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## Variability in the redox status of plant 2-Cys peroxiredoxins in relation to species and light cycle

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1 **Variability in the redox status of plant 2-Cys peroxiredoxins in relation to species and**  
2 **light cycle**

3  
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30 **Variability in the redox status of plant 2-Cys peroxiredoxins in relation to species and**  
31 **light cycle**

32

33 **Running title**

34 Variability in the redox status of plant 2-Cys peroxiredoxins

35

36

37 **Highlight**

38 Based on the variability in the redox forms of plant 2-Cys peroxiredoxins, we propose that  
39 their functions in redox homeostasis are differentially modulated as a function of species.

40 **Abstract**

41 Plant 2-Cys peroxiredoxins (2-CysPRXs) are abundant plastidial thiol-peroxidases involved  
42 in key signaling processes such as photosynthesis deactivation at night. Their functions rely  
43 on the redox status of their two cysteines and on the enzyme quaternary structure, features for  
44 which the knowledge remains poor in plant cells. Using *ex vivo* and biochemical approaches,  
45 we thoroughly characterized the 2-CysPRX dimer/monomer distribution, hyperoxidation  
46 level, and thiol content in Arabidopsis, barley and potato, in relation to light cycle. Our data  
47 reveal that the enzyme hyperoxidation level and its distribution in dimer and monomer vary  
48 along the light cycle in a species-dependent manner. A differential susceptibility to  
49 hyperoxidation was observed for the two Arabidopsis 2-CysPRX isoforms and among the  
50 proteins of the three species, and was associated to sequence variation in hyperoxidation  
51 resistance motifs. Alkylation experiments indicate that only a minor fraction of the 2-CysPRX  
52 pool carries one free thiol in the three species, and that this content does not change during the  
53 light period. We conclude that most plastidial 2-CysPRX forms are oxidized and propose that  
54 there is a species-dependent variability in their functions since dimer and hyperoxidized forms  
55 fulfill distinct roles regarding direct oxidation of partners and signal transmission.

56

57 **Keywords**

58 Cysteine, peroxiredoxin, plant, plastid, redox status, signaling, thiol content

59

60 **Introduction**

61 Peroxiredoxins (PRXs), first identified in yeast (Kim *et al.*, 1989), are ubiquitous thiol-  
62 peroxidases reducing H<sub>2</sub>O<sub>2</sub> and organic peroxides. The substrate reduction results in oxidation  
63 of the thiol group in a sulfenic acid form, and the activity is generally regenerated via the  
64 oxidation of thioredoxins (TRXs) (Rhee, 2016). PRXs are classified based on the number  
65 (one or two) of redox-active cysteines (Cys) and on the catalytic form (monomer or dimer).  
66 The most represented, 2-CysPRXs, are active as a dimer and harbor two conserved Cys  
67 termed CysP and CysR for peroxidatic and resolving, respectively (Rhee, 2016). The head-to-  
68 tail dimers are formed thanks to one or two covalent bounds between the two Cys. Pro-  
69 oxidative conditions lead to peroxidase activity inactivation due to CysP hyperoxidation to a  
70 sulfinic acid form, which can be reversed by sulfiredoxin (SRX) (Biteau *et al.*, 2003; Jönsson  
71 *et al.*, 2008). In yeast exposed to severe oxidative or heat stresses, 2-CysPRX hyperoxidation  
72 results in formation of high molecular weight (HMW) multimers and functional switch from  
73 peroxidase to chaperone activity (Jang *et al.*, 2004). Signaling roles have been also unveiled  
74 for yeast and animal 2-CysPRXs via the control of peroxide concentration or via direct and  
75 sensitive thiol oxidation in protein partners (Day *et al.*, 2012; Rhee and Woo, 2011; Stöcker *at*  
76 *al.*, 2017).

77 In plants, four PRX types are present (Dietz, 2011), three being plastidial: 2-CysPRXs,  
78 PRXII-E and PRXQ (Baier and Dietz, 1997; Lamkemeyer *et al.*, 2006; Gama *et al.*, 2008).  
79 Typical 2-CysPRXs, first characterized in barley (Baier and Dietz, 1996), are the most  
80 abundant since they represent *ca* 1% of plastidial proteins (Dietz *et al.*, 2006). Their  
81 hyperoxidation is reversed by SRX (Liu *et al.*, 2006; Rey *et al.*, 2007) and several TRX types  
82 reduce them *in vitro*: i) NTRC, NADPH-dependent TRX Reductase C, that contains one TRX  
83 domain and one NADPH-dependent TRX reductase domain (Moon *et al.*, 2006; Perez-Ruiz *et*  
84 *al.*, 2006; Perez-Ruiz and Cejudo, 2009); ii) a TRX-like protein termed CDSP32  
85 (Chloroplastic Drought-induced Stress Protein of 32 kDa) (Rey *et al.*, 1998; Broin *et al.*,  
86 2002; Rey *et al.*, 2005; Cerveau *et al.*, 2016a) ; iii) a typical TRX termed TRX x (Collin *et*  
87 *al.*, 2003). In the last years, several functions have been uncovered for plant 2-CysPRXs.  
88 Pulido *et al.*, (2010) reported that an Arabidopsis mutant with less than 5% protein displays  
89 reduced growth and altered redox homeostasis. Based on the high light sensitivity of a fully  
90 knocked-out line, these enzymes were proposed to take part in an alternative water-water  
91 cycle to protect photosynthetic membranes (Awad *et al.*, 2015). Dangoor *et al.*, (2012)  
92 reported that oxidization by 2-CysPRX of the Atypical Cysteine Histidine rich TRX, ACHT1,  
93 is associated with altered production of peroxides upon moderate light intensity. They

94 concluded that ACHT1 could sense and transmit a light-related signal regulating  
95 photosynthetic activity. Using a genetic approach, Perez-Ruiz *et al.*, (2017) proposed that the  
96 2-CysPRX redox balance controls photosynthetic metabolism. Consistently, Yoshida *et al.*,  
97 (2018), Ojeda *et al.*, (2018), and Vaseghi *et al.*, (2018) showed that the oxidation dynamics of  
98 several photosynthetic enzymes was delayed in the Arabidopsis mutant lacking 2-CysPRXs  
99 during the light-dark transition. Further, Yoshida *et al.*, (2018) showed that these  
100 photosynthetic enzymes are oxidized by the atypical TRX-like2, which can reduce 2-CysPRX  
101 *in vitro*, and suggested that the TRX-like2/2-CysPRX redox cascade supports photosynthesis  
102 deactivation at night.

103 The multiple 2-CysPRX functions upon environmental constraints, ageing or diseases  
104 (Dietz, 2011; Rhee and Woo, 2011; Rhee, 2016; Liebthal *et al.*, 2018) rely on the redox status  
105 of the two Cys residues that greatly condition the protein structural and biochemical features.  
106 Of note, human 2-CysPRXs exhibit differential properties with regard to hyperoxidation,  
107 likely conferring them distinct functions (Haynes *et al.*, 2013; Bolduc *et al.* 2018). Few data  
108 are currently available about the redox status of plant 2-CysPRXs and its possible variability.  
109 The two recombinant Arabidopsis isoforms share a similar behavior *in vitro* (Kirchsteiger *et al.*,  
110 2009; Puerto-Galan *et al.*, 2015). The dimer/monomer distribution likely varies depending  
111 on species since low and high monomer abundances were noticed in potato and Arabidopsis,  
112 respectively (Broin and Rey, 2003; Baier and Dietz 1999). Here, we performed a thorough  
113 analysis of plant 2-CysPRX redox forms in three representative model and cultivated species  
114 that have been the subject of most studies on these thiol-peroxidases, two dicotyledons  
115 (Arabidopsis, and potato) and one monocotyledon (barley). Our data reveal that only a minor  
116 fraction of the 2-CysPRX pool carries one free thiol and that the dimer/monomer distribution  
117 and the hyperoxidation level substantially vary as a function of species and light cycle.

## 118 **Materials and methods**

### 119 **Plant materials and growth conditions**

120 Arabidopsis plants were grown from sowing in soil in standard conditions under an 8-  
121 h photoperiod and a photon flux density of 200  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 22/18°C (day/night) and  
122 55% relative humidity. Plants were alternatively watered with tap water and nutritive solution  
123 (Coic and Lesaint, 1971) every two days. The genotypes used were wild-type Col-0, T-DNA  
124 homozygous mutants for *SRX* (SALK\_015324) (Rey *et al.*, 2007), *NTRC* (SALK\_096776,  
125 SALK\_012208) (Serrato *et al.*, 2004; Lepisto *et al.*, 2009), *2-CysPRXA* (GK\_295C05) and *2-*  
126 *CysPRXB* (SALK\_017213). These two last lines were crossed to generate one double mutant

127 deficient in both 2-CysPRX genes (Cerveau *et al.*, 2016b). Potato (*Solanum tuberosum* cv.  
128 Désirée) plantlets were propagated *in vitro* for 3 weeks and transferred in soil in a phytotron  
129 under a 12-h photoperiod, 250  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and 24/19°C (day/night). WT and five  
130 lines modified for *CDSP32* expression either co-suppressed or over-expressing WT or active-  
131 site mutated forms were used (Broin *et al.*, 2002; Rey *et al.*, 2005). *Hordeum vulgare* L.  
132 plants (cv. Express) were cultivated for 3 weeks as described in Marok *et al.*, (2013). All  
133 plants were cultivated in the “Phytotec” platform (CEA, DRF, BIAM).

134

### 135 **Protein preparation**

136 Leaves were ground in liquid nitrogen and the powder suspended in 50 mM Tris-HCl  
137 pH 8, 1 mM phenylmethylsulfonyl fluoride, PMSF, and 50 mM  $\beta$ -mercaptoethanol to prepare  
138 soluble proteins (Rey *et al.*, 2005). In non-reducing conditions, the powder was suspended in  
139 50 mM Tris-HCl pH 8 and 1 mM PMSF. Poly(vinylpyrrolidone), PVPP, was added in the  
140 extraction buffer (5%) when specified. Following vigorous shaking at 4°C for 20 min and  
141 centrifugation (20 min, 15,000 rpm, 4°C), the supernatant was precipitated using two volumes  
142 of acetone at -20°C and, when non-reduced, used rapidly for subsequent analyses. Protein  
143 concentration was quantified using the “Protein Quantification BCA Assay” kit (Interchim).

144

### 145 **Alkylation experiments**

146 Leaf soluble proteins were prepared in PBS pH 7.2, 1 mM PMSF in the absence of  
147 reductant and alkylated using mPEG-maleimide-2000 (Laysan Bio Arab, AL, USA) as  
148 reported in Rey *et al.*, (2017). Control experiments were performed on proteins extracted in  
149 the presence of 50 mM  $\beta$ -mercaptoethanol or 50 mM Tris(2-carboxyethyl)phosphine  
150 hydrochloride, TCEP). Proteins were precipitated using two acetone volumes at -20°C for 1 h  
151 and immediately used for labelling to approach the Cys redox status in a reliable manner.  
152 Following centrifugation, an aliquot of 200  $\mu\text{g}$  protein was suspended in PBS pH 7.2, 1%  
153 SDS and 1.7  $\text{mg}\cdot\text{mL}^{-1}$  mPEG-maleimide-2000 and incubated at room temperature for 3 h.  
154 Then, the reaction mixture was added with loading buffer devoid or not of reductant for SDS-  
155 PAGE and Western blot analyses.

156

### 157 **Electrophoresis and immunoblot analysis**

158 Proteins were separated using SDS-PAGE in reducing or non-reducing conditions and  
159 electroblotted onto 0.45  $\mu\text{m}$  nitrocellulose (Pall Corporation) to perform immunoblot analysis.  
160 Antibodies against hyperoxidized 2-CysPRX forms (Rabbit anti-peroxiredoxin-SO<sub>3</sub>, reference

161 LF-PA0004) were purchased from AbFrontier (Seoul, Korea) and used at a dilution of  
162 1:3,000. The At2-CysPRX antiserum was raised against the recombinant protein (Broin *et al.*,  
163 2002) and used diluted 1:10,000. Bound antibodies were detected using either a goat anti-  
164 rabbit secondary antibody coupled to a fluorescent molecule at a dilution of 1:10,000 (Alexa  
165 Fluor 680, Invitrogen) using the ‘Odyssey Infrared Imager’ at 680 nm (Licor, Lincoln, NE,  
166 USA) or an anti-rabbit immunoglobulin G coupled to alkaline phosphatase (Sigma) for  
167 chromogenic detection. For assessing the level of hyperoxidation, membranes were probed  
168 first with the serum raised against hyperoxidized 2-CysPRX for fluorescent detection, and  
169 subsequently probed with the serum raised against At2-CysPRX for chromogenic detection.  
170 Quantification of band intensity was performed using the software associated with the imager.

171

### 172 **Protein sequence and structure analysis**

173 Sequence alignments were performed using the softwares “LALIGN” and “ClustalW” at the  
174 ExPASy resource portal. Protein structure predictions were done using the Phyre2 web portal  
175 (Kelley *et al.*, 2015). The best hit from the Phyre2 search carried out on the sequence of  
176 mature At2-CysPRXB and the mature Hv2-CysPRX was for both sequences the 3D structure  
177 of At2-CysPRXA C119S (PDB code 5ZTE) (Yang *et al.*, 2018) (96% alignment coverage (7-  
178 200aa), 100% confidence, 96% identity and 96% alignment coverage (8-201aa), 100%  
179 confidence, 93% identity, respectively). Structural comparisons and three-dimensional  
180 structure images were generated using PyMOL (PyMOL Molecular Graphics System,  
181 Version 2.0 Schrödinger, LLC).

182

## 183 **Results**

### 184 **Variability of dimer/monomer distribution in plant 2-CysPRXs**

185 Relatively few data are currently available regarding the plant 2-CysPRX dimer/monomer  
186 distribution that is revealed in non-reducing SDS-PAGE. We first analyzed by Western  
187 analysis the protein amount in Arabidopsis mutants deficient in one 2-CysPRX isoform (A or  
188 B), SRX or NTRC following extraction and migration in reducing conditions (Fig. 1A). The  
189 peroxidase was found almost exclusively as a 22-kDa monomer, the dimer band being faintly  
190 detected due to incomplete reduction. Compared to WT, the enzyme amount was not  
191 modified in *srx* plants as previously observed (Rey *et al.*, 2007), reduced by 25 to 30% in  
192 lines lacking 2-CysPRXB or NTRC and by *ca* 75% in the mutant lacking 2-CysPRXA. When  
193 extracted and migrated in the absence of reductant (Fig. 1B), 2-CysPRX appears as a  
194 monomer at 22 kDa and as a dimer at *ca* 45 kDa in WT extracts as previously reported

195 (Cerveau *et al.*, 2016b). Quantification of signal intensity revealed that in WT the monomer  
196 represents more than 50% of the total protein amount. A ratio in the same range was observed  
197 in lines lacking one PRX isoform or SRX. As observed by Pulido *et al.*, (2010) and Puerto-  
198 Galan *et al.*, (2015), this ratio was lower than 20% in *ntrc* plants due to a substantially lower  
199 monomer amount, clearly showing the decisive role of the electron donor in the maintenance  
200 of 2-CysPRX redox status.

201 Some data indicate that the dimer/monomer distribution of plant 2-CysPRXs could  
202 vary as a function of species. Indeed, a much lower monomer amount was observed in potato  
203 (Broin and Rey, 2003) compared to Arabidopsis (Baier and Dietz 1999; Cerveau *et al.*,  
204 2016b). As this discrepancy might come from different preparation procedures, we compared  
205 the dimer/monomer distribution in extracts simultaneously and similarly prepared from  
206 Arabidopsis, potato and barley leaves. Of note, a lower 2-CysPRX total amount, was found  
207 in both cultivated species compared to Arabidopsis (Fig. 1C). In non-reducing conditions, the  
208 monomer proportion was much lower in barley (less than 20%) than in Arabidopsis and  
209 comparable to that in *ntrc* (Fig. 1D). Strikingly, this proportion was even lower in potato (less  
210 than 10%), the 22-kDa band being sometimes barely detected (Fig. 1D, data not shown)  
211 consistently with previous findings (Broin and Rey, 2003). When adding 5%  
212 polyvinylpyrrolidone in the extraction buffer to adsorb phenolic compounds, that are  
213 abundant in Solanaceae and promote oxidation of cell compounds, similar results were  
214 obtained (data not shown). These data highlight a strong variability in the 2-CysPRX  
215 dimer/monomer distribution among plant species.

216

### 217 **Plant 2-CysPRX thiol content**

218 The presence of free thiols in 2-CysPRX *in planta* is a parameter very likely underlying its  
219 peroxidase and/or signaling activities, which has not been thoroughly investigated so far. We  
220 analyzed the Cys redox status by carrying out alkylation experiments using mPEG-  
221 maleimide-2000, which forms stable thioether bonds with thiol groups, allowing the detection  
222 of reduced Cys due to a 2-kDa size increase per free thiol. Following incubation with this  
223 compound in the absence of reductant and Western analysis, 2-CysPRX bands displaying  
224 slower mobility were specifically revealed in WT compared to *2-cysprxa 2-cysprxb* (Fig. 2A).  
225 One additional monomer band at *ca* 25 kDa and another faint one at *ca* 23.5 were observed.  
226 Regarding dimer, one supplementary band at *ca* 48 kDa and another higher faint band were  
227 detected. We then performed the same experiments using protein extracts from mutants  
228 deficient in one 2-CysPRX, SRX or NTRC (Fig. 2A). We did not observe any qualitative



229 difference in the alkylation patterns compared to WT, except in *ntrc* where no higher  
230 monomer band was detected due to the very low abundance of the 22-kDa form in this  
231 genotype (Fig. 2A).

232 The patterns in non-reducing conditions did not allow accurately determining the  
233 number of free thiols in 2-CysPRX monomers or dimers, notably due to the presence of  
234 several bands in non-alkylated extracts. To better approach this parameter, alkylated WT  
235 samples prepared in non-reducing conditions were subjected to SDS-PAGE in the presence of  
236 reductant (Fig. 2B). No dimer was detected as expected and one supplementary band at *ca* 25  
237 kDa was observed compared to non-alkylated samples. The intensity of this band was low  
238 compared to that revealed in reduced non-alkylated extracts (Fig. 2B, lanes 1 and 3). As a  
239 control, we analyzed the number of free thiols in 2-CysPRX using protein extracts prepared  
240 directly in reducing conditions in the presence of  $\beta$ -mercaptoethanol. The revelation pattern  
241 was very similar to that obtained using oxidized proteins, but with a higher intensity of the  
242 supplementary 25-kDa band (Fig. 2B, lanes 1-2). This is consistent with the fact that dimer  
243 reduction leads to the appearance of free thiols available for alkylation. Nonetheless, the  
244 intensity of this band remained lower than that of the 22-kDa isoforms. When using extracts  
245 from *ntrc* or *srx* plants (Fig. 2B, lanes 4-9), very similar data were obtained, indicating that  
246 the absence of one of these two 2-CysPRX partners does not substantially alter the protein  
247 thiol content.

248 Two Cys residues are present in the sequence of mature 2-CysPRX. The appearance of  
249 only one supplementary alkylated band, even in extracts reduced using  $\beta$ -mercaptoethanol,  
250 raised questions and prompted us to use another reductant. TCEP, tris(2-  
251 carboxyethyl)phosphine) is a powerful non-thiol reductant, compared to DTT and  $\beta$ -  
252 mercaptoethanol, that proved more appropriate for Cys labeling with maleimides, since it is  
253 less deleterious to conjugation with these compounds (Getz *et al.*, 1999). Reduction by TCEP  
254 led to a very distinct pattern compared to that observed with  $\beta$ -mercaptoethanol. Indeed, a  
255 very intense 28-kDa band was revealed, while the previously detected 25-kDa band was still  
256 present and the bands at *ca* 22 kDa almost absent (Fig. 2C, lane 3). The appearance of two  
257 major bands shifted by *ca* 2.5 and 5 kDa, is consistent with the presence of two free thiols in  
258 reduced 2-CysPRX that can be alkylated only in the presence of TCEP. These data lead us to  
259 conclude that since only the 25-kDa alkylated band is revealed in proteins prepared in non-  
260 reducing conditions, 2-CysPRX from leaf extracts probably harbors only one reduced Cys  
261 residue. Further, the lower intensity of this band compared to those at 22 kDa suggests that a  
262 limited protein fraction carries one free thiol. Alkylation experiments were finally carried out

263 on leaf extracts from barley and potato. In non-reduced extracts, a lower intensity of the  
264 additional 25-kDa band was noticed in both species compared to Arabidopsis (Fig. 2D, lanes  
265 1, 4 and 7). Following alkylation of TCEP-reduced extracts, two major bands at *ca* 25 and 28  
266 kDa were revealed. Note that the abundance of the 25-kDa band appeared much higher in  
267 barley and potato than in Arabidopsis (Fig. 2D, lanes 2, 5 and 8). Altogether, these data  
268 suggest that in leaf extracts only a limited 2-CysPRX pool harbors one reduced Cys.

269

### 270 **Redox status of 2-CysPRX as a function of light cycle in Arabidopsis.**

271 2-CysPRXs fulfill a key role in the regulation of photosynthesis upon the light-dark transition  
272 (Yoshida *et al.*, 2018). In other respects, 2-CysPRX hyperoxidation follows a circadian  
273 rhythm (O'Neill *et al.*, 2011; Cerveau *et al.*, 2016b). Therefore, we investigated whether the  
274 dimer/monomer distribution and the proportion of protein carrying one free thiol varies as a  
275 function of light cycle. Arabidopsis WT leaves were collected at five time points (Fig. S1):  
276 before and after the dark-light transition (D1 and L1, respectively), middle of the light period  
277 (L2), before and after the light-dark transition (L3 and D2, respectively). No change was  
278 observed in the total 2-CysPRX abundance while a substantially higher amount of the  
279 hyperoxidized form was detected following the dark-light transition and at the middle of the  
280 light period (Fig. 3A). In the absence of reductant, the amounts of both dimer and monomer  
281 forms in WT noticeably decreased along the light period, the lowest ones being observed after  
282 the light-dark transition (Fig. 3B). Quantification of band intensities indicated that the 2-  
283 CysPRX amount detected as both monomer and dimer forms was decreased by more than  
284 25% at the D2 time point compared to L1 (Fig. 3C). Similar data were obtained when  
285 analyzing *srx* proteins (Fig. 3D). In *ntrc*, where the monomer amount is very low, the dimer  
286 abundance decreased along the light phase and the monomer amount slightly increased  
287 following the dark-light transition and then decreased (Fig. 3D). Finally, we performed  
288 alkylation experiments on WT leaf extracts collected at the five time points and prepared  
289 without reductant. No noticeable variation in the abundance of the supplementary 25-kDa  
290 band was observed as a function of light phase (Fig. 3E). In *ntrc* alkylated extracts, a slight  
291 increase in the abundance of the 25-kDa band was noticed before the light-dark transition  
292 (Fig. S2). Altogether, these data reveal that light cycle in addition to regulating  
293 hyperoxidation, strongly influences the 2-CysPRX amount detected in the range from 20 to 50  
294 kDa in non-reducing conditions, while it does not provoke any substantial change in the total  
295 protein amount and thiol content.

296

297 **Redox status of 2-CysPRX as a function of light cycle in barley and potato.**

298 We then investigated the effect of light cycle on 2-CysPRX redox status in barley and potato.  
299 In the presence of reductant, no change was observed regarding the protein abundance in both  
300 species (Fig. 4A-B). In barley, similarly to what observed in Arabidopsis, we noticed a strong  
301 increase in the amount of the hyperoxidized form following the dark-light transition and a  
302 noticeable decrease at the beginning of the dark phase (Figs. 3A, 4A). A distinct pattern was  
303 noticed in potato, since the highest amounts of hyperoxidized 2-CysPRX were observed  
304 before and following the light phase (Fig. 4B). In non-reducing conditions, a low amount of  
305 2-CysPRX monomer was detected in both species as shown above (Figs. 1D, 4C-D). In  
306 barley, the dimer abundance slightly decreased during the light period, the lowest amount  
307 being observed at the beginning of the dark phase, while a substantially higher monomer  
308 abundance was observed at the middle of the light period (Fig. 4C). In potato, a gradual  
309 decrease in the dimer amount was observed along the light period, the lowest amount being  
310 noticed at the beginning of the dark phase. Of note, a noticeably higher monomer abundance  
311 was revealed at the end of the light period (Fig. 4D). Alkylation experiments revealed no  
312 change in the abundance of the supplementary 25-kDa band in both species as a function of  
313 light cycle (Fig. 4E-F).

314

315 **Redox status of 2-CysPRX in potato lines modified for *CDSP32* expression**

316 We previously generated potato lines modified for the expression of the CDSP32 TRX that  
317 interacts with 2-CysPRX and reduces it (Broin *et al.*, 2002; Broin and Rey, 2003). Here, we  
318 analyzed the redox status of 2-CysPRX in lines co-suppressed (CS), or over-expressing WT  
319 (OE) or active-site mutated (OE-M) CDSP32. In all lines, a similar peroxidase abundance was  
320 found (Fig. 5A). We noticed that the CDSP32-deficient line exhibits the lowest level of  
321 hyperoxidized PRX (Fig. 5A) as already observed (Cerveau *et al.*, 2016b). In non-reducing  
322 conditions, plants co-suppressed for *CDSP32* displayed a higher monomer amount than WT  
323 (Fig. 5B) as reported in Broin and Rey (2003). Consistently, the lowest monomer level was  
324 observed in the line over-expressing *CDSP32*. Of note, both lines expressing a CDSP32 form  
325 lacking CysP displayed a monomer abundance noticeably higher than that in plants  
326 overexpressing the WT TRX. Finally, we examined the 2-CysPRX thiol content by  
327 performing alkylation experiments. As observed in Figs. 2D and 4F, a very low abundance of  
328 the 25-kDa additional band was detected in WT (Fig. 5C). However, a higher band intensity  
329 was noticed in all lines modified for *CDSP32* expression, particularly CS and OE-M,

330 indicating that the absence of functional CDSP32 is associated with an increased thiol content  
331 in 2-CysPRX.

332

### 333 **Differential behavior of plant 2-CysPRXs towards hyperoxidation**

334 Finally, we investigated whether plant 2-CysPRXs exhibit variability regarding  
335 hyperoxidation as observed for human 2-CysPRXs. We first compared the amount of 2-  
336 CysPRXs A and B in young, adult and old leaves of Arabidopsis WT and mutants deficient in  
337 one of the two isoforms (Fig. 6A). 2-CysPRXs A and B were found to account for *ca* 75%  
338 and 25 % of the WT level whatever the developmental stage (Fig. 6B) in agreement with  
339 Kirchsteiger *et al.*, (2009). No variation in protein abundance was observed as a function of  
340 leaf age in the three genotypes. We previously reported that 2-CysPRX hyperoxidation level  
341 decreases with leaf age in WT (Cerveau *et al.*, 2016b). The age-dependent hyperoxidation  
342 pattern in plants expressing only 2-CysPRXA was very similar to that observed in WT. In  
343 contrast, only a faint band corresponding to hyperoxidized 2-CysPRXB was detected in young  
344 leaves of 2-cysprxa plants, and this form was not revealed in old leaves (Fig. 6A).  
345 Quantification revealed that hyperoxidized 2-CysPRXB in young, adult and old leaves  
346 represents 10, 3 and 0%, respectively, of that in WT, whereas this isoform represents 25 to  
347 30% of the total protein amount (Fig. 6B). Consistently, the level of hyperoxidized 2-  
348 CysPRXA reaches values in the range of 100% of those in WT at all developmental stages.  
349 These data indicate that At2-CysPRXB is substantially less prone to hyperoxidation than At2-  
350 CysPRXA. We then compared the abundance of the hyperoxidized form in Arabidopsis,  
351 barley and potato. In reducing conditions, a similar 2-CysPRX amount, lower than that in  
352 Arabidopsis, was noticed in both cultivated species (Figs. 6C, 1C). Strikingly, a much higher  
353 abundance of the hyperoxidized form was observed in barley compared to Arabidopsis and  
354 potato, revealing a higher sensitivity in this species (Fig. 6C).

355 We thus searched for the sequence determinants possibly underlying the differential behavior  
356 of At2-CysPRXB and Hv2-CysPRX within or in the proximity of the motifs A and B that are  
357 involved in hyperoxidation resistance (Bolduc *et al.*, 2018). When aligning the four sequences  
358 (Fig. 7A), we observed very few differences, but noticed that an Ile residue instead of Val just  
359 precedes the motif B in At2-CysPRXB. Regarding the sequence of Hv2-CysPRX, two  
360 residues in the motif A differ compared to the other PRXs (Ile-136 and Lys-164 instead of  
361 Val and Asn, respectively). These two residues, which exhibit different steric hindrance or  
362 charge, are located in close vicinity of the CysP residue, the GGLG motif and the dimer-  
363 dimer interface (Bolduc *et al.*, 2018). Within the 3D Phyre2 model of Hv2-CysPRX, Ile 136

364 and Lys-164 are also close to each other (distance between 7.5 to 10 Å) (Figs. 7B-C).  
365 Furthermore, structural comparison between the 3D structure of At2-CysPRXA C119S (PDB  
366 code 5ZTE) (Yang *et al.*, 2018) and the 3D model of Hv2-CysPRX shows that the two residue  
367 differences within motif A induce electrostatic surface potential changes very close to the  
368 GGLG motif.

369

## 370 **Discussion**

371 Plant 2-CysPRXs display a high abundance and limited variations in gene expression in  
372 relation to developmental factors and environmental constraints (Broin and Rey, 2003; Dietz  
373 *et al.*, 2006; Cerveau *et al.*, 2016b). Comparatively, more important variations were observed  
374 regarding hyperoxidation level or quaternary structure (Broin and Rey, 2003; König *et al.*,  
375 2003; Cerveau *et al.*, 2016b). The present report provides new information particularly  
376 regarding protein hyperoxidation, thiol content and dimer/monomer distribution in relation  
377 with light cycle, but also species type.

378

### 379 **Plant 2-CysPRX hyperoxidation**

380 In eukaryotic organisms, 2-CysPRX hyperoxidation follows a circadian rhythm while no  
381 change occurs in transcription rate and protein amount (O'Neil *et al.*, 2011; Edgar *et al.*,  
382 2012). In Arabidopsis, our previous data (Cerveau *et al.*, 2016b) and this work (Fig. 3A)  
383 reveal higher levels of hyperoxidation following the dark-light transition. A similar pattern  
384 was observed in barley, but in potato the highest level was noticed after the light-dark  
385 transition (Fig. 4A-B). Arabidopsis and barley were cultivated under short photoperiod and  
386 potato under longer day length, raising the question of the influence of this parameter. When  
387 examining 2-CysPRX hyperoxidation in Arabidopsis plants grown under a 16-h photoperiod,  
388 Puerto-Galan *et al.*, (2015) observed a higher level following the dark-light transition. Lastly,  
389 Edgar *et al.*, (2012) showed persistent oscillations of hyperoxidation in Arabidopsis seedlings  
390 grown under a 12-h photoperiod and then exposed to continuous light, the highest levels  
391 occurring at Zeitgeber time points corresponding to the beginning of the light phase. These  
392 data indicate that in Arabidopsis 2-CysPRX hyperoxidation peaks following the dark-light  
393 transition whatever the photoperiod length. However, as shown in potato, such a pattern  
394 cannot be extended to all plant species.

395 Eukaryote 2-CysPRXs exhibit two motifs (GGLG and YF) considered as a signature of  
396 hyperoxidation sensitivity (Wood *et al.*, 2003). However, human 2-CysPRXs 1, 2 and 3  
397 exhibit differential susceptibility to hyperoxidation (Cox *et al.*, 2009). The higher resistance

398 of human PRX3 is linked to the presence of the two motifs A and B also found in bacterial 2-  
399 Cys PRXs (Bolduc *et al.*, 2018). Interestingly, plant 2-CysPRXs display both motifs A and B  
400 (Fig. 7), but still exhibit differential susceptibility to hyperoxidation, as clearly shown for  
401 At2-CysPRXB and Hv2-CysPRX, which are less and more sensitive to this redox  
402 modification, respectively (Fig. 6). This differential behavior might be linked to sequence  
403 differences within or in the proximity of motifs A and B (Fig. 7A). Plant 2-CysPRXs are  
404 highly conserved and differ by only a limited number of residues (Fig. 7A). Interestingly an  
405 Ile residue, instead of Val in the three other proteins, precedes the motif B in At2-CysPRXB.  
406 Structural comparison between the 3D structure of At2-CysPRXA C119S and the 3D model  
407 of At2-CysPRXB shows that this Ile, which has a higher steric hindrance, is located between  
408 motif A and B (Fig. S3). This modification could explain the better resistance of At2-  
409 CysPRXB to hyperoxidation, and this residue could be included in the motif B. Concerning  
410 the barley enzyme, that shows a higher level of sensitivity, its motif A contains two specific  
411 residues (Ile-136 and Lys-164) that induce electrostatic surface changes very close to the  
412 GGLC motif (Figs. 7B-C). This modification could alter the property of this motif, which is a  
413 key determinant in hyperoxidation sensitivity. A survey of motifs A and B in various plant 2-  
414 CysPRXs reveals even more sequence divergence within these two motifs, notably in the first  
415 part of motif A, which is associated with the  $\alpha$  helix leading to CysP, and regarding the last  
416 residue in motif B that can also be an Ala instead of Ser in some species (Fig. S4). Based on  
417 these findings, we propose that such variations in motifs A and B fine-tune differential  
418 hyperoxidation in plant 2-CysPRXS (Fig. 8) and confer them distinct physiological functions.  
419 Indeed, hyperoxidation results in inactivation of peroxidase activity and initiation of signaling  
420 responses (Wood *et al.*, 2003; Rey *et al.*, 2007; Bolduc *et al.*, 2018). Up to now, no data  
421 support distinct functions since Arabidopsis mutants deficient in one 2-CysPRX do not  
422 exhibit any obvious phenotype in standard conditions (Kirchsteiger *et al.*, 2009). Further  
423 investigations in challenging conditions are thus needed. Finally, we can also hypothesize that  
424 the subtle sequence divergence among plant 2-Cys PRXs underlie specific structural features,  
425 such as the dimer/monomer distribution, as observed in Arabidopsis and potato (Fig. 1D).

426

### 427 **Thiol content in plant 2-CysPRXs**

428 The content in free thiols is likely a critical determinant underlying 2-CysPRX functions.  
429 Very few data are available regarding this parameter even outside the plant kingdom. By  
430 performing alkylation experiments on leaf proteins, we show that a minor proportion of the  
431 peroxidase displays shifted migration to 25 kDa (Fig. 2). This *ex vivo* approach suggests that

432 only a limited pool of 2-CysPRX carries one free thiol in leaf cells. Perez-Ruiz *et al.*, (2017),  
433 when performing alkylation experiments using methyl-maleimide polyethylene glycol24 on  
434 Arabidopsis extracts, observed the appearance of one major upper band and another very faint  
435 one very close in size to the latter. These two bands were attributed to 2-CysPRX forms  
436 harboring one or two free thiols. However, no marker size and no control on fully reduced  
437 proteins were shown to unambiguously identify the one- and two-free-thiol forms. When  
438 carefully examining and comparing the pattern from Fig. 2C to that reported by Perez-Ruiz *et*  
439 *al.*, (2017), it seems that the two very close bands revealed by this group might correspond to  
440 one unique alkylated form. The high proportion of oxidized Cys in 2-CysPRX observed here  
441 is in agreement with the conclusion of Vaseghi *et al.*, (2018), which was based on the  
442 monomer abundance in Arabidopsis extracts migrated in non-reducing conditions. To perform  
443 peroxidase activity, typical 2-CysPRXs assemble in obligate homodimers that are presumed  
444 to be reduced or bound with one or two disulfide bounds (Dietz, 2011). Our experiments  
445 revealed that most Cys are oxidized (Fig. 2), indicating that a high proportion of dimers likely  
446 display two disulfide bounds. Of note, the abundance of the alkylated 25-kDa band was found  
447 higher in Arabidopsis than in barley and potato (Fig. 2D). Since the protein is revealed mainly  
448 as a dimer in non-reducing SDS-PAGE in the two cultivated species compared to Arabidopsis  
449 (Fig. 1D), we might infer that dimer forms are bound by two disulfide bridges and that there  
450 is a positive relationship between the monomer amount and the presence of free thiols in 2-  
451 CysPRX, as hypothesized by Vaseghi *et al.*, (2018).

452 In the three species studied, no substantial change was observed in the amount of the  
453 additional 25-kDa band along the light cycle (Figs. 3E, 4E-F). Consistently, Perez-Ruiz *et al.*,  
454 (2017) reported no variation in the alkylation pattern following the dark-light transition in WT  
455 Arabidopsis. However, the abundance of this band increased in *ntrc* at the beginning of the  
456 light period (Perez-Ruiz *et al.*, 2017). In our experiments, we noticed increased intensity of  
457 the 25-kDa alkylated band in *ntrc* at the end of the light phase (Fig. S1). Moreover, potato  
458 plants lacking the CDSP32 TRX or those expressing a non-active form exhibit a higher  
459 abundance of the 2-CysPRX form carrying one free thiol (Fig. 5C), further highlighting the  
460 importance of reductants in the maintenance of 2-CysPRX thiol content.

461

#### 462 **Relationship between 2-CysPRX redox status, quaternary structure and functions**

463 2-CysPRXs display several quaternary structures such as monomer, dimer and HMW  
464 complexes including tetramer and decamer (Dietz, 2011; Cerveau *et al.*, 2016b). Of note, the  
465 monomer level revealed by SDS-PAGE in the absence of reductant is non-representative of

466 the original level, partly due to SDS dissociation of non-covalently bound forms. Nonetheless,  
467 this migration feature is a valuable indicator of protein structure and reveals a striking  
468 species-dependent variability. Surprisingly, a very low monomer abundance was recorded in  
469 potato and barley (König *et al.*, 2002; Broin and Rey, 2003; König *et al.*, 2003) while a much  
470 higher amount was noticed in Arabidopsis and tomato (Baier and Dietz 1999; Cerveau *et al.*  
471 2016b; Puerto-Galan *et al.*, 2015; Pulido *et al.*, 2010; Xia *et al.*, 2018). When preparing  
472 proteins from Arabidopsis, barley and potato in a simultaneous and similar manner, we get  
473 results highly consistent to those reported in the literature *i.e.* a monomer proportion of *ca*  
474 50% in Arabidopsis and lower than 20% in the two other species (Fig. 1B). We thus conclude  
475 that the 2-CysPRX dimer/monomer distribution revealed in non-reducing SDS-PAGE  
476 strongly depends on species type. This feature might be an indicator of preferential PRX  
477 functions among the plant kingdom.

478 Based on Arabidopsis data, Puerto-Galan *et al.*, (2015) and Cerveau *et al.*, (2016b) concluded  
479 to a correlation between hyperoxidation level and monomer abundance in non-reducing SDS-  
480 PAGE. However, barley is characterized by a low monomer amount and a much higher  
481 abundance of hyperoxidized enzyme (Figs. 6C, 1D) and potato plants co-suppressed for  
482 *CDSP32* concomitantly display compared to other lines the highest monomer level and the  
483 lowest amount of hyperoxidized protein (Fig. 5 A-B). This clearly demonstrates the absence  
484 of relationship between plant 2-CysPRX monomer abundance and hyperoxidation level. Of  
485 note, the absence of reductants such as NTRC in Arabidopsis and *CDSP32* in potato is  
486 associated with altered dimer to monomer proportion (Figs. 1B, D; 5B), highlighting the  
487 importance of physiological reductants in the maintenance of enzyme quaternary structure.  
488 Within a species, the dimer/monomer ratio could be a marker of proper redox homeostasis.  
489 Indeed, potato plants modified for *CDSP32* expression exhibit no phenotype in standard  
490 conditions, but increased sensitivity to oxidative stress (Broin *et al.*, 2002; Rey *et al.*, 2005).

491 In other respects, the 2-CysPRX distribution in dimer and monomer forms is very likely  
492 related to environmental conditions. Indeed, a higher monomer proportion was observed in  
493 potato and barley upon osmotic or oxidative constraints (König *et al.*, 2002; Broin and Rey,  
494 2003), and this proportion decreased in tomato in response to chilling (Xia *et al.*, 2018).  
495 Further evidence of environment influence on monomer and dimer distribution is deduced  
496 from the patterns along the light cycle. In barley, the monomer amount is substantially higher  
497 during the light period and in potato, the highest amount is recorded at the end of the light  
498 phase (Fig. 4 C-D). Intriguingly in WT Arabidopsis, the protein detected as both dimer and  
499 monomer in the 20-50 kDa range in non-reducing conditions is 25% lower at the beginning of



500 the dark period compared to the beginning of the light phase (Fig. 3C). Similar data were  
501 observed in *ntrc* and *srx* mutants (Fig. 3D) and in potato (Fig. 4D). In barley, no such a  
502 change was noticed, but the monomer amount substantially decreased following the light-dark  
503 transition (Fig. 4C). The variations observed in Arabidopsis and potato appear at first sight  
504 contradictory to the patterns in reducing conditions, since no change in the total protein  
505 abundance occurs depending on light cycle (Figs. 3A, 4A-B). This discrepancy might  
506 originate from 2-CysPRX involvement in HMW complexes or covalent binding to partners.  
507 The enzyme is indeed susceptible to interact with numerous partners as reported in  
508 Arabidopsis (Cerveau *et al.*, 2016a). These homo- or heteromeric complexes could be poorly  
509 detected by antibodies or migrate outside the 20-100 kDa gel area. Thus, we can presume that  
510 2-CysPRX binding occurs at specific time points, such as at the end of the light phase, where  
511 this enzyme fulfills a critical role in re-oxidation of TRXs and deactivation of photosynthetic  
512 enzymes (Yoshida *et al.*, 2014; Yoshida *et al.*, 2018).

513 The data presented here reveal a striking complexity of 2-CysPRX hyperoxidation level and  
514 quaternary structure that depend on environmental factors such as light cycle, but also on  
515 species. In contrast, the low thiol content observed in three species does not vary along the  
516 light cycle, indicating that most of the 2-CysPRX pool is oxidized. This enzyme, which is  
517 considered as a major plastidial regulatory hub (Muthuramalingam *et al.*, 2009), could play a  
518 role of oxidant buffer allowing the maintenance of proper plastidial redox homeostasis in  
519 concert with diverse TRX types (Vieira Dos Santos and Rey, 2006) depending on  
520 physiological context (Broin *et al.*, 2002; Perez-Ruiz *et al.*, 2006; Dangoor *et al.*, 2012 ;  
521 Eliyahu *et al.*, 2015; Yoshida *et al.*, 2018). For instance, the function of photosynthesis  
522 deactivation during the light-dark transition would be ensured via the dimer peroxidase  
523 activity and subsequent regeneration by the atypical TRX-like2 (Yoshida *et al.*, 2018). But,  
524 oxidized 2-CysPRX could also directly interact with reduced non-TRX partners as inferred  
525 from the Arabidopsis PRX interactome (Cerveau *et al.*, 2016a) and the capacity of the human  
526 enzyme to oxidize proteins (Stocker *et al.*, 2017). Finally, the other oxidized PRX forms, such  
527 as hyperoxidized monomer, are very likely involved in sensing and transmitting redox  
528 information (Liebthal *et al.*, 2018) in relation for instance with light cycle. This signal could  
529 be transduced via the control of peroxide concentration as proposed in the floodgate theory  
530 (Poole *et al.*, 2011) or interaction with specific partners remaining to be identified.

531

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536

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708



709 **Legends to figures**

710 **Figure 1. 2-CysPRX monomer and dimer distribution in Arabidopsis, barley and potato**  
711 **leaves.**

712 **A and B.** Western blot analysis of 2-CysPRX abundance in leaves from 6-week Arabidopsis  
713 old plants prepared and migrated in reducing (**A**) or non-reducing conditions (**B**) (10 µg per  
714 lane).

715 WT, wild type Arabidopsis Col-0 plants ; *a* and *b*, *2-cysprxa* and *2-cysprxb* homozygous  
716 plants from the GK\_295C05 and SALK\_017213 lines, respectively; *ab*, *2-cysprxa 2-cysprxb*  
717 double mutant generated from the crossing of single mutant lines; *ntrc*, SALK\_096776  
718 homozygous plants; *srx*, SALK\_015324 homozygous plants.

719 **C and D.** Western blot analysis of 2-CysPRX abundance in leaves from 6-week old  
720 Arabidopsis plants and 3-week-old barley and potato plants prepared and migrated in  
721 reducing (**C**) or non-reducing conditions (**D**) (12 µg per lane).

722 At, Arabidopsis WT Col-0; *ntrc*, SALK\_096776 homozygous Arabidopsis mutant; Hv, barley  
723 WT cv. “Express”; St, potato WT cv. “Désirée”. CB, Coomassie-blue stained gel in the 50-  
724 kDa range as a loading control; WB, Western blot.

725

726 **Figure 2. Thiol content in 2-CysPRXs from Arabidopsis, barley and potato leaves.**

727 **A.** Western blot analysis of 2-CysPRX abundance in leaves from 6-week Arabidopsis old  
728 plants prepared in non-reducing conditions, incubated with mPEG-maleimide-2000 and  
729 migrated in non-reducing conditions (50 µg per lane).

730 **B.** Western blot analysis of 2-CysPRX abundance in leaves from 6-week Arabidopsis old  
731 plants prepared in reducing or non-reducing conditions, incubated or not in the presence of  
732 mPEG-maleimide-2000 and migrated in the presence of reductant (12 µg per lane).

733 WT, wild type Col-0 ; *2-cysprxa* and *2-cysprxb*, homozygous plants from the GK\_295C05  
734 and SALK\_017213 lines, respectively; *2-cysprxa 2-cysprxb*, double mutant generated from  
735 the crossing of the single mutant lines; *ntrc*, SALK\_096776 homozygous plants; *srx*,  
736 SALK\_015324 homozygous plants.

737 **C.** Western blot analysis of 2-CysPRX abundance in leaves from 6-week old WT Arabidopsis  
738 plants prepared in reducing (either TCEP or β-mercaptoethanol) or non-reducing conditions,  
739 incubated or not in the presence of mPEG-maleimide-2000 and migrated in the presence of  
740 reductant (12 µg per lane).

741 **D.** Western blot analysis of 2-CysPRX abundance in leaves from 6-week old Arabidopsis  
742 plants and 3-week-old barley and potato plants prepared in reducing (50 mM TCEP) or non-  
743 reducing conditions, incubated or not in the presence of mPEG-maleimide-2000 and  
744 migrated in the presence of reductant (12  $\mu$ g per lane). At, Arabidopsis WT Col-0; Hv, barley  
745 WT cv. “Express”; St, potato WT cv. “Désirée”.  
746 Asterisks and arrows on the right indicate additional bands.

747

748 **Figure 3. Redox status of 2-CysPRXs in Arabidopsis leaves as a function of light cycle.**

749 **A and B.** Western blot analysis of 2-CysPRX abundance and hyperoxidation level in leaves  
750 from 6-week old Arabidopsis WT plants as a function of light cycle. Proteins were prepared  
751 and migrated in the presence of reductant (**A**) or not (**B**). (10 and 12  $\mu$ g per lane in **A** and **B**,  
752 respectively).

753 **C.** Relative abundance of 2-CysPRX abundance detected in reducing and non-reducing  
754 conditions following the light-dark transition compared to the dark-light transition. Band  
755 intensities from L1 and D2 light time points were quantified as described in Material and  
756 Methods. Data are means of values  $\pm$  SD originating from three and five independent  
757 experiments in reducing and non-reducing conditions, respectively. \*\*, values significantly  
758 different with  $P < 0.01$  (*t*-test).

759 **D.** Western blot analysis of 2-CysPRX abundance in leaves from 6-week old *ntrc* and *srx*  
760 plants as a function of light cycle. Proteins were prepared and migrated in the absence of  
761 reductant (12  $\mu$ g per lane).

762 **E.** Western blot analysis of 2-CysPRX abundance following alkylation in leaves from 6-week  
763 old WT Arabidopsis plants as a function of light cycle. Proteins were prepared in non-  
764 reducing conditions, incubated with mPEG-maleimide-2000 and migrated in the presence of  
765 reductant (12  $\mu$ g per lane). The arrow on the right indicates additional bands.

766 Light time points: D1 and L1, 45 min before and after the dark-light transition, respectively;  
767 L2, middle of the 8-h light period; L3 and D2, 45 min before and after the light-dark  
768 transition, respectively. CB, Coomassie-blue stained gels in the 50-kDa (**A-B, D**) or 25-kDa  
769 (**E**) ranges as loading controls; WB, Western blot.

770

771

772

773

774 **Figure 4. Redox status of 2-CysPRXs in barley and potato leaves as a function of light**  
775 **cycle.**

776 **A and B.** Western blot analysis of 2-CysPRX abundance and hyperoxidation level in leaves  
777 from 3-week old barley (**A**) and potato (**B**) WT plants as a function of light cycle. Proteins  
778 were prepared and migrated in the presence of reductant (10 µg per lane).

779 **C and D.** Western blot analysis of 2-CysPRX abundance in leaves from 3-week old barley (**C**)  
780 and potato (**D**) WT plants as a function of light cycle. Proteins were prepared and migrated in  
781 the absence of reductant (12 µg per lane).

782 **E and F.** Western blot analysis of 2-CysPRX abundance following alkylation in leaves from  
783 3-week old barley (**E**) and potato (**F**) WT plants as a function of the light time point. Proteins  
784 were prepared in non-reducing conditions, incubated with mPEG-maleimide-2000 and  
785 migrated in reducing conditions (12 µg per lane). The arrow on the right indicates additional  
786 bands.

787 Light time points: D1 and L1, 45 min before and after the dark-light transition, respectively;  
788 L2, middle of the 8-h and 12-h light periods for barley and potato, respectively; L3 and D2,  
789 45 min before and after the light-dark transition, respectively.

790 CB, Coomassie Blue-stained gels in the 50-kDa (**A-D**) or 25-kDa (**E-F**) ranges as loading  
791 controls; WB, Western blot.

792

793 **Figure 5. Redox status of 2-CysPRXs in potato plants modified for *CDSP32* expression.**

794 **A.** Western blot analysis of 2-CysPRX abundance and hyperoxidation level in leaves from 3-  
795 week old potato plants. Proteins were prepared and migrated in the presence of reductant (10  
796 µg per lane).

797 **B.** Western blot analysis of 2-CysPRX abundance in leaves from 3-week old potato plants.  
798 Proteins were prepared and migrated in the absence of reductant (12 µg per lane).

799 **C.** Western blot analysis of 2-CysPRX abundance following alkylation in leaves from 3-week  
800 old potato plants. Proteins were prepared in non-reducing conditions, incubated with mPEG-  
801 maleimide-2000 and migrated in reducing conditions (12 µg per lane). The arrow on the right  
802 indicates additional bands.

803 WT, wild type cv. "Désirée"; CS, line co-suppressed for *CDSP32* expression; OE, line over-  
804 expressing WT *CDSP32*; OE-M1 and OE-M2, two independent lines over-expressing a  
805 *CDSP32* gene coding for a mutated active site form. Leaf samples were collected at the L3  
806 light time point (45 min before the light-dark transition). CB, Coomassie Blue-stained gels in  
807 the 50-kDa (**A-B**) or 25-kDa (**C**) ranges as loading controls; WB, Western blot.

808

809 **Figure 6. Hyperoxidation of plant 2-CysPRXs.**

810 **A.** Western blot analysis of 2-CysPRX abundance and hyperoxidation level in Arabidopsis  
811 leaves as a function of developmental stage in 6-week old plants using sera raised against  
812 At2-CysPRX and hyperoxidized 2-CysPRX (10 µg per lane).

813 **B.** Relative abundances compared to WT of total and hyperoxidized 2-CysPRX in leaves from  
814 6-week old Arabidopsis *2-cysprxa* and *2-cysprxb* plants. Band intensity was quantified as  
815 described in Material and Methods. Data are means of values ± SD originating from three  
816 independent plants. \*, \*\* and \*\*\*, values significantly different with  $P < 0.05$ ,  $P < 0.01$  and  $P$   
817  $< 0.001$ , respectively (*t*-test).

818 **C.** Western blot analysis of 2-CysPRX abundance and hyperoxidation level in leaves from 6-  
819 week old Arabidopsis plants and 3-week-old barley and potato plants (10 µg per lane).  
820 Arabidopsis and barley leaves were collected 45 min following the dark-light transition and  
821 potato leaves 45 min before the light-dark transition,  
822 WT and At, wild type Arabidopsis Col-0; *2-cysprxa* and *2-cysprxb*, homozygous plants from  
823 the GK\_295C05 and SALK\_017213 lines, respectively. Y, young leaf; A, adult leaf; O, old  
824 leaf. Hv, barley WT cv. “Express”; St, potato WT cv. “Désirée”. CB, Coomassie-blue stained  
825 gel in the 50-kDa range as a loading control; WB, Western blot.

826

827 **Figure 7. Sequence and structural comparisons of plant 2-Cys peroxiredoxins.**

828 **A. Sequence alignment of Arabidopsis, potato and barley 2-CysPRXs.** NCBI references for  
829 Arabidopsis 2-Cys PRXs A and B: NP\_187769.1 and NP\_568166.1, respectively. NCBI  
830 reference for potato: XP\_006339159.1. GenBank reference for barley: BAJ98505.1. The  
831 motifs A and B, involved in hyperoxidation resistance (Bolduc *et al.*, 2018), are highlighted in  
832 blue and yellow, respectively. The GGLG and YF motifs that are considered as a signature of  
833 sensitivity to hyperoxidation are highlighted in gray. The two catalytic Cys are highlighted in  
834 purple. Residues highlighted in red and green are specific to Hv2-CysPRX and to At2-  
835 CysPRXB, respectively, within motifs A and B. Two-by-two sequence alignments were made  
836 using At2-CysPRXA as a reference.

837 **B. and C. Structural comparison of At2-CysPRXA and Hv2-CysPRX.** Ribbon  
838 representation of At2-CysPRXA C119A (PDB code 5ZTE monomer C) and Hv2-CysPRX  
839 (3D structural model) showing motifs A, B, GGLG and YF and position of the two different  
840 residues within motif A highlighted in red in Fig. 7A (left). Surface representation displaying  
841 atoms color-coded according to the surface potential from red (negative) to blue (positive)

842 (right). All images were created using PyMOL.

843

844 **Figure 8. Model for the hierarchy of the resistance of plant 2-CysPRXs to**  
845 **hyperoxidation.** The model is adapted from Bolduc *et al.*, (2018). Based on the presence or  
846 not of GGLG/YF, A and B motifs, the bacterial AhpC and the human HsPRX2 are the most  
847 resistant and sensitive, respectively. Human HsPRX3 like plant 2-Cys-PRXs displays motifs  
848 A and B. The proposed graded resistance of plant 2-CysPRXs is based on the sequence  
849 features of At2-CysPRXB and Hv2-CysPRx within or very close to motifs A and B.

850

851 **Supplementary data**

852 **Figure S1: Time points for collecting leaf samples during the light cycle.** D1 and L1  
853 correspond to 45 min before and after the dark-light transition, respectively, L2 to the middle  
854 of the light period, and L3 and D2 to 45 min before and after the light-dark transition. The  
855 photoperiod length is 8 h for Arabidopsis and barley, and 12 h for potato.

856

857 **Figure S2. Thiol content in 2-CysPRX in Arabidopsis *ntrc* leaves as a function of light**  
858 **cycle.**

859 Western blot analysis of 2-CysPRX abundance following alkylation in leaves from 6-week  
860 old Arabidopsis *ntrc* plants as a function of light cycle. Proteins were prepared in non-  
861 reducing conditions, incubated in the presence of mPEG-maleimide-2000 and migrated in the  
862 presence of reductant (12 µg per lane). The arrow on the right indicates additional bands.

863 Light time points: D1 and L1, 45 min before and after the dark-light transition, respectively;  
864 L3 and D2, 45 min before and after the light-dark transition, respectively. CB, Coomassie-  
865 blue stained gel in the 25-kDa range as a loading control; WB, Western blot.

866

867 **Figure S3. Structural comparison of At2-CysPRXA (A) and At2-CysPRXB (B).** Ribbon  
868 (left) and ball (right) representation of At2-CysPRXA C119A (PDB code 5ZTE dimer BC)  
869 and At2-CysPRXB (3D structural model) showing motifs A and B and the position of the  
870 different residue in green preceding the motif B. All images were created using PyMOL.

871

872 **Figure S4: Sequence alignment of plant 2-Cys peroxiredoxins.**

873 NCBI or Genbank references: *Arabidopsis thaliana* 2-Cys PRXs A and B, NP\_187769.1 and  
874 NP\_568166.1, respectively; *Camelina sativa* (Cs), XP\_010464879.1; *Raphanus sativus* (Rs),  
875 XP\_018493095.1; *Papaver somniferum* (Ps), XP\_026426631.1; *Prunus persica* (Pp),  
876 XP\_007202444.1; *Glycine max* (Gm), NP\_001341836.1; *Spinacia oleracea* (St),  
877 XP\_021867340.1; *Solanum tuberosum* (St): XP\_006339159.1; *Helianthus annuus* (Ha),  
878 XP\_021984163.1; *Hordeum vulgare* (Hv), BAJ98505.1; *Triticum aestivum* (Ta),  
879 SPT18356.1; *Zea mays* (Zm), NP\_001137046.1. The motifs A and B, involved in  
880 hyperoxidation resistance of 2-Cys peroxiredoxins (Bolduc *et al.*, 2018), are highlighted in  
881 blue and yellow, respectively. The GGLG and YF motifs considered as a signature of  
882 sensitivity to hyperoxidation are highlighted in gray. The two catalytic Cys are highlighted in  
883 purple. Black bars indicate sequence divergence within motifs A and B.

Fig. 1

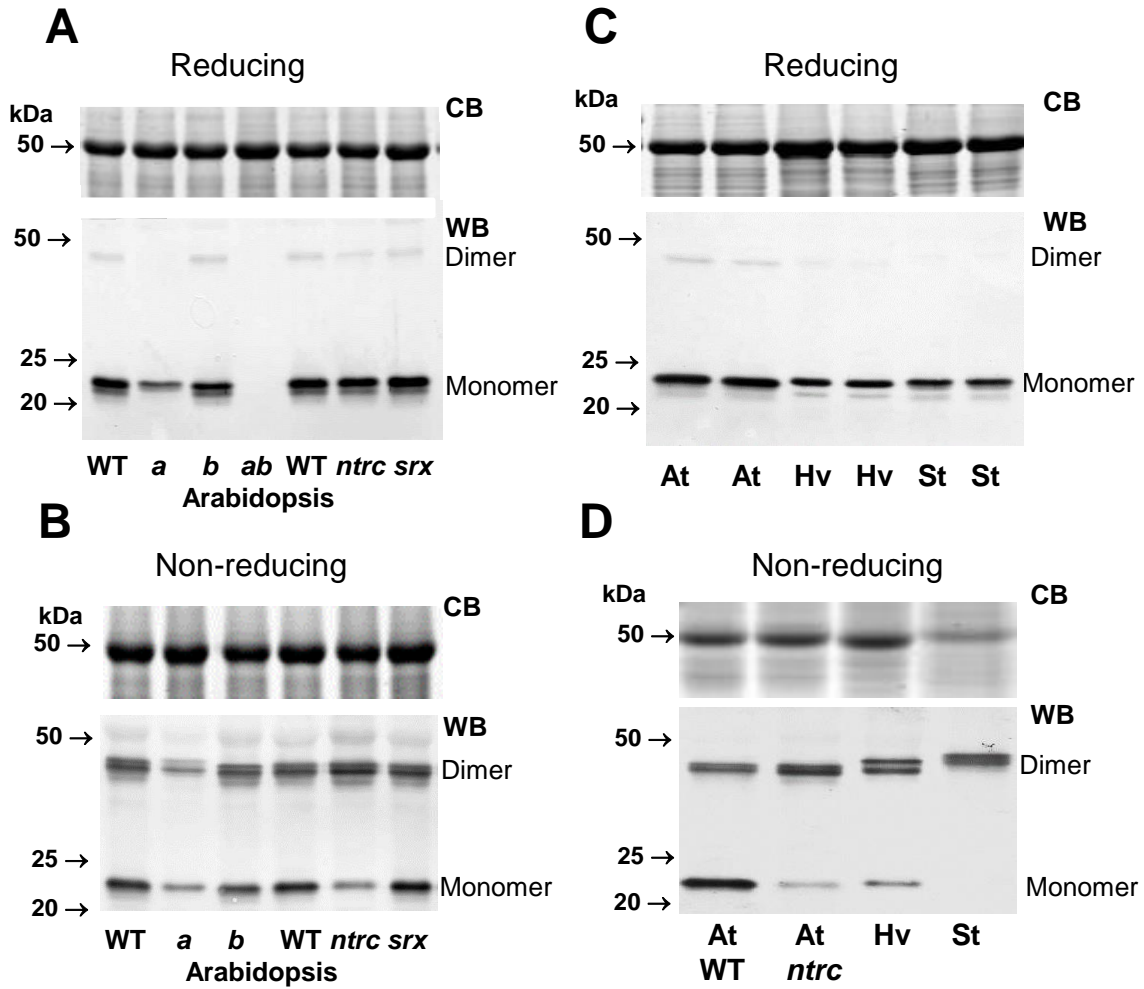
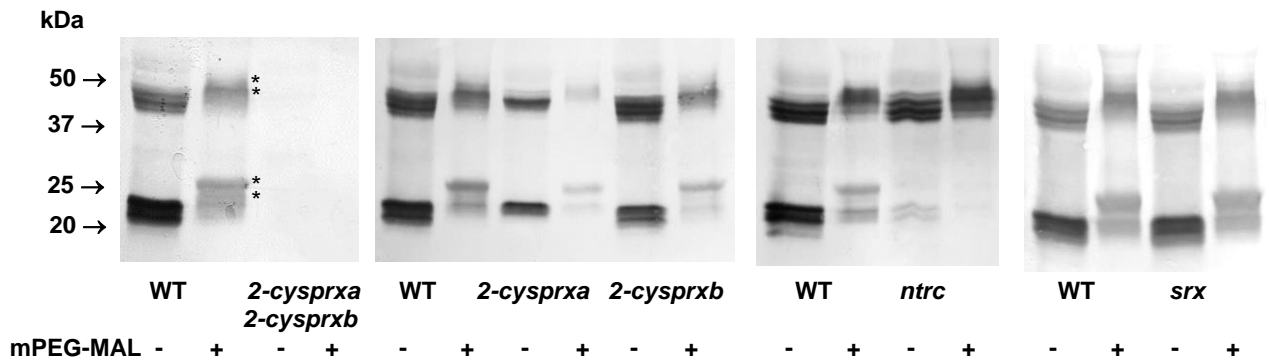
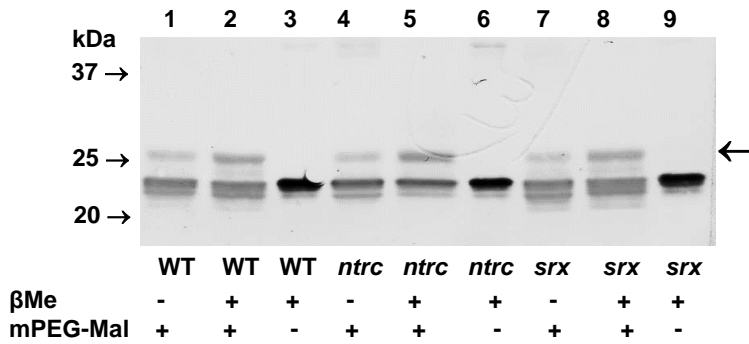


Fig.2

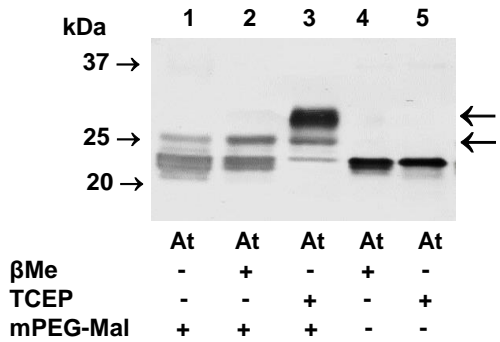
**A**



**B**



**C**



**D**

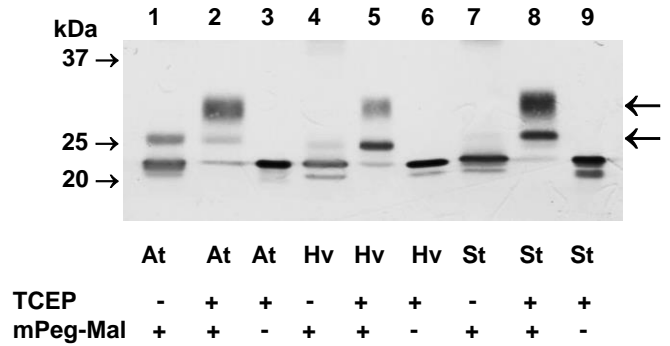




Fig. 3

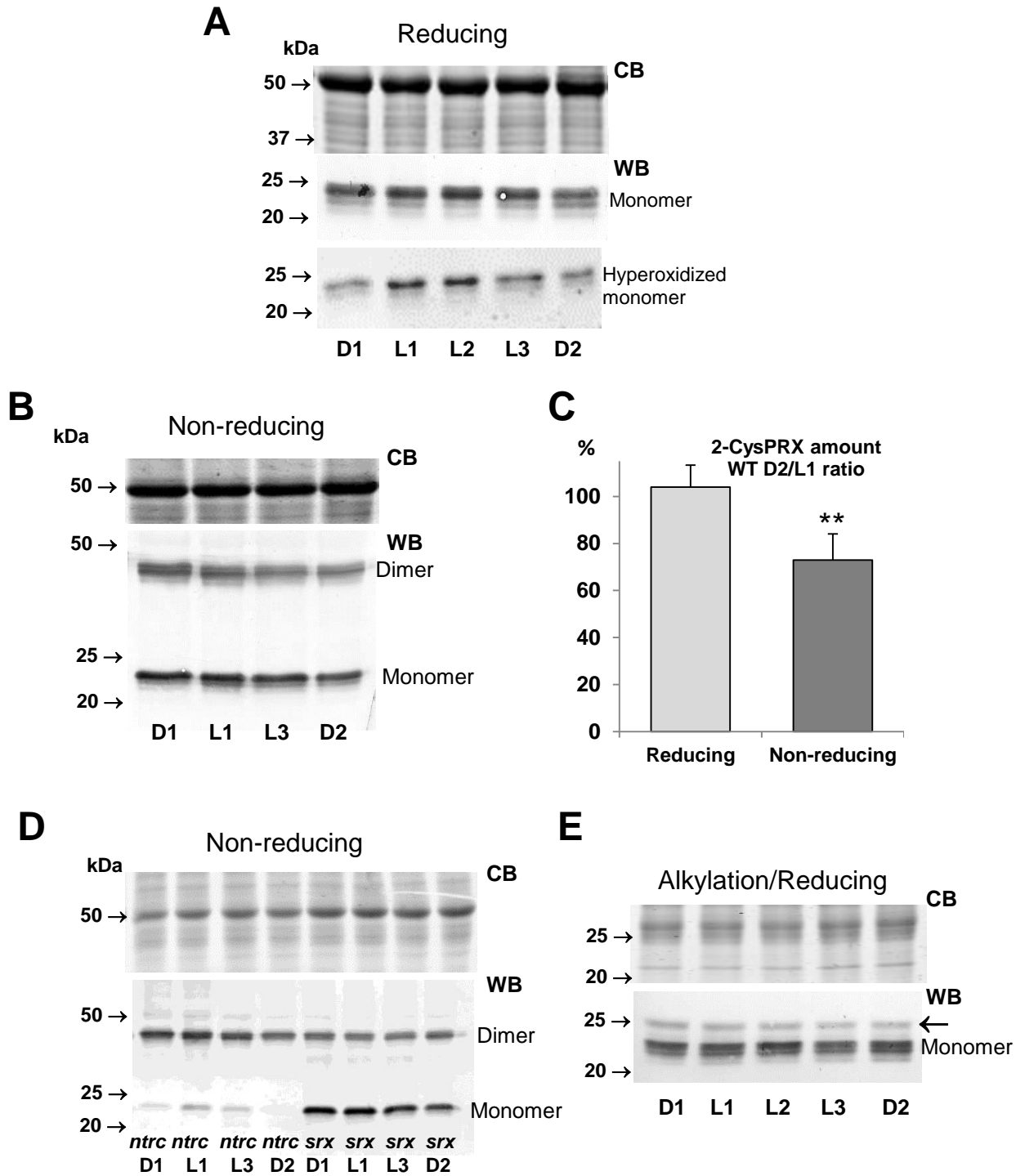


Fig. 4

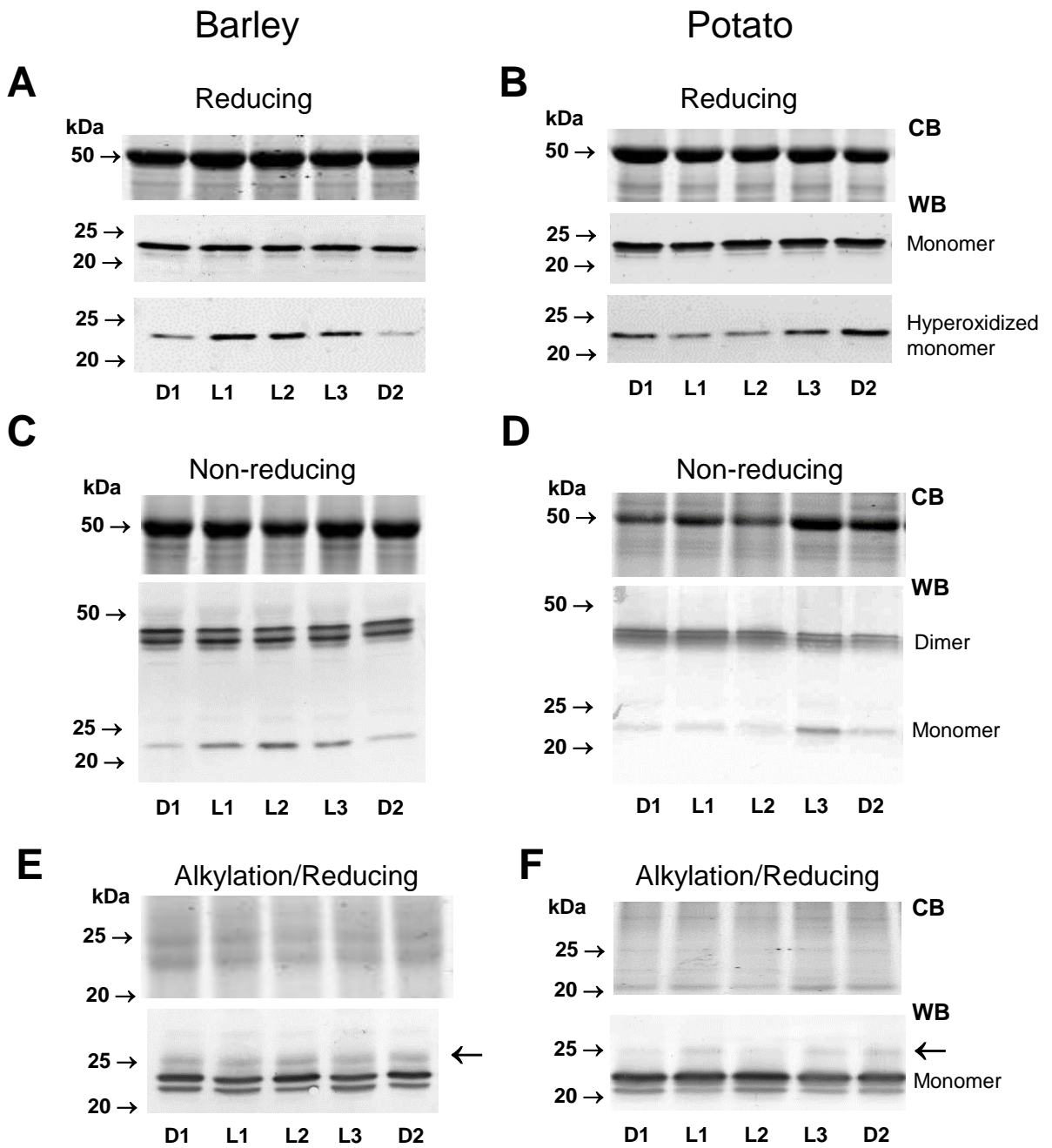


Fig. 5

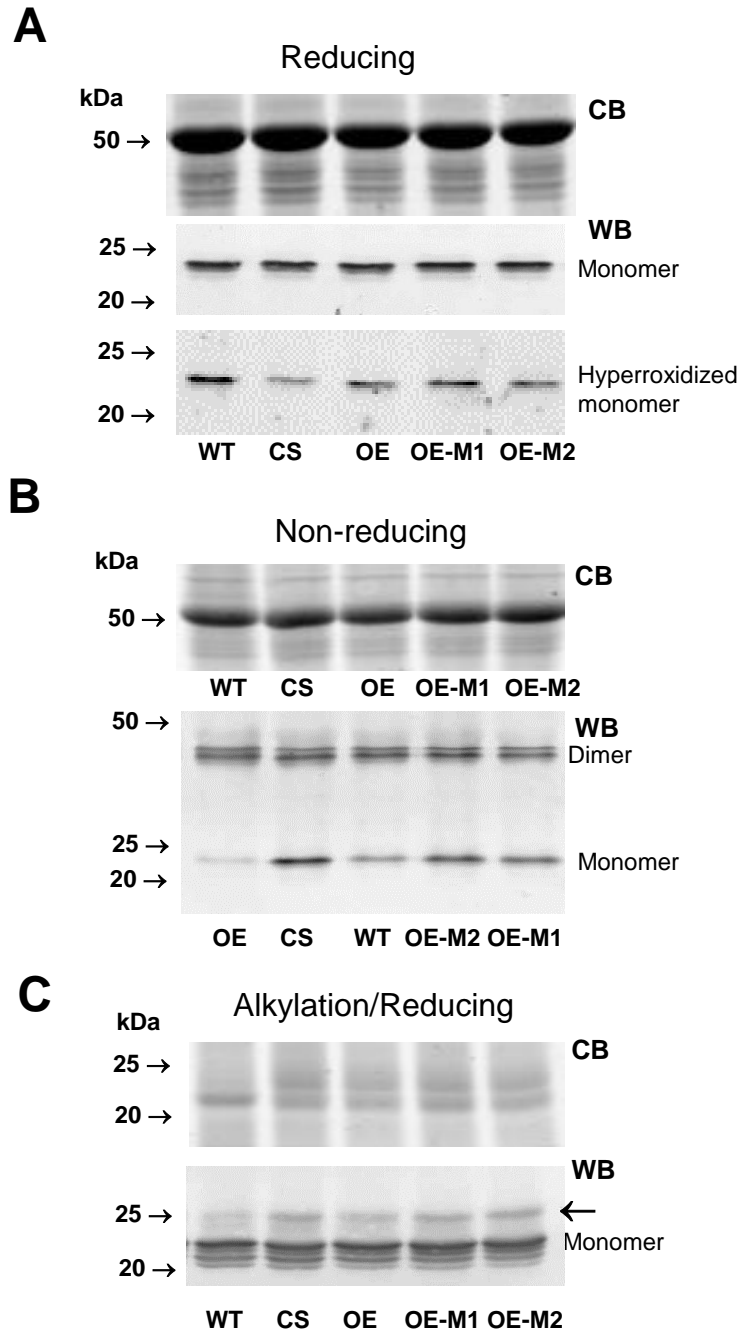


Fig.6

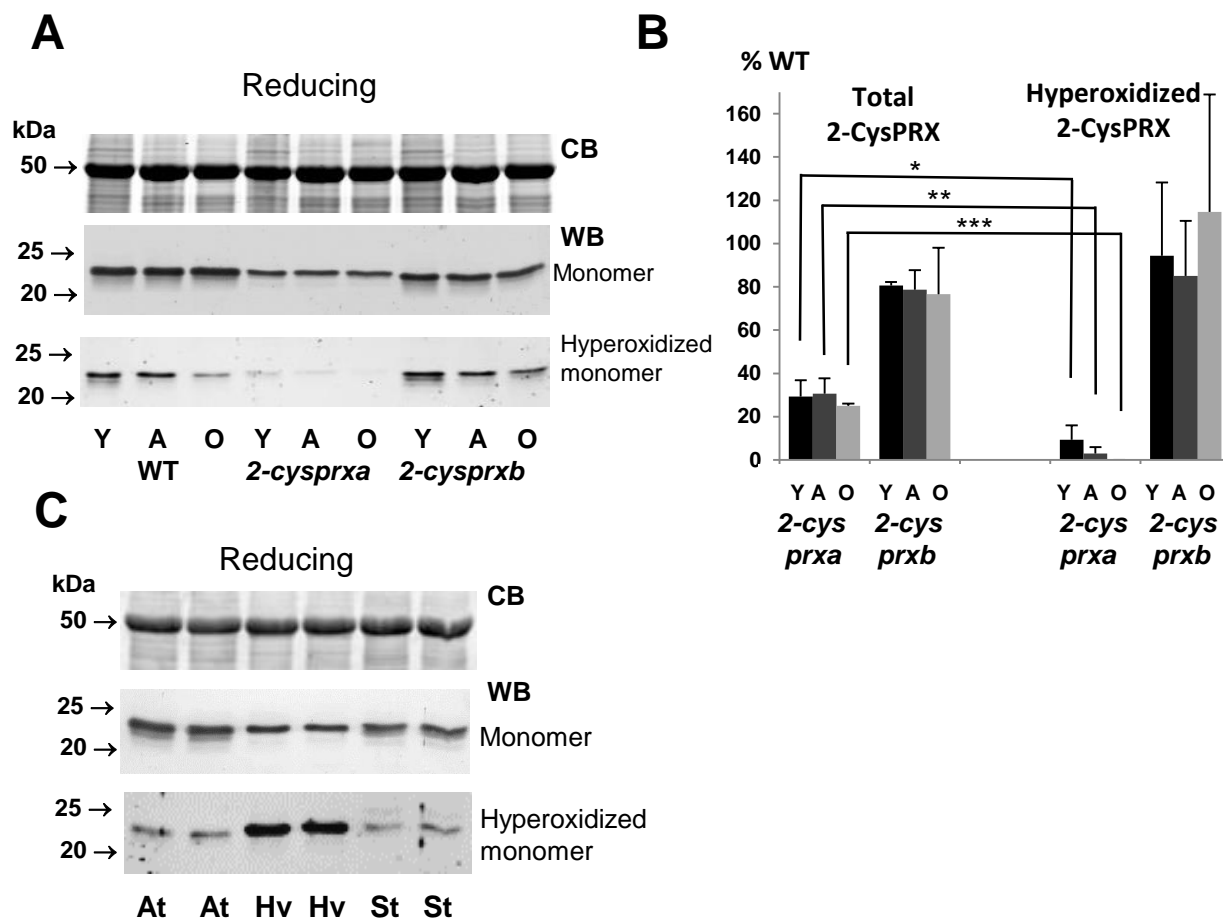


Fig. 7

A

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At2-CysPRXA 67 -AQADDLPLVGNKAPDFEAEAVFDQEFIKVKLSDYIGKKYVILFFYPDFTFVCPTEITA
At2-CysPRXB 74 -AQADDLPLVGNKAPDFEAEAVFDQEFIKVKLSEYIGKKYVILFFYPDFTFVCPTEITA
St2-CysPRX 69 --ASSELPVGNQAPDFEAEAVFDQEFIKVKLSEYIGKKYVILFFYPDFTFVCPTEITA
Hv2-CysPRX 62 AAAEYDLPVGNKAPDFAAEAVFDQEFINVKLSDYIGKKYVILFFYPDFTFVCPTEITA
          :*****:**** *****:****:*****:*****
    
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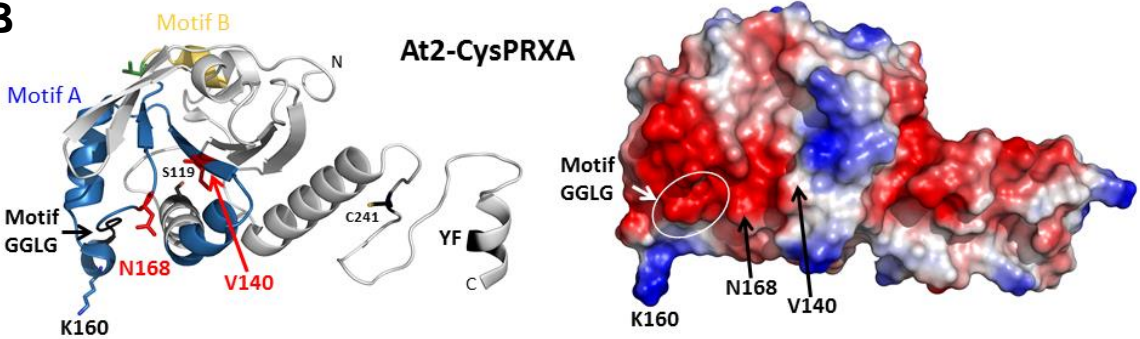
At2-CysPRXA 126 FSDRHSEFEKLNTEVLGVSVDVFSHLAWVQTRKSGGLGDLNYPLISDVTKSISKSGV
At2-CysPRXB 133 FSDRYEEFEKLNTEVLGVSVDVFSHLAWVQTRKSGGLGDLNYPLVSDTKSISKSGV
St2-CysPRX 127 FSDRYEEFEKVNTEVLGVSVDVFSHLAWVQTRKSGGLGDLNYPLISDVTKSISKSYNV
Hv2-CysPRX 122 FSDRHEEFEKINTEVLGVSVDVFSHLAWVQTRKSGGLGDIYPLVSDTKSISKSGV
          *****:****:*****:*****:*****:****:****:*****:..*
          Motif A: D-X8-N/G-X10-H-X27-S/G Motif B: T-X3-S
    
```

```

At2-CysPRXA 186 LIHQGIALRGLFIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYIQENPDEVPAGW
At2-CysPRXB 193 LIPDQGIALRGLFIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYVQENPDEVPAGW
St2-CysPRX 187 LIPDQGIALRGLFIIDKEGVIQHSTINNLGIGRSVDETLRTLQALQYVQENPDEVPAGW
Hv2-CysPRX 182 LIPDQGIALRGLFIIDKEGVIQHSTINNLGIGRSVDETLRTLQALQYVQENPDEVPAGW
          ** *****:*****:*****:*****:*****:*****:*****
    
```

			Overlap	Identity	Similarity
At2-CysPRXA	246	KPGEKSMKPDPKLSKEYFSAI 266			
At2-CysPRXB	253	KPGEKSMKPDPKLSKEYFSAI 273	200 AA	96.5%	99.0%
St2-CysPRX	247	KPGEKSMKPDPKGSKEYFASI 267	198 AA	91.4%	98.5%
Hv2-CysPRX	242	KPGEKSMKPDPKGSKEYFAAI 262	200 AA	92.0%	96.5%
		*****:****:*****:*****:*****:*****:*****:..*			

B



C

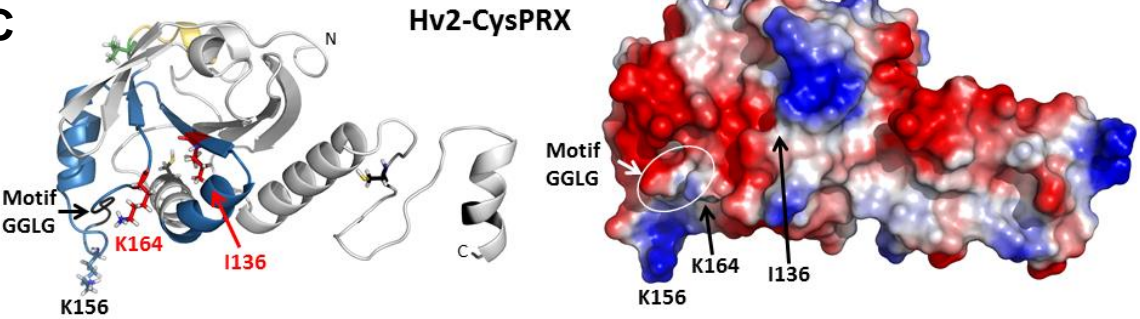
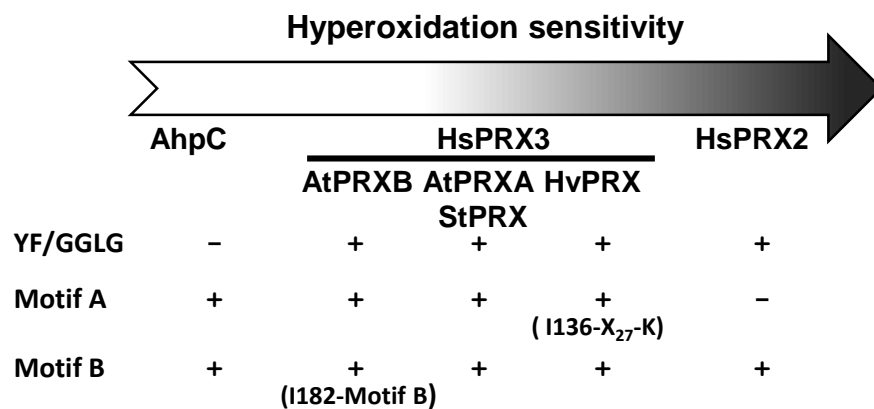


Fig. 8



1 **Variability in the redox status of plant 2-Cys peroxiredoxins in relation to species and**  
2 **light cycle**

3

4 **Delphine Cerveau<sup>1</sup>, Patricia Henri<sup>1</sup>, Laurence Blanchard<sup>2</sup> and Pascal Rey<sup>1,\*</sup>**

5

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24 **Date of submission:** April 9, 2019

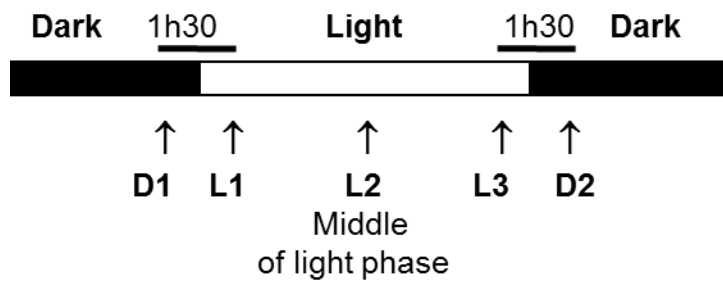
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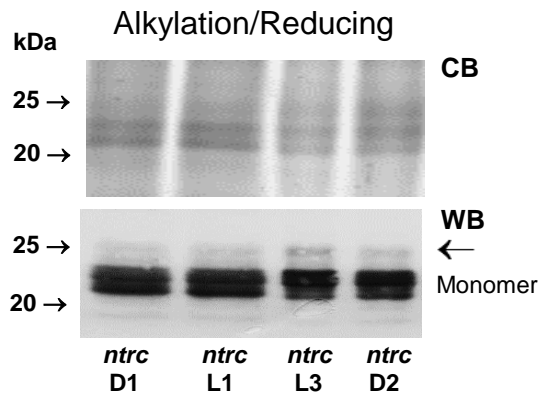
29 **Supplementary data:** Four figures



**Figure S1: Time points for collecting leaf samples during the light cycle.** D1 and L1 correspond to 45 min before and after the dark-light transition, respectively, L2 to the middle of the light period, and L3 and D2 to 45 min before and after the light-dark transition. The photoperiod length is 8 h for Arabidopsis and barley, and 12 h for potato.



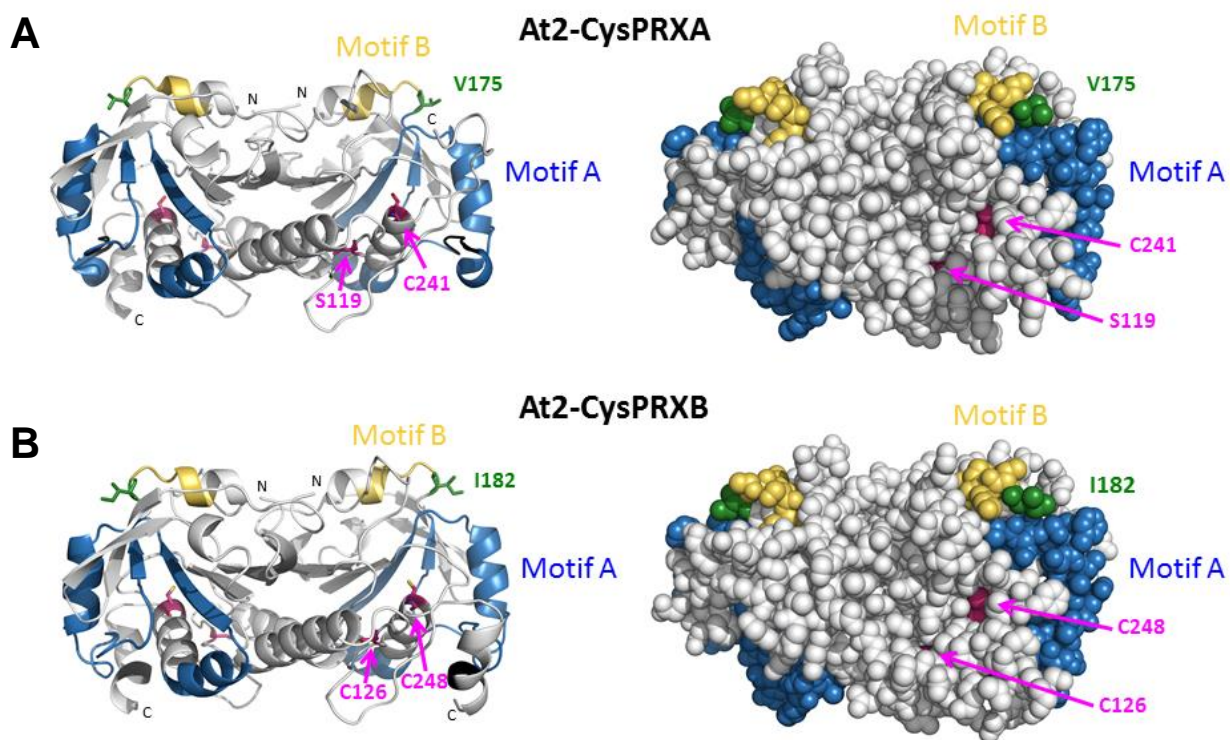
Fig. S2



**Figure S2. Thiol content in 2-CysPRX in Arabidopsis *ntrc* leaves as a function of light cycle.**

Western blot analysis of 2-CysPRX abundance following alkylation in leaves from 6-week old Arabidopsis *ntrc* plants as a function of light cycle. Proteins were prepared in non-reducing conditions, incubated in the presence of mPEG-maleimide-2000 and migrated in the presence of reductant (12  $\mu$ g per lane). The arrow on the right indicates additional bands. Light time points: D1 and L1, 45 min before and after the dark-light transition, respectively; L3 and D2, 45 min before and after the light-dark transition, respectively. CB, Coomassie-blue stained gel in the 25-kDa range as a loading control; WB, Western blot.

Fig. S3



**Figure S3. Structural comparison of At2-CysPRXA (A) and At2-CysPRXB (B).** Ribbon (left) and ball (right) representation of At2-CysPRXA C119A (PDB code 5ZTE dimer BC) and At2-CysPRXB (3D structural model) showing motifs A and B and the position of the different residue in green preceding the motif B. All images were created using PyMOL.

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At2-CysPRXA 1 ---MASVASSTTLISSPSSRVFPKSSLSPPSVSFLRTLSSP--SASASLRSGFARRSSLSS---TSRRSFAVKAQADDLPLVGNKAPDFEAEAVFDQEF
At2-CysPRXB 1 MSMASIASSSSTLLSSSRVLLPSKSSLLSPTVVSFPRIIPSSSASSSSSLCSGFSSLGSLTNRASRRNFVAVKAQADDLPLVGNKAPDFEAEAVFDQEF
Cs2-CysPRX 1 ---MASVASSTTLISSPSSRVFPVKSSLSPPSVSFLRTLSSPSASAVALRSGFARRSSLTS---TSRRSFAVKAQADELPLVGNKAPDFEAEAVFDQEF
Rs2-CysPRX 1 ---MASVASSTTLISS--STRALPAKSPLPSPSISFLPTLSSPLRSGFSQRS---SLTSIRS---TSRRSFAVKAQTDLPLVGNKAPDFEAEAVFDQEF
Ps2-CysPRX 1 ----MACSASSTVISSNPSSIKFPKPMASLTSSLPFSQTLNVPKSFNGLRNSFQSRASRSISTNQSKRSLVVKASAGELPLVGNKAPDFEAEAVFDQEF
Pp2-CysPRX 1 ---MAASTALISSTPSRAFSSKSTPLVASSSISKPISQTLTFPKSFNGLRRLPRVAHSVLSRGAHSRRSFLVKASVDELPLVGNVAPDFEAEAVFDQEF
Gm2-CysPRX 1 -----MACSATSASLFSANPTPLFSPKSS-----LSLPNNSLHNLPLTRPSSLTRPSSHTRRSFVVKASSELPVGNTPADDFEAEAVFDQEF
So2-CysPRX 1 ---MACAASSSAILSPNPRVFAAKSHAPMAAASVSSLPKPFSQTLTLSSNFNGVRKSFQSPRAQSSRSSFFVVRASAEPLVGNVAPDFEAEAVFDQEF
St2-CysPRX 1 -----MACSASSSTALLSSTSRASISPKSHISQSISVPSAFNGLRNCKPFVSRVARISSTRVAQSERRRFVAVCASSELPVGNQAPDFEAEAVFDQEF
Ha2-CysPRX 1 ---MASLSASAALLSSNPRYISPKSSNLSQTLFGLSSSVNFRSKSLRS-----ALAVRPSASRCNGSLIKAALPLVGNKAPDFEAEAVFDQEF
Hv2-CysPRX 1 -----MACAISASTVSTAAALVASPKTSGAPQCLSFPRAFGGAAARPARLAAAGSRTARARSFVARAAAEYDLPLVGNKAPDFAAEAVFDQEF
Ta2-CysPRX 1 -----MACAFSASTVSTAAALVASPKPAGVPQCLSFP----RAARPSRLAAAGSRTARARSFVARAAAEYDLPLVGNKAPDFAAEAVFDQEF
Zm2-CysPRX 1 -----MACSFAAATVSSAPTPAARPLAVAPQSVSVRSVAVATAARPLRLVAS--RSARATRLVARAGGVDDLPLVGNKAPDFEAEAVFDQEF
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At2-CysPRXA 93 IKVKLSDIYIGKKYVILFFYPLDFTFVCPTEITAFSDRHSEFEKLNTEVLGVSVDVSFVSHLAWVQTDKSGGLGDLNYPLISDVTKSISKSFVGLIHDQG
At2-CysPRXB 102 IKVKLSEYIGKKYVILFFYPLDFTFVCPTEITAFSDRYEEFEKLNTEVLGVSVDVSFVSHLAWVQTDKSGGLGDLNYPLVSDITKSISKSFVGLIPDQG
Cs2-CysPRX 94 IKVKLSEYIGKKYVILFFYPLDFTFVCPTEITAFSDRYSEFEKLNTEVLGVSVDVSFVSHLAWVQTDKSGGLGDLNYPLVSDVTKSIKSFVGLIHDQG
Rs2-CysPRX 90 IKVKLSEYIGKKYVILFFYPLDFTFVCPTEITAFSDRYAEFEKLNTEVLGVSVDVSFVSHLAWVQTDKSGGLGDLNYPLVSDVTKSISKSFVGLIPDQG
Ps2-CysPRX 96 IKVKLSDIYIGKKYVILFFYPLDFTFVCPTEITAFSDRHAEFEKLDTEILGVSVDVSFVSHLAWVQTDKSGGLGDLNYPLVSDVTKSISKAYDVLIIADQG
Pp2-CysPRX 97 IKVKLSEYIGKKYVILFFYPLDFTFVCPTEITAFSDRHAEFEELNTEILGVSVDVSFVSHLAWVQTDKSGGLGDLNYPLISDVTKSISKSYDVLIPDQG
Gm2-CysPRX 85 INVKLSDIYIGKKYVILFFYPLDFTFVCPTEITAFSDRHAEFEALNTEILGVSVDVSFVSHLAWIQTDKSGGLGDLNYPLISDVTKSISKSYGVLIPDQG
So2-CysPRX 97 INVKLSDIYIGKKYVILFFYPLDFTFVCPTEITAFSDRHGEFEKLNTEILGVSVDVSFVSHLAWVQTERKSGGLGDLNYPLVSDVTKSISKAFNVLIIPDQG
St2-CysPRX 94 IKVKLSEYIGKKYVILFFYPLDFTFVCPTEITAFSDRYEEFEKVNTEVLGVSVDVSFVSHLAWVQTERKSGGLGDLNYPLISDVTKSISKSYNVLIIPDQG
Ha2-CysPRX 87 IKVKLSDIYIGKKYVILFFYPLDFTFVCPTEITAFSDRYAEFEKINTEILGVSVDVSFVSHLAWVQTDKSGGLGDLNYPLVSDVTKSIKAFNVLIEDQG
Hv2-CysPRX 89 INVKLSDIYIGKKYVILFFYPLDFTFVCPTEITAFSDRHEEFEKINTEILGVSVDVSFVSHLAWVQTERKSGGLGDLKYPLVSDVTKSISKSFVGLIPDQG
Ta2-CysPRX 85 INVKLSDIYIGKKYVILFFYPLDFTFVCPTEITAFSDRHEEFEKINTEILGVSVDVSFVSHLAWVQTERKSGGLGDLKYPLVSDVTKSISKSFVGLIPDQG
Zm2-CysPRX 87 INVKLSDIYIGKKYVILFFYPLDFTFVCPTEITAFSDRYEEFEKLNTEVLGVSIDSFVSHLAWVQTDKSGGLGDLKYPLISDVTKSISKAFVGLIPDQG
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At2-CysPRXA 192 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYIQENPDEVCPAGWKPGGEKSMKDPKLSKEYFSAI 266
At2-CysPRXB 199 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYVQENPDEVCPAGWKPGGEKSMKDPKLSKEYFSAI 273
Cs2-CysPRX 193 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYIQENPDEVCPAGWKPGGEKSMKDPKLSKDYFAAI 267
Rs2-CysPRX 189 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYIQENPDEVCPAGWKPGGEKSMKDPKLSKEYFSAI 263
Ps2-CysPRX 195 IALRGLFIIIDKEGIIQHSTINNLGIGRSVDETMRTLQALQYVQDNPDEVCPAGWKPGGETMKPDTKLSKEYFSAI 269
Pp2-CysPRX 196 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETKRTLQALQYVQDNPDEVCPAGWKPGGEKSMKDPKLSKEYFSAI 270
Gm2-CysPRX 184 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETKRTLQALQYVQENPDEVCPAGWKPGGEKSMKDPKLSKDYFAAV 258
So2-CysPRX 196 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETLRTLQALQVQENPDEVCPAGWKPGGEKSMKDPKLSKEYFAA- 269
St2-CysPRX 193 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETLRTLQALQYVQENPDEVCPAGWKPGGEKSMKDPKLSKEYFSAI 267
Ha2-CysPRX 186 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYVQENPDEVCPAGWKPGGEKSMKDPKLSKEYFAAV 260
Hv2-CysPRX 188 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETLRTLQALQYVQENPDEVCPAGWKPGGEKSMKDPKLSKEYFAAI 262
Ta2-CysPRX 184 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETLRTLQALQYVQENPDEVCPAGWKPGGEKSMKDPKLSKEYFAAI 258
Zm2-CysPRX 186 IALRGLFIIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYVQENPDEVCPAGWKPGERSMKPDPKLSKEYFAAV 260
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**Figure S4: Sequence alignment of plant 2-Cys peroxiredoxins.**

NCBI or Genbank references: *Arabidopsis thaliana* 2-Cys PRXs A and B, NP\_187769.1 and NP\_568166.1, respectively; *Camelina sativa* (Cs), XP\_010464879.1; *Raphanus sativus* (Rs), XP\_018493095.1; *Papaver somniferum* (Ps), XP\_026426631.1; *Prunus persica* (Pp), XP\_007202444.1; *Glycine max* (Gm), NP\_001341836.1; *Spinacia oleracea* (St), XP\_021867340.1; *Solanum tuberosum* (St): XP\_006339159.1; *Helianthus annuus* (Ha), XP\_021984163.1; *Hordeum vulgare* (Hv), BAJ98505.1; *Triticum aestivum* (Ta), SPT18356.1; *Zea mays* (Zm), NP\_001137046.1. The motifs A and B, involved in hyperoxidation resistance of 2-Cys peroxiredoxins (Bolduc *et al.*, 2018), are highlighted in blue and yellow, respectively. The GGLG and YF motifs considered as a signature of sensitivity to hyperoxidation are highlighted in gray. The two catalytic Cys are highlighted in purple. Black bars indicate sequence divergence within motifs A and B.