Intersection of the tocopherol and plastoquinol metabolic pathways at the plastoglobule

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Plastoglobules, lipid–protein bodies in the stroma of plant chloroplasts, are enriched in non-polar lipids, in particular prenyl quinols. In the present study we show that, in addition to the thylakoids, plastoglobules also contain a considerable proportion of the plastidial PQ-9 (plastoquinol-9), the redox component of photosystem II, and of the cyclized product of PQ-9, PC-8 (plastochromanol-8), a tocochromanol with a structure similar to γ-tocopherol and γ-tocotrienol, but with a C-40 prenyl side chain. PC-8 formation was abolished in the Arabidopsis thaliana tocopherol cyclase mutant vte1, but accumulated in VTE1-overexpressing plants, in agreement with a role of tocopherol cyclase (VTE1) in PC-8 synthesis. VTE1 overexpression resulted in the proliferation of the number of plastoglobules which occurred in the form of clusters in the transgenic lines. Simultaneous overexpression of VTE1 and of the methyltransferase VTE4 resulted in the accumulation of a compound tentatively identified as 5-methyl-PC-8, the methylated form of PC-8. The results of the present study suggest that the existence of a plastoglobular pool of PQ-9, along with the partial conversion of PQ-9 into PC-8, might represent a mechanism for the regulation of the antioxidant content in thylakoids and of the PQ-9 pool that is available for photosynthesis.

Key words: Arabidopsis, chloroplast, plant, plastochromanol, plastoquinone, tocochromanol.

INTRODUCTION

Organisms performing oxygenic photosynthesis harbour a unique set of prenyl quinols, i.e. phyloquinol (vitamin K1), PQ-9 (plastoquinol-9) and tocochromanols (tocopherol, tocotrienol and vitamin E), which serve as electron carriers in PSI and PSII (photosystems I and II), and as lipid antioxidant respectively. Eight forms of tocochromanols are commonly considered to occur in plants, i.e. four forms each of tocopherol and tocotrienol, carrying a phytol or geranylgeranyl side chain respectively [1]. The individual forms of tocopherols and tocotrienols are distinguished by the number and positions of methyl substituents on the chromanol ring. Tocopherols are synthesized from homogentisate and phytol-diphosphate by homogentisate phytyltransferase (HPT1 and VTE2) [2, 3] (Figure 1). After methylation by VTE3, the second ring of DMPBQ (2,3-dimethyl-5-phytyl-1,4-benzoquinol) is closed by tocopherol cyclase (VTE1) yielding γ-tocopherol and γ-tocotrienol [4–7]. Final methylation by γ-tocopherol methyltransferase (VTE4) results in formation of α-tocopherol [8–11] (Figure 1). In many species, including Arabidopsis thaliana, α-tocopherol is the most abundant form in leaves, whereas γ-tocopherol is predominant in seeds [8, 9]. Tocopherol is found in the thylakoid membranes of chloroplasts, where it presumably protects the photosynthetic apparatus against oxidative stress. Another fraction of tocopherol is associated with the plastoglobules, which is in agreement with the finding that VTE1 has been identified as a plastoglobular protein [12–15].

The synthesis of PQ-9 depends on the prenylation of homogentisate [16] employing a long hydrocarbon side chain derived from solanesyl-diphosphate (nonaprenyl-diphosphate). An A. thaliana gene, HST, presumably involved in the transfer of the solanesyl group on to homogentisate, has been described [17, 18]. Methylation by VTE3, a methyltransferase shared by the tocopherol and PQ-9 pathways, results in the formation of PQ-9 [4–6]. Two molecules of PQ-9 are associated with PSII, one (QA) which is firmly bound to the PSII reaction centre, and a second one (QB) which can be exchanged with the mobile pool of PQ-9 in the thylakoid membranes [19]. Furthermore, PQ-9 was identified in the plastoglobules of chloroplasts, but the pool size of plastoglobular PQ-9 and its function remained unknown [20].

Whittle et al. [21] were the first to describe a derivative of PQ-9 in plants which was designated PC-8 (‘plastochochrome-8’). PC-8 contains the very same head group as γ-tocopherol and γ-tocotrienol (‘plastochochrome-3’), but differs with respect to its prenyl side chain which contains 40 carbon atoms (eight isoprene units: Figure 1). PC-8 was detected in rubber trees and in the seed oils of flax, rape and maize [22–26], and later in the seed oils of Turkish pine, hemp and caper [27–29]. The fact that γ-tocopherol and PC-8 share the same head group suggested that PC-8 is derived from a cyclization reaction of PQ-9 catalysed by VTE1 (Figure 1). In agreement with this hypothesis,
it became clear that a considerable proportion of these two prenyl quinol lipids is associated with plastoglobules. Measurements of prenyl quinols in different A. thaliana mutants and transgenic lines revealed that PQ-9/PC-8 metabolism is closely linked with tocopherol metabolism by sharing a number of enzymatic steps.

EXPERIMENTAL

Plant material

A. thaliana WT (wild-type; Columbia), transgenic and mutant plants were grown with 16 h of light (120 μmol·s⁻¹·m⁻²) per day. Leaf material for chloroplast isolation and fractionation was obtained from 6- to 8-week-old plants grown under an 8 h daylight regime. The following lines were used: vte1, ethylmethane sulfonate mutant carrying a premature stop codon in the tocopherol cyclase gene [7]; vte2-1, ethylmethane sulfonate mutant with an amino-acid exchange in the homogentisate phytyltransferase gene [32]; vte2-3, mutant allele carrying a T-DNA (transferred DNA) insertion (line GABI_1676G08; GABI Kat Collection, Bielefeld, Germany) [33]. The position of the T-DNA insertion in vte2-3 was confirmed by PCR using primers for amplification of genomic DNA (PD294, 5'-TGTTCTGGGCTCTTTTTGTA-3'; PD256, 5'-AAATTGGAGGGCAATAAAAGGCAGTA-3') and of the sequence flanking the insertion (T-DNA left-border primer, 5'-CCCATTGGGACGTGAATGTAGACAC-3'; PD256). Sequencing of the flanking DNA amplified by PCR confirmed the position of the T-DNA in the 11th exon after Val193.

Overexpression of VTE1 and VTE4 in A. thaliana leaves

Overexpression of the VTE1 cDNA under the control of the cauliflower mosaic virus 35S promoter in A. thaliana has been described previously [34]. Two lines (WT-VTE1#40 and vte1-VTE1#1 in WT and vte1 background respectively) showing strong expression of VTE1 in Northern and Western blot analysis were selected for further analyses. The full-length VTE4 cDNA was released from clone RAFL07-18-C14 (RIKEN BioResource Center) with EcoRI and BamHI, and ligated into pBluescriptIIISK+ (Stratagene). After digestion with KpnI and XbaI, the VTE4 cDNA was cloned behind the 35S promoter of the binary vector pBINAR-Hyg [35]. The VTE4 construct was transferred into the line WT-VTE1#40 by floral dip [36]. Double-transgenic lines selected for hygromycin B resistance were screened by fluorescence HPLC for alterations in tocochromanol composition.

Measurement of tocochromanols and PQ-9

Tocochromanols were isolated from plant leaves with diethyl ether and 1 M KCl/0.2 M H₃PO₄, or from plant seeds with ethanol [34,37]. Tocotrienol (500 ng) was added as an internal standard. Tocopherol concentrations were adjusted using absorption coefficients as published previously [38]. The tocochromanols were dissolved in hexane and quantified by fluorescence HPLC [39]. Briefly, tocochromanols were injected on to a Lichrospher 100 diol, 5 μm, 3 mm × 25 cm column (Knauer) attached to an Agilent 1100 HPLC System. The solvent was n-hexane/tertiary butylmethyl ether (96:4) at a flow rate of 0.75 ml·min⁻¹. Fluorescence was recorded with an excitation of 320 nm and emission of 390 nm.

PC-8 was quantified together with tocopherols by fluorescence HPLC (diol column, see above) using tocol as an internal standard [39]. The linearity of PC-8 quantification by HPLC...
was determined using PC-8 isolated from line 6 via HPLC. The response factor of PC-8 relative to tocol was determined after adjusting the concentration of PC-8 (isolated from line 6) using the absorption coefficient [24].

PQ-9 was isolated from frozen leaves by extraction with 1 M KCl/0.2 M H3PO4 and hexane and subsequently with diethyl ether. Ubiquinone-4 (500 ng; Sigma) was added as an internal standard. The combined organic phases were evaporated with air and the residue dissolved in hexane. PQ-9 was quantified by HPLC on a diol column (see above) using n-hexane/tertiary butylmethyl ether (90:10; flow rate, 0.75 ml · min⁻¹) by recording the absorption at 255 nm. The identity of PQ-9 was confirmed by MS and by comparing its UV absorption spectrum with that of PQ-9 isolated from A. thaliana leaves by reversed-phase HPLC [40]. The content of PQ-9 was calculated based on the amount of the internal standard (ubiquinone-4) taking into account the difference in absorption coefficients [41].

Ubiquinone-4 (500 ng; Sigma) was added as an internal standard. (90:10; flow rate, 0.75 ml · min⁻¹) by recording the absorption at 255 nm. The identity of PQ-9 was confirmed by MS and by comparing its UV absorption spectrum with that of PQ-9 isolated from A. thaliana leaves by reversed-phase HPLC [40].

**Analysis of quinol lipids by MS**

Quinol lipids were isolated by HPLC as described above and mass spectra were recorded using a Q-TOF (quadrupole-time-of-flight) mass spectrometer (Q-TOF 6530; Agilent). The lipids were dissolved in chloroform/methanol/ammonium acetate (300:665:35) and directly infused at a flow rate of 1 μl · min⁻¹ using a chip-based nanospray ion source (HPLC Chip/MS 1200 with infusion chip; Agilent Technologies). Samples were analysed in positive mode with a fragmentor voltage of 270 V. Molecular ions were selected in the quadrupole, fragmented in the collision cell with nitrogen gas and a collision energy of 33 V (γ-tocopherol) and 40 V (PC-8). Data were processed with the Mass Hunter Workstation software (Version B.02.00; Agilent Technologies).

**Fractionation of A. thaliana chloroplasts**

Chloroplasts were isolated from leaves of 6- or 8-week-old A. thaliana plants, as indicated, hypotonically ruptured and subplastidial compartments were isolated by centrifugation using a standard sucrose-density gradient [15]. The different subfractions were pooled into five fractions according to Western blot analysis using antibodies against AtPGL35 (A. thaliana plastoglobulin 35; a plastoglobule marker), AtTOC75 (A. thaliana translocon at the outer envelope membrane 75; an envelope marker) and CAB (chlorophyll a binding protein; a thylakoid marker). The gradient fractions F1 and F2 contained mostly plastoglobules, fraction F3 envelopes, and fractions F4 and F5 thylakoids [15]. Tocochromanols were extracted from the five fractions with chloroform/methanol (2:1), and were quantified by fluorescence HPLC.

**Subcellular localization of VTE4 using GFP (green fluorescent protein)**

The coding region of VTE4 was amplified from the cDNA clone RAFL07-18-C14 (RIKEN) using the primers PD590 (5'−AGGGATCCAATGAAACGACTCTAGCAG-3') and PD591 (5'-ATCCATGGAGAGCTCTGCTGGCAGTG-3') adding flanking BamHI and Ncol restriction sites. The PCR product was cloned into pCL60, 5' and in-frame to the GFP sequence under the control of the 35S promoter [42]. The other GFP constructs [AtPGL35-GFP, AtTIC110 (A. thaliana translocon at the outer envelope membrane of chloroplasts 110)—GFP and pSSU—GFP] have been described previously [15,43]. A. thaliana leaves were transiently transformed by particle bombardment as follows. Gold particles (1.0 μm, Bio-Rad Laboratories; 1.5–3 μm, Aldrich) were sterilized in ethanol and re-suspended in water to a final concentration of 60 μg/μl. Plasmid DNA (5 μg) was precipitated on the gold carrier (50 μl aliquots consisting of a 1:1 mixture of microcarrier from Bio-Rad Laboratories and Aldrich) by adding 50 μl of 2.5 M CaCl2 and 20 μl of 0.1 M spermidine-free base under continuous vortex-mixing. After washing with ethanol, DNA-coated gold particles were suspended in ethanol, spread on four macrocarrier discs (Bio-Rad Laboratories) and used for ballistic transformation. Leaves of mature A. thaliana plants grown on soil for 4–7 weeks were placed upside-down on 0.5 × MS medium (Murashige and Skoog medium) and bombarded with a PDS-1000/He BIONISTIC R Particle Delivery System (Bio-Rad Laboratories) operating at 1100 p.s.i. (1 p.s.i. = 6.9 kPa) He pressure. At 24–48 h after transformation, leaves were placed on to glass slides with lanolin. Transient transformation of protoplasts was performed with poly(ethylene glycol) [44], but reducing cellulase and macerozyme (Serva) contents to 1% and 0.25% respectively. Fluorescence in transiently transformed leaves and protoplasts was monitored 48 h after transformation by confocal laser scanning microscopy (LEICA TCS 4D microscope). The FITC (488 nm) laser line was used to detect GFP fluorescence, and chlorophyll autofluorescence was monitored using the TRITC (tetramethylrhodamine β-isothiocyanate) (568 nm) excitation wavelength.

**Electron microscopy**

Chloroplast ultrastructures of 4-week-old WT, vte1 and WT-VTE1#40 A. thaliana plants were examined by electron microscopy. Leaves were fixed in 1% glutaraldehyde in 0.05 M phosphate buffer overnight at 4 °C, and post-fixed in osmium tetroxide for 2 h at room temperature (22–25 °C). After dehydration and embedding in LRWhite resin, ultrathin sections (80–90 nm) were obtained on a Reichert Ultracut S microtome and mounted on to copper grids. Ultrathin sections were post-stained with uranyl acetate and lead citrate and observed with a Philips CM 100 transmission electron microscope at 60 kV. Chloroplast area and plastoglobule diameter were measured using the ImageJ software.

**RESULTS**

**Accumulation of PC-8 in A. thaliana plants affected the expression of genes of tocopherol synthesis**

PC-8, an unusual prenyl quinol lipid, was originally identified in the leaves of rubber trees [21] and later in the seeds of other plant species [22]. To address the question of its distribution, we analysed different additional plants for the occurrence of PC-8. PC-8 was detected in leaves of tobacco, rice, pumpkin, potato, tomato and Lotus japonicus, and in tomato fruits in various amounts (results not shown). This suggests that PC-8 is widely distributed in the plant kingdom, and it occurs both in monocot and dicot plants, and in different plant organs. The occurrence of PC-8...
Table 1  Tocochromanol content in A. thaliana tocopherol mutants and overexpressing lines

Tocochromanol compositions of leaves and seeds of A. thaliana WT, tocopherol mutants (vte1, vte2-1 and vte2-3) and plants overexpressing VTE1 (WT-VTE1#40 and vte1-VTE1#1) were determined by diol column HPLC. Values are means ± S.D. of three measurements each. n.d., not detectable.

<table>
<thead>
<tr>
<th>Plant line</th>
<th>α-Tocopherol (nmol · g⁻¹ of FW)</th>
<th>β-Tocopherol (nmol · g⁻¹ of FW)</th>
<th>γ-Tocopherol (nmol · g⁻¹ of FW)</th>
<th>δ-Tocopherol (nmol · g⁻¹ of FW)</th>
<th>PC-8 (nmol · g⁻¹ of FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaves</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>25.2 ± 4.5</td>
<td>0.5 ± 0.1</td>
<td>3.0 ± 0.7</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>vte1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>vte2-3</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>WT-VTE1#40</td>
<td>29.7 ± 4.3</td>
<td>4.3 ± 0.7</td>
<td>7.2 ± 0.7</td>
<td>1.9 ± 0.6</td>
<td>7.2 ± 0.4</td>
</tr>
<tr>
<td>vte1-VTE1#1</td>
<td>37.8 ± 5.2</td>
<td>1.0 ± 0.5</td>
<td>4.1 ± 0.6</td>
<td>5.7 ± 0.6</td>
<td>6.79 ± 13.5</td>
</tr>
<tr>
<td><strong>Seeds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>23.1 ± 4.3</td>
<td>0.4 ± 0.1</td>
<td>7.2 ± 0.7</td>
<td>1.9 ± 0.6</td>
<td>7.2 ± 0.4</td>
</tr>
<tr>
<td>WT-VTE1#40</td>
<td>21.4 ± 2.1</td>
<td>0.6 ± 0.1</td>
<td>2.2 ± 0.7</td>
<td>0.9 ± 0.1</td>
<td>19.0 ± 1.0</td>
</tr>
<tr>
<td>vte1-VTE1#1</td>
<td>0.8 ± 0.2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Figure 2  Tocochromanol composition in leaves of A. thaliana plants altered in VTE1 or VTE4 expression

Tocochromanols were separated by (A) HPLC on a diol column as described previously [39], or (B) by reversed-phase HPLC with the reversed-phase chromatogram is shown which contains PC-8 and MPC-8. Individual peaks were identified by standards for α-, β-, γ-, and δ-tocopherol. PC-8 was identified by co-elution with PC-8 from a linseed oil standard (results not shown). A second chromatography of the peak collected at 33 min in the diol HPLC chromatogram of WT-VTE1#40 resulted in a peak with a retention time of 33 min in reversed-phase HPLC, indicating that it is PC-8. The peak collected at 8 min from the diol chromatogram of VTE1-VTE4 gave rise to a peak at 32 min in reverse-phase conditions indicating that it was MPC-8.

in A. thaliana WT leaves was confirmed by co-elution with PC-8 from linseed oil in fluorescence HPLC at 23 min (diol column) (Figure 2A, top panel). PC-8 in A. thaliana leaves constitutes approx. 1.2 nmol · g⁻¹ of FW (fresh weight) (equivalent to 5–10 mol% of total tocochromanols, i.e. all tocopherols and PC-8) depending on growth conditions and age (Figure 2A, top panel; and Table 1). Therefore the abundance of PC-8 in leaves is in the range of that of γ-tocopherol, indicating that it represents an important form of tocochromanols in leaves. Owing to the very high content of γ-tocopherol in WT seeds (approx. 1200 nmol · g⁻¹ of FW), the amount of PC-8 (approx. 13 nmol · g⁻¹ of FW) was equivalent to only 1 mol% of total tocochromanols (Table 1).

Previously, overexpression of tocopherol cyclase (VTE1) under the control of the 35S promoter in A. thaliana leaves was reported to result in a strong increase of tocochromanols [34]. After separation of tocochromanols from the VTE1-overexpressing line WT-VTE1#40 by diol column HPLC [39], it became clear that the PC-8 peak was drastically increased, whereas the γ-tocopherol peak remained similar to WT (Figure 2A, middle panel). High amounts of PC-8 were also found in leaves of a second line overexpressing VTE1 (vte1-VTE1#1; Table 1). The amounts of individual tocopherols in leaves of WT-VTE1#40 and vte1-VTE1#1 were only slightly increased (Table 1). In addition to the increase in PC-8 in leaves of lines WT-VTE1#40 and vte1-VTE1#1, PC-8 also accumulated in the seeds of the transgenic lines (Table 1).

To unambiguously identify PC-8 in A. thaliana leaves, fractions containing PC-8 were collected from HPLC and subjected to MS via Q-TOF MS. Figure 3 shows the mass spectra of PC-8 samples collected from linseed oil and A. thaliana WT-VTE1#40 and γ-tocopherol collected from WT leaves. All quinol lipids were ionized as cation radicals [M]+. The cation radical for PC-8 from line WT-VTE1#40 was detected at 750.6294 m/z in agreement with the calculated mass of 750.6309 m/z (CėH₇₀O₂). After selecting the molecular ions in the quadrupole and fragmentation by collision-induced dissociation, the mass spectra of PC-8 and γ-tocopherol were recorded. All quinol lipids showed peaks at m/z 151.07 and 191.10, which is in good agreement with the calculated masses of two fragments derived from the PC-8/γ-tocopherol head groups (see also [25,45]).

Tocopherol and PQ-9/PC-8 synthesis are closely linked because the two biosynthetic pathways depend on the availability of a common precursor molecule, homogentisate, and in part employ the same biochemical reactions (VTE3 and VTE1; Figure 1). To study the role of the enzymes of tocopherol synthesis in PC-8 production, two A. thaliana mutants were selected: the vte1 mutant carrying a null mutation in tocopherol cyclase [7,46], and two alleles of vte2 affected in homogentisate phytyltransferase (VTE2; [46]): vte2-1 contains very low amounts of tocopherol [32] due to an amino-acid exchange in VTE2, and the T-DNA mutant vte2-3 is completely devoid of tocopherol (the present study).
Tocochromanols were absent from vte1 leaves or seeds (Table 1). This is in agreement with the scenario that PC-8 synthesis depends on VTE1. Leaves and seeds of vte2-1 and of vte2-3 contained low amounts of PC-8 similar to WT, indicating that PC-8 synthesis is independent of VTE2 (Figures 3A and 3B). Therefore, although the vte2-3 mutant is devoid of tocopherol, it still contains PC-8 as a residual form of tocochromanol.

The amount of tocopherol increases in leaves during abiotic stress, e.g. high light and during senescence [4,7,47,48]. The accumulation of tocopherol during stress is correlated with an increase in expression of tocopherol synthesis genes, e.g. HPPD (p-hydroxyphenylpyruvate dioxygenase), VTE2 and VTE1 [34,47]. Table 2 shows that the amount of PC-8 also accumulates in leaves of A. thaliana plants exposed to high light. The proportion of PC-8 relative to total tocochromanols during high light remains unchanged.

Quantification of PQ-9 in A. thaliana lines altered in tocochromanol metabolism

The synthesis of PC-8 by VTE1 depends on the availability of the precursor PQ-9. Therefore the accumulation of PC-8 in VTE1-overexpressing lines and the increase during light stress might result in a depletion of the PQ-9 pool in the chloroplasts. PQ-9 was quantified by HPLC in different A. thaliana lines (Table 2). PQ-9 content was not altered in the vte1 and vte2 mutants, indicating that a decrease in tocopherol or PC-8 does not affect PQ-9 synthesis. Similar to tocopherol, PQ-9 was increased under high light. In the lines WT-VTE1#40 and vte1-VTE1, PQ-9 content was slightly decreased (Table 2). The changes in PQ-9 and PC-8 were recorded in separate HPLC experiments because the separation conditions were optimized for tocochromanols or PQ-9 respectively (Table 2; see Experimental section). However, PQ-9 can also be measured via UV absorption during chromatography of tocochromanols which are recorded by fluorescence. Thus the ratios of peak areas of PQ-9 (measured by UV absorption) and of PC-8 (measured by fluorescence) were recorded simultaneously in one HPLC experiment. The ratios of PQ-9 to PC-8 peak areas are drastically decreased upon VTE1 overexpression when measured in the very same chromatogram (2.12 ± 0.67, 0.20 ± 0.08 and 0.12 ± 0.05 for WT, WT-VTE1#40 and vte1-VTE1#1 respectively; n = 3, means ± S.D.). This is in good accordance with the relative changes in PC-8 and PQ-9 measured in individual chromatograms as shown in Table 2. In conclusion, the strong accumulation of PC-8 in VTE1-overexpressing lines resulted in a decrease in PQ-9 content, which is in agreement with the role of PQ-9 as a precursor for PC-8 synthesis.

PC-8 and PQ-9 accumulate in thylakoids and plastoglobules of A. thaliana chloroplasts

Most tocopherol synthesis enzymes localize to the envelope membranes of chloroplasts, whereas tocopherol cyclase (VTE1) was found in the plastoglobules [12,14,15]. To study the localization of PC-8 experimentally, different fractions were isolated from ruptured chloroplasts of 8-week-old plants by sucrose-density centrifugation. The gradient subfractions were pooled into five fractions enriched in plastoglobules (fractions F1 and F2), envelope membranes (fraction F3) and thylakoids (fractions F4 and F5) according to the distribution of marker proteins as visualized by immunoblot analysis (Figure 4A) [15]. Tocochromanols were measured by fluorescence HPLC and calculated as nmol per ml of fraction volume [39]. The amount of

Table 2  PC-8 and PQ-9 contents of A. thaliana leaves of tocopherol mutants and overexpressing plants

<table>
<thead>
<tr>
<th>Leaves</th>
<th>PC-8 (nmol g⁻¹ of FW)</th>
<th>PQ-9 (nmol g⁻¹ of FW)</th>
<th>MPC-8 (nmol g⁻¹ of FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.2 ± 0.2</td>
<td>23.8 ± 3.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>vte1</td>
<td>n.d.</td>
<td>22.8 ± 1.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>vte1-1</td>
<td>2.2 ± 0.3</td>
<td>23.8 ± 2.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>vte1-3</td>
<td>0.2 ± 0.0</td>
<td>26.2 ± 3.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>WT-VTE1#40</td>
<td>67.9 ± 13.5</td>
<td>19.8 ± 1.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>vte1-VTE1#1</td>
<td>77.2 ± 13.4</td>
<td>17.6 ± 1.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>WT-VTE1-VTE4</td>
<td>29.2 ± 7.2</td>
<td>11.9 ± 2.1</td>
<td>33.5 ± 5.7</td>
</tr>
<tr>
<td>Low light</td>
<td>1.2 ± 0.2</td>
<td>21.6 ± 1.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>High light</td>
<td>4.0 ± 0.6</td>
<td>36.9 ± 2.0</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

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PC-8 in each of the five fractions was obtained after multiplication with the fraction volume. A large proportion of plastidial PC-8 localizes to plastoglobules (fractions F1 and F2), whereas the remainder is mostly associated with thylakoids (fractions F4 and F5) (Figure 4B, top panel). The amount of PC-8 in envelope membranes is very low. The tocochromanol distribution in plants overexpressing VTE1 (line WT-VTE1#40) was assessed to study the subplastidial compartmentation of the extra amount of PC-8 accumulating in the transgenic line. As shown in the bottom panel of Figure 4(B), the content of PC-8 relative to the other tocochromanols remained similar in all chloroplast fractions. A large fraction of PC-8 accumulated in the plastoglobule fractions F1 and F2, and the amount of PC-8 associated with thylakoids (fractions F4 and F5) was also increased (Figure 4B).

To study the subplastidial distribution of the PC-8 precursor, PQ-9 was also quantified by HPLC in the chloroplast sucrose-gradient fractions from WT. The middle panel of Figure 4(B) shows that a large proportion of total PQ-9 is associated with plastoglobules and with thylakoids. PC-8 was previously shown to harbour antioxidant activity comparable with that of tocopherols [26]. Therefore the accumulation of PC-8 in the transgenic line WT-VTE1#40 might contribute to the antioxidant activity of leaves. To analyse the effect of overexpression of VTE1 on photosynthesis, the two lines WT-VTE1#40 and vte1-VTE1#1 were exposed to high light for 4 days and used for the measurement of chlorophyll fluorescence [49]. Quantum yield at normal- and high-light conditions in the PC-8-accumulating plants was similar to WT (results not shown), indicating that PC-8 accumulation had no major impact on photosynthesis under normal or high light at ambient temperature.

**Chloroplast ultrastructure in PC-8-accumulating A. thaliana lines**

The fact that PC-8 accumulates in plastoglobules (Figure 4) suggested that plastoglobule number or size might be altered in chloroplasts after PC-8 accumulation. Analysis by transmission electron microscopy revealed that leaves of 4-week-old plants accumulating PC-8 (WT-VTE1#40) showed a high increase in plastoglobule number (Figure 5A, bottom panel). Although the diameter of the individual plastoglobules remained similar to WT, the number of plastoglobules per chloroplast cross-sectional area in WT-VTE1#40 (2.60 ± 0.30) was strongly increased as compared with WT (1.05 ± 0.10), and this number was significantly reduced in the vte1 mutant (0.80 ± 0.08) (n = 20 chloroplasts analysed for each line). In line WT-VTE1#40, PC-8 increases to 68 nmol·g⁻¹ of FW, whereas the content of its precursor, PQ-9 (20 nmol·g⁻¹ of FW), is only slightly reduced as compared with WT (24 nmol·g⁻¹ of FW) (Table 2). Therefore the increase in plastoglobule number presumably originates from the accumulation of PC-8. However, it is also possible that the accumulation of the VTE1 protein itself affects plastoglobule size because VTE1 also localizes to the plastoglobules.

The plastoglobules in WT-VTE1#40 are organized in grape-like clusters and localize to the stroma in proximity to the thylakoid membranes, suggesting that they are interconnected (Figure 5A, bottom panel; see [14]). The number of plastoglobules in clusters was strongly increased in line WT-VTE1#40. Although WT and vte1 contained single plastoglobules or clusters of two or three plastoglobules, line WT-VTE1#40 contained clusters containing up to 17 plastoglobules (Figure 5B). The count of plastoglobules grouped in a cluster was performed on two-dimensional images of ultrathin sections, probably underestimating the actual number of plastoglobules per cluster.

**Overexpression of γ-tocopherol methyltransferase (VTE4) in PC-8-accumulating plants**

PC-8 and γ-tocopherol each lack one methyl group as compared with the head group of α-tocopherol which contains three methyl groups (Figure 1). γ-Tocopherol is converted into α-tocopherol by γ-tocopherol methyltransferase (VTE4) [8]. The fact that PC-8 occurs in the non-methylated form in WT and WT-VTE1#40 suggested that VTE4 activity is limiting, or that this enzyme is not specific for PC-8. Therefore VTE4 was co-overexpressed in line WT-VTE1#40, and double-transgenic lines
clusters of two, three or more plastoglobules, often in grape-like structures. Scale bars overexpressing line WT-VTE1#40. In WT-VTE1#40, a large number of plastoglobules exist in (line. Similar results were obtained with line vte1-VTE1#1. in WT-VTE1#40. A total of 20 chloroplasts from three different plants were analysed for each plastoglobules occur in an isolated form. Clusters of four or more plastoglobules are frequent no clusters with more than three plastoglobules were observed. In WT-VTE1#40, only 37 % of α considered that –tocopherol and MPC-8 might co-elute during peak as compared with the parental line WT-VTE1#40 (Figure 2A, bottom panel). Because methylation of PC-8 in line WT-VTE1-VTE4 was expected to result in the accumulation of MPC-8, it was considered that α-tocopherol and MPC-8 might co-elute during HPLC analysis on the diol column. Therefore the peak eluting at 9 min in diol column HPLC of WT-VTE1-VTE4 (Figure 2A, bottom panel) was collected and separated by reversed-phase HPLC. It became clear that this peak contains two substances, α-tocopherol, and a second non-polar compound eluting at 32 min, tentatively identified as MPC-8 (results not shown). Direct injection of a WT-VTE1-VTE4 leaf extract on to reversed-phase HPLC confirmed this result, because it revealed the presence of two peaks co-eluting at 32 min (MPC-8) and 33 min (PC-8) (Figure 2B). The identity of the PC-8 peak eluting at 33 min on reversed-phase HPLC was confirmed by co-elution with authentic PC-8 isolated from linseed via diol column HPLC (results not shown).

MPC-8 was isolated from line WT-VTE1-VTE4 via diol column HPLC and subjected to Q-TOF MS in positive mode (Supplementary Figure S1 at http://www.BiochemJ.org/bj/425/bj4250389add.htm). MPC-8 was ionized as a radical cation with a mass of 764.7166 m/z in agreement with the calculated mass of 764.6466 m/z (C₃₀H₄₄O₂). After fragmentation, a prominent peak at m/z 382.3086 appeared which can be explained by a cleavage of the molecular ion into two fragments of m/z 382.2866 (C₂₅H₃₄O₂) and m/z 382.3594 (C₂₅H₃₂O). The peak at m/z 165.0848 which can also be observed in the α-tocopherol spectrum is derived from a headgroup fragment (C₁₀H₁₃O₂, 165.0916 m/z).

To study the impact of PC-8 and MPC-8 accumulation on the amount of the precursor molecule, PQ-9 was measured in leaves of the transgenic line WT-VTE1-VTE4. Analogous to the single overexpressing lines WT-VTE1#40 and vte1-VTE1#1, the amount of PQ-9 in line WT-VTE1-VTE4 was decreased, however, to an even stronger extent (Table 2). Taken together, these results strongly suggest that overexpression of VTE4 in line WT-VTE1#40 results in the partial conversion of PC-8 into MPC-8 via methylation.

A possible explanation for the low degree of PC-8 methylation in leaves might be the localization of the corresponding enzymes to different subplastidial compartments. Although the VTE1 protein was previously localized to the plastoglobules of A. thaliana [12,15], Soll et al. [2] suggested that the γ-tocopherol methyltransferase (VTE4) activity is associated with the envelope membranes of spinach chloroplasts. To study the localization of A. thaliana VTE4, a VTE4 fusion protein with GFP was transiently expressed in leaves, and the fluorescence analysed by confocal microscopy (Figure 6). VTE4–GFP fluorescence was observed as a peripheral ring around the chloroplasts (Figure 6A, top panel). This pattern was similar to the GFP fusion of the envelope membrane marker protein TIC110, but different from that of PGL35 and VTE1 which both localize to plastoglobules and show a dotted pattern inside the chloroplasts (Figure 6A) [15]. Transient expression of the VTE4–GFP fusion protein in A. thaliana protoplasts also revealed a ring-like pattern of fluorescence surrounding the chloroplasts (Figure 6B). Western blot analysis using anti-GFP antibodies demonstrated the expression of the VTE4–GFP fusion protein with the correct size in the protoplast expression system. These results suggest that the VTE4 protein localizes to the envelope membranes in accordance with the localization of γ-tocopherol methyltransferase activity previously determined [2].

**DISCUSSION**

**Accumulation of PQ-9 and PC-8 in plastoglobules**

Figure 4 suggests that considerable proportions of the plastidial pools of PQ-9 and PC-8 are associated with plastoglobules. The separation of plastidial subfractions by sucrose-density
with anti-GFP antibodies (lane 2). The molecular mass in kDa is indicated on the left-hand side.

expressing VTE4–GFP. Proteins on the membrane were visualized with amidoblack (lane 1) or merge, overlap of chlorophyll and GFP fluorescence. (Figure 6) Subcellular localization of γ-tocopherol methyltransferase (VTE4)

(A) A. thaliana leaf epidermal cells expressing fusion proteins with GFP after biolistic transformation: VTE4–GFP (γ-tocopherol methyltransferase), PGL35–GFP (plastoglobules), AtTIC110–GFP (inner envelopes) and pSSU–GFP (precursor for the Rubisco (ribulose-1, 5-bisphosphate carboxylase/oxygenase) small subunit, stroma). (B) Transient expression of VTE4–GFP in A. thaliana protoplasts. Scale bars=5 μm. Fluorescence was observed by confocal microscopy. Chlorophyll, autofluorescence after excitation at 568 nm; GFP, excitation at 488 nm; merge, overlap of chlorophyll and GFP fluorescence. (C) Western blot analysis of protoplasts expressing VTE4–GFP. Proteins on the membrane were visualized with amidoblack (lane 1) or with anti-GFP antibodies (lane 2). The molecular mass in kDa is indicated on the left-hand side.

centrifugation was followed by analysing marker proteins via Western blotting (Figure 4A). The individual plastidial compartments (plastoglobules, envelopes and thylakoids) are distributed across several gradient fractions which is reflected by the accumulation of marker proteins in the different fractions. For example, PGL35 and VTE1, which are considered as markers for plastoglobules, are enriched in fractions F1, F2 and F3, but also occur in fractions F4 and F5, albeit at a lower level. The presence of these two proteins in fractions F4 and F5 might indicate that they also occur in thylakoids, or that some of the plastoglobules remain connected to the thylakoids, possibly through membrane filaments [50], and thus are co-isolated with thylakoid fractions. The low levels of the thylakoid marker CAB in fractions F1 and F2 suggest that the prenyl quinols found in these fractions originate from plastoglobules themselves, rather than from thylakoid contamination. Thus, although an exact quantification of the lipid distribution is not possible, these results suggest that the quinol lipids PC-8 and PQ-9 are enriched in thylakoids and plastoglobules of chloroplasts [15,51]. This finding is in full agreement with results previously obtained for tocopherols, PQ-9 and phylloquinol [15,20,51,52]. The proportions of the two prenyl quinol lipids in the plastoglobules relative to the thylakoids might depend on plant development, because only relatively low numbers of plastoglobules were found in chloroplasts of younger 4-week-old leaves (Figure 5A), and the plastoglobule number and size is known to increase in older leaves and during stress or senescence. For this reason, the amount of PQ-9 and PC-8 in the plastoglobule fraction might correlate with the number of plastoglobules per chloroplast, and therefore with plant age, development and stress. We obtained similar distributions of PC-8 or PQ-9 to the different subplastidial fractions in chloroplasts isolated from 6- or 8-week-old plants (results not shown). Lower proportions of prenyl quinols might be found in leaves from younger plants or plants grown in tissue culture, since these leaves are likely to contain fewer plastoglobules (e.g. [53]). The pool of PQ-9 in plastoglobules not associated with thylakoids might provide a reservoir for antioxidant molecules and electron carriers for photosynthetic electron transport. It is known that the numbers of PSII, as well as PQ-9, in chloroplasts are present in excess and therefore are not limiting for photosynthesis [54].

Accumulation of PC-8 in WT and in overexpressing plants

PC-8 was first discovered in rubber tree leaves (Hevea brasiliensis) more than 40 years ago [21]. Since that time, PC-8 was identified in the seeds of a number of plant species [22–24,27,28,31,39]. However, reports on the occurrence of PC-8 in plant leaves remained scarce. PC-8 was not measured in the previous studies on tocopherol synthesis and function in A. thaliana leaves [7,46,55,56]. The two tocochromanols, PC-8 and γ-tocopherol, are difficult to separate because they share the same head group and, therefore, have very similar physical characteristics. During normal-phase HPLC, PC-8 can co-elute with γ-tocopherol, whereas in reversed-phase HPLC, PC-8 is eluted under highly non-polar conditions at the end of the gradient (Figure 2). Using diol column HPLC [39], all four forms of tocopherol and PC-8 can be separated and quantified. Previous studies and results shown in the present study clearly demonstrate that PC-8 represents an authentic form of tocochromanol in leaves, seeds and other organs of plants, including A. thaliana, in considerable amounts of approx. 5–10 mol% of total tocochromanols. Previously, overexpression of VTE1 in A. thaliana leaves was found to result in an apparent increase in the γ-tocopherol peak in normal-phase HPLC [34]. Re-evaluation of the tocochromanol composition of line WT-VTE1#40 by diol column HPLC revealed that this peak contains two compounds, γ-tocopherol and PC-8 (Table 1). Thus it is now clear that VTE1 overexpression in A. thaliana leaves causes a drastic increase in PC-8 content from approx. 1–70 nmol·g FW, such that PC-8 becomes the most abundant tocochromanol. The amounts of the individual tocopherol forms were only slightly increased in the leaves of the two lines WT-VTE1#40 and vte1-VTE1#1 (Table 1; [34]). In seeds of transgenic plants, PC-8 also accumulates, whereas the amounts of the different tocopherol forms are comparable with WT levels. Overexpression of VTE1
under the control of the 35S promoter presumably results in the accumulation of low levels of VTE1 in seeds which can explain the fact that less pronounced tocochromanol changes are observed in seeds. Previous studies have demonstrated that seed-specific overexpression of corn or A. thaliana VTE1 in rape using the napin promoter resulted in a strong accumulation in PC-8 accompanied with moderate increases in other tocopherol forms, particularly δ-tocopherol [30,31]. The increase in PC-8 and tocopherols was observed in lines overexpressing VTE1 as a single construct, or VTE1 in combination with VTE2 or HPPD. In contrast with VTE1, the overexpression of VTE2 in A. thaliana leaves resulted in a much stronger increase in total tocopherols by a factor of 4.4, whereas PC-8 was not measured [57]. Taken together, these results indicate that VTE2 is limiting for tocopherol accumulation because it is required for the conversion of homogentisate into MPBQ (2-methyl-6-phytyl-1,4-benzoquinol) (Figure 1). The impact of VTE1 overexpression on tocopherol synthesis is much lower due to the limitation in supply of the precursors MPBQ/DMPBQ. However, VTE1 overexpression results in a strong increase in PC-8 by cyclization of PQ-9 which itself is present in chloroplasts in high amounts (Table 2).

During a 12-day high-light stress experiment, the transcripts of HPPD and VTE2 were found to be strongly up-regulated via real-time PCR analysis, whereas the expression patterns of VTE1 and VTE4 were less affected with a moderate induction of the two latter genes at approx. day 3 (see Figure 5 of [47]). It was concluded that HPPD and VTE2 are limiting for tocopherol synthesis during high-light stress. On the other hand, Northern blot analysis revealed that VTE1 expression is up-regulated during the first 4 days of high-light stress [34]. Expression profiling of a 24 h time course experiment of abscisic acid-treated A. thaliana seedlings revealed a co-ordinated up-regulation of expression of several tocopherol synthesis genes, i.e. HPPD, VTE2, VTE1 and VTE4 [58]. Taken together, these results suggest that the increase in tocopherol synthesis during abiotic stress is mediated via the induction of several genes, possibly with different stress-dependent induction kinetics.

**Overexpressing VTE4 in VTE1-expressing plants**

In A. thaliana leaves, α-tocopherol carrying three methyl groups is the predominant form of tocopherols, indicating that the largest portion of γ-tocopherol is methylated by VTE4. The two tocochromanols γ-tocopherol and PC-8 contain two methyl groups in their head group. VTE1 was previously suggested to localize to the plastoglobules of chloroplasts [12,15], whereas the other enzymes of tocopherol synthesis, including VTE4, are mostly associated with the envelope membranes [2]. Transient expression of VTE4–GFP under the control of the 35S promoter resulted in the accumulation of fluorescence in a ring at the boundary of the plastoglobules indicating envelope localization (Figure 6). It is possible that the distribution of tocopherols between plastoglobules, thylakoids and envelopes is in a diffusion-dependent equilibrium. Furthermore, it is possible that the enzymes (VTE1 and VTE4) involved in the final biosynthetic steps are distributed between the different compartments, i.e. plastoglobules, stroma and envelopes of chloroplasts. Thus a small amount of VTE4 might localize to plastoglobules or might be mobile, thereby having direct access to γ-tocopherol for methylation. After strong overexpression of VTE4, a fraction of PC-8 was converted into a compound tentatively identified as MPC-8. This suggests that PC-8 can be used by VTE4 as a substrate, but with lower activity. Furthermore, these results are in agreement with the fact that VTE4 from tocopherol-containing plants prefers γ-tocopherol, whereas VTE4 from tocotrienol-containing species uses γ-tocotrienol as the preferred substrate [59]. As PC-8 contains an unsaturated side chain similar to γ-tocotrienol, it is possible that the A. thaliana enzyme is more active with γ-tocopherol than with PC-8. This scenario implies that the rate of conversion of PC-8 into MPC-8 is low because PC-8 is not a good substrate for VTE4.

**Prenyl quinol lipids in the vte1 and vte2 mutants**

Results of the present study clearly show that the vte1 mutant which lacks tocopherol cyclase is not only devoid of tocopherol, as described previously [7,55], but also lacks PC-8, another tocochromanol form. PC-8 was described to harbour antioxidant activity, comparable with that of tocopherols [26]. Therefore the additional loss of PC-8 in the vte1 mutant should be taken into consideration in physiological studies employing this mutant, e.g. ([7,32,34,46,56,60–62], but see [63]). Given the fact that PC-8 amounts to only 5–10 mol% of total tocochromanols in WT leaves, it is likely that most of the effects observed in vte1 mutant plants are indeed caused by the deficiency in tocopherol synthesis, rather than by the additional lack of PC-8.

Similarly to vte1, the vte2 mutant is totally deficient in tocopherol. Table 1 shows that vte2 contains an amount of PC-8 very similar to that of WT leaves, i.e. 5–10 mol% of tocopherol in WT. The presence of a residual amount of PC-8 in vte2 suggests that this compound might exert some antioxidant functions. Given the fact that the amount of PC-8 is rather low, it is likely that the physiological effects observed in vte2 mutant plants predominantly originate from tocopherol deficiency [32,46,56,61]. However, it should be considered that vte2 plants do contain PC-8, and this might partially suppress some physiological effects of tocopherol deficiency (e.g. [32]).

**AUTHOR CONTRIBUTION**

Felix Kessler, Claire Bréhélin and Peter Dörmann designed the research. Anna Maria Zbierzak, Marion Kanwischer, Christina Wille, Pierre-Alexandre Vidi, Patrick Giavalisco, Antje Lohmann, Isabel Briesen, Svetlana Potirova and Claire Bréhélin performed the research. Anna Maria Zbierzak, Pierre-Alexandre Vidi, Isabel Briesen, Svetlana Potirova, Claire Bréhélin, Felix Kessler and Peter Dörmann analysed the data. Anna Maria Zbierzak, Felix Kessler and Peter Dörmann wrote the manuscript.

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SUPPLEMENTARY ONLINE DATA
Intersection of the tocopherol and plastoquinol metabolic pathways at the plastoglobule

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Figure S1 Identification of MPC-8 by Q-TOF MS

(A) Mass spectrum of MPC-8 from A. thaliana line WT-VTE1-VTE4. (B) Mass spectrum of α-tocopherol purified from A. thaliana WT leaves. Lipids were extracted and purified by diol column HPLC prior to analysis by Q-TOF MS. The spectra were obtained by collision-induced dissociation after selecting the molecular ion peaks in the quadrupole (M⁺=764.6466 and 430.3805 for MPC-8 and α-tocopherol respectively).

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