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Hydrogen sulfide: A novel component in *Arabidopsis* peroxisomes which triggers catalase inhibition[FA]

Research Article

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Abstract Plant peroxisomes have the capacity to generate different reactive oxygen and nitrogen species (ROS and RNS), such as H_2O_2, superoxide radical ($O_2^{\cdot -}$), nitric oxide and peroxynitrite ($ONOO^-$). These organelles have an active nitro-oxidative metabolism which can be exacerbated by adverse stress conditions. Hydrogen sulfide (H_2S) is a new signaling gasotransmitter which can mediate the posttranslational modification (PTM) persulfidation. We used *Arabidopsis thaliana* transgenic seedlings expressing cyan fluorescent protein (CFP) fused to a canonical peroxisome targeting signal 1 (PTS1) to visualize peroxisomes in living cells, as well as a specific fluorescent probe which showed that peroxisomes contain H_2S. H_2S was also detected in chloroplasts under glyphosate-induced oxidative stress conditions. Peroxisomal enzyme activities, including catalase, photorespiratory H_2O_2-generating glycolate oxidase (GOX) and hydroxypyruvate reductase (HPR), were assayed *in vitro* with a H_2S donor. In line with the persulfidation of this enzyme, catalase activity declined significantly in the presence of the H_2S donor. To corroborate the inhibitory effect of H_2S on catalase activity, we also assayed pure catalase from bovine liver and pepper fruit-enriched samples, in which catalase activity was inhibited. Taken together, these data provide evidence of the presence of H_2S in plant peroxisomes which appears to regulate catalase activity and, consequently, the peroxisomal H_2O_2 metabolism.

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INTRODUCTION

Multifunctional hydrogen peroxide (H_2O_2) and nitric oxide (NO) molecules are involved in physiological processes, as well as responses to adverse environmental conditions (Neill et al. 2002; Corpas 2015; del Río 2015; da Silva et al. 2017; Corpas and Palma 2018). Though characterized by a very simple chemical structure, both these molecules are capable of generating reactive oxygen and nitrogen species (ROS and RNS).

Hydrogen sulfide (H_2S), the simplest thiol found in animal and plant cells, has been known to be toxic for some time. However, it has recently been found to have properties similar to those of multifunctional signaling molecules (NO and H_2O_2) (Lisjak et al. 2013; Gotor et al. 2015; Hancock and Whiteman 2016; Li et al. 2016; Yamasaki and Cohen 2016; Filipovic and Jovanovic 2017). As with NO and H_2O_2, exogenous applications of H_2S in plants have been shown to counteract the toxic effects of stresses, such as heavy metal and salinity (Zhang et al. 2010; Chen et al. 2014, 2015, 2017; Ali et al. 2014; Bharwana et al. 2014; Kharbech et al. 2017; Corpas et al. 2019). In plant systems, several enzymes are capable of generating H_2S, which is part of the cysteine (Cys) metabolism. These enzymes, including L- and D-cysteine desulfhydrase (L-DES/D-DES), sulfite reductase (SiR), cyano alanine synthase (CAS) and cysteine synthase (CS) (Li et al. 2013; Calderwood and Kopriva 2014; Hancock and Whiteman 2014), are present in different subcellular compartments, such as cytosol, chloroplasts

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and mitochondria (Gotor et al. 2015; Hancock and Whiteman 2016). For example, endogenous H_2S content has recently been reported to increase during sweet pepper fruit ripening, which is associated with an increase in cytosolic L-cysteine desulfhydrase (L-DES) activity (Muñoz-Vargas et al. 2018).

With their active nitro-oxidative metabolisms, plant peroxisome organelles are necessary in multiple biochemical pathways in all phases of plant development, from seed germination to plant senescence and also in response to adverse environmental conditions (del Río et al., 2002; Hu et al. 2012; Corpas et al. 2017; Kao et al. 2018; Palma et al. 2018). To our knowledge, no information exists on the presence of H_2S in plant or animal peroxisomes. However, proteomic studies of animals and plants have identified catalase as a potential target of persulfidation (Mustafa et al. 2009; Aroca et al. 2015), which is a post-translational modification (PTM) mediated by H_2S that modulates the function of target proteins (Iciek et al. 2015; Ju et al. 2017). Thus, this study mainly aims to evaluate the presence of H_2S in plant peroxisomes and its potential biochemical implications.

RESULTS

In 2014, Peng et al. devised a series of pyridine disulfide-based fluorescent Washington State Probes (WSP1 to WSP5) to detect H_2S in both aqueous solutions and cell images. H_2S can undergo dual nucleophilicity which facilitates a tandem nucleophilic substitution-cyclization reaction, enables fluorophore release and activates fluorescence. These WSPs also exhibited greater sensitivity and selectivity in relation to H_2S as compared to other cellular sulfur species, such as cysteine (Cys), and reduced glutathione (GSH). Using fluorescence microscopy, Peng et al. (2014) observed the presence of H_2S in human HeLa living cells pre-incubated with these WSP fluorescence probes.

Using confocal laser scanning microscope (CLSM) to analyze 10-d-old *Arabidopsis thaliana* transgenic seedlings expressing the cyan fluorescent protein (CFP) fused to canonical peroxisome targeting signal 1 (PTS1), we observed peroxisomes in the form of spherical spots (green color) distributed randomly throughout *Arabidopsis* root tip cells (Figure 1A). We used the WSP-5 fluorescent probe to specifically detect cellular H_2S (Peng et al. 2014; Yu et al. 2014). Using this probe and CLSM with the CFP-PTS1 *Arabidopsis* transgenic line, we observed intense red fluorescence corresponding to H_2S in spherical spots on the root tip cells (Figure 1B), with a pattern analogous to that of CFP-PTS1 (Figure 1A). Figure 1C shows a merged image of panels A and B, with a significant correspondence of both punctate distributions, indicating that H_2S is present in *Arabidopsis* peroxisomes. A similar analysis was carried out on the guard cells of green *Arabidopsis* cotyledons (Figure 1D, G), with chlorophyll autofluorescence also enabling us to observe the presence of chloroplasts (Figure 1F). Figure 1G shows the merged image of panels D to F, which indicate that H_2S is located in peroxisomes and the cytosol.

Although CFP excitation/emission wavelengths do not theoretically overlap in WSP-5 when used to detect H_2S, this was evaluated at the experimental level in order to rule out this possibility. 10-d-old *Arabidopsis* seedlings expressing CFP-PTS1 were, therefore, used without pre-incubation with fluorescent probe WSP-5 to detect H_2S. Thus, Figure S1A shows the detection by CLSM of peroxisomes in the root tip of an *Arabidopsis* seedling expressing CFP-PTS1 using excitation and emission wavelengths of 459 and 475 nm, respectively. On the other hand, Figure S1B shows the same *Arabidopsis* root area with excitation and emission wavelengths set at 502 nm and 525 nm, respectively. Under these conditions, no fluorescent signal was observed, which corroborated the absence of overlap between CFP and WSP-5.

In order to evaluate the specificity and efficiency of WSP-5 in the detection of H_2S in *Arabidopsis* seedlings, additional controls using *Arabidopsis* wild-type (WT) seedlings were carried out. Figure 2A–D shows a primary root of 10-d-old *Arabidopsis* seedlings incubated with increasing concentrations (0.0, 0.1, 0.5 and 2 mmol/L) of sodium hydrosulfide NaHS which is a recognized H_2S donor molecule in animal and plant systems (Wang et al. 2010; Aroca et al. 2015; Yang et al. 2016; Kharbech et al. 2017; Zhang et al. 2017; Muñoz-Vargas et al. 2018). Thus, an increase in the red fluorescence signal corresponding to the presence of H_2S was also observed. Furthermore, when *Arabidopsis* seedlings were pre-incubated with 0.1 mmol/L NaHS and 0.1 mmol/L hypotaurine (a H_2S scavenger), the fluorescence attributable to H_2S decreased significantly in the root (Figure 2E) as compared to the seedling treated

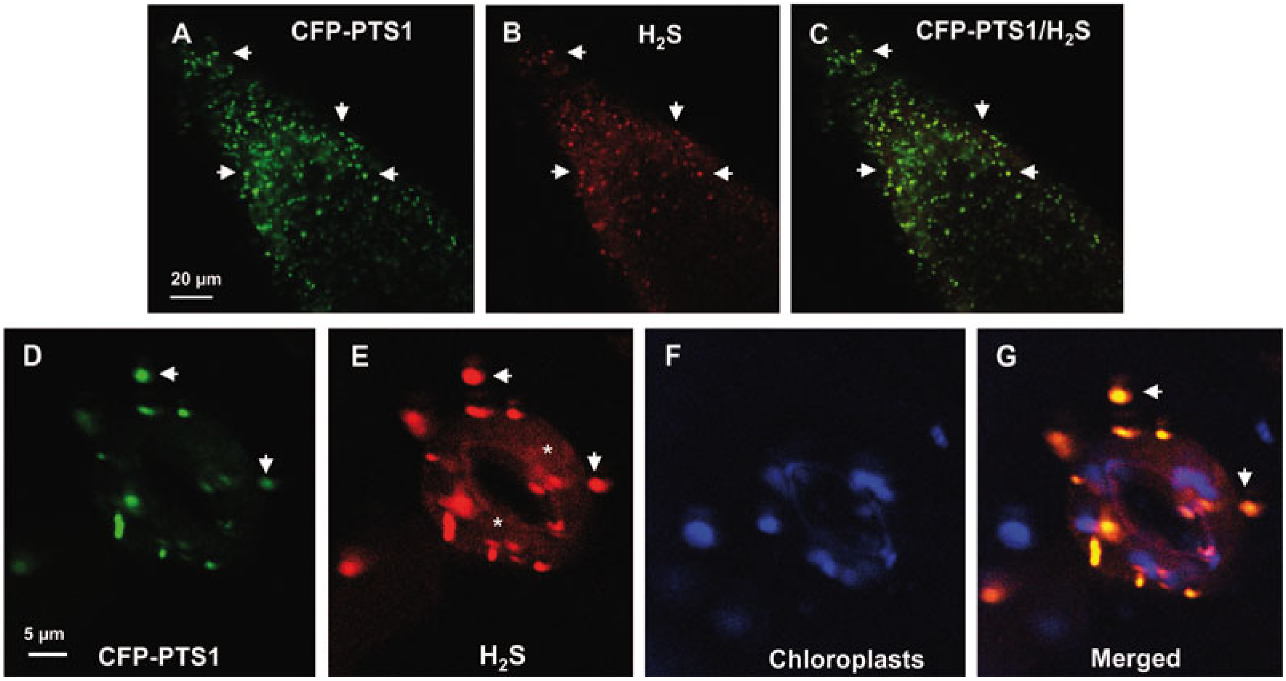

Figure 1. Representative images illustrating the CLSM *in vivo* detection of H_2S (red color) and peroxisomes (green color) in root tips (A to C) and guard cells (D to G) of 10-d-old *Arabidopsis* seedlings expressing CFP-PTS1
(**A**) and (**D**), Fluorescence punctates (green) attributable to CFP-PTS1, indicating the localization of peroxisomes. (**B**) and (**E**), Fluorescence punctates (red) attributable to H2S detection in the same area. (**C**) Merged image of (**A**) and (**B**) showing colocalized fluorescence punctates (yellow). (**F**) Chlorophyll autofluorescence (blue) demonstrating location of chloroplasts. (**G**) Merged images of (**D**) to (**F**). H_2S (red color) was detected by using 5 µM WSP-5. Arrows indicate representative punctate spots corresponding to the colocalization of H_2S with peroxisomes. Asterisks indicate localization of H_2S in the cytosol.

with 0.1 mmol/L NaHS alone (Figure 2B). Figure 2F shows the change in the fluorescence intensity of H_2S (red color) in the primary roots of *Arabidopsis* seedlings (Figure 2A–E). These data confirm that WSP-5 is a reliable tool for analyzing H_2S in *Arabidopsis* seedlings.

To assess how H_2S content and cellular distribution are affected under stress conditions, *Arabidopsis* seedlings were grown in the presence of glyphosate, a herbicide which triggers oxidative stress in peroxisomal metabolism (de Freitas-Silva et al. 2017). Figure 3 shows that H_2S detected in the green cotyledons (Figure 3A–H) and roots (Figure 3I–N) of 14-d-old *Arabidopsis* seedlings grown under optimal conditions or in the presence of 20 µM glyphosate. Under optimal conditions, as previously described (Figure 1), H_2S was mainly detected in peroxisomes and the cytosol. However, under glyphosate-induced oxidative stress, red fluorescence corresponding to H_2S content increased significantly in green cotyledons and roots (Figure 3D, L). In addition to its presence in green cotyledons and peroxisomes, H_2S was also detected in other rounded structures corresponding to chloroplasts (Figure 3D, F, H).

We used catalase to evaluate the potential impact of H_2S on the peroxisomal metabolism. Proteomic analysis has identified catalase, which is the main peroxisomal antioxidant enzyme, as a target of persulfidation in mouse liver (Mustafa et al. 2009) and *Arabidopsis* leaves (Aroca et al. 2015, 2017). Therefore, we analyzed enzymatic catalase activity under *in vitro* conditions using a NaHS concentration gradient. Figure 4A shows the impact of H_2S inhibition on catalase activity, with doses of NaHS ranging from 0.1 to more than 4 mmol/L, which led to reductions of 29% and 88% in catalase activity, respectively. Accordingly, the H_2S donor inhibited catalase activity by 50% (IC_{50}) at a concentration of approximately 0.9 mmol/L. We also studied photorespiratory H_2O_2-generating glycolate oxidase (GOX), another peroxisomal enzyme

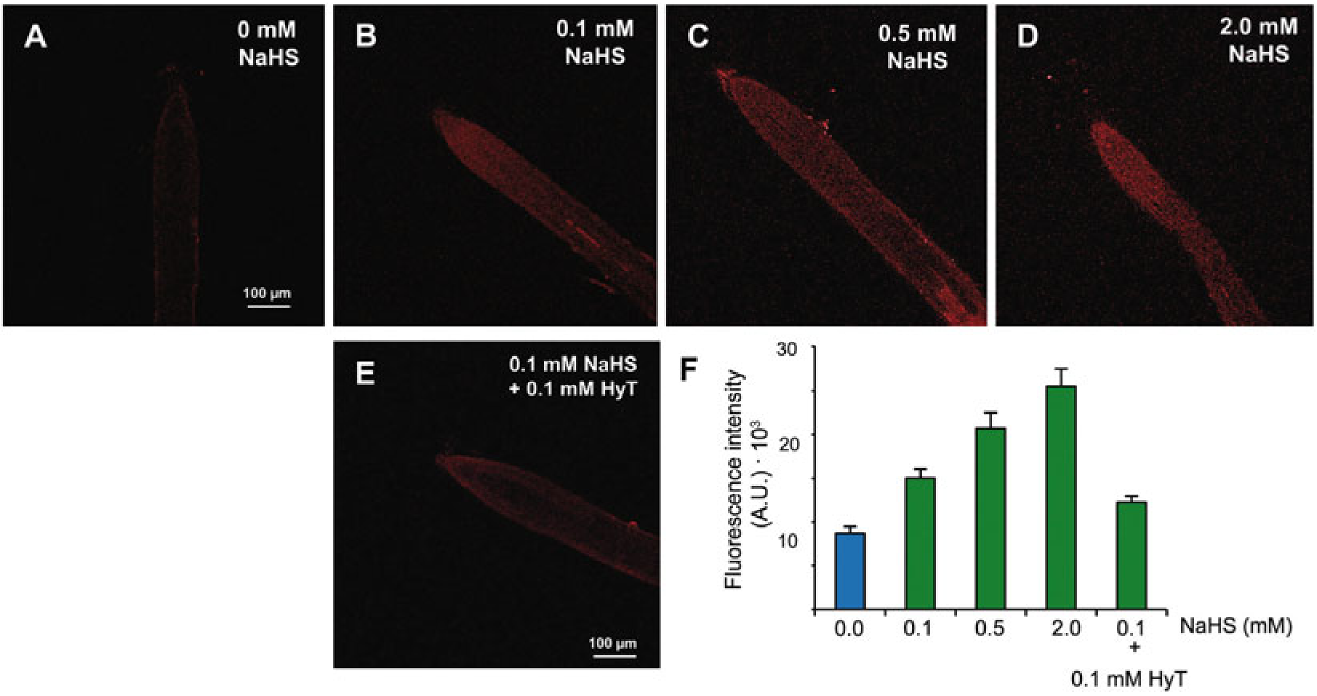

Figure 2. Representative images illustrating the CLSM in vivo detection of H_2S (red color) in primary roots of 10-d-old *Arabidopsis thaliana* wild-type seedlings pre-incubated with different concentrations of NaHS (0.0, 0.1, 0.5 and 2 mmol/L) (A to D, respectively) and pre-incubated with 0.1 mmol/L NaHS plus 0.1 mmol/L hypotaurine (HyT), a H_2S scavenger (E)

The fluorescence intensity of the red WSP-5 signal in roots of panels (**A**) to (**E**) was determined using Image J software and is expressed as arbitrary units (A.U.) (**F**).

(Oikawa et al. 2015; Hagemann and Bauwe 2016), whose activity decreased by 22% at a concentration of 4 mmol/L NaHS (Figure 4B). In addition, hydroxypyruvate reductase (HPR) activity, which is also involved in the photorespiratory pathway, was barely affected by these concentrations (Figure 4C). To corroborate the inhibitory effect of H_2S on *Arabidopsis* catalase activity, catalase from green pepper fruit-enriched samples and pure catalase from bovine liver were also assayed (Figure 5A, B, respectively). In both cases, catalase activity was inhibited in the presence of NaHS, with an IC_{50} of 0.51 mmol/L for the pepper fruit catalase and 0.98 mmol/L for bovine liver catalase.

DISCUSSION

Persulfidation is a post-translational modification (PTM) in which the thiol group (-SH) of a specific cysteine (Cys) residue is converted into a persulfide group (-SSH). Thus, protein persulfidation is an oxidative PTM that can mediate signaling events caused by H_2S (Filipovic and Jovanovic 2017; Filipovic et al. 2018; Corpas et al. 2019). Using the modified biotin switch method, 106 putative target proteins, which can be persulfidated in Cys residues in 30-d-old *Arabidopsis thaliana* leaves, were initially identified (Aroca et al. 2015). *In vitro* assays in the presence of the H_2S donor NaHS in selected proteins showed inactivated glutamine synthetase (GS) activity, while ascorbate peroxidase (APX) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity increased significantly (Aroca et al. 2015). Using an improved tag switch method with a biotin-linked cyanoacetate (CN-biotin) to form stable thiol-ether conjugates (Zhang et al. 2014) combined with LC-MS/MS analysis, Aroca et al. (2017) found that the number of putative persulfidated proteins in *Arabidopsis* leaves increased to 2,015. After inspecting the reported list of *Arabidopsis* persulfidated proteins, 17 peroxisomal proteins, representing 0.8% of the total, were identified, including three catalase isozymes, several fatty acid β-oxidation enzymes and a few enzymes involved in photorespiration pathways (see

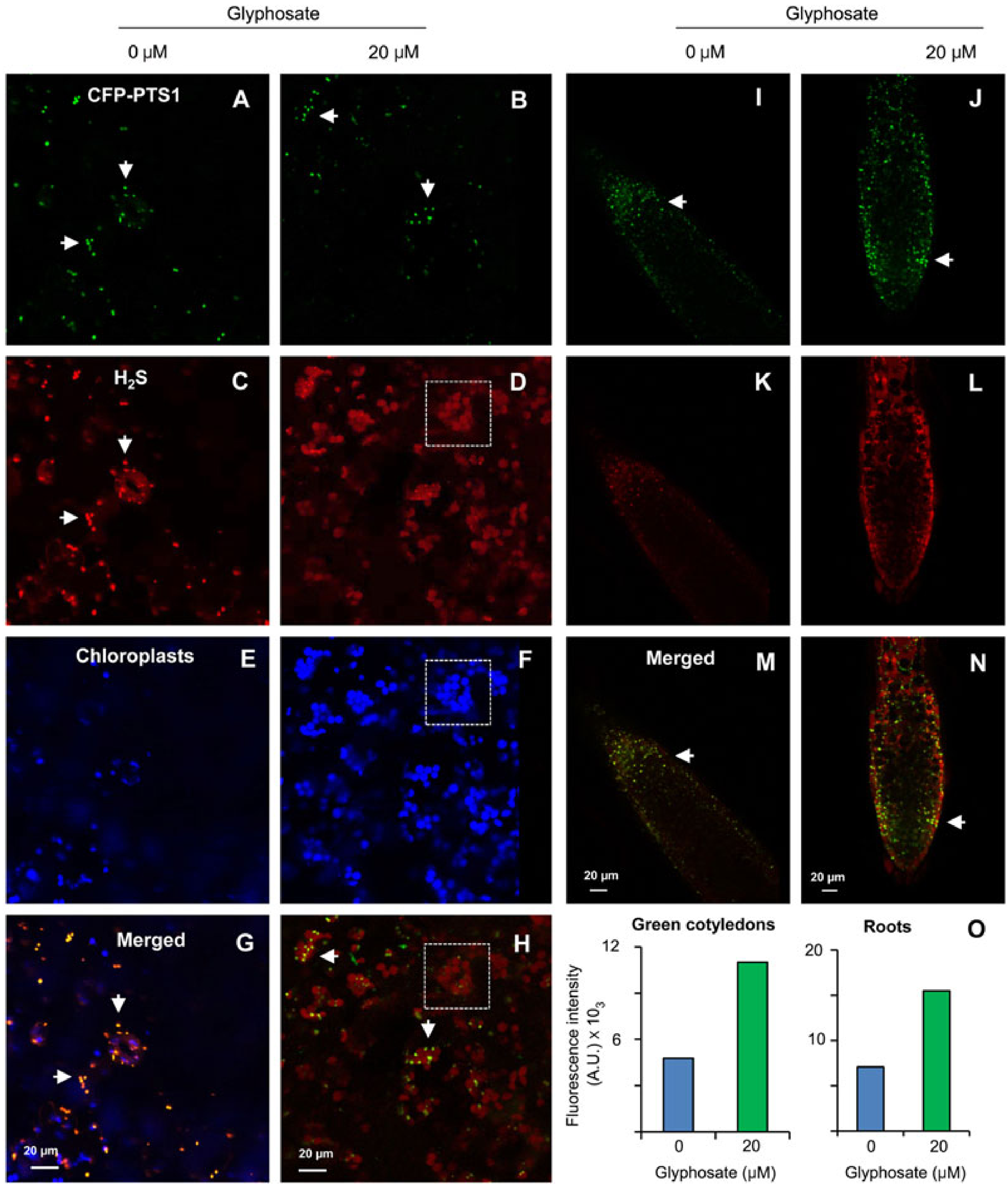

Figure 3. Representative images illustrating the CLSM *in vivo* detection of H_2S (red) in green cotyledons (panels A to H) and roots (panels I to N) of 14-d-old *Arabidopsis* seedlings expressing CFP-PTS1 (green) grown in the presence of 20 μM glyphosate

(**A**), (**B**), (**I**) and (**J**), Fluorescence punctates (green) attributable to CFP-PTS1, indicating the localization of peroxisomes. (**C**), (**D**), (**K**) and (**L**), Red fluorescence attributable to H_2S detection in the same area. (**E**) and (**F**), Chlorophyll autofluorescence (blue) demonstrating location of chloroplasts. (**G**), (**H**), (**M**) and (**N**) merged images. (O), fluorescence intensity of the red WSP-5 signal in green cotyledons (**C** and **D**) and in roots (**K** and **L**)

Table 1). However, the effect of persulfidation was not analyzed in any of these proteins. We therefore evaluated the possible presence of H_2S in *Arabidopsis* peroxisomes using cell imaging techniques and, with the aid of *in vitro* assays, we also studied the potential impact of H_2S on some of the peroxisomal proteins identified, including antioxidant catalase, photorespiratory H_2O_2-generating GOX and hydroxypyruvate reductase (HPR).

H_2S is present in plant cell cytosols, peroxisomes and chloroplasts

In previous studies, using *Arabidopsis* seedlings expressing CFP-PTS1 and specific fluorescent probes, we detected the presence of various molecules involved in the peroxisomal metabolism of ROS and RNS, such as superoxide radicals ($O_2^{\cdot-}$), peroxynitrite ($ONOO^-$) and nitric oxide (NO), as well as the presence of calcium which is essential for NO generation (Corpas and Barroso 2014, 2018; Corpas et al. 2009). On the other hand, WSP fluorescent probes have proven useful to visualize H_2S in animal HeLa cells (Peng et al. 2014) and tomato roots (Li et al. 2014). We used fluorescent WSP-5, which has a faster fluorescence turn-on rate and more sensitive detection limits as compared to other WSPs, to study the potential presence of H_2S in plant peroxisomes. The data obtained, together with those from controls (Figure 2), indicate that the WSP-5 fluorescent probe is useful for detecting H_2S in plant systems. Similar observations have been made in relation to tomato roots using fluorescent probe WSP-1 (Li et al. 2014). In addition, using *Arabidopsis* seedlings expressing CFP-PTS1, which enable peroxisomes to be visualized in living cells incubated with fluorescent probe WSP-5, as well as CLSM, we observed a red fluorescent signal attributable to H_2S which was present in the cytosol and peroxisomes of green cotyledons and roots of *Arabidopsis* seedlings grown under optimal conditions. The presence of H_2S in other cell compartments cannot be ruled out, as several enzymatic sources of H_2S present in cytosols, chloroplasts and mitochondria are involved in the cysteine metabolism (Papenbrock et al. 2007; Alvarez et al. 2010; Birke et al. 2015; Li et al. 2016), although none appear to be located in peroxisomes. To explore this possibility, *Arabidopsis* seedlings were grown in the presence of glyphosate which affects the shikimate pathway, reduces aromatic amino acid synthesis and disturbs plant growth and secondary metabolites which compromises auxin and salicylate biosynthesis occurs (Tzin and Galili 2010; Peek and Christendat 2015). We previously demonstrated that, in *Arabidopsis* seedlings, glyphosate triggers oxidative stress in the peroxisomal metabolism and in the oxidative phase of the pentose phosphate pathway (OxPPP) (de Freitas-Silva et al. 2017). Under these oxidative conditions, CLSM showed that H_2S content increased in both green cotyledons and roots. However, interestingly, its location in chloroplasts is very much in line with that of H_2S-generating sulfite reductase (Khan et al. 2010). On the other hand, a recent study identified a novel pathway that produces H_2S from D-cysteine in animals, involving D-amino acid oxidase (DAO) and 3-mercaptopyruvate sulfurtransferase (3MST), which are present in peroxisomes and mitochondria, respectively (Kimura 2015). H_2S could be present in other subcellular compartments of *Arabidopsis* due to the chemical properties of this highly lipophilic molecule which easily spreads throughout the lipid bilayer of cell membranes (Mathai et al. 2009; Cuevasanta et al. 2017). Thus, given an estimated H_2S mobility of 0.5 cm/s in the lipid bilayer, or around four orders of magnitude faster than water (Mathai et al. 2009; Riahi and Rowley 2014), H_2S accumulated in *Arabidopsis* peroxisomes could originate from cytosol and chloroplasts. It has not been determined whether peroxisomal H_2S is endogenously generated or imported, although both are possible. It is worth noting that plant peroxisomes contain enzymatic and non-enzymatic components such as glutathione reductase (GR), sulfite oxidase (SO), glutathione (GSH), S-nitrosoglutathione (GSNO) and sulfite (Jiménez et al. 1997; Hänsch and Mendel 2005; Hänsch et al. 2006; Corpas and Barroso 2015), which are involved in the sulfur metabolism.

determined using Image J software and is expressed as arbitrary units (A.U). H_2S (red color) was detected by using 5 μM WSP-5. Arrows indicate representative punctuate spots corresponding to the colocalization of H_2S with peroxisomes. Squares with broken lines indicate localization of H_2S in the chloroplasts.

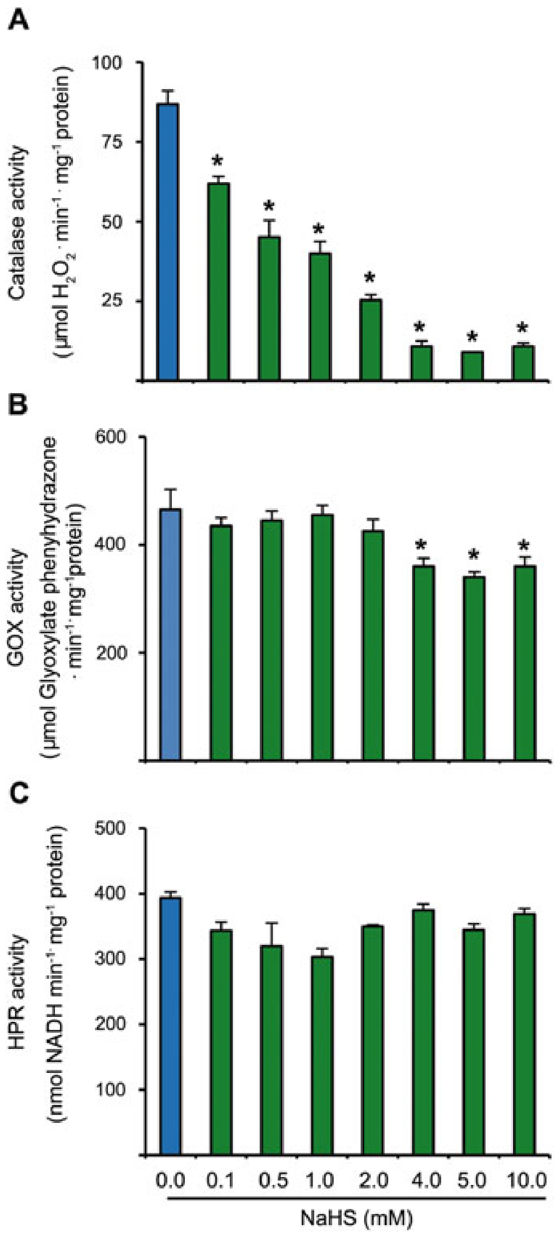

Figure 4. Effect of NaHS (H_2S donors) on several peroxisomal enzymes of *Arabidopsis* seedlings

Peroxisomal enzymatic activity assays for (**A**) catalase expressed as μmol H_2O_2 · min^{-1} · mg^{-1} protein, (**B**) glycolate oxidase (GOX) expressed as μmol Glyoxylate phenyhydrazone · min^{-1} · mg^{-1} protein, and (**C**) hydroxypyruvate reductase (HPR) expressed as nmol NADH · min^{-1} · mg^{-1} protein. Data are means ±*SE* of at least three replicates. Differences from control values were significant at $P < 0.05$.

Inhibition of catalase by H_2S

The presence of H_2S in specific organelles is also evidenced by the identification of the protein post-translational modification persulfidation (also known as *S*-sulfhydration) which affects susceptible reactive cysteine residues and converts Cys-SH groups into Cys-SSH groups (Filipovic 2015; Paul and Snyder 2015; Filipovic et al. 2018). Using a modified biotin switch method combined with liquid chromatography-tandem mass spectrometry, 39 and 2,015 endogenously persulfidated proteins have been identified in mouse liver (Mustafa et al. 2009) and *A. thaliana* leaves (Aroca et al. 2015, 2017), respectively. In both these studies, catalase, which is exclusively located in peroxisomes, was observed to be a target for persulfidation, a finding that corroborates the functional presence of H_2S in these organelles. However, to our knowledge, no information exists on the specific effects of persulfidation on catalase activity. It is important to point out that we used high concentrations of the H_2S donor, as NaHS, which is immediately hydrolyzed in aqueous solution, establishes an equilibrium between H_2S, HS^- and S_2^- species. Moreover, after this equilibrium is established, H_2S is volatilized, which reduces the concentration of sulfur species in solution. Air oxidation of HS^- catalyzed by the presence of trace metals in aqueous solution also reduces the actual concentration of H_2S in solution (Hughes et al. 2009). However, as mentioned above, NaHS is the most commonly used H_2S donor in animal and plant systems.

Catalase, which regulates peroxisomal H_2O_2 content, is exclusively located in peroxisomes; the inhibition of catalase by persulfidation is in line with previous studies which indicate that catalase is also targeted by other PTMs mediated by catalase- inhibiting NO-derived molecules, such as *S*-nitrosylation and nitration (Clark et al. 2000; Begara-Morales et al. 2013; Chaki et al. 2015; Hu et al. 2015; Titov and Osipov 2017). This suggests that catalase is strictly regulated by H_2S and NO, which is particularly important under adverse conditions given the nitro-oxidative environment inside peroxisomes (Corpas et al. 2017). The other peroxisomal enzymes assayed under similar *in vitro* conditions behaved differently, with GOX experiencing an inhibition of 22% at higher concentrations of NaHS, while HPR was virtually unaffected, indicating that the inhibitory effect on catalase is specific.

It is possible to conclude that, although plant peroxisomes are known to contain biologically active ROS and RNS metabolisms, the presence of H_2S opens up new questions about their role under physiological and stress conditions. Catalase, an essential enzyme in living organisms and one of the first peroxisomal

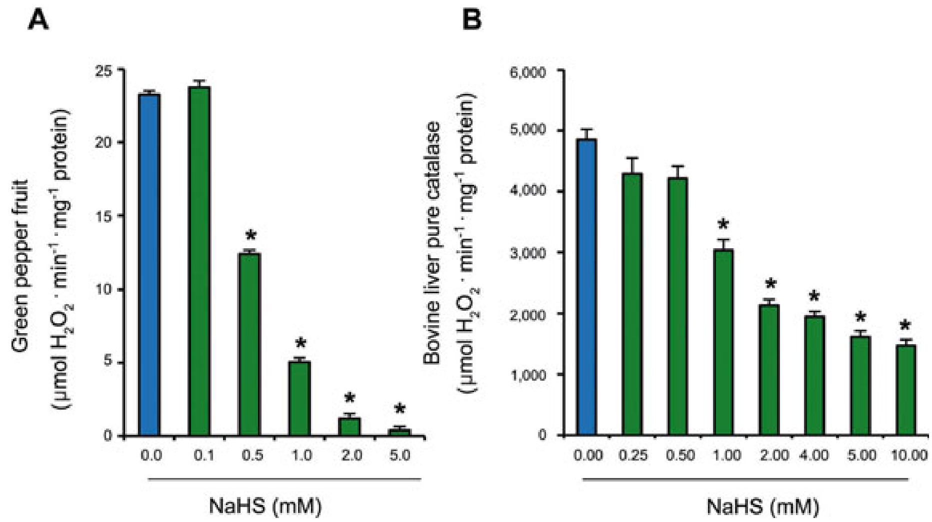

Figure 5. Effect of NaHS (H_2S donors) on catalase activity from different origins
(**A**) 0–50% enriched (NH4)2SO4 protein fraction obtained from green pepper fruits. (**B**) Commercial pure catalase from bovine liver. Catalase activity is expressed as μmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ protein. Data are means $\pm$ *SE* of at least four replicates. *Differences from control values were significant at $P < 0.05$.

Table 1. List of peroxisomal persulfidated proteins found in leaves of *Arabidopsis thaliana* (Aroca et al. 2015, 2017) with its corresponding Uniprot accession number

Peroxisomal protein	Uniprot accession number
Catalase 1	Q96528
Catalase 2	P25819
Catalase 3	Q42547
Glycerate dehydrogenase HPR, peroxisomal	Q9C9W5
Glycolate oxidase 1	Q9LRR9
3-ketoacyl-CoA thiolase 1	Q8LF48
3-ketoacyl-CoA thiolase 2	Q56WD9
3-ketoacyl-CoA thiolase 5	Q570C8
Peroxisomal fatty acid β-oxidation multifunctional protein AIM1	Q9ZPI6
Peroxisomal acyl-coenzyme A oxidase 1	O65202
Acetate/butyrate–CoA ligase AAE7	Q8VZF1
NADP-isocitrate dehydrogenase	Q9SLK0
Fatty acid β-oxidation multifunctional protein MFP2	Q9ZPI5
Acyl-coenzyme A oxidase 3	P0CZ23
Acyl-coenzyme A oxidase 4	Q96329
β-glucosidase 26	O64883
Enoyl-CoA hydratase 2	Q8VYI3

antioxidant enzymes to be characterized, catalyzes the decomposition of hydrogen peroxide ($2\,H_2O_2 \rightarrow 2\,H_2O + O_2$) (Mhamdi et al. 2012; Corpas 2015; Su et al. 2018). The presence of H_2S, which regulates catalase activity, supports the hypothesis that plant peroxisomes contain an active metabolism of reactive sulfur species (RSS) (Corpas and Barroso 2015). Previous studies have reported the presence of other enzymes, such as glutathione reductase (GR) and sulfite oxidase (SO), involved in the sulfur metabolism (Nowak et al. 2004; Hänsch et al. 2006, 2007). SO, which catalyzes the conversion of sulfite to sulfate with a concomitant generation of H_2O_2, appears to protect catalase, which is inhibited by low concentrations of sulfite (Veljovi-Jovanovic et al. 1998; Hänsch et al. 2007). In summary, our findings provide new evidence of the complexity of the peroxisomal metabolism in plants, in which H_2S can be regarded as a new regulatory molecule that may be involved in crosstalk between peroxisomes and other subcellular compartments, especially under nitro-oxidative stress conditions. These data could also corroborate the potential presence of H_2S in animal peroxisomes, as animal catalase is also reported to be persulfidated (Mustafa et al. 2009) and, consequently, inhibited, as shown in this study (Figure 4B). Thus, this situation could be similar to that in relation to peroxisomal superoxide dismutase (SOD) which was first described in plant peroxisomes, a discovery which was not questioned until CuZn-SOD was detected in animal peroxisomes (Corpas et al. 2017).

MATERIALS AND METHODS

Arabidopsis growth conditions

Wild-type and transgenic seeds of *Arabidopsis thaliana* expressing cyan fluorescent protein (CFP) fused to a canonical peroxisome targeting signal 1 (PTS1) (Nelson et al. 2007) were surface-sterilized for 5 min using a solution of 70% ethanol containing 0.1% SDS. Then, the seeds were kept in sterile water containing 20% bleach and 0.1% SDS for 20 min and washed several times in sterile water. The seeds were sown for 2 d at 4°C in the dark on Petri plates containing 4.32 g/L Murashige and Skoog basal medium (Sigma), 1% sucrose and 0.8% phytoagar, with a pH of 5.5 (Corpas and Barroso 2014b). The *Arabidopsis* seeds were then grown for 10 d at 16 h light, 22°C/8 h dark, at 18°C (long day conditions) under a light intensity of 100 $\mu E/m^2/s$ (Corpas and Barroso 2017). California-type green sweet pepper (*Capsicum annuum* L.) fruits were provided for Syngenta Seeds Ltd. (El Ejido, Almería, Spain). Pure catalase from bovine liver was a product of Sigma. For the experiments with glyphosate stress, seeds were grown directly on MS medium plates with and without 20 μM glyphosate for 14 d under long-day conditions (de Freitas-Silva et al. 2017).

Crude extracts from plant samples

Arabidopsis seedlings were collected, pooled and frozen in liquid nitrogen. Then, the seedlings were ground in a mortar with a pestle and the obtained powder was suspended in a medium containing 50 mmol/L Tris-HCl (pH 7.8, ratio 1:4; w/v), 0.1 mmol/L EDTA, 0.2% (v/v) Triton X-100 and 10% (v/v) glycerol. Homogenates were filtered through two layers of Miracloth and centrifuged at 27,000 g for 20 min. These supernatants were collected and utilized for the enzymatic *in vitro* assays.

Green pepper (*C. annuum* L.) fruit extracts were obtained in similar way but the resulting powder was resuspended in 0.1 M Tris-HCl buffer (pH 8.0, ratio 1:1; w/v) containing 1 mmol/L EDTA, 0.1% (v/v) Triton X-100 and 10% (v/v) glycerol. Homogenates were also filtered through two layers of Miracloth and centrifuged at 27,000 g for 20 min. The supernatants were used for protein enrichment by ammonium-sulfate [$(NH_4)_2SO_4$] to a quantity of 50% saturation. The obtained pellet was suspended in the same previous buffer and then loaded on a PD-10 desalting column containing Sephadex^{TM} G-25 to eliminate the $(NH_4)_2SO_4$.

Enzyme activity assays in the presence of H_2S

Catalase (EC 1.11.1.6) enzymatic activity was spectrophotometrically assayed by following the disappearance of H_2O_2 at 240 nm (Aebi 1984). Glycolate oxidase (EC 1.1.3.1) activity was also determined spectrophotometrically by the formation of a glyoxylate-phenylhydrazone complex at 324 nm (Kerr and Groves 1975). NADH-dependent hydroxypyruvate reductase (HPR; EC 1.1.1.29) activity was assayed by monitoring the NADH oxidation at 340 nm (Schwitzguébel and Siegenthaler 1984).

For *in vitro* assays of persulfidation, the samples (*Arabidopsis* extracts, enriched pepper fruits and pure catalase from bovine liver) were previously treated in the absence and presence of sodium hydrosulfide (0.1, 0.5, 1.0, 2.0, 4.0, 5.0 and 10.0 mmol/L NaHS) as H_2S donor for 60 min at 4°C in darkness (Aroca et al. 2015).

Detection of hydrogen sulfide (H_2S) in transgenic *Arabidopsis* seedlings expressing CFP-PTS1 and using CLSM technology

H_2S was identified using 5 μM WSP-5 (Washington State Probe-5, Cayman Chemical) fluorescence probe dissolved in 10 mmol/L Tris-HCl (pH 7.4) buffer (Peng et al. 2014; Yu et al. 2014). The *Arabidopsis* seedlings were kept with this fluorescence probe (5 μM WSP-5 final concentration) in darkness conditions at 25°C for 1 h. Then, seedlings were washed twice in the same solution (10 mmol/L Tris-HCl, pH 7.4) for 15 min and placed on a microscopic slide. For examination, the confocal laser scanning microscope (Leica TCS SP5 II) was set up with the follow conditions: WSP-5 was excited at 502 nm and emission was collected at 525 nm and a 40 nm band pass width (490–530 nm); cyan fluorescent protein (CFP) was excited at 458 nm and emission was collected at 475 nm and a 40-nm band pass width (465–505 nm). As internal control, it was evaluated the potential overlap between the excitation and emission wavelengths of cyan fluorescent protein (CFP) with the fluorescent probe WSP-5 used to detect H_2S. Additional controls were done to evaluate the efficiency and specificity of WSP5 to detect H_2S in *Arabidopsis* samples. In this sense, 10-d-old *Arabidopsis* wild-type seedlings were incubated with increasing concentrations of NaHS (0.1, 0.5 and 2 mmol/L) for 60 min at 25°C in the presence of 5 μM WSP5 and then observed by CLSM. In some cases, *Arabidopsis* seedlings were also incubated with 0.1 mmol/L hypotaurine, a specific H_2S scavenger (Li et al. 2014; Shi et al. 2015) for 1 h at 25°C in the presence of 5 μM WSP5 and then observed by CLSM.

Other assays

The measure of protein concentration was determined at 595 nm using the Bio-Rad protein assay (Hercules, CA) and a bovine serum albumin solution was used to prepare the standard curve. Relative fluorescence was quantified by using ImageJ software.

The results were the mean values ± standard errors (*SE*) obtained from a minimum of three independent biological replicates. The Student's *t*-test was used to determine the statistical significance between means. The half maximal inhibitory concentration (IC_{50}) value represents the concentration at which a substance exerts half of its maximal inhibitory effect, and IC_{50} for H_2S was determined by on-line and easy-to-use software (https://www.aatbio.com/tools/ic50-calculator/).

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AUTHOR CONTRIBUTIONS

F.J.C. designed the experiments, supervised the study, and wrote the manuscript. F.J.C. and J.B.B. carried out confocal scanning laser microscopy analysis. S.G-G and M.A.M-V performed enzymatic assays. F.J.C and J.M.P discussed the data and revised the manuscript. All authors read and approved the contents of this manuscript.

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SUPPORTING INFORMATION

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Figure S1. Control to evaluate the potential overlap between the excitation and emission wavelengths of cyan fluorescent protein (CFP) with the fluorescent probe WSP-5

Representative images illustrating the CLSM *in vivo* detection of peroxisomes (green) in root tips of transgenic *Arabidopsis* 10-d-old seedlings expressing CFP-PTS1 without the presence of WSP-5, fluorescent probe used to detect H_2S, and observed under two conditions. (**A**) Shows fluorescence punctuates (green) attributable to CFP-PTS1 (excitation 458 nm; emission 475 nm) indicating the localization of peroxisomes. (**B**) Shows the absence of any fluorescence in the same area observed in (**A**), respectively, without any fluorescence probe WSP-5 (negative control) using the wavelength conditions to detect H_2S with WSP-5 (excitation 502 nm and emission 525 nm).