

1 Function and regulation of chloroplast 2 peroxiredoxin IIE

3 Anna Dreyer^{1§}, Patrick Treffon^{1§§}, Daniel Basiry¹, Anna Maria Jozefowicz², Andrea Matros^{2‡},
4 Hans-Peter Mock², Karl-Josef Dietz^{1*}

5 ¹ Department of Biochemistry and Physiology of Plants, Faculty of Biology, University of Bielefeld, 33615
6 Bielefeld, Germany; ptreffon@umass.edu (PT); anna.dreyer@uni-bielefeld.de (AD); danielbasiry@gmx.de
7 (DB)

8 ² Applied Biochemistry Group, Leibniz Institute for Plant Genetics and Crop Plant Research (IPK), 06466
9 Seeland, OT Gatersleben, Germany; jozefowicz@ipk-gatersleben.de (AMJ); andrea.matros@icloud.com
10 (AM); mock@ipk-gatersleben.de (HPM)

11
12 [§] shared first authorship due to equal contribution to this research

13 ^{§§} current affiliation PT: Department of Biochemistry and Molecular Biology, University of Massachusetts
14 Amherst, USA

15 [‡] current affiliation AM: Australian Research Council Centre of Excellence in Plant Cell Walls, University of
16 Adelaide, SA 5064 Adelaide, Australia

17 ^{*} Correspondence: karl-josef.dietz@uni-bielefeld.de; Tel. +49-521-106-5589 (KJD)

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19 **Abstract:** Peroxiredoxins (PRX) are thiol peroxidases which are highly conserved throughout all
20 biological kingdoms. Increasing evidence suggests that their high reactivity toward peroxides has a
21 function not only in antioxidant defense but in particular in redox regulation of the cell.
22 Peroxiredoxin IIE is one of three PRX types found in plastids and has previously been linked to
23 pathogen defense and protection from protein nitration. However, its posttranslational regulation
24 and its function in the chloroplast protein network remained to be explored. Using recombinant
25 protein, it was shown that the peroxidatic Cys121 is subjected to multiple posttranslational
26 modifications, namely disulfide formation, S-nitrosation, S-glutathionylation and hyperoxidation.
27 Slightly oxidized glutathione fostered S-glutathionylation and inhibited activity *in vitro*.
28 Immobilized recombinant PRX-IIE allowed trapping and subsequent identification of interaction
29 partners by mass spectrometry. Interaction with the 14-3-3 ν protein was confirmed *in vitro* and was
30 shown to be stimulated under oxidizing conditions. Interactions did not depend on
31 phosphorylation as revealed by testing phospho-mimicry variants of PRX-IIE. Based on these data
32 it is proposed that 14-3-3 ν guides PRX-IIE to certain target proteins, possibly for redox regulation.
33 These findings together with the other identified potential interaction partners of type II PRXs
34 localized to plastids, mitochondria and cytosol provide a new perspective on the redox regulatory
35 network of the cell.

36 **Keywords:** Peroxiredoxin, AT3G52960, glutathione, S-glutathionylation, glutaredoxin, 14-3-3
37 protein, phosphorylation, posttranslational modification, redox regulatory network
38

39 1. Introduction

40 Chloroplasts of cormophytes contain three types of peroxiredoxins (PRXs), namely classical 2-
41 cysteine peroxiredoxin (2-CysPRX), a bacteroferritin-comigratory protein homologue PRX-Q and a
42 type II peroxiredoxin named PRX-IIE [1,2]. The basic PRX complement of plastids is subjected to
43 variation by the presence of isoforms, e.g., 2-CysPRX-A and -B in *Arabidopsis thaliana* (At3g11630,
44 At5g06290), and PRX-IIE-1 and PRX-IIE-2 in *Oryza sativa* (Os06g42000, Os02g09940) [3].

45 PRXs are thiol peroxidases. They possess a peroxidatic cysteinyl thiol (Cys_P) with a very low pK
46 value, and thus expose the deprotonated thiolate anion in a conserved catalytic environment. Because
47 of this particular feature, PRXs function as highly affine and efficient thiol peroxidases [4]. The
48 catalytic activity of 2-CysPRX, PRX-Q and type II PRX relies on a conserved second cysteine, which

49 acts as resolving thiol (Cys_R). Upon reaction with the peroxide substrate, the Cys_P-thiol forms a
50 sulfenic acid derivative which immediately is attacked by the Cys_R. A disulfide bond is formed
51 between Cys_P and Cys_R. Prior to the next catalytic cycle, the disulfide bond needs to be reduced to
52 dithiols by electron donors like thioredoxin (TRX) or glutathione/glutaredoxin (GRX). The different
53 PRX forms show variation in their primary sequence, as well as the presence and position of Cys_R
54 and the regeneration mechanism [2].

55 The best understood plant PRX is the 2-CysPRX, which has been scrutinized with respect to its
56 catalytic properties, redox-dependent conformational dynamics, regeneration by electron
57 transmitters such as TRX and GRX/GSH and its role in the redox regulatory network of the
58 chloroplast [5–8]. In contrast PRX-Q and PRX-IIIE have been studied for their peroxidatic property,
59 regeneration mechanism and their function in mutant plants with decreased protein amounts [9,10].
60 PRX-IIIE belongs to the group of highly conserved atypical 2-cysteine peroxiredoxins, initially
61 discovered in the phloem of *poplar* [11]. PRX-II type PRX are found in, e.g., some but not all
62 photosynthetic cyanobacteria [12], animals/humans (PRDX5) [13] and lower and higher plants [14].

63 Previous studies showed PRXIIIE to be localized in the soluble fraction of plastids. Its transcript
64 amount in *A. thaliana* leaves slightly increased in response to high light, decreased upon ascorbate
65 and NaCl treatment and remained unchanged with leaf age [1,15]. The redox titration of the dithiol-
66 disulfide transition gave a midpoint redox potential of -288 mV which is 19 and 34 mV, respectively,
67 less negative than 2-CysPRX-A and -B [1].

68 Re-reduction of the oxidized PRX-IIIE from *poplar* was most efficient with GRX/glutathione
69 system, and low with glutathione and TRX [14]. The catalytic efficiency was 10⁵ M⁻¹s⁻¹ with tertiary
70 butylhydroperoxide (t-BOOH), fourfold less with H₂O₂, and activity was absent with cumene
71 hydroperoxide (CuOOH) [14]. Among the three GRXs (GRX-S12, -S14 and -S16) targeted to the
72 plastid in *poplar*, GRX-S12 efficiently regenerated oxidized PRX-IIIE [14]. The authors suggested a
73 catalytic mechanism where the sulfenic acid derivative, formed by reaction with the peroxide
74 substrate, reacts with reduced glutathione to form a S-glutathionylated intermediate. GRX reduces
75 the Cys_P-SG and become itself S-glutathionylated. Another glutathione molecule then regenerates
76 GRX-SG, leading to the formation of oxidized glutathione(GSSG)[14,16].

77 This study aimed for the biochemical characterization of PRX-IIIE, with focus on redox-
78 dependent posttranslational modifications of its critical cysteine residues and their impact on protein
79 function. In the light of the presumed function in redox signaling, it appeared timely to address the
80 interactome of PRX-IIIE by affinity chromatography and mass-spectrometric identification. The
81 interaction with 14-3-3 *ν* was further validated and shown to slightly affect the peroxidase activity.
82 These novel findings point out to an additional role in redox signaling apart from its peroxidase
83 activity.

84 2. Materials and Methods

85 2.1. Plant material and growth conditions

86 *A. thaliana* Col-0 plants were grown on soil (SM Max Planck Köln, project 187509, Stender,
87 Germany) in a greenhouse with 14 h day and 10 h night at 25°C.

88 2.2. Cloning

89 PRX-IIIE (AT3G52960), GRX-S12 (AT2G20270) and sulfiredoxin (SRX; AT1G31170) were cloned
90 into pET28a (Novagen, Darmstadt, Germany). Forward and reverse primer were designed with *Nde*I
91 and *Bam*HI restriction sites, respectively (Table S1). The variants C121S, C146S, C121S/C146S, S82D,
92 T108E and T223E of PRX-IIIE were generated by site-directed mutagenesis with specific primers
93 (Table S1). Correctness of all constructs was confirmed by DNA sequencing.

94 2.3. Heterologous expression and purification

95 *E. coli* BL21 (DE3)pLysS cells (Invitrogen, La Jolla, CA, USA) were transformed with plasmid
96 DNA. Overnight cultures were used to inoculate 2 L lysogeny broth medium supplemented with

97 50 µg/mL kanamycin and 20 µg/mL chloramphenicol. Protein expression was induced in the
98 exponential phase by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of
99 0.4 mM. Induced cells were grown at 37°C and 140 rpm for 4 h. Cells were harvested for 15 min at
100 5,000 xg and resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole,
101 pH 8.0). Cells were disrupted using lysozyme digestion followed by sonication (HF-Generator
102 GM2070 in combination with an ultrasonic converter UW2070, standard horn SH 70G and microtip
103 MS73, Bandelin, Berlin, Germany). The soluble and insoluble fractions were separated by
104 centrifugation at 20,000 xg for 45 min. His-tagged proteins were incubated with Nickel-nitrilotriacetic
105 acid (NTA) sepharose (Qiagen, Hilden, Germany) at 4°C with slight shaking for 1 h. Washing was
106 performed with washing buffer I (50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole, pH 8.0)
107 and subsequently washing buffer 2 (50 mM NaH₂PO₄, 300 mM NaCl, 20 (v/v) glycerol and 50 mM
108 imidazole, pH 8.0) until the OD₂₈₀ reached zero. Elution was achieved with elution buffer (50 mM
109 NaH₂PO₄, 300 mM NaCl and 250 mM imidazole, pH 8.0). Protein containing fractions were pooled
110 and dialyzed against 40 mM K-Pi pH 7.2. After dialysis, protein concentrations were determined
111 using a molar extinction coefficient of 8480 M⁻¹ cm⁻¹ for PRX-IIIE and its variants and 9970 M⁻¹·cm⁻¹
112 and 4470 M⁻¹·cm⁻¹ for GrxS12 and Srx, respectively. GrxC5 (At4g28730) was purified following
113 established procedures [17,18].

114 2.4. Xylenol orange assay

115 The ferrous-dependent xylenol orange assay (FOX) was used to analyze the activity of PRX-IIIE
116 and its phosphomimic variants with DTT as electron donor. The reaction mixture contained 2 µM
117 PRX-IIIE and 4 mM dithiothreitol (DTT) in 40 mM K-Pi pH 7.2. The measurement was started by
118 addition of peroxides (400 µM H₂O₂, 200 µM t-BOOH or 200 µM CuOOH) in a time course of 90 sec
119 at 15 s intervals. The remaining peroxides were detected by ferrous-dependent oxidation of xylenol
120 orange as reported previously [19].

121 2.5. NADPH-dependent peroxidase activity measurement

122 Reduction of peroxides by PRX-IIIE was monitored with the GRX system as reductant. The
123 activity was measured using a Cary 300 Bio UV/VIS spectrometer (Varian, Middelburg, Netherlands)
124 following NADPH oxidation at 340 nm. The assay was performed at 25 °C in quartz cuvettes with
125 2 µM PRX-IIIE, 0.5 units glutathione reductase (GR), 200 µM NADPH, 1 mM EDTA, 1 mM GSH,
126 varying amounts of GrxS12 and of peroxides (H₂O₂, t-BOOH, CuOOH) in 40 mM K-Pi, pH 7.2.

127 2.6. Hyperoxidation of PRX-IIIE

128 Hyperoxidation of PRX-IIIE was assayed as described above using the FOX assay with 400 µM
129 H₂O₂ as substrate and increasing CuOOH concentrations. Furthermore, the oxidation state was
130 investigated by electrospray ionization coupled with mass spectrometry (ESI-MS; Esquire 3000,
131 Bruker Daltonics, Bremen, Germany). 10-20 µM of prereduced protein in 100 mM Tris-HCl, pH 8.0,
132 was incubated with 5 mM DTT and different CuOOH and 0.5 mM DTT and increasing H₂O₂
133 concentrations for 1 h at room temperature (RT). Excess low molecular weight reagents were
134 removed by acetone precipitation and proteins were resuspended in H₂O. Dilutions were prepared
135 in 30 % EtOH, 0.1 % formaldehyde (FA) and the mixture was introduced into the ESI-MS.
136 Instrumental settings: Capillary voltage = 4.000 V. Nebulizer gas pressure = 15 psi. Drying gas flow =
137 4.0 L/min. Drying gas temperature = 300 °C. Mass-to-charge (m/z) values: 650-1200. Mass spectra
138 were deconvoluted using the software provided by the manufacturer (DataAnalysis, Bruker
139 Daltonics, Bremen, Germany).

140 2.7. S-glutathionylation

141 10 – 30 µM PRX-IIIE in 100 mM Tris-HCl, pH 8, was reduced for 30 min at room temperature
142 (RT) with 4 mM DTT. Desalting was achieved by passing the solution through PD10 columns. S-
143 glutathionylation was carried out by disulfide exchange with oxidized glutathione (GSSG) for 1 h at

144 RT. Excess GSSG was removed via acetone precipitation. Afterwards, S-glutathionylation was
145 detected by Western blot using monoclonal anti-GSH antibody (Thermo Scientific, Schwerte,
146 Germany). In addition, molecular masses of modified and unmodified proteins were assessed by ESI-
147 MS as mentioned before. For deglutathionylation, 10 μ M glutathionylated PRX-IIIE was incubated
148 with 10 μ M of GrxS12, GrxC5 or SRX and 0.5 mM GSH at 25 °C. The decrease of glutathionylated
149 PRX-IIIE was determined using Western Blot with anti-GSH antibodies. The spot intensities were
150 analysed using ImageJ.

151 2.8. 2-Dimensional SDS-PAGE

152 6-week-old *A. thaliana* Col_0 plants were sprayed with 300 μ M methylviologen (MV) and 0.1%
153 (v/v) Tween-20 as control, respectively. After 3 h the plants were harvested and immediately frozen
154 in liquid nitrogen and grinded. Proteins were isolated and used for the separation in the first
155 dimension with Immobiline Dry Stripes (pH range 3-10 NL, 18 cm, GE Healthcare, Uppsala, Sweden)
156 [20]. To this end 250 μ g protein were dissolved in 340 μ L rehydration buffer (0.01% ampholyte;
157 0002 % (w/v) bromophenolblue) and applied to the Immobiline Dry Stripe. The rehydration and
158 focusing consisted of the following steps: 1 h 0 V, 12 h 30 V, 2 h 60 V, 1 h 500 V, 1 h 1000 V and 8000 V
159 until 42000 Vh were reached. Separation in the second dimension was done with 12 % non-reductive
160 SDS-PAGE. Afterwards, the gel was blotted to nitrocellulose membrane and subjected to Western
161 blotting with PRX-IIIE antibody. and peroxidase-labeled secondary antibodies. Detection was done
162 with ECL Substrate (GE Healthcare, Chicago, IL, USA) and X-ray films.

163 2.9. Subcellular localisation of PRX-IIIE

164 The open reading frame of PRX-IIIE from *A. thaliana* was cloned into the 35S-EYFP-NosT vector
165 using specific primers (Table S1) for *in vivo* subcellular localization of PRX-IIIE [21]. The resulting
166 construct consists of the PRX-IIIE preprotein fused to EYFP as reporter under control of the CaMV35S-
167 promoter. Transient expression in mesophyll protoplasts and confocal laser scanning microscopy
168 were performed as described by Seidel et al. [22]. Chloroplast isolation and fractionation for
169 subplastidial Western blot analysis with antibodies raised against PRX-IIIE were carried out according
170 to Muthuramalingam et al. [23].

171 2.10. Affinity chromatography and mass spectrometry

172 Reduced His-tagged PRX-IIIE (3 mg) or PRX-IIIE C146S (3 mg) were bound to 1 mL Ni-NTA resin
173 (Qiagen, Hilden, Germany) and used as affinity matrix. Ni-NTA matrix without PRX-IIIE served as
174 control. Leaves of about 5-week-old plants were homogenized in 50 mM Tris-HCl, pH 8.0, 1 mM
175 PMSF, filtrated through Miracloth. Clear protein extract was obtained via centrifugation (30 min at
176 20.000 rpm and 4°C). The supernatant (about 40 mg protein) was applied to the matrix and incubated
177 at RT with gentle agitation for 1.5 h. Non-bound material was removed by washing the column with
178 20 mL of 50 mM Tris-HCl, pH 8.0, and 20 mL of 50 mM Tris-HCl, pH 8.0, 200 mM NaCl. The first
179 elution step was achieved with 1 ml of 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 50 mM DTT and
180 incubation for 15 min at RT. The eluted fraction was collected and stored. Afterwards the columns
181 were washed with 10 mL 50 mM Tris-HCl, pH 8.0, 200 mM NaCl. During the second elution step the
182 material was incubated for 15 min at RT in 1 mL 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 50 mM DTT
183 and 500 mM imidazole. The samples of the first and second elution were trypsinated after
184 chloroform/methanol precipitation [24] and dissolved in 0.1 % TCA, 1 % acetonitrile. Peptides were
185 separated by reverse-phase nano LC and analyzed by electrospray ionization-mass spectrometry
186 (ESI-QTOF-MS) as described elsewhere [25]. Data were searched against the entries of UP000006548
187 3702 ARATH *A. thaliana* of the UniProt database using ProteinLynx Global Server 3.0.2. Proteins,
188 which were found in two out of three biological experiments with at least two peptides were accepted
189 for further analysis. In addition, proteins, which were identified in control sample (nonspecific
190 binding), were removed from protein lists. The LC-MS data are deposited using the e!DAL system of
191 IPK Gatersleben [26] and available at: the submission is under progress and will be available soon.

192 2.11. Far Western blot

193 Dilution series of recombinant 14-3-3 v protein were spotted on nitrocellulose membrane,
 194 together with a dilution series of PRX-IIE as calibration curve. The membrane was blocked with Tris-
 195 buffered saline (TBS), pH 7.5, containing 1 % (w/v) milk powder. The membrane was incubated with
 196 PRX-IIE or its phospho-mimicry variants in TBS with either 1 mM DTT or 100 μ M H₂O₂ over night at
 197 4°C. After three times washing with TBS for 5 min, proteins were detected using specific anti-PRX-
 198 IIE antibody, peroxidase-labeled secondary antibody against rabbit, ECL[®] substrate (GE Healthcare,
 199 Chicago, IL, USA) and X-ray films. Intensity quantification of the spots after documentation was done
 200 using ImageJ. The relative amounts of bound PRX-IIE were determined in the linear range of the
 201 blots.

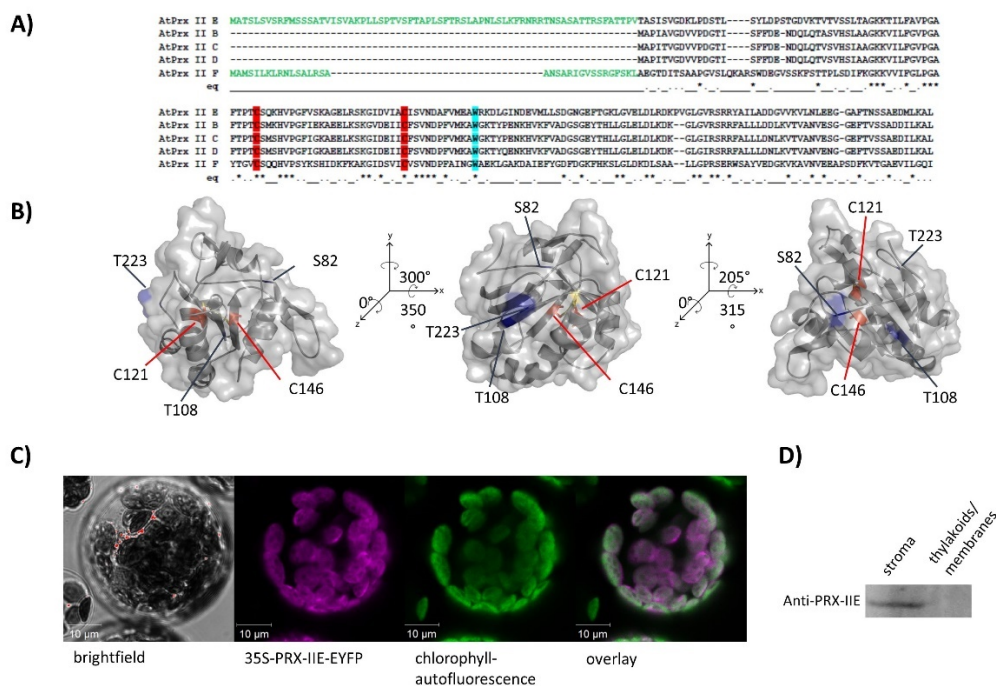
202 2.12. Structural modeling

203 PRX-IIE structure was obtained from SWISS-MODEL [27] based on the structure of *Populus*
 204 *tremula* PRX D type II (pdb: 1tp9A). Further analysis was done with PyMOL version 2.4.0 [28].

205 3. Results

206 3.1. PRX-IIE is localized to the chloroplast stroma

207 The PRX family in *A. thaliana* consists of 10 ORFs, of which 9 members are described to be
 208 expressed [29]. Besides the mitochondrial PRX-IIF, RRX-IIE is the only type II PRX in Arabidopsis
 209 which displays a putative transit peptide. Bioinformatic analysis of the preprotein sequence predicted
 210 an N-terminal chloroplastic transit peptide for the first 70 amino acids (Figure 1A). The remaining
 211 amino acids form the stable TRX-like structure with seven β -sheets and five α -helices (Figure 1B). To
 212 study the subcellular localization of PRX-IIE, a plasmid encoding the PRX-IIE-EYFP fusion protein
 213 was transfected into mesophyll protoplasts. Confocal laser scanning microscopy of the transfected
 214 protoplasts revealed the plastidial localization, as indicated in the overlay of the EYFP signal with
 215 the chlorophyll autofluorescence (Figure 1C). Western blot analysis of fractionated chloroplasts with
 216 anti-PRX-IIE antibody revealed a signal exclusively in the stromal fraction, whereas the
 217 thylakoid/membrane fraction lacked any PRX-IIE signal (Figure 1D).



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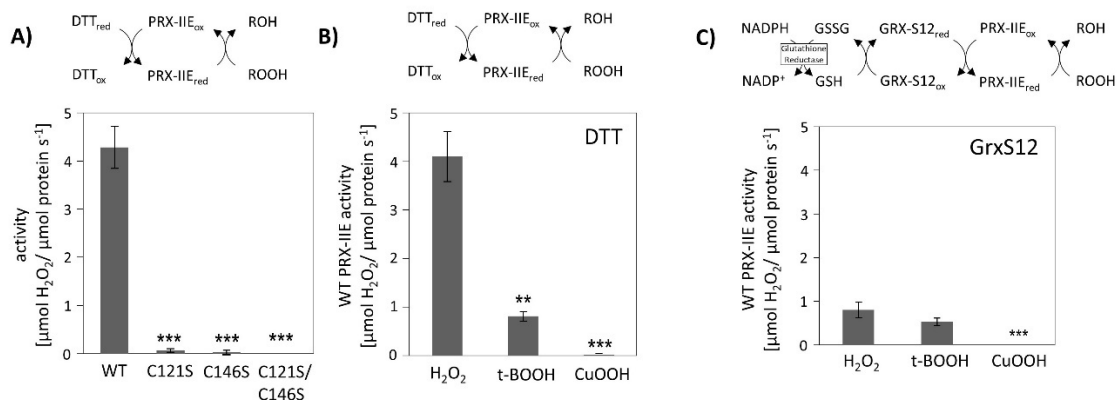
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Figure 1 Subcellular localization of PRX-IIE. A) Amino acid sequence alignment of *A. thaliana* type II PRXs. The signal peptides of PRX-IIE and PRX-IIF are highlighted in green as well as the highly

222 conserved cysteines (C: red) and tryptophan (W: blue). B) Predicted 3D structure of mature PRX-IIIE
 223 from three different points of view. The model is based on the PRX D type II from *P. tremula*
 224 (pdb:1tp9A) and further analysis was done using PyMOL 2.4. Both cysteines, C121 and C146 are
 225 marked in red, whereas the possible phosphorylation sites S82, T108 and T223 are marked in blue.
 226 The distance between the cysteines are 7.83 Å. The accessible surface area of the peroxidatic cysteine
 227 at position 121 is 0.795 Å². C) The coding sequence of PRX-IIIE was fused in frame to EYFP as reporter
 228 and used for transient expression in *A. thaliana* mesophyll protoplasts. Confocal laser scanning images
 229 reveal chlorophyll autofluorescence (green) and fluorescence of the PRX-IIIE-EYFP construct (purple).
 230 White areas in the overlay indicate chloroplastic localization of PRX-IIIE. D) Subplastidal localization
 231 of PRX-IIIE was analyzed in isolated intact chloroplasts fractionated into stroma and membrane
 232 fraction by ultracentrifugation. Equal amounts of both fractions (50 µg protein) were loaded per lane
 233 and PRX-IIIE was identified in chloroplast stroma using specific anti PRX-IIIE antibody.

234 3.2. PRXIIIE detoxifies H₂O₂ using the GRX system for regeneration

235 Peroxiredoxins reduce a broad range of peroxides and their activities rely on the conserved
 236 cysteine residues. Reduction of H₂O₂ by PRX-IIIE and its cysteine variants was determined using the
 237 FOX assay. The removal of either the Cys_P as well as the Cys_R residue has a negative impact on
 238 peroxidase activity, constraining that both thiol groups are necessary for reactive oxygen species
 239 (ROS) scavenging (Figure 2A). Substrate specificity with H₂O₂, t-BOOH and CuOOH of wild-type
 240 (WT) protein is depicted in Figure 2B. PRX-IIIE showed the highest rate of activity with H₂O₂ as
 241 substrate and DTT as reductant. Reduction rates of t-BOOH and CuOOH relative to H₂O₂ were about
 242 20 % and 0.5 %, respectively. This indicates that H₂O₂ is the preferred substrate for PRX-IIIE.
 243 Furthermore, peroxide reduction by PRX-IIIE in the presence of chloroplastic glutaredoxin-S12 (GRX-
 244 S12) as reductant was determined (Figure 2C) and confirmed H₂O₂ as preferred substrate, but unlike
 245 with DTT, lower activities were recorded.
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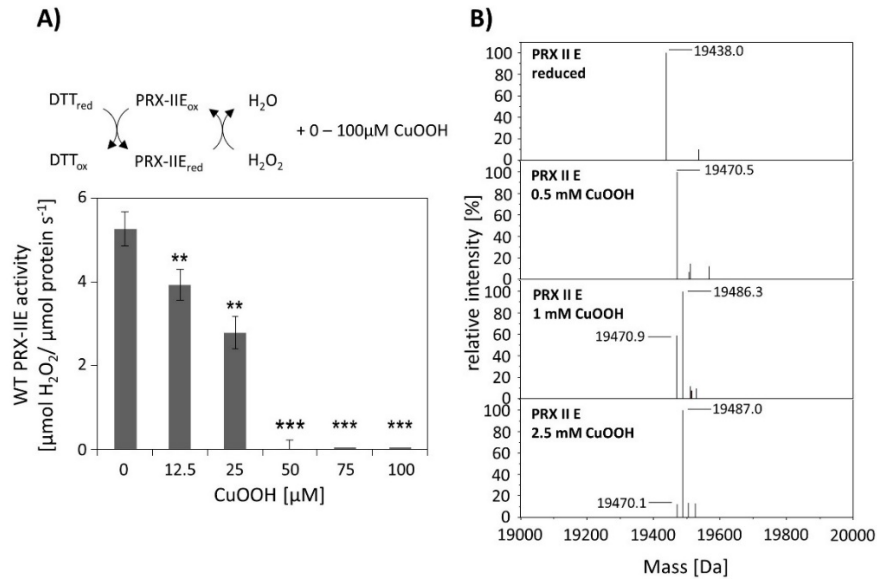


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 248 **Figure 2 Peroxidase activity.** A) Peroxide reduction by wild-type PRX-IIIE and its cysteine variants
 249 was measured with 400 µM H₂O₂, 4 mM DTT and 2 µM protein. The decrease in H₂O₂ was quantified
 250 with the FOX assay. Data are means ± SD, n = 30 with protein of three independent protein
 251 purifications. ***: p < 0.001. B) Substrate specificity was tested with the FOX assay with 4 mM DTT as
 252 reductant and different peroxides (400 µM H₂O₂, 200 µM t-BOOH or 200 µM CuOOH). Data are
 253 means ± SD, n = 18-28 with protein from three independent protein purifications. **: p < 0.01; ***: p <
 254 0.001. C) PRX-IIIE activity in the presence of GRX-S12 as monitored as NADPH oxidation at 340 nm
 255 in an enzyme-coupled reduction. Data are means ± SD, n = 9 with protein from three independent
 256 protein purifications. **: p < 0.01; ***: p < 0.001.

257 3.3. CuOOH- and H₂O₂-dependent hyperoxidation

258 CuOOH is a strong oxidizing agent and based on activity measurements (Figure 2B) it was
 259 assumed that PRX-IIIE is hyperoxidized by CuOOH. This hypothesis was tested *in vitro* using the FOX
 260 assay at increasing CuOOH concentrations (Figure 3A). A significant inhibition of H₂O₂ detoxification

261 could be detected in the presence of 12.5 μM CuOOH and the peroxidase activity was undetectable
262 at high CuOOH concentration. To further address the possibility for overoxidation of PRX-IIIE by
263 CuOOH, ESI-MS analysis was carried out (Figure 2 B). In contrast to reduced PRX-IIIE with a
264 molecular mass of 19438.0 Da, the sample treated with 0.5 mM CuOOH showed a mass increase of
265 ~32 Da which corresponds to the formation of the sulfinic acid derivative (-SO₂H). Higher CuOOH
266 concentrations lead to further oxidation to the sulfonic acid (-SO₃H).



267

268 **Figure 3 Hyperoxidation of PRX-IIIE.** A) Activity of 2 μM PRX-IIIE was determined with 400 μM H₂O₂
269 as substrate and 4 mM DTT using the FOX assay. CuOOH concentrations >50 μM completely
270 inhibited the peroxidase activity. Data are means \pm SD, n = 9 with protein of three independent protein
271 purifications. **: p < 0.01; ***: p < 0.001. B) Deconvoluted ESI-MS spectra of wild-type PRX-IIIE after
272 treatment with CuOOH as described in Materials and Methods. 19438.0 is the expected mass of the
273 reduced (-SH), His-tagged PRX-IIIE protein. The peak at 19470.5 Da after incubation with 0.5 mM
274 CuOOH shows a mass increase by 32 Da, which corresponds to the formation of the sulfinic acid
275 derivative (-SO₂H). Higher CuOOH concentrations lead to further oxidation to the sulfonic acid
276 (-SO₃H). Data are representative spectra of n = 15 measurements with protein of three independent
277 protein purifications.

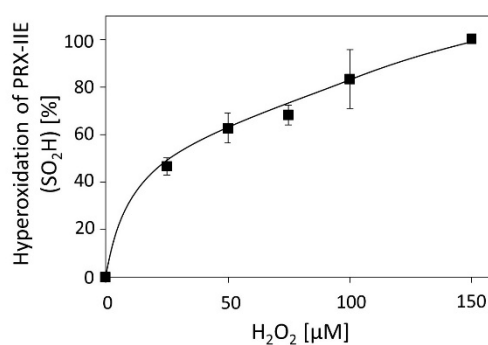
278 ESI-MS analysis of cysteine variants of PRX-IIIE was performed to elucidate which of the two
279 cysteines was modified (Table 1). Deconvoluted data revealed that hyperoxidation occurs in the
280 C146S variant, lacking the Cys_R, while the PRX-IIIE protein variant lacking Cys_P (C121S) showed only
281 a slight oxidation at high CuOOH concentrations.

282 **Table 1 CuOOH-dependent thiol modifications of PRX-IIIE variants.** Proteins were treated with
283 DTT and CuOOH for 1 h at RT and then analyzed by ESI-MS. Generation of the oxidized (-SOH) and
284 hyperoxidized (-SO₂H, -SO₃H) forms increased the mass of the protein by 16, 32 or 48 Da. Data are
285 means \pm SD, n = 10 with protein from two independent purifications.

Treatment	PRX-IIIE C121S		PRX-IIIE C146S	
	Mass [Da]	Cys modification	Mass [Da]	Cys modification
5 mM DTT (control)	19,422.93 ± 0.61	None (SH)	19,422.22 ± 0.52	None (SH)
0.5 mM CuOOH	19,422.75 ± 0.57	None (SH)	19,422.57 ± 0.55 19,454.61 ± 0.44	None (SH) Hyperoxidation (SO ₂ H)
1 mM CuOOH	19,423.13 ± 0.22	None (SH)	19,422.86 ± 0.87 19,454.93 ± 0.62	None (SH) Hyperoxidation (SO ₂ H)
2.5 mM CuOOH	19,423.31 ± 0.17	None (SH)	19,422.46 ± 0.86 19,454.51 ± 0.92 19,471.37 ± 0.81	None (SH) Hyperoxidation (SO ₂ H) Hyperoxidation (SO ₃ H)
5 mM CuOOH	19,422.95 ± 0.54	None (SH)	19,422.52 ± 0.41 19,453.86 ± 0.53 19,470.75 ± 0.96	None (SH) Hyperoxidation (SO ₂ H) Hyperoxidation (SO ₃ H)
10 mM CuOOH	19,422.97 ± 0.57 19,438.11 ± 0.92	None (SH) Oxidation (SOH)	19,423.25 ± 0.69 19,454.14 ± 0.98 19,470.73 ± 0.81	None (SH) Hyperoxidation (SO ₂ H) Hyperoxidation (SO ₃ H)

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Activity measurements for PRX-IIIE revealed that H₂O₂ is the preferred substrate (Figure 2B), but H₂O₂ is also known to catalyze hyperoxidation of proteins [18]. To test this for PRX-IIIE, peroxide-mediated hyperoxidation was analyzed by ESI-MS (Figure 4). The extent of sulfinic acid formation was tentatively estimated from the ratio of the peak intensities for the reduced (-SH, 19438.0 Da) and hyperoxidized (-SO₂H, 19470.0 Da) protein in the deconvoluted ESI-MS spectra. Incubation of PRX-IIIE with 25 μM H₂O₂ resulted in an hyperoxidation rate of 46 %, and higher peroxide concentrations lead to further hyperoxidation of the protein. Masses that correspond to the sulfonic acid (-SO₃H) could not be detected, suggesting that H₂O₂-mediated hyperoxidation is limited to the formation of the sulfinic acid derivative at Cys_{SP}.



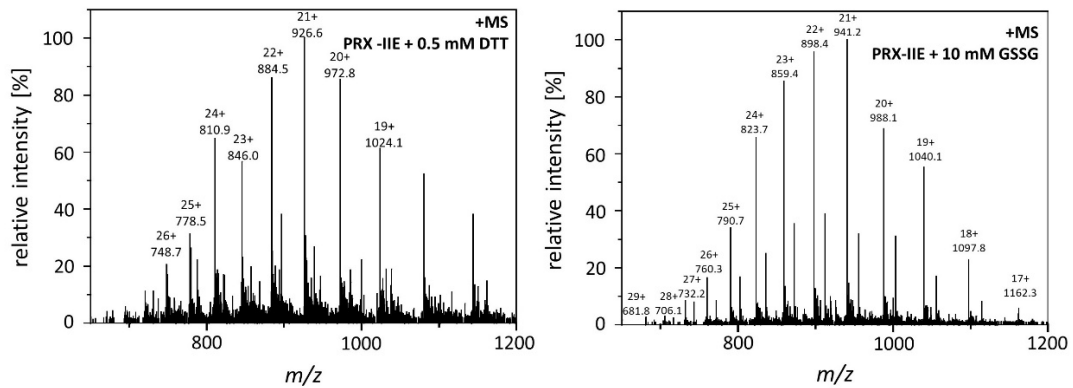
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Figure 4 H₂O₂-dependent hyperoxidation of PRX-IIIE. The extent of sulfinic acid formation in percent of total was estimated from the ratio of the peak intensities for the reduced (SH; 19.438 Da) and hyperoxidized (SO₂H; 19.470 Da) protein in the deconvoluted ESI-MS spectra. Data are means of n = 10 ± SD with recombinant protein from two independent purifications.

302 3.4. S-Glutathionylation of PRX-IIIE occurs at Cys_{SP}

303 The results shown above revealed a lower peroxidase activity for PRX-IIIE with GRXs as electron
304 donor in comparison to DTT as reductant (Figure 2C). The antioxidant glutathione is one component

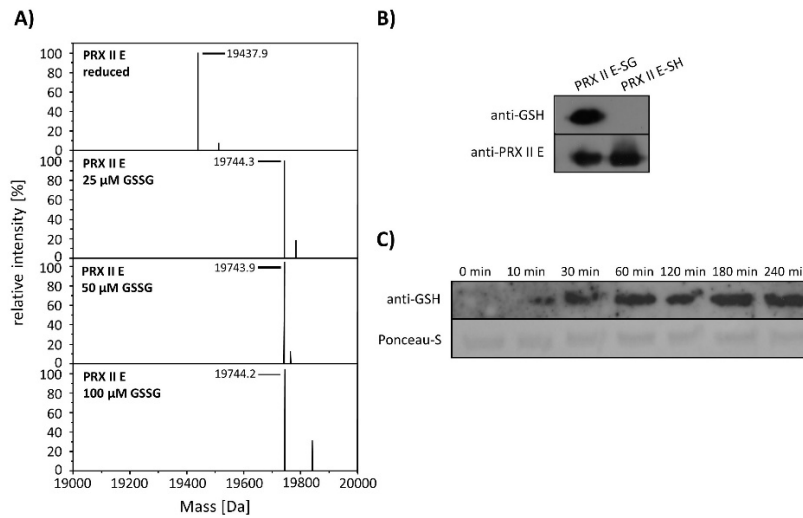
305 of the assay. In addition, H₂O₂-dependent hyperoxidation of PRX-III-E could be observed (Figure 4).
306 For *A. thaliana* and *T. brucei* reversible S-glutathionylation, the addition of one glutathione molecule
307 to specific cysteine residues, has been shown to prevent 2-Cys PRX to hyperoxidize and thereby
308 regulates its function [30,31]. In order to analyze the possibility for this redox-related
309 posttranslational modification, reduced PRX-III-E was incubated with either 0.5 mM DTT or 10 mM
310 oxidized glutathione (GSSG) overnight at 4°C. Following acetone precipitation samples were
311 subjected to ESI-MS and intact masses of the protein were obtained (Figure 5)
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313

314 **Figure 5 Mass spectra of reduced and S-glutathionylated PRX-III-E.** S-glutathionylation was carried
315 out by disulfide exchange with GSSG. The reaction mixtures containing 30 μM reduced PRX-III-E
316 without (left diagram) or with 10 mM GSSG (right diagram) in Tris buffer, pH 8.0, were incubated at
317 4°C for 18 h. The protein products were precipitated and subsequently subjected to ESI-MS analysis.

318 Deconvoluted data revealed the addition of one glutathione residue that increased the mass of
319 the protein by 306 Da (Figure 6A). The PTM of PRX-III-E was further proven with specific anti-GSH
320 antibody (Figure 6B). In addition, S-glutathionylation of PRX-III-E at low physiological GSSG
321 concentrations could be observed (Figure 6A). The time-dependent S-glutathionylation *in vitro*
322 was detected after incubation with GSSG for different time periods (Figure 6C). Thiol modification was
323 observed already after 10 min and reached a maximum at 60 min. The results demonstrate the fast S-
324 glutathionylation of PRX-III-E at physiological relevant concentrations *in vitro*. Not only incubation of
325 PRX-III-E with GSSG results in S-glutathionylation, but also incubation of pre-reduced PRX-III-E with
326 1 or 5 mM S-nitrosoglutathione. Besides this, formation of N-nitrosation (19.469 kDa, -SNO) and S-
327 nitrosoglutathionylation (19.772 kDa, -SSGNO) was observed, using ESI-MS.
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Figure 6 S-glutathionylation of PRX-III E. A) Deconvoluted mass spectra of reduced and S-glutathionylated PRX-III E after treatment with oxidized glutathione (GSSG). Reduced PRX-III E (19,438 Da) was treated with indicated GSSG concentrations and the degree of modification was estimated from the mass shift of 306 Da to 19744 Da, which could be assigned to monoglutathionylated protein. Data are representative spectra of $n = 12$, with protein from three independent protein purifications. B) Western blot analyses of reduced and S-glutathionylated PRX-III E (treatment with 10 mM GSSG) using specific anti-GSH and anti-PRX-III E antibody. C) Time dependence of S-glutathionylation with recombinant PRX-III E *in vitro*.

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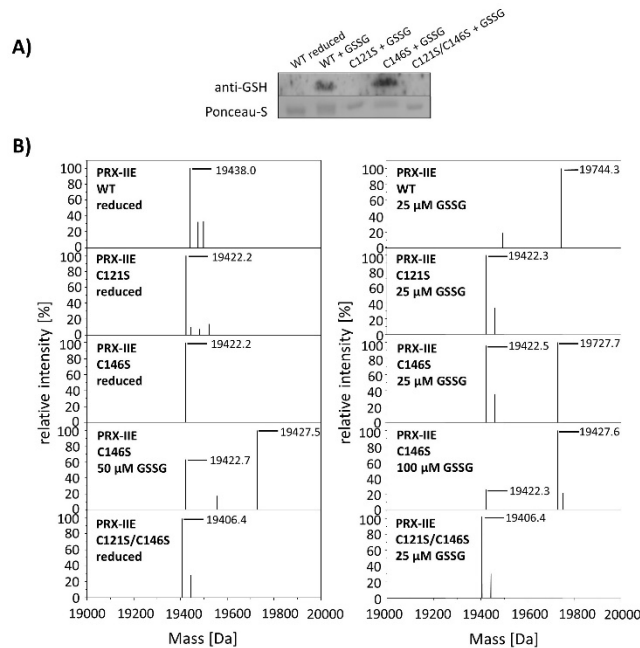
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To test for the particular residue that is prone to S-glutathionylation, Cys to Ser variants were analyzed using Western Blot with specific anti-GSH antibody. Results showed S-glutathionylation of WT PRX-III E and the C146S variant, but not on the C121S variant (Figure 7). Furthermore, mass spectrometry was done with the WT and the Cys→Ser variants. 19422 Da correspond to the reduced variants with single mutated cysteine residues (C121S or C146S) and 19406 Da to the double mutant. Only the C146S protein, lacking the Cys_R at position 146, was S-glutathionylated in a concentration-dependent manner of GSSG (Figure 7B).



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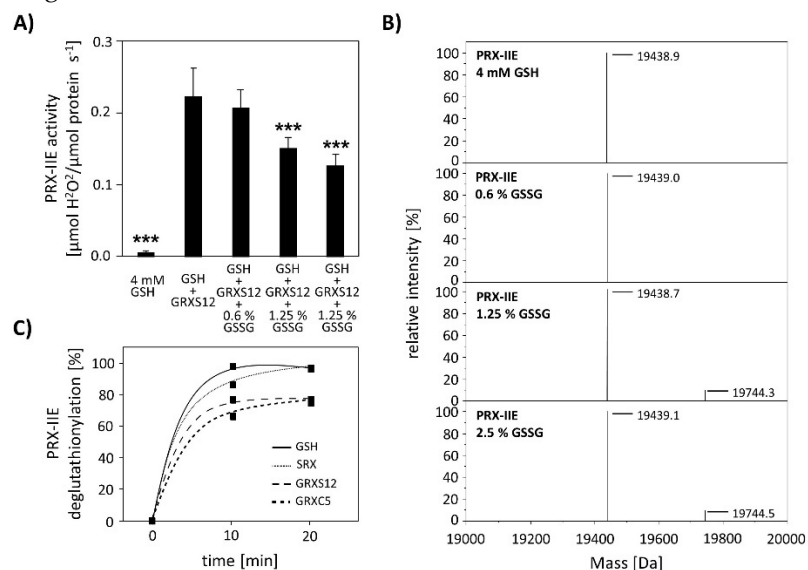
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Figure 7 Identification of S-glutathionylated Cys residue in PRX-III E using Cys→Ser variants. To test for the site of glutathionylation, Cys→Ser mutated PRX-III E variants were analyzed by Western

349 blot with specific anti-GSH antibody and by mass spectroscopy. A) 10 μ M reduced WT PRX-IIIE and
350 variants (C121S; C146S; C121S/C146S) were incubated with 10 mM GSSG for 1 h at RT and subjected
351 for Western blot analysis. B) Representative deconvoluted ESI-MS spectra of PRX-IIIE and variants
352 treated with GSSG. 19438 Da corresponds to the theoretical mass of the reduced and His-tagged
353 protein, whereas a shift of 306 Da to 19744 was observed for the glutathionylated version of PRX-IIIE
354 with a single bound glutathione molecule. 19422 Da correspond to the reduced variants with single
355 mutated cysteinyl residues (C121S; C146S) and 19406 Da to the double mutant. Only the C146S
356 protein, lacking the Cys_{SR}, was glutathionylated in a concentration-dependent manner. The figure
357 shows representative spectra of n = 6 determinations, using two independent protein purifications.

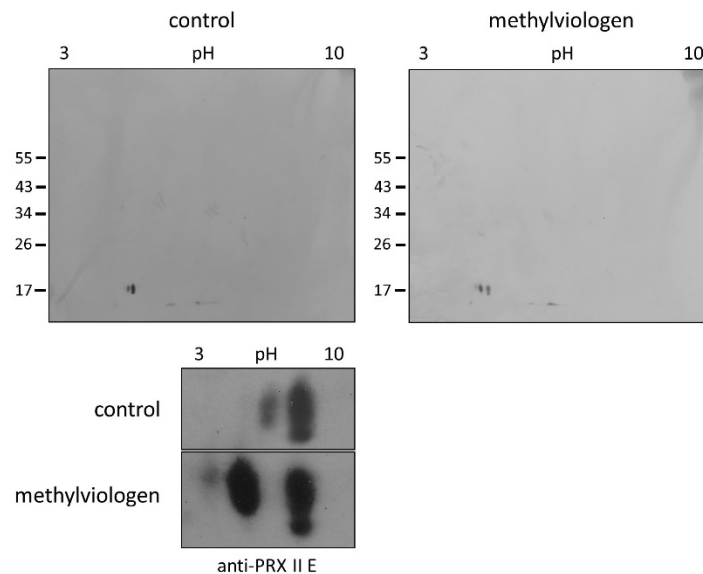
358 Next, the effect of S-glutathionylation on peroxidase activity was investigated using a modified
359 FOX assay (Figure 8A). Peroxidase activity in the presence of GRX-S12, GSH and H₂O₂ as substrate
360 decreased in the presence of GSSG amounts as low as < 2.5 % GSSG of total glutathione. The extent
361 of glutathionylation was tested by ESI-MS at different GSH/GSSG ratios (Figure 8B). Deconvoluted
362 spectra showed the presence of PRX-IIIE-SG already at 1.25 % GSSG, which is in line with the
363 decreased peroxidase activity. Protein-SG forms through several mechanisms *in vitro*, however the
364 precise reaction mechanism *in vivo* remains unclear [32]. In contrast, deglutathionylation reaction is
365 reported to be catalyzed by GRXs and SRXs [33,34]. To monitor the deglutathionylation of PRX-IIIE,
366 PRX-IIIE-SG was incubated with GSH in the presence or absence of GRX-S12, GRX-C5 and SRX
367 (Figure 8C). Western blot analysis with specific GSH antibody revealed a decrease in signal intensity
368 for PRX-IIIE-SG after 20 min of reaction time with GSH alone. The addition of SRX or GRX did not
369 increase the deglutathionylation reaction, leading to the conclusion that the glutathione pool itself is
370 capable of modulating the redox state of PRX-IIIE.



371
372 **Figure 8 Effect of glutathionylation on peroxidase activity and deglutathionylation reaction.** A)
373 Titration of peroxidase activity of PRX-IIIE in the presence of GRX-S12, GSH and H₂O₂ and increasing
374 amounts of GSSG using the FOX assay. Glutathionylation and inhibition of activity occurred at <2.5 %
375 GSSG of total glutathione. Data are means of n=10 ± SD with protein of two independent protein
376 purifications; *** p ≤ 0.001. B) PRX-IIIE was treated with different GSH/GSSG ratios and
377 glutathionylation was determined via ESI-MS. Deconvoluted spectra reveal the presence of
378 glutathionylated PRX-IIIE already at 1.25 % GSSG. (C) Time course of the deglutathionylation reaction.
379 10 μ M PRX-IIIE-SG were incubated together with 0.5 mM GSH and equal amounts of sulfiredoxin
380 (SRX), GRX-S12 or GRX-C5 in 100 mM Tris-HCl, pH 8.0, at 37 °C for indicated time and the decrease
381 in signal intensity was monitored over time using anti-glutathione antibody.

382 Protein S-glutathionylation ensures protection of critical protein thiols against irreversible
383 overoxidation *in vivo* and is therefore considered as biomarker for oxidative stress [35,36]. To test
384 redox-dependent posttranslational modifications of PRX-IIIE *in vivo*, *A. thaliana* plants were sprayed

385 with a single dose of 300 μ M methylviologen, harvested after 3 h and analyzed using non-reducing
386 two-dimensional gel electrophoresis following Western blot with specific anti-PRX-IIIE antibody. In
387 contrast to the mock treated samples, which showed two protein spots representing reduced (-SH)
388 and presumably oxidized protein species, plants stressed with MV exhibited three distinct protein
389 spots, two of them differed in acidity and molecular mass (Figure 9). Mature PRX-IIIE-SH displays a
390 molecular mass of 17260 Da with a theoretical pI value of 5.02, whereas for PRX-IIIE-SG a mass shift
391 to 17566 Da and a more acidic pI value of 4.91 are predicted. Molecular mass and pI values were
392 obtained using the ExPASy ProtParam tool [37]. Theoretical pI calculations and correlation with the
393 pI values observed on the 2D gels together with the mass change, suggest that the acidic spots of the
394 triplet correspond to the glutathionylated protein.

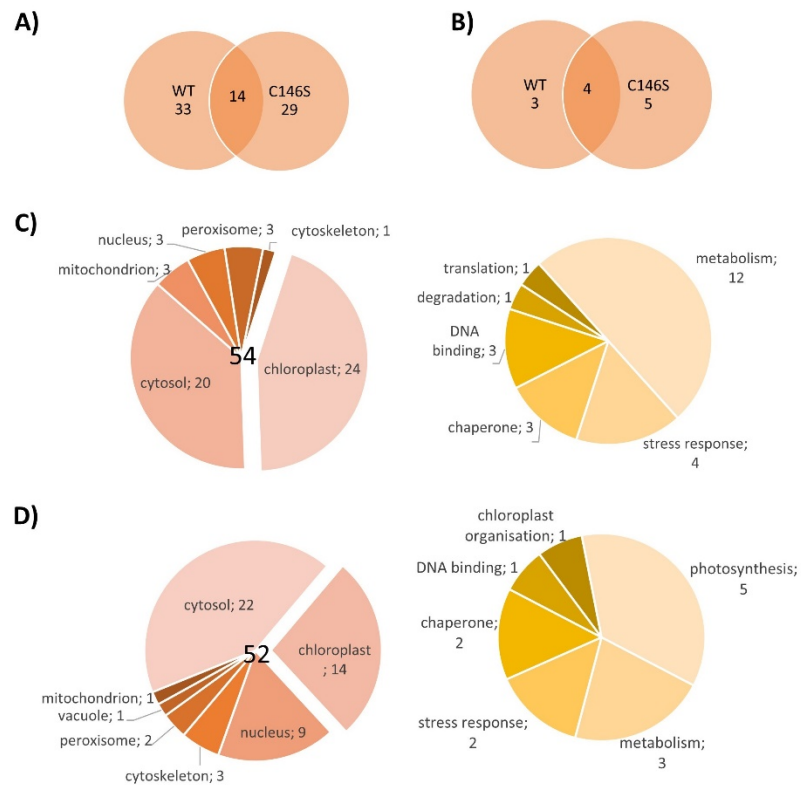


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396 **Figure 9 Detection of glutathionylated PRX-IIIE species *in vivo*.** *A. thaliana* plants were stressed for
397 3 h with 300 μ M MV or 0.1 % (v/v) Tween-20 as control. S-glutathionylation was detected with specific
398 anti-PRX-IIIE antibody following separation by non-reducing 2D-SDS-PAGE and blotting. The figure
399 shows representative Western blots from two independent experiments.

400 3.5. Identification of PRX-IIIE interaction partners

401 The next experiments aimed to elucidate, whether PRX-IIIE exclusively functions as a peroxidase
402 or if it might be involved in cell signaling by direct protein-protein interactions. For that, an affinity
403 chromatography approach with immobilized His₆-tagged PRX-IIIE and total *A. thaliana* leaf protein
404 was used to identify interacting proteins. After washing, bound proteins were eluted with buffer
405 containing DTT or imidazole. They were subsequently identified by mass spectrometry. In addition,
406 a thiol-trapping experiment with the C146S variant was used in order to identify redox-regulated
407 proteins that preferentially interact through the Cys_p. The reductive elution with DTT resulted in the
408 identification of 47 proteins, 14 of which were also identified to interact with the C146S variant
409 (Figure 10A). To address proteins that interact with PRX-IIIE electrostatically, a second elution was
410 done with imidazole. Seven proteins co-eluted with PRX-IIIE, and 9 with the PRX-IIIE C146S variant
411 (Figure 10B). Out of the 54 proteins that interacted with PRX-IIIE, 24 are localized within the
412 chloroplast and half of them are involved in metabolic pathways (Figure 10C). However, in
413 comparison to the WT protein, almost the same number of proteins were trapped by the C146S
414 variant, but only 14 of them reside in the chloroplast (Figure 10D).



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Figure 10 Localization and function of PRX-IIIE interaction partners. Venn diagrams depicting unique and overlapping interactors of PRX-IIIE WT and C146S variant after A) elution with DTT and B) second elution with imidazole. Identified interacting proteins with C) PRX-IIIE WT or D) PRX-IIIE C146S during first and second elution, were grouped according to their localization and function. See also supplementary file.

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Interestingly, 4 out of the 24 identified chloroplast proteins trapped by the WT PRX-IIIE protein belong to the 14-3-3 family (Table 2). In total 6 different 14-3-3 proteins were identified regardless of the bait protein (Table S 2). Plant 14-3-3 proteins are reported to be involved in multiple developmental and stress related processes, such as apoptosis, leaf shape and salt stress tolerance [38–40]. They normally occur as homodimers or heterodimers and are able to bind two different targets at the same time and, therefore, act as scaffold proteins [41]. Furthermore, they are involved in signaling processes regulated by phosphorylation [42–44]. Since PRX-IIIE has three experimentally reported phosphorylation sites [45], an interaction between PRX-IIIE and a 14-3-3 protein could be part of a signaling process. To study the interaction between PRX-IIIE and 14-3-3 proteins in more detail, 14-3-3 *v* was chosen as a representative for the 14-3-3 protein family and used in further experiments.

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Table 2 Chloroplast localized interaction partners of PRX-IIIE WT. Listed are the proteins identified from elution with DTT (above the line) and imidazole (below the line), with AGI code and uniprot accession number

AGI code	Protein accession	Protein name
AT4G09000	P42643	14-3-3 χ
AT5G10450	P48349	14-3-3 λ
AT3G02520	Q96300	14-3-3 <i>v</i>
AT5G16050	P42645	14-3-3 <i>v</i>
AT3G60880	Q9LZX6	4-Hydroxy-tetrahydrodipicolinate synthase 1
AT1G02560	Q9S834	ATP-dependent Clp protease proteolytic subunit 5
AT5G03690	F4KGQ0	Fructose-bisphosphate aldolase 4
AT4G26530	O65581	Fructose-bisphosphate aldolase 5

AT5G49910	Q9LTX9	Heat shock 70 kDa protein 7
AT2G24200	P30184	Leucine aminopeptidase 1
AT5G45930	Q5XF33	Magnesium-chelatase subunit ChII-2
AT1G70890	Q9SSK5	MLP-like protein 43
AT5G26000	P37702	Myrosinase 1
AT3G62030	P34791	Peptidyl-prolyl cis-trans isomerase CYP20-3
AT2G29630	O82392	Phosphomethylpyrimidine synthase
AT5G52920	Q9FLW9	Plastidial pyruvate kinase 2
AT1G32440	Q93Z53	Plastidial pyruvate kinase 3
AT5G52520	Q9FYR6	Proline tRNA ligase
AT2G21170	Q9SKP6	Triosephosphate isomerase
AT4G17090	O23553	β -amylase 3
AT3G01500	P27140	β -carbonic anhydrase 1
AT5G14740	P42737	β -carbonic anhydrase 2
AT5G64460	Q9FGF0	Phosphoglycerate mutase-like protein 1

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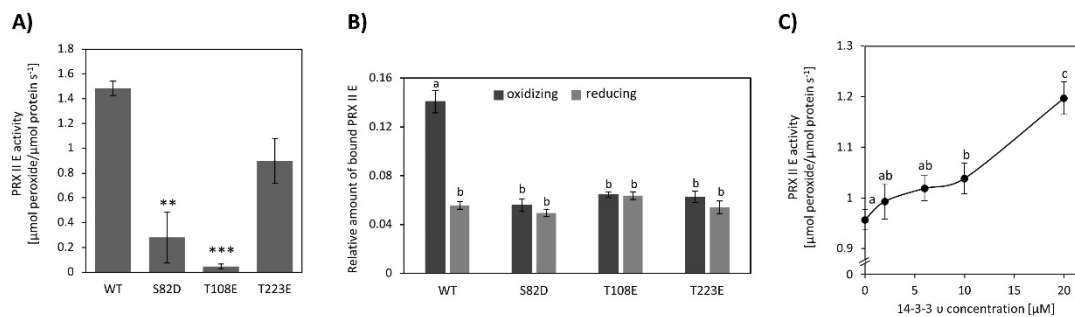
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To characterize the interaction between 14-3-3 ν and PRX-IIIE, phospho-mimicry variants of PRX-IIIE were generated and their peroxidase activity was analyzed using the FOX-Assay. Interestingly, all variants revealed lower peroxidase activity in comparison to WT PRX-IIIE, whereas S82D and T108E showed a significant lower activity (Figure 11A). Binding between PRX-IIIE or its phosphomimic variants and 14-3-3 ν under defined redox-conditions conditions was assessed in an overlay approach (Figure 11B). Binding was similar under all conditions apart from the 2.5-fold improved binding of WT PRX-IIIE to 14-3-3 ν under oxidizing conditions (Figure 11B). Supplementation with 14-3-3 ν had a beneficial effect on peroxidase activity albeit a five-fold excess of 14-3-3 ν was necessary to observe a significant increase of PRX-IIIE peroxidase activity (Figure 11C).



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Figure 11 Influence of phospho-mimicry point-mutations on the activity of PRX-IIIE and the interaction with 14-3-3 ν . A) Peroxide reduction by wildtype PRX-IIIE and its phospho-mimicry variants were measured with 400 μ M H₂O₂, 4 mM DTT and 2 μ M protein. The decrease in H₂O₂ was quantified with the FOX assay. Data are means of $n = 10-16 \pm$ SE with protein from three independent protein purifications. Significant differences to the WT protein were calculated using Student T-Test whereas **: $p < 0.01$; *** $p < 0.001$. B) Overlay assay of 14-3-3 ν with PRX-IIIE. A higher affinity of PRX-IIIE to 14-3-3 ν was observed under oxidizing conditions. Data are means of $n = 13-22 \pm$ SD. Significant differences were calculated using Student T- Test and are indicated by different letters $p \leq 0.05$. C) Influence of 14-3-3 ν on the peroxidase activity of PRX-IIIE. The activity of PRX-IIIE was measured in the presence of different concentrations of 14-3-3 ν with FOX Assay. Data are means of $n = 12-18 \pm$ SE. Different letters indicate significant differences calculated with Student T-Test at $p \leq 0.05$.

457

458 4. Discussion

459 PRX-IIIE is a thiol dependent peroxidase that is localized to the chloroplast stroma (Figure 1C, D).
460 Two cysteinyl residues are highly conserved within the type II PRX (Figure 1A) and mutation of
461 either one or both cysteines to serine results in a loss of peroxidase activity (Figure 2A). Therefore,
462 both Cys, namely Cys_p121 and Cys_{sr}146, are essential for the PRX-IIIE peroxidase activity.

463 The human homologue peroxiredoxin 5 (PRDX5) more readily reduces t-BOOH [46], while PRX-
464 IIE from *A. thaliana* showed the highest activity with H₂O₂ as substrate (Figure 2B, C). The differences
465 in substrate specificity could be due to the different accessible surface areas of Cys_p of both enzymes.
466 The accessible surface area of the Cys_p of PRDX5 is 1.305 Å², whereas the accessible surface area of
467 the PRX-IIIE Cys_p is just 0.795 Å² (Figure 1B). Therefore, PRX-IIIE seems more likely to detoxify smaller
468 peroxides in comparison to the human analogue PRDX5.

469 4.1. Regeneration of reduced PRX-IIIE limits catalytic turnover

470 The GRX-S12-coupled assay revealed the same substrate preference as the FOX assay, however,
471 the catalytic activities were lower in comparison to the DTT-driven activity, especially in case of H₂O₂
472 as substrate (Figure 2C). Since activity measurements with t-BOOH showed almost the same values,
473 it seems that PRX-IIIE regeneration by GRX-S12 is the rate-limiting step in the catalytic cycle of
474 peroxide reduction, disulfide formation and regeneration.

475 4.2. Bulky substrates favour hyperoxidation and inhibition of PRX-IIIE

476 Activity was undetectable in both assays using CuOOH as substrate, and rather bulky substrates
477 like CuOOH inhibit peroxidase activity, which was already reported for *poplar* PRX-IIIE [14]. The
478 inhibitory effect of CuOOH on the peroxidase activity of PRX-IIIE was revealed by the decreased
479 H₂O₂-reduction activity in the presence of increasing CuOOH concentrations. Already the presence
480 of 12.5 μM CuOOH resulted in a significant decrease in PRX-IIIE peroxidase activity (Figure 3A) and
481 the inhibition of activity correlated with hyperoxidation of PRX-IIIE (Figure 3B).

482 ESI-MS measurements of CuOOH-treated PRX-IIIE C121S and C146S variants proved
483 hyperoxidation of the C121 to sulfinic (-SO₂H) and sulfonic acid (SO₃H) at low amounts of CuOOH
484 (Table 1). Oxidation of C146 to sulfenic acid (-SOH) only occurred after treatment with relatively high
485 CuOOH concentrations. H₂O₂ treatment only resulted in oxidation of PRX-IIIE to the sulfenic acid
486 derivative in contrast to 2-CysPRX, where hyperoxidation occurs after about 250 peroxidase cycles
487 (Figure 4) [47]. Oxidized sulfenylated 2-CysPRX functions in proximity-based oxidation. Sobotta et
488 al [48] reported this type of signaling cascade, where human PRDX2 gets oxidized by ROS and
489 afterwards oxidizes STAT3. Disulfide-bonded 2-CysPRX from *A. thaliana* oxidizes chloroplast TRXs
490 which in turn oxidize target proteins in the Calvin-Benson cycle or malate dehydrogenase [8]. This
491 type of regulation was termed TRX oxidase function of 2-CysPRX and participates in adjustment of
492 enzyme activity to decreased light intensity. PRX-IIIE could also be part of a ROS-induced signaling
493 cascade, whereas PRX-IIIE oxidizes a nearby protein.

494 Hyperoxidized PRX may also function in cell signaling, e.g. if the change in redox-state affects
495 its conformational state which in turn allows for binding to other proteins and alters their activity
496 [49]. Pea mitochondrial PRX-IIIF adopts a hexameric conformation in addition to its dimeric form and
497 tightly binds thioredoxin-o [50].

498 4.3. Besides hyperoxidation, Cys121 of PRX-IIIE is subject to multiple posttranslational modifications

499 Apart from oxidation and hyperoxidation, PRX-IIIE is also S-glutathionylated (Figure 5 and
500 Figure 6). Although glutathionylation of PRX-IIIE inhibited its peroxidase activity (Figure 8A),
501 S-glutathionylation of Cys_p (Figure 7) may prevent PRX-IIIE from hyperoxidation, like it was already
502 shown for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) from spinach and isocitrate lyase
503 from *C. reinhardtii* [51,52]. Reversal of this type of regulation of PRX-IIIE could be achieved by
504 deglutathionylation via GRXs, TRXs and SRXs [53–55]. However, the presence of GRX-S12, SRX or

505 GRX-C5 failed to increase the de-glutathionylation rate in comparison to GSH alone, indicating that
506 the GSH/GSSG ratio could be the main route for the regulation of this PTM *in vivo*.

507 Under normal physiological relevant concentrations of ~1 mM glutathione [56] and a ratio of
508 0.002 % oxidized glutathione [57], PRX-IIIE should not be glutathionylated. Application of stresses to
509 the plant, like exposure to methylviologen or arsenic treatment can, however, alter the GSSG ratio
510 [58,59] and, therefore, may result in S-glutathionylation of PRX-IIIE. This is consistent with the results
511 shown in Figure 9, where application of severe stress resulted in S-glutathionylation of PRX-IIIE. This
512 post-translational modification could prevent PRX-IIIE from hyperoxidation. In addition, reversible
513 S-glutathionylation of PRX-IIIE could take part in PRX-dependent signal transduction and regulation
514 of the redox homeostasis [60].

515 4.4. PRX-IIIE binds to target proteins

516 Besides post-translational control of activity, protein-protein interactions alter functions and
517 properties of binding partners. PRX-IIIE protein interactions mostly seem to be redox-regulated, since
518 most of the trapped proteins were eluted with DTT (Figure 10A, B). In total 54 proteins were
519 identified to interact with PRX-IIIE. Since PRX-IIIE is located in plastids, we focused on the 24 proteins
520 with plastidial localization. However also the other proteins deserve attention since they might
521 interact with the cytosolic type II PRXs which display high similarity with PRX-IIIE (Figure 1A). Most
522 of the identified proteins are known targets of redox regulation [61] like cyclophilin 20-3 [55] or β -
523 carbonic anhydrase [62].

524 4.5. 14-3-3 proteins as binding partner of PRX-IIIE open up new perspectives

525 14-3-3 proteins function as molecular adapters and their identification as binding partners of
526 PRX-IIIE appeared interesting and novel. They are present in various isoforms in plant genomes and
527 act as homo- and heterodimers [63]. 14-3-3 proteins are able to integrate and control multiple
528 pathways like the abscisic acid-dependent transcription of embryo-specific target genes [64]. They
529 participate in regulation of salt stress tolerance and apoptotic signaling transduction [38,39].
530 Furthermore, they function in development of cotyledons [40]. The 14-3-3 ν isoform co-controls the
531 cell proliferation cycle and induces the division of chloroplasts, which results in an increased plastid
532 number, chlorophyll content and photosynthetic activity [40].

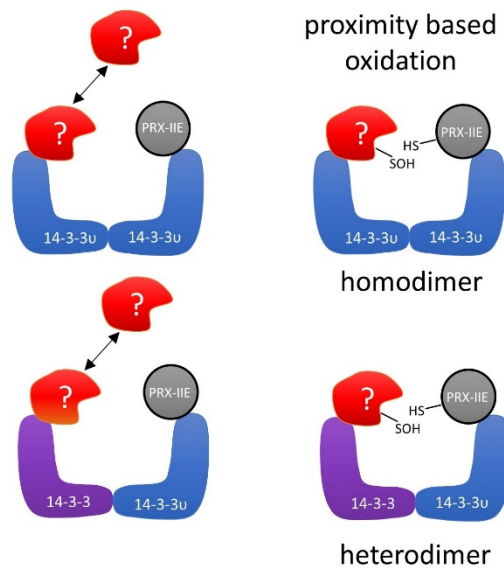
533 In this study, four 14-3-3 proteins could be identified to interact with PRX-IIIE WT. 14-3-3
534 proteins are known to preferentially bind to phosphorylated motifs containing phosphoserine
535 residues [65,66]. In addition, pThr-dependent binding as well as non-phosphorylation dependent
536 interactions with target proteins were reported [67]. Phospho-mimetic variants of PRXIIIE have been
537 used to further address the binding properties to 14-3-3 ν under defined redox-conditions. However,
538 preferential binding of 14-3-3 ν to these variants could not be detected under reducing as well as
539 oxidizing conditions. Instead, the highest binding could be observed for WT PRX-IIIE under oxidizing
540 conditions (Figure 11B). In addition, introducing negative charges at positions S82, T108 and T223,
541 resulted in an inhibition of the thiol peroxidase activity (Figure 11A). Similar results have been
542 reported for human PRX2, where Cdk5-derived phosphorylation at T89 had a negative effect on its
543 activity [68].

544 To check, if the interaction of 14-3-3 ν and PRX-IIIE under oxidizing conditions may alter the
545 peroxidase activity, the H₂O₂ reduction by PRX-IIIE was determined using the FOX assay in the
546 presence or absence of 14-3-3 ν (Figure 11C). A fivefold excess of 14-3-3 ν increased the peroxidase
547 activity of PRX-IIIE significantly. Since such a high amount of 14-3-3 ν is necessary to alter the PRX-IIIE
548 activity, it is more likely, that the interaction is important during signaling processes.

549 4.6. Hypothetical outlook and where to go

550 Several mechanistic scenarios may be hypothesized. Formation of regulatory assemblies of PRX-
551 IIE with homo- or heterodimers of 14-3-3 proteins may recruit additional binding partners. In such a
552 regulatory complex, proximity-based oxidation between oxidized PRX-IIIE and reduced 14-3-3 ν or

553 the bound third partner could be the regulatory mechanism that leads to changes in function and
554 regulation of cellular processes (Figure 12).



555

556 **Figure 12 Schematic depiction of the hypothetical interaction between PRX-IIIE and 14-3-3 v.**
557 Binding of oxidized PRX-IIIE to 14-3-3 v could induce association of a third partner. Formation of such
558 a complex could facilitate redox regulation, e.g. by proximity-based oxidation. The hypothetical
559 assembly may involve homo- or heterodimers of 14-3-3 isoforms.

560 As described previously, oxidative stress not just induces new interactions of 14-3-3 proteins
561 with protein partners, but also results in a loss of homeostatic interactions [69]. Under oxidative stress
562 in humans, the selenoprotein W binds to 14-3-3 with an intermolecular disulfide bridge. This process
563 results in a release of apoptosis signal-regulating kinase-1 (ASK1) from the 14-3-3-ASK1 complex.
564 ASK1 then activates the Jun N-terminal kinase and p38 MAP kinase pathways, which in turn activates
565 caspases and thereby apoptosis [69,70]. Therefore, PRX-IIIE may affect several functions of 14-3-3 v.
566 As indicated in figure 12, PRX-IIIE could induce dimerization of 14-3-3 v and, thereby, mediate the
567 binding or release of other proteins. Further studies are needed to address the importance of PRX-IIIE
568 on 14-3-3 complex formation and their associated signaling pathways. It will also be important to
569 scrutinize the interaction of 14-3-3 proteins with the cytosolic PRX-IIB, C and D in the same functional
570 scenario of facilitating redox regulatory assemblies.

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572 **Supplementary Materials:** Table S1 Primers used in this study for cloning. Table S 2 Identified 14-3-3 proteins
573 and their unique peptide. Supplement file list of identified proteins.

574 **Author Contributions:** Conceptualization, PT, AD; HPM. and KJD.; methodology, PT, AD, AM, AMJ.;
575 validation, AD and PT.; formal analysis, AD, PT and AMJ.; investigation, PT, AD, DB and AMJ.; resources, AD,
576 PT, AMJ, AM; data curation, AD, PT, AMJ and AM.; writing—original draft preparation, PT, AD and KJD.;
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588

589 **Table S1 Primers used in this study for cloning.**

Gene	AGI	Restriction enzyme		Primer Sequence 5'→3'
GRX-S12	AT2G20270	NdeI	Forward	TTTCCAACATATGGGATCGACATTGGAGGAGACTG
		BamHI	Reverse	TTTCAGGATCCCTAGGTCTGACCGTTTTTCC
SRX	AT1G31170	NdeI	Forward	ACATATGAACGGTTCGCCCGGTGAT
		BamHI	Reverse	AAAGGATCCTCAGCGAAGATGATGCCTTA
PRX-IIIE	AT3G52960	NdeI	Forward	ATATACATATGGCCTCCATTTCCGTCGG
		BamHI	Reverse	ATATAGGATCCTCAGAGAGCTTTAAGCATATC
PRX-IIIE C121S	AT3G52960		Forward	GGCGCATTACACCAACAAGCTCAC
			Reverse	GTGAGCTTGTTGGTGTGAATG
PRX-IIIE C146S	AT3G52960		Forward	AATCGCAAGTATCTCCGTCAAC
			Reverse	GACGGAGATACTTGCGATTACAT
PRX-IIIE S82D	AT3G52960		Forward	GGTAGGAGAGAGTGTCTGTTGGGAGCTTGT
			Reverse	ACAAGCTCCCAGACGACACTCTCTCCTACC
PRX-IIIE T108E	AT3G52960		Forward	GAACGGCGAATAGGATTTCTTTCTTCCCGGCG
			Reverse	CGCCGGGAAGAAAGAAATCCTATTGCGCGTTC
PRX-IIIE T223E	AT3G52960		Forward	CTCAGCACTACTATTTTCAAAGCACCTCCTTC
			Reverse	GAAGGAGGTGCTTTTGAAAATAGTAGTGCTGAG
PRX-IIIE- EYFP	AT3G52960	BamHI	Forward	ATATAGGATCCATGGCGACTTCTCTCCGTTTC
		AgeI	Reverse	AAACCGGTGGGAGAGCTTTAAGCATATCCTCAG

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Table S 2 Identified 14-3-3 proteins and their unique peptide.

Protein	AGI	Unique identified peptides	Identified peptides	Unique Peptide sequences	Localization	reference
14-3-3 χ	AT4G09000	11	25	DEFVYMAKLAEQAER DEFVYMAKLAEQAERYEEMVEFMEK DNLTWTSDMQDDVADDIK DSTLIMQLLRDNLTWTSDMQDDVADDIK DSTLIMQLLRDNLTWTSDMQDDVADDIKEAAPAAAKPADEQQS EESRGNDHVS LIRDYR GNDDHVSLIR GNDDHVSLIRDYR IETELSDICDGILK KDAAEHTLTAYK MATPGASSARDEFVYMAKLAEQAER	Cytosol, nucleus, chloroplast, golgi, vacuole	[71–75]
14-3-3 ω	AT1G78300	0	8		Cytosol, nucleus	[76]
14-3-3 φ	AT1G78300	4	14	DNLTWTSDMQDESPEEIK EEFVYLAKLAEQAERYEEMVEFMEK GNDDHVTTIR LAEQAERYEEMVEFMEKVAEAVDK	Cytosol, nucleus, plasma membrane	[72,77,78]
14-3-3 ν	AT5G16050	8	17	ASWRIISSIEQKEDSR DNLTWTS DLNDEAGDDIKEAPK DSTLIMQLLRDNLTWTS DLNDEAGDDIK EDSRGNSDHVSIK ICDGILNLEAHLIPAASLAESK LGLALNFSVFYYEILNSSDR SAQDIALADLAPTHPIRLGLALNFSVFYYEILNSSDRACSLAK VDEQAQPPPSQ	Chloroplast, cytosol	[43]

Protein	AGI	Unique identified peptides	Identified peptides	Unique Peptide sequences	Localization	reference
14-3-3 λ	AT5G10450	5	7	DQYVYMAKLAEQAERYEEMVQFMEQLVTGATPAEELTVEER QAFEEAIAELDTLGEESYK QAFEEAIAELDTLGEESYKDSTLIMQLLR YEEMVQFMEQLVTGATPAEELTVEER YMAEFK	Nucleus, cytosol, plasma membrane, chloroplast	[79–81]
14-3-3 ν	AT3G02520	2	12	MSSSREENVYLAK TVDTDELVEERNLLSVAYK	Chloroplast, Cytosol	[43]

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References

1. Horling, F.; Lamkemeyer, P.; König, J.; Finkemeier, I.; Kandlbinder, A.; Baier, M.; Dietz, K.-J. Divergent light-, ascorbate-, and oxidative stress-dependent regulation of expression of the peroxiredoxin gene family in Arabidopsis. *Plant Physiol.* **2003**, *131*, 317–325, doi:10.1104/pp.010017.
2. Dietz, K.-J. Plant peroxiredoxins. *Annu. Rev. Plant Biol.* **2003**, *54*, 93–107, doi:10.1146/annurev.arplant.54.031902.134934.
3. Dietz, K.-J.; Jacob, S.; Oelze, M.-L.; Laxa, M.; Tognetti, V.; Miranda, S.M.N. de; Baier, M.; Finkemeier, I. The function of peroxiredoxins in plant organelle redox metabolism. *J. Exp. Bot.* **2006**, *57*, 1697–1709, doi:10.1093/jxb/erj160.
4. Brigelius-Flohé, R.; Flohé, L. Basic principles and emerging concepts in the redox control of transcription factors. *Antioxid. Redox Signal.* **2011**, *15*, 2335–2381, doi:10.1089/ars.2010.3534.
5. Baier, M.; Dietz, K.J. The plant 2-Cys peroxiredoxin BAS1 is a nuclear-encoded chloroplast protein: its expressional regulation, phylogenetic origin, and implications for its specific physiological function in plants. *Plant J.* **1997**, *12*, 179–190, doi:10.1046/j.1365-313x.1997.12010179.x.
6. Collin, V.; Issakidis-Bourguet, E.; Marchand, C.; Hirasawa, M.; Lancelin, J.-M.; Knaff, D.B.; Miginiac-Maslow, M. The Arabidopsis plastidial thioredoxins: new functions and new insights into specificity. *J. Biol. Chem.* **2003**, *278*, 23747–23752, doi:10.1074/jbc.M302077200.
7. Muthuramalingam, M.; Dietz, K.-J.; Ströher, E. Thiol - Disulfide Redox Proteomics in Plant Research. In *Plant Stress Tolerance: Methods and Protocols*; Sunkar, R., Ed.; Humana Press: Totowa, NJ, 2010; pp 219–238, ISBN 978-1-60761-702-0.
8. Vaseghi, M.-J.; Chibani, K.; Telman, W.; Liebthal, M.F.; Gerken, M.; Schnitzer, H.; Mueller, S.M.; Dietz, K.-J. The chloroplast 2-cysteine peroxiredoxin functions as thioredoxin oxidase in redox regulation of chloroplast metabolism. *Elife* **2018**, *7*, doi:10.7554/eLife.38194.
9. Lamkemeyer, P.; Laxa, M.; Collin, V.; Li, W.; Finkemeier, I.; Schöttler, M.A.; Holtkamp, V.; Tognetti, V.B.; Issakidis-Bourguet, E.; Kandlbinder, A.; et al. Peroxiredoxin Q of Arabidopsis thaliana is attached to the thylakoids and functions in context of photosynthesis. *Plant J.* **2006**, *45*, 968–981, doi:10.1111/j.1365-313X.2006.02665.x.
10. Romero-Puertas, M.C.; Laxa, M.; Mattè, A.; Zaninotto, F.; Finkemeier, I.; Jones, A.M.E.; Perazzolli, M.; Vandelle, E.; Dietz, K.-J.; Delledonne, M. S-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration. *Plant Cell* **2007**, *19*, 4120–4130, doi:10.1105/tpc.107.055061.
11. Rouhier, N.; Gelhaye, E.; Sautiere, P.E.; Brun, A.; Laurent, P.; Tagu, D.; Gerard, J.; Faÿ, E. de; Meyer, Y.; Jacquot, J.P. Isolation and characterization of a new peroxiredoxin from poplar sieve tubes that uses either glutaredoxin or thioredoxin as a proton donor. *Plant Physiol.* **2001**, *127*, 1299–1309.
12. Stork, T.; Michel, K.-P.; Pistorius, E.K.; Dietz, K.-J. Bioinformatic analysis of the genomes of the cyanobacteria Synechocystis sp. PCC 6803 and Synechococcus elongatus PCC 7942 for the presence of peroxiredoxins and their transcript regulation under stress. *J. Exp. Bot.* **2005**, *56*, 3193–3206, doi:10.1093/jxb/eri316.
13. Declercq, J.P.; Evrard, C.; Clippe, A.; Stricht, D.V.; Bernard, A.; Knoops, B. Crystal structure of human peroxiredoxin 5, a novel type of mammalian peroxiredoxin at 1.5 Å resolution. *J. Mol. Biol.* **2001**, *311*, 751–759, doi:10.1006/jmbi.2001.4853.
14. Gama, F.; Bréhélin, C.; Gelhaye, E.; Meyer, Y.; Jacquot, J.-P.; Rey, P.; Rouhier, N. Functional analysis and expression characteristics of chloroplastic Prx IIE. *Physiol Plant* **2008**, *133*, 599–610, doi:10.1111/j.1399-3054.2008.01097.x.
15. Horling, F.; König, J.; Dietz, K.-J. Type II peroxiredoxin C, a member of the peroxiredoxin family of Arabidopsis thaliana: its expression and activity in comparison with other peroxiredoxins. *Plant Physiology and Biochemistry* **2002**, *40*, 491–499, doi:10.1016/S0981-9428(02)01396-7.
16. Couturier, J.; Ströher, E.; Albetel, A.-N.; Roret, T.; Muthuramalingam, M.; Tarrago, L.; Seidel, T.; Tsan, P.; Jacquot, J.-P.; Johnson, M.K.; et al. Arabidopsis chloroplastic glutaredoxin C5 as a model to explore molecular determinants for iron-sulfur cluster binding into glutaredoxins. *J. Biol. Chem.* **2011**, *286*, 27515–27527, doi:10.1074/jbc.M111.228726.
17. Ströher, E.; Dietz, K.-J. The dynamic thiol-disulphide redox proteome of the Arabidopsis thaliana chloroplast as revealed by differential electrophoretic mobility. *Physiol Plant* **2008**, *133*, 566–583, doi:10.1111/j.1399-3054.2008.01103.x.

- 647 18. König, J.; Baier, M.; Horling, F.; Kahmann, U.; Harris, G.; Schürmann, P.; Dietz, K.-J. The plant-specific
648 function of 2-Cys peroxiredoxin-mediated detoxification of peroxides in the redox-hierarchy of photosynthetic
649 electron flux. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 5738–5743, doi:10.1073/pnas.072644999.
- 650 19. Finkemeier, I.; Goodman, M.; Lamkemeyer, P.; Kandlbinder, A.; Sweetlove, L.J.; Dietz, K.-J. The
651 mitochondrial type II peroxiredoxin F is essential for redox homeostasis and root growth of *Arabidopsis thaliana*
652 under stress. *J. Biol. Chem.* **2005**, *280*, 12168–12180, doi:10.1074/jbc.M413189200.
- 653 20. Méchin, V.; Damerval, C.; Zivy, M. Total protein extraction with TCA-acetone. *Methods Mol. Biol.* **2007**, *355*,
654 1–8, doi:10.1385/1-59745-227-0:1.
- 655 21. Seidel, T.; Gollmack, D.; Dietz, K.-J. Mapping of C-termini of V-ATPase subunits by in vivo-FRET
656 measurements. *FEBS Letters* **2005**, *579*, 4374–4382, doi:10.1016/j.febslet.2005.06.077.
- 657 22. Seidel, T.; Kluge, C.; Hanitzsch, M.; Ross, J.; Sauer, M.; Dietz, K.-J.; Gollmack, D. Colocalization and FRET-
658 analysis of subunits c and a of the vacuolar H⁺-ATPase in living plant cells. *Journal of biotechnology* **2004**, *112*,
659 165–175, doi:10.1016/j.jbiotec.2004.04.027.
- 660 23. Muthuramalingam, M.; Matros, A.; Scheibe, R.; Mock, H.-P.; Dietz, K.-J. The hydrogen peroxide-sensitive
661 proteome of the chloroplast in vitro and in vivo. *Frontiers in plant science* **2013**, *4*, 54, doi:10.3389/fpls.2013.00054.
- 662 24. Wessel, D.; Flügge, U.I. A method for the quantitative recovery of protein in dilute solution in the presence
663 of detergents and lipids. *Anal. Biochem.* **1984**, *138*, 141–143, doi:10.1016/0003-2697(84)90782-6.
- 664 25. Jozefowicz, A.M.; Hartmann, A.; Matros, A.; Schum, A.; Mock, H.-P. Nitrogen Deficiency Induced
665 Alterations in the Root Proteome of a Pair of Potato (*Solanum tuberosum* L.) Varieties Contrasting for their
666 Response to Low N. *Proteomics* **2017**, *17*, doi:10.1002/pmic.201700231.
- 667 26. Arend, D.; Lange, M.; Chen, J.; Colmsee, C.; Flemming, S.; Hecht, D.; Scholz, U. e!DAL--a framework to
668 store, share and publish research data. *BMC Bioinformatics* **2014**, *15*, 214, doi:10.1186/1471-2105-15-214.
- 669 27. Waterhouse, A.; Bertoni, M.; Bienert, S.; Studer, G.; Tauriello, G.; Gumienny, R.; Heer, F.T.; Beer, T.A.P. de;
670 Rempfer, C.; Bordoli, L.; et al. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic*
671 *Acids Res.* **2018**, *46*, W296-W303, doi:10.1093/nar/gky427.
- 672 28. Schrödinger, L.L.C. *The PyMOL Molecular Graphics System, Version 2.4.0*, 2015.
- 673 29. Dietz, K.-J. Peroxiredoxins in plants and cyanobacteria. *Antioxid. Redox Signal.* **2011**, *15*, 1129–1159,
674 doi:10.1089/ars.2010.3657.
- 675 30. Melchers, J.; Dirdjaja, N.; Ruppert, T.; Krauth-Siegel, R.L. Glutathionylation of trypanosomal thiol redox
676 proteins. *J. Biol. Chem.* **2007**, *282*, 8678–8694, doi:10.1074/jbc.M608140200.
- 677 31. Park, J.W.; Piszczek, G.; Rhee, S.G.; Chock, P.B. Glutathionylation of peroxiredoxin I induces decamer to
678 dimers dissociation with concomitant loss of chaperone activity. *Biochemistry* **2011**, *50*, 3204–3210,
679 doi:10.1021/bi101373h.
- 680 32. Zaffagnini, M.; Bedhomme, M.; Marchand, C.H.; Morisse, S.; Trost, P.; Lemaire, S.D. Redox regulation in
681 photosynthetic organisms: focus on glutathionylation. *Antioxid. Redox Signal.* **2012**, *16*, 567–586,
682 doi:10.1089/ars.2011.4255.
- 683 33. Couturier, J.; Jacquot, J.-P.; Rouhier, N. Toward a refined classification of class I dithiol glutaredoxins from
684 poplar: biochemical basis for the definition of two subclasses. *Frontiers in plant science* **2013**, *4*, 518,
685 doi:10.3389/fpls.2013.00518.
- 686 34. Park, J.W.; Mieyal, J.J.; Rhee, S.G.; Chock, P.B. Deglutathionylation of 2-Cys peroxiredoxin is specifically
687 catalyzed by sulfiredoxin. *J. Biol. Chem.* **2009**, *284*, 23364–23374, doi:10.1074/jbc.M109.021394.
- 688 35. Giustarini, D.; Rossi, R.; Milzani, A.; Colombo, R.; Dalle-Donne, I. S-glutathionylation: from redox
689 regulation of protein functions to human diseases. *J. Cell. Mol. Med.* **2004**, *8*, 201–212, doi:10.1111/j.1582-
690 4934.2004.tb00275.x.
- 691 36. Popov, D. Protein S-glutathionylation: from current basics to targeted modifications. *Archives of Physiology*
692 *and Biochemistry* **2014**, *120*, 123–130, doi:10.3109/13813455.2014.944544.
- 693 37. Gasteiger, E.; Hoogland, C.; Gattiker, A.; Duvaud, S.'e.; Wilkins, M.R.; Appel, R.D.; Bairoch, A. Protein
694 Identification and Analysis Tools on the ExpASY Server. In *The Proteomics Protocols Handbook*; Walker, J.M., Ed.;
695 Humana Press: Totowa, NJ, 2005; pp 571–607, ISBN 978-1-59259-890-8.
- 696 38. Ferl, R.J.; Manak, M.S.; Reyes, M.F. The 14-3-3s. *Genome Biol.* **2002**, *3*, REVIEWS3010, doi:10.1186/gb-2002-
697 3-7-reviews3010.
- 698 39. Zhou, H.; Lin, H.; Chen, S.; Becker, K.; Yang, Y.; Zhao, J.; Kudla, J.; Schumaker, K.S.; Guo, Y. Inhibition of
699 the *Arabidopsis* salt overly sensitive pathway by 14-3-3 proteins. *Plant Cell* **2014**, *26*, 1166–1182,
700 doi:10.1105/tpc.113.117069.

- 701 40. Vercruyssen, L.; Tognetti, V.B.; Gonzalez, N.; van Dingenen, J.; Milde, L. de; Bielach, A.; Rycke, R. de; van
702 Breusegem, F.; Inzé, D. GROWTH REGULATING FACTOR5 stimulates Arabidopsis chloroplast division,
703 photosynthesis, and leaf longevity. *Plant Physiol.* **2015**, *167*, 817–832, doi:10.1104/pp.114.256180.
- 704 41. Chevalier, D.; Morris, E.R.; Walker, J.C. 14-3-3 and FHA domains mediate phosphoprotein interactions.
705 *Annu. Rev. Plant Biol.* **2009**, *60*, 67–91, doi:10.1146/annurev.arplant.59.032607.092844.
- 706 42. Dougherty, M.K.; Morrison, D.K. Unlocking the code of 14-3-3. *J. Cell Sci.* **2004**, *117*, 1875–1884,
707 doi:10.1242/jcs.01171.
- 708 43. Sehnke, P.C.; Henry, R.; Cline, K.; Ferl, R.J. Interaction of a plant 14-3-3 protein with the signal peptide of a
709 thylakoid-targeted chloroplast precursor protein and the presence of 14-3-3 isoforms in the chloroplast stroma.
710 *Plant Physiol.* **2000**, *122*, 235–242, doi:10.1104/pp.122.1.235.
- 711 44. Cao, A.; Jain, A.; Baldwin, J.C.; Raghothama, K.G. Phosphate differentially regulates 14-3-3 family members
712 and GRF9 plays a role in Pi-starvation induced responses. *Planta* **2007**, *226*, 1219–1230, doi:10.1007/s00425-007-
713 0569-0.
- 714 45. Aryal, U.K.; Krochko, J.E.; Ross, A.R.S. Identification of phosphoproteins in Arabidopsis thaliana leaves
715 using polyethylene glycol fractionation, immobilized metal-ion affinity chromatography, two-dimensional gel
716 electrophoresis and mass spectrometry. *J. Proteome Res.* **2012**, *11*, 425–437, doi:10.1021/pr200917t.
- 717 46. Trujillo, M.; Clippe, A.; Manta, B.; Ferrer-Sueta, G.; Smeets, A.; Declercq, J.-P.; Knoops, B.; Radi, R. Pre-
718 steady state kinetic characterization of human peroxiredoxin 5: taking advantage of Trp84 fluorescence increase
719 upon oxidation. *Archives of Biochemistry and Biophysics* **2007**, *467*, 95–106, doi:10.1016/j.abb.2007.08.008.
- 720 47. König, J.; Galliardt, H.; Jütte, P.; Schäper, S.; Dittmann, L.; Dietz, K.-J. The conformational bases for the two
721 functionalities of 2-cysteine peroxiredoxins as peroxidase and chaperone. *J. Exp. Bot.* **2013**, *64*, 3483–3497.
- 722 48. Sobotta, M.C.; Liou, W.; Stöcker, S.; Talwar, D.; Oehler, M.; Ruppert, T.; Scharf, A.N.D.; Dick, T.P.
723 Peroxiredoxin-2 and STAT3 form a redox relay for H₂O₂ signaling. *Nat. Chem. Biol.* **2015**, *11*, 64–70,
724 doi:10.1038/nchembio.1695.
- 725 49. Liebthal, M.; Maynard, D.; Dietz, K.-J. Peroxiredoxins and Redox Signaling in Plants. *Antioxid. Redox Signal.*
726 **2018**, *28*, 609–624.
- 727 50. Barranco-Medina, S.; Krell, T.; Bernier-Villamor, L.; Sevilla, F.; Lázaro, J.-J.; Dietz, K.-J. Hexameric
728 oligomerization of mitochondrial peroxiredoxin PrxIIIF and formation of an ultrahigh affinity complex with its
729 electron donor thioredoxin Trx-o. *J. Exp. Bot.* **2008**, *59*, 3259–3269.
- 730 51. Sparla, F.; Fermani, S.; Falini, G.; Zaffagnini, M.; Ripamonti, A.; Sabatino, P.; Pupillo, P.; Trost, P. Coenzyme
731 site-directed mutants of photosynthetic A4-GAPDH show selectively reduced NADPH-dependent catalysis,
732 similar to regulatory AB-GAPDH inhibited by oxidized thioredoxin. *J. Mol. Biol.* **2004**, *340*, 1025–1037,
733 doi:10.1016/j.jmb.2004.06.005.
- 734 52. Bedhomme, M.; Zaffagnini, M.; Marchand, C.H.; Gao, X.-H.; Moslonka-Lefebvre, M.; Michelet, L.;
735 Decottignies, P.; Lemaire, S.D. Regulation by glutathionylation of isocitrate lyase from *Chlamydomonas*
736 *reinhardtii*. *J. Biol. Chem.* **2009**, *284*, 36282–36291, doi:10.1074/jbc.M109.064428.
- 737 53. Rouhier, N.; Lemaire, S.D.; Jacquot, J.-P. The role of glutathione in photosynthetic organisms: emerging
738 functions for glutaredoxins and glutathionylation. *Annu. Rev. Plant Biol.* **2008**, *59*, 143–166,
739 doi:10.1146/annurev.arplant.59.032607.092811.
- 740 54. Michelet, L.; Zaffagnini, M.; Marchand, C.; Collin, V.; Decottignies, P.; Tsan, P.; Lancelin, J.-M.; Trost, P.;
741 Miginiac-Maslow, M.; Noctor, G.; et al. Glutathionylation of chloroplast thioredoxin f is a redox signaling
742 mechanism in plants. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 16478–16483, doi:10.1073/pnas.0507498102.
- 743 55. Park, S.-W.; Li, W.; Viehhauser, A.; He, B.; Kim, S.; Nilsson, A.K.; Andersson, M.X.; Kittle, J.D.; Ambavaram,
744 M.M.R.; Luan, S.; et al. Cyclophilin 20-3 relays a 12-oxo-phytyldienoic acid signal during stress responsive
745 regulation of cellular redox homeostasis. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 9559–9564,
746 doi:10.1073/pnas.1218872110.
- 747 56. Hartmann, T.N.; Fricker, M.D.; Rennenberg, H.; Meyer, A.J. Cell-specific measurement of cytosolic
748 glutathione in poplar leaves. *Plant Cell Environ.* **2003**, *26*, 965–975, doi:10.1046/j.1365-3040.2003.01031.x.
- 749 57. Meyer, A.J.; Brach, T.; Marty, L.; Kreye, S.; Rouhier, N.; Jacquot, J.-P.; Hell, R. Redox-sensitive GFP in
750 Arabidopsis thaliana is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer.
751 *Plant J.* **2007**, *52*, 973–986, doi:10.1111/j.1365-313X.2007.03280.x.
- 752 58. König, K.; Vaseghi, M.J.; Dreyer, A.; Dietz, K.-J. The significance of glutathione and ascorbate in modulating
753 the retrograde high light response in Arabidopsis thaliana leaves. *Physiol Plant* **2018**, *162*, 262–273,
754 doi:10.1111/ppl.12644.

- 755 59. Kumar, V.; Vogelsang, L.; Seidel, T.; Schmidt, R.; Weber, M.; Reichelt, M.; Meyer, A.; Clemens, S.; Sharma,
756 S.S.; Dietz, K.-J. Interference between arsenic-induced toxicity and hypoxia. *Plant Cell Environ.* **2019**, *42*, 574–590,
757 doi:10.1111/pce.13441.
- 758 60. Chae, H.Z.; Oubrahim, H.; Park, J.W.; Rhee, S.G.; Chock, P.B. Protein glutathionylation in the regulation of
759 peroxiredoxins: a family of thiol-specific peroxidases that function as antioxidants, molecular chaperones, and
760 signal modulators. *Antioxid. Redox Signal.* **2012**, *16*, 506–523, doi:10.1089/ars.2011.4260.
- 761 61. Buchanan, B.B.; Balmer, Y. Redox regulation: a broadening horizon. *Annu. Rev. Plant Biol.* **2005**, *56*, 187–
762 220, doi:10.1146/annurev.arplant.56.032604.144246.
- 763 62. Dreyer, A.; Schackmann, A.; Kriznik, A.; Chibani, K.; Wesemann, C.; Vogelsang, L.; Beyer, A.; Dietz, K.-J.
764 Thiol Redox Regulation of Plant β -Carbonic Anhydrase. *Biomolecules* **2020**, *10*, doi:10.3390/biom10081125.
- 765 63. Jones, D.H.; Ley, S.; Aitken, A. Isoforms of 14-3-3 protein can form homo- and heterodimers in vivo and in
766 vitro: implications for function as adapter proteins. *FEBS Letters* **1995**, *368*, 55–58, doi:10.1016/0014-
767 5793(95)00598-4.
- 768 64. Schultz, T.F.; Medina, J.; Hill, A.; Quatrano, R.S. 14-3-3 proteins are part of an abscisic acid-VIVIPAROUS1
769 (VP1) response complex in the Em promoter and interact with VP1 and EmBP1. *Plant Cell* **1998**, *10*, 837–847,
770 doi:10.1105/tpc.10.5.837.
- 771 65. Chung, H. The 14-3-3 proteins: cellular regulators of plant metabolism. *Trends in Plant Science* **1999**, *4*, 367–
772 371, doi:10.1016/S1360-1385(99)01462-4.
- 773 66. Liang, X.; Da Paula, A.C.; Bozóky, Z.; Zhang, H.; Bertrand, C.A.; Peters, K.W.; Forman-Kay, J.D.; Frizzell,
774 R.A. Phosphorylation-dependent 14-3-3 protein interactions regulate CFTR biogenesis. *Mol. Biol. Cell* **2012**, *23*,
775 996–1009, doi:10.1091/mbc.E11-08-0662.
- 776 67. van Heusden, G.P.H. 14-3-3 proteins: regulators of numerous eukaryotic proteins. *IUBMB Life* **2005**, *57*,
777 623–629.
- 778 68. Qu, D.; Rashidian, J.; Mount, M.P.; Aleyasin, H.; Parsanejad, M.; Lira, A.; Haque, E.; Zhang, Y.; Callaghan,
779 S.; Daigle, M.; et al. Role of Cdk5-mediated phosphorylation of Prx2 in MPTP toxicity and Parkinson's disease.
780 *Neuron* **2007**, *55*, 37–52, doi:10.1016/j.neuron.2007.05.033.
- 781 69. Pennington, K.L.; Chan, T.Y.; Torres, M.P.; Andersen, J.L. The dynamic and stress-adaptive signaling hub
782 of 14-3-3: emerging mechanisms of regulation and context-dependent protein-protein interactions. *Oncogene*
783 **2018**, *37*, 5587–5604, doi:10.1038/s41388-018-0348-3.
- 784 70. Ichijo, H.; Nishida, E.; Irie, K.; Dijke, P. ten; Saitoh, M.; Moriguchi, T.; Takagi, M.; Matsumoto, K.; Miyazono,
785 K.; Gotoh, Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38
786 signaling pathways. *Science* **1997**, *275*, 90–94, doi:10.1126/science.275.5296.90.
- 787 71. Bae, M.S.; Cho, E.J.; Choi, E.-Y.; Park, O.K. Analysis of the Arabidopsis nuclear proteome and its response
788 to cold stress. *Plant J.* **2003**, *36*, 652–663, doi:10.1046/j.1365-313x.2003.01907.x.
- 789 72. Ito, J.; Bathth, T.S.; Petzold, C.J.; Redding-Johanson, A.M.; Mukhopadhyay, A.; Verboom, R.; Meyer, E.H.;
790 Millar, A.H.; Heazlewood, J.L. Analysis of the Arabidopsis cytosolic proteome highlights subcellular
791 partitioning of central plant metabolism. *J. Proteome Res.* **2011**, *10*, 1571–1582, doi:10.1021/pr1009433.
- 792 73. Helm, S.; Dobritzsch, D.; Rödiger, A.; Agne, B.; Baginsky, S. Protein identification and quantification by
793 data-independent acquisition and multi-parallel collision-induced dissociation mass spectrometry (MS(E)) in the
794 chloroplast stroma proteome. *J. Proteomics* **2014**, *98*, 79–89, doi:10.1016/j.jprot.2013.12.007.
- 795 74. Heard, W.; Sklenář, J.; Tomé, D.F.A.; Robatzek, S.; Jones, A.M.E. Identification of Regulatory and Cargo
796 Proteins of Endosomal and Secretory Pathways in Arabidopsis thaliana by Proteomic Dissection. *Mol. Cell.*
797 *Proteomics* **2015**, *14*, 1796–1813, doi:10.1074/mcp.M115.050286.
- 798 75. Yoshida, K.; Ohnishi, M.; Fukao, Y.; Okazaki, Y.; Fujiwara, M.; Song, C.; Nakanishi, Y.; Saito, K.; Shimmen,
799 T.; Suzuki, T.; et al. Studies on vacuolar membrane microdomains isolated from Arabidopsis suspension-
800 cultured cells: local distribution of vacuolar membrane proteins. *Plant Cell Physiol.* **2013**, *54*, 1571–1584,
801 doi:10.1093/pcp/pct107.
- 802 76. Fakih, Z.; Ahmed, M.B.; Letanneur, C.; Germain, H. An unbiased nuclear proteomics approach reveals
803 novel nuclear protein components that participates in MAMP-triggered immunity. *Plant Signal. Behav.* **2016**, *11*,
804 e1183087, doi:10.1080/15592324.2016.1183087.
- 805 77. Mitra, S.K.; Walters, B.T.; Clouse, S.D.; Goshe, M.B. An efficient organic solvent based extraction method
806 for the proteomic analysis of Arabidopsis plasma membranes. *J. Proteome Res.* **2009**, *8*, 2752–2767,
807 doi:10.1021/pr801044y.

- 808 78. Iglesias, J.; Trigueros, M.; Rojas-Triana, M.; Fernández, M.; Albar, J.P.; Bustos, R.; Paz-Ares, J.; Rubio, V.
809 Proteomics identifies ubiquitin-proteasome targets and new roles for chromatin-remodeling in the Arabidopsis
810 response to phosphate starvation. *J. Proteomics* **2013**, *94*, 1–22, doi:10.1016/j.jprot.2013.08.015.
- 811 79. Rienties, I.M.; Vink, J.; Borst, J.W.; Russinova, E.; Vries, S.C. de. The Arabidopsis SERK1 protein interacts
812 with the AAA-ATPase AtCDC48, the 14-3-3 protein GF14lambda and the PP2C phosphatase KAPP. *Planta* **2005**,
813 *221*, 394–405, doi:10.1007/s00425-004-1447-7.
- 814 80. Yasuda, S.; Sato, T.; Maekawa, S.; Aoyama, S.; Fukao, Y.; Yamaguchi, J. Phosphorylation of Arabidopsis
815 ubiquitin ligase ATL31 is critical for plant carbon/nitrogen nutrient balance response and controls the stability
816 of 14-3-3 proteins. *J. Biol. Chem.* **2014**, *289*, 15179–15193, doi:10.1074/jbc.M113.533133.
- 817 81. Ferro, M.; Brugière, S.; Salvi, D.; Seigneurin-Berny, D.; Court, M.; Moyet, L.; Ramus, C.; Miras, S.; Mellal,
818 M.; Le Gall, S.; et al. AT_CHLORO, a comprehensive chloroplast proteome database with subplastidial
819 localization and curated information on envelope proteins. *Mol. Cell. Proteomics* **2010**, *9*, 1063–1084,
820 doi:10.1074/mcp.M900325-MCP200.