*Running head:* S-sulfhydration in *Arabidopsis*

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# S-sulfhydration: a new post-translational modification in plant systems

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*Summary*:

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

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## ABSTRACT

Hydrogen sulfide ($H_2S$) 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 $H_2S$, 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 thaliana* proteins modified by S-sulfhydration under physiological conditions. The presence of an S-sulfhydration-modified cysteine residue on cytosolic ascorbate peroxidase (APX) was demonstrated using LC-MS/MS analysis, and a total of 106 S-sulfhydrated proteins were identified. This constitutes the first report of S-sulfhydration as a posttranslational modification in plants. 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.

## INTRODUCTION

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 (CO) (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 gamma-lyase (CGL), cystathionine beta-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MTS). 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 $H_2O_2$. 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; Zhang et al., 2010). In addition, $H_2S$ pretreatment alleviates cadmium toxicity in alfalfa (*Medicago sativa* L.) (Li et al., 2012a), improves heat tolerance in tobacco (*Nicotiana tabacum*) suspension-cultured cells (Li et al., 2012b), and protects Bermuda grass (*Cynodon dactylon* (L). Pers.) 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 (Garcia-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-cysteine desulfhydrase (DES1) is involved in the degradation of cysteine 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 cysteine-

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 ATG8 protein, which is associated with the process of autophagy (Alvarez et al., 2012). 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 reverse ATG8 protein accumulation and lipidation, even in wild-type plants, when autophagy is induced by carbon starvation (Alvarez 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 cysteine residues to form a persulfide group (R-SSH) (Mustafa et al., 2009b; Paul and Snyder, 2012). This process is called S-sulfhydration, as opposed to S-nitrosylation, i.e., the posttranslational modification of protein cysteine residues by NO to form S-nitrosocysteine residues (R-SNO).

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 cysteine residue determines its dissociation constant (pKa) to form a thiolate anion ($R\text{-}S^-$) and therefore determines its susceptibility to oxidation by reactive oxygen species (ROS) 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 ($S^0$), which interacts with the –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 NaHS solutions, and not NaHS itself, are the oxidizing species when lipid phosphatase PTEN is used as the

model protein. These authors presented evidence that sulfane sulfur is added to the active site PTEN cysteine residues. Other posttranslational protein-cysteine modifications that have been described are the reversible addition of glutathione or NO. Additionally, the direct oxidation of the cysteine residue by $H_2O_2$ to form a sulfenic (R-SOH), sulfinic (R-$SO_2H$) or sulfonic (R-$SO_3H$) 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*. Several proteins involved in signal perception and transduction events, protein degradation and redox regulations processes have been identified (Waszczak et al., 2014).

Although 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., 2009b; Vandiver et al., 2013), or inactivate enzymatic activities, as has been reported for Tyr phosphatase 1B (Krishnan et al., 2011). In other cases, S-sulfhydration has been shown to modify protein-protein interactions, such as in the case of Keap1c, which acts as a negative regulator of 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 cysteine residue modification through S-sulfhydration

The biotin switch method (BSM) has been widely used for the detection of post-translational modifications of proteins by S-nitrosylation, the covalent attachment of NO to cysteine residues (Sell et al., 2008). This assay consists 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 post-translational modifications by S-sulfhydration, i.e.,

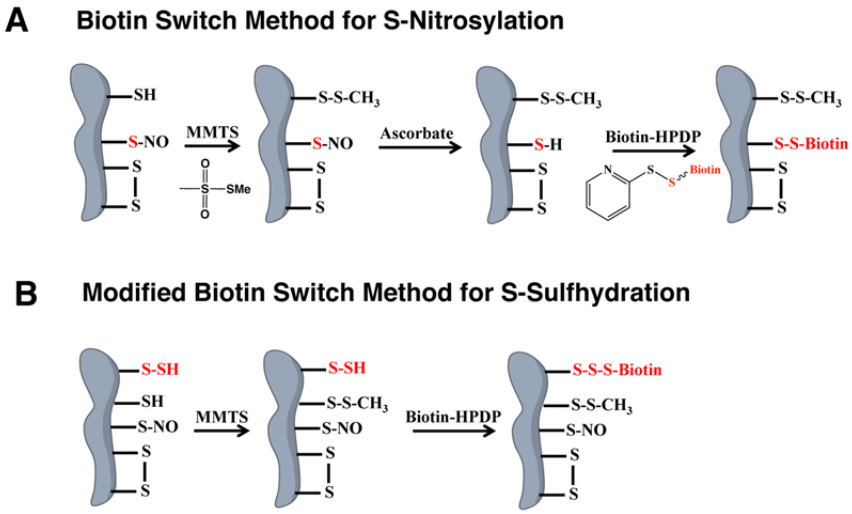



**Figure 1.** (A) Schematic representation of the biotin switch method for the detection of post-translational modification of proteins by S-nitrosylation, where free thiols are blocked by methyl methanethiosulfonate (MMTS), the S-NO bonds are reduced by ascorbate to form free thiols, and finally, these 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 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.

the conversion of cysteine –SH residues to persulfide (-SSH), can also be detected using a modified biotin switch method that was first described for protein analysis in mouse liver lysates (Mustafa et al., 2009b). In the modified biotin switch method, 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., 2009b).

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 protein 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 biotin switch method 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

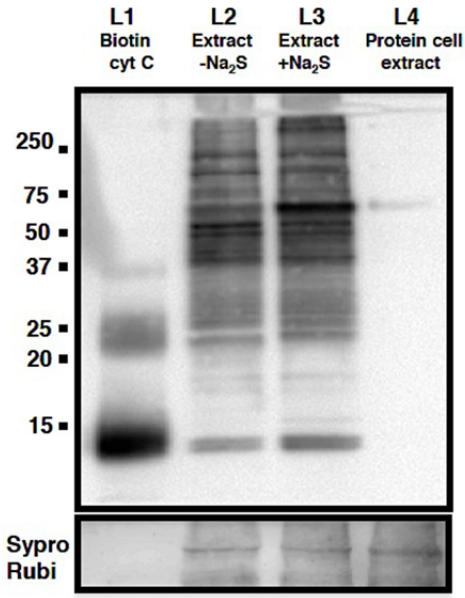



**Figure 2.** Immunoblot analysis of the total S-sulfhydrated proteins. Protein cell extracts from 1 g of leaf tissue were exogenously untreated (L2) or treated (L3) using 200 µM $Na_2S$ for 30 min at 4ºC and were subjected to the modified biotin switch method. The labeled proteins were detected using 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.

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 (FDR < 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 cysteine residues.

Based on UniProt, several of the proteins identified in *Arabidopsis* have also been described in mammalian systems, such as actin, catalase, glutamine synthetase, glyceraldehyde 3-phosphate dehydrogenase, leucine aminopeptidase, ATP synthase, ß-tubulin, and UDP-glucose dehydrogenase (Supplemental Table S3) (Mustafa et al., 2009b). Furthermore, the identities of some of the candidate plant proteins were confirmed through immunoblot analysis. 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 (A-1, 42 kDa) and GapB (48 kDa) isoforms and the cytosolic GapC (37 kDa) isoform of glyceraldehyde 3-phosphate dehydrogenase, the chloroplastic glutamine synthetase GS2 isoform (43 kDa) and cytosolic ascorbate peroxidase APX1 (27 kDa) 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 these plant proteins underwent S-sulfhydration.

The recombinant cytosolic proteins ascorbate peroxidase 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 similar intensity to that in the sample untreated with NaHS, which was endogenously S-sulfhydrated (Supplemental Fig. S1). When the proteins were pretreated with 1 mM 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 cysteine residues of cytosolic APX using mass spectrometry**

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

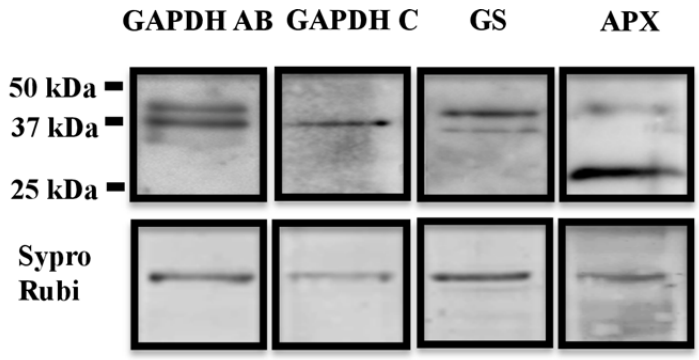



**Figure 3.** Immunoblot analysis of specific S-sulfhydrated candidate proteins. Biotinylated proteins obtained from the leaf extracts subjected to the modified biotin switch assay were purified using streptavidin-agarose beads and analyzed using four different immunoblots with the following antibodies: 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.

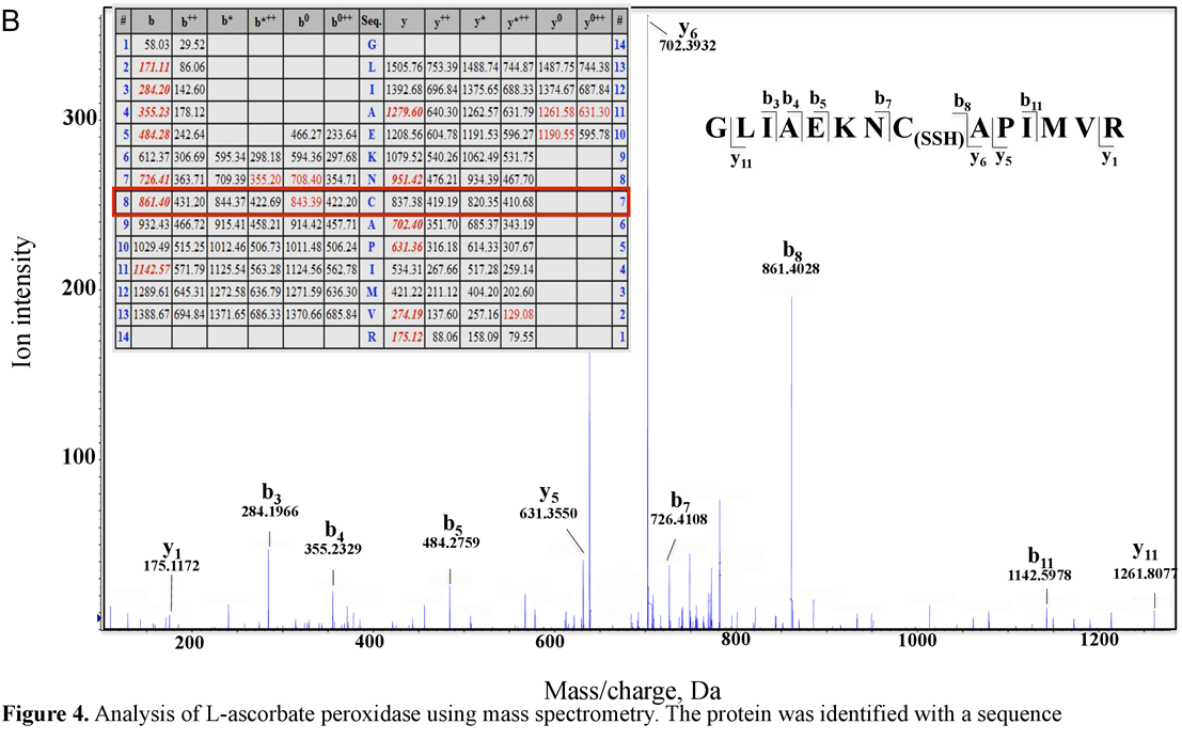
reduction of persulfide residues. However, under this condition, disulfide bridges between digested peptides cannot be avoided. The digested peptides were analyzed using LC-MS/MS for a 32-Da mass increase in the fragmentation spectrum. As illustrated in Figure 4, cytosolic L-ascorbate peroxidase 1 was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

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

A

MTKNYPTVSEDYKKAVEKCRRKLRGLIAEKNCAPIMVRLAWHSAGTFDCQSRTGGPFGTMRFDAEQAHGANSGIHIAL
RLLDPIREQFPTISFADFHQLAGVVAVEVTGGPDIPFHPGREDKPQPPPEGRLPDATKGCDHLRDVFAKQMGLSDKDIVALS
GAHTLGRCHKDRSGFEGAWTSNPLIFDNSYFKELLSGEKEGLLQLVSDKALLDDPVFRPLVEKYAADEDAFFADYAEAHM
KLSELGFADA

B




| # | 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 L-ascorbate peroxidase using mass spectrometry. 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 (A). 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 (Biemann, 1988) (B).

## S-sulfhydration regulates enzyme activity

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

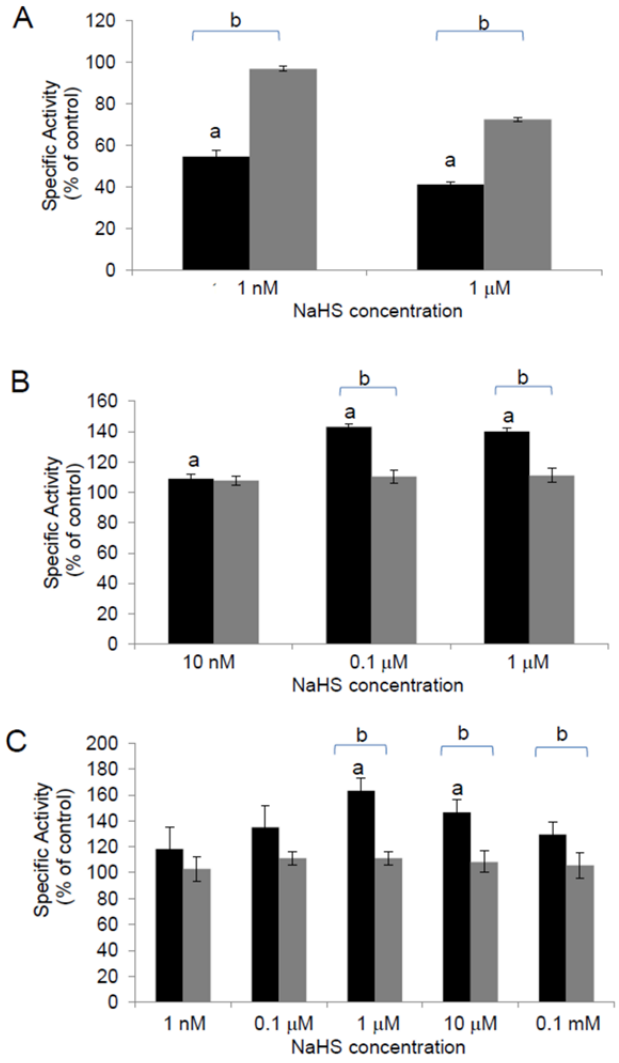



**Figure 5.** Enzyme activity regulation of glutamine synthetase, ascorbate peroxidase and glyceraldehyde-3-phosphate dehydrogenase by S-sulfhydration in *Arabidopsis thaliana*. The protein leaf extracts were treated in the absence or presence of NaHS at the indicated concentrations for 30 min at 4ºC (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 ± 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" ($P<0.05$)

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 1µM of NaHS was the minimum concentration required to detect a significant increase in activity (Fig. 4B and 4C). 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, similarly to mammalian systems, where the biological function of S-sulfhydration is well established. For example, it has been reported that the protein tyrosine phosphatase PTP1B is reversibly inactivated by sulfide, with PTP1B S-sulfhydration playing a role in the response to endoplasmic reticulum (ER) stress (Krishnan et al., 2011). Similarly, it has been shown that $H_2S$ acts as an endogenous inhibitor of phosphodiesterase (PDE) activity, suggesting that some of the critical cysteine residues are S-sulfhydrated and impair PDE activity (Bucci and Cirino, 2011). However, incubation with NaHS increases glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity, which is reversed by DTT (Mustafa et al., 2009b; 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., 2009b; Mustafa et al., 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., 2009a; 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

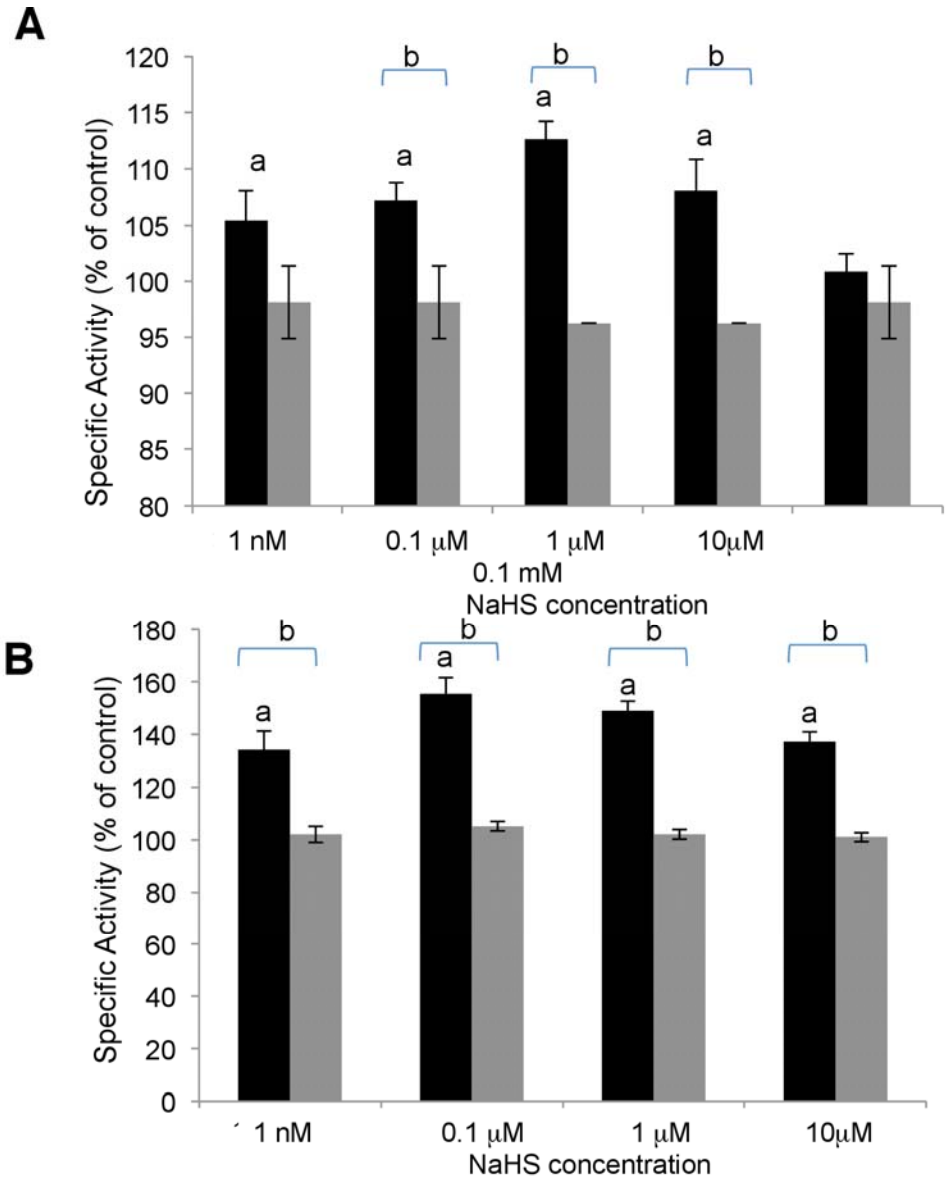



**Figure 6.** Enzyme activity regulation of recombinant cytosolic ascorbate peroxidase (APX1) and cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC1) by S-sulfhydration. Purified proteins were treated in the absence or presence of NaHS at the indicated concentrations for 30 min at 4ºC (black bars), 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 ± 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" ($P<0.05$).

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

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

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

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

### Modified Biotin Switch Method

The modified biotin switch assay was adapted from a previously described protocol (Mustafa et al., 2009b). Frozen *Arabidopsis* leaves collected from 30-day-old plants were ground to a fine powder in a mortar under liquid nitrogen, homogenized in HEN buffer containing 250 mM Hepes-NaOH (pH 7.7), 1 mM EDTA and 0.1 mM neocuproine supplemented with 100 µM deferoxamine and 1X protease inhibitor cocktail (Roche) and centrifuged at 14,000 rpm for 10 min at 4ºC. Two volumes of blocking buffer (HEN buffer supplemented with 2.5% SDS and 20 mM methyl methanethiosulfonate (MMTS)) were added to the leaf extract, and the solution was incubated at 50ºC for 20 min to block free sulfhydryl groups. The MMTS was then removed, and the proteins were precipitated using acetone at -20ºC for 20 min. The acetone was removed, and the proteins were resuspended in HENS buffer (HEN buffer supplemented with 1% SDS). The S-sulfhydrated proteins were then labeled using 4 mM N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (Biotin-HPDP) for 3 hours at 25ºC 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 4ºC and buffer 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 methyl methanethiosulfonate (MMTS) during the modified biotin switch assay for comparison with the blocked proteins. The biotinylated proteins were detected using an immunoblot assay with anti-biotin 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 HENS buffer. To purify the biotinylated proteins, the solution was incubated with streptavidin beads for 1 hour at room temperature with frequent vortexing. The streptavidin beads were intensively washed five times using ten volumes of 20 mM Hepes-NaOH (pH 7.7), 600 mM NaCl, 1 mM EDTA and 0.5% Triton X-100 and then centrifuged at 3000 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 NaCl, 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 cDNAs of cytosolic ascorbate peroxidase APX1 (At1g07890) and the cytosolic GapC isoform of glyceraldehyde 3-phosphate dehydrogenase GAPC1 (At3g04120) were cloned into the pDEST17 vector (Invitrogen) to express an N-terminal 6-His-tagged protein using the *E. coli* expression system with Gateway Technology (Invitrogen). For APX1 and GAPC1 protein expression, transformed *E. coli* BL21(DE3) cell cultures at an $OD_{600}$ of 0.6 were treated with 0.1 and 0.5 mM of I*PTG* (isopropyl-beta-D-thiogalactopyranoside), respectively; the cell cultures were incubated for 4 hours at 30ºC. Purification was performed by nickel resin binding under non-denaturing 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 Cysteine Residues of Recombinant Cytosolic APX using Mass Spectrometry**

Cytosolic ascorbate peroxidase APX1 was separated using non-reducing SDS-PAGE on 12% 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, Bremen, Germany). 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 ACN, and then dried under a stream of nitrogen. Then, proteomics-grade trypsin (Sigma Aldrich) at a final concentration of 16 ng/µl in 25% ACN/50 mM ammonium bicarbonate solution was added and digestion took place at 37ºC for 5 h. The reaction was stopped by adding 50% ACN/0.5% TFA for peptide extraction. The tryptic eluted peptides were dried using speed-vacuum centrifugation and were resuspended in 6 µl of 0.1% FA in water.

Digested peptides were subjected to 1D-nano LC ESI-MSMS analysis using a nano liquid chromatography system (nanoLC Ultra 1D plus, Eksigent Technologies) coupled to a high speed Triple TOF 5600 mass spectrometer (AB SCIEX, Foster City, CA) with a duo spray ionization source. Data acquisition was performed using a TripleTOF 5600 System (AB SCIEX, Concord, ON). MS 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 ppm and 0.05 Da for fragment masses, and only 1 or 2 missed cleavages were allowed. Peptides with an individual MOWSE score $\geq$ 20 were considered correctly identified.

**Immunoblot Analysis**

The biotinylated proteins were separated using non-reducing SDS-PAGE on 12% 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% polyacrylamide gels using the following antibodies: i) 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; ii) anti-GS antibodies raised against recombinant homopolymeric GS from *Phaseolus vulgaris*, recognizing mainly the *Arabidopsis* chloroplastic isoform and also to a minor extent the cytosolic isoform (Betti et al., 2006) diluted 1:1,000; and iii) and anti-cytosolic 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% 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 -80ºC. A label-free analysis was performed as described in (Schroder et al., 2012). Briefly, peptides were separated by reverse-phase chromatography using an Eksigent Ultra 2D+ pump fitted with a 75-µm ID column (nanoLC column, 75 µm id x 15 cm, C18, 3 µm, 120 Å, ChromXP); the samples were first loaded into a 2 cm long, 100-µm ID pre-column, packed using the same chemistry as the separating column, for desalting and concentrating. The mobile phases were 100% water/0.1% formic acid (buffer A) and 100% acetonitrile/0.1% formic acid (buffer B). The column gradient was developed using a 60-min, two-step run from 5% B to 30% B in 30 min and 30% B to 70% B in 10 min. The column was equilibrated with 95% B for 5 min and 5% B for 15 min. During all processes, the pre-column was in-line with the separating column, and the flow was maintained along the entire gradient at 300 nl/min. 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 m/z to 1250 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 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 ProteinPilotTM 4.5 software from AB
406 Sciex, which implements the algorithms ParagonTM for database searching and
407 ProgroupTM 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 PeakViewTM 1.1 Software from AB Sciex using the
412 combined database search results generated in the ProteinPilotTM 4.5 software. The peak
413 list matrix generated was exported to MarkerViewTM 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 4ºC, 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 $H_2O_2$, and the oxidation of ascorbate was
433 determined by the decrease in absorbance at 290 nm ($\varepsilon$= 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 approx. 2 mg of total protein) or 0.05 ml of purified GAPDH cytosolic isoform C recombinant protein (containing approx. 0.1 mg of total protein). The reaction was initiated by the addition of 1 mM glyceraldehyde 3-phosphate, and the absorbance at 340 nm was recorded for 1 minute ($\varepsilon$= 6.2 $mM^{-1}$ $cm^{-1}$).

The GS transferase activity was measured by the formation of γ-glutamylhydroxamate (Merida 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-glutamine, 4 µmol of $MnCl_2$, 60 µmol of hydroxylamine, 1 µmol of ADP and 0.05 ml of leaf extract (containing approx. 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 ($\varepsilon$= 0.89 $mM^{-1}$ $cm^{-1}$).

**Statistical Analysis**

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

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## FIGURE LEGENDS

**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 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.

**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 $Na_2S$ for 30 min at 4ºC 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.

**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.

**Figure 4.** Analysis of L-ascorbate peroxidase using mass spectrometry. 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 (A). 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 (Biemann, 1988) (B).

**Figure 5.** Enzyme activity regulation of glutamine synthetase, ascorbate peroxidase and glyceraldehyde-3-phosphate dehydrogenase by S-sulfhydration in *Arabidopsis thaliana*. The protein leaf extracts were treated in the absence or presence of NaHS at the indicated concentrations for 30 min at 4ºC (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 ± 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" ($P<0.05$).

**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 4ºC (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 ± 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" ($P<0.05$).

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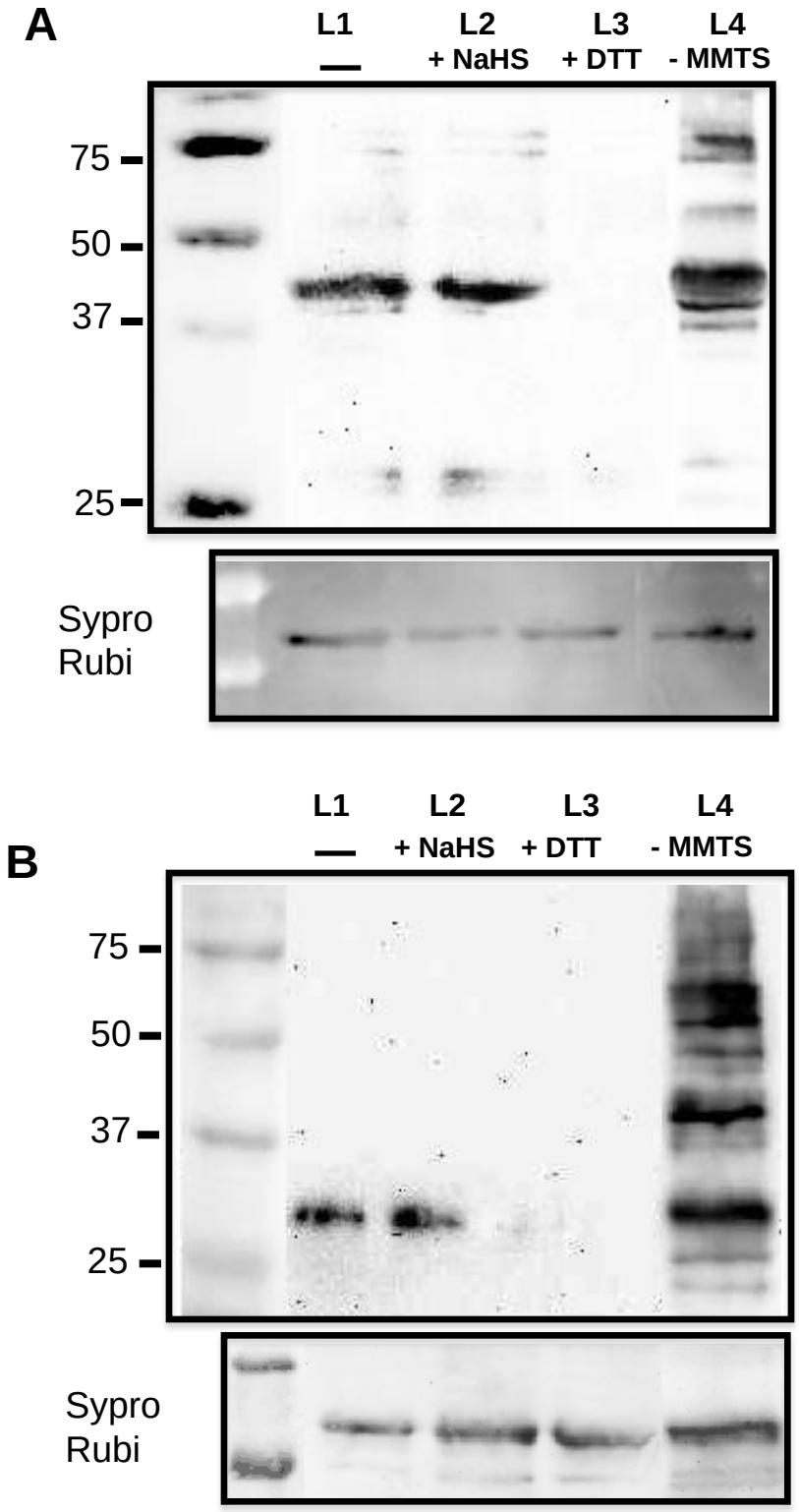
**Supplemental Data**

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

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

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

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




**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 µM 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 inmunoblotted 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 |

## Parsed Citations


**Abe, K., Kimura, H. (1996). The possible role of hydrogen sulfide as an endogenous neuromodulator. J Neurosci 16: 1066-1071.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Alvarez, C., Calo, L., Romero, L.C., Garcia, I., Gotor, C. (2010). An O-acetylserine(thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis. Plant Physiol 152: 656-669.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Alvarez, C., Bermudez, M.A., Romero, L.C., Gotor, C., Garcia, I. (2012a). Cysteine homeostasis plays an essential role in plant immunity. New Phytol 193: 165-177.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Alvarez, C., Lozano-Juste, J., Romero, L.C., Garcia, I., Gotor, C., Leon, J. (2011). Inhibition of Arabidopsis O-acetylserine(thiol)lyase A1 by tyrosine nitration. J Biol Chem 286: 578-586.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Alvarez, C., Garcia, I., Moreno, I., Perez-Perez, M.E., Crespo, J.L., Romero, L.C., Gotor, C. (2012b). Cysteine-generated sulfide in the cytosol negatively regulates autophagy and modulates the transcriptional profile in Arabidopsis. Plant Cell 24: 4621-4634.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Bedhomme, M., Adamo, M., Marchand, C.H., Couturier, J., Rouhier, N., Lemaire, S.D., Zaffagnini, M., Trost, P. (2012). Glutathionylation of cytosolic glyceraldehyde-3-phosphate dehydrogenase from the model plant Arabidopsis thaliana is reversed by both glutaredoxins and thioredoxins in vitro. Biochem J 445: 337-347.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Begara-Morales, J.C., Sanchez-Calvo, B., Chaki, M., Valderrama, R., Mata-Perez, C., Lopez-Jaramillo, J., Padilla, M.N., Carreras, A., Corpas, F.J., Barroso, J.B. (2014). Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and S-nitrosylation. J Exp Bot 65: 527-538.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Betti, M., Arcondéguy, T., Márquez, A.J. (2006). Molecular analysis of two mutants from Lotus japonicus deficient in plastidic glutamine synthetase: Functional properties of purified GLN2 enzymes. Planta 224: 1068-1079.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Biemann, K. (1988). Contributions of mass spectrometry to peptide and protein structure. Biomed Environ Mass Spectrom 16: 99-111.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Bucci, M., Cirino, G. (2011). Hydrogen sulphide in heart and systemic circulation. Inflamm Allergy Drug Targets 10: 103-108.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Chen, J., Wu, F.H., Wang, W.H., Zheng, C.J., Lin, G.H., Dong, X.J., He, J.X., Pei, Z.M., Zheng, H.L. (2011). Hydrogen sulphide enhances photosynthesis through promoting chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification in Spinacia oleracea seedlings. J Exp Bot 62: 4481-4493.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Fukuto, J.M., Carrington, S.J., Tantillo, D.J., Harrison, J.G., Ignarro, L.J., Freeman, B.A., Chen, A., Wink, D.A. (2012). Small molecule signaling agents: the integrated chemistry and biochemistry of nitrogen oxides, oxides of carbon, dioxygen, hydrogen sulfide, and their derived species. Chem Res Toxicol 25: 769-793.**
Pubmed: Author and Title

CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Gadalla, M.M., Snyder, S.H. (2010). Hydrogen sulfide as a gasotransmitter. J Neurochem 113: 14-26.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Garcia, I., Rosas, T., Bejarano, E.R., Gotor, C., Romero, L.C. (2013). Transient transcriptional regulation of the CYS-C1 gene and cyanide accumulation upon pathogen infection in the plant immune response. Plant Physiol 162: 2015-2027.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**García-Limones, C., Hervás, A., Navas-Cortés, J.A., Jiménez-Díaz, R.M., Tena, M. (2002). Induction of an antioxidant enzyme system and other oxidative stress markers associated with compatible and incompatible interactions between chickpea (Cicer arietinum L.) and Fusarium oxysporum f. sp. ciceris. Physiol Mol Plant Pathol 61: 325-337.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Garcia-Mata, C., Lamattina, L. (2010). Hydrogen sulphide, a novel gasotransmitter involved in guard cell signalling. New Phytol 188: 977-984.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Gotor, C., Garcia, I., Crespo, J.L., Romero, L.C. (2013). Sulfide as a signaling molecule in autophagy. Autophagy 9: 609-611.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Greiner, R., Palinkas, Z., Basell, K., Becher, D., Antelmann, H., Nagy, P., Dick, T.P. (2013). Polysulfides link H2S to protein thiol oxidation. Antioxid Redox Signal 19: 1749-1765.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Gruhlke, M.C., Slusarenko, A.J. (2012). The biology of reactive sulfur species (RSS). Plant Physiol Biochem 59: 98-107.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Hess, D.T., Stamler, J.S. (2012). Regulation by S-Nitrosylation of Protein Post-translational Modification. J Biol Chem 287: 4411-4418.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Jin, Z., Xue, S., Luo, Y., Tian, B., Fang, H., Li, H., Pei, Y. (2013). Hydrogen sulfide interacting with abscisic acid in stomatal regulation responses to drought stress in Arabidopsis. Plant Physiol Biochem 62: 41-46.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Kabil, O., Banerjee, R. (2010). Redox biochemistry of hydrogen sulfide. J Biol Chem 285: 21903-21907.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Kitajima, S., Kurioka, M., Yoshimoto, T., Shindo, M., Kanaori, K., Tajima, K., Oda, K. (2008). A cysteine residue near the propionate side chain of heme is the radical site in ascorbate peroxidase. FEBS J 275: 470-480.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Klie, S., Nikoloski, Z (2012). The Choice between MapMan and Gene Ontology for Automated Gene Function Prediction in Plant Science. Front Genet 3: 115.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Kolluru, G.K., Shen, X., Bir, S.C., Kevil, C.G. (2013). Hydrogen sulfide chemical biology: Pathophysiological roles and detection. Nitric Oxide 35C: 5-20.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Krishnan, N., Fu, C., Pappin, D.J., Tonks, N.K. (2011). H2S-Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. Sci Signal 4: ra86.**
Pubmed: Author and Title

CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Krueger, S., Niehl, A., Lopez Martin, M.C., Steinhauser, D., Donath, A., Hildebrandt, T., Romero, L.C., Hoefgen, R., Gotor, C., Hesse, H. (2009). Analysis of cytosolic and plastidic serine acetyltransferase mutants and subcellular metabolite distributions suggests interplay of the cellular compartments for cysteine biosynthesis in Arabidopsis. Plant Cell Environ 32: 349-367.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Laureano-Marín, A.M., García, I., Romero, L.C., Gotor, C. (2014). Assessing the transcriptional regulation of L-CYSTEINE DESULFHYDRASE 1 in Arabidopsis thaliana. Front Plant Science 5:683.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Li, L., Rose, P., Moore, P.K. (2011). Hydrogen sulfide and cell signaling. Annu Rev Pharmacol Toxicol 51: 169-187.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Li, L., Wang, Y., Shen, W. (2012a). Roles of hydrogen sulfide and nitric oxide in the alleviation of cadmium-induced oxidative damage in alfalfa seedling roots. BioMetals 25: 617-631.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Li, Z.G., Gong, M., Xie, H., Yang, L., Li, J. (2012b). Hydrogen sulfide donor sodium hydrosulfide-induced heat tolerance in tobacco (Nicotiana tabacum L) suspension cultured cells and involvement of Ca(2+) and calmodulin. Plant Sci 185-186: 185-189.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Lisjak, M., Srivastava, N., Teklic, T., Civale, L., Lewandowski, K., Wilson, I., Wood, M.E., Whiteman, M., Hancock, J.T. (2010). A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation. Plant Physiol Biochem 48: 931-935.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Merida, A., Candau, P., Florencio, F.J. (1991). Regulation of glutamine synthetase activity in the unicellular cyanobacterium Synechocystis sp. strain PCC 6803 by the nitrogen source: effect of ammonium. J Bacteriol 173: 4095-4100.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Mustafa, A.K., Gadalla, M.M., Snyder, S.H. (2009a). Signaling by gasotransmitters. Sci Signal 2: re2.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Mustafa, A.K., Gadalla, M.M., Sen, N., Kim, S., Mu, W., Gazi, S.K., Barrow, R.K., Yang, G., Wang, R., Snyder, S.H. (2009b). H2S signals through protein S-sulfhydration. Sci Signal 2: ra72.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Mustafa, A.K., Sikka, G., Gazi, S.K., Steppan, J., Jung, S.M., Bhunia, A.K., Barodka, V.M., Gazi, F.K., Barrow, R.K., Wang, R., Amzel, L.M., Berkowitz, D.E., Snyder, S.H. (2011). Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels. Circ Res 109: 1259-1268.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Nagy, P., Palinkas, Z., Nagy, A., Budai, B., Toth, I., Vasas, A. (2014). Chemical aspects of hydrogen sulfide measurements in physiological samples. Biochim Biophys Acta 1840: 876-891.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Paul, B.D., Snyder, S.H. (2012). H2S signalling through protein sulfhydration and beyond. Nat Rev Mol Cell Biol 13: 499-507.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Roepstorff, P., Fohlman, J. (1984). Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed Mass Spectrom 11: 601.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Romero, L.C., Garcia, I., Gotor, C. (2013). L-Cysteine Desulfhydrase 1 modulates the generation of the signaling molecule sulfide in**

**plant cytosol. Plant Signal Behav 8: e24007.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Schroder, P.C., Fernandez-Irigoyen, J., Bigaud, E., Serna, A., Renandez-Alcoceba, R., Lu, S.C., Mato, J.M., Prieto, J., Corrales, F.J. (2012). Proteomic analysis of human hepatoma cells expressing methionine adenosyltransferase I/III: Characterization of DDX3X as a target of S-adenosylmethionine. J Proteomics 75: 2855-2868.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Scuffi, D., Álvarez, C., Laspina, N., Gotor, C., Lamattina, L., García-Mata, C. (2014). Hydrogen Sulfide Generated by l-Cysteine Desulfhydrase Acts Upstream of Nitric Oxide to Modulate Abscisic Acid-Dependent Stomatal Closure. Plant Physiol 166: 2065-2076.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Sell, S., Lindermayr, C., Durner, J. (2008). Identification of S-nitrosylated proteins in plants. Methods Enzymol 440: 283-293.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Shevchenko, A., Wilm, M., Vorm, O., Mann, M. (1996). Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Analytical chemistry 68: 850-858.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Shi, H., Ye, T., Chan, Z. (2013). Exogenous application of hydrogen sulfide donor sodium hydrosulfide enhanced multiple abiotic stress tolerance in bermudagrass (Cynodon dactylon (L). Pers.). Plant Physiol Biochem 71C: 226-234.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Shilov, I.V., Seymour, S.L., Patel, A.A., Loboda, A., Tang, W.H., Keating, S.P., Hunter, C.L., Nuwaysir, L.M., Schaeffer, D.A. (2007). The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics 6: 1638-1655.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Tang, W.H., Shilov, I.V., Seymour, S.L. (2008). Nonlinear fitting method for determining local false discovery rates from decoy database searches. Journal of proteome research 7: 3661-3667.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Thimm, O., Blasing, O., Gibon, Y., Nagel, A., Meyer, S., Kruger, P., Selbig, J., Muller, L.A., Rhee, S.Y., Stitt, M. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J 37: 914-939.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Toohey, J.I. (2011). Sulfur signaling: is the agent sulfide or sulfane? Anal Biochem 413: 1-7.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Toohey, J.I. (2012). The conversion of H(2)S to sulfane sulfur. Nat Rev Mol Cell Biol 13: 803; author reply p 803.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Valverde, F., Ortega, J.M., Losada, M., Serrano, A. (2005). Sugar-mediated transcriptional regulation of the Gap gene system and concerted photosystem II functional modulation in the microalga Scenedesmus vacuolatus. Planta 221: 937-952.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Vandiver, M., Snyder, S. (2012). Hydrogen sulfide: a gasotransmitter of clinical relevance. J. Mol. Med 90: 1-9.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Vandiver, M.S., Paul, B.D., Xu, R., Karuppagounder, S., Rao, F., Snowman, A.M., Ko, H.S., Lee, Y.I., Dawson, V.L., Dawson, T.M., Sen, N., Snyder, S.H. (2013). Sulfhydration mediates neuroprotective actions of parkin. Nat Commun 4: 1626.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Wang, B.L., Shi, L., Li, Y.X., Zhang, W.H. (2010). Boron toxicity is alleviated by hydrogen sulfide in cucumber (Cucumis sativus L.) seedlings. Planta 231: 1301-1309.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Wang, R. (2012). Physiological Implications of Hydrogen Sulfide: A Whiff Exploration That Blossomed. Physiol Rev 92: 791-896.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Waszczak, C., Akter, S., Eeckhout, D., Persiau, G., Wahni, K., Bodra, N., Van Molle, I., De Smet, B., Vertommen, D., Gevaert, K., De Jaeger, G., Van Montagu, M., Messens, J., Van Breusegem, F. (2014). Sulfenome mining in Arabidopsis thaliana. Proc Natl Acad Sci U S A 111: 11545-11550.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Yang, G., Zhao, K., Ju, Y., Mani, S., Cao, Q., Puukila, S., Khaper, N., Wu, L., Wang, R. (2013). Hydrogen sulfide protects against cellular senescence via s-sulfhydration of keap1 and activation of Nrf2. Antioxid Redox Signal 18: 1906-1919.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Zachgo, S., Hanke, G.T., Scheibe, R. (2013). Plant cell microcompartments: a redox-signaling perspective. Biol Chem 394: 203-216.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Zaffagnini, M., Morisse, S., Bedhomme, M., Marchand, C.H., Festa, M., Rouhier, N., Lemaire, S.D., Trost, P. (2013). Mechanisms of nitrosylation and denitrosylation of cytoplasmic glyceraldehyde-3-phosphate dehydrogenase from Arabidopsis thaliana. J Biol Chem 288: 22777-22789.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Zhang, H., Hu, L.Y., Hu, K.D., He, Y.D., Wang, S.H., Luo, J.P. (2008). Hydrogen sulfide promotes wheat seed germination and alleviates oxidative damage against copper stress. J Integr Plant Biol 50: 1518-1529.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

**Zhang, H., Tan, Z.Q., Hu, L.Y., Wang, S.H., Luo, J.P., Jones, R.L. (2010). Hydrogen sulfide alleviates aluminum toxicity in germinating wheat seedlings. J Integr Plant Biol 52: 556-567.**
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title