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4 **The plastoquinone pool outside the thylakoid membrane serves in plant**
5 **photoprotection as a reservoir of singlet oxygen scavengers**

6

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29

30 **Abstract**

31 The Arabidopsis *vte1* mutant is devoid of tocopherol and plastochromanol (PC-8). When exposed to
32 excess light energy, *vte1* produced more singlet oxygen ($^1\text{O}_2$) and suffered from extensive oxidative
33 damage compared to the wild type. Here we show that overexpressing the *SOLANESYL DIPHOSPHATE*
34 *SYNTHASE 1 (SPS1)* gene in *vte1* induced a marked accumulation of plastoquinone (PQ-9) and rendered
35 the *vte1 SPS1oex* plants tolerant to photooxidative stress, indicating that PQ-9 can replace tocopherol
36 and PC-8 in photoprotection. High PQ-9 levels were associated with a noticeable decrease in $^1\text{O}_2$
37 production and with higher levels of PQ-C, a $^1\text{O}_2$ -specific PQ-9 oxidation product. The extra PQ-9
38 molecules in the *vte1 SPS1oex* plants were stored in the plastoglobules and the chloroplast envelopes,
39 rather than in the thylakoid membranes, whereas PQ-C was found almost exclusively in the thylakoid
40 membranes. Upon exposure of wild-type plants to high light, the thylakoid PQ-9 pool decreased, while
41 the extra-thylakoid pool remained unchanged. In *vte1* and *vte1 SPS1oex* plants, the PQ-9 losses in high
42 light were strongly amplified, affecting also the extra-thylakoid pool, and PQ-C was found in high
43 amounts in the thylakoids. We conclude that the thylakoid PQ-9 pool acts as a $^1\text{O}_2$ scavenger and is
44 replenished from the extra-thylakoid stock.

45

46 **KEYWORDS**

47 plastoquinone, antioxidant, reactive oxygen species, photoprotection, tocopherol

48

49

50 **1 INTRODUCTION**

51 Plastoquinone-9 (PQ-9) is essentially viewed as a component of the photosynthetic electron transport
52 chain in chloroplasts, carrying electrons from photosystem II (PSII) to cytochrome *b6/f* (Amesz, 1973).
53 It is thus essential for the photosynthetic activity of plants, and mutations inhibiting PQ-9 accumulation
54 led to a lethal phenotype in Arabidopsis and maize (Cook & Miles, 1992; Norris, Barrette, & DellaPenna,
55 1995). Through its redox state, the PQ-9 pool can also play a role in the regulation of gene expressions
56 and enzyme activities (Pfannschmidt et al., 2009; Rochaix, 2014). However, several observations
57 obtained in recent *in vitro* and *in vivo* studies have raised the idea that the physiological role of PQ-9
58 can be more diverse than just electron shuttling from PSII to PSI. In particular, *in vitro* studies have
59 revealed that PQ-9 has antioxidant properties, being able to quench singlet oxygen ($^1\text{O}_2$) and to inhibit
60 oxidation of lipid membranes (Gruszka, Pawlak, & Kruk, 2008; Nowicka, Gruszka, & Kruk, 2013; Yadav,
61 Kruk, Sinha, & Pospisil, 2010). In isolated thylakoids, maintenance of the PQ-9 pool in the reduced state
62 with an electron transport inhibitor was associated with a lowering of high light-induced lipid

63 peroxidation, suggesting an antioxidant role for reduced PQ-9 (Hundal, Forsmark-Andrée, Ernster, &
64 Andersson, 1995).

65 Tocopherol and plastoquinone are synthesized through a common pathway until
66 homogentisate (HGA) where SOLANESYL DIPHOSPHATE SYNTHASE 1 (SPS1) introduces the bifurcation
67 towards PQ-9 and PC-8. Accumulation of PQ-9 in leaves by overexpression of the *SPS1* gene in *SPS1oex*
68 *Arabidopsis* plants reduced lipid peroxidation and increased the tolerance to photooxidative stress
69 (Ksas, Becuwe, Chevalier, & Havaux, 2015). However, because the *SPS1oex* plants also accumulated
70 plastochromanol-8 (PC-8), a PQ-9 metabolite with known antioxidative activities (Kruk, Szymanska,
71 Cela, & Munne-Bosch, 2014), photoprotection could not be unambiguously and exclusively attributed
72 to PQ-9. Nevertheless, sudden exposure of *Arabidopsis* plants to high light stress resulted in a marked
73 loss of PQ-9 (Ksas et al., 2015) and to an accumulation of oxidized derivatives formed by the interaction
74 between PQ-9 and $^1\text{O}_2$ (Szymanska & Kruk, 2010), supporting the idea that PQ-9 can act as a $^1\text{O}_2$
75 scavenger *in vivo*. Similarly, in the green microalga *Chlamydomonas*, PQ-9 was observed to decrease
76 in high light when its re-synthesis was blocked by an inhibitor of hydroxylphenylpyruvate dioxygenase
77 (Kruk & Trebst, 2008).

78 In this context, an interesting observation was the various localizations of PQ-9 in thylakoid
79 membranes, plastoglobules (Lichtenthaler & Weinert, 1970; Zbierzak et al., 2009) and chloroplast
80 envelopes (Soll, Schultz, Joyard, Douce, & Block, 1985), suggesting that PQ-9 could play diverse roles
81 in the chloroplasts. To investigate these roles, we have crossed the *vte1* *Arabidopsis* mutant, deficient
82 in both tocopherols and PC-8 (Porfiriva, Bergmuller, Tropf, Lemke, & Dörmann, 2002), with *SPS1oex*
83 lines previously described (Ksas et al., 2010) to generate *vte1 SPS1oex* plants that selectively
84 accumulate PQ-9 in the absence of PC-8. The responses of *vte1 SPS1oex* plants to high light together
85 with the analyses of PQ-9 localization and concentration demonstrate that PQ-9 is a $^1\text{O}_2$ -scavenging
86 antioxidant *in planta* and show the participation of the extra-thylakoid PQ-9 pool in this process
87 through delivery of PQ-9 to the thylakoids in order to replace oxidized PQ-9 molecules.

88

89 **2 MATERIAL AND METHODS**

90 **2.1 Plant material and growth conditions**

91 *Arabidopsis* plants (*Arabidopsis thaliana*, Columbia-0 ecotype) were grown in a phytotron under
92 controlled conditions of light ($150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, photoperiod 8 h), temperature ($20^\circ\text{C}/18^\circ\text{C}$,
93 day/night) and relative air humidity (60%). Several mutant/transformed plants were examined in this
94 work: the tocopherol cyclase mutant *vte1* deficient in tocopherols (Porfirova et al., 2002), *SPS1*-
95 overexpressing lines described in a previous work (lines #12 and #14 in Ksas et al. (2015)) and a crossing
96 between them (*vte1 SPS1oex*). Selection of homozygous *vte1 SPS1oex* plants was achieved first by

97 screening the progeny for resistance to the herbicide BASTA and then by screening for total absence
98 of tocopherol and PC-8 by HPLC analyses (see below for HPLC method). Photooxidative stress was
99 induced by transferring plants to a growth chamber at 7°C/15°C (day/night, air temperature) under a
100 PFD of 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a photoperiod of 8 h, as previously explained (Ksas et al., 2015).
101 Depending of the experiment, the duration of the treatment was 48 h (photooxidative stress
102 experiments) or 8 h (dynamics of the PQ-9 pools), as indicated in the legend of the figures.

103

104 **2.2 Prenyl lipid determinations**

105 Leaf discs (3 discs, diameter 1 cm) were grinded in ethyl acetate. After centrifugation, the supernatant
106 was filtered and evaporated on ice under a stream of N_2 . The residue was recovered in
107 methanol/hexane (17/1) and analyzed by HPLC, as described elsewhere (Ksas et al., 2015; Szymanska,
108 Nowicka, & Kruk, 2014; Kruk & Karpinski, 2006). The column was a Phenomenex Kinetex 2.6 μm , 100
109 x 4.6 mm, 100 A. Separation of tocopherols, plastoquinone-9 and plastochromanol-8 was done in the
110 isocratic mode with methanol/hexane (17/1) as solvent system and a flow rate of 0.8 ml min^{-1} . All
111 prenyl lipids, except oxidized plastoquinone-9, were detected by their fluorescence at 330 nm with an
112 excitation at 290 nm. Plastoquinone-9 in the oxidized state was measured by its absorbance at 255
113 nm. Typical chromatograms are shown in Supplemental Fig. S1. Plastochromanol-8 and
114 plastoquinone-9 standards were a kind gift from Dr. J. Kruk. Tocopherol standards were purchased
115 from Sigma. Unless specified otherwise, the PQ-9 data given in this article correspond to the total
116 content (oxidized PQ-9 + reduced PQH₂-9).

117 Determination of the photoactive and non-active fractions of plastoquinone-9 was done
118 following the protocol described in Kruk & Karpinski (2006) modified by Ksas et al. (2015). Leaf samples
119 were frozen in liquid nitrogen as quickly as possible to avoid changes in the plastoquinone redox state
120 during sample preparation. Two discs (diameter, 0.8 cm) were punched out from each leaf. To
121 determine the pool of photoactive plastoquinone-9, we first measured the amount of reduced
122 plastoquinone-9 (plastoquinol-9) in one disc after a dark-adaptation period of 2 h. The dark-adapted
123 samples were frozen in liquid nitrogen after 3-s illumination with far-red light to ensure full oxidation
124 of the photoactive pool of plastoquinone. Then, plastoquinol-9 was re-quantified in the disc that had
125 been exposed to a high PFD of white light (2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 15 s. Illumination was done
126 in a mortar that was filled with liquid nitrogen after the 15-s illumination in order to freeze the samples
127 in the light. The size of the photoactive pool was calculated as the difference between the plastoquinol-
128 9 content in the light and the plastoquinol-9 content in the dark. The size of the non-photoactive pool
129 of plastoquinone-9 was the sum of the amount of plastoquinol-9 in the dark (not reoxidable in the
130 dark) and the amount of oxidized plastoquinone-9 in the light (not reducible by high light). This pool

131 corresponds mainly to the plastoquinone molecules present in the plastoglobules and the chloroplast
132 envelope (Lichtenthaler, 2007) which are not involved in photosynthetic electron transport.

133

134 **2.3 PQ-C determination by UPLC-MS/MS**

135 Hydroxyplastoquinone (PQ-C) was analyzed using an UPLC-MS/MS system (UPLC ultimate RS 3000;
136 Thermo Fisher with QTOF 5600; AB Sciex) connected to a Kinetex C18 2.1 × 150 mm column
137 (Phenomenex). The APCI source was operated in negative mode. A binary solvent system was used, in
138 which mobile phase A consisted of acetonitrile:water (60:40, v/v) and 10 mM ammonium formate and
139 mobile phase B consisted of isopropanol:acetonitrile (90:10, v/v), 10 mM ammonium formate, and
140 0.1% formic acid (v/v). The gradient started with 27% of solution B and was increased to 97% of solution
141 B within 20 min at a speed of 0.3 ml min⁻¹, and then maintained at 97% for 5 min. Solution B was then
142 decreased to a 27% enrichment during 7 min for column re-equilibration. Column temperature was
143 set at 45°C. Oxidized and reduced PQ-9 and PQ-C identification was achieved based on the comparison
144 of retention times and accurate masses with standards. Reduced PQ-9 and PQ-C were obtained by
145 reducing the oxidized PQ-9 and PQ-C standards with sodium borohydride. In samples, PQ-C and PQ-9
146 contents were measured in selected reaction monitoring (SRM) mode using the following transition:
147 m/z 764.6 → m/z 202.095 ± 0.025 (CE = -70 eV) and m/z 748.6 → m/z 149.058 ± 0.025 (CE = -70 eV)
148 respectively. Examples of extracted-ion chromatograms (XIC) for PQ-C are shown in Supplementary
149 Fig. S2.

150

151 **2.4 Chloroplast fractionation**

152 Chloroplasts, isolated from leaves of 6-week-old plants, were hypotonically ruptured and subplastidial
153 compartments were isolated by centrifugation using sucrose-density gradient following the protocol
154 described in (Besagni, Eugeni Piller, & Bréhélin, 2011), except that plants were not pre-adapted to
155 darkness. 32 fractions of 1 ml were collected from the centrifuged tubes (from top to bottom), and
156 400 µl of each fraction were diluted to obtain a final sucrose concentration of 5%. The diluted fractions
157 were then grinded in mortar glass tube with 5 ml of cold ethyl acetate and sonicated (Digital Sonifer,
158 Brandon, USA) for 1 min with a 50% 'duty cycle' at 40% of power in an ice bath. The extracts were
159 centrifuged for 13 min at 9600 g at 4°C. 500 µl of supernatant was evaporated in a stream of nitrogen
160 and dissolved in 300 µl of methanol/hexane (17/1, v/v). 100 µl were immediately analyzed by HPLC, as
161 described above.

162

163 **2.5 Chlorophyll fluorescence**

164 The maximal quantum yield of PSII photochemistry was measured on attached leaves with a PAM-
165 2000 fluorometer (Walz) as $F_v/F_m = (F_m - F_o)/F_m$ where F_m is the maximal fluorescence level obtained

166 with a 800-ms pulse of intense white light and F_0 is the initial level obtained after a 2-s pulse of far-red
167 light. Measurements were done in the dark in leaves dark-adapted for 30 min.

168 Chlorophyll fluorescence induction curves were measured with a JTS-10 spectrophotometer/
169 fluorimeter (BioLogic, France), and the number of PSII 2-electron acceptors was estimated as described
170 in Joliot & Joliot (2002) as the area over the fluorescence rise measured in dark-adapted leaves divided
171 by the area over the curve for leaves infiltrated with DCMU (one electron transferred to Q_A in the light).
172 Chlorophyll fluorescence imaging was done with a laboratory-built instrument described in Johnson et
173 al. (2009).

174

175 **2.6 Lipid peroxidation**

176 Lipids were extracted from approximately 0.5 g of leaves frozen in liquid nitrogen. The leaves were
177 grinded in an equivolume methanol/chloroform solution containing 5mM Triphenyl Phosphine, 1 mM
178 2,6-tert-butyl-p-cresol (BHT) (5 ml g^{-1} fresh weight) and citric acid (2.5 ml g^{-1} fresh weight), using an
179 Ultraturax blender. Internal standard 15-HEDE was added to a final concentration 100 nmol g^{-1} fresh
180 weight, and mixed properly. After centrifugation at 700 rpm and 4°C for 5 min, the lower organic phase
181 was carefully taken out with the help of a glass syringe into a 15 ml glass tube. The syringe was rinsed
182 with approximately 2.5 ml chloroform and transferred back into the tube. The process was repeated
183 and the lower layer was again collected and pooled to the first collection. The solvent was evaporated
184 under N_2 gas at 40 °C. The residues were recovered in 1.25 ml absolute ethanol and 1.25 ml of 3.5 N
185 NaOH and hydrolyzed at 80°C for 30 min. The ethanol was evaporated under N_2 -gas at 40 °C for ~10
186 min. After cooling to room temperature, pH was adjusted to 4-5 with 2.1 ml citric acid. Hydroxy fatty
187 acids were extracted with hexane/ether 50/50 (v/v). The organic phase was analyzed by straight phase
188 HPLC-UV, as previously described (Montillet et al., 2004). The hydroxyoctadecatrienoic acid (HOTE)
189 isomers (9-, 12-, 13- and 16-HOTE derived from the oxidation of the main fatty acid in Arabidopsis
190 leaves, linolenic acid) were quantified based on the 15-HEDE internal standard.

191 Lipid peroxidation was also visualized in whole plants by autoluminescence imaging. Stressed
192 plants were dark adapted for 2 h, and the luminescence emitted from the spontaneous decomposition
193 of lipid peroxides was captured by a highly sensitive liquid N_2 -cooled charge-coupled device (CCD)
194 camera, as previously described (Birtic et al., 2011). The images were treated using Image J software
195 (NIH, USA)

196

197 **2.7 SOSG-EP fluorescence**

198 Production of 1O_2 was measured in attached leaves from the fluorescence of the 1O_2 -specific SOSG
199 (Singlet Oxygen Sensor Green) fluorescent probe (Invitrogen), as described previously (Ramel et al.,

200 2012). With the help of a 1-ml syringe (without the needle), 100 μM SOSG was pressure-infiltrated into
201 the leaves through the lower surface. Plants with SOSG-infiltrated leaves were exposed to a PFD of
202 1500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ at 7 °C for 20 min. As a control treatment, plants with SOSG-infiltrated leaves
203 were placed in the dark at room temperature for 20 min. SOSG-EP fluorescence was then measured
204 from leaf discs punched from the SOSG-infiltrated leaves using a fiberoptics-equipped Perkin-Elmer
205 spectrofluorometer (LS 50B) with a 475 nm excitation light. SOSG-EP fluorescence at 524 nm (F524)
206 was normalized to chlorophyll fluorescence at 680 nm (F680) for each leaf disc. SOSG-EP fluorescence
207 was calculated as: $([F524 \text{ in the light} - F524 \text{ in the dark}]/F680) \times 10$.

208

209 **2.8 EPR spectroscopy**

210 For the preparation of thylakoid membranes, 7 g of leaves (fresh weight) were grinded for 2 s in 50 ml
211 of extraction buffer (330 mM sorbitol, 50 mM Tricine, 2 mM EDTA(Na₂), 1 mM MgCl₂, 2 mM Ascorbate,
212 pH 7.7) with 5 mM dithiothreitol (DTT) in a Warring blender at low speed. The liquid phase was
213 removed and set aside, and 50 ml of extraction buffer was added for a second extraction. The extracts
214 were filtered onto 4 Miracloth layers, and the filtrate was centrifuged for 4 min at 1500 g at 4°C. The
215 pellet was washed twice with the extraction buffer and centrifuged for 4 min at 1500 g at 4°C. The
216 washed pellet was resuspended in 21 ml of lysis buffer pH 7.8 (10 mM Tricine, 10 mM NaCl, 10 mM
217 MgCl₂) with 1 mM PMSF (phenyl methylsulfonyl) with occasional stirring for 15 min. The sample was
218 centrifuged at 48400 g for 15 min. The pellet was resuspended in 1.75 ml of storage buffer (100 mM
219 Tricine, 10 mM NaCl, 10 mM MgCl₂, 400 mM sucrose, pH 7.8) and stored at -80°C before analyses.

220 Singlet oxygen formation was monitored by electron paramagnetic resonance (EPR)
221 spectroscopy using the spin probe 2,2,6,6-tetramethyl-4-piperidone (TEMPD) purified by vacuum
222 distillation. The oxidation of TEMPD by ¹O₂ forms 2,2,6,6-tetramethyl-4-piperidone-1-oxyl (TEMPONE)
223 detectable by EPR spectroscopy. Thylakoid membranes (25 $\mu\text{g Chl ml}^{-1}$) were illuminated in the
224 presence of 50 mM TEMPD and 40 mM MES-NaOH buffer (pH = 6.5) for 30 min under 1000 μmol
225 $\text{photons m}^{-2}\text{s}^{-1}$. After illumination, thylakoid membranes were centrifuged at 1 000 x g for 2 min to
226 separate sample from spin probe. The EPR spectra were recorded using an EPR spectrometer Mini
227 Scope MS400 (Magnettech GmbH, Berlin, Germany). EPR measurement conditions were as follows:
228 microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 G; scan rate, 1.62
229 G s⁻¹.

230

231

232 **3 RESULTS**

233 **3.1 Overexpression of the *SPS1* gene in the *vte1* mutant leads to a marked accumulation of PQ-9,**
234 **mainly in the plastoglobules and the chloroplastic envelopes**

235 Ksas et al. (2015) previously showed that overexpressing the *SPS1* gene in Arabidopsis brings about a
236 noticeable increase in the leaf content in both PQ-9 and PC-8. This was confirmed here with one of the
237 *SPS1oex* lines previously described, with a 50%-increase in the total PQ-9 content and a 3-fold increase
238 in PC-8 compared to WT (Fig. 1A). Please note that throughout the text the term PQ-9 is used as a
239 generic term for total PQ-9, i.e. oxidized PQ-9 + reduced PQH₂-9 (plastoquinol). In all genotypes, PQ-9
240 was highly reduced, with PQH₂-9 (plastoquinol) representing around 75% of the total pool
241 (Supplemental Fig. S3). The tocopherol cyclase mutant *vte1* is devoid of both tocopherols and PC-8
242 (Zbierzak et al., 2009). The loss of those two lipid-soluble antioxidants in *vte1* leaves was accompanied
243 by a significant decrease in the total PQ-9 levels, as previously reported (Ksas et al, 2015). When the
244 *vte1* mutant was crossed with the *SPS1*-overexpressing line (line #12 in Ksas et al., 2015) to generate
245 a double mutant *vte1 SPS1oex*, the total PQ-9 content of the leaves was strongly increased, reaching
246 values above the WT levels. Compared to *vte1*, *vte1 SPS1oex* contained approximately 3 times more
247 PQ-9. The changes in tocopherols observed in *vte1*, *SPS1oex* and *vte1 SPS1oex* had no significant
248 effect on the growth phenotype of the plants in low light (Fig. 1B) and did not affect significantly the
249 efficiency of photosynthetic electron transport in young and mature leaves as measured by the
250 chlorophyll fluorescence parameter Φ_{PSII} (Fig. 1C). Compared to the *SPS1* overexpressors, a decrease
251 in Φ_{PSII} was nevertheless observed in the older leaves of *vte1* and, to a lesser extent, of WT. Thus, high
252 PQ-9 levels appear to be helpful to maintain the efficiency of PSII-mediated electron flow in old, pre-
253 senescing leaves.

254 To determine the localization of the extra PQ-9 that accumulated in *SPS1*-overexpressing
255 plants, chloroplast membranes were fractionated by ultracentrifugation on sucrose gradient (Besagni
256 et al., 2011). As expected (Besagni et al., 2011; Vidi et al., 2006), 3 pigmented fractions were obtained
257 (Fig. 2A): plastoglobules (PG) were found floating at the top of the tubes and forming a whitish layer,
258 while an intermediate yellowish zone corresponding to the outer and inner chloroplast envelope
259 membranes (ENV) separated from the green zone at the bottom containing chlorophyll-binding
260 thylakoid membranes. 32 fractions were collected from top (fraction 1) to bottom (fraction 32) and
261 analyzed for their PQ-9 content (Fig. 2B, Supplemental Fig. S4). PQ-9 was found in all fractions, with an
262 almost equivalent partitioning between the thylakoids and the two other fractions
263 (envelopes+plastoglobules) in WT leaves (Supplemental Fig. S4). We checked the abundance of the
264 plastoglobule marker protein Plastoglobulin 35 (PGL35) in the different fractions by Western blotting.
265 Compared to the plastoglobule fraction, the abundance of PGL35 in the envelope fraction was very
266 low (Supplemental Fig. S5). Consequently, the almost similar amounts of PQ-9 found in the
267 plastoglobules and the envelopes (Supplemental Fig. S4) are not attributable to a contamination of the
268 envelopes by plastoglobules. Surprisingly, fractions 24-28 contain very little PQ-9 although they are
269 green. It is possible that those fractions contain photosynthetic pigmented complexes released from

270 the thylakoids during preparation. In *vte1*, the loss of PQ-9 concerned exclusively the PG and envelope
271 fractions (fractions 1-4 and 5-14, respectively), with the levels in the thylakoids (fractions 26-32) being
272 virtually unchanged relative to WT. Similarly, the accumulation of total PQ-9 in *SPS1oex* and *vte1*
273 *SPS1oex* leaves impacted mainly the PG and envelope fractions. Thus, thylakoid membranes appear to
274 accommodate a relatively constant amount of PQ-9 (presumably ensuring optimum electron
275 transport) while the PG and envelope fractions function as PQ-9 storage sites that can absorb large
276 variations in total PQ-9 in the chloroplasts. This was confirmed by the estimation of the
277 photochemically active and non-active pools of PQ-9 using a previously described procedure based on
278 HPLC analysis of reduced and oxidized PQ-9 in the dark and upon intense illumination (Ksas et al.,
279 2015). For each genotype, we found that the pool sizes of the active plus non-active pools (Fig. 2C)
280 were matching, within experimental accuracy, the total pool size (Fig. 1A; in nmol cm^{-2} , 0.91 ± 0.07 in
281 WT, 1.43 ± 0.15 in *SPS1oex*, 0.60 ± 0.04 in *vte1* and 1.73 ± 0.08 in *vte1 SPS1oex*). Figure 2C shows that
282 the photoactive pool (reflecting the thylakoid PQ-9 pool involved in photosynthetic electron transport)
283 showed relatively little variations in the different genotypes while the non-photoactive pool (reflecting
284 the pool, located outside the thylakoid membranes, not involved in electron transport) exhibited
285 strong variations, with *SPS1oex* and *vte1 SPS1oex* having by far the largest pools of extra-thylakoid PQ-
286 9 (Fig. 2C), in agreement with the analyses of Fig. 2B and Supplemental Fig. S4.

287 It is also possible to estimate the size of the functional pool of PSII electron acceptors by *in vivo*
288 chlorophyll fluorescence measurements (Malkin & Kok, 1966; Forbush & Kok, 1968). The chlorophyll
289 fluorescence induction curves of Fig. 2D showed little differences between the different genotypes,
290 confirming that the functional pool in the thylakoids was not much affected by the total PQ-9 levels.
291 From the fluorescence induction curves of Fig. 2D and the corresponding curves obtained in the
292 presence of DCMU, we estimated that the number of PSII two-electron acceptors is in the range 13-16
293 molecules (insert of Fig. 2D), which is consistent with previous estimations in various photosynthetic
294 organisms (Forbush & Kok, 1968; Kolber & Falkowski, 1993).

295

296 **3.2 Accumulation of total PQ-9 in the *vte1 SPS1oex* mutant compensated for the lack of tocopherols** 297 **and PC-8 and increased tolerance to photooxidative stress**

298 Plants grown in low light ($150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) were transferred to photooxidative stress
299 conditions ($1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, 7°C). After 2 d, this treatment led to leaf bleaching (Fig. 3A),
300 low Fv/Fm ratio indicative of PSII photoinhibition (Fig. 3D) and enhanced lipid peroxidation (Fig. 3B and
301 C) in WT leaves. These symptoms of photodamage were markedly attenuated in *SPS1oex* plants, as
302 previously shown (Ksas et al., 2015). In striking contrast, *vte1* exhibited a very high sensitivity to
303 photooxidative stress, showing dramatic effects on leaves (severe bleaching and loss of turgescence),
304 intense lipid peroxidation and drastic inhibition of PSII activity relative to WT. This confirms the

305 photoprotective role of tocopherol and/or PC-8 which are absent in *vte1* (Havaux, Eymery, Porfirova,
306 Rey & Dörmann, 2005). Interestingly, overexpression of *SPS1* suppressed the photobleaching
307 phenotype of *vte1*. Furthermore, *vte1 SPS1oex* was more tolerant to lipid peroxidation than *vte1*. The
308 data of Fig. 3 thus demonstrate that total PQ-9 can compensate for the lack of tocopherol and PC-8 in
309 *vte1* leaves, leading to a markedly increased tolerance to photooxidative stress. This effect was
310 confirmed in another *SPS1*-overexpressing *vte1* mutant and under longer stress exposure (4 d)
311 (Supplemental Figure S6).

312

313 **3.3 Plastoquinone as a singlet oxygen scavenger in planta**

314 $^1\text{O}_2$ production by illuminated leaves was monitored by measuring the intensity of SOSG-endoperoxide
315 (EP) fluorescence at 525 nm (Flors et al., 2006; Kim, Fujitsuka, & Majima, 2013). As shown in Fig. 4A,
316 the photosensitivity of *vte1* leaves correlated with an increased production of $^1\text{O}_2$ compared to WT, in
317 line with the known function of tocopherols as $^1\text{O}_2$ quenchers (Fahrenholtz, Doleiden, Trozzolo, &
318 Lamola, 1974; Di Mascio, Devasagayam, Kaiser, & Sies, 1990; Krieger-Liszkay, Fufezan, & Trebst, 2008;
319 Choe, 2107). It was decreased in *vte1 SPS1oex*, showing that replacement of tocopherols by total PQ-
320 9 restores the $^1\text{O}_2$ quenching capacities. This finding was confirmed by the analysis of $^1\text{O}_2$ production
321 by illuminated thylakoid membranes using EPR spectroscopy and the $^1\text{O}_2$ -specific spin probe TEMPONE
322 (Fig. 4C-D). The production rate of $^1\text{O}_2$ was the highest in *vte1* thylakoid membranes, whereas
323 accumulation of PQ-9 by *SPS1* overexpression in both WT and *vte1* backgrounds resulted in the lowest
324 $^1\text{O}_2$ production levels in the light, emphasizing the role of total PQ-9 in the quenching of $^1\text{O}_2$ *in vivo*.

325 Oxidation of PQ-9 by $^1\text{O}_2$ has been shown to produce some specific derivatives, such as the
326 hydroxy derivative PQ-C (Szymanska et al., 2014). The latter compound was shown to be a stable
327 product of $^1\text{O}_2$ action in plants, particularly when $^1\text{O}_2$ production is elevated during short-term
328 exposure to high light stress. Quantification of PQ-C in control Arabidopsis leaves showed the presence
329 of this compound in all genotypes (Fig. 4B). Similarly to PQ-9, PQ-C was predominantly in the reduced
330 state (PQH₂-OH) (Supplemental Fig. S3). PQ-C accumulation is consistent with the attribution of a $^1\text{O}_2$
331 scavenging function to PQ-9: the amount of this PQ-9 hydroxy derivative, formed by $^1\text{O}_2$, is the highest
332 in *vte1 SPS1oex* where total PQ-9 is the most abundant and is the major lipid-soluble antioxidant in
333 the absence of tocopherol and PC-8.

334

335 **3.4 High light stress preferentially consumes the thylakoidal PQ-9 pools**

336 Arabidopsis plants were exposed for 2.5 h to high PFD (1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low
337 temperature (7°C). The total PQ-9 concentration was decreased by around 30% in WT leaves after this
338 treatment (Fig. 5A). Fig. 5B shows that the PQ-9 losses were particularly marked for the thylakoid-
339 located photochemically active PQ-9 pool (-60%) while the extra-thylakoid, photochemically non-

340 active pool was not significantly affected. Since the photosystems in the thylakoids are the main source
341 of ROS in the light, a preferential oxidation of this photochemically active pool is expected. This is
342 actually confirmed by the analysis of $^1\text{O}_2$ -specific PQ-C compound in the different membrane fractions
343 of the chloroplasts: PQ-C was predominantly found in the thylakoids, with the concentration in the
344 plastoglobules and the envelopes being very low (Fig. 5C). In *vte1*, the photoinduced decrease in total
345 PQ-9 content was very marked (>50%) (Fig. 5A), and the photochemically active pool was almost
346 completely depleted after the high light treatment (Fig. 5B). However, contrary to WT, the size of the
347 extra-thylakoid, photochemically non-active PQ-9 pool was also decreased in the mutant, although to
348 a lesser extent (-40%) than the active pool (Fig. 5B). Fig. 5C shows that, similarly to WT, PQ-C in *vte1*
349 chloroplasts was present mainly in the thylakoids, confirming that PQ-9 oxidation occurred at this level.
350 As shown at the leaf level (Fig. 3), the PQ-C concentration was noticeably higher in *vte1* thylakoids
351 compared to WT thylakoids, indicating enhanced oxidation of PQ-9 in this mutant. In *vte1 SPS1oex*
352 leaves which rely on high levels of PQ-9 for their phototolerance, a considerable loss of total PQ-9 was
353 observed during high light stress (Fig. 5A, 5B), with a very strong accumulation of PQ-C in the thylakoids
354 (Fig. 5C). Thus, our data suggest that, under conditions of massive oxidative degradation of PQ-9 in the
355 thylakoids of *vte1* and *vte1 SPS1oex* chloroplasts, there is a transfer of PQ-9 molecules from the
356 plastoglobules and/or the envelope to the thylakoid membranes in order to compensate for PQ-9
357 photooxidation, hence lowering the PQ-9 levels in the storage sites outside the thylakoid membranes.
358 Owing to their high accumulation levels of total PQ-9, *vte1 SPS1oex* leaves were able to maintain the
359 total PQ-9 content close to the WT levels after the high light treatment (Fig. 5A), and this was
360 associated with a high resistance to photooxidative stress (Fig. 3).

361

362 **4 DISCUSSION**

363 The essential role of PQ-9 in photosynthesis as an electron carrier has long been recognized (Amesz,
364 1973; Crane, 2010). However, it is clear from this study that PQ-9 also fulfills an important
365 photoprotective function that is not directly related to photosynthetic electron transport. Actually,
366 only a fraction of the total PQ-9 pool is located in the thylakoid membranes where it can participate in
367 linear and cyclic electron fluxes. We estimated that, in WT Arabidopsis leaves under normal growth
368 conditions, the photochemically active PQ-9 corresponds to around 15 molecules per PSII and 50% of
369 the total PQ-9 content. The latter value is higher than previous reports (ca. 30%, Kruk & Karpinski,
370 2006; Szymanska & Kruk 2010), possibly due to different growth conditions. The size of the total
371 thylakoid-associated PQ-9 pool was found to be stable in control conditions, showing relatively little
372 variation among the different genotypes studied here or in response to changes in the total PQ-9
373 content of the leaves. This stability contrasts with the non-photochemically active pool, the size of
374 which exhibited large variations. As confirmed in this study, the latter PQ-9 pool partitions between

375 the plastoglobules (Zbierzak et al., 2009) and the envelope membranes (Soll et al., 1985). The size of
376 the extra-thylakoid PQ-9 pool drastically decreased in the photosensitive *vte1* mutant, whereas it was
377 selectively increased when PQ-9 biosynthesis was stimulated. The highest PQ-9 levels were found in
378 *vte1 SPS1oex* leaves in which approximately two-thirds of the total PQ-9 pool was not in the thylakoids.
379 In contrast, only 40% of the total pool was outside the thylakoid membrane in the *vte1* single mutant
380 that contains the lowest levels of total PQ-9. Somehow, the PQ-9 reservoir stored in the plastoglobules
381 and the chloroplast envelopes functions as a buffer that can manage large variations in the total PQ-9
382 levels in the leaves.

383 The multi-location storage of PQ-9 suggests a multi-functionality for this compound.
384 Previously, PQ-9 was reported to be involved in biosynthesis pathways, as a component of phytoene
385 desaturase in the carotenoid biosynthesis pathway (Norris et al., 1995) and of NDC1, a type-II NAD(P)H
386 quinone oxidoreductase involved in vitamin K1 synthesis (Eugeni Piller et al., 2011). This work shows
387 that it is also a ROS scavenger protecting the chloroplasts against photodamage by a mechanism that
388 could possibly be similar to the antioxidant activity of tocopherol. Indeed, overexpression of *SPS1* in
389 the tocopherol-deficient *vte1* mutant concomitantly enhanced leaf PQ-9 levels and decreased plant
390 photosensitivity, indicating that PQ-9 can replace tocopherols in photoprotection. Tocopherol has
391 been shown to act as a $^1\text{O}_2$ quencher (Fahrenholtz et al., 1974; Di Mascio et al., 1990; Krieger-Liszkay
392 et al., 2008; Choe, 2017) and a terminator of lipid peroxidation (Burton & Ingold, 1986; Ham & Liebler,
393 1995). PQ-9 can also do both. Indeed, Nowicka et al. (2013) showed that reduced or oxidized PQ-9
394 incorporated into liposomes precludes lipid oxidation, and the present work demonstrates that high
395 total PQ-9 levels lowered $^1\text{O}_2$ accumulation levels. Moreover, high light stress leads to a loss of PQ-9
396 and a concomitant formation of PQ-C indicating $^1\text{O}_2$ oxidation of PQ-9. As expected, the accumulation
397 of PQ-C was the highest in *vte1 SPS1oex* leaves which rely the most on total PQ-9 for their resistance
398 to photooxidative stress. PQ-C was found almost exclusively in the thylakoids where ROS are produced
399 in the light, either by excitation energy transfer ($^1\text{O}_2$) or by electron transfers (superoxide anion radical,
400 hydrogen peroxide or hydroxyl radical) (Asada, 2006; Krieger-Liszkay et al., 2008; Li, Wakao, Fischer &
401 Niyogi, 2009; Pospisil, 2014).

402 PQ-9, especially when it is in the reduced state, possesses a number of features that are likely
403 to make this compound an excellent antioxidant. Firstly, it is present in high amounts as a diffusible
404 molecule in thylakoids. As shown in Fig. 1, the concentration of total PQ-9 in Arabidopsis leaves is
405 higher than tocopherol + plastochromanol levels. Leaf enrichment in total PQ-9 relative to tocopherols
406 can be even amplified during long-term acclimation of plants to high light intensities (Ksas et al., 2015;
407 Lichtenthaler, 2007). Second, the PQ-9 side chain bears several double bonds contrary to tocopherols.
408 Double bonds are known to be preferential targets of $^1\text{O}_2$ for oxidation (Triantaphylidès & Havaux,
409 2009), and therefore the unsaturated side-chain of PQ-9 could provide a possibility for PQ-9 to quench

410 $^1\text{O}_2$ by a chemical mechanism. Nevertheless, the antioxidant activity is mainly beard by the phenolic
411 group of reduced PQ-9 (Kim & Min, 2008), so that reduced PQ-9 is a better $^1\text{O}_2$ quencher than oxidized
412 PQ-9. Third, in thylakoid membranes, photosynthetic electron transport relies on the interaction
413 between PQ-9 and the PSII reaction centers which are the main $^1\text{O}_2$ generators during photosynthesis
414 (Krieger-Liszkay et al., 2008). It was initially hypothesized that, in the thylakoid membranes, PQ-9 is
415 located in the fluid bilayer-midplane region allowing rapid lateral movements between cytochrome
416 b6/f and PSII complexes (Millner & Barber, 1984). However, more recent data favor a close
417 compartmentization of PSII, PQ-9 and cytochrome b6/f complex in membrane microdomains
418 (Lavergne & Joliot, 1991; Johnson, Vasilev, Olsen, & Hunter, 2014). Moreover, PQ-9 diffusion
419 towards/from PSII was recently proposed to occur via several entries/exits in the PSII reaction center
420 with an exchange cavity where PQ-9 can diffuse around (Van Eerden, Melo, Frederix, Periole, &
421 Marrink, 2017). These characteristics of the interactions between PSII and PQ-9 are likely to augment
422 the opportunities for PQ-9 to scavenge $^1\text{O}_2$ produced in the reaction centers. Consequently, PQ-9 could
423 constitute a first line of defense against photosynthesis-produced ROS, supplementing the action of
424 the β -carotene molecules bound to the PSII centers.

425 Chemical quenching involves oxidation of the quencher and therefore total PQ-9 is expected
426 to be consumed during its antioxidant activity, as indeed observed in plants exposed to photooxidative
427 stress conditions (Ksas et al., 2015; Fig. 5). In the *vte1* mutant, the total PQ-9 levels are constitutively
428 lower than in WT, and this can be attributed to the chronic oxidation of PQ-9 in the absence of
429 tocopherol. The increased levels of PQ-C in the *vte1* mutants compared to WT corroborate this
430 interpretation. This phenomenon concerns mainly the extra-thylakoid PQ-9 pool, with the total
431 thylakoid-located PQ-9 levels being virtually unchanged relative to WT levels under control conditions.
432 Since ROS are produced in the light in the thylakoids, a dynamic exchange of PQ-9 molecules must exist
433 between the thylakoids and their storage sites in the plastoglobules and the envelopes to replace
434 photooxidized PQ-9 molecules in the thylakoid membranes with new PQ-9 molecules and to maintain
435 a constant pool in the thylakoid membranes. Accordingly, plastoglobules have been shown to be
436 physically coupled to thylakoids in a way allowing bidirectional channeling of lipid metabolites (Austin,
437 Frost, Vidi, Kessler, & Staehelin, 2006, Bréhélin & Kessler, 2008). However, experimental data
438 measuring the nature and the rate of this metabolite exchange are not available. When Arabidopsis
439 plants were suddenly exposed to high light stress, total PQ-9 levels drastically decreased in the
440 thylakoids, as expected when excess light energy leads to high $^1\text{O}_2$ production levels by the
441 photosystems. Moreover, PQ-C was detected predominantly in the thylakoid fractions, indicating that
442 $^1\text{O}_2$ oxidation of PQ-9 occurs at this level. This is in agreement with a previous study by Szymanska and
443 Kruk (2010) who showed a selective decrease in the photochemically active PQ-9 in Arabidopsis leaves
444 exposed to high light stress (Szymanska & Kruk, 2010). However, we also observed a significant

445 reduction of the total PQ-9 concentration, though less pronounced, in the photochemically non-active
446 pool when the photostress was severe and PQ-9 degradation was very pronounced such as in the *vte1*
447 background. This effect of high light stress is consistent with a transfer of PQ-9 molecules from the
448 plastoglobules and/or envelopes to the thylakoids to compensate the oxidative modification of PQ-9
449 during its scavenging activity. In this context, it is worth mentioning that reduced PQ-9 showed a high
450 mobility in lipid membranes compared to α -tocopherol (Jemiola-Rzeminska, Kruk, & Strzalka, 2003).
451 Nevertheless, the rate of this transfer appeared to be slow compared to the rate of PQ-9 oxidation in
452 high light since it could not maintain the pool size of photochemically active PQs to the control levels
453 measured in low light.

454 Because PQ-9 somehow functions as a sacrificial antioxidant consumed during
455 oxidative stress, it must be re-synthesized for the pool being re-filled. Accordingly, most genes of the
456 PQ-9 biosynthesis pathway are strongly upregulated during acclimation of Arabidopsis to high light
457 intensities (Ksas et al., 2015; Block et al., 2013). This induction of PQ-9 biosynthesis genes was
458 accompanied by a concomitant rise in the size of the total PQ-9 pool while there was less effect on the
459 tocopherol content (Ksas et al., 2015; Szymanska & Kruk, 2010). The selective accumulation of PQ-9
460 molecules during growth in high light environments was reported in other species such as beech or fig
461 trees (Lichtenthaler, 2007), consistently with the photoprotective role described for this compound in
462 the present work.

463

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473

474 CONFLICT OF INTEREST

475 The authors claim no conflict of interest

476

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621

622 Figure legends

623 **FIGURE 1** Accumulation of PQ-9 in the Arabidopsis *vte1 SPS1oex* plants. A) α -tocopherol (α -Toc), PC-
624 8 and PQ-9 concentrations in leaves of WT Arabidopsis, the single mutant *vte1*, the *SPS1* overexpressor
625 *SPS1oex* and the double mutant *vte1 SPS1oex* (obtained by crossing *vte1* with the *SPS1oex* line #12
626 previously described in Ksas et al. (2015)). Data are mean values of 4 separate measurements \pm SD. B)
627 Picture of the different genotypes at the age of 4 weeks. C) Images of the efficiency of PSII-mediated
628 electron transport Φ_{PSII} in plants illuminated with red light of PFD 230 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. * and ***,
629 different from WT at $P < 0.01$ and 0.001 (Student's t-test).

630

631 **FIGURE 2** Localization of PQ-9 in the chloroplasts. A) Fractionation of chloroplast membranes by
632 ultracentrifugation on sucrose gradient showing zones corresponding to the plastoglobules (PG), the
633 envelopes and the thylakoids. The scale on the right shows the position of the different fractions
634 collected for the PQ-9 analyses. B) Quantification of PQ-9 in 1-ml fractions from top (fraction 1) to
635 bottom (fraction 32) for WT, *vte1*, *SPS1oex* and *vte1 SPS1oex* leaves. The same amounts of chlorophyll
636 were loaded on the sucrose gradient. C) Estimation of the photochemically active and non-active pools
637 of PQ-9 in Arabidopsis leaves. Data are mean values of 4 separate measurements \pm SD. D) Induction
638 of chlorophyll fluorescence in intact leaves. Insert: pool sizes of PSII 2-electron acceptors estimated
639 from the fluorescence induction curves in the presence and absence of DCMU. ** and ***, different
640 from WT at $P < 0.005$ and $p < 0.001$ (Student's t-test).

641

642 **FIGURE 3** PQ-9 accumulation increases plant tolerance to photooxidative stress. Plants were exposed
643 for 2 d to high light stress ($1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low temperature (7°C). A) Picture of the
644 plants after the high light treatment. B) Autoluminescence imaging of lipid peroxidation after high light
645 treatment. The color palette shows the luminescence signal intensity from low (black) to high values
646 (white). C) HOTE levels in leaves (in nmoles per mg fresh weight (F.W.)) before and after the high light
647 stress. Data are mean values of 3 or 4 separate measurements \pm SD. D) Maximal PSII photochemical
648 efficiency, as measured by the Fv/Fm chlorophyll fluorescence ratio, before and after the high light
649 stress. data are mean values of 8-to-12 separate measurements \pm SD. *, ** and ***, different from WT
650 and $P < 0.01$, 0.005 and 0.001 (Student's t-test).

651

652 **FIGURE 4** $^1\text{O}_2$ scavenging by PQ-9. A) $^1\text{O}_2$ production by leaves in the light as measured by SOSG-EP
653 fluorescence. Attached leaves infiltrated with SOSG were exposed for 20 min to light ($1500 \mu\text{mol}$
654 $\text{photons m}^{-2} \text{s}^{-1}$) at 7°C . Data are mean values of minimum 4 or 5 measurements. B) Total PQ-C levels
655 in leaves before and after high light stress ($1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 2d at 7°C). Data are mean

656 values (in nmoles per mg leaf fresh weight) of 3 separate measurements \pm SD. C-D) $^1\text{O}_2$ production by
657 thylakoids as measured by the spin probe TEMPONE and EPR spectroscopy. No signal was detected in
658 the dark. C) TEMPONE EPR traces and D) quantification of the TEMPONE EPR signal. Data are mean
659 values of 3 separate measurements \pm SD. *, ** and ***, different from WT at $P < 0.01$, $P < 0.005$ and P
660 < 0.001 (Student's t-test).

661

662 **FIGURE 5** Dynamics of the PQ-9 pools. Effects of high light stress (HL = 2.5 h at $1500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) on A) the PQ-9 levels in Arabidopsis leaves and B) on the repartition of PQ-9 between the
663 photochemically active pool (in the thylakoids) and the non-active pool (in the plastoglobules and the
664 envelopes). Data are mean values of mimum 3 separate measurements \pm SD. C) PQ-C concentration
665 in different chloroplast fractions, plastoglobules (PG), envelopes (ENV) and thylakoids separated by
666 ultracentrifugation on sucrose gradient (fractions 1-4, 5-10 and 30-32, respectively, see Fig. 2B). LL
667 corresponds to the growth light conditions ($150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Data are mean values of 3
668 experiments \pm SD. *, ** and ***, different from LL at $P < 0.01$, $P < 0.005$ and $P < 0.001$ (Student's t-
669 test).
670

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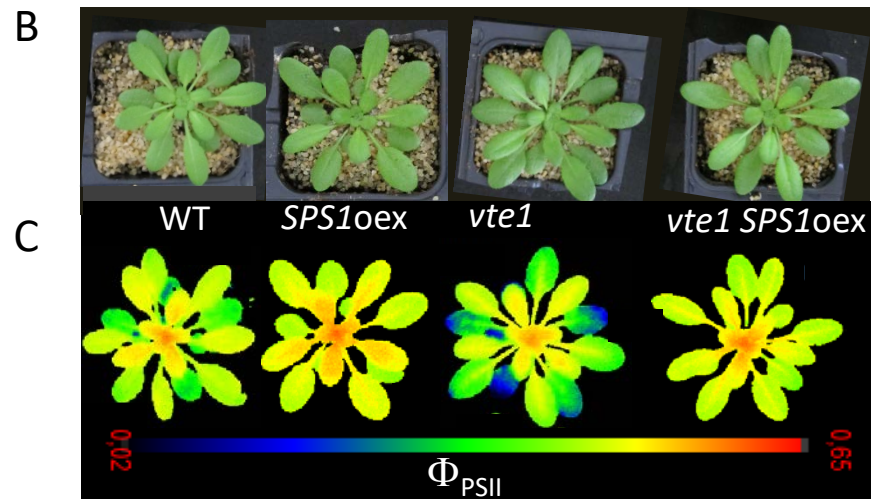
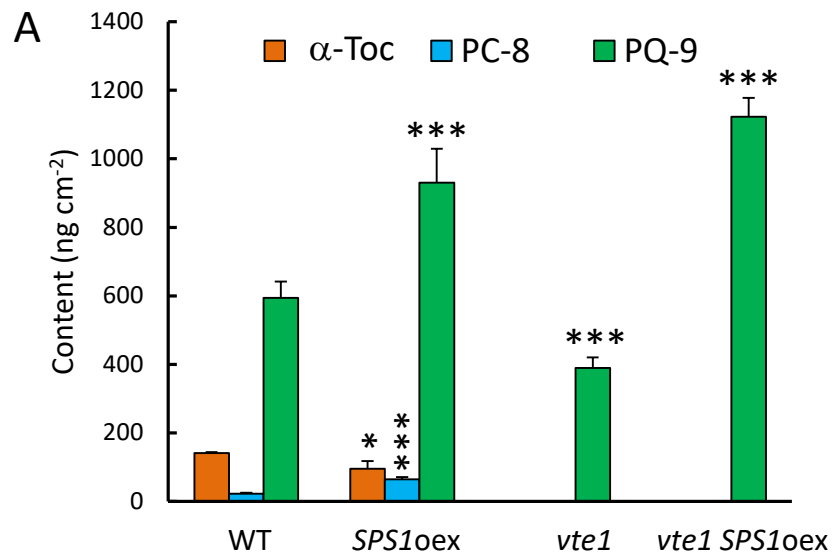


Figure 1

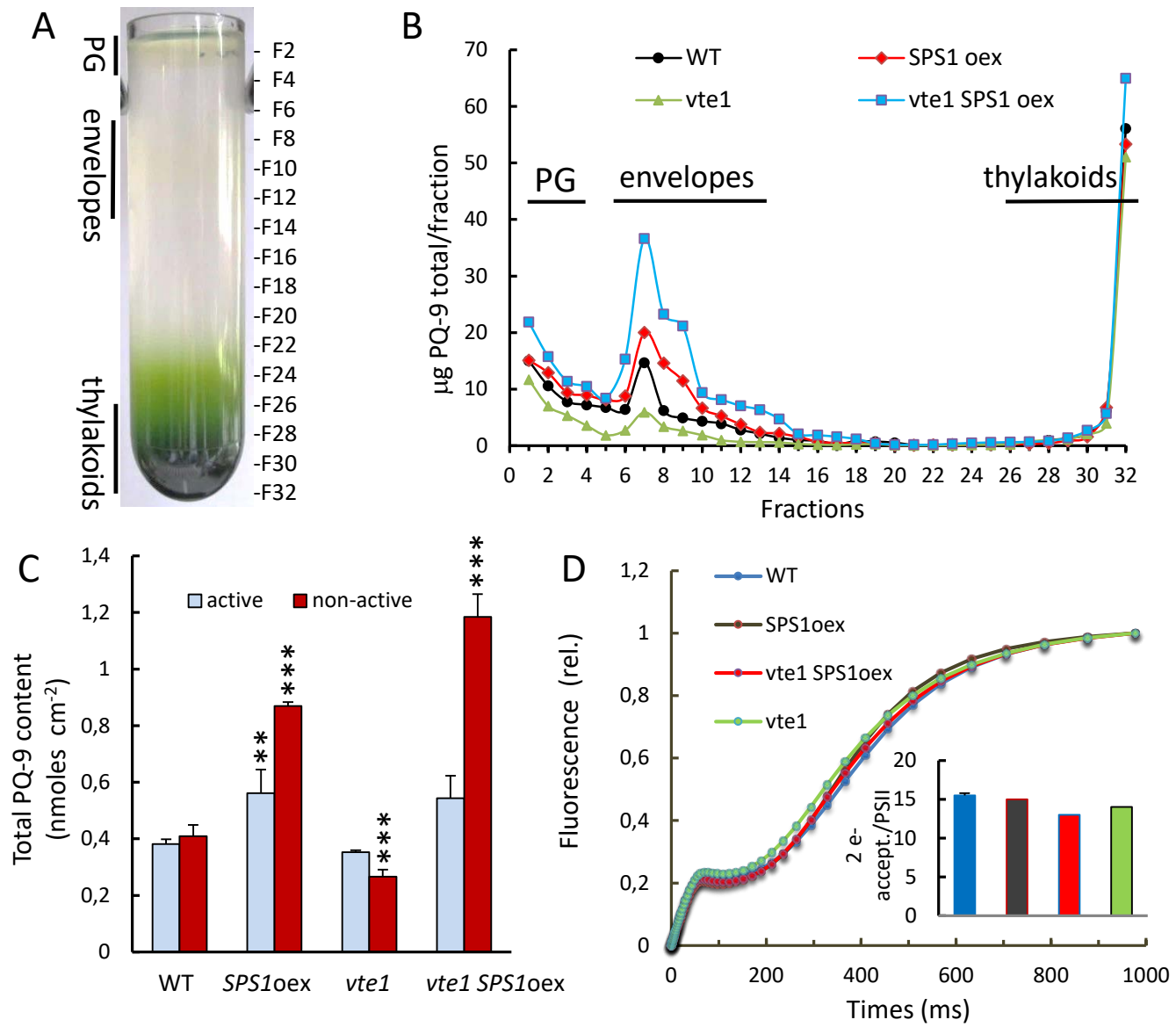


Figure 2

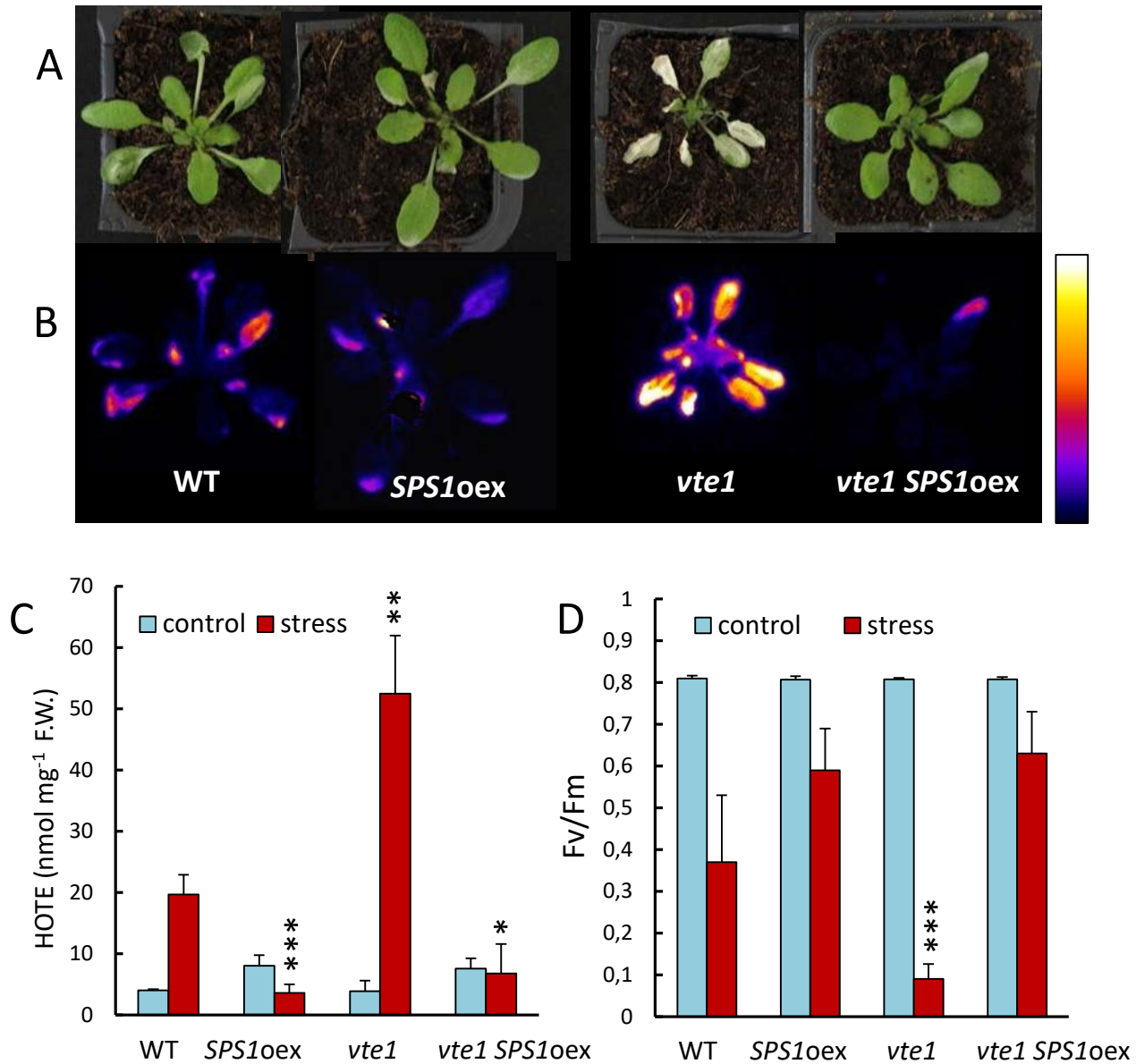


Figure 3

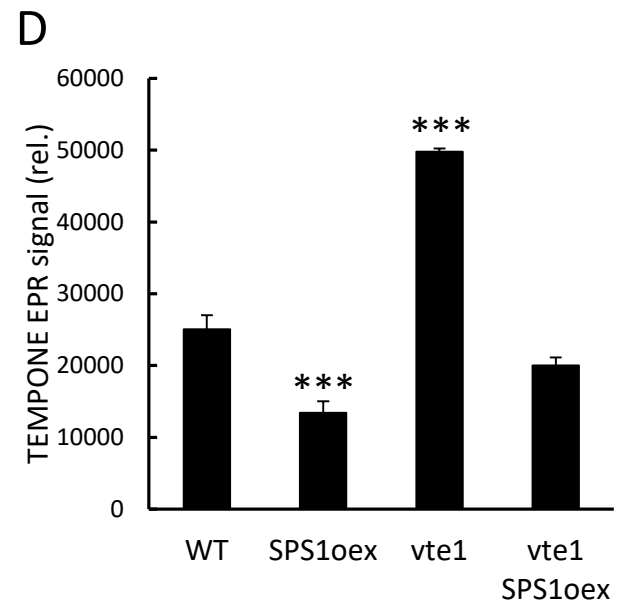
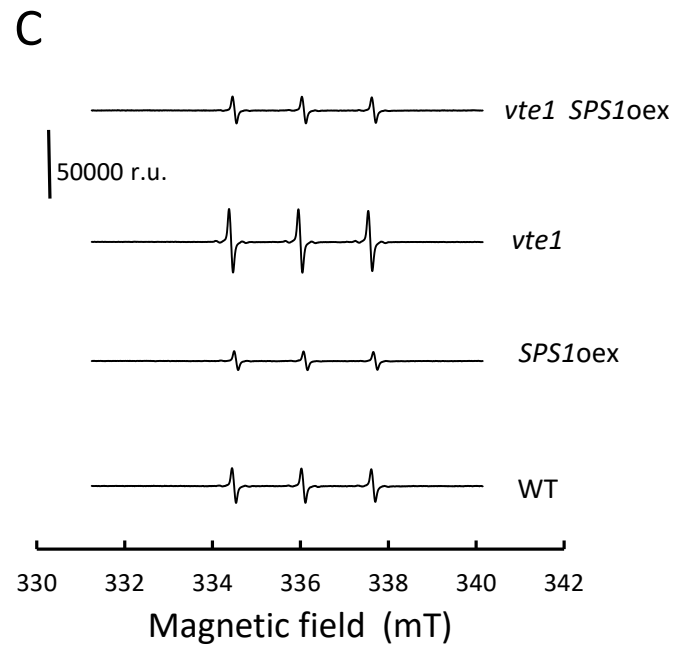
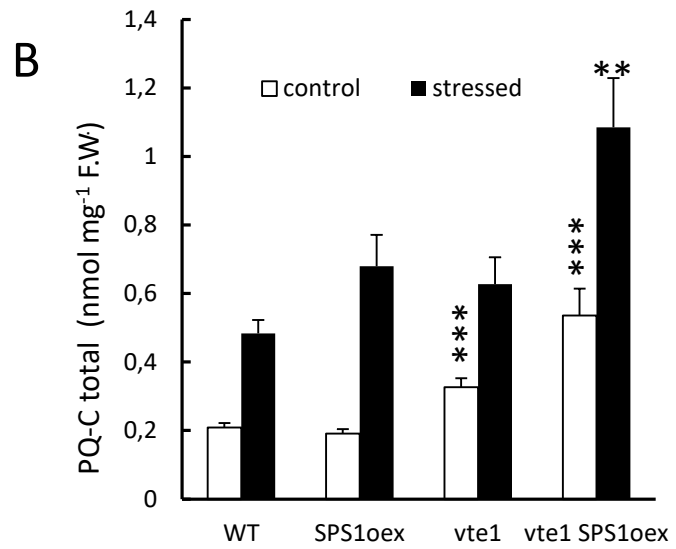
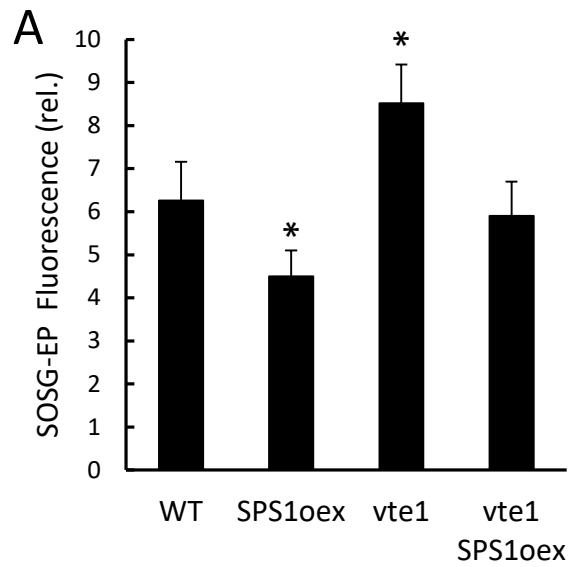


Figure 4

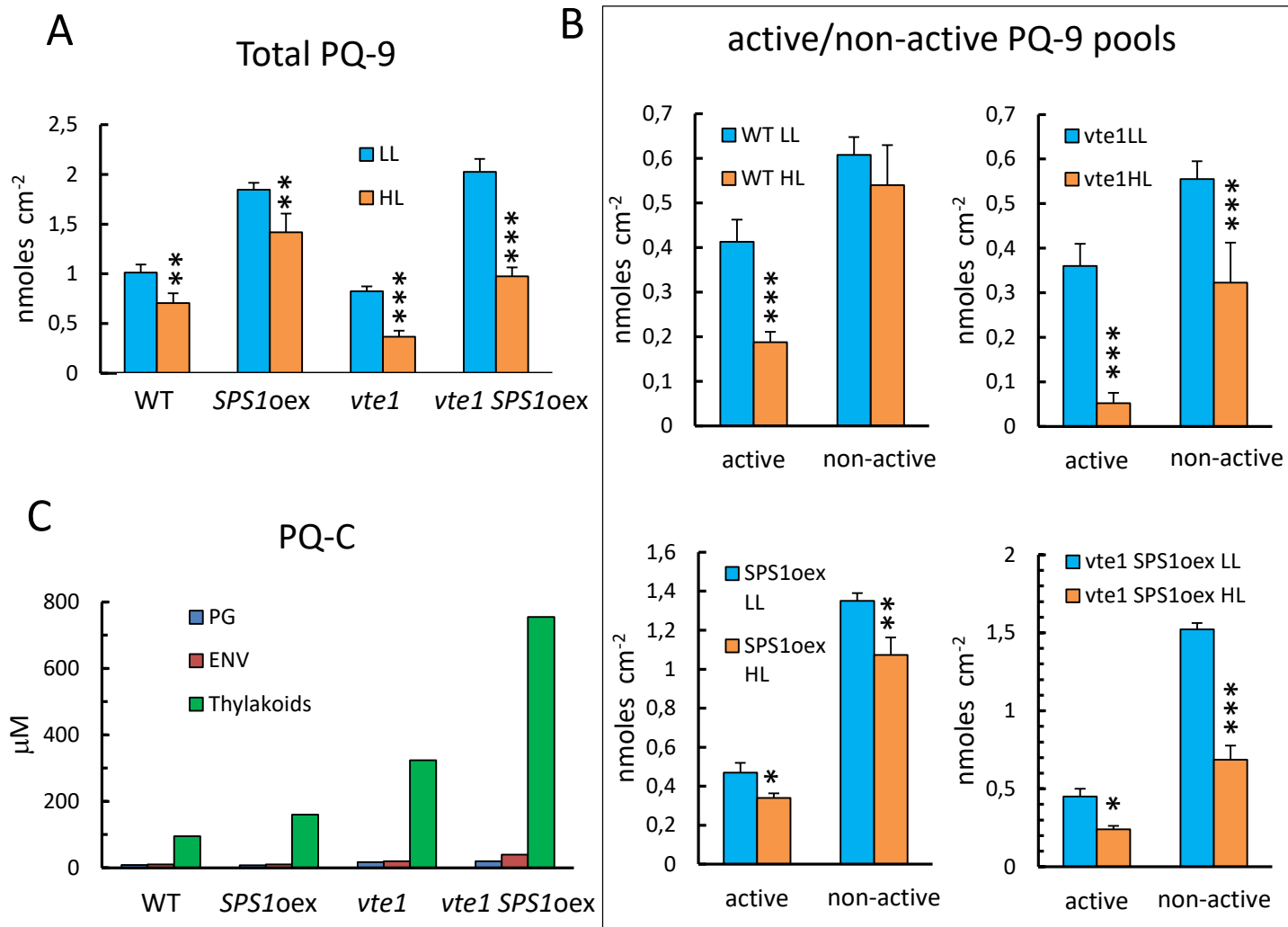


Figure 5