gene At1g23360 was isolated by PCR from an Arabidopsis (Columbia) cDNA library prepared from inflorescences, stems and leaves using the primers At1g23360-forward (5′-ATC TTA TAC ATA TGG CGG CTC TAC TCG GTA TCG) and At1g23360-reverse (5′-TAT GGA TTC TTT ACC TCA TAG CGA CCA AAT CC). The amplification product, which contained added flanking NdeI and BamHI restriction sites (underlined) was subcloned into the EcoRV site of pBlue-scriptIISK+ (Stratagene) to generate pBlue-script-At1g23360. Sequencing revealed that this clone contains an authentic full-length open reading frame derived from the locus At1g23360. A Synechocystis menG knock-out mutant was prepared by replacement of the sll1653 gene with a spectinomycin resistance marker through homologous recombination (17). A menG-specific knock-out vector was generated using procedures similar to those described previously (18). Initially, sequences flanking sll1653 were amplified from Synechocystis sp. PCC6803 genomic DNA. Primers used for amplification of a 541-base pair 5′-flanking sequence were: 5′-AAC TTT GGA TTG CGG CGC CAG ATG-3′ (primer 5′-A) and 5′-GCT GTA CGA TCT CCT GAG TCG-3′ (primer 5′-B). Primers used for amplification of a 612-base pair 3′-flanking sequence were: 5′-GAC TCA GGA GAG CGT ACA CGC TAC GAC CGG TTT CCC ACT GGC C-3′ (primer 3′-A) and 5′-CTG AGT GAC ACT TTC CGC C-3′ (primer 3′-B). The purified PCR products were combined and reamplified along with primers 5′-A and 3′-B to generate a contiguous sequence that contained an added BsiWI restriction site (underlined nucleotides in primer 3′-A) between the 5′- and 3′-flanking sequences. This product was cloned into the pGEM-T Easy vector (Promega) to generate the plasmid pMenG-KO. An adaA gene encoding a spectinomycin resistance marker was cloned into the BsiWI site of pMenG-KO to generate pMenG-Spec-KO. This plasmid was introduced into Synechocystis sp. PCC6803 as described (17), and homologous recombinant lines were selected for spectinomycin resistance. Replacement of the sll1653 gene with the spectinomycin resistance marker was confirmed by PCR analyses of genomic DNA. Complementation of the Synechocystis menG knock-out mutant was conducted by expression of the full-length AtmenG cDNA using the vector pSyn-Exp-2 (18). The AtmenG cDNA was isolated as an NdeI/BamH restriction fragment from pBlue-script-At1g23360 and cloned into the NdeI/BglIII sites of pSyn-Exp-2 downstream of the psbA2 promoter. The resulting plasmid was introduced into the Synechocystis menG knock-out mutant, and recombinant lines were selected by chloramphenicol and spectinomycin resistance. Incorporation of the AtmenG cDNA expression cassette was confirmed by PCR analyses of genomic DNA. Total lipids were extracted as previously described (18) from wild type (WT) cells, menG knock-out lines, and AtmenG-complemented knock-out lines that were maintained on solid BG11 medium (17) with appropriate antibiotic selection.

Phylloquinone Measurements—Phylloquinone was quantified according to Jakob and Elmadfa (20). Briefly, ca. 100 mg of leaf material was ground in liquid nitrogen. Lipids were extracted from leaves or cyanobacteria with 0.8 ml of isopropyl alcohol/hexane (3:1) after addition of menaquinone-4 as internal standard (250 ng in 50 μl of ethanol, Sigma). After vortexing, samples were centrifuged at 14,000 × g for 2 min, and the green lipid phase transferred to a new vial. The remaining pellet was again extracted with 0.6 ml of hexane. To the combined organic phases, 0.6 ml of methanol/water (9:1) was added, and after vortexing and centrifugation, the upper hexane phase transferred to a glass vial. The solvent was evaporated with nitrogen gas and the residue dissolved in 100 μl of methanol/ dichloromethane (9:1). Phylloquinone was quantified on a 1100 Series HPLC (Agilent). Lipids were separated by isocratic chromatography (flow rate: 1 ml min⁻¹) on a reversed phase RP18
column (Europhos-100, 250 × 4.6 mm, Knauer, Berlin, Germany) equipped with a post-column derivatization cartridge (30 × 4 mm, filled with Zink powder, 63-μm particle size, VWR) to reduce all quinones to their respective hydroquinone forms. The solvent was composed of 900 ml of methanol, 100 ml of dichloromethane, and 5 ml of a methanolic solution of 1.37 g of ZnCl₂, 0.41 g of sodium acetate, and 0.30 g of acetic acid. Phylloquinone was measured by fluorescence (excitation, 243 nm; emission, 430 nm) using menaquinone-4 as an internal standard.

**Subcellular Localization of the At1g23360 Protein**—The entire open reading frame of the At1g23360 cDNA was amplified by PCR (primers: PD592, 5′-AGG GAT CCT ATG GGC GCT CTA CTC GGT A-3′; PD593, 5′-ATC CAT GGA CCT CAT AGC GACCAA ATT C-3′) from pBlueScript-At1g23360 (see above). The PCR fragment was ligated into the BamHI and Ncol sites of pCL60 (21) in translational fusion with the N terminus of the green fluorescent protein (GFP) sequence. Plasmid DNA was coated onto 1.0-μm gold microcarriers (Bio-Rad) and transferred into Arabidopsis leaves in a Biolistic PSD-1000/He particle delivery system (Bio-Rad) using 1100 psi rupture discs. After bombardment, leaves were incubated on Murashige and Skoog (22) medium in the dark for 2 days. Fluorescence signals were analyzed under a Leica DM-IRBE confocal microscope (Leica, Wetzlar, Germany) for GFP (excitation, 488 nm; emission, 518–524 nm) or for chlorophyll fluorescence (excitation, 488 nm; emission, 680–690 nm).

**Isolation and Complementation of the Arabidopsis AtmenG Mutant**—An Arabidopsis line carrying a T-DNA insertion in the At1g23360 locus (GABI_565F06) was obtained from the GABI-Kat collection in Cologne, Germany (23). PCR using gene-specific primers (PD464, 5′-AGA AAT GTG TGT TTT ACG TIT GGA-3′) with genomic DNA from wild type or a heterozygous line resulted in amplification of a 1630-bp fragment, in contrast to homozygous lines where no band was produced. A 670-bp fragment containing part of the T-DNA insertion could be amplified by PCR with the primers PD464 and PD394 (T-DNA left boarder primer; 5′-CCC ATT TAG ACG TGA ATG TAG ACA C-3′) in homozygous or heterozygous lines. For complementation, the ORF was released from pBlueScript-At1g23360 with KpnI and BamHI, ligated into the corresponding sites of the binary vector pBINAR containing a CaMV 35S promoter (24) and transferred into the Arabidopsis AtmenG mutant via Agrobacterium-mediated transformation (25). Transformed plants were selected on kanamycin-containing medium, and At1g23360 expressing lines identified by Northern blot analysis. Total RNA was isolated from leaves and used for Northern blot hybridization with a 32P-labeled At1g23360 cDNA as a probe following standard protocols (26).

**Incorporation of [1-3H]Phytol into Arabidopsis Seedlings**—Seedlings of Arabidopsis (10 days old) were incubated in 20 mM MES-KOH, pH 6.8, 0.2% Tween 80 with 100 pmol of [1-3H]phytol (20 Ci/mmole, American Radiolabeled Company/ BioTrend, Cologne, Germany) and lipids extracted as previously described (27). Radioactive lipids were separated by TLC (hexane/diethylether/acetic acid; 85:15:1) including standards (phytol, α-tocopherol, phylloquinone) and visualized with a β-scanner (Automatic TLC Linear Analyzer, Berthold, Wildbad, Germany).

**Quantification of Chlorophyll, Photosynthetic Pigments, Anthocyanin, and Tocopherol**—Chlorophyll in leaves was measured photometrically in 80% acetone (28). Photosynthetic pigments were extracted from leaves with acetone and quantified by HPLC (29). Anthocyanin was extracted from leaves with 1-propanol/1% HCl/water (18:1:81), the extract boiled for 3 min, and incubated in darkness for 2 h. Subsequently, anthocyanin was quantified photometrically according to the equation \( A_{535} = 2 \times A_{650} \) per g fresh weight (30). Tocopherol was measured by fluorescence HPLC (31).

**Chloroplast Isolation and Fractionation**—Chloroplasts were isolated from Arabidopsis WT and AtmenG leaves by homogenization (32). After hypotonic rupture of chloroplasts, subplastidial compartments were separated by centrifugation using a standard sucrose density gradient, and subfractions of 1 ml were collected (32). Immunoblot analysis (with antibodies against plastoglobulin AtPGL35, the translocator of the outer envelope AtToc75, chlorophyll a binding protein, CAB) was performed on subfractions of the sucrose gradient to assess the distribution of plastoglobules, envelopes, and thylakoids. The results of immunoblots were analogous to those published in Fig. 2 of Vidi et al. (32), and the gradient subfractions were pooled accordingly into five fractions (F1–F5). The upper gradient fractions F1 (subfractions 1–6 or 1–5 for WT or AtmenG, respectively) and F2 (subfractions 7–13 or 6–17) contained mostly plastoglobules. Envelopes and low amounts of thylakoids were found in F3 (subfractions 14–19 or 18–21). Fraction F4 (subfractions 20–23 or 22–25) contained envelopes and thylakoids, and F5 (subfractions 24–29 or 26–29) mostly thylakoids (32). Lipids were extracted from the five fractions with chloroform/methanol (2:1), and total fatty acids and phylloquinone were quantified by GC (33) and fluorescence HPLC (see above), respectively.

**Differential Chlorophyll Absorption Measurement of PSI in Leaf Discs**—As a measure for the content of photochemically active PSI, the transmission change at 830–870 nm, resulting from photo-oxidation of P₇₀₀, was determined in leaf discs of Arabidopsis. The transmission changes (ΔI/I) were corrected for the contribution of plastocyanin, determined at 870–950 nm (34), but using an optimized new generation pulse amplified modulation (PAM) system (Dual-PAM, plastocyanin-P₇₀₀ version, Heinz Walz, Effeltrich, Germany), which allows simultaneous determination of the difference transmission signals arising from plastocyanin and P₇₀₀. Using pre-illuminated Arabidopsis leaves with fully active Calvin cycle to avoid an acceptor-side limitation of PSI oxidation, P₇₀₀ and plastocyanin were pre-oxidized by 10-s illumination with weak far-red light selectively exciting PSI, followed by a strong saturating red light pulse (100-ms duration, 6000 μmol m⁻² s⁻¹), resulting in complete photo-oxidation of P₇₀₀ and plastocyanin and their subsequent reduction after the end of the light pulse. The maximal transmission changes between fully oxidized and fully reduced states were calculated. After transmission measurement, the

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leaf segment was punched, frozen in liquid nitrogen, and chlorophyll content determined (28). Transmission changes were normalized to a chlorophyll content of 1 mg.

Thylakoid Isolation and Quantification of PSI, PSII, and Cyt b₆/f—Thylakoid membranes were isolated as described (36). Thylakoid proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to immunoblot membranes, and blots were probed with polyclonal antiserum raised against PsaC (PS I), PsbD (PS II), or PetA (Cyt b₆/f).

PSI, PSII, or Cyt b₆/f were determined in isolated thylakoids by difference absorption measurements (36). Briefly, PSI was measured from P₇₀₀ redox changes after thylakoid membrane solubilization with 0.2% (w/v) β-dodecylmaltoside (50 μg Chl ml⁻¹). Ascorbate was used as electron donor to maintain P₇₀₀ in a fully reduced state in the dark, and methylviologen was used as electron acceptor during photooxidation after application of a saturating red light pulse (6000 μmol m⁻² s⁻¹, 200-ms duration).

For measurements of the cytochromes in PSI and Cyt b₆/f, thylakoid membranes were de-stacked in a low salt buffer containing 0.03% (w/v) β-dodecylmaltoside to eliminate light scattering effects. The cytochromes were oxidized with ferricyanide and subsequently reduced with ascorbate and dithionite, resulting in reduction of Cyt f and the high potential form of Cyt b₅₅₉ (HP, ascorbate-ferricyanide difference absorption spectrum) and reduction of cytochrome b₅₆ and the low potential form of Cyt b₅₅₉ (LP, dithionite-ascorbate), respectively. Absorption spectra were recorded using a Jasco J-550 spectrophotometer with head-on photomultiplier between 575 and 540 nm, and difference absorption spectra were deconvoluted using reference spectra and difference absorption coefficients for the cytochromes. PSI contents were calculated from the sum of the Cyt b₅₅₉ HP and LP difference absorption signals (37).

Measurement of PSI Electron Transfer Rate—PSI electron transfer activity was measured in thylakoids by recording oxygen consumption in continuous white light (5000 μmol m⁻² s⁻¹, halogen lamp) with a Clark-type electrode. Briefly, PSI activity in thylakoids was inhibited with 3′-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU, 100 μM), and electron transfer from ascorbate (10 mM) via the mediator N,N,N′,N′-tetramethylphenylenediamine dihydrochloride (TMPD, 100 μM) and plastocyanin to PSI was measured. Methylviologen (100 μM) was added as electron acceptor, resulting in the reduction of O₂ to H₂O₂. Because catalase activity was inhibited by the addition of 1 mM NaN₃, PSI activity could be calculated from O₂ reduction (38).

Low Temperature (77 K) Chlorophyll Fluorescence—Low temperature (77 K) chlorophyll a fluorescence emission spectra were recorded with a Jasco F6500 fluorometer with a red-sensitive photomultiplier. Isolated thylakoids were diluted to a chlorophyll concentration of 10 μg of Chl ml⁻¹, fluorescence was excited at 430 nm and emission spectra measured from 660 to 800 nm. Spectra were corrected for the instrumental response of the photomultiplier and normalized to the PSI emission signal at 685 nm.

Measurements of PSII Quantum Yield—Chlorophyll fluorescence was measured with a pulse amplitude modulation fluorometer (Imaging PAM; Heinz Walz, Effeltrich, Germany) with dark-adapted plants. Fluorescence light response curves were recorded after a 5-min exposure of the leaves to the photosynthetically active radiation (PAR) as indicated. PSII quantum yield was calculated according to the equation (Fₘ⁺−F)/Fₘ⁺ (39).

RESULTS

Identification of the Gene Encoding PNQ Methyltransferase in Arabidopsis—The pathways of menaquinone and phytolquinone biosynthesis have been studied in bacteria, and most of the genes have been functionally characterized (Refs. 7, 10, 15 and Fig. 1). The ultimate step of phytolquinone synthesis, the methylation of 2-phytly-1,4-naphthoquinone, is encoded by the menG gene (sll1653) in Synechocystis (10). A gene survey performed for genes of isoprenoid metabolism revealed the existence of up to four menG-like sequences in the Arabidopsis
The At1g23360 Protein Localizes to Chloroplasts—The enzymatic activity of PNQ methyltransferase was previously localized to the envelope membranes of spinach chloroplasts (11). Therefore, it was expected that the At1g23360 protein localizes to chloroplasts. As compared with the orthologous Synechocystis sequence sll1653, the At1g23360 protein contains an N-terminal extension of 37 amino acids. This N-terminal extension was predicted to contain a signal peptide for import into chloroplasts with a putative cleavage site C-terminal to Lys30 according to the ChloroP1.1 program (Ref. 41). To determine the subcellular localization experimentally, the entire At1g23360 coding sequence was translationally fused to the N-terminal sequence of the GFP gene and transiently transferred into Arabidopsis leaf mesophyll cells (Fig. 2B). Examination by confocal microscopy revealed that the green fluorescence of the At1g23360-GFP fusion protein co-localized with chlorophyll fluorescence, indicating that the At1g23360 protein localizes to chloroplasts (Fig. 2B).

Isolation and Complementation of an Arabidopsis AtmenG Mutant—To study the functional significance of the last step of phylloquinone synthesis in higher plants, an Arabidopsis mutant carrying a T-DNA insertion in the gene At1g23360 was obtained. A homozygous line was isolated by PCR screening using primer combinations for the genomic At1g23360 locus or for a T-DNA fragment containing flanking genomic sequences (Fig. 3A). Sequence analysis confirmed that the mutant harbors a T-DNA insertion in the 7th exon of the At1g23360 gene. The At1g23360 mRNA was not detectable in homozygous AtmenG mutant plants, in contrast to WT, where a weak band at around 800 bases was observed (Fig. 3B). To test whether the AtmenG mutant can be phenotypically complemented by expression of the candidate Arabidopsis menG gene product, the mutant was transformed with the At1g23360 ORF under the control of the CaMV 35S promoter, and transgenic plants with high levels of At1g23360 mRNA were identified by Northern blot analysis (Fig. 3B).

Fluorescence HPLC measurements revealed that AtmenG mutant leaves were totally devoid of phylloquinone (Fig. 3C). A new peak with shorter retention time was detected, which was tentatively identified as PNQ. The amount of PNQ in AtmenG was even higher than that of phylloquinone in WT leaves (5.71 ± 0.39 and 4.56 ± 0.48 nmol g⁻¹ FW, respectively; Fig. 3C). Furthermore, fluorescence HPLC analysis showed that all PNQ was converted into phylloquinone by overexpression of the At1g23360 cDNA in the AtmenG mutant background.

During phylloquinone synthesis, the phytyl group of phytodiphosphate is transferred onto DHNA by DHNA phytlytransferase (Refs. 11 and 15 and Fig. 1). Free phytol can serve for the production of phytyl containing lipids (tocopherol, fatty acid phytyl esters) in Arabidopsis after phosphorylation to phytlydiphosphate (27, 42). To test whether free phytol can be employed for phylloquinone synthesis and to identify different phytyl-containing reaction products, seedlings of Arabidopsis WT, AtmenG, and AtmenG-At1g23360 were incubated with radioactive [1-³H]phytol, followed by lipid separation by TLC. In wild type and complemented plants, a radioactive band was observed that co-migrated with a phylloquinone standard (Fig. 3D). This band was absent in the AtmenG mutant, but another
substantial and significant difference in the distribution of plastoglobules and envelopes, and thylakoid membranes. Subsequently, total fatty acids and phylloquinone were measured in these five fractions, and the ratio of phylloquinone to fatty acids and the relative distribution of phylloquinone fraction after lower and higher activity.

Furthermore, the distribution of PNQ was measured in chloroplast fractions derived from AtmenG plants. The amount of PNQ in thylakoids (F4, F5) was about 25%, and in plastoglobules (F1, F2) ca. 70% (Fig. 4B). The ratio of PNQ to total fatty acids was ca. 0.016 in the plastoglobules fraction F1, and much lower in the other gradient fractions. Therefore, the relative distribution of PNQ in AtmenG differs from that of phylloquinone in WT, because PNQ is highly enriched in plastoglobules compared with thylakoids.

The AtmenG Mutation Affects Growth and Anthocyanin Accumulation—AtmenG plants showed significantly reduced growth when raised under normal light of 150 μmol m⁻² s⁻¹ (Fig. 5A). Growth reduction of AtmenG was restored after transformation with the At1g23360 cDNA indicating that it was caused by a block in PNQ methyltransferase activity. High light conditions are known to affect the efficiency of photosynthesis, resulting in photoinhibition of PSI and stimulation of different photoprotective mechanisms. For example, anthocyanin, a flavonoid pigment localized to the vacuoles of mesophyll cells, accumulates at high light (43, 44). Plants were transferred to high light (550 μmol m⁻² s⁻¹) for 4 days to study the effect of the replacement of phylloquinone with PNQ on photosynthesis and overall plant physiology. WT Arabidopsis plants showed a red leaf color at high light (Fig. 5A). This color originated from anthocyanin accumulation as demonstrated by photometric measurements (Fig. 5B). By contrast, the accumulation of anthocyanin was compromised in AtmenG plants. Chalcone synthase represents one of the key enzymes in anthocyanin synthesis, and the CHS1 gene is known to be strongly expressed during high light exposure (43). Northern analysis confirmed that CHS1 expression in WT increased under high light, but it was reduced in AtmenG compared with WT and the complemented line. Therefore, phylloquinone deficiency affects the
light-dependent stimulation of anthocyanin synthesis in AtmenG, presumably by interfering with transcriptional activation of key pathway genes (CHS1).

Photosystem I Abundance Is Decreased in AtmenG—Phylloquinone is an integral constituent of PSI where it serves as electron carrier in the $P_{700}$ reaction center. Thus, it was expected that replacement of phylloquinone with its unmethylated form might affect PSI stability and abundance. To analyze the composition of photosynthetic units in thylakoid membranes, the amounts of chlorophylls and carotenoids were quantified (Table 1). In plants raised under normal light (150 $\mu$mol m$^{-2}$ s$^{-1}$), the contents of chlorophyll and carotenoids were very similar in WT and AtmenG. To assess the abundance of PSI (PsAC), PSII (PsBD), and Cyt $b_{6}/f$ (PetA) complexes, immunoblot analysis was done for subunits known to be essential for the assembly of the individual complexes in photosynthetic eukaryotes (Fig. 6A). The band intensity for these three proteins was very similar in WT, AtmenG, and in complemented mutant plants raised under normal light. Low temperature (77 K) chlorophyll fluorescence of thylakoids can be employed to estimate the relative chlorophyll distribution between PSI and PSII. When 77 K fluorescence was normalized to 1 at 685 nm, a characteristic wavelength for PSI fluorescence, differences in PSI abundance relative to PSII can be observed at the peak of 730 nm, which is indicative for PSI. As shown in Fig. 6B (upper panel) the amount of PSI was very similar for WT, AtmenG, and complemented plants raised at normal light.

After high light exposure for 4 days, the amount of chlorophyll in AtmenG decreased to 963 $\mu$g g$^{-1}$ FW as compared with 1172 and 1162 in wild type and complemented plants, respectively (Table 1). Western analysis revealed that the amount of PsAC protein was reduced in AtmenG plants exposed to high light, but the amounts of PsBD and PetA remained similar to wild type and complemented plants. Furthermore, the PSI maximum at 730 nm in 77K fluorescence was reduced in AtmenG plants raised at high light (Fig. 6B, lower panel). Taken together, the reduction in chlorophyll content observed in AtmenG under high light can predominantly be attributed to a decrease in PSI abundance, while the other components of the photosynthetic apparatus (PSII, Cyt $b_{6}/f$) remain similar to wild type.

The content of active PSI reaction centers ($P_{700}$ content) was measured by difference absorption spectroscopy in leaf discs of Arabidopsis. As shown in Fig. 7A, the difference absorption signal for $P_{700}$ per chlorophyll was slightly reduced in AtmenG
TABLE 1
Photosynthetic pigments and lipid antioxidants in the AtmenG mutant

Plants (WT, AtmenG, complemented mutant plants) were raised at normal or high light as indicated in the legend to Fig. 5. Chlorophyll a+b (Chl a+b, in g⁻¹ FW), the chlorophyll a-to-b ratio (Chl a/b) in leaves was measured photometrically. The carotenoid contents were determined by HPLC and are presented in mmol mol⁻¹ Chl. Values show means and S.D. from at least three measurements.

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<td>Chl a+b</td>
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<td>1342 ± 136</td>
<td>1334 ± 175</td>
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<tr>
<td>Lutein</td>
<td>105.5 ± 5.4</td>
<td>108.8 ± 15.3</td>
<td>107.3 ± 15.8</td>
<td>121.9 ± 18.2</td>
<td>119.4 ± 5.7</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>69.2 ± 3.4</td>
<td>65.0 ± 5.2</td>
<td>69.6 ± 10.2</td>
<td>77.8 ± 11.0</td>
<td>74.1 ± 2.6</td>
</tr>
<tr>
<td>V + A + Zc</td>
<td>34.4 ± 1.9</td>
<td>41.5 ± 9.3</td>
<td>39.8 ± 7.1</td>
<td>55.7 ± 11.5</td>
<td>73.4 ± 4.4</td>
</tr>
<tr>
<td>(A + Z)/(V + A + Z)</td>
<td>0.186 ± 0.013</td>
<td>0.186 ± 0.030</td>
<td>0.165 ± 0.004</td>
<td>0.224 ± 0.084</td>
<td>0.451 ± 0.102</td>
</tr>
<tr>
<td>Tocopherol&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.0 ± 2.0</td>
<td>6.0 ± 1.0</td>
<td>8.2 ± 2.5</td>
<td>27.4 ± 7.3</td>
<td>19.8 ± 3.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> V + A + Z, sum of xanthophyll-cycle pigments (V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; mmol mol⁻¹ Chl.

<sup>bc</sup>(A + Z)/(V + A + Z), de-epoxidation status of xanthophyll-cycle pigments (ratio).

<sup>d</sup> Tocopherol was measured by fluorescence HPLC (µg g⁻¹ FW).

![FIGURE 6. Photosystem I abundance is affected in AtmenG plants under high light.](image)

A. Western blots of thylakoid proteins probed with antibodies against PsaC (PS II), PsbD (PS II), or PetA (Cyt b6/f). The first three lanes contain 100, 50, and 25% of wild-type thylakoid protein. Lane 1, WT; lane 2, AtmenG mutant; lane 3, AtmenG complemented with At1g23360. B. Low temperature (77 K) fluorescence spectra of thylakoids derived from plants raised at normal or high light. Chlorophyll fluorescence at 685 nm (PS II band) was normalized to 1. WT (continuous line); AtmenG (dotted line), AtmenG-At1g23360 (dashed line). Plants were raised at normal or high light as described in the legend to Fig. 5.

as compared with WT and complemented plants (AtmenG-At1g23360), and this effect became more severe when plants were exposed to high light. The contents of the different photosynthetic complexes, PSI, PSII, and Cyt b6/f were quantified in thylakoids by differential absorption spectroscopy. In accordance with data obtained for leaf discs (Fig. 7A), the amount of PSI per chlorophyll was slightly decreased in thylakoids exposed to normal light (Fig. 7B), and it was strongly reduced at high light. On the other hand, the PSII and Cyt b6/f contents were very similar at normal light, and slightly increased at high light in AtmenG (Fig. 7, C and D), a consequence of the reduced chlorophyll distribution to PSI.

To obtain a measure for the activity of electron flow through PSI, thylakoids were purified from chloroplasts and used for electron transport assays with ascorbate/TMPD and methylviologen as electron donor and acceptor, respectively, while PSII activity was inhibited with DCMU. Oxygen consumption was measured with a Clark-type electrode. This assay is strongly dependent on plastocyanin availability per PSI, as the P₇₇₀ reduction by plastocyanin is the rate-limiting step of the ascorbate/TMPD assay. Whereas the plastocyanin-PSI ratios were identical in all low light grown lines (not shown), in high light, the ratio of plastocyanin to PSI was strongly increased in AtmenG, because of the reduced PSI content (data not shown). Therefore, the assays were only conducted with thylakoids from plants grown at normal light. As shown in Fig. 7E, PSI electron transfer rates (on a chlorophyll basis) were slightly decreased in AtmenG plants raised at normal light. Considering the slight reduction in PSI content per chlorophyll (Fig. 7, A and B), electron transfer rates per PSI of WT and AtmenG were indistinguishable. In conclusion, differential absorption measurements of leaf discs and thylakoids, and PSI electron transport activity determinations revealed a slight reduction in PSI abundance in AtmenG plants at normal light, and this decrease became even more severe when plants were exposed to high light. However, the electron transport capacity per PSI unit was not affected by replacement of phyloquinone in AtmenG. The amounts of the other photosynthetic complexes were not decreased.

Exposure to High Light Affects PSII Quantum Yield and Xanthophyll Cycle Pigment Composition in AtmenG—To obtain a measure for the efficiency of light conversion into chemical energy, chlorophyll fluorescence was measured with plants exposed to different light intensities and the photosynthetic quantum yield of PSII was calculated. No difference in quantum yield was detected for dark adapted plants (Fig. 8), indicating that PSII efficiency was not altered in AtmenG. The quantum
yield measured at light intensities of 50–200 μmol m⁻² s⁻¹ was slightly reduced in AtmenG as compared with wild type and complemented lines (Fig. 8A). AtmenG plants raised at high light showed a severe decrease in PSII quantum yield already at low light intensities, indicating a strong restriction of linear electron flux because of the reduced PSI content (Fig. 8B).

To address the question whether changes in photosynthetic characteristics of the thylakoid membrane in AtmenG affect the antioxidant status of the plant, tocopherol was measured in leaves of plants exposed to normal or high light (Table 1). The amount of tocopherol in AtmenG plants raised at normal light was not different from WT. High light resulted in a strong increase in tocopherol in all three lines, but tocopherol content in AtmenG was not different from WT. These data suggest that the AtmenG mutation does not result in overall oxidative stress.

High light exposure resulted in an apparent increase in the pool size of xanthophyll cycle pigments violaxanthin, antheraxanthin, and zeaxanthin in AtmenG when calculated on a chlorophyll basis (Table 1). This apparent increase can be explained by the decrease in PSI and total chlorophyll content in AtmenG plants exposed to high light (Table 1). The de-epoxidation status of xanthophyll cycle pigments (A+Z)/(V+A+Z) was clearly increased at high light, from 0.224 in WT to 0.451 in AtmenG. In conclusion, phylloquinone deficiency in AtmenG affected PSI abundance and PSII efficiency at high light. As a consequence, the xanthophyll cycle de-epoxidation status was increased, indicating the stimulation of photoprotective mechanisms in AtmenG.

**DISCUSSION**

The initial goal of this study was the identification of the gene encoding PNQ methyltransferase from Arabidopsis. Several lines of evidence support the conclusion that the gene At1g23360 encodes a genuine PNQ methyltransferase: (i) The At1g23360 cDNA is capable of complementing a Synechocystis menG (sll1653) loss-of-function mutant. (ii) An Arabidopsis...
AtmenG mutant carrying a T-DNA insertion in the gene At1g23360 displays phylloquinone deficiency and contains increased amounts of PNQ. (iii) Transformation of the AtmenG mutant with the At1g23360 ORF results in the conversion of PNQ to phylloquinone. (iv) Radioactive phytol was incorporated into phylloquinone in WT and complemented AtmenG plants, but into PNQ in AtmenG (Fig. 3D). Complete absence of phylloquinone from AtmenG suggests that Arabidopsis has only one functional menG gene and that the other three ORFs identified as weakly homologous to menG (38) are unlikely to specify functional PNQ methyltransferases. Our finding that even massive overexpression of AtmenG in the complemented mutant plants (Fig. 3B) does not result in phylloquinone accumulation beyond wild-type levels (Fig. 3C) indicates that PNQ methylation does not constitute a rate-limiting step in phylloquinone synthesis.

Two molecules of phylloquinone localize to each PSI monomer in the thylakoid membranes (1–3). Mutations in the isochoerismate synthase genes of Arabidopsis (ics1, ics2) result in partial phylloquinone depletion, but PSI activity was affected to a lesser extent (16). It was concluded that a fraction of phylloquinone is not associated with PSI reaction centers (16). Calculations based on our results (Table 1, Figs. 3C and 7C) indicate that the ratio (mmol per mmol) of phylloquinone to PSI is about 3.1 in WT, whereas the PNQ to PSI ratio is ca. 4.4 in AtmenG. These two ratios exceed the theoretical value of 2.0. The excess amounts of phytynaphthoquinones might be localized to other compartments of the chloroplast, i.e. the thylakoid lipid matrix, envelope membranes or to plastoglobules. Indeed, the proportion of phylloquinone in thylakoids and plastoglobules of WT was ca. 60 and 30% of total phylloquinone (Fig. 4), respectively, confirming previous data on the identification of phylloquinone in plastoglobules (45). Therefore, the excess amount of phylloquinone not associated with PSI localizes to the plastoglobules of chloroplasts. In this respect it seems unlikely that significant amounts of phylloquinone not associated with the thylakoids exist outside of plastids (e.g. plasma membrane) as previously suggested (16). Furthermore, the increase in PNQ in AtmenG as compared with phylloquinone in WT (Fig. 3C) can be explained by a preferred accumulation in plastoglobules (Fig. 4D). These results indicate that plastoglobules serve as a sink for the deposition of excess amounts of phylloquinone and its biosynthetic precursor. Furthermore, lipid trafficking must be involved in the transport of prenyl quinones from their site of synthesis, the envelope membranes, to thylakoids and plastoglobules.

The block in PNQ methyltransferase activity in AtmenG affects anthocyanin accumulation during high light exposure, and this effect is mediated by interfering with expression of anthocyanin synthesis genes (CHS1). Interestingly, a similar effect, i.e. the reduced accumulation of anthocyanin during high light exposure, was previously observed for other Arabidopsis mutants, e.g. vte1 and vte2, affected in tocopherol and ascorbate synthesis, respectively (46–48). The impact of a mutation in chloroplast lipid synthesis (AtmenG, vte1) on anthocyanin production is unusual given the fact that anthocyanin is synthesized in the cytosol and localizes to a different compartment, the vacuoles. Nevertheless, the accumulation of anthocyanin is believed to be crucial in protecting the photosynthetic membrane against excess light (44). However, alterations in photosynthesis and antioxidant content do not necessarily result in anthocyanin increase (47). We presume that the altered anthocyanin accumulation in AtmenG originates from changes in chloroplast-to-nucleus signaling in response to high light, potentially as a consequence of the metabolic status of the cell. The photosynthetic capacity of the AtmenG mutant is clearly reduced at high light, and therefore, carbon assimilation presumably is also affected (Fig. 8). As chalcone synthase expression is well known to be sucrose-dependent (49), the high light-stimulated synthesis of anthocyanins in wild type and complemented plants might be explained by their higher photosynthetic efficiency, as this should result in more pronounced photoassimilate accumulation than in the AtmenG line.

Total absence of phylloquinone results in a drastic reduction in growth and photosynthetic efficiency in the AtmenA mutant of Arabidopsis, which is deficient in DHNA phytyltransferase (15). However, in the AtmenG mutant, which is also phylloquinone-deficient, growth, total chlorophyll and PSI contents, and photosynthetic efficiency were only moderately affected. This suggests that PNQ can partially replace phylloquinone as electron carrier in PSI of AtmenG. Similarly, in the phylloquinone-deficient Synechocystis menG mutant, PNQ was detected in PSI complexes where it functionally replaced phylloquinone (10). The replacement of phylloquinone with its unmethylated precursor in the PSI reaction center affects electron flow from P700 to the iron-sulfur cluster Fx in the Synechocystis menG mutant (10). The lifetime for electron transfer from Q to Fx is slowed from 290 ns (WT) to 600 ns (AtmenG), and the redox potential of PNQ is slightly more oxidizing (about 50–60 mV) than that of phylloquinone (10).

Photosynthesis in AtmenG was only slightly affected in plants raised at normal light (Figs. 6–8 and Table 1). This is not surprising, because in wild type the rate-limiting step of electron flux through PSI is plastocyanin binding to PSI and electron transfer to P700+ (50, 51). Therefore, only very drastic changes at the PSI acceptor side could have a significant impact on electron transfer rates through PSI. This is definitely not the case in the AtmenG mutant, as electron transfer from ascorbate via TMPD and plastocyanin to methylviologen/O2 was unaltered. However, it is possible that very minor changes in the properties of the PSI acceptor side, such as those reported by Sakuragi et al. (10) could slightly affect the rate of side reactions, e.g. electron transfer to O2 instead of ferredoxin reduction. This might lead to local oxidative damage at PSI, which is known to result in loss of the stromal PSI subunits PsaC, PsaD and PsaE from the complex, and subsequent disassembly and degradation of the entire PSI complex (35). In this context, the more pronounced decrease of PSI units in AtmenG under high light (Figs. 6 and 7) might be caused by accelerated PSI degradation because of oxidative damage, which cannot be compensated by de novo synthesis in mature Arabidopsis leaves (35). An alternative explanation for the reduced PSI content is decreased de novo synthesis, which could be caused by down-regulation of expression of nuclear PSI genes by an unknown, phylloquinone-dependent mecha-
nism. However, this scenario seems to be unlikely, as it would not explain the differential phenotype of AtmenG under high versus normal light conditions. Therefore, local oxidative damage at the PSI reaction center, because of slight alterations in the function of the PSI acceptor side, is a more likely explanation for the observed alterations in AtmenG photosynthesis. This scenario is also supported by the observation that PSI abundance is selectively reduced in AtmenG, whereas the other photosynthetic complexes accumulate to normal or slightly increased amounts, relative to wild type.

Further evidence for the scenario that oxidative stress is involved in PSI decrease can be obtained from the apparent discrepancy of PSI quantification. While PsAC immunoblot analysis (PsAC signal reduced to less than 50% of wild type at high light; Fig. 6A) and in vivo difference transmission signals originating from P700 (Fig. 7A) on the one hand indicate a strong decrease in PSI, the 77 K chlorophyll α fluorescence emission (Fig. 6B) and in vitro P700 quantification on the other hand suggest a far less pronounced decrease of PSI (Fig. 7B). This apparent discrepancy can be explained by close interactions of the FeS clusters F1 and F8 bound to PsAC with phyloquinone during electron transfer. Changes in phyloquinone structure in AtmenG that presumably cause local oxidative stress might result in damage to PsAC, as observed in the diminished immunoblot signal. Furthermore, PsAC damage can strongly affect in vivo P700 difference transmission, as it is known that PsAC is required for PSI charge separation. PSI abundance as estimated from in vitro difference absorption spectroscopy of thylakoids is affected to a lesser extent. In this experiment, electrons from the first FeS cluster F1 bound to the reaction center dimer PsA and PsB of PSI can be directly transferred onto methylviologen, i.e. by circumventing the FeS clusters of PsAC. Furthermore, 77 K fluorescence spectroscopy reflects changes in the antenna size of PSI because low temperature fluorescence emission originates from the LHca antenna proteins. Therefore, our data indicate that the amount of PsAC is strongly decreased in AtmenG plants under high light, whereas the PSI reaction center core and the PSI antenna size seem to be affected to a lesser extent.

The decrease in chlorophyll amount observed during high light can mostly be attributed to a lower content of PSI units. PSII quantum yield of AtmenG was similar to wild type in dark-adapted plants (Fig. 8, A and B), but was already decreased at low light intensities, indicating that the plastoquinone pool of AtmenG becomes overreduced during light exposure. The lack of functional PSI complexes might be the cause for the decreased electron flow through PSII. The observed change in de-epoxidation status of xanthophyll-cycle pigments indicates an increase in photoprotective mechanisms.

In conclusion, our results clearly demonstrate that methyla-
tion of phyloquinone is important for maximal photosynthetic efficiency and optimal growth when plants are raised under normal light conditions. At high light, the replacement of phyloquinone with PNQ becomes even more significant causing a decrease in PSI complexes. This in turn leads to lowered PSII efficiency and thus negatively affects the performance of the entire photosynthetic electron transfer chain.

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REFERENCES