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Detection and quantification of vitamin K_1 quinol in leaf tissues

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Abstract

Phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone; vitamin K_1) is vital to plants. It is responsible for the one-electron transfer at the A_1 site of photosystem I, a process that involves turnover between the quinone and semi-quinone forms of phylloquinone. Using HPLC coupled with fluorometric detection to analyze Arabidopsis leaf extracts, we detected a third redox form of phylloquinone corresponding to its fully reduced – quinol–naphthoquinone ring ($PhQH_2$). A method was developed to quantify $PhQH_2$ and its corresponding oxidized quinone (PhQ) counterpart in a single HPLC run. $PhQH_2$ was found in leaves of all dicotyledonous and monocotyledonous species tested, but not in fruits or in tubers. Its level correlated with that of PhQ, and represented 5–10% of total leaf phylloquinone. Analysis of purified pea chloroplasts showed that these organelles accounted for the bulk of $PhQH_2$. The respective pool sizes of $PhQH_2$ and PhQ were remarkably stable throughout the development of Arabidopsis green leaves. On the other hand, in Arabidopsis and tomato senescing leaves, $PhQH_2$ was found to increase at the expense of PhQ, and represented 25–35% of the total pool of phylloquinone. Arabidopsis leaves exposed to light contained lower level of $PhQH_2$ than those kept in the dark. These data indicate that $PhQH_2$ does not originate from the photochemical reduction of PhQ, and point to a hitherto unsuspected function of phylloquinone in plants. The putative origin of $PhQH_2$ and its recycling into PhQ are discussed.

Keywords: Phylloquinone, Vitamin K_1, Naphthoquinone, Quinone, Quinol, Chlororespiration, Photosystem I

Abbreviations: Gla, γ-carboxyglutamate; MK-4, menaquinone-4; MK-$4H_2$, menaquinone-4 quinol form; PhQ, phylloquinone quinone form; $PhQH_2$, phylloquinone quinol form

1. Introduction

Phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone) **1** or vitamin K_1, is a conjugated isoprenoid consisting of a naphthoquinone ring attached to a C-20 phytyl side chain (Figure 1A). It is synthesized exclusively by plants, algae, and cyanobacteria. Facultative anaerobic bacteria synthesize a closely related form called menaquinone (vitamin K_2). Vertebrates, that cannot synthesize vitamin K, require it as an essential micronutrient. For humans, plant-based foods represent the main dietary source of this vitamin (Damon et al., 2005).

Vitamin K **1** plays very different roles in the organisms that cannot synthesize it compared to those that can. In vertebrates, for instance, it serves as a cofactor for certain carboxylases that convert specific glutamate residues in target proteins into γ-carboxyglutamates (Gla) (Ulrich et al., 1988). Such Gla-containing proteins are involved in blood coagulation, bone homeostasis, and the maintenance of vascular integrity (Vermeer et al., 2004). The biologically active form of the vitamin required for such processes is vitamin K quinol **3**, *i.e.* the one with a fully reduced naphthoquinone moiety (Figure 1B). The homeostasis of vitamin K quinol **3** is therefore key to the function of the vitamin K-dependent carboxylases, and vertebrates posses an enzyme that is capable of reducing the naphthoquinone **1** ring to its quinol **3** form (Chu et al., 2006). Vitamin K-synthesizing organisms, on the other hand, use vitamin K **1** as an electron transporter. In photosynthetic organisms, for instance, phylloquinone **1** is the electron carrier at the A_1 site of photosystem I. There, it transfers a single electron at a time from chlorophyll A to the iron–sulfur center of ferredoxin reductase (Sigfridsson et al., 1995). This one-electron transfer thus involves the quinone **1** and semiquinone **2** forms of phylloquinone (Figure 1B), and typically occurs in the nanosecond time-scale. The quinone/semiquinone (1:2) turnover is classically studied by electron paramagnetic resonance (Xu et al., 2003), while the comparatively stable quinone **1** can be analyzed by HPLC coupled with fluorometric or electrochemical detection (Booth and Sadowski, 1997; McCarthy et al., 1997).

Taking advantage of the sensitivity and selectivity of HPLC–fluorometry, we report here the existence of the fully reduced form of phylloquinone **3** ($PhQH_2$) in leaf tissues. After showing that $PhQH_2$ **3** is localized in chloroplast, we surveyed its respective pool sizes and its oxidized quinone counterpart **1** (PhQ) in Arabidopsis leaves of different developmental stages, and subjected to light and dark conditions.

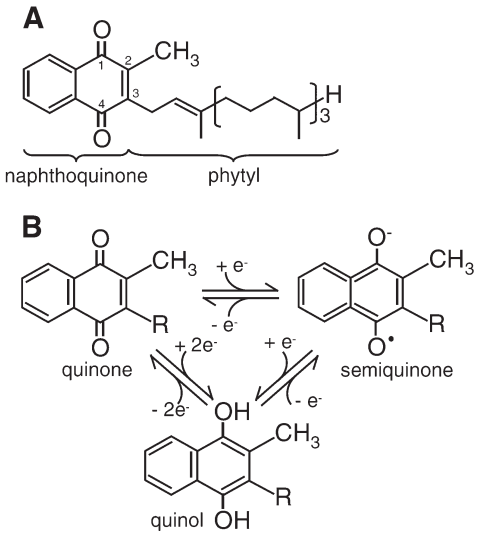

Figure 1. Structure and redox forms of phylloquinone **1** (vitamin K_1). (A) The phytyl side-chain of phylloquinone **1** consists of one isopentenyl unit followed by three isopentyl units. (B) Interconversion between the quinone **1** (oxidized), semiquinone **2** (hemi-reduced), and quinol **3** (fully reduced) forms of phylloquinone **1**. The latter is known only in vertebrates, and appears to originate from the direct enzymatic bi-reduction of the quinone. R = phytyl.

2. Results and discussion

2.1. Detection and quantification of $PhQH_2$3 in leaf tissues

Usual methods for the quantification of phylloquinone **1** in plant samples involve the preparation of a lipid-enriched fraction prior to analysis. These classically include extraction in an organic solvent, phase partitioning, evaporation of an organic phase and sample resolubilization. Pilot experiments using Arabidopsis leaves showed that HPLC–fluorometry provided sufficient selectivity and sensitivity to render these pre-treatments unnecessary, and that conjugated naphthoquinones could be directly detected in ethanolic extracts. The detection method used is based on the property of the naphthoquinone ring to fluoresce when reduced to its quinol form. In our HPLC system, this reduction was achieved on-line in a post-column dry reactor packed with zinc dust as described in the Experimental. Using this simpler and shorter protocol (~5 min from extraction to injection), we detected an additional peak of fluorescence at a shorter retention time than PhQ **1** (Figure 2A). The corresponding chemical species appeared to be labile, for it could be detected only if the samples were injected within a few minutes of extraction. This is probably why this peak was not observed with standard phylloquinone **1** analytical procedures. Surprisingly, the unknown compound displayed the same excitation and emission maxima of fluorescence than PhQ **1** after post-column reduction (data not shown). However, unlike PhQ **1** it retained its fluorescence properties in absence of post-column reduction (data not shown). Such characteristics suggested that it could correspond to the quinol **3** form of phylloquinone **1** present in the extract before the post-column reduction. Further investigations confirmed this hypothesis: the unknown compound co-chromatographed with authentic $PhQH_2$ **3** (Figure 2A), and displayed the same characteristic spectrum of absorption (Figure 2B). Moreover, when the peak was collected and exposed to air, and then re-chromatographed, its absorbance spectrum (Figure 2C) and retention time (Figure 2D) were converted to that of PhQ **1**.

During these pilot experiments, the question arose as to whether $PhQH_2$**3** detection could be an artifact due to the reaction of PhQ **1** with reducing compounds (e.g. ascorbate, glutathione, tocopherols) released from the tissue during its homogenization. It was especially a concern since it had been shown that another conjugated plant quinone, plastoquinone, could be reduced nonspecifically in ethanolic solution by ascorbate (Kruk and Karpinski, 2006). Therefore, as a control experiment we spiked the Arabidopsis leaf tissue before homogenization with 450 pmol of menaquinone-4 (MK-4; vitamin K_2). MK-4 is not synthesized by plants; however, it contains the same naphthoquinone ring as phylloquinone, and thus displays similar redox properties. Due to its structurally different polyisoprenyl side chain, MK-4 can also be easily distinguished from phylloquinone **1** by reversed phase analysis. After extraction and HPLC separation, while $PhQH_2$**3** was detected, MK-$4H_2$ was not (data not shown). Nor did we detect any $PhQH_2$**3** when pure PhQ **1** was mixed prior to extraction with ascorbate (30 mM), conditions that are known to cause significant reduction of plastoquinone (Kruk and Karpinski, 2006). From these control experiments, it can be concluded that the detection of $PhQH_2$**3** in plant extracts was not caused by the non-specific reduction of PhQ **1** during tissue homogenization. In addition, the tissues were spiked at the beginning of the extraction with an internal standard consisting of a mixture of MK-$4H_2$/MK-4, whose ratio had been quantified. By comparing the initial MK-$4H_2$/MK-4 ratio with that measured in the sample after extraction and HPLC separation, the respective $PhQH_2$**3** and PhQ **1** levels could be corrected for reoxidation of the naphthoquinone moiety. The same internal standard was used for the calculation of the recovery of total phylloquinone (PhQ + $PhQH_2$). Reoxidation levels ranged from 5% to 40%, and total recovery varied from 76% to 98%.

$PhQH_2$**3** was readily detected in the leaves of all dicotyledonous and monocotyledonous species examined (Table 1). Its level correlated with that of PhQ **1**, and was found to represent ~5% to 10% of the total pool of leaf PhQ **1**. On the other hand, in tubers and fruits, whose PhQ **1** content was very low, $PhQH_2$**3** was below the detection limit (~0.01 pmol/mg FW) if present at all in these organs.

2.2. $PhQH_2$3 is mainly—if not exclusively—localized in chloroplasts

As phylloquinone **1** biosynthesis has been shown to occur in chloroplasts (Schulze-siebert et al., 1987), we investigated the distribution of $PhQH_2$**3** and PhQ **1** in this organelle. Cell fractionation was done using young pea leaves as those have been established to be one of the best sources for obtaining highly pure preparations of intact chloroplasts (Cline, 1986). Marker enzyme assays confirmed that indeed isolated chloroplasts were devoid of detectable contamination by other fractions (Table 2). The PhQ/chlorophyll and $PhQH_2$/chlorophyll ratios of the crude extract were consistent with that of purified chloroplasts, indicating that these organelles account for virtually the entire cellular content of both PhQ **1** and $PhQH_2$**3** (Table 2). Such a distribution is in agreement with the previous report that the bulk of phylloquinone **1** in Arabidopsis leaves is localized in the chloroplast (Lohmann et al., 2006). In that regard, these data and ours question the proposal that phylloquinone could act as an electron donor for certain redox proteins located in the plant plasma membrane (Lochner et al., 2003).

2.3. $PhQH_2$3 levels increase in senescent leaves and in the dark

As it has been reported that phylloquinone **1** content of several plant species depends of the age of the tissue (Ferland and Sadowski, 1992), we surveyed $PhQH_2$ **3** and PhQ **1** levels of Arabidopsis cotyledons, rosette leaves harvested at different days after emergence, and senescent leaves. The specific content – expressed per mg of fresh weight- of total phylloquinone, $PhQH_2$ **3** and PhQ **1**, did not vary significantly be-

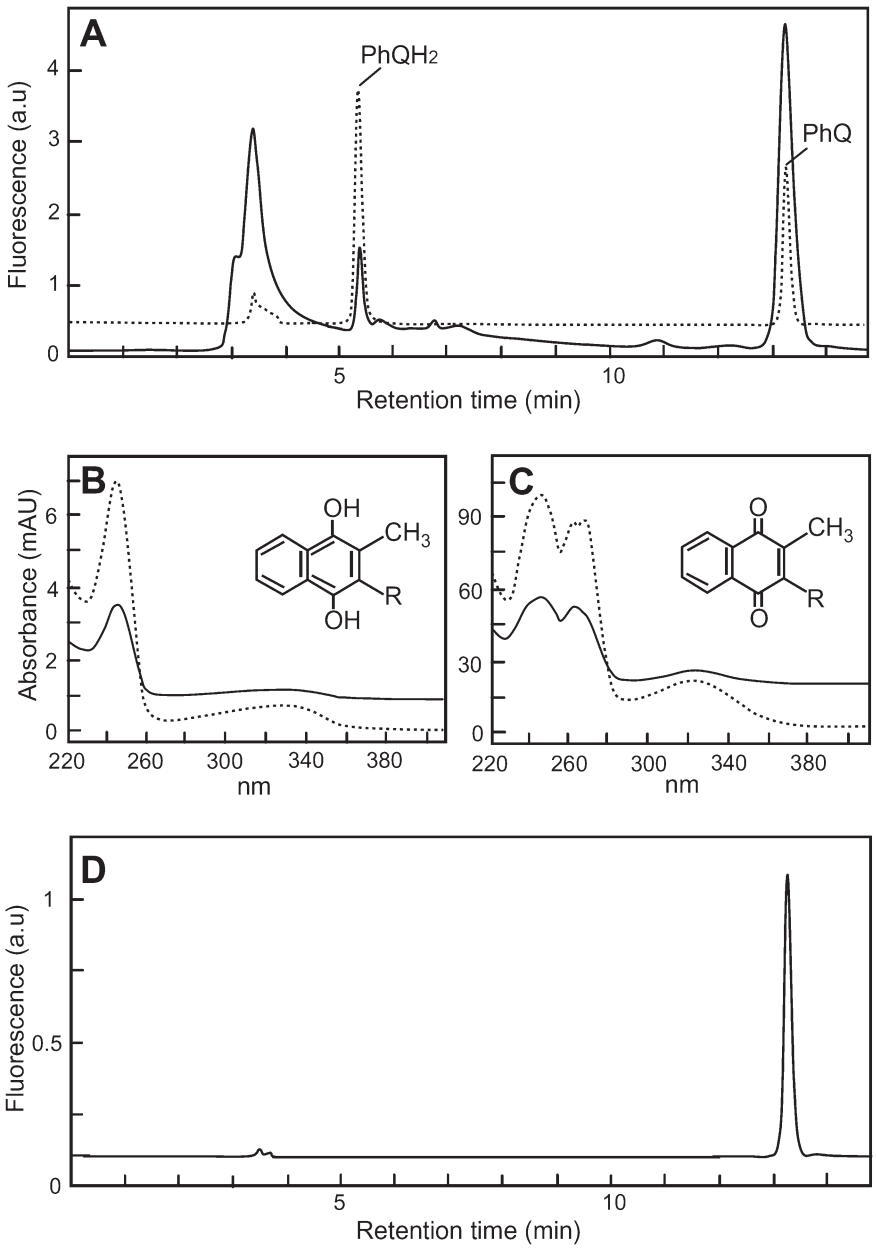

Figure 2. Detection of $PhQH_2$**3** and PhQ **1** in Arabidopsis leaves.

(A) HPLC fluorescence analysis of phylloquinone **1** in an Arabidopsis leaf ethanol extract (solid line). $PhQH_2$**3** and PhQ **1** external standards (dotted line). Traces have been offset for clarity. Retention times of $PhQH_2$**3** and PhQ **1** were 5.4 and 13.6 min, respectively.

(B) On-line absorption spectra of the unknown compound (solid line) and of authentic $PhQH_2$**3** (dotted line).

(C) Absorption spectra of the unknown compound after oxidation (solid line) and of authentic PhQ **3** (dotted line). Insets show the corresponding redox states of the naphthoquinone ring.

(D) Fluorescence trace of the unknown compound after oxidation and re-chromatography. Fluorescence traces in (A) and (D) have been obtained with post-column chemical reduction. R = phytyl.

Table 1. $PhQH_2$**3** and PhQ **1** contents of different plant tissues

Species/tissue	$PhQH_2$ (pmol mg^{-1} FW)	% of total phylloquinone	PhQ (pmol mg^{-1} FW)	% of total phylloquinone
A. thaliana (green leaf)	0.9 ± 0.2	11	7.2 ± 0.4	89
S. lycopersicum (green leaf)	1.4 ± 0.1	5	25.6 ± 0.3	95
(green fruit)	n.d	–	0.43 ± 0.1	100
(red ripe fruit)	n.d	–	0.18 ± 0.1	100
P. sativum (green leaf)	1.4 ± 0.6	9	14.4 ± 1.5	91
Z. mays (green leaf)	3 ± 1	9	30.6 ± 6	91
O. sativa (green leaf)	1 ± 0.1	7	13.7 ± 4.5	93
S. tuberosum (tuber)	n.d	–	0.03 ± 0.01	100
D. carota (tuber)	n.d	–	0.06 ± 0.02	100

PhQ **1** and $PhQH_2$**3** contents of ethanolic extracts were quantified by HPLC-fluorometry with post-column chemical reduction, and were corrected for recovery and re-oxidation. Values are the means of data from 3 to 29 extractions ± SE. n.d: not detected. The detection limit is approximately 0.01 pmol mg^{-1} FW.

tween cotyledons and green rosette leaves of different ages (Figure 3A, B). The steadiness of the PhQ/$PhQH_2$ ratio (5.6 ± 0.9) throughout various developmental stages of photosynthetically active leaves is remarkable (Figure 3B), and indicates that the relative pool sizes of $PhQH_2$ **3** and PhQ **1** are precisely regulated in these tissues. By contrast, in senescent leaves, whose total phylloquinone **1** level slightly decreased (Figure 3A), the PhQ/$PhQH_2$ ratio was found to decrease significantly

Table 2. Localization of PhQ **1** and $PhQH_2$**3** in pea leaf chloroplasts by subcellular fractionation

	Fumarase	Catalase	GAPDH	PhQ	$PhQH_2$
	(nkat mg^{-1} protein)			(nmol mg^{-1} chlorophyll)	
CE	5.0 ± 2.6	2921 ± 374	13 ± 0.8	3.4 ± 1.0	0.7 ± 0.3
CP	n.d	n.d	21.5 ± 1.7	4.0 ± 1.1	0.9 ± 0.2

Chloroplasts (CP) from pea leaf crude extract (CE) were purified on a Percoll gradient. Specific activities of marker enzymes (nkat mg^{-1} protein), and PhQ **1** and $PhQH_2$**3** contents (nmol mg^{-1} chlorophyll) were measured in crude extract and purified chloroplasts. Marker enzymes were fumarase for mitochondrion, catalase for peroxysome (+ cytosol), and NADP-linked glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for chloroplast. Values are the means of data from 3 independent preparations ± SE. n.d: not detected.

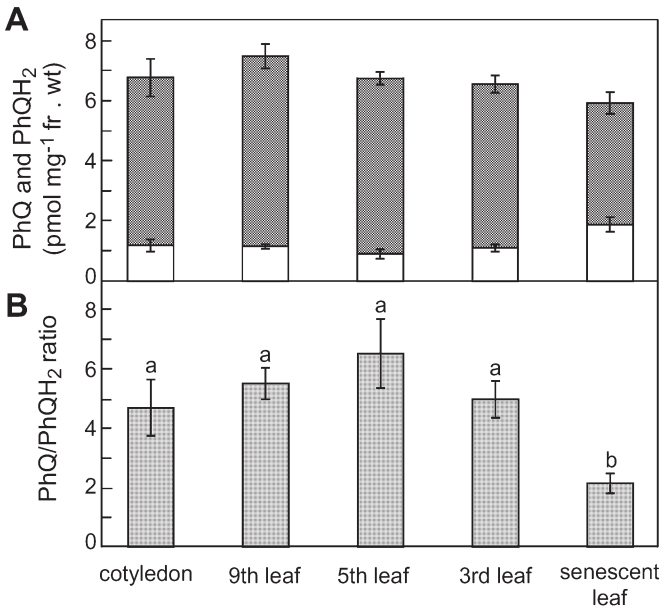

Figure 3. Redox status of phylloquinone **1** in Arabidopsis leaves. (A) $PhQH_2$**3** (white bars) and PhQ **1** (grey bars) contents of Arabidopsis cotyledons, and green and senescent rosette leaves. 9th, 5th, and 3rd leaves correspond to green rosette leaves harvested 8, 15, 18 days, respectively, after emergence. (B) Corresponding $PhQ/PhQH_2$ (1:3) ratios. The letters a or b above the bars indicate that the corresponding values are significantly different using Student's *t*-test ($P < 0.001$).

(2.9 ± 0.7; Student's *t*-test) as a consequence of a decrease in PhQ **1** and an increase in $PhQH_2$ (Figure 3A and B). This phenomenon was even more pronounced in tomato, whose $PhQ/PhQH_2$ ratio of 18.2 in green leaves (Table 1) dropped to a value of 3.1 in senescent leaves, while the total PhQ **1** level did not change significantly (data not shown).

In parallel, the levels of $PhQH_2$ **3** and PhQ **1** of Arabidopsis leaves exposed to light were compared with that of those kept in the dark. No statistically significant difference in total phylloquinone **1** content was observed between light and dark treatments. Nor were there any statistically significant changes of $PhQH_2$ **3** and PhQ **1** contents within each treatment. On the other hand, an analysis of variance revealed that the ratio of PhQ **1** to $PhQH_2$ **3** of leaves kept in the dark was significantly lower than that of leaves exposed to light (Figure 4). Such a difference, which corresponded to a 55% increase of the $PhQH_2$ **3** pool (1.4 pmol mg^{-1} in the dark vs. 0.9 pmol mg^{-1} in the light), was too small to significantly impact the PhQ **1** pool size. In other words, the natural variation of the PhQ **1** pool –which is ten times bigger than that of $PhQH_2$**3** masked the variation caused by the increased level of reduction of PhQ **1**

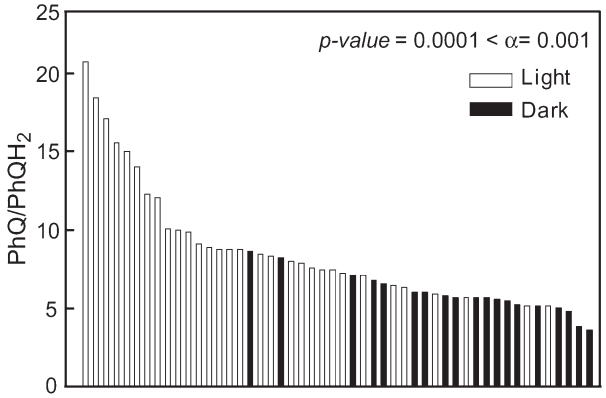

Figure 4. $PhQ/PhQH_2$ (1:3) ratios of Arabidopsis leaves subjected to light or dark. Each bar represents an individual experiment. One-way ANOVA, significant at *p*-value = 0.0001 < *a* = 0.001.

in the dark. It is noteworthy that in this experiment the tissues were sampled at different times after the beginning of the light and dark periods. As it has been shown that pre-illumination conditions impact the extent and duration of the dark reduction of plastoquinone (Groom et al., 1993), it is possible that a similar phenomenon occurs with phylloquinone and would cause the observed scattering of the data.

3. Concluding remarks

$PhQH_2$ **3** is well known in mammals, where it actually represents the biologically active form of the cofactor for certain carboxylases that catalyze the formation of Gla residues. Its detection in leaf tissues is a priori surprising, since there is no ortholog of the mammalian vitamin K-dependent carboxylases in the plant genomic databases, nor are there any reports of the existence of Gla-containing proteins outside the animal kingdom. In fact, the sole established role of phylloquinone **1** in plants is the quinone/semiquinone turnover in photosystem I. The very low stability constant of the semiquinone radical at physiological pH precludes the detection of this form of phylloquinone in our system. However, the detection of $PhQH_2$ **3** in leaves in the dark, imply that the bi-reduced $PhQH_2$ **3** is probably not part of the photoactive pool of phylloquinone **1**. Reinforcing this view, estimates from two recent studies show that about 50% of plant phylloquinone is not associated with photosystem I (Gross et al., 2006; Lohmann et al., 2006). Moreover, subplastidial fractionation experiments indicate that most of this unbound phylloquinone is probably localized into plastoglobules (Lohmann et al., 2006). Our finding of $PhQH_2$ in chloroplasts suggests that part of the non-photoactive phylloquinone **1**, more than a mere storage pool, is functionally involved in redox reactions. It would be now informative to determine if the presence of $PhQH_2$ **3** in photosynthetic organisms is restricted to plants, or if it can also be found in cyanobacteria and green algae, which like plants synthesize phylloquinone **1** (Johnson et al., 2000; Lefebvre-Legendre et al., 2007).

The detection of $PhQH_2$ **3** in leaves in the dark is reminiscent of the pool of reduced plastoquinone **1** that has been observed in similar conditions (Asada et al., 1993; Groom et al., 1993; Kruk and Karpinski, 2006). This pool of reduced plastoquinone whose formation does not depend on photochemical processes, has been attributed to chlororespiration (Carol and Kuntz, 2001). In this pathway, a plastid terminal oxidase (PTOX) is thought to couple the reoxidation of reduced plastoquinone with the reduction of oxygen (Joët et al., 2002). The existence of $PhQH_2$**3** in leaves subjected to darkness suggests that phylloquinone **1** is also connected to chlororespiration. If it

is so, $PhQH_2$ **3** would possibly be reoxidized by a different oxidase than PTOX, for this enzyme has been shown to display strict specificity for plastoquinone, at least *in vitro* (Josse et al., 2003). Other plastidial quinone oxidases, such as cytochrome b559 (Kruk and Strzalka, 2001; Bondarava et al., 2003) and a thylakoid-associated peroxidase (Casano et al., 2000), whose activities with $PhQH_2$ **3** as a substrate have not been tested, are obvious candidates for the recycling of $PhQH_2$ into PhQ.

Similarly, it is possible that at least one of the plastidial quinone oxidoreductases, which are thought to mediate plastoquinone reduction (Rumeau et al., 2007), is at the origin of $PhQH_2$ **3**. In that regard, it is noteworthy that the purified NAD(P)H-plastoquinone oxidoreductase complex has been shown to use menadione (vitamin K_3) – *i.e.* the unconjugated naphthoquinone ring – as electron acceptor *in vitro* (Sazanov et al., 1998).

There is also compelling evidence that reduced plastoquinone functions as a signal for the regulation of photosynthetic efficiency via the activation of cytochrome b_6-f-dependent kinase (Vener et al., 1995; Karpinski et al., 1997; Yang et al., 2001). However, the observation that the kinase activity does not always correlate with the reduction state of plastoquinone **1** *in vivo*, has led to the proposal that another redox sensor must act in conjunction with reduced plastoquinone **1** (Kruk and Karpinski, 2006). The existence of a chloroplastic pool of $PhQH_2$ **3**, which oscillates between light and dark, opens the exciting possibility that $PhQH_2$ **3** could function as such an additional regulatory molecule.

4. Experimental

4.1. General experimental procedures

MK-4 (vitamin K_2) was from Sigma (Saint-Louis, MO), and PhQ **1** (vitamin K_1) was from MP biomedicals (Illkirch, France). Calibration solutions of MK-4 and PhQ **1** were quantified spectrophotometrically using the absortivity value E_{1cm} (1%) at 248 nm = 420 (Booth and Sadowski, 1997). HPLC analyses were carried out on an Agilent 1200 series instrument (Agilent Technologies, Santa Clara, CA) equipped with diode array and fluorometer detection modules, and employing Chemstation Software. For experiments involving naphthoquinone species, the samples were protected from light to avoid photodegradation and photoreduction.

4.2. Synthesis of $PhQH_2$3 and MK-$4H_2$

Ten to 20 nmole of PhQ **1** or MK-4 were reduced with 0.1 M sodium dithionite in EtoH:H_2O (85:15, v/v) (final volume 1 ml) at room temperature in a pyrex screw-cap tube. After 1 h incubation, H_2O (600 µl) was added, and the EtoH/H_2O phase was extracted once with hexane (2.5 ml). The hexane phase was evaporated to dryness under a gentle stream of nitrogen, and the residue was resuspended in EtoH:H_2O (1 ml, 95:5, v/v). Typical preparations yielded a mixture of ~80% quinol form and ~20% quinone **1** form. The rate of reoxidation of quinol **3** into quinone **1** was ~2%/day at −20 °C.

4.3. Plant material

Tomato (*Solanum lycopersicum* cv. Micro-Tom), pea (*Pisum sativum* cv. Laxton's Progress 9), and *Arabidopsis thaliana* (ecotype Col-0) were grown in potting soil in a chamber (photosynthetic photon flux density 150 µmol quanta m^{-2} s^{-1}, 22 °C, 8 h night).

Maize (*Zea mays* cv. H99) and rice (*Oryza sativa* cv. Japonica 'Kitaake') were grown in a greenhouse under natural light. Carrot (*Daucus carota*) and potato (*Solanum tuberosum*) tubers were purchased locally.

4.4. Phylloquinone analyses

Tissues (10–30 mg of fresh weight) were sampled at the time of extraction, then spiked with 0.5–1 nmole of a MK-$4H_2$/MK-4 mixture as an internal standard and homogenized in EtoH:H_2O (0.9 ml, 95:5, v/v) using a 5-ml pyrex tissue grinder (# 7724-5 Corning Inc., NY). It was particularly important that the tissues were disrupted as quickly as possible; in our set-up the total time from sample spiking to complete extraction did not exceed 30 s. After a brief centrifugation (5 min at 14,000*g*) to pellet debris, the samples were immediately analyzed by HPLC on a 5 µM Discovery C-18 column (250 × 4.6 mm, Supelco) thermostated at 30 °C. Samples were eluted in isocratic mode at a flow rate of 1 ml min^{-1} with MeoH:EtoH (80:20, v/v) containing 1 mM sodium acetate, 2 mM acetic acid, and 2 mM $ZnCl_2$. Naphthoquinone species were detected fluorometrically (238 nm and 426 nm for excitation and emission, respectively) after their on-line reduction into a post-column chemical reactor (70 × 1.5 mm) packed with −100 mesh zinc dust (Aldrich). Retention times of MK-$4H_2$, $PhQH_2$, MK-4, and PhQ were 4.4 min, 5.4 min, 8.7 min and 13.6 min, respectively. PhQ **1** and MK-4 were quantified according to external calibration standards. Data were corrected for recovery and reoxidation of the MK-$4H_2$/MK-4 spikes added to the plant samples at the beginning of extraction.

4.5. Chloroplast isolation from pea leaves

Chloroplasts were purified on a Percoll gradient as previously described (Cline, 1986), except that ascorbate and bovine serum albumin were omitted from the extraction and wash buffers. For the fumarase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activities, protein extracts were desalted on Sephadex G-25 pre-equilibrated with Tricine–NaOH buffer, 50 mM, pH 8.4. GAPDH activity was measured in a two-step procedure. For the first step (enzyme activation), desalted protein extracts (75 µl) were incubated with 0.2 M DTT (5 µl), 0.1 M ATP (5 µl), 0.4 M Pi (5 µl) (pH 8.4), and Tricine–NaOH (10 µl) desalting buffer for 10 min at room temperature. For the second step (assay), reaction mixtures (100 µl) contained Tricine–NaOH desalting buffer (4 µl), 0.8 M $MgCl_2$ (1.5 µl), 0.2 M DTT (1 µl), 0.1 M ATP (4.5 µl), freshly prepared 12 mM NADPH (1 µl), 2 units of phosphoglycerate kinase, 0.2 M 3-phosphoglycerate (2.5 µl), and H_2O (75 µl). Reactions were started with the addition of activated extract (10 µl), and the decrease in absorbance at 340 nm was monitored. Activities were calculated using the molar absorption coefficient of NADPH at 340 nm (6200 M^{-1} cm^{-1}). For the fumarase assay, reaction mixtures (final volume of 100 µl) contained Tricine–NaOH desalting buffer (90 µl), and desalted protein extracts (5 µl). Reactions were started with the addition of 1 M malate (5 µl), and the increase in absorbance at 240 nm was monitored. Activities were quantified according to a standard curve of known concentrations of fumarate. For the catalase assay, reaction mixtures (final volume of 100 µl) contained 70 mM KPi buffer (94 µl) (pH 7.5), protein extracts desalted on Sephadex G-25 pre-equilibrated with KPi buffer (5 µl), and 3% (v/v) H_2O_2 (1 µl) as substrate. The decrease in absorbance at 240 nm was monitored, and the activity was quantified according to a standard curve of known concentrations of H_2O_2. For chlorophyll determination, 50 µl of extract were mixed with acetone/H_2O (5 ml, 80:20, v/v). Cell debris were removed by centrifugation (2000 g, 10 min), and the OD of the supernatant was measured at 645 and 663 nm. Chlorophyll concentration was calculated using the formula: chlorophyll concentration (mg ml^{-1}) = (OD_{645} × 0.0202 + OD_{663} × 0.00865) × 100 (Arnon, 1949).

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