

1 *Running head: S-sulfhydration in Arabidopsis*

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5 *Correspondence:* Luis C. Romero. Instituto de Bioquímica Vegetal y Fotosíntesis. Consejo

6 Superior de Investigaciones Científicas and Universidad de Sevilla. Avenida Américo

7 Vespucio, 49, 41092 Sevilla, Spain. Tel: 34.954489516. E-mail: lromero@ibvf.csic.es

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12 **S-sulphydration: a new post-translational modification in plant systems**

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16 **Ángeles Aroca<sup>1</sup>, Antonio Serna<sup>2</sup>, Cecilia Gotor<sup>1</sup>, Luis C. Romero<sup>1</sup>**

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18 <sup>1</sup>Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones  
19 Científicas y Universidad de Sevilla, Avenida Américo Vespucio, 49, 41092 Sevilla, Spain.

20 <sup>2</sup>AB Sciex. Valgrande 8, Edificio Thanworth II, Nave B1A 28108 Alcobendas, Madrid,  
21 Spain

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26 *Summary:*

27 A new protein post-translational modification in plants, consisting of the S-sulphydration of  
28 cysteine residues by sulfide, is reported.

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30 **Footnotes:**

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32 This work was funded in part by the European Regional Development Fund through the  
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37 **ABSTRACT**

38

39 Hydrogen sulfide (H<sub>2</sub>S) is a highly reactive molecule that is currently accepted as a  
40 signaling compound. This molecule is as important as carbon monoxide in mammals and  
41 hydrogen peroxide in plants, as well as nitric oxide in both eukaryotic systems. Although  
42 many studies have been conducted on the physiological effects of H<sub>2</sub>S, the underlying  
43 mechanisms are poorly understood. One of the proposed mechanisms involves the  
44 posttranslational modification of protein cysteine residues, a process called S-sulfhydration.  
45 In this work, a modified biotin switch method was used for the detection of *Arabidopsis*  
46 *thaliana* proteins modified by S-sulfhydration under physiological conditions. The presence  
47 of an S-sulfhydration-modified cysteine residue on cytosolic ascorbate peroxidase (APX)  
48 was demonstrated using LC-MS/MS analysis, and a total of 106 S-sulfhydrated proteins  
49 were identified. This constitutes the first report of S-sulfhydration as a posttranslational  
50 modification in plants. Immunoblot and enzyme activity analyses of some of these proteins  
51 showed that the sulfide added through S-sulfhydration reversibly regulates the functions of  
52 plant proteins in a manner similar to that described in mammalian systems.

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## 56 INTRODUCTION

57

58 Hydrogen sulfide (H<sub>2</sub>S) is a highly reactive and toxic molecule that has recently  
59 emerged as an important signaling compound with many physiological functions in both  
60 health and disease (Li et al., 2011; Kolluru et al., 2013). The possible role of H<sub>2</sub>S as an  
61 endogenous neuromodulator was first described in 1996, and the molecule is now accepted  
62 as the third most prevalent gasotransmitter after nitric oxide (NO) and carbon monoxide  
63 (CO) (Abe and Kimura, 1996; Vandiver and Snyder, 2012). In animal systems, the  
64 biosynthesis of H<sub>2</sub>S occurs through the action of three enzymes that are involved in the  
65 metabolism of sulfur-containing amino acids: cystathionine gamma-lyase (CGL),  
66 cystathionine beta-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MTS).  
67 These enzymes are typically localized either to specific organs or to subcellular  
68 components such as the mitochondria and cytosol (Wang, 2012).

69 In plant systems, emerging data in recent years also suggest that H<sub>2</sub>S may function as an  
70 important signaling molecule, similar to NO or H<sub>2</sub>O<sub>2</sub>. With regard to certain stresses, H<sub>2</sub>S  
71 treatment alleviates the inhibitory effect of boron on cucumber (*Cucumis sativus*) root  
72 elongation (Wang et al., 2010) and the inhibitory effects of copper and aluminum stress on  
73 wheat (*Triticum aestivum*) germination (Zhang et al., 2008; Zhang et al., 2010). In addition,  
74 H<sub>2</sub>S pretreatment alleviates cadmium toxicity in alfalfa (*Medicago sativa* L.) (Li et al.,  
75 2012a), improves heat tolerance in tobacco (*Nicotiana tabacum*) suspension-cultured cells  
76 (Li et al., 2012b), and protects Bermuda grass (*Cynodon dactylon* (L.) Pers.) from saline,  
77 osmotic and freezing stresses (Shi et al., 2013). H<sub>2</sub>S also plays a role in the regulation of  
78 drought stress and has been described as a component of the abscisic acid signaling  
79 network in guard cells (Garcia-Mata and Lamattina, 2010; Lisjak et al., 2010; Jin et al.,  
80 2013; Scuffi et al., 2014). Moreover, H<sub>2</sub>S has been shown to modulate photosynthesis  
81 through the promotion of chloroplast biogenesis, photosynthetic enzyme expression, and  
82 thiol redox modification in *Spinacia oleracea* seedlings (Chen et al., 2011).

83 At the cellular level, cytosolic enzyme L-cysteine desulphydrase (DES1) is involved in  
84 the degradation of cysteine and is therefore responsible for the generation of H<sub>2</sub>S in this  
85 cellular compartment (Alvarez et al., 2010; Romero et al., 2013). The detailed  
86 characterization of *DES1* null mutants has provided insight into the role of cysteine-

87 generated sulfide as a signaling molecule that regulates the process of autophagy in the  
88 cytosol. Furthermore, DES1 deficiency promotes the accumulation and lipidation of the  
89 ATG8 protein, which is associated with the process of autophagy (Alvarez et al., 2012). In  
90 addition, the transcriptional profile of the *DES1* null mutant, in which different *ATG* genes  
91 are upregulated, confirms its autophagy-induced phenotype. Restoring the capacity of  
92 sulfide generation through exogenous sources or by genetic complementation eliminates the  
93 phenotypic differences between the null mutants and wild-type plants. Interestingly, sulfide  
94 is also able to reverse ATG8 protein accumulation and lipidation, even in wild-type plants,  
95 when autophagy is induced by carbon starvation (Alvarez et al., 2012b; Gotor et al., 2013).

96 Although many studies have been conducted on the physiological effects of H<sub>2</sub>S in  
97 mammals and more recently in plants, the underlying mechanisms are poorly understood.  
98 Nonetheless, two mechanisms have been proposed based on the chemical properties of H<sub>2</sub>S.  
99 The nucleophilic properties of this molecule and its capacity to react with oxygen, H<sub>2</sub>O<sub>2</sub> or  
100 peroxyxynitrite suggest that it acts by reducing cellular oxidative stress (Kabil and Banerjee,  
101 2010; Fukuto et al., 2012). The second mechanism involves the posttranslational  
102 modification of protein cysteine residues to form a persulfide group (R-SSH) (Mustafa et  
103 al., 2009b; Paul and Snyder, 2012). This process is called S-sulfhydration, as opposed to S-  
104 nitrosylation, i.e., the posttranslational modification of protein cysteine residues by NO to  
105 form S-nitrosocysteine residues (R-SNO).

106 The biochemical processes underlying protein S-sulfhydration remain controversial, and  
107 it is most likely that several chemical processes can result in the modification of protein  
108 sulfhydryl groups to form a persulfide. The local environment of the cysteine residue  
109 determines its dissociation constant (pK<sub>a</sub>) to form a thiolate anion (R-S<sup>-</sup>) and therefore  
110 determines its susceptibility to oxidation by reactive oxygen species (ROS) to generate a  
111 sulfenic residue (R-SOH) (Gruhlke and Slusarenko, 2012). This residue can further react  
112 with HS<sup>-</sup> or H<sub>2</sub>S to ultimately form a persulfide residue, as has been described for the  
113 protein Tyr phosphatase 1B (PTP1B) (Krishnan et al., 2011). Other authors have suggested  
114 that H<sub>2</sub>S reacts with oxygen to form sulfane sulfur (S<sup>0</sup>), which interacts with the -SH  
115 groups of proteins to form a persulfide bridge (Toohey, 2011, 2012). Deeper investigation  
116 of this aspect by Greiner et al. (2013) revealed that polysulfides formed in NaHS solutions,  
117 and not NaHS itself, are the oxidizing species when lipid phosphatase PTEN is used as the

118 model protein. These authors presented evidence that sulfane sulfur is added to the active  
119 site PTEN cysteine residues. Other posttranslational protein-cysteine modifications that  
120 have been described are the reversible addition of glutathione or NO. Additionally, the  
121 direct oxidation of the cysteine residue by H<sub>2</sub>O<sub>2</sub> to form a sulfenic (R-SOH), sulfinic (R-  
122 SO<sub>2</sub>H) or sulfonic (R-SO<sub>3</sub>H) group is well established (Zachgo et al., 2013). Oxidation to  
123 sulfenic acid is also a reversible process involved in many redox regulatory mechanisms in  
124 plants and recently, the H<sub>2</sub>O<sub>2</sub>-dependent sulfenome has been reported in *Arabidopsis*.  
125 Several proteins involved in signal perception and transduction events, protein degradation  
126 and redox regulations processes have been identified (Waszczak et al., 2014).

127 Although nitrosylation typically inhibits protein function (Hess and Stamler, 2012;  
128 Zaffagnini et al., 2013), the effect of S-sulphydration can either activate, as has been  
129 described for glyceraldehyde-3-phosphate dehydrogenase and Parkin E3 ligase activity  
130 (Mustafa et al., 2009b; Vandiver et al., 2013), or inactivate enzymatic activities, as has been  
131 reported for Tyr phosphatase 1B (Krishnan et al., 2011). In other cases, S-sulphydration has  
132 been shown to modify protein-protein interactions, such as in the case of Keap1c, which  
133 acts as a negative regulator of Nrf2, a master regulator of the antioxidant response in mice  
134 (Yang et al., 2013).

135 In this work, we studied protein modifications by S-sulphydration in plants and the effect  
136 of this type of modification on protein function.

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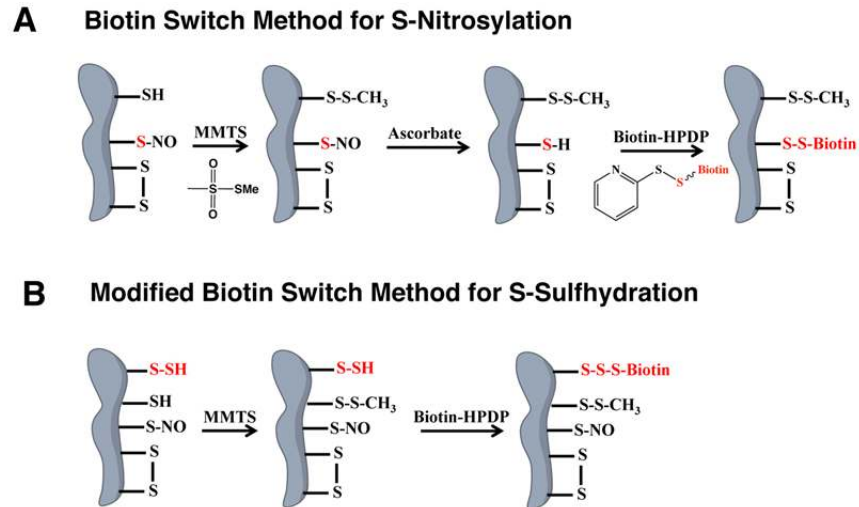
## 138 **RESULTS AND DISCUSSION**

139

### 140 **Covalent cysteine residue modification through S-sulphydration**

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142 The biotin switch method (BSM) has been widely used for the detection of post-  
143 translational modifications of proteins by S-nitrosylation, the covalent attachment of NO to  
144 cysteine residues (Sell et al., 2008). This assay consists of three steps: first, free thiols are  
145 blocked by the thiol-blocking reagent methyl methanethiosulfonate (MMTS); next, the S-  
146 NO bonds are reduced by ascorbate to form free thiols; finally, these thiols are ligated with  
147 N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide (biotin-HPDP) to form biotin-  
148 labeled proteins (Fig. 1A). Protein post-translational modifications by S-sulphydration, i.e.,



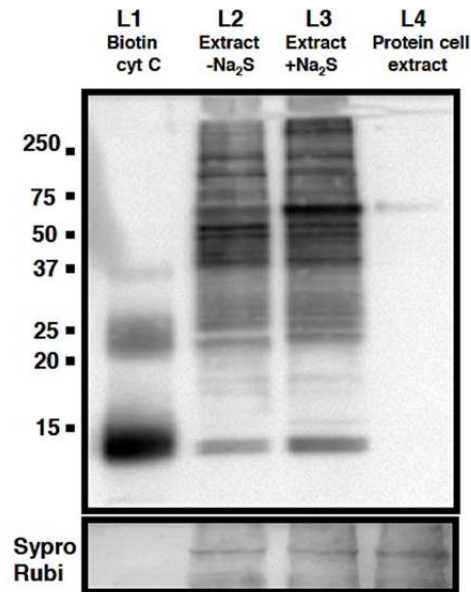
**Figure 1.** (A) Schematic representation of the biotin switch method for the detection of post-translational modification of proteins by S-nitrosylation, where free thiols are blocked by methyl methanethiosulfonate (MMTS), the S-NO bonds are reduced by ascorbate to form free thiols, and finally, these new thiols are ligated with the thiol-specific biotinylating agent biotin-HPDP to form biotin-labeled proteins. (B) Schematic representation of the modified biotin switch method for the detection of post-translational modification of proteins by S-sulfhydration, where free thiol residues are first blocked with MMTS; the persulfide residues remain unreacted and are available for subsequent reaction with biotin-HPDP to form biotin-labeled proteins. A sketch of a protein with different cysteine residues is shown. Additional details are described in the text.

149 the conversion of cysteine –SH residues to persulfide (-SSH), can also be detected using a  
 150 modified biotin switch method that was first described for protein analysis in mouse liver  
 151 lysates (Mustafa et al., 2009b). In the modified biotin switch method, free thiol residues are  
 152 first blocked with MMTS; the persulfide residues remain unreacted and are therefore  
 153 available for subsequent reaction with the thiol-specific biotinylating agent biotin-HPDP  
 154 (Fig. 1B) (Mustafa et al., 2009b).

155 Total leaf protein extracts from mature *Arabidopsis* plants grown under physiological  
 156 conditions, in the absence of oxidative stress or chemical treatments, were subjected to the  
 157 modified protein switch method to detect S-sulfhydrated proteins. The method selected  
 158 only biotin-labeled proteins, corresponding to proteins that contained persulfide residues,  
 159 which were analyzed using immunoblotting with antibodies against biotin (Fig. 2, lane L2).  
 160 A large array of proteins was clearly detected by the antibody, and the intensities of several  
 161 of the labeled proteins increased in protein extracts that were previously treated  
 162 exogenously using 200  $\mu$ M  $\text{Na}_2\text{S}$  for 30 min (Fig. 2, lane L3). Crude protein extracts that  
 163 were not subjected to the modified biotin switch method did not show any biotin-labeled  
 164 proteins (Fig. 2, lane L4).

165 The biotin-labeled proteins obtained using the modified BSM were further isolated using  
 166 a streptavidin-based affinity purification process. Three independent crude extracts from





**Figure 2.** Immunoblot analysis of the total S-sulfhydrated proteins. Protein cell extracts from 1 g of leaf tissue were exogenously untreated (L2) or treated (L3) using 200  $\mu$ M  $\text{Na}_2\text{S}$  for 30 min at 4°C and were subjected to the modified biotin switch method. The labeled proteins were detected using protein blot analysis with antibodies against biotin. Biotin labeled-cytochrome C protein (L1) and a protein cell extract that was not subjected to the modified biotin switch method (BSM) (L4) were used for the positive and negative control, respectively. Sypro Ruby fluorescent staining is shown as the protein loading control.

167 leaf tissue that were treated using the modified BSM were incubated with streptavidin  
 168 beads and then washed several times to avoid nonspecific bead binding. The eluted proteins  
 169 from the streptavidin beads were digested with trypsin and subsequently analyzed using  
 170 mass spectrometry. A total of 106 S-sulfhydrated proteins were identified with high  
 171 confidence (FDR < 1%) (Supplemental Table S1). This list of proteins represents the first  
 172 version of the group of plant proteins that are endogenously modified by S-sulfhydration.

173 The biological processes in which these proteins are involved were classified into 26  
 174 groups based on MapMan Classification (Thimm et al., 2004; Klie and Nikoloski, 2012).  
 175 The most abundant groups contained proteins involved in photosynthesis, protein synthesis  
 176 and cell organization (Supplemental Table S2). Many of the proteins identified are involved  
 177 in enzymatic processes related to primary metabolism, such as the Calvin cycle and the  
 178 tricarboxylic acid cycle, and many are regulated by thioredoxins, suggesting that these  
 179 proteins contain highly reactive cysteine residues.

180 Based on UniProt, several of the proteins identified in *Arabidopsis* have also been  
181 described in mammalian systems, such as actin, catalase, glutamine synthetase,  
182 glyceraldehyde 3-phosphate dehydrogenase, leucine aminopeptidase, ATP synthase,  $\beta$ -  
183 tubulin, and UDP-glucose dehydrogenase (Supplemental Table S3) (Mustafa et al., 2009b).  
184 Furthermore, the identities of some of the candidate plant proteins were confirmed through  
185 immunoblot analysis. Leaf protein extracts were subjected to the modified BSM assay and  
186 purified using streptavidin-agarose beads, and the retained proteins were separated using  
187 SDS-PAGE for immunoblot analysis. The chloroplastic GapA (A-1, 42 kDa) and GapB (48  
188 kDa) isoforms and the cytosolic GapC (37 kDa) isoform of glyceraldehyde 3-phosphate  
189 dehydrogenase, the chloroplastic glutamine synthetase GS2 isoform (43 kDa) and cytosolic  
190 ascorbate peroxidase APX1 (27 kDa) were identified in the eluted biotin-labeled protein  
191 pool by their expected molecular masses using polyclonal antibodies against homolog  
192 proteins (Fig. 3). Therefore, we demonstrated that all these plant proteins underwent S-  
193 sulfhydrylation.

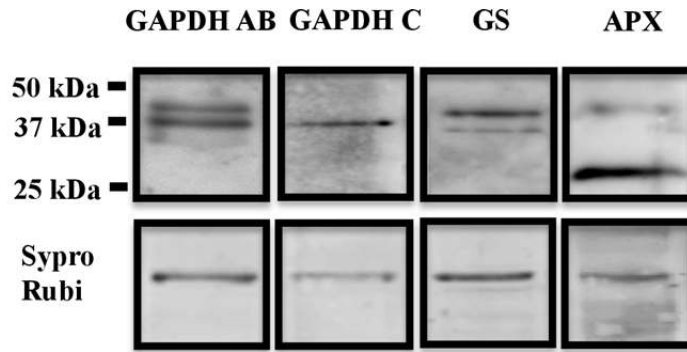
194 The recombinant cytosolic proteins ascorbate peroxidase and glyceraldehyde 3-  
195 phosphate dehydrogenase were purified and used to distinguish whether the proteins  
196 identified within the eluted biotin-labeled protein pool had been identified as a consequence  
197 of uncompleted MMTS blocking or whether they were indeed endogenously S-sulfhydrylated  
198 proteins. Recombinant APX1 and GAPC1 pretreated with 200  $\mu$ M NaHS showed a band of  
199 similar intensity to that in the sample untreated with NaHS, which was endogenously S-  
200 sulfhydrylated (Supplemental Fig. S1). When the proteins were pretreated with 1 mM DTT,  
201 no bands were detected because all disulfide bonds were reduced and then blocked by  
202 MMTS. The unblocked samples showed several bands of greater intensity than in the  
203 blocked samples, indicating that the MMTS blocking conditions were optimized.

204

#### 205 **Identification of S-sulfhydrylated cysteine residues of cytosolic APX using mass** 206 **spectrometry**

207

208 To demonstrate the presence of cysteine residues modified by S-sulfhydrylation and the  
209 target sites of a representative protein identified in this work, we carried out LC-MS/MS  
210 analysis on the cytosolic APX enzyme. Recombinant cytosolic APX enzyme was purified  
211 from a bacterial extract and trypsin-digested under non-reducing conditions to avoid the



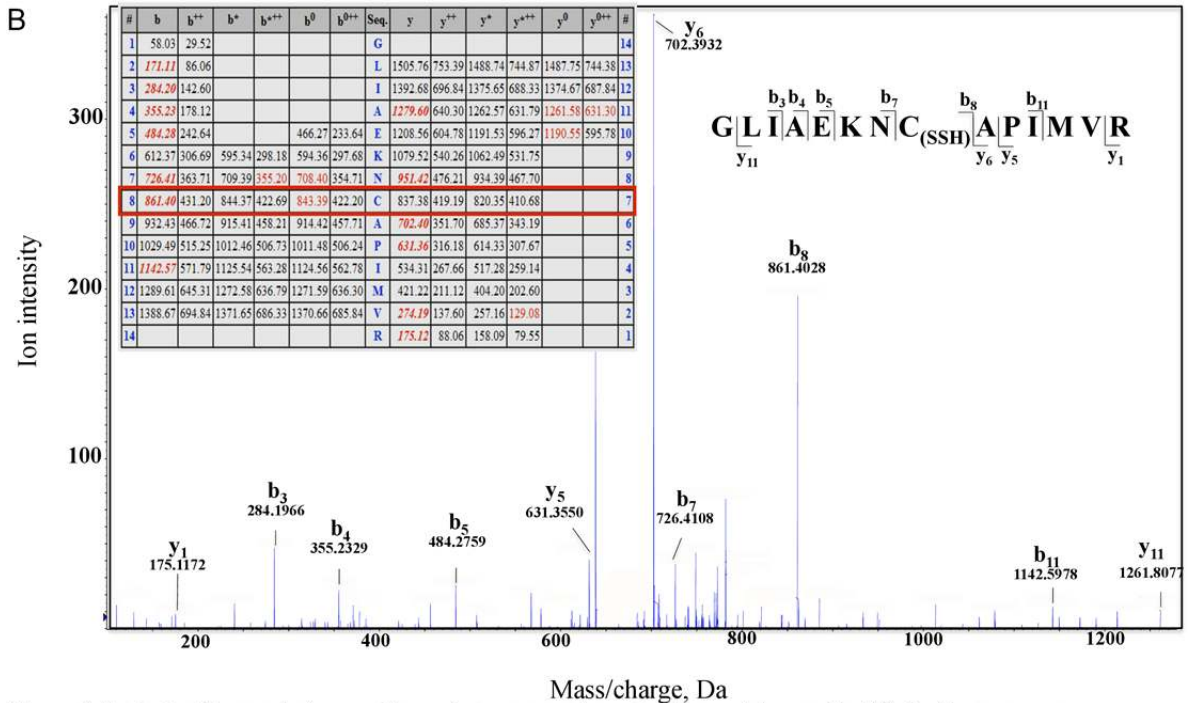
**Figure 3.** Immunoblot analysis of specific S-sulfhydrated candidate proteins.

Biotinylated proteins obtained from the leaf extracts subjected to the modified biotin switch assay were purified using streptavidin-agarose beads and analyzed using four different immunoblots with the following antibodies: anti-chloroplastic GAPDH antibodies that recognized the chloroplastic isoforms A and B; anti-cytosolic GAPDH antibodies that recognized the cytosolic isoform C; anti-GS antibodies that recognized both the chloroplastic and cytosolic isoforms, and anti-cytosolic APX antibodies. Sypro Ruby fluorescent staining is shown as the protein loading control.

212 reduction of persulfide residues. However, under this condition, disulfide bridges between  
 213 digested peptides cannot be avoided. The digested peptides were analyzed using LC-  
 214 MS/MS for a 32-Da mass increase in the fragmentation spectrum. As illustrated in Figure  
 215 4, cytosolic L-ascorbate peroxidase 1 was identified with a sequence coverage of 74%.  
 216 Among the peptides identified, only one, GLIAEKNCAPIMVR, containing Cys32, showed  
 217 a sulfhydryl modification. Putative peptides containing the two other cysteine residues were  
 218 not detected in the analysis, most likely because they formed a disulfide bridge under the  
 219 non-reducing conditions utilized.

220 The oxidation of Cys32 causes APX1 inactivation, and it has been suggested that  
 221 glutathionylation protects the enzyme from irreversible oxidation (Kitajima et al., 2008).  
 222 The active site Cys32 of APX1 can also be S-nitrosylated by nitric oxide, which increases  
 223 the activity of the enzyme, and it has been hypothesized that this PTM might be involved in  
 224 the specific case of salinity stress, which is accompanied by both oxidative stress and an  
 225 increase in SNOs (Begara-Morales et al., 2014). The fact that Cys32 is altered by different  
 226 posttranslational modifications suggests that this enzyme must be finely regulated under  
 227 specific environmental stress conditions.

A MTKNYPTVSE~~DK~~YKKA~~VEK~~CRRKLRGLIAEKNCAPIMVRLAWHSAGTFDCQSRTGGPFGTMRFD~~AEQA~~HGANS~~GIHIAL~~  
**RLLDPIRE**QFPPTISFAD~~FHQLAGVVAVEVTGGDPDIPFHPGR~~**EDKPQPPPEGR**LPDATKGC~~DHLRDVFAK~~**QMGLSDKDI**VALS  
**GAHTLGR**CHKDRS~~GFEGAWTSNPLIFD~~NSYFK**ELLSGEKEGLLQLVSDK**ALLDDP**VFRPLVEKYAA**DEDAFFADYAEAHM  
**KLSELGFADA**

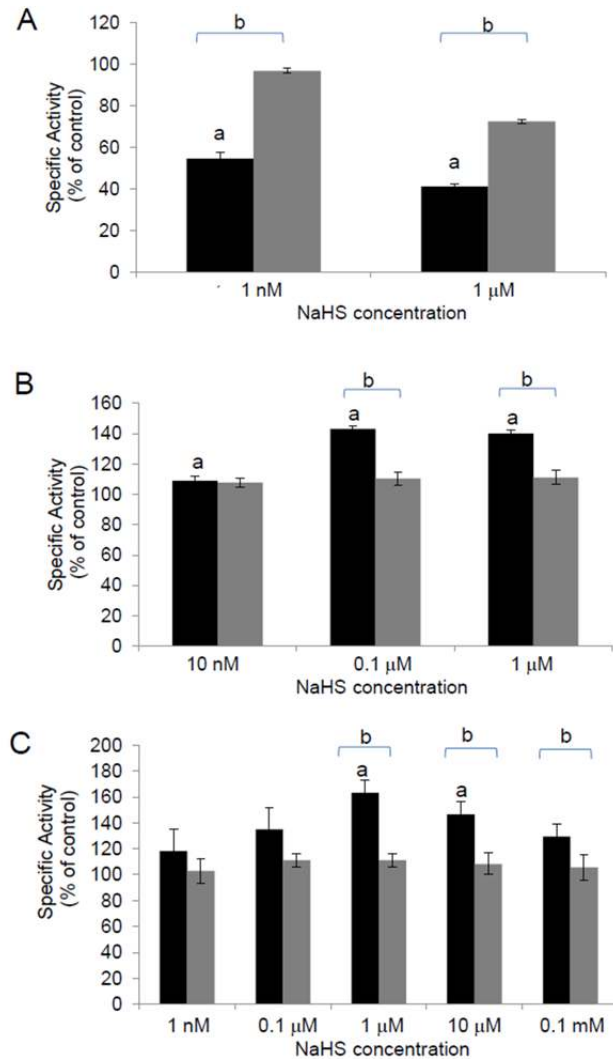


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## 229 S-sulfhydration regulates enzyme activity

230

231 To determine whether this protein modification has a biological role in plant systems,  
 232 we performed enzyme activity assays using total leaf protein extracts. Protein extracts were  
 233 treated with different concentrations of NaHS as a sulfide donor, and then GS, APX and  
 234 GAPDH activities were measured (Fig. 5). We clearly observed a significant inactivation of  
 235 GS activity, even in the presence of very low concentrations (1 nM) of NaHS in which only  
 236 half the level of the measured activity in the absence of sulfide was reached (Fig. 5A).  
 237 Curiously, incubation with NaHS produced the opposite response for APX and GAPDH  
 238 activities. In these cases, we observed an increase in APX and GAPDH activities of  
 239 approximately 40% and 60%, respectively, compared with the activity level in the absence  
 240 of sulfide. However, the activity of the APX enzyme appeared less sensitive to sulfide



**Figure 5.** Enzyme activity regulation of glutamine synthetase, ascorbate peroxidase and glyceraldehyde-3-phosphate dehydrogenase by S-sulphydration in *Arabidopsis thaliana*. The protein leaf extracts were treated in the absence or presence of NaHS at the indicated concentrations for 30 min at 4°C (black bars), and an additional treatment with DTT 50 mM was performed for 10 min in some cases (grey bars). Then, glutamine synthetase (A), ascorbate peroxidase (B) or glyceraldehyde-3-phosphate dehydrogenase (C) enzyme activity was measured as described in Materials and Methods. All results are shown as the mean  $\pm$  SD. Significant differences between the treatments with and without NaHS are indicated by the letter “a” ( $P < 0.05$ ). Significant differences between samples with or without DTT are indicated by the letter “b” ( $P < 0.05$ )

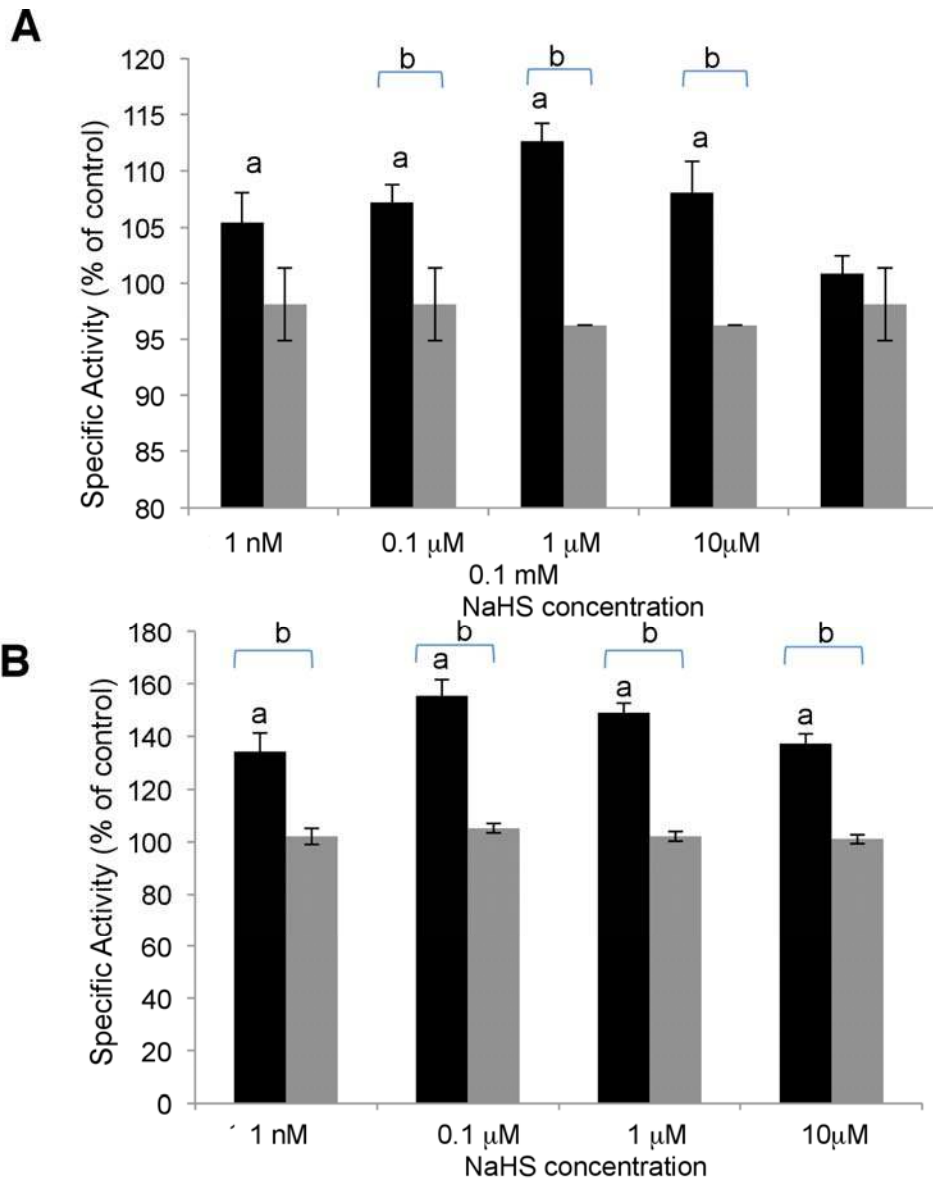
241 regulation than that of GS because the lowest concentration of NaHS required to observe  
 242 increased activity was 10 nM. In the case of GAPDH activity, the addition of 1  $\mu$ M of  
 243 NaHS was the minimum concentration required to detect a significant increase in activity  
 244 (Fig. 4B and 4C). Interestingly, the addition of DTT to the sulfide-treated extracts reversed  
 245 the effect of sulfide in these enzyme activity assays. In the case of APX and GAPDH, the

246 activity levels decreased following the addition of DTT and fell to the untreated value. In  
247 the case of GS, reactivation to the same level as that of the untreated extract was observed  
248 following the addition of DTT (Fig. 5).

249 The enzymatic activity assay was also performed with recombinant APX1 and GAPC1  
250 (Fig. 6). Recombinant GAPC1 showed higher activity than the leaf protein extract (data not  
251 shown) but the same sensitivity to the addition of NaHS, reaching an increased activity of  
252 approximately 60% when the protein was pretreated with NaHS. In contrast, recombinant  
253 APX1 appeared to be less sensitive to the addition of NaHS, showing an increased activity  
254 of almost 15% in response to NaHS pretreatment. Nevertheless, 1 nM NaHS was the  
255 minimal concentration required to observe a significant increase in the activity of both  
256 enzymes. The addition of DTT to the NaHS-pretreated samples had the same effect as in  
257 the leaf protein extract assay: both recombinant enzymes showed a reduction in activity  
258 after the addition of DTT, decreasing to the same value as the untreated protein (Fig. 6).

259 Because very low concentrations of NaHS were sufficient to produce an  
260 inactivation/activation effect on enzyme activities and because this effect was reversible, it  
261 is possible that sulfide has a biological role in plants, most likely through S-sulfhydration  
262 protein modification, similarly to mammalian systems, where the biological function of S-  
263 sulfhydration is well established. For example, it has been reported that the protein tyrosine  
264 phosphatase PTP1B is reversibly inactivated by sulfide, with PTP1B S-sulfhydration  
265 playing a role in the response to endoplasmic reticulum (ER) stress (Krishnan et al., 2011).  
266 Similarly, it has been shown that H<sub>2</sub>S acts as an endogenous inhibitor of phosphodiesterase  
267 (PDE) activity, suggesting that some of the critical cysteine residues are S-sulfhydrated and  
268 impair PDE activity (Bucci and Cirino, 2011). However, incubation with NaHS increases  
269 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity, which is reversed by DTT  
270 (Mustafa et al., 2009b; Gadalla and Snyder, 2010). Similarly, NaHS specifically enhances  
271 actin polymerization and activates ATP-sensitive potassium channels, effects that are also  
272 both reversed by DTT (Mustafa et al., 2009b; Mustafa et al., 2011). Finally, the median  
273 effective physiological concentration of sulfide for sulfhydrating protein targets in most  
274 mammalian tissues is considered to be in the micromolar range (Mustafa et al., 2009a;  
275 Nagy et al., 2014), similar to the average concentration of 50 μM calculated for the cytosol  
276 in *Arabidopsis* leaves (Krueger et al., 2009). However, we must consider that the level of





**Figure 6.** Enzyme activity regulation of recombinant cytosolic ascorbate peroxidase (APX1) and cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC1) by S-sulfhydration. Purified proteins were treated in the absence or presence of NaHS at the indicated concentrations for 30 min at 4°C (black bars), and in some cases an additional treatment with DTT 1 mM was performed (grey bars). Then, APX1 (A) or GAPC1 (B) enzyme activity was measured as described in Materials and Methods. All results are shown as the mean  $\pm$  SD. Significant differences between treatments with and without NaHS are indicated by the letter “a” ( $P < 0.05$ ). Significant differences between samples with or without DTT are indicated by the letter “b” ( $P < 0.05$ ).

277 sulfide in the cytosol is determined via the coordinated activities of both cytosolic enzymes,  
 278 i.e., O-acetylserine(thiol)lyase OAS-A1 that incorporates sulfide to form cysteine, and L-  
 279 cysteine desulfhydrase DES1. Therefore, the level of sulfide may change under the

280 developmental and stress conditions that may regulate these activities (Alvarez et al., 2011;  
281 Alvarez et al., 2012a; Laureano-Marín et al., 2014).

282

## 283 **MATERIALS AND METHODS**

284

### 285 **Plant Material and Growth Conditions**

286

287 *Arabidopsis* (*Arabidopsis thaliana*), wild-type ecotype Col-0, was grown in soil under a  
288 photoperiod of 16 h of white light ( $120 \mu\text{E m}^{-2} \text{s}^{-1}$ ) at 20 °C and 8 h of dark at 18 °C (Garcia  
289 et al., 2013).

290

### 291 **Modified Biotin Switch Method**

292

293 The modified biotin switch assay was adapted from a previously described protocol  
294 (Mustafa et al., 2009b). Frozen *Arabidopsis* leaves collected from 30-day-old plants were  
295 ground to a fine powder in a mortar under liquid nitrogen, homogenized in HEN buffer  
296 containing 250 mM Hepes-NaOH (pH 7.7), 1 mM EDTA and 0.1 mM neocuproine  
297 supplemented with 100  $\mu\text{M}$  deferoxamine and 1X protease inhibitor cocktail (Roche) and  
298 centrifuged at 14,000 rpm for 10 min at 4°C. Two volumes of blocking buffer (HEN buffer  
299 supplemented with 2.5% SDS and 20 mM methyl methanethiosulfonate (MMTS)) were  
300 added to the leaf extract, and the solution was incubated at 50°C for 20 min to block free  
301 sulfhydryl groups. The MMTS was then removed, and the proteins were precipitated using  
302 acetone at -20°C for 20 min. The acetone was removed, and the proteins were resuspended  
303 in HENS buffer (HEN buffer supplemented with 1% SDS). The S-sulfhydrated proteins  
304 were then labeled using 4 mM N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide  
305 (Biotin-HPDP) for 3 hours at 25°C in the dark.

306 Purified recombinant proteins were used to optimize the blocking conditions to avoid  
307 incomplete blocking. Thus, purified recombinant APX1 and GAPC1 were pretreated with  
308 200  $\mu\text{M}$  NaHS to increase the concentration of S-sulfhydrated proteins or with 1 mM DTT  
309 to reduce all disulfide bonds; both treatments were carried out for 30 min at 4°C and buffer  
310 exchanged to eliminate residual DTT or NaHS before performing the modified biotin



311 switch assay. An untreated aliquot of purified proteins did not undergo the blocking stage  
312 with methyl methanethiosulfonate (MMTS) during the modified biotin switch assay for  
313 comparison with the blocked proteins. The biotinylated proteins were detected using an  
314 immunoblot assay with anti-biotin antibodies (Abcam antibodies) as described below.

315

### 316 **Streptavidin-Based Affinity Purification Process**

317

318 The labeled proteins were precipitated using acetone, and the washed pellet was  
319 resuspended in HENS buffer. To purify the biotinylated proteins, the solution was  
320 incubated with streptavidin beads for 1 hour at room temperature with frequent vortexing.  
321 The streptavidin beads were intensively washed five times using ten volumes of 20 mM  
322 Hepes-NaOH (pH 7.7), 600 mM NaCl, 1 mM EDTA and 0.5% Triton X-100 and then  
323 centrifuged at 3000 rpm for 5 s at room temperature between each wash. To recover the  
324 bound proteins, the beads were incubated with 20 mM Hepes-NaOH (pH 7.7), 100 mM  
325 NaCl, 1 mM EDTA and 100 mM 2-mercaptoethanol for 10 min at room temperature. The  
326 total amount of purified proteins was determined using Bradford's method (Bradford, 1976).

327

### 328 **Expression and Purification of Recombinant His-Tagged Proteins**

329

330 The complete cDNAs of cytosolic ascorbate peroxidase APX1 (At1g07890) and the  
331 cytosolic GapC isoform of glyceraldehyde 3-phosphate dehydrogenase GAPC1  
332 (At3g04120) were cloned into the pDEST17 vector (Invitrogen) to express an N-terminal 6-  
333 His-tagged protein using the *E. coli* expression system with Gateway Technology  
334 (Invitrogen). For APX1 and GAPC1 protein expression, transformed *E. coli* BL21(DE3)  
335 cell cultures at an OD<sub>600</sub> of 0.6 were treated with 0.1 and 0.5 mM of IPTG (isopropyl-beta-  
336 D-thiogalactopyranoside), respectively; the cell cultures were incubated for 4 hours at 30°C.  
337 Purification was performed by nickel resin binding under non-denaturing conditions using  
338 the Ni-NTA Purification System (Invitrogen) according to the manufacturer's  
339 recommendations. Recombinant protein production and purification were assessed by SDS-  
340 PAGE using 12% (w/v) polyacrylamide gels and Coomassie Brilliant Blue staining.

341

342 **Identification of S-Sulfhydrated Cysteine Residues of Recombinant Cytosolic APX**  
343 **using Mass Spectrometry**  
344

345 Cytosolic ascorbate peroxidase APX1 was separated using non-reducing SDS-PAGE on  
346 12% polyacrylamide gels and the band corresponding to APX1 was excised manually from  
347 Coomassie-stained gels, deposited in 96-well plates and processed automatically in a  
348 Proteiner DP (Bruker Daltonics, Bremen, Germany). The digestion protocol used was  
349 based on Shevchenko et al. (1996) without the reduction or alkylation steps: gel plugs were  
350 washed twice, first using 50 mM ammonium bicarbonate and second using ACN, and then  
351 dried under a stream of nitrogen. Then, proteomics-grade trypsin (Sigma Aldrich) at a final  
352 concentration of 16 ng/ $\mu$ l in 25% ACN/50 mM ammonium bicarbonate solution was added  
353 and digestion took place at 37°C for 5 h. The reaction was stopped by adding 50%  
354 ACN/0.5% TFA for peptide extraction. The tryptic eluted peptides were dried using speed-  
355 vacuum centrifugation and were resuspended in 6  $\mu$ l of 0.1% FA in water.

356 Digested peptides were subjected to 1D-nano LC ESI-MSMS analysis using a nano liquid  
357 chromatography system (nanoLC Ultra 1D plus, Eksigent Technologies) coupled to a high  
358 speed Triple TOF 5600 mass spectrometer (AB SCIEX, Foster City, CA) with a duo spray  
359 ionization source. Data acquisition was performed using a TripleTOF 5600 System (AB  
360 SCIEX, Concord, ON). MS and MS/MS data obtained for individual samples were  
361 processed using Analyst® TF 1.5.1 Software (AB SCIEX). Peptide mass tolerance was set  
362 to 25 ppm and 0.05 Da for fragment masses, and only 1 or 2 missed cleavages were  
363 allowed. Peptides with an individual MOWSE score  $\geq$  20 were considered correctly  
364 identified.

365  
366 **Immunoblot Analysis**

367 The biotinylated proteins were separated using non-reducing SDS-PAGE on 12%  
368 polyacrylamide gels before being transferred to polyvinylidene fluoride membranes (Bio-  
369 Rad) according to the manufacturer's instructions. Anti-biotin (Abcam antibodies) and  
370 secondary antibodies were diluted 1:500,000 and 1:100,000, respectively, and an ECL  
371 Select Western Blotting Detection Reaction (GE Healthcare) was used to detect the proteins  
372 using horseradish peroxidase-conjugated anti-rabbit secondary antibodies. The streptavidin-

373 purified biotinylated proteins were also subjected to an immunoblot analysis using SDS-  
374 PAGE on 15% polyacrylamide gels using the following antibodies: i) polyclonal antibodies  
375 raised against different forms of GAPDH from the microalga *Scenedesmus vacuolatus*,  
376 anti-GAP2 and anti-GAP3, which recognize the *Arabidopsis* chloroplastic isoforms A and  
377 B and the cytosolic isoform C, respectively (Valverde et al., 2005), diluted 1:10,000; ii)  
378 anti-GS antibodies raised against recombinant homopolymeric GS from *Phaseolus vulgaris*,  
379 recognizing mainly the *Arabidopsis* chloroplastic isoform and also to a minor extent the  
380 cytosolic isoform (Betti et al., 2006) diluted 1:1,000; and iii) and anti-cytosolic APX  
381 antibodies (Agrisera) diluted 1:10,000. Prior to immunodetection, the membrane was  
382 stained using SYPRO Ruby (Life Technologies) as a protein loading control.

383

### 384 **Mass Spectrometry Analysis**

385

386 The purified protein samples were precipitated using 10% trichloroacetic acid and were  
387 acetone-washed before tryptic digestion. The samples were then purified using Pierce®  
388 C18 spin columns and evaporated in a speed vacuum prior to storage at -80°C. A label-free  
389 analysis was performed as described in (Schroder et al., 2012). Briefly, peptides were  
390 separated by reverse-phase chromatography using an Eksigent Ultra 2D+ pump fitted with  
391 a 75- $\mu$ m ID column (nanoLC column, 75  $\mu$ m id x 15 cm, C18, 3  $\mu$ m, 120 Å, ChromXP);  
392 the samples were first loaded into a 2 cm long, 100- $\mu$ m ID pre-column, packed using the  
393 same chemistry as the separating column, for desalting and concentrating. The mobile  
394 phases were 100% water/0.1% formic acid (buffer A) and 100% acetonitrile/0.1% formic  
395 acid (buffer B). The column gradient was developed using a 60-min, two-step run from 5%  
396 B to 30% B in 30 min and 30% B to 70% B in 10 min. The column was equilibrated with  
397 95% B for 5 min and 5% B for 15 min. During all processes, the pre-column was in-line  
398 with the separating column, and the flow was maintained along the entire gradient at 300  
399 nl/min. The peptides eluted from the column were analyzed using an AB Sciex 5600  
400 TripleTOFTM+ system. Data-dependent acquisition occurred during a 250-ms survey  
401 sampling performed over a mass range from 350 m/z to 1250 m/z. The top 20 peaks were  
402 selected for fragmentation. The minimum accumulation time for MS/MS was set to 50 ms,  
403 for a total cycle time of 1250 ms. The product ions were surveyed during a 15-s period over

404 a mass range from 100 m/z to 1500 m/z and excluded from further fragmentation. After the  
405 MS/MS analysis, the data files were processed using ProteinPilot™ 4.5 software from AB  
406 Sciex, which implements the algorithms Paragon™ for database searching and  
407 Progroup™ for data grouping (Shilov et al., 2007), and were searched against the Uniprot  
408 *Arabidopsis*-specific database. A false discovery rate was performed using a non-linear  
409 fitting method (Tang et al., 2008), and the results displayed are those with a 1% global false  
410 discovery rate or better. The data were analyzed using three technical replicas for each  
411 sample. Peak lists were generated in PeakView™ 1.1 Software from AB Sciex using the  
412 combined database search results generated in the ProteinPilot™ 4.5 software. The peak  
413 list matrix generated was exported to MarkerView™ 1.2.1 software for Principal  
414 Component Analysis (PCA). Sample comparison was performed using the first two  
415 components, which explained a total of 75% of the variance between samples. Sample  
416 dispersion was measured using a *t* test, and proteins with extreme *t* values were chosen as  
417 candidates for validation.

418

#### 419 **Enzyme Activity Assays**

420

421 Plant leaf material from 30-day-old plants was ground using a mortar and pestle with  
422 liquid nitrogen in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1  
423 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM sodium ascorbate and 5%  
424 polyvinylpyrrolidone (PVPP) (w/v) for APX activity, in 50 mM Tris-HCl (pH 7.5) for  
425 GAPDH activity and in 20 mM HEPES-NaOH (pH 7.0) for GS activity. All buffers were  
426 supplemented with a protease inhibitor cocktail (Roche). The leaf extracts were centrifuged  
427 at 14,000 rpm for 10 min at 4°C, and the supernatant was used as the soluble extract.

428 The APX activity was determined as previously described (García-Limones et al., 2002).  
429 The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.25 mM  
430 sodium ascorbate and 0.05 ml of leaf extract (containing approx. 0.5 mg of total protein) or  
431 0.05 ml of purified APX recombinant protein (containing approx. 0.1 mg of total protein).  
432 The reaction was initiated by adding 5 mM H<sub>2</sub>O<sub>2</sub>, and the oxidation of ascorbate was  
433 determined by the decrease in absorbance at 290 nm ( $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

434 The GAPDH activity was monitored spectrophotometrically at room temperature by  
435 following the glycolytic reaction assays, as described previously (Bedhomme et al., 2012).  
436 The glycolytic reaction was measured in an assay containing 50 mM Tris-HCl (pH 7.5), 1  
437 mM NAD<sup>+</sup>, 10 mM sodium arsenate and 0.05 ml of leaf extract (containing approx. 2 mg  
438 of total protein) or 0.05 ml of purified GAPDH cytosolic isoform C recombinant protein  
439 (containing approx. 0.1 mg of total protein). The reaction was initiated by the addition of 1  
440 mM glyceraldehyde 3-phosphate, and the absorbance at 340 nm was recorded for 1 minute  
441 ( $\epsilon= 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

442 The GS transferase activity was measured by the formation of  $\gamma$ -glutamylhydroxamate  
443 (Merida et al., 1991). The assay was performed in a final volume of 1 ml that contained 60  
444  $\mu\text{mol}$  of HEPES-NaOH buffer (pH 7.0), 40  $\mu\text{mol}$  of L-glutamine, 4  $\mu\text{mol}$  of  $\text{MnCl}_2$ , 60  
445  $\mu\text{mol}$  of hydroxylamine, 1  $\mu\text{mol}$  of ADP and 0.05 ml of leaf extract (containing approx. 2  
446 mg of total protein). The reaction was initiated by the addition of 20  $\mu\text{mol}$  of sodium  
447 arsenate, and the amount of  $\gamma$ -glutamylhydroxamate formed after 10 min of incubation at  
448 30°C was determined spectrophotometrically at 500 nm ( $\epsilon= 0.89 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

449

## 450 **Statistical Analysis**

451

452 All results are shown as the mean  $\pm$  standard deviation of three biological replicas. The  
453 data were analyzed by ANOVA using Microsoft Excel ( $P<0.05$ ).

454

455

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457

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462 and Dr. M. Isabel Muro-Pastor for helping with the GS activity assay.

463

464

465 **FIGURE LEGENDS**

466

467 **Figure 1.** (A) Schematic representation of the biotin switch method for the detection of  
468 post-translational modification of proteins by S-nitrosylation, where free thiols are blocked  
469 by methyl methanethiosulfonate (MMTS), the S-NO bonds are reduced by ascorbate to  
470 form free thiols, and finally, these new thiols are ligated with the thiol-specific biotinylating  
471 agent biotin-HPDP to form biotin-labeled proteins. (B) Schematic representation of the  
472 modified biotin switch method for the detection of post-translational modification of  
473 proteins by S-sulfhydration, where free thiol residues are first blocked with MMTS; the  
474 persulfide residues remain unreacted and available for subsequent reaction with biotin-  
475 HPDP to form biotin-labeled proteins. A sketch of a protein with different cysteine residues  
476 is shown. Additional details are described in the text.

477

478 **Figure 2.** Immunoblot analysis of the total S-sulfhydrated proteins. Protein cell extracts  
479 from 1 g of leaf tissue were exogenously untreated (L2) or treated (L3) using 200  $\mu$ M  $\text{Na}_2\text{S}$   
480 for 30 min at 4°C and were subjected to the modified biotin switch method. The labeled  
481 proteins were detected using protein blot analysis with antibodies against biotin. Biotin  
482 labeled-cytochrome C protein (L1) and a protein cell extract that was not subjected to the  
483 modified biotin switch method (BSM) (L4) were used for the positive and negative control,  
484 respectively. Sypro Ruby fluorescent staining is shown as the protein loading control.

485

486

487 **Figure 3.** Immunoblot analysis of specific S-sulfhydrated candidate proteins. Biotinylated  
488 proteins obtained from the leaf extracts subjected to the modified biotin switch assay were  
489 purified using streptavidin-agarose beads and analyzed using four different immunoblots  
490 with the following antibodies: anti-chloroplastic GAPDH antibodies that recognized the  
491 chloroplastic isoforms A and B; anti-cytosolic GAPDH antibodies that recognized the  
492 cytosolic isoform C; anti-GS antibodies that recognized both the chloroplastic and cytosolic  
493 isoforms, and anti-cytosolic APX antibodies. Sypro Ruby fluorescent staining is shown as  
494 the protein loading control.

495

496 **Figure 4.** Analysis of L-ascorbate peroxidase using mass spectrometry. The protein was  
497 identified with a sequence coverage of 74%; the identified peptides are shown in bold red  
498 and the peptide containing S-sulfhydrated Cys<sup>32</sup> is shown underlined (A). LC-MS/MS  
499 analysis of the tryptic peptide containing Cys<sup>32</sup> of APX1. The table inside the spectrum  
500 contains the predicted ion types for the modified peptide, and the ions detected in the  
501 spectrum (Biemann, 1988) are highlighted in red color. Nomenclature of the fragment ions  
502 and types corresponds to that proposed by Roepstorff and Fohlman (1984) and modified by  
503 Biemann (Biemann, 1988) (B).

504

505 **Figure 5.** Enzyme activity regulation of glutamine synthetase, ascorbate peroxidase and  
506 glyceraldehyde-3-phosphate dehydrogenase by S-sulfhydration in *Arabidopsis thaliana*.  
507 The protein leaf extracts were treated in the absence or presence of NaHS at the indicated  
508 concentrations for 30 min at 4°C (black bars), and an additional treatment with DTT 50 mM  
509 was performed for 10 min in some cases (grey bars). Then, glutamine synthetase (A),  
510 ascorbate peroxidase (B) or glyceraldehyde-3-phosphate dehydrogenase (C) enzyme  
511 activity was measured as described in Materials and Methods. All results are shown as the  
512 mean ± SD. Significant differences between the treatments with and without NaHS are  
513 indicated by the letter “a” (P<0.05). Significant differences between samples with or  
514 without DTT are indicated by the letter “b” (P<0.05).

515

516 **Figure 6.** Enzyme activity regulation of recombinant cytosolic ascorbate peroxidase  
517 (APX1) and cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC1) by S-  
518 sulfhydration. Purified proteins were treated in the absence or presence of NaHS at the  
519 indicated concentrations for 30 min at 4°C (black bars), and in some cases an additional  
520 treatment with DTT 1 mM was performed (grey bars). Then, APX1 (A) or GAPC1 (B)  
521 enzyme activity was measured as described in Materials and Methods. All results are  
522 shown as the mean ± SD. Significant differences between treatments with and without  
523 NaHS are indicated by the letter “a” (P<0.05). Significant differences between samples  
524 with or without DTT are indicated by the letter “b” (P<0.05).

525

526





528 **Supplemental Data**

529

530 **Supplemental Figure S1.** Immunoblot analysis of specific S-sulfhydrated candidate  
531 proteins.

532

533 **Supplemental Table S1.** S-sulfhydrated proteins from *Arabidopsis thaliana*.

534

535 **Supplemental Table S2.** Gene ontology classification of S-sulfhydrated proteins.

536

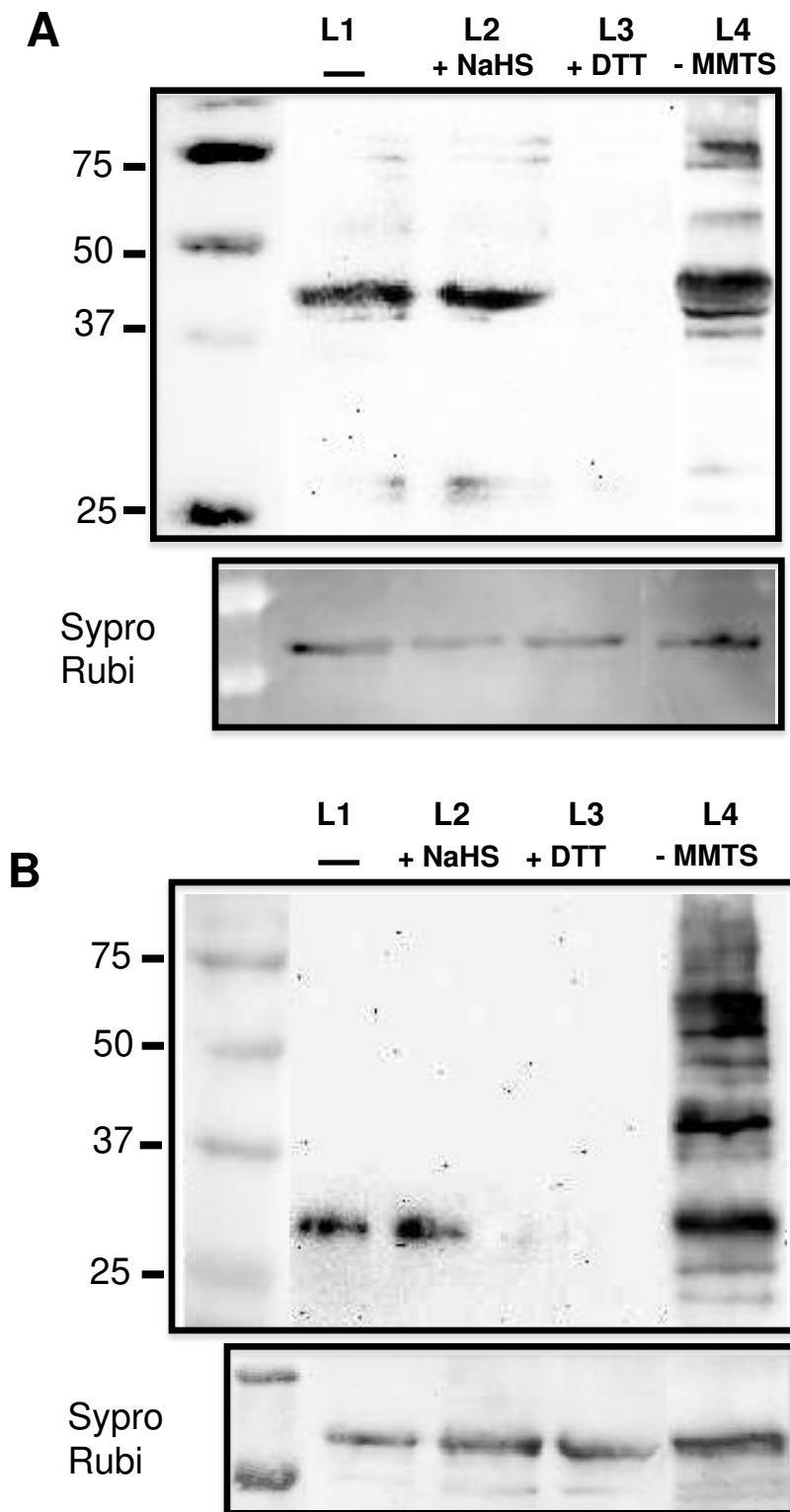
537 **Supplemental Table S3.** Common candidates for protein S-sulfhydration from  
538 *Arabidopsis thaliana* and liver mammalian cells.

539

540

541

542



**Supplemental Figure S1.** Analysis of the specificity of the modified biotin switch method using recombinant proteins. Purified recombinant GAPC1 (A) and APX1 (B) proteins were untreated (L1) or treated with 200  $\mu$ M NaHS (L2) or 1 mM DTT (L3) prior to perform the modified biotin switch assay as described in Materials and Methods. As a negative control of the blocking treatment, the modified biotin switch assay without MMTS treatment was performed on the untreated sample (L4). Biotinylated proteins were separated by SDS-PAGE and immunoblotted with antibodies anti-Biotin. The Sypro Rubi fluorescent staining is shown as the protein loading control.

**Supplemental Table S3.** Common candidates for protein S-sulfhydration from *Arabidopsis thaliana* and liver mammalian cells.

S-sulfhydrated proteins in mouse liver	S-sulfhydrated proteins in Arabidopsis leaves	Arabidopsis locus
Actin	Actin-1	At2g37620
	Actin-2	At3g18780
	Actin-3	At3g53750
	Actin-4	At5g59370
	Actin-7	At5g09810
	Actin-11	At3g12110
	Actin-12	At3g46520
Catalase	Catalase-3	At1g20620
Glutamine synthetase	Glutamine synthetase, chloroplastic/mitochondrial	At5g35630
Glyceraldehyde 3 phosphate dehydrogenase (GAPDH)	Glyceraldehyde-3-phosphate dehydrogenase A	At3g26650
	Glyceraldehyde-3-phosphate dehydrogenase B	At1g42970
	Glyceraldehyde-3-phosphate dehydrogenase, GapC1	At3g04120
	Glyceraldehyde-3-phosphate dehydrogenase, GapC2	At1g13440
ATP synthase, mitochondrial	ATP synthase subunit alpha	At2g07698
	ATP synthase subunit alpha, chloroplastic	AtCg00120
	ATP synthase subunit beta, chloroplastic	AtCg00480
Tubulin beta	Tubulin beta-9 chain	At4g20890

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