Plastoquinol is the Main Prenyllipid Synthesized During Acclimation to High Light Conditions in Arabidopsis and is Converted to Plastochromanol by Tocopherol Cyclase

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Plants have evolved various strategies to acclimate to high light conditions at different levels of organization. High light stress stimulates synthesis of different antioxidant enzymes and low molecular weight antioxidants, mainly in chloroplasts. In the present studies we showed that plastoquinol, in addition to \( \alpha \)-tocopherol, is the main lipid-soluble antioxidant synthesized during acclimation of Arabidopsis plants to high light conditions. The level of plastoquinol increased >10-fold and independently of tocopherols, as revealed using tocopherol biosynthetic mutants. The high light-induced increase in plastoquinol level was mainly attributable to the photochemically non-active fraction of this compound localized in plastoglobuli, which are the storage site of prenyllipids for their antioxidant action. Our data also revealed that tocopherol cyclase is required for plastochromanol biosynthesis from plastoquinol in vivo. Plastochromanol accumulated in increasing amounts in leaves during growth and it was also identified in seeds. The obtained data suggest that plastochromanol may, similarly to other prenyllipids, fulfill antioxidant function in leaves and seeds, especially during aging.

Keywords: Antioxidants • Arabidopsis • Light stress • Plastochromanol • Plastoquinol • Tocopherol.

Abbreviations: PC, plastochromanol; PQ, plastoquinone, WT, wild type.

Introduction

High light stress is probably the environmental stress most frequently experienced by plants under natural conditions. Even though the photosynthetic apparatus shows various adaptive strategies to cope with such unfavorable conditions, high light generates an excess of reactive oxygen species in chloroplasts and, in response, plants synthesize various antioxidant enzymes and low molecular weight antioxidants, both water soluble (ascorbate and glutathione) and lipid soluble, such as carotenoids or tocopherols (vitamin E). Although tocopherols are regarded as the main membrane-localized antioxidants (Munne-Bosch and Alegre 2002), the tocopherol biosynthetic mutants of Arabidopsis (vte4, vte2 and vte1) have been shown not to be affected significantly under high light stress (Havaux et al. 2005, Maeda et al. 2006). This has often been explained by the compensatory action of other photoprotective mechanisms (Havaux et al. 2005). In this respect, not much attention has been devoted in recent years to other membrane-localized antioxidants such as plastoquinol.

In the past, the group of Lichtenthaler showed that massive accumulation of total plastoquinone (PQ), preferentially plastoquinol, takes place under high light conditions in sun-exposed leaves of beech, as compared with shade leaves (Lichtenthaler 1971, Lichtenthaler 1977). The increase in \( \alpha \)-tocopherol was much less pronounced in that case. The site of the strong accumulation of total plastoquinone and \( \alpha \)-tocopherol was identified to be the large osmophilic plastoglobuli of chloroplasts (Lichtenthaler and Sprey 1966). However, to date, similar experiments using currently available highly sensitive and accurate HPLC methods performed on Arabidopsis plants under controlled growth conditions are lacking. Moreover, the mechanism and site of antioxidant action of the prenyllipids remain to be elucidated.

The main reactive oxygen species generated in thylakoid membranes during high light stress are singlet oxygen and superoxide, formed in PSII (Krieger-Liszay 2004) and PSI (Asada 1999), respectively. It has been proposed that the specific function of \( \alpha \)-tocopherol in chloroplasts is the protection of PSII against singlet oxygen formed by the reaction center triplet chlorophyll in Chlamydomonas reinhardtii (Trebst et al. 2002, Kruk et al. 2005). It has been also shown in Arabidopsis thaliana that tocopherol plays a specific role in the maintenance of PSII function (Havaux et al. 2005) and that this function supplements the photoprotective function of non-photochemical quenching.

Plastoquinol, which is a component of the electron transport chain in photosynthesis, was shown to have antioxidant activity similar to or even higher than that of tocopherols.
in vitro (Kruk et al. 1994, Kruk et al. 1997) and it was also demonstrated that in vivo it may inhibit membrane lipid peroxidation (Hundal et al. 1995), as well as scavenge singlet oxygen (Kruk and Trebst 2008) and superoxide (Kruk et al. 2003) formed in thylakoid membranes. In contrast to tocopherols, both the head group and the isoprenoid side chain of plastoquinol are able to scavenge singlet oxygen (Gruszka et al. 2008) which makes this compound an excellent membrane-localized antioxidant. In recent years, additional functions of plastoquinol as a trigger of gene expression (Karpinski et al. 1997, Fey et al. 2005) and as a redox activator (Rochaix 2007) have been revealed. This indicates multiple functions of plastoquinol in chloroplasts.

Plastochromanol (PC) is a naturally occurring γ-tocotrienol homolog with a longer side chain composed of eight isoprenoid units, which is probably synthesized from reduced PQ by tocopherol cyclase (Kumar et al. 2005), the key enzyme in tocopherol biosynthesis. PC is found in the greatest amounts in flax and rape seed oils (Gruszka and Kruk 2007), as well as in minor amounts in seed oils of camelina and others (Gruszka and Kruk 2007). In contrast to tocotrienols, whose occurrence is limited to non-photosynthetic organs (Horvath et al. 2006), PC was also found as a minor component in leaves of rubber trees (Whittle et al. 1965) and some other plant species (Dunphy et al. 1966, Strzalka et al. 2009). Its function in seeds and leaves seems to be similar to that of tocopherols, i.e. an antioxidant action, but the literature data on that subject are very limited (Olejnik et al. 1997). It could also have a similar action to that of tocotrienols for human health (Theriault et al. 1999, Sen et al. 2006), but this issue requires further study.

Since there are no data in the literature on plastoquinol response to high light stress in Arabidopsis, we performed such studies on Arabidopsis wild-type (WT) plants and tocopherol-deficient mutants (vte4 and vte1). These mutants were examined because both plastoquinol and tocopherol biosynthesis share the same substrate (homogentisate), both kinds of molecules fulfill a similar antioxidant function under high light stress and the deficiency in one pathway could affect the other (Fig. 1). Moreover, taking into account that Arabidopsis belongs to the same Brassicaceae family as rape, we examined Arabidopsis leaves and seeds for PC content, in both low and high light-grown plants, as well as in terms of dependence on the age of leaves. In order to investigate the question of PC synthesis by tocopherol cyclase, besides the WT plants, tocopherol-deficient mutants have also been examined for PC content.

Results

When WT Arabidopsis plants and the tocopherol mutants (vte4 and vte1) are grown under low light conditions, all these lines are indistinguishable (Fig. 2). However, under high light the tocopherol mutants showed inhibited growth. Under low light, the chlorophyll content was the same among the lines, being higher in young leaves (Table 1). However, under high light the chlorophyll content was lower in both tocopherol mutants than in the WT, especially in young leaves of the vte1 mutant.

An analysis of the prenyllipids in plants grown in both low and high light revealed that high light conditions dramatically increased the content of α-tocopherol and especially that of plastoquinol (Fig. 3). The level of α-tocopherol increased >4-fold while that of plastoquinol increased >16-fold in older leaves. In young rosette leaves, the increase in the α-tocopherol level was considerably lower, while for plastoquinol it was still high, i.e. >9-fold. The total level of PQ (oxidized plus reduced) increased nearly 8- and 11-fold in old and young leaves, respectively. In the vte4 mutant, γ-tocopherol substitutes for α-tocopherol and a similar response of γ-tocopherol to that of α-tocopherol in the WT under high light, in both old and young leaves, can be observed (Fig. 3). High light caused an increase in the plastoquinol level in the tocopherol-free vte1 mutant similar to that in the WT in old leaves but to a considerably lower extent in young leaves (Fig. 3).
membranes and taking part in the electron transport chain, was determined by illumination of leaves under the conditions that provide full reduction and oxidation of the PQ pool in two independent experiments (see Materials and Methods for details). The redox state of PQ was calculated from both conditions and this allowed calculation of the extent of the photochemically active PQ pool and the photochemically non-active PQ fraction in leaves, as well as the redox state of the latter fraction. The results obtained show (Table 2) that under low light conditions, the PQ pool represented about 30% of the total PQ in chloroplasts while under high light conditions it corresponded to only 8% of the total PQ. In this case, the photochemically non-active PQ was mostly in a reduced state. The increase in the proportion of the PQ pool to chlorophyll (Table 2) under high light is certainly due to different organization of the chloroplast structure under such conditions. It is known that high light chloroplasts have less grana and less antenna chlorophyll per electron transport chain than low light chloroplasts (Lichtenthaler 2007). These presented results demonstrate that most of the plastoquinol was photochemically non-active under high light and it was localized outside thylakoids in plastoglobuli, which are the storage site for the plastoquinol acting as an antioxidant in the thylakoids. The electron micrographs show that the number and size of plastoglobuli in the chloroplasts of plants grown in low light were small, while in plants grown in high light the plastoglobuli were extremely large (Fig. 4), which is in line with the biochemical analysis of PQ/plastoquinol distribution. The plastoglobuli fraction isolated from leaves of high light-grown plants was greatly enriched in both total PQ and \( \alpha \)-tocopherol (Table 3). The prenyllipid composition of plastoglobuli strongly resembled that of the leaves. The only significant difference is that the level of reduced PQ was diminished at the expense of the oxidized form in plastoglobuli. This was probably due to the oxidation of plastoquinol during the isolation procedure of the plastoglobuli fraction. Thus, the results obtained confirm that the majority of the photochemically non-active PQ in leaves of high light-grown plants was localized in plastoglobuli.

In the present studies, PC was identified in both leaves and seeds of the WT, accounting for 30% of the \( \alpha \)-tocopherol in leaves of plants grown under low light (Fig. 3) and 10% of all tocopherols in seeds (Table 4). The identity of PC was confirmed by absorption spectroscopy, HPLC and mass spectrometry studies. PC isolated from the WT leaves showed absorption maxima at 294.5 and 300.5 nm in both cyclohexane and iso-octane, and these maxima were identical to those of authentic PC standards (natural and synthetic), as well as to the \( \gamma \)-tocopherol standard which shows the same chromanol ring structure as PC, and therefore it is expected to have the same absorption maxima. The obtained absorption maxima of PC are also in agreement with the literature data (Whittle et al. 1965, Thies 1997). The HPLC retention time of PC in the extract from the WT was the same as that of the PC standard. The mass spectral analysis of both the PC isolated from the WT and the PC standard showed the presence of a peak with \( m/e = 790.0 \)
Fig. 3 Prenyl lipid content in older and younger leaves of 4- to 5-week-old Arabidopsis WT plants, the α-tocopherol-deficient mutant vte4 and the tocopherol-free mutant vte1 grown under low light (LL) and high light (HL) conditions. Data are means ± SE (n = 3–5).
Table 2  The size of the PQ pool and of the photochemically non-active PQ (PQ_{NP}), as well as its redox state in Arabidopsis WT leaves grown under low light (LL) and high light (HL) conditions

<table>
<thead>
<tr>
<th></th>
<th>PQ pool (% total)</th>
<th>PQ_{NP} (% total)</th>
<th>PQ_{NP} redox state (% reduced)</th>
<th>PQ pool (µg mg Chl(^{-1}))</th>
<th>PQ_{NP} (µg mg Chl(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT–LL</td>
<td>28.2 ± 3.4</td>
<td>71.8 ± 3.4</td>
<td>71.1 ± 6.7</td>
<td>1.7</td>
<td>4.4</td>
</tr>
<tr>
<td>WT–HL</td>
<td>8.2 ± 1.4</td>
<td>91.8 ± 1.4</td>
<td>91.8 ± 2.4</td>
<td>3.9</td>
<td>44.3</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 3–5).

Table 3  Tocopherol and plastochromanol (PC) content in older leaves of the Arabidopsis WT grown under high light conditions and in plastoglobuli isolated from these leaves

<table>
<thead>
<tr>
<th>Prenyllipid</th>
<th>% Total</th>
<th>Leaves</th>
<th>Plastoglobuli</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol</td>
<td>36.5 ± 4.8</td>
<td>34.3 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>2.5 ± 0.2</td>
<td>2.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>PQ</td>
<td>9.3 ± 0.4</td>
<td>26.5 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Plastoquinol-9 (PQH(_2)-9)</td>
<td>51.1 ± 3.8</td>
<td>35.9 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>Total PQ</td>
<td>60.4 ± 4.2</td>
<td>62.4 ± 5.9</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 3–5).

which corresponds to the potassium ion adduct of PC. The occurrence of such adducts has been observed recently in the analysis of prenyllipids (Gruszka et al. 2008).

The biosynthesis of PC was not affected in the vte4 mutant (Fig. 3), while this prenyllipid was not detected in the tocopherol-deficient vte1 mutant, indicating that tocopherol cyclase is required for PC synthesis from plastoquinol. In contrast to other prenyllipids, the level of PC in leaves was not significantly influenced by high light conditions (Fig. 3) but its level increased gradually with the age of leaves of plants grown in low light, accounting for 50% of α-tocopherol and 60% of the total PQ in 3-month-old leaves (Fig. 5). During the investigated period of growth, the total (oxidized and reduced) level of PQ in leaves increased gradually, as did the contribution of plastoquinol,
The literature data and the present results suggest that plastoglobuli are the storage site of plastoquinol for its antioxidant action in thylakoids. When plastoquinol is irreversibly degraded by reactive oxygen species in thylakoid membranes, it can be replaced by plastoquinol from plastoglobuli which have been shown to be permanently coupled to thylakoid membranes (Austin et al. 2006) and, under such conditions, diffusion of prenyllipids from plastoglobuli to thylakoids is possible.

During acclimation of Arabidopsis to high light conditions, the levels of water-soluble chloroplast antioxidants, such as ascorbate or glutathione, have also been shown to increase (Havaux et al. 2005, Golan et al. 2006). However, this increase was not so pronounced as that observed for plastoquinol and α-tocopherol in the present study.

Our data also reveal for the first time that PC is a native component of Arabidopsis leaves and provide evidence that PC is synthesized from plastoquinol by tocopherol cyclase (Fig. 1) and this reaction probably proceeds in plastoglobuli where the cyclase is localized (Vidi et al. 2006). The identification of PC in Arabidopsis leaves suggests that this compound, previously not recognized as an antioxidant in leaves, may perform a similar function to tocopherols and plastoquinol, especially in older leaves and seeds. For example, PC has been recently shown to be an efficient singlet oxygen scavenger and that in the scavenging process not only the head group of the molecule is active but also the unsaturated side chain (Gruszka et al. 2008). PC was also identified as an efficient inhibitor of lipid peroxidation (Olejnik et al. 1997). Interestingly, the level of PC was not significantly increased during high light stress either in the WT or in the vte4 mutant, but its level increased clearly during aging of leaves in plants grown under low light. This observation also suggests that PC is not a side-product of tocopherol cyclase action in plastoglobuli where the strong accumulation of plastoquinol takes place during high light conditions, but it is rather specifically synthesized from plastoquinol during aging of leaves. The significance of this process requires further study.

Taking into account that the structure of PC is similar to that of tocotrienols, compounds of plant origin that are supposed to have anti-cancer and anti-atherosclerotic action in humans (Theriault et al. 1999, Sen et al. 2006), it will also be of interest and practical importance to investigate in the future both plastoquinol and PC in this respect.

**Materials and Methods**

**Plant material and growth conditions**

*Arabidopsis thaliana* plants (WT Col-0, vte4 and vte1 mutants) were grown under low light conditions (100 µmol m⁻² s⁻¹, 22°C, 12/12 h light/dark period), and under high light conditions (380 µmol m⁻² s⁻¹, 26/17°C, 12/12 h light/dark period). Strong light was applied by 400 W metal halide lamps (Kolorarc, Budapest, Hungary) with double thermal glass filters to minimize overheating of plants. For analysis, 4- to 5-week-old plants
were used, unless otherwise stated. The old (−4-week-old) and young (1–2-week-old) leaves were analyzed from the rosettes.

**Prenyllipid analysis**

For tocochromanols and plastoquinol analysis, 25–130 mg of Arabidopsis leaves was ground in a mortar with 1.5 ml of cold ethyl acetate. Then, 400 µl of the extract was transferred to an Eppendorf tube and evaporated under nitrogen. The evaporated extract was re-dissolved in HPLC solvent (methanol/hexane, 340/20, v/v), briefly centrifuged on a benchtop centrifuge (10,000 × g for 10 s) and analyzed immediately by HPLC. The HPLC measurements were performed using a 100 µl loop, Jasco PU-980 pump and UV-VIS detector system UV-970, a Shimadzu RF10-AXL fluorescence detector (excitation/emission detection at 290/330 nm) and a Teknokroma (Barcelona, Spain) C18 reverse-phase column (Nucleosil 100, 250 × 4 mm, 5 µm) with an isocratic solvent system: methanol/hexane (340/20, v/v) at a flow rate of 1.5 ml min⁻¹. Oxidized PQ was followed by absorption at 255 nm while other prenyllipids were followed using fluorescence detection.

**Determination of the photochemically active and non-active PQ fractions in leaves**

The size of the PQ pool and the redox state of the photochemically non-active PQ in Arabidopsis WT leaves were measured as described in detail by Kruk and Karpinski (2006). For the experiments fully grown leaves of a similar size (older leaves) were used. In brief, one part of the leaves was illuminated briefly with strong light (2000 µmol m⁻² s⁻¹ for 15 s) to fully reduce the PQ pool, and immediately homogenized in a mortar with cold ethyl acetate. The extract was transferred to an Eppendorf tube, evaporated in a stream of nitrogen and analyzed by HPLC. The other part of the leaves was infiltrated with DCMU, illuminated briefly (500 µmol m⁻² s⁻¹ for 15 s) to fully oxidize the PQ pool and the leaves were then treated as before. The redox state of PQ in both conditions was compared and this allows calculation of the extent of the photochemically active PQ pool and the photochemically non-active PQ fraction in leaves, as well as the redox state of the latter fraction.

**Isolation of plastoglobuli**

The plastoglobuli fraction was isolated from high light-grown WT Arabidopsis plants as described by Szymanśka and Kruk (2007). In brief, the older leaves from two rosettes were homogenized for 5 s in HB buffer (0.45 M sorbitol, 20 mM Tricine-KOH pH 8.4, 10 mM EDTA, 10 mM NaHCO₃, 5 mM ascorbate, 1 mM MnCl₂), filtered through nylon cloth and centrifuged at 1,000 × g for 2 min. The sedimented chloroplasts were suspended in 10 ml of deoxygenated (with nitrogen) distilled water and sonicated (model 4710, Cole Parmer Instruments, Vernon Hills, IL, USA) for 60 s with a 50% ‘duty cycle’ in an ice bath and the microtip at full power. Then, the suspension was centrifuged at 100,000 × g for 4 min. The sedimented extract was re-dissolved in HPLC solvent (methanol/hexane, 340/20, v/v), briefly centrifuged on a benchtop centrifuge (10,000 × g for 10 s), then 400 µl of the extract was transferred to an Eppendorf tube, evaporated in a stream of nitrogen and analyzed by HPLC.

**Electron microscopy**

For electron microscopy, several small pieces of the leaves of 6-week-old Arabidopsis WT plants (from low and high light) were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, 2 mM calcium chloride, in a 0.1 M cacodylate buffer (pH 7.0) for 48 h at 4°C. Post-fixation was carried out in 1% OsO₄ in the same buffer for 1 h at 4°C. After post-fixation, the samples were washed several times and then ‘en bloc’ dyeing (1% uranyl acetate for 1 h at room temperature) was performed. Following that procedure, dehydration and embedding in epoxy resin were carried out by protocols described by Glauert and Lewis (1998). Ultrathin section (60–70 nm) were cut with diamond knives. Transmission electron microscopy observations were performed using a Jeol Jem-100SX electron microscope (Jeol, Tokyo, Japan).

**Standards and chlorophyll determination**

Tocopherol homologs of HPLC grade (≥99.5%) were purchased from Merck (Darmstadt, Germany). Oxidized and reduced PQ standards were obtained as described by Kruk (1988). The PC standard was purified from linseed oil according to the published procedure (Gruszka et al. 2008), and it was also synthesized from natural PQ as described by Gruszka and Kruk (2007). Chlorophyll content was determined spectrophotometrically in an acetone extract of leaves according to Lichtenthaler (1987).

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**References**


