

Identification of hydroxy-plastochromanol in *Arabidopsis* leaves

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In the present study we have identified hydroxy-plastochromanol in plants for the first time. This compound was found both in low light and high light-grown *Arabidopsis* plants, however, under high light stress its level was considerably increased. Hydroxy-plastochromanol accumulated also during ageing of leaves of low light-grown plants, similarly as in the case of other prenyllipids. Our results indicate that hydroxy-plastochromanol found in leaves is probably formed as a result of plastochromanol oxidation by singlet oxygen generated in photosystem II during photosynthesis. These data also support the hypothesis that plastochromanol is an efficient antioxidant *in vivo*, similarly as tocopherols and plastoquinol.

Keywords: *Arabidopsis*, hydroxy-plastochromanol, plastochromanol, plastoquinol, tocopherol, light stress

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INTRODUCTION

Plastochromanol (PC) is a naturally occurring γ -tocotrienol homologue with a longer side chain of eight isoprenoid units, which is synthesized from a reduced plastoquinone by tocopherol cyclase (Kumar *et al.*, 2005; Szymańska & Kruk, 2010; Zbierzak *et al.*, 2010), the key enzyme in tocopherol biosynthesis. PC was first identified as a minor component in leaves of rubber tree (Whittle *et al.*, 1965) and later in leaves of other plant species (Dunphy *et al.*, 1966; Strzalka *et al.*, 2009). It is found at the highest amounts in flax and rape seed oils (Balz *et al.*, 1992; Gofmann & Möllers, 2000; Velasco & Goffman, 2000; Kalique *et al.*, 2005; Gruszka & Kruk, 2007), as well as in minor amounts in seed oils of camelina (Zubr & Matthäus 2002; Gruszka & Kruk, 2007), maize (Gruszka & Kruk, 2007), hemp (Kriese *et al.*, 2004), many Vietnamese plants (Matthäus *et al.*, 2003), salvia (Bagci & Karaagacli, 2004), black pine (Bagci *et al.*, 2004) and others (Gruszka & Kruk, 2007). Its function in seeds seems to be similar to that of tocopherols, i.e., antioxidant action but the literature data on that subject is very limited (Olejnik *et al.*, 1997). Recently, PC has been found in substantial amounts in *Arabidopsis* leaves and seeds (Szymańska & Kruk, 2010; Zbierzak *et al.*, 2010) and it has been suggested to play there the role of an antioxidant (Szymańska & Kruk, 2010). On the other hand, it has been shown that PC is an efficient singlet oxygen quencher *in vitro*, being even more active than γ -tocopherol or γ -tocotrienol in hydrophobic solvents (Gruszka *et al.*, 2008). In the process of singlet oxygen quenching, not only the aromatic ring of plastochromanol is active but also the long unsaturated

side-chain. It is known that compounds containing conjugated double bonds, like carotenoids, are very efficient singlet oxygen quenchers (Di Mascio *et al.*, 1990). Even molecules with an isolated double bond can be efficient quenchers, especially if the double bond is at a tertiary carbon atom (Bonesi *et al.*, 2006), like in prenyllipids with an unsaturated side chain (Gruszka *et al.*, 2008). In contrast to tocopherols and tocotrienols, the main scavenging products of plastochromanol were monohydroxy- (PC-OH) and trihydroxy-derivatives (Gruszka *et al.*, 2008), although 8-hydroperoxy- γ -tocopherones of all these compounds were also found.

If PC is a singlet oxygen scavenger *in vivo*, its hydroxyl-derivatives should be detectable in leaves. Until now, there are no reports in the literature on the occurrence of such derivatives. In the present study we analyzed *Arabidopsis* leaves grown under different light intensity conditions for oxidation products of plastochromanol.

MATERIALS AND METHODS

Plant material and growth conditions. *Arabidopsis thaliana* plants (wild-type Col-0, *vte4* and *vte1* mutants) were grown under low light conditions (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 22°C, 12/12h light/dark period) as well as high light conditions (380 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 26°C/17°C, 12/12h light/dark period). Strong light was applied by 400 W metal halide lamps (Kolorarc, Hungary) with double thermal glass filters to minimize overheating of plants. The higher temperature during the photoperiod of high light conditions was a consequence of heat emission from the light source even though the heat filters were used. The average temperature of high light conditions corresponds to low light conditions and therefore we do not expect any significant influence of these differences on the composition of prenyllipids.

The *vte1* mutant (GABI_111D07, line 2) carries T-DNA insertion in intron 3 of the At4g32770 gene, while the *vte4* mutant (SALK_036736) shows T-DNA insertion in intron 1 of the At1g64970 gene (Mène-Saffrané & DellaPenna, 2009).

For analysis, 4–5 week-old plants were used, unless otherwise stated. The old and young leaves were analyzed separately.

Prenyllipid analysis. For quantitative analysis of prenyllipids, three *Arabidopsis* leaves were ground in a mortar with 1.5 ml of cold ethyl acetate. Then, 400 μl

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Abbreviations: HL, high light; LL, low light; PC, plastochromanol; PC-OH, hydroxy-plastochromanol; PQ-9, plastoquinone; PQH₂-9, plastoquinol; Toc, tocopherol; WT, wild type.

of the extract was transferred to an Eppendorf tube and evaporated under nitrogen. The evaporated extract was dissolved in HPLC solvent (methanol/hexane, 340:20, v/v), centrifuged shortly on a benchtop centrifuge ($10000g \times 10s$) and analyzed immediately. The HPLC measurements were performed using a 100 μ l loop, Jasco PU-980 pump and UV-VIS detector system UV-970 connected in line with a Shimadzu RF10-AXL fluorescence detector (excitation/emission detection at 290/330 nm), a Teknokroma (Barcelona, Spain) C_{18} reverse-phase column (Nucleosil 100, 250 mm \times 4 mm, 5 μ m), isocratic solvent system — methanol/hexane (340:20, v/v) at the flow rate of 1.5 ml/min. Oxidized PQ was followed by absorbance at 255 nm while other prenyllipids were monitored using fluorescence detection as described in detail by Kruk and Karpinski (2006). Taking into account that plastoquinone and its hydroxyl-derivatives have the same chromanol ring structure (chromophore) as that of γ -tocopherol, for quantitative HPLC analysis the response of the fluorescence detector, using excitation at 290 nm and emission detection at 330 nm, was taken to be the same for both these compounds.

For preparative analysis, ethyl acetate extracts from 10 rosettes were pooled and separated on a Nucleosil column (as above) in methanol at the flow rate of 1.5 ml/min and the peak corresponding to PC-OH was collected.

Absorbance spectra were recorded on a Varian Cary 400 Bio UV-VIS spectrophotometer.

Mass spectrometry analysis was performed using an Esquire 3000 mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a standard electrospray ion source. The prenyllipid was dissolved in a solution of methanol/formic acid (99.9:0.1, v/v). Samples were introduced into the ion source using a syringe pump (KD Scientific, USA, model KDS100) with the flow rate set to 3 microliters per minute. Voltage between capillaries was set to 4500 V, temperature of heated capillary to 280°C. Additional parameters (voltages on skimmer 1, skimmer 2, octopole, trap drive potential, etc.) were optimized according to the scan range used during analyses.

Prenyllipid standards. Tocopherol homologues of HPLC grade ($\geq 99.5\%$) were purchased from Merck. Oxidized and reduced plastoquinone standards were obtained as described by Kruk (1988). The plastoquinone standard was purified from linseed oil according to the published procedure (Gruszka *et al.*, 2008) or synthesized from natural PQ as described by Gruszka and Kruk (2007).

RESULTS

In the HPLC chromatogram of leaf extracts of *Arabidopsis* wild type (WT), using fluorescence detection for tocopherols and reduced prenyllipids, apart from peaks of the known compounds, an additional peak was found with the retention time of 6 min (Fig. 1). The compound corresponding to this peak was also present in the *vte4* mutant lacking α -tocopherol (α -Toc) but it was not found for the *vte1* mutant lacking all tocopherols and plastoquinone. This indicates that this peak could be a PC derivative. The subsequent preparative isolation of this compound and spectroscopic analysis showed that it has identical absorption spectra to those of PC and γ -Toc. The absorbance maxima were at 294.5 nm and 300.5 nm with a minimum at about 260 nm both in cyclohexane

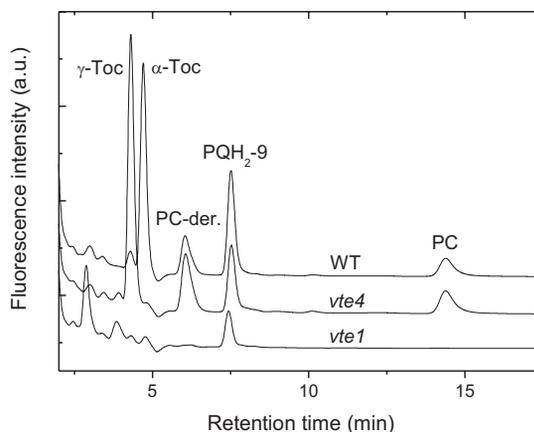


Figure 1. HPLC chromatograms of leaf extracts from *Arabidopsis*

The extracts from wild type (WT) and tocopherol biosynthetic mutants (*vte4*, *vte1*) were analyzed using fluorescence detection (ex/em. = 290/330 nm). PC-der., PC-derivative.

and iso-octane. The obtained absorbance maxima of the investigated compound are the same as those reported for PC and γ -Toc in the literature (Whittle *et al.*, 1965; Thies, 1997). This indicates that all three compounds have the same structure of the chromanol ring. The considerably shorter retention time of the investigated compound

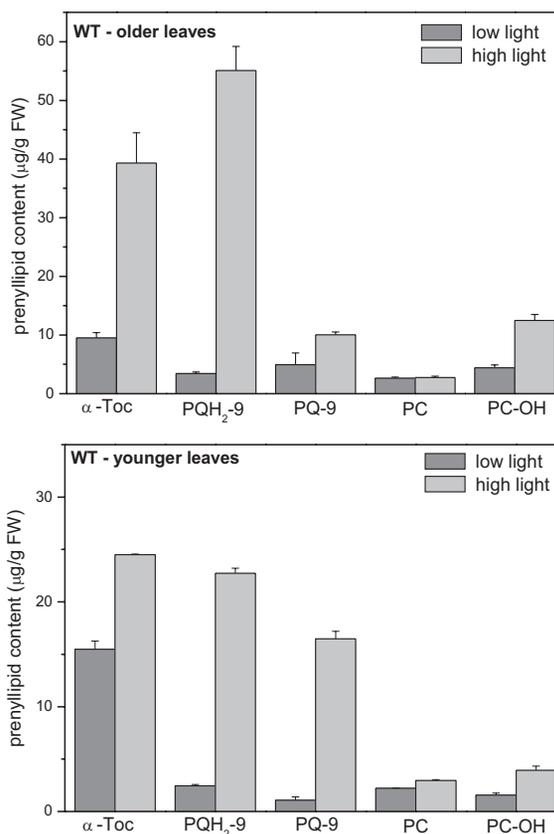


Figure 2. Prenyllipid content in older and younger leaves of *Arabidopsis*

Four- to five-week-old WT plants grown under low and high light conditions were analyzed. Data are means \pm S.E. (n = 3–5).

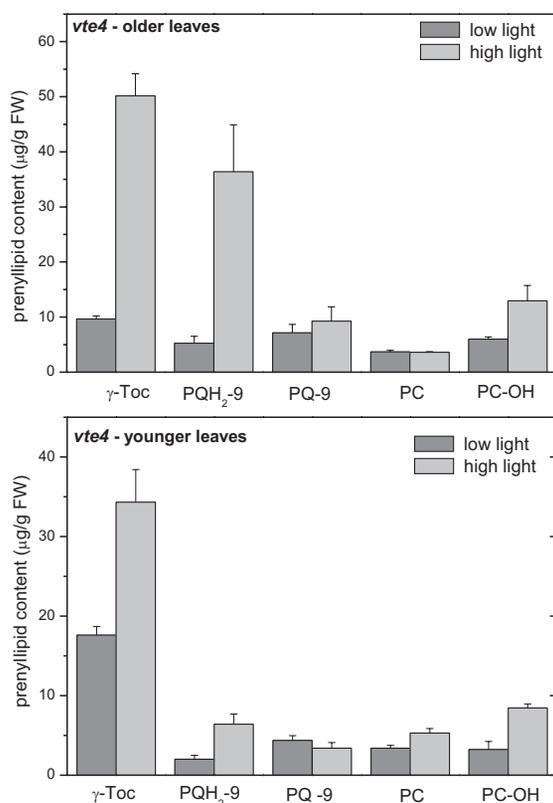


Figure 3. Prenyllipid content in older and younger leaves of *Arabidopsis*

Four- to five-week-old plants of α -Toc-deficient mutant *vte4* grown under low and high light conditions were analyzed. Data are means \pm S.E. (n=3–5).

as compared to PC, together with the identical spectroscopic properties, indicates that the new compound is a hydroxyl-derivative of PC with the hydroxyl group(s) located in the side-chain. A mass spectrometry analysis of this compound showed a $m/z = 806.0$ corresponding to $[PC+O+K]^+$ ion, which is in agreement with the molecular mass of a potassium adduct of PC having one hydroxyl group (PC-OH). An analogous peak for the oxidation product of PC by singlet oxygen was observed *in vitro* (Gruszka *et al.*, 2008). Observation of the PC-OH pseudomolecular ion as an adduct with potassium most probably results from the high affinity of hydroxylated PC for potassium ions originating from the glass vessels used for sample preparation.

Figure 2 shows that high light stress stimulates strongly the synthesis of α -Toc and of plastoquinol (PQH₂-9) both in older and younger leaves of the WT, while the level of PC remains relatively low under both light conditions. In the older leaves of low-light-grown plants, the PC-OH level is slightly higher than the level of PC but nearly four-fold higher under high light conditions. In the younger leaves, the content of both compounds was similar.

In the *vte4* mutant lacking α -Toc, the level of γ -Toc is similar to that of α -Toc in the WT (Fig. 3). The response of all the prenyllipids to high light is similar to that observed for the WT plants when the older leaves are concerned, while in the case of the younger leaves the increase in the level of both PQ forms is less pronounced.

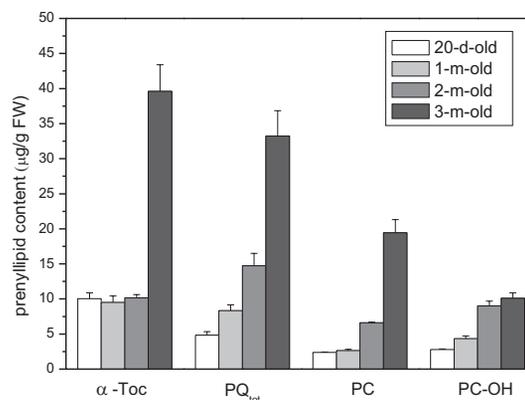


Figure 4. Prenyllipid content in leaves of *Arabidopsis* in the course of aging

The WT plants grown under low-light conditions were analyzed. Data are means \pm S.E. (n=3–5).

When the content of prenyllipids in leaves of WT plants grown under low light conditions was analyzed in the course of aging (Fig. 4), the content of both PC and PC-OH increased. When 2- and 3-month old leaves were compared, the level of PC-OH was similar, while in the case of PC it increased several-fold. Within the analyzed growth period, the total level of PC and PC-OH was similar to that of total (oxidized and reduced) PQ.

In seeds of the WT, PC-OH was not detected in contrast to PC which amounts to 10% of all the prenyllipids (Szymańska & Kruk, 2010).

DISCUSSION

The obtained results demonstrate a strong increase in the levels of tocopherols and plastoquinol, the known lipophilic antioxidants, under high light conditions, which is supposed to be the response of *Arabidopsis* plants to oxidative stress. It has been already shown that massive accumulation of total plastoquinone (mainly plastoquinol) takes place at high light conditions in sun-exposed leaves of beech, as compared to shade leaves (Lichtenthaler 1971; 1977; 2007). However, the accumulation of α -tocopherol was less pronounced in those studies than in our case. It was also found that in high light-grown *Arabidopsis* plants the level of α -tocopherol increased several-fold as compared to low light-grown plants (Golan *et al.*, 2006). In a recent paper of Zbierzak *et al.* (2010) it was shown that the level of PC increased over 3-fold under high-light stress applied for 4 days, which is in contrast to our results. However, in our experiments the plants were acclimated to high light conditions for a long period of growth and during this time some of the PC formed was probably converted to PC-OH, whose level clearly increased under high light conditions.

In the present study we have identified for the first time hydroxyl-plastochromanol in plants. Regarding the position of the hydroxyl group in the molecule, there is a question of the order of the carbon atom and the position of the isoprene unit where the group is attached. If the hydroxyl group is formed due to the oxidation of a double bond in the side chain by singlet oxygen, and the mechanism of the side chain oxidation is the same as that of the double bond in cholesterol (Gruszka *et*

al., 2008), then the hydroxyl group will be of the second order. Taking into account that the oxidation is a non-enzymatic reaction, theoretically all the double bonds in the PC side chain could be oxidized and a mixture of isomers formed. This could be the case for the PC-OH isolated in our study. The HPLC system applied would be probably unable to resolve individual isomers differing in the position of the hydroxyl group at different isoprene units.

Since PC-OH has been identified *in vitro* as the main oxidation product of PC during singlet oxygen scavenging (Gruszka *et al.*, 2008), our results indicate that such a reaction possibly also occurs *in vivo* and indicates that the main function of PC in leaves is quenching and scavenging of singlet oxygen generated in photosystem II during high light stress, similarly as in the case of tocopherol and plastoquinol (Krieger-Liszkay, 2004; Kruk *et al.*, 2005; Kruk & Trebst, 2008). The absence of PC-OH in seeds of *Arabidopsis*, where no formation of singlet oxygen is expected, also supports this hypothesis. The origin of PC-OH as the oxidation product of PC by singlet oxygen or by other reactive oxygen forms, is also indicated by its increased level in leaves of *Arabidopsis* grown under high light conditions. Nevertheless, PC-OH is also formed under low light conditions, where the formation of reactive oxygen forms is expected to be considerably less pronounced. It also cannot be excluded that PC-OH is formed enzymatically in leaves from PC to some extent by a still unidentified oxidase. It is also possible that PC-OH has some other metabolic function. It should be also pointed out that PC-OH itself can be potentially a singlet oxygen scavenger because of the presence of the chromanol ring. Experiments on the singlet oxidation of PC *in vitro* (Gruszka *et al.*, 2008) showed that besides the hydroxylated products of PC, also PC-OH homologue of 8-hydroperoxy- γ -tocopherone was formed showing that this compound is a singlet oxygen scavenger at least in the investigated system. It is also worth mentioning that also other hydroxylated derivatives of prenyllipids, such as plastoquinone-C, have been detected in higher plants and algae (Kruk & Strzalka, 1998; Kruk & Trebst, 2008) and could be products of the singlet oxygen scavenging activity of prenyllipids (Gruszka *et al.*, 2008). However, in the case of *Arabidopsis*, only trace amounts of plastoquinone-C were detected (Kruk, unpublished).

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