S-Sulfhydration: A Cysteine Posttranslational Modification in Plant Systems[1]

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Hydrogen sulfide is a highly reactive molecule that is currently accepted as a signaling compound. This molecule is as important as carbon monoxide in mammals and hydrogen peroxide in plants, as well as nitric oxide in both eukaryotic systems. Although many studies have been conducted on the physiological effects of hydrogen sulfide, the underlying mechanisms are poorly understood. One of the proposed mechanisms involves the posttranslational modification of protein cysteine residues, a process called *S*-sulfhydration. In this work, a modified biotin switch method was used for the detection of Arabidopsis (*Arabidopsis thaliana*) proteins modified by *S*-sulfhydration under physiological conditions. The presence of an *S*-sulfhydration-modified cysteine residue on cytosolic ascorbate peroxidase was demonstrated using liquid chromatography-tandem mass spectrometry analysis, and a total of 106 *S*-sulfhydrated proteins were identified. Immunoblot and enzyme activity analyses of some of these proteins showed that the sulfide added through *S*-sulfhydration reversibly regulates the functions of plant proteins in a manner similar to that described in mammalian systems.

Hydrogen sulfide (H_2S) is a highly reactive and toxic molecule that has recently emerged as an important signaling compound with many physiological functions in both health and disease (Li et al., 2011; Kolluru et al., 2013). The possible role of H_2S as an endogenous neuromodulator was first described in 1996, and the molecule is now accepted as the third most prevalent gasotransmitter after nitric oxide (NO) and carbon monoxide (Abe and Kimura, 1996; Vandiver and Snyder, 2012). In animal systems, the biosynthesis of H_2S occurs through the action of three enzymes that are involved in the metabolism of sulfur-containing amino acids: cystathionine γ-lyase, cystathionine β-synthase, and 3-mercaptopyruvate sulfurtransferase. These enzymes are typically localized either to specific organs or to subcellular components such as the mitochondria and cytosol (Wang, 2012).

In plant systems, emerging data in recent years also suggest that H_2S may function as an important signaling molecule, similar to NO or hydrogen peroxide (H_2O_2). With regard to certain stresses, H_2S treatment alleviates the inhibitory effect of boron on cucumber (*Cucumis sativus*) root elongation (Wang et al., 2010) and the inhibitory effects of copper and aluminum stress on wheat (*Triticum aestivum*) germination (Zhang et al., 2008, 2010). In addition, H_2S pretreatment alleviates cadmium toxicity in alfalfa (*Medicago sativa*; Li et al., 2012a), improves heat tolerance in tobacco (*Nicotiana tabacum*) suspension-cultured cells (Li et al., 2012b), and protects bermudagrass (*Cynodon dactylon*) from saline, osmotic, and freezing stresses (Shi et al., 2013). H_2S also plays a role in the regulation of drought stress and has been described as a component of the abscisic acid signaling network in guard cells (García-Mata and Lamattina, 2010; Lisjak et al., 2010; Jin et al., 2013; Scuffi et al., 2014). Moreover, H_2S has been shown to modulate photosynthesis through the promotion of chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification in *Spinacia oleracea* seedlings (Chen et al., 2011).

At the cellular level, cytosolic enzyme L-Cys desulfhydrase (DES1) is involved in the degradation of Cys and is therefore responsible for the generation of H_2S in this cellular compartment (Alvarez et al., 2010; Romero et al., 2013). The detailed characterization of *DES1* null mutants has provided insight into the role of Cys-generated sulfide as a signaling molecule that regulates the process of autophagy in the cytosol. Furthermore, DES1 deficiency promotes the accumulation and lipidation of the AUTOPHAGY RELATED8 (ATG8) protein, which is associated with the process of autophagy (Álvarez et al., 2012b). In addition, the transcriptional profile of the *DES1* null mutant, in which different *ATG* genes are upregulated, confirms its autophagy-induced phenotype. Restoring the capacity of sulfide generation through exogenous sources or by genetic complementation eliminates the phenotypic differences between the null mutants and wild-type plants. Interestingly, sulfide is also able to

[1] This work was supported in part by the European Regional Development Fund through the Ministerio de Economía y Competitividad (MOLCIS; grant no. BIO2013-44648-P), the Junta de Andalucía (grant no. CVI–190), and the Consejo Superior de Investigaciones Científicas (postdoctoral program of the Junta de Ampliación de Estudios to A.A.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.15.00009

reverse ATG8 protein accumulation and lipidation, even in wild-type plants, when autophagy is induced by carbon starvation (Álvarez et al., 2012b; Gotor et al., 2013).

Although many studies have been conducted on the physiological effects of H_2S in mammals and, more recently, in plants, the underlying mechanisms are poorly understood. Nonetheless, two mechanisms have been proposed based on the chemical properties of H_2S. The nucleophilic properties of this molecule and its capacity to react with oxygen, H_2O_2, or peroxynitrite suggest that it acts by reducing cellular oxidative stress (Kabil and Banerjee, 2010; Fukuto et al., 2012). The second mechanism involves the posttranslational modification of protein Cys residues to form a persulfide group (R-SSH; Mustafa et al., 2009a; Paul and Snyder, 2012). This process is called *S*-sulfhydration, as opposed to *S*-nitrosylation (i.e. the posttranslational modification of protein Cys residues by NO to form *S*-nitroso-Cys residues).

The biochemical processes underlying protein *S*-sulfhydration remain controversial, and it is most likely that several chemical processes can result in the modification of protein sulfhydryl groups to form a persulfide. The local environment of the Cys residue determines its dissociation constant (pK_a) to form a thiolate anion ($R\text{-}S^-$), and therefore determines its susceptibility to oxidation by reactive oxygen species to generate a sulfenic residue (R-SOH; Gruhlke and Slusarenko, 2012). This residue can further react with HS^- or H_2S to ultimately form a persulfide residue, as has been described for the protein Tyr phosphatase 1B (PTP1B; Krishnan et al., 2011). Other authors have suggested that H_2S reacts with oxygen to form sulfane sulfur, which interacts with the thiol (-SH) groups of proteins to form a persulfide bridge (Toohey, 2011, 2012). Deeper investigation of this aspect by Greiner et al. (2013) revealed that polysulfides formed in sodium hydrogen sulfide (NaHS) solutions, and not NaHS itself, are the oxidizing species when lipid phosphatase and tensin homolog (PTEN) is used as the model protein. These authors presented evidence that sulfane sulfur is added to the active-site PTEN Cys residues. Other posttranslational protein-Cys modifications that have been described are the reversible additions of glutathione and NO. Additionally, the direct oxidation of the Cys residue by H_2O_2 to form a sulfenic (R-SOH), sulfinic ($R\text{-}SO_2H$), or sulfonic ($R\text{-}SO_3H$) group is well established (Zachgo et al., 2013). Oxidation to sulfenic acid is also a reversible process involved in many redox regulatory mechanisms in plants, and recently, the H_2O_2-dependent sulfenome has been reported in Arabidopsis (*Arabidopsis thaliana*). Several proteins involved in signal perception and transduction events, protein degradation, and redox regulation processes have been identified (Waszczak et al., 2014).

Although *S*-nitrosylation typically inhibits protein function (Hess and Stamler, 2012; Zaffagnini et al., 2013), the effect of *S*-sulfhydration can either activate, as has been described for glyceraldehyde-3-phosphate dehydrogenase and Parkin E3 ligase activity (Mustafa et al., 2009a; Vandiver et al., 2013), or inactivate enzymatic activities, as has been reported for PTP1B (Krishnan et al., 2011). In other cases, *S*-sulfhydration has been shown to modify protein-protein interactions, such as in the case of Kelch-like ECH-associated protein1 (Keap1), which acts as a negative regulator of Nuclear factor (erythroid-derived2)-like2 (Nrf2), a master regulator of the antioxidant response in mice (Yang et al., 2013).

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

RESULTS AND DISCUSSION

Covalent Cys Residue Modification through *S*-Sulfhydration

The biotin switch method (BSM) has been widely used for the detection of posttranslational modifications of proteins by *S*-nitrosylation, the covalent attachment of NO to Cys residues (Sell et al., 2008). This assay consists

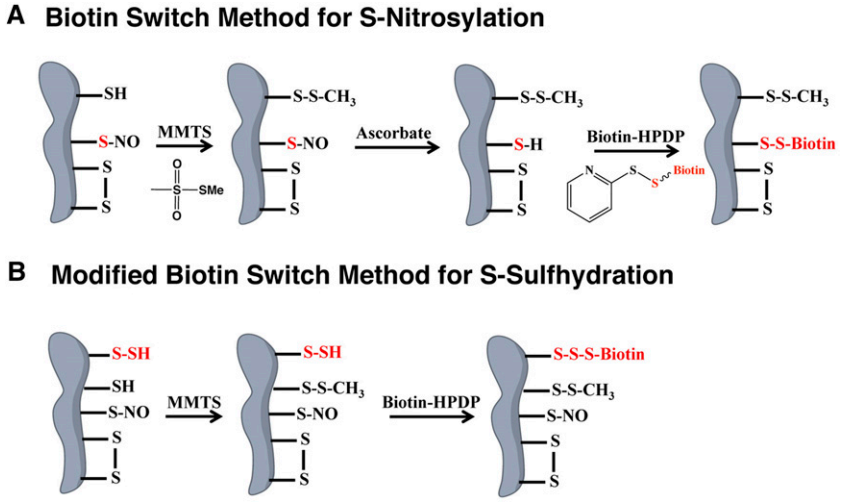


Figure 1. A, Schematic representation of the BSM for the detection of posttranslational modification of proteins by *S*-nitrosylation, where free thiols are blocked by MMTS, the *S*-NO bonds are reduced by ascorbate to form free thiols, and finally, these new thiols are ligated with the thiol-specific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

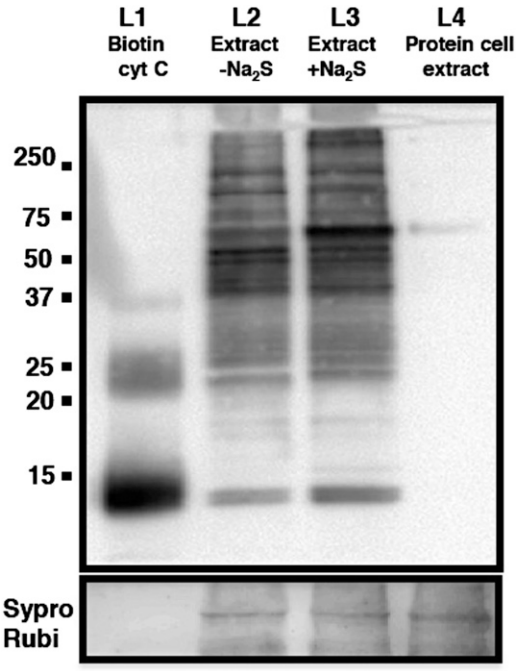


Figure 2. Immunoblot analysis of the total *S*-sulfhydrated proteins. Protein cell extracts from 1 g of leaf tissue were exogenously untreated (L2) or treated (L3) using 200 μM hydrogen sulfide (Na_2S) for 30 min at 4°C and were subjected to the modified BSM. The labeled proteins were detected using 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 BSM (L4) were used for the positive and negative control, respectively. Sypro Ruby fluorescent staining is shown as the protein loading control.

of three steps: first, free thiols are blocked by the thiol-blocking reagent methyl methanethiosulfonate (MMTS); next, the *S*-NO bonds are reduced by ascorbate to form free thiols; finally, these thiols are ligated with *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide (biotin-HPDP) to form biotin-labeled proteins (Fig. 1A). Protein posttranslational modifications by *S*-sulfhydration (i.e. the conversion of Cys –SH residues to persulfide [-SSH]), can also be detected using a modified BSM that was first described for protein analysis in mouse liver lysates (Mustafa et al., 2009a). In the modified BSM, free thiol residues are first blocked with MMTS; the persulfide residues remain unreacted and are therefore available for subsequent reaction with the thiol-specific biotinylating agent biotin-HPDP (Fig. 1B; Mustafa et al., 2009a).

Total leaf protein extracts from mature Arabidopsis plants grown under physiological conditions, in the absence of oxidative stress or chemical treatments, were subjected to the modified biotin switch method to detect *S*-sulfhydrated proteins. The method selected only biotin-labeled proteins, corresponding to proteins that contained persulfide residues, which were analyzed using immunoblotting with antibodies against biotin (Fig. 2, lane L2). A large array of proteins was clearly detected by the antibody, and the intensities of several of the labeled proteins increased in protein extracts that were previously treated exogenously using 200 μM Na_2S for 30 min (Fig. 2, lane L3). Crude protein extracts that were not subjected to the modified BSM did not show any biotin-labeled proteins (Fig. 2, lane L4).

The biotin-labeled proteins obtained using the modified BSM were further isolated using a streptavidin-based affinity purification process. Three independent crude extracts from leaf tissue that were treated using the modified BSM were incubated with streptavidin beads and then washed several times to avoid nonspecific bead binding. The eluted proteins from the streptavidin beads were digested with trypsin and subsequently analyzed using mass spectrometry. A total of 106 *S*-sulfhydrated proteins were identified with high confidence (false discovery rate $< 1\%$; Supplemental Table S1). This list of proteins represents the first version of the group of plant proteins that are endogenously modified by *S*-sulfhydration.

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

Based on UniProt, several of the proteins identified in Arabidopsis have also been described in mammalian systems, such as actin, catalase, Gln synthetase, glyceraldehyde 3-phosphate dehydrogenase, Leu aminopeptidase, ATP synthase, β-tubulin, and UDP-Glc dehydrogenase (Supplemental Table S3; Mustafa et al., 2009a). Furthermore, the identities of some of the candidate plant proteins were confirmed through immunoblot analysis.

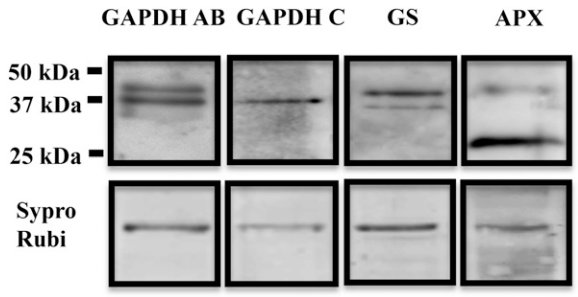


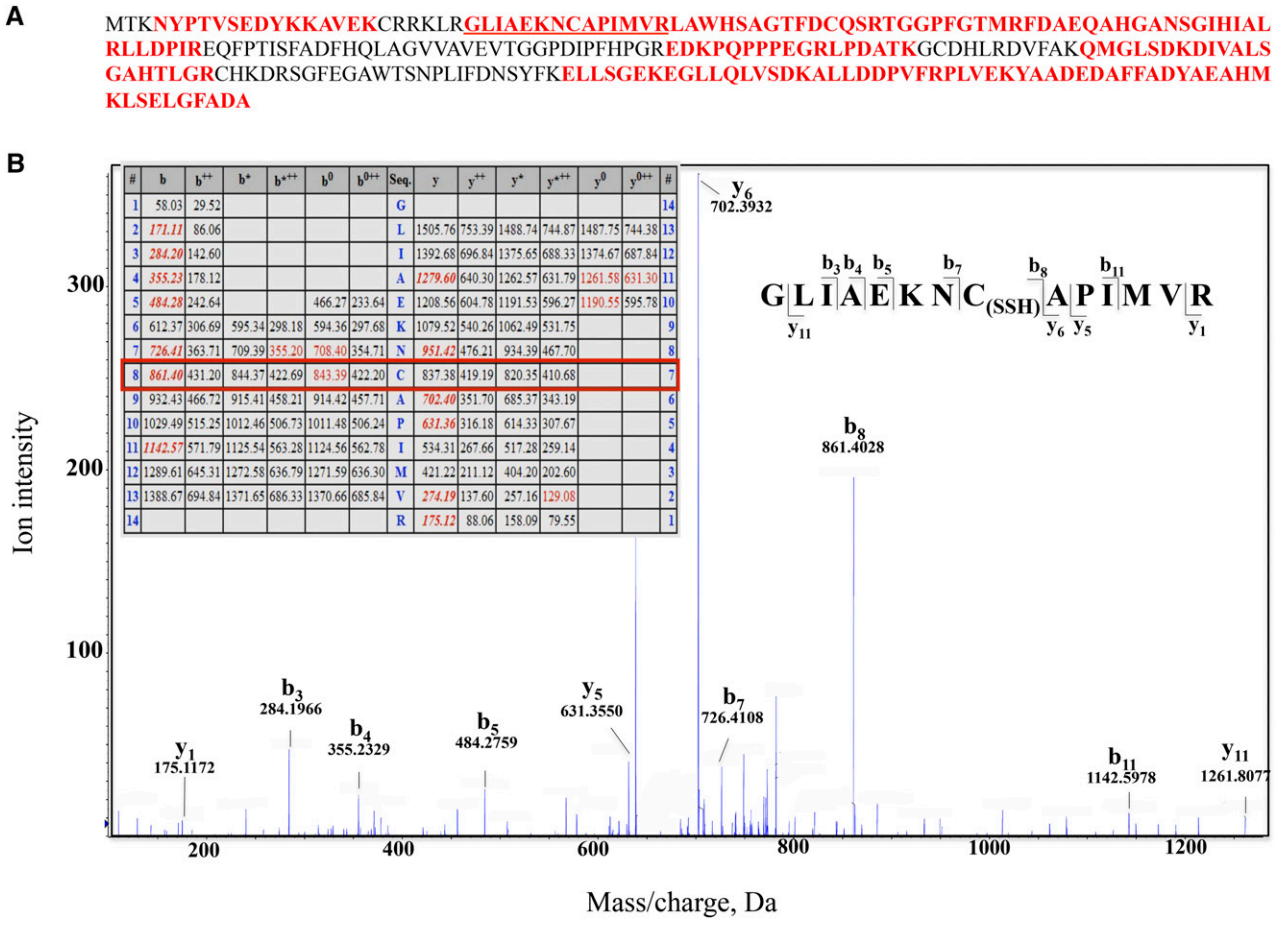
Figure 3. Immunoblot analysis of specific *S*-sulfhydrated candidate proteins. Biotinylated proteins obtained from the leaf extracts subjected to the modified biotin switch assay were purified using streptavidin-agarose beads and analyzed using four different immunoblots with the following antibodies: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

Leaf protein extracts were subjected to the modified BSM assay and purified using streptavidin-agarose beads, and the retained proteins were separated using SDS-PAGE for immunoblot analysis. The chloroplastic GAPA (42 kD) and GAPB (48 kD) isoforms and the cytosolic GAPC (37 kD) isoform of glyceraldehyde 3-phosphate dehydrogenase, the chloroplastic Gln synthetase GS2 isoform (43 kD), and cytosolic ascorbate peroxidase1 (APX1; 27 kD) were identified in the eluted biotin-labeled protein pool by their expected molecular masses using polyclonal antibodies against homolog proteins (Fig. 3). Therefore, we demonstrated that all of these plant proteins underwent *S*-sulfhydration.

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

Identification of *S*-Sulfhydrated Cys Residues of Cytosolic APX Using Mass Spectrometry

To demonstrate the presence of Cys residues modified by *S*-sulfhydration and the target sites of a representative protein identified in this work, we carried out liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis on the cytosolic APX enzyme. Recombinant cytosolic APX1 enzyme was purified from a bacterial extract and trypsin digested under nonreducing conditions to avoid the reduction of



#	b	b++	b*	b*++	b0	b0++	Seq.	y	y++	y*	y*++	y0	y0++	#
1	58.03	29.52					G							14
2	*171.11*	86.06					L	1505.76	753.39	1488.74	744.87	1487.75	744.38	13
3	*284.20*	142.60					I	1392.68	696.84	1375.65	688.33	1374.67	687.84	12
4	*355.23*	178.12					A	*1279.60*	640.30	1262.57	631.79	1261.58	631.30	11
5	*484.28*	242.64			466.27	233.64	E	1208.56	604.78	1191.53	596.27	1190.55	595.78	10
6	612.37	306.69	595.34	298.18	594.36	297.68	K	1079.52	540.26	1062.49	531.75			9
7	*726.41*	363.71	709.39	*355.20*	*708.40*	354.71	N	*951.42*	476.21	934.39	467.70			8
8	*861.40*	431.20	844.37	422.69	*843.39*	422.20	C	837.38	419.19	820.35	410.68			7
9	932.43	466.72	915.41	458.21	914.42	457.71	A	*702.40*	351.70	685.37	343.19			6
10	1029.49	515.25	1012.46	506.73	1011.48	506.24	P	*631.36*	316.18	614.33	307.67			5
11	*1142.57*	571.79	1125.54	563.28	1124.56	562.78	I	534.31	267.66	517.28	259.14			4
12	1289.61	645.31	1272.58	636.79	1271.59	636.30	M	421.22	211.12	404.20	202.60			3
13	1388.67	694.84	1371.65	686.33	1370.66	685.84	V	*274.19*	137.60	257.16	129.08			2
14							R	*175.12*	88.06	158.09	79.55			1

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

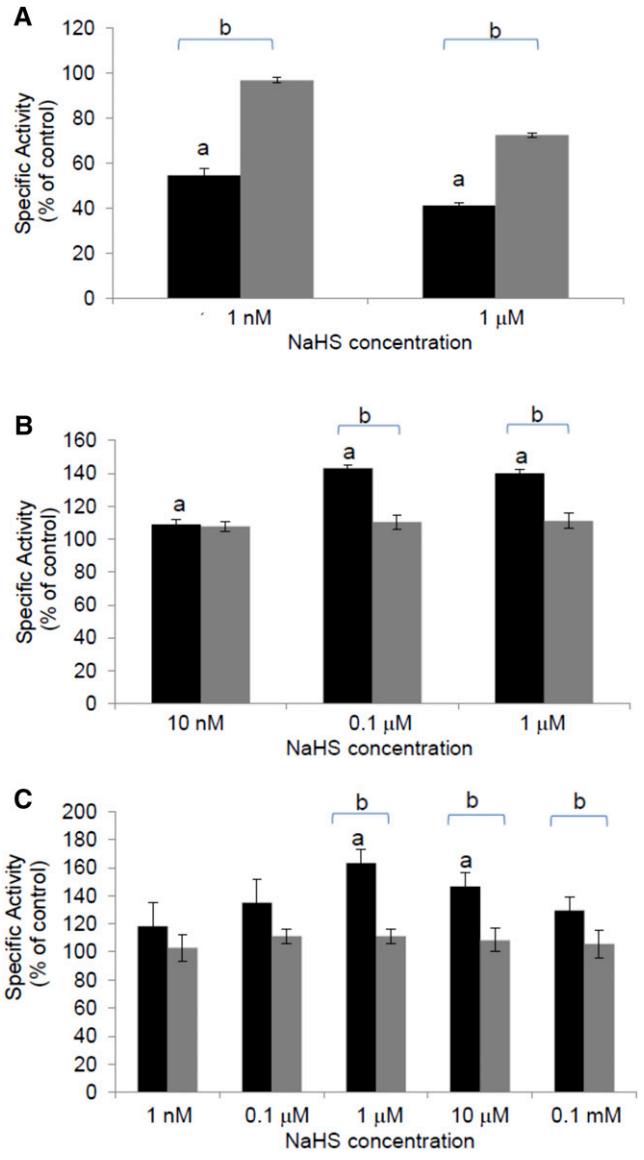


Figure 5. Enzyme activity regulation of Gln synthetase, APX, and GAPDH by *S*-sulfhydration in Arabidopsis. The protein leaf extracts were treated in the absence or presence of NaHS at the indicated concentrations for 30 min at 4°C (black bars), and an additional treatment with 50 mM DTT was performed for 10 min in some cases (gray bars). Then, Gln synthetase (A), APX (B), or GAPDH (C) enzyme activity was measured as described in "Materials and Methods." All results are shown as the mean ± SD. a, Significant differences between treatments with and without NaHS ($P < 0.05$); b, significant differences between samples with or without DTT ($P < 0.05$).

persulfide residues. However, under this condition, disulfide bridges between digested peptides cannot be avoided. The digested peptides were analyzed using LC-MS/MS for a 32-D mass increase in the fragmentation spectrum. As illustrated in Figure 4, cytosolic APX1 was identified with a sequence coverage of 74%. Among the peptides identified, only one, GLIAEKNCAPIMVR, containing Cys-32, showed a sulfhydryl modification. Putative peptides containing the two other Cys residues were not detected in the analysis, most likely because they formed a disulfide bridge under the nonreducing conditions utilized.

The oxidation of Cys-32 causes APX1 inactivation, and it has been suggested that glutathionylation protects the enzyme from irreversible oxidation (Kitajima et al., 2008). The active-site Cys-32 of APX1 can also be *S*-nitrosylated by NO, which increases the activity of the enzyme, and it has been hypothesized that this posttranslational modification might be involved in the specific case of salinity stress, which is accompanied by both oxidative stress and an increase in *S*-nitrosothiols (Begara-Morales et al., 2014). The fact that Cys-32 is altered by different posttranslational modifications suggests that this enzyme must be finely regulated under specific environmental stress conditions.

S-Sulfhydration Regulates Enzyme Activity

To determine whether this protein modification has a biological role in plant systems, we performed enzyme

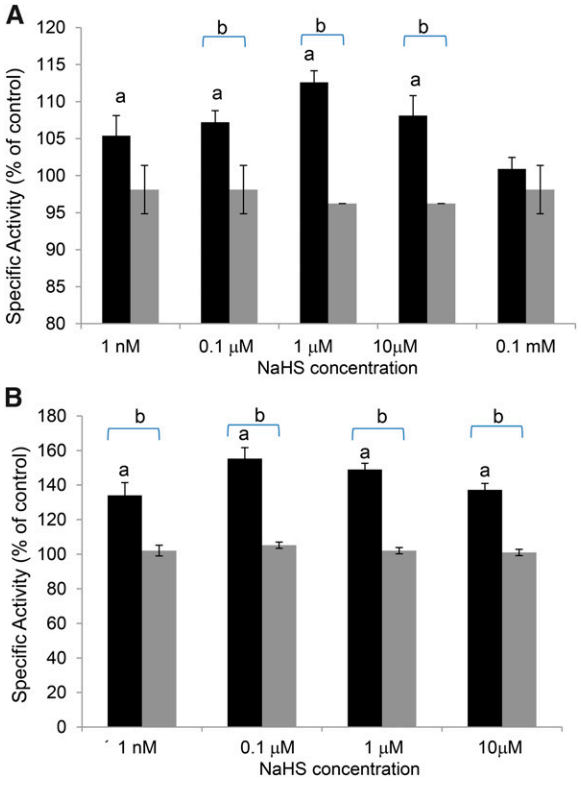


Figure 6. Enzyme activity regulation of recombinant cytosolic APX1 and cytosolic glyceraldehyde 3-phosphate dehydrogenase, isoform C1 (GAPC1) by *S*-sulfhydration. Purified proteins were treated in the absence or presence of NaHS at the indicated concentrations for 30 min at 4°C (black bars), and in some cases an additional treatment with 1 mM DTT was performed (gray bars). Then, APX1 (A) or GAPC1 (B) enzyme activity was measured as described in "Materials and Methods." All results are shown as the mean ± SD. a, Significant differences between treatments with and without NaHS ($P < 0.05$); b, significant differences between samples with or without DTT ($P < 0.05$).

activity assays using total leaf protein extracts. Protein extracts were treated with different concentrations of NaHS as a sulfide donor, and then GS, APX, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activities were measured (Fig. 5). We clearly observed a significant inactivation of GS activity, even in the presence of very low concentrations (1 nM) of NaHS in which only one-half the level of the measured activity in the absence of sulfide was reached (Fig. 5A). Curiously, incubation with NaHS produced the opposite response for APX and GAPDH activities. In these cases, we observed an increase in APX and GAPDH activities of approximately 40% and 60%, respectively, compared with the activity level in the absence of sulfide. However, the activity of the APX enzyme appeared less sensitive to sulfide regulation than that of GS because the lowest concentration of NaHS required to observe increased activity was 10 nM. In the case of GAPDH activity, the addition of 1 μM NaHS was the minimum concentration required to detect a significant increase in activity (Fig. 5, B and C). Interestingly, the addition of DTT to the sulfide-treated extracts reversed the effect of sulfide in these enzyme activity assays. In the case of APX and GAPDH, the activity levels decreased following the addition of DTT and fell to the untreated value. In the case of GS, reactivation to the same level as that of the untreated extract was observed following the addition of DTT (Fig. 5).

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

Because very low concentrations of NaHS were sufficient to produce an inactivation/activation effect on enzyme activities, and because this effect was reversible, it is possible that sulfide has a biological role in plants, most likely through *S*-sulfhydration protein modification, similar to mammalian systems, where the biological function of *S*-sulfhydration is well established. For example, it has been reported that the protein PTP1B is reversibly inactivated by sulfide, with PTP1B *S*-sulfhydration playing a role in the response to endoplasmic reticulum stress (Krishnan et al., 2011). Similarly, it has been shown that H_2S acts as an endogenous inhibitor of phosphodiesterase activity, suggesting that some of the critical Cys residues are *S*-sulfhydrated and impair phosphodiesterase activity (Bucci and Cirino, 2011). However, incubation with NaHS increases GAPDH activity, which is reversed by DTT (Mustafa et al., 2009a; Gadalla and Snyder, 2010). Similarly, NaHS specifically enhances actin polymerization and activates ATP-sensitive potassium channels, effects that are also both reversed by DTT (Mustafa et al., 2009a, 2011). Finally, the median effective physiological concentration of sulfide for sulfhydrating protein targets in most mammalian tissues is considered to be in the micromolar range (Mustafa et al., 2009b; Nagy et al., 2014), similar to the average concentration of 50 μM calculated for the cytosol in Arabidopsis leaves (Krueger et al., 2009). However, we must consider that the level of sulfide in the cytosol is determined via the coordinated activities of both cytosolic enzymes, i.e. *O*-acetyl-Ser (thiol)lyase A1, which incorporates sulfide to form Cys, and DES1. Therefore, the level of sulfide may change under the developmental and stress conditions that may regulate these activities (Alvarez et al., 2011; Álvarez et al., 2012a; Laureano-Marín et al., 2014).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*), wild-type ecotype Columbia-0, was grown in soil under a photoperiod of 16 h of white light (120 μE m^{-2} s^{-1}) at 20°C and 8 h of dark at 18°C (García et al., 2013).

Modified BSM

The modified biotin switch assay was adapted from a previously described protocol (Mustafa et al., 2009a). Frozen Arabidopsis leaves collected from 30-d-old plants were ground to a fine powder in a mortar under liquid nitrogen, homogenized in HEN buffer (250 mM HEPES-NaOH [sodium hydroxide; pH 7.7], 1 mM EDTA, and 0.1 mM neocuproine) supplemented with 100 μM deferoxamine and 1× protease inhibitor cocktail (Roche), and centrifuged at 14,000 rpm for 10 min at 4°C. Two volumes of blocking buffer (HEN buffer supplemented with 2.5% [w/v] SDS and 20 mM MMTS) was added to the leaf extract, and the solution was incubated at 50°C for 20 min to block free sulfhydryl groups. The MMTS was then removed, and the proteins were precipitated using acetone at −20°C for 20 min. The acetone was removed, and the proteins were resuspended in HEN buffer supplemented with 1% (w/v) SDS. The *S*-sulfhydrated proteins were then labeled using 4 mM biotin-HPDP for 3 h at 25°C in the dark.

Purified recombinant proteins were used to optimize the blocking conditions to avoid incomplete blocking. Thus, purified recombinant APX1 and GAPC1 were pretreated with 200 μM NaHS to increase the concentration of *S*-sulfhydrated proteins or with 1 mM DTT to reduce all disulfide bonds; both treatments were carried out for 30 min at 4°C, and buffer was exchanged to eliminate residual DTT or NaHS before performing the modified biotin switch assay. An untreated aliquot of purified proteins did not undergo the blocking stage with MMTS during the modified biotin switch assay for comparison with the blocked proteins. The biotinylated proteins were detected using an immunoblot assay with antibiotin antibodies (Abcam antibodies) as described below.

Streptavidin-Based Affinity Purification Process

The labeled proteins were precipitated using acetone, and the washed pellet was resuspended in HEN buffer supplemented with 1% (w/v) SDS. To purify the biotinylated proteins, the solution was incubated with streptavidin beads for 1 h at room temperature with frequent vortexing. The streptavidin beads were intensively washed five times using 10 volumes of 20 mM HEPES-NaOH (pH 7.7), 600 mM sodium chloride, 1 mM EDTA, and 0.5% (v/v) Triton X-100, and then centrifuged at 3,000 rpm for 5 s at room temperature between each wash. To recover the bound proteins, the beads were incubated with 20 mM HEPES-NaOH (pH 7.7), 100 mM sodium chloride, 1 mM EDTA, and 100 mM

2-mercaptoethanol for 10 min at room temperature. The total amount of purified proteins was determined using Bradford's method (Bradford, 1976).

Expression and Purification of Recombinant His-Tagged Proteins

The complete complementary DNAs of cytosolic APX1 (At1g07890) and the cytosolic isoform of GAPDH GAPC1 (At3g04120) were cloned into the pDEST17 vector (Invitrogen) to express an N-terminal 6-His-tagged protein using the *Escherichia coli* expression system with Gateway Technology (Invitrogen). For APX1 and GAPC1 protein expression, transformed *E. coli* BL21 (DE3) cell cultures at an optical density at 600 nm of 0.6 were treated with 0.1 and 0.5 mM isopropyl-β-D-thiogalactopyranoside, respectively; the cell cultures were incubated for 4 h at 30°C. Purification was performed by nickel resin binding under nondenaturing conditions using the Ni-NTA Purification System (Invitrogen) according to the manufacturer's recommendations. Recombinant protein production and purification were assessed by SDS-PAGE using 12% (w/v) polyacrylamide gels and Coomassie Brilliant Blue staining.

Identification of *S*-Sulfhydrated Cys Residues of Recombinant Cytosolic APX Using Mass Spectrometry

Cytosolic APX1 was separated using nonreducing SDS-PAGE on 12% (w/v) polyacrylamide gels, and the band corresponding to APX1 was excised manually from Coomassie-stained gels, deposited in 96-well plates, and processed automatically in a Proteineer DP (Bruker Daltonics). The digestion protocol used was based on Shevchenko et al. (1996) without the reduction or alkylation steps: Gel plugs were washed twice, first using 50 mM ammonium bicarbonate and second using acetonitrile (ACN), and then dried under a stream of nitrogen. Then, proteomics-grade trypsin (Sigma-Aldrich) at a final concentration of 16 ng μL^{-1} in 25% (v/v) ACN/50 mM ammonium bicarbonate solution was added, and digestion took place at 37°C for 5 h. The reaction was stopped by adding 50% (v/v) ACN/0.5% (v/v) trifluoroacetic acid for peptide extraction. The tryptic eluted peptides were dried using speed-vacuum centrifugation and were resuspended in 6 μL of 0.1% (v/v) formic acid in water.

Digested peptides were subjected to one-dimensional nanoliquid chromatography electrospray ionization tandem mass spectrometry analysis using a nano liquid chromatography system (nanoLC Ultra 1D plus; Eksigent Technologies) coupled to a high-speed TripleTOF 5600 mass spectrometer (AB SCIEX) with a duo spray ionization source. Data acquisition was performed using a TripleTOF 5600 System (AB SCIEX). Mass spectrometry and MS/MS data obtained for individual samples were processed using Analyst TF 1.5.1 Software (AB SCIEX). Peptide mass tolerance was set to 25 $\mu D\ D^{-1}$ and 0.05 D for fragment masses, and only one or two missed cleavages were allowed. Peptides with an individual M_r search score ≥ 20 were considered correctly identified.

Immunoblot Analysis

The biotinylated proteins were separated using nonreducing SDS-PAGE on 12% (w/v) polyacrylamide gels before being transferred to polyvinylidene fluoride membranes (Bio-Rad) according to the manufacturer's instructions. Anti-biotin (Abcam antibodies) and secondary antibodies were diluted 1:500,000 and 1:100,000, respectively, and an ECL Select Western Blotting Detection Reaction (GE Healthcare) was used to detect the proteins using horseradish peroxidase-conjugated anti-rabbit secondary antibodies. The streptavidin-purified biotinylated proteins were also subjected to an immunoblot analysis using SDS-PAGE on 15% (w/v) polyacrylamide gels using the following antibodies: (1) polyclonal antibodies raised against different forms of GAPDH from the microalga *Scenedesmus vacuolatus*, anti-GAP2 and anti-GAP3, which recognize the Arabidopsis chloroplastic isoforms A and B and the cytosolic isoform C, respectively (Valverde et al., 2005), diluted 1:10,000; (2) anti-GS antibodies raised against recombinant homopolymeric GS from *Phaseolus vulgaris*, recognizing mainly the Arabidopsis chloroplastic isoform and to a minor extent the cytosolic isoform (Betti et al., 2006) diluted 1:1,000; and (3) and anticytosolic APX antibodies (Agrisera) diluted 1:10,000. Prior to immunodetection, the membrane was stained using SYPRO Ruby (Life Technologies) as a protein-loading control.

Mass Spectrometry Analysis

The purified protein samples were precipitated using 10% (v/v) trichloroacetic acid and were acetone washed before tryptic digestion. The samples were then purified using Pierce C18 spin columns and evaporated in a speed vacuum prior to storage at −80°C. A label-free analysis was performed as described in Schröder et al. (2012). In brief, peptides were separated by reverse-phase chromatography using an Eksigent Ultra 2D+ pump fitted with a 75-μm i.d. column (nanoLC column, 75-μm i.d. × 15 cm, C18, 3 μm, 120 Å, ChromXP); the samples were first loaded into a 2-cm-long, 100-μm i.d. precolumn, packed using the same chemistry as the separating column, for desalting and concentrating. The mobile phases were 100% (v/v) water/0.1% (v/v) formic acid (buffer A) and 100% (v/v) acetonitrile/0.1% (v/v) formic acid (buffer B). The column gradient was developed using a 60-min, two-step run from 5% (v/v) B to 30% (v/v) B in 30 min and 30% (v/v) B to 70% (v/v) B in 10 min. The column was equilibrated with 95% (v/v) B for 5 min and 5% (v/v) B for 15 min. During all processes, the precolumn was in line with the separating column, and the flow was maintained along the entire gradient at 300 nL min^{-1}. The peptides eluted from the column were analyzed using an AB SCIEX 5600 TripleTOFTM+ system. Data-dependent acquisition occurred during a 250-ms survey sampling performed over a mass range from 350 to 1,250 *m/z*. The top 20 peaks were selected for fragmentation. The minimum accumulation time for MS/MS was set to 50 ms, for a total cycle time of 1,250 ms. The product ions were surveyed during a 15-s period over a mass range from 100 to 1,500 *m/z* and excluded from further fragmentation. After the MS/MS analysis, the data files were processed using ProteinPilotTM 4.5 software from AB SCIEX, which implements the algorithms ParagonTM for database searching and ProgroupTM for data grouping (Shilov et al., 2007), and were searched against the UniProt Arabidopsis-specific database. A false discovery rate was performed using a nonlinear fitting method (Tang et al., 2008), and the results displayed are those with a 1% global false discovery rate or better. The data were analyzed using three technical replicas for each sample. Peak lists were generated in PeakView version 1.1 software from AB SCIEX using the combined database search results generated in the ProteinPilotTM version 4.5 software. The peak list matrix generated was exported to MarkerViewTM version 1.2.1 software for principal component analysis. Sample comparison was performed using the first two components, which explained a total of 75% of the variance between samples. Sample dispersion was measured using a *t* test, and proteins with extreme *t* values were chosen as candidates for validation.

Enzyme Activity Assays

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

The APX activity was determined as previously described (García-Limones et al., 2002). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.25 mM sodium ascorbate, and 0.05 mL of leaf extract (containing approximately 0.5 mg of total protein) or 0.05 mL of purified APX recombinant protein (containing approximately 0.1 mg of total protein). The reaction was initiated by adding 5 mM H_2O_2, and the oxidation of ascorbate was determined by the decrease in A_{290} (ε = 2.8 $mM^{-1}\ cm^{-1}$).

The GAPDH activity was monitored spectrophotometrically at room temperature by following the glycolytic reaction assays, as described previously (Bedhomme et al., 2012). The glycolytic reaction was measured in an assay containing 50 mM Tris-HCl (pH 7.5), 1 mM NAD^+, 10 mM sodium arsenate, and 0.05 mL of leaf extract (containing approximately 2 mg of total protein) or 0.05 mL of purified GAPDH cytosolic isoform C recombinant protein (containing approximately 0.1 mg of total protein). The reaction was initiated by the addition of 1 mM glyceraldehyde 3-phosphate, and the A_{340} was recorded for 1 min (ε = 6.2 $mM^{-1}\ cm^{-1}$).

The GS transferase activity was measured by the formation of γ-glutamylhydroxamate (Mérida et al., 1991). The assay was performed in a final volume of 1 mL that contained 60 μmol of HEPES-NaOH buffer (pH 7.0), 40 μmol of L-Gln, 4 μmol of $MnCl_2$, 60 μmol of hydroxylamine, 1 μmol of ADP, and 0.05 mL of leaf extract (containing approximately 2 mg of total protein). The reaction was initiated by the addition of 20 μmol of sodium arsenate, and the amount of γ-glutamylhydroxamate formed after 10 min of incubation at 30°C was determined spectrophotometrically at 500 nm (ε = 0.89 $mM^{-1}\ cm^{-1}$).

Statistical Analysis

All results are shown as the mean ± SD of three biological replicas. The data were analyzed by ANOVA using Microsoft Excel ($P < 0.05$).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers APX1 (At1g07890) and GAPC1 (At3g04120).

Supplemental Data

The following supplemental materials are available.

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

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

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

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

ACKNOWLEDGMENTS

We thank Drs. Federico Valverde and Antonio J. Márquez for providing the anti-GAPDH and anti-GS antibodies, respectively, and Dr. M. Isabel Muro-Pastor for helping with the GS activity assay.

Received January 8, 2015; accepted March 24, 2015; published March 25, 2015.

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