Negative regulation of autophagy by sulfide in *Arabidopsis thaliana* is independent of reactive oxygen species

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Authors’ contribution: A.M.L-M. performed the experiments and analyzed the data; I.M. provided technical assistance to A.M.L-M.; L.C.R. conceived the project, designed experiments, analyzed the data and revised the article; and C.G. conceived the project, designed experiments, analyzed the data and wrote the article.

Summary:

Sulfide, but not other sulfur-containing molecules, represses autophagy irrespective of the redox conditions.
Footnotes:

This work was funded in part by the European Regional Development Fund through the Ministerio de Economia y Competitividad (grant MOLCIS, no. BIO2013-44648-P) and the Junta de Andalucia (grant no. CVI-7190). A.M.L.-M. thanks the Ministerio de Economia y Competitividad for fellowship support through the program of Formación de Personal Investigador.
Accumulating experimental evidence in mammalian - and recently plant - systems has led to a change in our understanding of the role played by hydrogen sulfide in life processes. In plants, hydrogen sulfide mitigates stress and regulates important plant processes such as photosynthesis, stomatal movement and autophagy, although the underlying mechanism is not well known. In this study, we provide new experimental evidence that, together with our previous findings, demonstrates the role of hydrogen sulfide in regulating autophagy. We used GFP fluorescence associated with autophagic bodies and immunoblot analysis of the ATG8 protein to show that sulfide (and no other molecules such as sulfur-containing molecules or ammonium) was able to inhibit the autophagy induced in *Arabidopsis thaliana* roots under nitrogen deprivation. Our results showed that sulfide was unable to scavenge reactive oxygen species generated by nitrogen limitation, in contrast to well-established reducers. In addition, reducers were unable to inhibit the accumulation of autophagic bodies and ATG8 protein forms to the same extent as sulfide. Therefore, we conclude that sulfide represses autophagy via a mechanism that is independent of redox conditions.
INTRODUCTION

Historically, sulfide has been considered to be a toxic molecule that is hazardous to life and the environment. However, hydrogen sulfide is currently recognized as an important signaling molecule that functions as a physiological gasotransmitter and is of comparable importance to NO and CO in mammalian systems. The number of biological roles of sulfide has rapidly expanded in recent times and is the subject of many reviews that have emphasized the physiological importance of sulfide because most mammalian cells produce and metabolize sulfide in a precise and regulated manner (Szabo, 2007; Li and Moore, 2008; Gadalla and Snyder, 2010; Kimura, 2011; Wang, 2012). Accumulating evidence from numerous studies in plant biology have also shown that hydrogen sulfide is a signaling molecule that can be as important as NO and H2O2; thus, hydrogen sulfide has also been the subject of several recent reviews (Garcia-Mata and Lamattina, 2013; Lisjak et al., 2013). Sulfide has been found to mediate increases in tolerance and protection against certain plant stresses, primarily through the increased performance of antioxidant defenses. For example, sulfide alleviates the inhibitory effects of copper and aluminum stress on wheat germination and barley seedlings (Zhang et al., 2008; Zhang et al., 2010; Dawood et al., 2012), the effect of boron on cucumber root elongation (Wang et al., 2010), and the toxicity of cadmium in Populus euphratica (Sun et al., 2013) and alfalfa seedlings (Li et al., 2012). Sulfide also improves drought and hypoxia resistance and heat and salinity tolerance (Jin et al., 2011; Li et al., 2012; Cheng et al., 2013; Christou et al., 2013). Furthermore, sulfide has been suggested to play a role in regulating photosynthesis and flower senescence and in prolonging the postharvest shelf life of fruits (Chen et al., 2011; Zhang et al., 2011; Hu et al., 2012). Interestingly, hydrogen sulfide has also been identified as a component of the abscisic acid signaling pathway in guard cells (Garcia-Mata and Lamattina, 2010; Lisjak et al., 2010). Recently, it has been demonstrated that sulfide acts upstream of nitric oxide to modulate abscisic acid-dependent stomatal closure (Scuffi et al., 2014). The cross-talk between NO and sulfide has also been suggested based on the induced alleviation of cadmium toxicity in alfalfa seedlings (Li et al., 2012), the improved heat tolerance of maize seedlings (Li et al., 2013) and the enhanced salinity tolerance of alfalfa seeds during germination (Wang et al., 2012). All of these findings clearly demonstrate the importance of sulfide as a signaling molecule that is involved in regulating numerous essential processes in plants.
The endogenous production of hydrogen sulfide in mammalian tissues occurs through the enzymatic reactions of L-cysteine (Szabo, 2007; Li and Moore, 2008; Gadalla and Snyder, 2010; Kimura, 2011; Wang, 2012). In plants, we identified an enzyme, DES1, with L-cysteine desulphydrase activity that is located in the cytosol of Arabidopsis thaliana (Alvarez et al., 2010). This is the only enzyme that has been unequivocally established to catalyze the desulfuration of L-cysteine to sulfide plus ammonia and pyruvate, and therefore, it is responsible for the release of hydrogen sulfide in the plant cytosol. Mutations in the DES1 gene impede the formation of sulfide and strongly affect plant metabolism and stress responses. Thus, the detailed characterization of des1 null mutants reveals premature leaf senescence, a dramatically altered plant transcriptional profile at the mature developmental stage and the induction of autophagy (Alvarez et al., 2012). The des1 mutants also present increased tolerance to conditions that promote oxidative stress, a high resistance to biotrophic and necrotrophic pathogens, salicylic acid accumulation and WRKY DOMAIN CONTAINING TRASSCRIPTION FACTOR54 and PATHOGENESIS RELATED1 induction; therefore, des1 mutants resemble constitutive systemic acquired resistance mutants (Alvarez et al., 2010; Alvarez et al., 2012). Moreover, in des1 mutants, stomata do not close in response to abscisic acid. This effect is restored by the application of exogenous sulfide or genetic complementation, demonstrating the involvement of DES1 in ABA signaling in guard cells. Further studies have shown that DES1 is required for ABA-dependent NO production (Scuffi et al., 2014). Thus, in plant cells, DES1 could be responsible for modulating the generation of sulfide for important signaling processes (Romero et al., 2013; Gotor et al., 2014) such as autophagy.

Autophagy is a universal mechanism with a pro-survival role in eukaryotic cells and involves the digestion of cell contents to recycle the necessary nutrients or to degrade damaged or toxic components. The most important feature of autophagy (we refer to the macroautophagy) is the de novo synthesis of double membrane-bound structures called autophagosomes, which engulf and deliver materials to the vacuole to be broken down. Proteins involved in autophagy (ATG proteins) have been used to monitor autophagic activity in plants; the most commonly used protein is ATG8, which is tethered to autophagosomes by lipidation (Thompson and Vierstra, 2005; Bassham et al., 2006; Bassham, 2007; Yoshimoto et al., 2010; Li and Vierstra, 2012; Yoshimoto, 2012). DES1 deficiency promotes the accumulation and lipidation of ATG8 isoforms in Arabidopsis leaves. Because mutation of the
DES1 gene impedes sulfide generation in the cytosol, ATG8 protein accumulation and lipidation is prevented in des1 mutants when sulfide is generated by genetic complementation or exogenous application. Interestingly, exogenous sulfide also rescues the activation of autophagy that results from dark-induced carbon starvation in wild type Arabidopsis leaf tissues (Alvarez et al., 2012).

The underlying mechanism for transforming the sulfide signal into a biological response is largely unknown. Two mechanisms of action have been proposed based on the chemical properties of H2S. The nucleophilic properties of sulfide and its capacity to react with different oxygen species and nitrogen oxides suggest that it can act as an antioxidant to reduce oxidative stress (Fukuto et al., 2012). The second mechanism consists of the post-translational modification of the –SH groups of protein cysteines to generate the persulfide group –SSH, and, in this way, alter protein activities or functions (Mustafa et al., 2009; Paul and Snyder, 2012; Aroca et al., 2015).

The aim of this work was to determine the mechanism underlying the regulation of autophagy by sulfide. For this purpose, we have investigated the role of sulfide in the induction of autophagy under nitrogen deprivation.

RESULTS

Sulfide Represses Autophagy when It Is Induced under Nitrogen Deprivation in Arabidopsis Roots

Previously, we showed that the application of exogenous sulfide rescues the induction of autophagy in Arabidopsis leaves, both in des1 mutant and in carbon-starved wild type plants, by analyzing the accumulation of ATG8 protein isoforms (Alvarez et al., 2012). These results prompted us to suggest that sulfide generated in the cytosol by DES1 behaves as a repressor of autophagy (Gotor et al., 2013; Romero et al., 2013; Gotor et al., 2014). To complement our previous studies and decipher the mechanism underlying the role of sulfide in autophagy, we established a new experimental system in which autophagy was induced by a different
condition, in a different tissue and by using a different molecular tool. Thus, wild type Arabidopsis plants expressing the GFP-ATG8a fusion protein were subjected to nitrogen limitation, and the effects of exogenous sulfide on seedling roots were analyzed by confocal microscopy of the GFP fluorescence. Seedlings were grown for 1 week on nitrogen-sufficient media, and a portion of the seedlings were transferred to fresh nitrogen-rich media. The other seedlings were transferred to media lacking nitrogen, and a third set of seedlings was placed on the same nitrogen-deficient media supplemented with 200 μM NaHS. After the transfer, the seedlings were grown for an additional 2 or 4 days, and then removed and treated with concanamycin A prior to observing them by confocal microscopy. In the roots of plants grown under nitrogen-rich conditions, we observed fluorescent punctate structures that were previously identified as GFP-ATG8-tagged autophagosomes and autophagic bodies (Yoshimoto et al., 2004; Contento et al., 2005; Thompson et al., 2005; Bassham, 2014; Merkulova et al., 2014). However, when the seedlings were subjected to nitrogen deprivation, independently of the time period, there was an increase in the number of autophagic bodies (Fig. 1), as previously demonstrated (Thompson et al., 2005; Phillips et al., 2008). Therefore, our results indicated that our nitrogen-limited conditions successfully induced autophagy. Interestingly, the presence of NaHS in the media significantly inhibited the accumulation of fluorescent vesicles observed during nitrogen deprivation (Fig. 1). Without concanamycin A treatment, we failed to detect dotted structures; in contrast, we observed diffuse fluorescent staining of only the cytoplasm, with no change in response to nitrogen limitation (Supplemental Fig. S1).

To determine whether the changes in the number of autophagic bodies observed by confocal microscopy were significant, these structures were quantified using the images of 25 to 35 different cells (Fig. 1). We quantified a large number of fluorescent vesicles per root cell in nitrogen-deprived seedlings, both of which were grown for 2 or 4 additional days, compared with those grown under nitrogen-rich conditions. The number of autophagic bodies was more than 3-fold higher in nitrogen-deficient media than in rich media after 4 additional days of growth. Moreover, the presence of exogenous sulfide produced an important decrease in the number of vesicles that was more significant during a treatment period of 4 days, reaching levels close to those observed in nitrogen-rich media. Both the increase in autophagic bodies under nitrogen-limited conditions and their reduction by exogenous sulfide were statistically significant.
It is important to note that the nitrogen-deficient media used in this study contained all of the sulfate salts at the same concentrations that were present in the MS media and were therefore sulfate-rich media. These results confirmed that sulfide represses autophagy in Arabidopsis independently of sulfur limitation.

To establish that the role of sulfide in regulating autophagy was specific to this molecule and therefore different from that of other inorganic sulfur-containing compounds, we performed a similar nitrogen limitation experiment in the presence of different sodium salts for 4 days (Fig. 2A). As expected, the sulfate and sulfite sodium salts that did not release sulfide were unable to reverse the accumulation of autophagic bodies under nitrogen deprivation to the same extent as sulfide. However, both the Na$_2$S and NaHS treatments produced a similar reduction in the number of autophagic bodies under nitrogen limitation. We concluded that only sulfide donor molecules are responsible for the inhibition of autophagic body accumulation induced by nitrogen deprivation in Arabidopsis roots. In addition, we included a treatment with the same concentration of ammonium and under the same nitrogen limitation conditions, and we did not observe a strong inhibition of fluorescent vesicle accumulation similar to that produced by sulfide (Fig. 2A). This result again confirmed that sulfide acts as a signaling molecule during the repression of autophagy. Furthermore, sulfide acted in a dose-dependent manner (Fig. 2B). The effects of sulfide were optimal at 100 to 200 $\mu$M NaHS; higher concentrations of the donor were less effective and even induced an increase in the accumulation of autophagic bodies. Thus, at 400 $\mu$M NaHS and nitrogen deprivation, we observed an increase of approximately 20% in the number of vesicles relative to roots under nitrogen limitation stress alone. This pattern may stem from sulfide toxicity; the generation of reactive sulfur and oxygen species after a certain concentration threshold that results in major oxidative damage, another condition that is known to induce autophagy (Xiong et al., 2007). We selected concentrations of 100 or 200 $\mu$M for further experiments to avoid any potential toxic effects on seedling growth that were not been observed in previous experiments.

**Sulfide Repression of Autophagy Is Also Observed at the ATG8 Protein Level**

We also studied the protein profiles of ATG8 in root protein extracts prepared from seedlings that had been subjected to nitrogen limitation in the presence or absence of sulfide,
using the same methods as presented above. We used polyclonal antibodies raised against the recombinant ATG8 protein from *Chlamydomonas reinhardtii*, a method that has been useful for the detection of ATG8 proteins in Arabidopsis (Perez-Perez et al., 2010; Alvarez et al., 2012), to detect the GFP-ATG8a fusion protein as well as endogenous ATG8 proteins, and commercial anti-GFP antibodies to detect the fusion protein and the free GFP to show the autophagic flux. Total protein extracts were electrophoresed in 10 % acrylamide gels to detect the GFP-ATG8a fusion protein, or alternatively in 15 % acrylamide gels to detect endogenous ATG8 proteins (Fig. 3). The immunoblot analysis revealed that the anti-GFP antibodies detected an intense protein band that corresponded to the fusion protein. Consistent with previous data, the ATG8 fusion protein level increased in seedling roots under nitrogen deprivation compared with those grown under nitrogen-replete conditions. The presence of sulfide in the nitrogen-deficient medium reduced the accumulation of the ATG8 fusion protein (Fig. 3A, left panel). This observation was reproducible in different experiments, considering the intensity of the bands relative to the total protein loading control. The amount of each immunodetected protein band relative to the loading control was quantified in the three replicates of the experiment and the average is shown in parentheses, with a value of 100 % assigned to the band corresponding to the +N sample. Multivariate analysis of variance (ANOVA) statistical analysis of the data was performed, demonstrating that effect of sulfide on the ATG8 fusion protein accumulation under nitrogen-deficient medium was statistically significant. Interestingly, the anti-GFP antibodies also detected the accumulation of free GFP as previously observed when the same GFP-ATG8a seedlings were subjected to nitrogen starvation conditions (Chung et al., 2010; Suttangkakul et al., 2011; Li et al., 2014). This free GFP released from the fusion protein after autophagic transport into the vacuole correlates with the autophagic flux and represents another marker for the autophagy process. We have to take into account that the GFP-ATG8a transgene is under the control of the strong cauliflower mosaic virus 35S promoter and therefore, the ratio of the fusion protein band intensity to that of the free GFP is higher than the one expected (Shin et al., 2014). We also found that the level of free GFP accumulation decreased in the presence of sulfide in the nitrogen-deficient medium (Fig. 3A, left panel), reflecting a repression of the autophagic flux by this molecule. To corroborate that the free GFP bands were specific and therefore were correlated with the autophagic fluxes, in parallel, immunoblot analysis of wild type seedling roots subjected to the same conditions was performed. None band was detected using the anti-GFP antibodies,
excluding the possibility of nonspecific bands (Fig. 3A, left panel).

Our results indicated that the GFP-ATG8a fusion protein accumulated in nitrogen-deficient conditions and that the presence of sulfide reduced its accumulation. However, we might expect that the GFP-ATG8a protein were degraded via autophagy and therefore observe a decrease in the amount of the fusion protein. The reason of this apparent contradiction is due to our experimental design in the following way. To perform the SDS-PAGE with the best loading control as possible, protein samples were diluted to the same concentration to ensure equal loading of the samples. However, a detailed observation of the protein concentration quantified in each extract showed that systematically the protein concentration of total protein extracts prepared from roots grown on nitrogen-deficient media were lower than of the extracts in nitrogen-rich conditions and of those in nitrogen-deficient conditions containing sulfide (Supplemental Table S1). Differences in protein concentrations were also observed when the protein extracts from the aerial part of the same seedlings were subjected to SDS-PAGE, mainly in the Rubisco large subunit protein band intensity (Supplemental Fig. S2). The net degradation of cellular proteins has been already observed in tobacco cells cultured in sucrose starvation and was concluded to be due to autophagy (Moriyasu and Ohsumi, 1996; Takatsuka et al., 2004). Our data again reinforced the conclusion that sulfide represses autophagy.

When the immunoblot analysis was performed to specifically detect endogenous ATG8 proteins in the root protein extracts from seedlings expressing GFP-ATG8a, nearly identical results were obtained (Fig. 3A, right panel). Two groups of ATG8 proteins were detected using the anti-Cr-ATG8 antibodies that should correspond to the unmodified ATG8 protein forms and the conjugated ATG8-PE proteins, as previously observed in Arabidopsis (Phillips et al., 2008; Chung et al., 2010; Alvarez et al., 2012). To confirm that the bands with faster mobility represented ATG8-PE adducts, we performed membrane fractionation on both the wild type and atg7-1 backgrounds. In the atg7-2 mutant, ATG8 lipidation is blocked and thus the ATG8-PE adducts are absent (Chung et al., 2010; Suttangkakul et al., 2011). By centrifugal separation of protein extracts prepared from wild type and atg7-1 seedlings that had been subjected to nitrogen limitation in the presence or absence of sulfide, we obtained membrane and soluble protein fractions that were subjected to immunoblot analysis (Fig. 3B). When the protein profiles were compared, we observed protein bands with faster mobility enriched in the membrane fraction of wild type that were absent in the atg7-1 background.
(shown by an asterisk). Moreover, consistent with these proteins representing the ATG8-PE adducts, they were absent in both the wild type and the \textit{atg7-1} soluble protein fractions. Other protein bands observed in the membrane fractions correspond to contamination of the soluble fractions. The SDS-PAGE profiles also showed the reversal of the accumulation of both types of ATG8 protein, in the soluble fraction and in the membrane fraction of wild type, in nitrogen-limited root seedlings when exogenous sulfide was present in the media. Curiously, the sulfide reduction of ATG8 protein accumulation was not observed in \textit{atg7-1} fractions. Collectively, these results provide evidence that sulfide regulates autophagy also under conditions of nitrogen deprivation. Our results confirmed this role at cellular and protein levels.

\textbf{The Mechanism of Autophagy Reversion via Sulfide is Not Dependent on its Antioxidant Activity}

Several reports have suggested that exogenous hydrogen sulfide increases the antioxidant capability of plant cells, thereby alleviating oxidative damage induced by plant stresses. This finding has led researchers to question whether sulfide functions as a signaling molecule (Hancock and Whiteman, 2014). Oxidative stress has been shown to induce autophagy (Xiong et al., 2007; Perez-Perez et al., 2012; Perez-Martin et al., 2014), especially under conditions of nutrient limitation, in which the induction of autophagy has been suggested to involve reactive oxygen species (ROS) (Liu et al., 2009). To determine whether the effect of sulfide on the induction autophagy was mediated by H$_2$O$_2$ scavenging, we performed the same nitrogen deprivation experiment in the presence of sulfide or any of the established antioxidants glutathione and ascorbate (Foyer and Noctor, 2011), except that the roots were specifically stained for hydrogen peroxide (Fig. 4). After 4 days of growth on nitrogen-deficient media, we clearly observed an increase in fluorescence emission (pseudocolored in green) in the roots of the seedlings that resulted from oxidation of the nonfluorescent 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) to the fluorescent product. This result suggested that H$_2$O$_2$ was being produced at high rates. In contrast, the fluorescence emission was nearly undetectable in roots from plants grown under nitrogen-rich conditions. Interestingly, the presence of 200 $\mu$M NaHS in the nitrogen-deficient growth media did not have any effect on the intensity of the fluorescence emission. A similar signal was observed compared with roots grown under conditions of nitrogen starvation. Furthermore, when the
nitrogen-deficient media were supplemented with either 200 μM of reduced glutathione or ascorbate, a decrease in the fluorescent signal was observed, reaching the same signal intensity as observed under nitrogen-sufficient conditions, as expected for well-known antioxidants. These findings showed that hydrogen sulfide does not behave as a H₂O₂ scavenger in our conditions. We cannot exclude that other ROS different to H₂O₂ might mediate the repressive effect of sulfide. Such is the case of the superoxide radical anion, whose function in root development is well established (Foreman et al., 2003). Consequently, superoxide production was also visualized by nitroblue tetrazolium staining of the same root tissues and basically the same results were obtained (Fig. 5). The dark-blue stain due to the superoxide was observed most intensively and extensively distributed in roots after 4 days of growth on nitrogen-deficient media alone and in the presence of sulfide by comparison with the other studied conditions, mainly in the root differentiation zone used for monitoring GFP-ATG8.

We further analyzed the effect of both GSH and ascorbate on the increased accumulation of autophagic bodies in response to nitrogen limitation in plants expressing the GFP-ATG8a fusion protein (Fig. 6). Only the presence of NaHS in the media significantly inhibited the accumulation of fluorescent vesicles, while GSH and ascorbate were unable to produce any effect (Fig. 6A). Representative single optical sections of the images visualized by confocal microscopy are shown in the Supplemental Material (Supplemental Fig. S3). Similar results were also obtained when the immunoblot analysis was performed. The presence of sulfide and no GSH and ascorbate significantly decreased the accumulation of the free GFP level, indicative of a repression of the autophagic flux by sulfide (Fig. 6B). These data clearly suggested that the effect of sulfide on the progression of autophagy is independent of ROS. In a previous work, however, a reduction of punctate structures was observed in the presence of antioxidants (Liu et al., 2009). Differences in methodological procedures, antioxidant concentrations and timing of the treatments make both investigations are not comparable. In the present work the antioxidant treatment is performed in solid medium at 200 μM for 4 days in nitrogen-deficient conditions, which are exactly the same conditions used to check the effect of sulfide as a possible antioxidant. On the contrary, in the previous work (Liu et al., 2009) the treatment is performed in liquid nitrogen-rich medium with the antioxidant at 20 mM in one case (imidazole) and 2 mM in other case (ascorbate) for 4 h.

Correlation between Autophagy and DES1
Previously, we demonstrated that DES1 deficiency leads to the accumulation and lipidation of ATG8 proteins. Through genetic complementation or exogenous application of sulfide, we were able to rescue autophagy induction in des1 null mutants (Alvarez et al., 2012). Consequently, we suggested that DES1 should modulate the generation of sulfide in the plant cytosol for autophagy signaling (Gotor et al., 2013; Romero et al., 2013). In addition, we wanted to determine whether stressful conditions that induce autophagy, such as nutrient limitation, had any effect on DES1. To test this hypothesis, we performed the same experiment as described above and compared seedlings grown under nitrogen-rich and nitrogen-deficient conditions in terms of DES1 gene expression and DES enzyme activity (Fig. 7). A strong reduction (73 %) in DES1 gene expression was observed in the roots of seedlings grown in nitrogen-deficient media compared with those grown in nitrogen-replete media. The total DES activity was also slightly decreased when the seedlings were transferred to nitrogen-deprived conditions, but this reduction was not statistically significant. Other enzymes with H2S-releasing activity, which is the method used to measure DES activity, are present in plants; therefore, the contribution of cytosolic DES1 protein to total DES activity is expected to be low.

The Effect of Sulfide Is Observable at the Phenotypic Level

To establish that particular concentrations of sulfide molecule donors did not have a toxic effect on seedling growth, we studied the phenotypic characteristics of seedlings expressing GFP-ATG8a and grown in the different conditions described above. When the seedlings were transferred from N-rich to N-deficient media, a decrease in shoot and root biomass was evident at the beginning of the transfer period. After 4 days, a change in leaf color was appreciable in the nitrogen-deficient media and reflected the repression of chlorophyll synthesis and the induction of anthocyanin synthesis, which are characteristic plant responses to nitrogen deprivation (Scheible et al., 2004; Peng et al., 2007). In contrast, the presence of NaHS apparently reduced the extent of browning, which was primarily observable in the abaxial portion of the leaves, resulting in a healthier phenotype (Fig. 8A). The level of anthocyanin was approximately 6-fold higher in the nitrogen-starved seedlings than in the nitrogen-sufficient seedlings. The presence of NaHS resulted in identical (or even slightly
lower) anthocyanin content compared to that in the nitrogen-sufficient seedlings (Fig. 8B).

Furthermore, the presence of sulfide in the nitrogen-limited medium was effective in alleviating the stress induced by the absence of a nitrogen source during the longer treatment periods (6 and 8 days). During these long periods of nitrogen deprivation, the application of lower concentrations of sulfide and the use of NaHS as a sulfide donor were more effective for reducing stress (Supplemental Fig. S4, S5). Therefore, we can conclude that hydrogen sulfide can play important roles in plants, with phenotypically observable outcomes.
DISCUSSION

The conceptualization of the role of hydrogen sulfide in both animal and plant systems has changed dramatically in recent years. Currently, hydrogen sulfide is viewed not as a toxic molecule to life, but rather as a regulator of essential life processes. In mammals, hydrogen sulfide has been considered to be a gasotransmitter with major physiological functions in different body systems (Gadalla and Snyder, 2010). Alterations of H$_2$S metabolism have important pathological consequences, establishing the clinical relevance of this molecule (Lowicka and Beltowski, 2007; Szabo, 2007; Wang, 2012). In addition to the protective effects of hydrogen sulfide against a wide array of different stresses that have been demonstrated in plants (Zhang et al., 2008; Wang et al., 2010; Zhang et al., 2010; Jin et al., 2011; Dawood et al., 2012; Li et al., 2012; Li et al., 2012; Cheng et al., 2013; Christou et al., 2013; Sun et al., 2013), this molecule has also been shown to regulate plant processes that are critical for plant performance, such as photosynthesis (Chen et al., 2011), stomatal movement (Garcia-Mata and Lamattina, 2010; Lisjak et al., 2010; Scuffi et al., 2014) and autophagy (Alvarez et al., 2012; Gotor et al., 2013). In the present study, we provide new experimental evidence that, together with our previous findings, demonstrates the role of hydrogen sulfide in regulating the progression of autophagy independently of the condition in which the autophagy originates. Most importantly, our findings contribute to our understanding of the mechanism underlying the regulation of autophagy by sulfide in plant systems, by confirming that its role is not dependent on the redox state.

As in other organisms, autophagy is constitutively active in plant cells under favorable growth conditions (Slavikova et al., 2005). However, when plants are exposed to adverse environmental conditions, one of their responses to cope with stress and survive is to increase the induction of autophagy. These stresses include nitrogen and carbon deprivation. Several studies have reported that autophagy can be induced by carbon and nitrogen deprivation, which is detected in many cases through a substantial increase in ATG8 protein levels (Doelling et al., 2002; Hanaoka et al., 2002; Thompson et al., 2005; Xiong et al., 2005; Chung et al., 2009; Guiboileau et al., 2013). In the present study, we observed basal levels of autophagy under nitrogen-sufficient conditions. However, under stressful growth conditions, increased levels of autophagy were observed under nitrogen deprivation. Interestingly, the presence of sulfide during stress prevented an increase in the activation of autophagy,
revealing the same level of basal autophagy as observed in the favorable growth conditions.

The observed effect of sulfide was unrelated to its availability as a nutrient, as the growth media contained sulfate salts as a source of sulfur nutrients. Furthermore, the presence of ammonium at the same concentration did not alter the activation of autophagy.

This study was performed in root cells from young seedlings overexpressing GFP-ATG8a under nitrogen deprivation using GFP fluorescence detection to monitor autophagy. Identical effects of sulfide on autophagy have been previously observed in different tissue (leaves), at different plant developmental stages (mature plants), under different stress conditions (carbon starvation) and in different genetic backgrounds (des1 mutant) (Alvarez et al., 2012). Therefore, the evidence indicates that sulfide regulates the progression of autophagy by acting as a repressor in plant systems. In mammals, the effect of sulfide on autophagy is unclear because recent findings have indicated that sulfide can both suppress and induce autophagy (Wang et al., 2012; Kundu et al., 2014). Recently, the sulfur amino acids cysteine and methionine have been shown to regulate the translational capacity and metabolic homeostasis in eukaryotic cells and to inhibit autophagy and promote cell growth (Laxman et al., 2013; Sutter et al., 2013). In the latter report, the importance was placed on methionine as a precursor of the methyl donor S-adenosylmethionine, which has been reported to inhibit autophagy through the action of methyltransferase enzymes, although cysteine has also been shown to suppress autophagy.

Deciphering the mechanism underlying the transformation of the sulfide signal into a biological response and identifying the molecular targets of sulfide remain challenging. It has been previously shown that the hydrogen sulfide anion (HS⁻) can generate cysteine and glutathione persulfides with antioxidant properties that are superior to GSH (Francoleon et al., 2011) (Ida et al., 2014). The persulfides have been shown to act as reactive reductants that are capable of reacting rapidly with the electrophilic H₂O₂; therefore, persulfides exhibit H₂O₂-scavenging activity. Moreover, several studies in photosynthetic organisms have demonstrated the activation of autophagy in response to several conditions that increase ROS generation, suggesting the presence of a strong link between autophagy and ROS (Perez-Perez et al., 2012). Thus, we sought to determine whether the mechanism underlying the repression of autophagy by sulfide involves some ROS, such as H₂O₂ and superoxide radical, this latter with well-established functions in roots. Our results showed that sulfide was unable to scavenge the ROS generated by nitrogen limitation, as observed for reduced glutathione and ascorbate.
Moreover, upon nitrogen limitation, we clearly differentiated the inhibitory effect of sulfide both on the accumulation of autophagic bodies visualized by GFP fluorescence and on the accumulation of ATG8 protein forms detected by immunoblotting, from the effect produced by reduced glutathione and ascorbate, which were unable to reverse to any extent the progression of autophagy. Therefore, our results suggest that the effect of sulfide on the progress of autophagy is independent of its capacity to react with H$_2$O$_2$ or with superoxide radical anion.

The second mechanism proposed in animal systems to explain the physiological effects of H$_2$S is the post-translational modification of proteins known as S-sulfhydration (Paul and Snyder, 2012). Previous studies have indicated that this modification can be regulated by competition between the nitrosylation and sulfhydration of the same cysteine residues. S-sulfhydration changes an –SH to an –SSH, modifying the chemical reactivity of enzymes and possibly their access to their respective targets, as observed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Gadalla and Snyder, 2010; Kabil and Banerjee, 2010; Paul and Snyder, 2012). Very recently, S-sulfhydration of proteins has been confirmed to occur in Arabidopsis leaf tissues under physiological conditions, and the presence of an S-sulfhydrated cysteine residue in a plant protein has recently been demonstrated for the first time (Aroca et al., 2015). Therefore, two questions arise: whether S-sulfhydration regulates autophagy and what are the specific targets of sulfide. The thiol redox state can profoundly influence autophagy, especially during the initiation and completion of autophagosomes, due to the ability of several proteins that are involved in autophagy to sense alterations in the cellular redox state by means of reactive cysteine residues (Filomeni et al., 2010). Examples of this are the ubiquitin-like systems ATG7-ATG10 and ATG7-ATG3, the function of which relies on the cysteine-based transfer of ATG12 and ATG8, respectively, which are essential proteins for autophagosome membrane elongation (Filomeni et al., 2010). Moreover, redox regulation of cysteine protease ATG4 has been characterized in detail in mammalian systems, and a cysteine residue located near the catalytic site has been found to be critical for this regulation (Scherz-Shouval et al., 2007). The ATG4 protein cleaves ATG8 near the C terminus, downstream of a conserved Gly, allowing the conjugation to PE to proceed through the exposed Gly. ATG4 also plays a role in the de-conjugation of ATG8-PE to release ATG8 from the autophagosome membrane. Because of its role as both a conjugating and de-conjugating enzyme, the activity of ATG4 is expected to be tightly regulated. A detailed study of the
ATG8-processing activity by two ATG4 isoforms from Arabidopsis has recently been reported. This study demonstrated a dose-dependent inhibition of Arabidopsis ATG4 cysteine protease activity under oxidative stress that was restored by DTT, indicating that this plant protein is also under redox regulation similarly to the mammalian protein (Woo et al., 2014). In addition, a study of yeast ATG4 demonstrated that this protein is regulated by the oxidoreduction of a single disulfide bond and that involves thioredoxin (Perez-Perez et al., 2014). Thus, both ubiquitin-like system proteins and the ATG4 protein could be specific targets of sulfide, and further studies are currently being developed.

In the present work, we provide more data that, together with previous studies, highlight the role of DES1 protein in autophagy. Not only does the deficiency in DES1 promote the induction of autophagy (Alvarez et al., 2012), but a conditions that induces autophagy also promotes the repression of DES1. Further research will be required to determine whether this reverse correlation can be generalized to other situations in which autophagy is activated and to ascertain the underlying regulatory mechanisms. In addition, this study, as well as previous ones, demonstrates that hydrogen sulfide plays an important signaling role in plants. This effect is observable phenotypically and is measurable by anthocyanin content because the presence of sulfide alleviated nitrogen limitation stress. When hydrogen sulfide is present below a toxic threshold, it plays a general role in improving plant performance. This finding is corroborated by a recent report that found that exposing plants to extremely low concentrations of sulfide results in significant increases in biomass (Dooley et al., 2013).
MATERIALS AND METHODS

Plant Material, Growth Conditions and Treatments

The *Arabidopsis thaliana* wild type plants expressing GFP-ATG8a used in this work have been described previously (Thompson et al., 2005), and the *atg7-1* mutant was obtained from NASC. *A. thaliana* seeds were sown on MS solid medium containing 0.8 % agar (w/v) and synchronized at 4 °C for 2 days. The plates were then incubated vertically in a growth chamber under a regime of 16 h light at 22 °C / 8 h dark at 20 °C. For exposure to N-starvation conditions, N-deficient MS media were prepared by replacing the nitrate salts with chlorate salts, without altering the sulfate salts. One-week-old seedlings were transferred to N-deficient MS solid media for an additional 2 or 4 days of growth. Alternatively, the 1-week-old seedlings were transferred to the same N-deficient MS media containing 200 or 100 μM of either Na₂S or NaHS as sources of exogenous sulfide. For the concanamycin A treatment, the seedlings were incubated in liquid N-deficient MS medium containing 0.5 μM concanamycin A (Santa Cruz Biotechnology) for 16 h at room temperature under agitation at 80 rpm. After the treatment, the roots were washed in water and the differentiation zone was observed under a confocal microscope. Concanamycin A was prepared as a 100 μM stock solution in dimethyl sulfoxide. To ensure that dimethyl sulfoxide did not produce any effect on the results of the experiments, the seedlings were also incubated in liquid N-deficient MS medium in the presence of dimethyl sulfoxide alone as a control.

Visualization and Quantification of GFP-Tagged Autophagic Bodies

Root cells were viewed using a TCS SP2 spectral confocal microscope (Leica Microsystems). GFP was excited using the 488 nm line of an argon ion laser, and emission was detected between 510 and 580 nm. Single optical images were processed with PDQuest software (Bio-Rad) as follows. First, a black and white image was automatically generated from the original image and a minimal intensity and size of the spot were defined. Second, a new image was generated with the selected spots encircled in red, a manual correction was performed to adjust the selected spots to the cells to be counted, and a file with automatic counting was obtained (Supplemental Fig. S6). The number of fluorescent vesicles within the
central vacuoles was counted in 25 to 35 different cells. The data represent the average number (±SD) from three independent experiments. Images were also processed using ImageJ software. In the latter, the mean fluorescence within the vacuoles was counted within a 400-μm² section from the same cells. The results, when expressed as a percentage relative to the value obtained in the N-rich medium, were the same using both software programs.

**Immunoblot Analysis**

Plant root material (20-100 mg) was ground in liquid nitrogen with 100-400 μL of extraction buffer (100 mM Tris-HCl, pH 7.5, 400 mM sucrose, 1 mM EDTA, 10 mg mL⁻¹ sodium deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg mL⁻¹ pepstatin A and 4 % [v/v] protease inhibitor cocktail [Roche]) using a pestle and mortar and centrifuged at 500 g for 10 min to obtain the supernatant fraction as described previously (Yoshimoto et al., 2004; Alvarez et al., 2012). The total amount of protein in the resulting supernatant was determined using a previously described method (Bradford, 1976). For subcellular fractionation, the supernatant was centrifuged at 13,000 g for 15 min to generate the membrane fraction and the supernatant (Yoshimoto et al., 2004). For the immunoblot analyses, 3 μg of root protein extracts was electrophoresed in 10 % acrylamide gels or, alternatively, 20 μg of root protein extracts were electrophoresed in 15 % acrylamide gels before transfer to polyvinylidene fluoride membranes (Bio-Rad) according to the manufacturer’s instructions. Anti-Cr-ATG8 (Perez-Perez et al., 2010; Alvarez et al., 2012), anti-GFP (eBioscience) and secondary antibodies were diluted 1:2,000, 1:1,000 and 1:50,000, respectively, in PBS containing 0.1 % Tween 20 (Sigma-Aldrich) and 5 % milk powder. The ECL Select Western Blotting Detection Reaction (GE Healthcare) was used to detect the proteins with horseradish peroxidase–conjugated anti-rabbit secondary antibodies. For a protein loading control, an identical gel was run in parallel and stained with Coomassie Brilliant Blue (Sigma) or, alternatively, the membrane before immunodetection was stained with SYPRO Ruby (Life Technologies) to detect all protein bands. The immunodetected protein bands were quantified relative to the Coomassie-stained gel or SYPRO-stained membrane using the Quantity One software (Bio-Rad).

**Detection of ROS**
For the fluorimetric detection of H$_2$O$_2$, roots were incubated for 5 min with 10 mM H$_2$DCFDA (2',7'-dichlorodihydrofluorescein diacetate, Life Technologies) in the presence of 10 mM PI (propidium iodide, Life Technologies) to visualize the cell walls. The samples were observed using a TCS SP2 spectral confocal microscope (Leica Microsystems) with the following settings: excitation, 488 nm; emission, 500 to 550 nm for fluorescein detection and 600 to 650 nm for propidium detection. For the detection of the superoxide radical anion, roots were stained with NBT (nitroblue tetrazolium chloride, Sigma-Aldrich) as previously described (Garcia et al., 2010). The seedlings were incubated in 0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl$_2$ and 0.5 mg mL$^{-1}$ NBT (pH 9.5) for 2 h at room temperature in the dark. After rinsing the roots were observed with an Olympus SZ-PT stereoscope equipped with a DFC300FX Leica camera.

Real-Time RT-PCR

Quantitative real-time RT-PCR was used to analyze the expression of the *DES1* gene as described previously (Laureano-Marin et al., 2014). The primer sequences used were as follows: qDES1-F, 5'-TCGAGTCAGTCAGATATGAAGCT-3' and qDES1-R, 5'-TGTAACCTTGTTACCAACA TCTCT-3' for the *DES1* gene; qUBQ-F, 5'-GGCCTTGTATAATCCCTGATGAATAAG-3' and qUBQ-R, 5'-AAAGAGATAACAGGAACGGAAACATAGT-3' for the constitutive *UBQ10* gene. The *DES1* expression levels were normalized to that of the constitutive *UBQ10* gene by subtracting the cycle threshold (CT) value of *UBQ10* from the CT value of the *DES1* gene (ΔCT). The results shown are the means ± SD of at least three independent RNA samples.

Determination of DES Activity

Plant root material was ground in 20 mM Tris-HCl (pH 8) using a mortar and pestle with liquid nitrogen. After centrifugation at 15,000 g for 15 min at 4 ℃, the resulting supernatant was used as a plant soluble extract for DES activity. The total amount of protein in the extracts was determined by the Bradford method using the Bio-Rad protein assay. DES activity was measured by the release of sulfide from L-Cys as described previously (Alvarez et al., 2010).
**Determination of Anthocyanin Content**

Approximately 40-70 mg of aerial tissues from seedlings was homogenized in 1 mL of propanol:HCl:water (18:1:81) and further extracted in a boiling water bath for 3 min. The mixture was centrifuged at 5,000 g for 40 min. The absorbance of the supernatant was measured at 535 and 650 nm, and the total anthocyanin content per gram fresh weight was determined using previously described methods (Lange et al., 1971).

**Statistical Analysis**

Multivariate analysis of variance (ANOVA) statistical analysis of the data was performed using the program Statgraphics Centurion.

**ACKNOWLEDGMENTS**

We thank Dr. Irene García for critical reading of the manuscript, Dr. Alicia Orea for confocal microscopy service, Dr. José Luis Crespo for providing the anti-ATG8 antibodies, and Dr. Richard Vierstra for providing seeds of wild type *A. thaliana* expressing GFP-ATG8a.
FIGURE LEGENDS

**Figure 1.** Effect of exogenous sulfide on autophagy induced by nitrogen deprivation in Arabidopsis roots. Wild type seedlings expressing GFP-ATG8a fusion were grown for 7 days on N-rich medium and then transferred to the same medium (+N), to a N-deficient medium (-N), or to a N-deficient medium containing 200 μM NaHS (-N + NaHS) for an additional 2 days (A) or 4 days (B). The root cells were visualized by confocal fluorescence microscopy and exposed to concanamycin A prior to observation as described in the Materials and Methods. Representative single optical section of fluorescence, visible and overlaid images are shown. The number of GFP-ATG8 puncta per cell section was determined using PDQuest software as described in the Materials and Methods. Values are the average number (±SD) of fluorescent vesicles within the central vacuoles per cell of 25-35 cells in each of the three independent experiments. Different letters indicate significant differences (P < 0.05).

**Figure 2.** Effect of different chemicals (A) and concentrations (B) on the autophagy induced by nitrogen starvation in Arabidopsis roots. Wild type seedlings expressing GFP-ATG8a fusion were grown for 7 days on N-rich medium and then transferred for an additional 4 days to the same medium (+N), to a N-deficient medium (-N), or to a N-deficient medium containing 200 μM of different salts as indicated in panel (A); or alternatively to the N-deficient medium containing different concentrations of NaHS as indicated in panel (B). Root cells were visualized by confocal fluorescence microscopy and, prior to observation, were exposed to concanamycin A as described in the Materials and Methods. The number of autophagic bodies per cell section was determined using the PDQuest software as described in the Materials and Methods. Values are the average number (±SD) of fluorescent vesicles within the central vacuoles per cell of 25-35 cells in each of the three independent experiments. Different letters indicate significant differences (P < 0.05).

**Figure 3.** Immunoblot analysis of GFP-ATG8a fusion and endogenous ATG8 protein accumulation in Arabidopsis roots. (A) Wild type and wild type seedlings expressing the GFP-ATG8a fusion were grown for 7 days on N-rich medium and then transferred to the same medium (+N), to a N-deficient medium (-N), or to a N-deficient medium containing 200 μM NaHS (-N + S) for an additional 4 days. Total protein extracts were prepared from roots as
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**Figure 4.** Detection of hydrogen peroxide in Arabidopsis roots. Wild type seedlings were grown for 7 days on N-rich medium and then transferred to the same medium (+N), to a N-deficient medium (-N), or to a N-deficient medium containing either 200 μM NaHS (-N + NaHS), or GSH (-N + GSH), or ascorbate (-N + ASC) for an additional 4 days. The roots were loaded with H2DCFDA for 5 min to detect H2O2 (pseudocolored in green) in the presence of PI to visualize cell walls (pseudocolored in red). Representative images of two different zones of the root for each sample are shown.

**Figure 5.** Detection of superoxide in Arabidopsis roots. Wild type seedlings were grown for 7 days on N-rich medium and then transferred to the same medium (+N), to a N-deficient
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Figure 6. Effect of exogenous sulfide, reduced glutathione and ascorbate on the autophagy induced by nitrogen deprivation in Arabidopsis roots. Wild type seedlings expressing GFP-ATG8a fusion were grown for 7 days on N-rich medium and then transferred to the same medium (+N), to a N-deficient medium (-N), or to a N-deficient medium containing either 200 μM NaHS (-N + NaHS), GSH (-N + GSH), or ascorbate (-N + ASC) for an additional 4 days. (A) Root cells were visualized by confocal fluorescence microscopy and, prior to observation, were exposed to concanamycin A as described in the Materials and Methods. The mean fluorescence inside the vacuoles was determined using ImageJ software. Values are expressed as relative units (±SD) of mean fluorescence within the central vacuoles of the three independent experiments. Representative single optical section of fluorescence, visible and overlaid images are shown in Supplemental Figure S3. (B) Immunoblot analysis of GFP-ATG8a fusion protein. Total protein extracts were prepared from roots as described in the Materials and Methods, and 8 μg of each extract was resolved by 10 % SDS-PAGE and subjected to immunoblot analysis with anti-GFP antibodies. As the protein loading control, a gel was run in parallel and stained with Coomassie Brilliant Blue. The experiment was repeated three times, and representative images are shown. The amount of the free GFP protein band relative to the loading control was quantified, and the average is shown in parentheses, with a value of 100 % assigned to the band corresponding to the -N sample. Different letters indicate significant differences (P < 0.05).

Figure 7. Effect of nitrogen starvation on DES1 gene expression and DES activity in Arabidopsis roots. Wild type seedlings were grown for 7 days on N-rich medium and then transferred to the same medium (+N) or to a N-deficient medium (-N) for an additional 4 days. Root samples were collected and used for quantitative real-time RT-PCR to analyze the expression of the DES1 gene and to measure DES activity as described in the Materials and Methods. Values are the means ±SD of three independent experiments. **P<0.01.
Figure 8. Phenotypes of wild type seedlings expressing GFP-ATG8a under different conditions. Wild type seedlings expressing the GFP-ATG8a fusion were grown for 7 days on N-rich medium and then transferred to the same medium (+N), to a N-deficient medium (-N), or to a N-deficient medium containing 200 μM NaHS for an additional 4 days. (A) Representative bright field images of whole seedlings are shown. The lower panels show images of the underside of the leaves (abaxial part of the leaves). Bars = 1 cm. (B) The anthocyanin content per gram fresh weight was measured as described in Materials and Methods. Values are the means ±SD of three independent experiments. Different letters indicate significant differences (P < 0.01).
**Supplemental Data**

**Supplemental Figure S1.** Autophagy induced by nitrogen deprivation in Arabidopsis roots. Wild type seedlings expressing the GFP-ATG8a fusion were grown for 7 days on N-rich medium and then transferred to the same medium (+N) or to a N-deficient medium (-N) for 4 additional days. The root cells were visualized by confocal fluorescence microscopy. Representative single optical section images of fluorescence, visible and overlaid images are shown.

**Supplemental Figure S2.** Representative SDS-PAGE of protein extracts. Wild type seedlings expressing the GFP-ATG8a fusion were grown for 7 days on N-rich medium and then transferred to the same medium (+N), to a N-deficient medium (-N), or to a N-deficient medium containing 200 μM NaHS for 4 additional days. Root (20 mg) and leaf (100 mg) plant materials were ground separately in liquid nitrogen with 100 and 300 μL of extraction buffer, respectively, as described in Materials and Methods, and 10 μL of the final supernatant fractions were electrophoresed in 10 % acrylamide gels and stained with Coomassie Brilliant Blue.

**Supplemental Figure S3.** Representative single optical section of fluorescence, visible and overlaid images visualized by confocal microscopy of the root cells corresponding to the experiment described in Figure 5.

**Supplemental Figure S4.** Phenotypes of wild type seedlings expressing GFP-ATG8a under different conditions. Representative bright field images of 7-day-old wild-type seedlings grown on N-rich medium and then transferred to a N-deficient medium (-N) or to a N-deficient medium containing NaHS or Na2S at 100 or 200 μM for 6 additional days. The lower panels show images of the abaxial part of the leaves. Bars = 1 cm.

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FIGURE 1

A

B

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FIGURE 3

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- GFP

α-GFP

Sypro

B

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- ATG8

α-ATG8

Soluble Fraction

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- ATG8

α-ATG8

Coomassie
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FIGURE 4

+N

-N

-N + NaHS

-N + GSH

-N + ASC

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<td>1.19 (100)</td>
<td>0.96 (80)</td>
<td>1.28 (107)</td>
</tr>
<tr>
<td>3</td>
<td>2.26 (100)</td>
<td>1.68 (74)</td>
<td>2.02 (89)</td>
</tr>
<tr>
<td>4</td>
<td>2.11 (100)</td>
<td>1.08 (51)</td>
<td>1.27 (60)</td>
</tr>
<tr>
<td>5</td>
<td>1.39 (100)</td>
<td>0.59 (42)</td>
<td>0.95 (68)</td>
</tr>
<tr>
<td>6</td>
<td>2.23 (100)</td>
<td>1.44 (64)</td>
<td>1.69 (76)</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>1.92 ± 0.49 (100)</strong></td>
<td><strong>1.22 ± 0.42 (64)</strong></td>
<td><strong>1.63 ± 0.59 (85)</strong></td>
</tr>
</tbody>
</table>