Unearthing The Alleviatory Mechanisms of Hydrogen Sulfide in Aluminum Toxicity in Rice

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Abstract

Hydrogen sulfide (H_2S) improves aluminum (Al) resistance in rice; however, the underlying molecular mechanism remains unclear. In the present study, treatment with 30-μM Al significantly inhibited rice root growth and increased the total Al content and apoplastic and cytoplasm Al concentration in the rice roots. However, pretreatment with NaHS (H_2S donor) reversed these negative effects. Transcriptomics and physiological experiments confirmed that H_2S increased the ATP, sucrose, glutathione, and ascorbic acid contents, which was accompanied by decreased $O_2^{\cdot-}$ and H_2O_2 contents, to alleviate Al toxicity. H_2S significantly inhibited ethylene emissions in the rice and then inhibited pectin synthesis and increased the pectin methylation degree to reduce cell wall Al deposition. The phytohormones indole-3-acetic and brassinolide were also involved in the alleviation of Al toxicity by H_2S. In addition, other pathways of material and energy metabolism, secondary metabolism, cell wall components, signal transduction, and transcriptional and translational pathways in the rice roots were also regulated by H_2S under Al toxicity conditions. These findings improve our understanding of how H_2S affects rice responses to Al toxicity, which will facilitate further studies on crop safety.

Introduction

Hydrogen sulfide (H_2S) is an important endogenous gasotransmitter that maintains a dynamic equilibrium with L-cysteine desulfhydrase, D-cysteine desulfhydrase, sulphite reductase, cyanoalanine synthase, cysteine synthase, and O-acetyl-l-serine(thiol)lyase in plants (Banerjee et al., 2018; Li, 2013; Sirko et al., 2004; Tai and Cook, 2000). Although a high H_2S concentration is harmful to plant growth, the appropriate H_2S concentrations acts as signaling molecule to regulate plant development and the response to environmental stress (Yamasaki and Cohen, 2016). For example, H_2S induces protein persulfidation to protect plants from oxidative damage (Filipovic, 2015), maintains a higher K^+/Na^+ ratio to ameliorate salt stress (Wang et al., 2012), reduces peroxidation damage to promote wheat seed germination under drought stress (Zhang et al., 2010c), and reduces electrolyte leakage in tobacco (*Nicotiana tabacum* L.) suspension-cultured cells to alleviate heat stress (Li et al., 2012b).

The amelioratory roles of H_2S on heavy metal stress in plants have been demonstrated in many studies (Liu et al., 2015; Rizwan et al., 2019; Singh et al., 2015; Zhang et al., 2010a; Zhang et al., 2008). The application of NaHS significantly enhanced the AsA-GSH cycle to alleviate As toxicity in pea seedlings (Singh et al., 2015), promoted the protoplast sequestering of Cd in *Arabidopsis* (Guan et al., 2018), boosted photosynthesis to alleviate Ni toxicity in rice, and reduced peroxidation damage to alleviate Cr, Cu, and Zn toxicity (Rizwan et al., 2019). About 30%–50% of arable land in the world is acidic and has limited crop production capacity due to Al toxicity (He et al., 2012). H_2S is involved in alleviating Al toxicity in plants. For example, treatment with NaHS significantly increased the germination of wheat seedlings (Zhang et al., 2010b), enhanced citrate secretion and inhibited reactive oxygen species (ROS) bursts in barely seedlings, enhanced the translocation of Al from the cytoplasm to the vacuole and reduced cell wall Al deposition in rice (Chen et al., 2013a; Dawood et al., 2012; Zhu et al., 2018). However, research into the mechanism by which H_2S alleviates Al toxicity has mainly focused on the physiological level, while the molecular mechanisms remain unclear.

Recently, transcriptomics has been used to explore Al response genes in plants; for instance, the possible transporter genes involved in Al resistance in buckwheat leaves were found following treatment with short-term moderate Al stress (Yokosho et al., 2014). In the present study, seedlings of the rice cultivar “Kasalath” were pretreated with or without the H_2S donor NaHS under Al toxicity conditions for one day, and the roots were collected for genome-wide transcriptome and physiological analysis.

Materials And Methods

The seeds of the *indica* rice "Kasalath" were soaked in distilled water for 24 h and then transferred to 0.5-mM $CaCl_2$ solution (pH 5.6) at 30°C under total darkness. The rice seedlings were treated with or without 2 μM of the H_2S donor NaHS in 0.5-mM $CaCl_2$ solution (pH 5.6) until the rice roots reached about 1 cm. After 8 h of treatment, the solution was discarded, and the roots were treated with or without 30-μM Al ($AlCl_3 \cdot 6H_2O$) in 0.5-mM $CaCl_2$ solution (pH 4.5). The treatments were set as CK (without NaHS and Al treatment), H_2S (only treated with NaHS), Al (only treated with Al), and Al+H_2S (treated with NaHS and Al). After 24 h of treatment, the fresh roots were collected and frozen in liquid nitrogen immediately. The root lengths were measured before and after Al treatment.

Measurement of Al content in rice

The fresh roots were collected and dried in the oven to the point where the weight no longer changed. The dry roots (0.1 g) were digested in 2 mL of HNO_3:$HClO_4$ (*v*:*v*, 4:1) at 120°C and then diluted to 50 mL with ultrapure water to measure the total Al content (Shen et al., 2002). The rice root tips (1 cm) were then placed into 1.5-mL ultra-free MC tubes (Millipore, Billerica, MA, USA) and stored at −80°C for 1 d, following which they were heated at 25°C to break the cells. The residues after centrifugation were used to extract an apoplastic solution with 1 mL of 2-M HCl. The Al concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS).

The cytoplasmic Al content was indicated by the intensity of the green fluorescence after staining with morin. One centimeter of the rice root tip was collected and stained in 0.01% morin for 30 min at room temperature, and the extra dye was washed off using deionized water. Images were captured using a fluorescence microscope (LEICA DM2500) (Li et al., 2016).

Transcriptome library construction, sequencing, and bioinformatics analysis

The total RNA was extracted by TRIzol reagent (Invitrogen, Germany) according to the manufacturer's instructions. The RNA, which met the experimental requirements, was enriched by Oligo(dT) beads and reverse transcribed to cDNA immediately after fragmenting into short fragments. The synthesis of second-strand cDNA, purification of cDNA fragments, amplification of cDNA, and sequencing by Illumina HiSeq^{TM} 2500 were completed by Gene Denovo Biotechnology Co. (Guangzhou, China) according to Yang et al. (2021). Reads were mapped to the ribosome RNA (rRNA) database by Bowtie2 (Langmead and Salzberg, 2012). TopHat2 was used to map the rRNA removed reads (version 2.0.3.12) (Kim et al., 2013a). Unmapped reads were then re-aligned with Bowtie2 and split into smaller segments (Trapnell et al., 2010). The reconstruction of transcripts was carried out with the Cufflinks software (Trapnell et al., 2012), which together with TopHat2 allowed for the identification of new genes and new splice variants of known genes.

The RSEM software was used to quantify gene abundances (Li and Dewey, 2011), and the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) method was used to normalize the gene expression levels. To identify differentially expressed genes (DEGs) across samples or groups, the edgeR package (http://www.rproject.org/) was used. We identified genes with a fold change $\log_2(fc) \geq 1$ and $P < 0.05$ in a comparison as a significant DEG. The Gene Ontology (GO) annotation (https://www.ebi.ac.uk/GOA/) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (https://www.kegg.jp/kegg/pathway.html) were used to analyze gene functions and enrichment.

Measurement of sucrose and ATP content

Fresh rice roots (0.1 g) were ground with 1 mL of 0.83-M cold perchloric acid (Sigma Aldrich, St. Louis, USA) on ice, and the supernatant was adjusted to a neutral pH with 3-M KOH. The BacTiter-Glo Microbial Cell Viability Assay (Promega, Madison, USA) was used to determine the ATP concentration in a white opaque-walled 96-well microplate (Greiner Bio-One, Frickenhausen, Germany) (Köpnick et al., 2018).

The rice roots were dried in the oven and ground into powder. The sucrose was extracted with 80% ethanol and measured using the resorcinol hydrochloric acid method (Li et al., 2012a).

Measurement of pectin demethylesterification degree

The extraction method of pectin and the concentration of pectin and methanol in the solution were in accordance with a previous study. The degree of pectin demethylesterification was calculated as (1–methanol concentration/pectin concentration) × 100% (Zhu et al., 2020).

Phytohormone measurement

Ethylene emissions: about 2 g of fresh roots were incubated in 20-mL glass vials sealed with a silica gel plug at 30°C for 2 h to collect the ethylene released from the roots. Then, 10 mL of incubated gas was injected into a gas chromatograph (GC-2010 Plus, SHIMADZU) to measure the ethylene concentration (Yu et al., 2016).

Indole-3-acetic acid (IAA) content measurement: fresh roots (about 0.5 g) were ground with liquid nitrogen and extracted with 80% (*v*/*v*) methanol. The extraction solution was filtered through a 0.22-μM membrane filter, and the IAA concentration was assayed by gas chromatography–mass spectrometry (GC–MS) (Zhou et al., 2010).

Brassinolide (BL) content measurement: the fresh roots (about 0.5 g) were ground with liquid nitrogen and extracted with 80% (*v*/*v*) methanol at 4°C for 2 h and then extracted through a Brond Elut and Strata-X column. The final extract was filtered through a 0.22-μM filter. The BL concentration was measured according to the method described by Bajguz et al. (2011).

Measurement of GSH, AsA, $O_2^{\cdot-}$, and H_2O_2 content

The glutathione (GSH) was extracted in 80% ethanol (v/v) containing 0.15-mM EDTA from 0.1 g of fresh rice roots and measured by a complex formed by the reaction of DTNB and GSH with a characteristic absorption peak at 412 nm (Sun et al., 2020).

The ascorbic acid was extracted in 15% metaphosphoric acid from 0.1 g of fresh rice roots and measured using a high-performance liquid chromatography (HPLC; Nanospace SI-1, Shiseido, Japan) system equipped with a PDA Detector (Thermo Fisher, Oklahoma, USA) at 254 nm (Muthusamy et al., 2019).

The content of $O_2^{\cdot-}$ was indicated by the intensity of red fluorescence after staining with dihydroethidium according to the manufacturer's instructions (S0063, Beyotime, China). The 1-cm rice root tips were collected and stained in 1-μM dihydroethidium for 30 min at room temperature, and the extra dye was washed off using deionized water. The fluorescence was captured at an emission wavelength of 300 nm and an excitation wavelength of 535 nm.

The content of H_2O_2 in the rice roots was indicated by the intensity of green fluorescence after staining with 2',7'-dichlorodihydrofluorescein diacetate. The 1-cm rice root tips were collected and stained in 50-μM 2',7'-dichlorodihydrofluorescein diacetate for 24 h at room temperature, following which the extra dye was washed off using HEPES-KOH buffer (20 mM, pH 7.8). The fluorescent image was captured by a fluorescence microscope (LEICA DM2500) (Chen et al., 2013b).

Real-time PCR

The total RNA was extracted by TRIzol reagent (Invitrogen, Germany) according to the instructions and reverse transcribed to cDNA immediately. The reaction mixture for real time (RT) PCR was according to Zhu et al. (2016). The

sequences of selected genes and reference gene primers are displayed in Table S1. The relative expression of selected genes was calculated according to a previous study (Livak and Schmittgen, 2001).

Western blot

The total protein was extracted, and the Bradford method was used to determine the protein content (Bradford, 1976). The SDS-PAGE method was used to separate proteins (20 μg for each sample) and transfer them to a 0.45 μm polyvinylidene difluoride (PVDF) membrane immediately (Laemmli, 1970). The protein blot was probed with a primary antibody of ascorbate peroxidase (APX; AS08 368, Agrisera, Sweden), catalase (CAT; AS09 501), ATP synthase (AS08 370, Agrisera, Sweden), and monoclonal anti-actin (plant) antibody produced in mice (A0480, Sigma, USA) at dilutions of 1:1000, 1:10000, and 1:1000 after resting on the PVDF membrane for 2 h at 25°C. We then probed the secondary antibody (HRP-labeled Goat Anti-Rabbit IgG (H+L) and HRP-labeled Goat Anti-Rat IgG (H+L) at a 1:1000 dilution for 2 h at 25°C. The enhanced chemiluminescence (Pierce, Waltham, WA, USA) was used to obtain blot images, and the Quantity One software (Bio-Rad, Hercules, CA, USA) was used to analyze the band intensities.

Statistical analysis

All experiments in the present study were performed with three independent biological replicates. A one-way ANOVA was used to analyze the data, and a post hoc Tukey's test was used to compare the mean values at $P < 0.05$ (SPSS 13.0). The R program (version 3.0.0; R Development Core Team) with the vegan package was used to perform multivariate analyses.

Results

Effect of H_2S on rice growth under Al toxicity conditions

The Al treatment significantly inhibited the elongation of the rice roots, amplified the total Al content in the rice roots and shoots, and increased the Al concentration in the rice apoplast and cytoplasm (Fig. 1). However, pretreatment with NaHS (H_2S donor) significantly reversed the inhibition of root growth induced by Al toxicity and also decreased the total Al content and the Al concentration in the rice apoplast and cytoplasm under Al conditions (Fig. 1), indicating that H_2S is involved in alleviating Al toxicity in rice.

Identification of DEGs by transcriptome sequencing

The results of the clean reads, Pearson's correlation coefficient analysis, principal components analysis (PCA), and hierarchical clustering analysis for each sample are provided in the supplementary material (Table S1–2, Fig. S2–3). The DEGs were obtained according to $P < 0.05$ and $\log_2FC > 1$ by the edgeR software, and the results showed that in the group of Al/CK, there were 529 upregulated genes and 282 downregulated genes. In the group of S/CK, there were 190 upregulated genes and 202 downregulated genes. In the group of Al+S/Al, there were 260 upregulated genes and 276 downregulated genes (Fig. 2A).

The Venn diagram indicated that 385, 584, and 236 genes were only differentially expressed in the Al-S/Al, Al/CK, and S/CK groups, 27 DEGs co-existed in the Al-S/Al and S/CK groups, 98 DEGs co-existed in the Al/CK and Al-S/Al groups, 103 DEGs co-existed in the Al/CK and S/CK groups, and 26 DEGs co-existed in three groups (Fig. 2B).

The GO classification in the biological process indicated that the top three DEGs belonged to metabolic process, cellular process, and single-organism process. For the molecular function category, the top three genes belonged to binding fractions, catalytic activity, and nucleic acid binding transcription factor activity. In addition, cellular component analysis found that the locations of the top three genes were the membrane, cell, and cell part (Fig. 3A).

The KEGG analysis also showed that most of the DEGs were involved in metabolism, such as carbohydrate metabolism, energy metabolism, and biosynthesis of other secondary metabolism. The other pathways included genetic information processing, environmental information processing, and cellular processes (Fig. 3B).

H_2S improved energy production and the antioxidant system in rice

The Al treatment significantly decreased the sucrose content, ATP content, and the protein abundance of ATP synthase in the rice roots compared with CK. However, pretreatment with NaHS significantly reversed these negative effects induced by Al toxicity (Fig. 4).

The Al treatment also affected the antioxidant system in the rice roots. GSH and AsA, which are involved in alleviating peroxidation damage, were induced by Al toxicity and further increased after applying NaHS to the rice roots. In addition, the $O_2^{\cdot-}$ and H_2O_2 content, indicated by the intensity of the red and green fluorescence, significantly increased under Al toxicity conditions and decreased following pretreatment with NaHS under Al toxicity conditions (Fig. 4). The protein abundance of APXs (sAPX and pAPX) decreased under Al toxicity conditions and increased following pretreatment with NaHS under Al toxicity (Fig. 4), suggesting that H_2S reduced the peroxidation stress induced by Al toxicity.

H_2S inhibited ethylene emission to reduce cell wall Al deposition in rice

Ethylene emissions increased under Al toxicity conditions and decreased after pretreatment with NaHS under Al toxicity (Fig. 5). A single application of the ethylene synthesis inhibitor AVG alone or in combination with NaHS significantly improved rice root growth, decreased the Al content in the rice roots and cell walls, and decreased the pectin content and pectin methylation degree; however, the application of the ethylene synthesis precursor ACC had an opposite tendency and even negated the positive role of NaHS in alleviating Al toxicity (Fig. 5).

In addition, the content of IAA and BL in the rice roots decreased under Al toxicity conditions and increased after pretreatment with NaHS under Al toxicity, and the exogenous application of IAA and BL both decreased the rice root Al content (Fig. S4).

Discussion

H_2S regulated material metabolism to alleviate Al toxicity

The Al stress significantly affected material metabolism in the plants, and exogenously applied signaling material alleviated the Al toxicity by regulating plant material metabolism. In our study, pretreatment with the H_2S donor NaHS induced a series of changes to material metabolism genes, including carbon metabolism (Nos. 1, 2), protein metabolism (Nos. 3, 4, 5), lipid metabolism (Nos. 6, 7, 8, 9, 10), energy production (Nos. 26, 27), and secondary metabolism (Nos. 56, 57, 58) (Table 1). Sucrose is responsible for energy supply and also acts as a signaling molecule in plants (Kühn and Grof, 2010). Producing enough sucrose and transporting sucrose to the right site is pivotal for plants to resist stress. A previous study demonstrated that sucrose transporters are involved in orchestrating sucrose allocation both intracellularly and at the whole plant level (Kühn and Grof 2010). In our study, the gene expression of the sucrose transporter (No. 2, Table 1) significantly decreased in the Al/CK set and increased in the Al+S/Al set, and this was accompanied by an increased sucrose content when the rice roots were pretreated with NaHS under Al toxicity conditions (Fig. 4B), suggesting that H_2S not only stimulates the production of sucrose but is also involved in regulating sucrose allocation to resist Al toxicity. ATP is the direct energy source for plant development, and most of the ATP in cells is produced in the mitochondria. Thus, maintaining the integrity and normal functioning of the mitochondria is important for producing enough energy in organisms (Harris and Das 1991). The GCN5-like protein 1 is responsible for the regulation of mitochondrial biogenesis and mitophagy (Scott et al. 2014). Kinesin superfamily proteins play an important

role in mitochondrial and lysosomal dispersion. In our study, the expression of GCN5-like 1 domain containing protein (No. 26) and kinesin heavy chain (No. 27) genes significantly increased in the Al+S/Al set (Table 1), and it was also accompanied by increased ATP content and ATP synthase abundance in the rice roots once pretreated with NaHS under Al toxicity conditions (Figs. 4 A and I). This indicated that H_2S maintained sufficient ATP in the rice roots to help the rice resist Al toxicity.

F-box proteins are responsible for catalyzing protein ubiquitination and maintaining the balance between protein synthesis and degradation (Boycheva et al., 2015); therefore, the increased expression of cyclin-like F-box domain containing protein (No. 3) in the Al+S/Al set in our study (Table 1) indicated that H_2S regulated the protein synthesis under Al toxicity. Previous studies have demonstrated that the carboxyl in the cell wall and phosphate in the plasma membrane are the main sites that bind with Al^{3+} and aggravate Al toxicity in plants (Kochian et al., 2005). Non-specific lipid transfer proteins (nsLTPs) are located in the plant cell wall and respond to the transfer of phospholipids (Thoma et al., 1993; Zhang et al., 2019). 3-Ketoacyl-coenzyme A synthase (KCS) and β-ketoacyl-CoA synthase are involved in the synthesis of long chain fatty acids in plants, including sphingolipids and phospholipids (Kim et al., 2013b; Li et al., 2018). In the present study, the expression of nonspecific lipid-transfer protein 3 (Nos. 6–8), 3-ketoacyl-CoA synthase (No. 9), and beta-ketoacyl-CoA synthase (No. 10) significantly decreased in the Al+S/Al set (Table 1), indicating that H_2S might decrease the transfer and synthesis of phospholipids to inhibit Al accumulation and then alleviate Al toxicity in rice.

Flavonoids are secondary metabolites present in higher plants that play an important role in plant development and abiotic and biotic stress resistance (Mo et al., 1992; Stapleton and Walbot, 1994). Flavanone 3-hydroxylase catalyzes the formation of dihydroflavonols from flavanones, therefore providing precursors for many classes of flavonoid compounds (Pelletier and Shirley, 1996). MYBL2 is a negative regulator of flavonoid synthesis in *Arabidopsis* (Dubos et al., 2008), and *OsWD40* family genes are co-expressed with MYB factors to initiate their diverse functions (Ouyang et al., 2012). In the present study, the expression of flavanone 3-hydroxylase 3 (No. 56) significantly increased; however, the expression of both MYBL2 (No. 57) and OsWD40-77 (No. 58) significantly decreased in the Al+S/Al set (Table 1), indicating that H_2S increased flavonoid synthesis to improve rice Al toxicity resistance.

H_2S alleviates Al toxicity by reducing cell wall deposition

Cell walls are the main site for Al deposition, and the removal of Al from the cell wall significantly improves plant growth. Pectin and hemicellulose are the major Al deposition sites in plant cell walls; for example, about 76% of Al in the tobacco cell wall is in the pectin (Chang et al., 1999) and most of cell wall Al in *Arabidopsis* roots is in the hemicellulose fraction (Yang et al., 2011). In our study, five DEGs (Nos. 11–15) associated with polysaccharide synthesis and construction modification were identified (Table 1). Among them, pectinesterase inhibitor is involved in blocking the process of demethylation in plants and thus reduces the negative charges in pectin in plants to decrease the binding of Al^{3+} (Van Beusichem et al., 1988). Glycoside hydrolase family proteins are involved in hemicellulose breakdown (Langston et al., 2011). Xylanase is responsible for catalyzing the degradation of the hemicellulose main composition material xylan and the glycoside hydrolase family 18-member xylanase inhibitor protein I (XIP-I), which are responsible for inhibiting the activity of xylanase (Anne et al., 2005; Jensen et al., 2018). Arabinogalactan-proteins (AGPs) participate in cell wall polysaccharide synthesis; for example, the mutant of *agp30* in *Arabidopsis* exhibited significantly decreased pectin content (Hengel and Roberts, 2003). In the present study, the expression of the pectinesterase inhibitor domain containing protein (No. 11) and glycoside hydrolase (No. 12) increased, while the expression of glycoside hydrolase family 18 (No. 13) and arabinogalactan protein 1 and 2 (Nos. 14, 15) decreased, following the application of NaHS under Al toxicity conditions (Table 1), which indicated that H_2S reduced the cell wall polysaccharide content to reduce cell wall Al deposition. Furthermore, we also found that two wall-associated kinase (WAK) genes (Nos. 18, 19), which are related to cell growth (Kim and Guerinot, 2007; Walker and Connolly, 2008), significantly increased after the application of NaHS

under Al toxicity conditions (Table 1). This implies that H_2S improved the expression of the WAK gene to improve rice root growth under Al toxicity conditions.

H_2S alleviates the peroxidation damage induced by Al toxicity

Al toxicity significantly increased the H_2O_2 and $O_2^{\cdot-}$ content in the rice roots (Figs. 4 E-G), suggesting that Al induced peroxidation damage in rice. To remove the excess oxidizing substances, the content of the most important antioxidants in rice roots, namely GSH and AsA (Jones, 2002), both significantly increased after NaHS application (Figs 4 C and D). In addition, enzymes belonging to the antioxidant system were also identified in the present study. Among them, germins and germin-like proteins (GLPs) possess the enzyme functions of superoxide dismutase (SOD) to produce H_2O_2 and play a pivotal role in plant development and defense, such as by improving blast disease resistance in rice (Manosalva et al., 2009). Thioredoxins are major cellular protein disulfide reductases that play a role in H_2O_2 removal (Chae et al., 1999). Peroxisomes are simple organelles present in most organisms and play an important role in ROS metabolism (Corpas et al., 2017). Ascorbate oxidase (AO) is responsible for converting ascorbate to monodehydroascorbate and reducing oxygen to water at the same time (Stevens et al., 2017). In our study, the expression of germin-like proteins (Nos. 28–34), thioredoxin domain 2 containing protein (No. 35), peroxisomal biogenesis factor 19 (No. 36), and L-ascorbate oxidase precursor (No. 39) all increased in the Al+S/Al set (Table1). However, the expression of benzothiadiazole-induced protein (No. 37), which participates in inhibiting CAT and APX to increase H_2O_2 content in plants (Iriti et al., 2003), decreased (Table 1). In addition, although the protein content of CAT did not change before or after the application of NaHS, the protein contents of sAPX and tAPX both significantly increased (Fig. 4 I), further confirming that H_2S alleviates peroxidation damage by reducing peroxides.

The phytohormones involved in H_2S alleviation of Al toxicity

In addition, H_2S often interacts with other signaling molecules to regulate the plant response to various stresses (Jin and Pei, 2015), such as nitric oxide, salicylic acid, abscisic acid, and Ca^{2+} (Fang et al., 2014; Li et al., 2015; Peng et al., 2016; Jin et al., 2013; Wang et al., 2018). In our study, we also identified some genes involved in the regulation of phytohormone synthesis, metabolism, and signal transduction. Ethylene emissions are induced by Al toxicity and aggravate Al toxicity in *Lotus japonicus* L. and *Arabidopsis* (Sun et al., 2007; Sun et al., 2010). The ACC oxidase is responsible for the final step of ethylene production, which catalyzes 1-aminocyclopropane carboxylic acid (ACC) to ethylene (Kende, 1993). GDSL lipase-like 1 regulates systemic resistance and is required for ethylene signaling in plants (Kwon et al., 2009). In the present study, ethylene emissions and the related gene ACC oxidase (No. 23) decreased in the Al+S/Al set and therefore decreased the expression of lipase and GDSL domain-containing protein (Nos. 24, 25) (Table 1). In addition, a previous study found that ethylene stimulates pectin synthesis in rice, and thus there is a hypothesis that H_2S decreases ethylene synthesis to reduce the pectin content, which then reduces the Al content in rice roots. In the present study, the application of the ethylene synthesis inhibitor AVG alone or together with NaHS significantly increased rice root growth, decreased cell wall Al and total Al content in the rice roots, and inhibited the degree of pectin synthesis and demethylation esterification, whereas the ethylene synthesis precursor ACC had an opposite tendency and even negated the positive role of H_2S in alleviating Al toxicity once applied with NaHS (Fig. 5).

Furthermore, the expression of some genes related to IAA (Nos. 21, 40–42) and BL (No. 43) also increased after pretreatment with H_2S under Al toxicity conditions, including tryptophan decarboxylase, which is responsible for catalyzing the first step of IAA synthesis that transfers tryptophan to tryptamine (Majerus et al. 2009) (Table 1). The small auxin-up RNA (SAUR) genes are early auxin-responsive genes that play a role in auxin-mediated cell elongation (Longnecker and Welch, 1990). Brassinosteroid insensitive 1 (BRI1)-Associated Kinase I (BAK1) is one of the key components in the brassinosteroid signal transduction pathway and is involved in regulating rice growth and development (Huang et al., 2012; Lei et al., 2014). In addition, the content of IAA and BL both increased in the rice roots

following pretreatment with NaHS under Al toxicity conditions and was accompanied by a decrease in the Al content in the rice root tips after exogenous application of IAA and BL under Al toxicity (Fig. S4). This further confirmed that H_2S cross-talks with IAA and BL to alleviate Al toxicity in rice.

H_2S regulated transcriptional and translational pathways to alleviate Al toxicity

Transcriptional and translational pathways that control the expression of stress-responsive genes are pivotal for the plant response to various stresses (Romheld and Marschner, 1986). Eukaryotic translation initiation factor 2 is involved in the initiation of polypeptide chain synthesis (Bienfait et al., 1985). Toll-interleukin-1 receptor domain (TIR) is associated with the response of growth factors in plants (Guerinot and Yi, 1994; Mori, 1999). Apetala2 (AP2) transcription factor genes belong to the ethylene response factor (ERF) subfamily (Jofuku et al., 1994; Ohme-Takagi and Shinshi, 1995). AP2/ERF family transcription factors, including the subfamily of dehydration-responsive element, play an important role in seed development (Boutilier et al., 2002) and response to environmental stress (Zhu et al., 2010). In the present study, the expression of eukaryotic translation initiation factor 2 (No. 48), toll-interleukin receptor domain containing protein (No. 49), AP2/ERF transcription factor (Nos. 50, 51), dehydration-responsive element (Nos. 52, 53), ethylene-responsive transcription factor 2 (No. 54), and ERF domain containing protein (No. 55) all significantly increased in the Al+S/Al set (Table 1), indicating that H_2S stimulates ethylene response transcription factors to increase Al resistance ability.

H_2S inhibited the transport of Al from the roots to the shoots by mediating genes related to ion uptake and transport

Heavy metal-associated (HMA) proteins are involved in heavy metal uptake and internal transportation in plants; for example, *OsHMA2* localizes to root pericycle cells and is associated with the transportation of heavy metal Cd^{2+} from the roots to shoots (Yamaji et al., 2013). In the present study, the expression of heavy-metal-associated domain-containing protein (No. 59) significantly decreased in the Al+S/Al set. This was concurrent with a decreasing shoot–root-Al-content ratio (Fig. 1E), indicating that H_2S not only decreased the Al uptake but also inhibited its transportation from the roots to the shoots.

Conclusion

As show in Fig. 9, H_2S improved energy production, reduced peroxidation damage, and decreased Al binding in the cell membrane to alleviate Al toxicity. In addition, H_2S inhibited ethylene emissions to reduce pectin content and improved the pectin methylation degree to decrease cell wall Al deposition. The phytohormones IAA and BL were also found to be involved in the alleviation of Al toxicity by H_2S. Additionally, H_2S also regulated secondary metabolism (such as flavonoids), transcriptional and translational pathways, or inhibited the transportation of Al from the roots to the shoots to alleviate Al toxicity in rice

Abbreviations

H_2S: Hydrogen sulfide;

Al: Aluminum;

ROS: reactive oxygen species;

DEGs: differentially expressed genes;

GO: Gene Ontology;

KEGG: Kyoto Encyclopedia of Genes and Genomes;

IAA: Indole-3-acetic acid;

BL: Brassinolide;

PVDF: polyvinylidene difluoride;

PCA: principal components analysis;

ACC: 1-aminocyclopropane carboxylic acid

Declarations

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

All authors agreed to the publication.

Availability of supporting data

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

C.Q.Z., W.J.H, Q.Q.W., and H.Z. performed research; C.Q.Z., X.C.C, L.F.Z. and Y.L.K. analyzed data, C.Q.Z. and X.J.X. wrote the draft; J.L., Q.Y.J. and J.H.Z. revised the article; C.Q.Z., X.J.X., and J.H.Z. designed the research.

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Tables

Table 1 Differentially expressed genes in the rice roots under different treatments.

No	ID	Name	Al+S/Al		Al/CK		S/CK	
			Log_2(fc)	*p*-value	Log_2(fc)	*p*-value	Log_2(fc)	*p*-value
1	Os03t0197200	Sorbitol transporter	1.01	0.00	NC		NC	
2	Os02t0576600	Sucrose transporter	1.87	0.01	-2.10	0.00	NC	
3	Os10t0136200	Cyclin-like F-box domain containing protein	1.87	0.05	NC		NC	
4	Os01t0729600	Pyridoxal phosphate-dependent transferase	-3.25	0.00	3.08	0.00	NC	
5	Os04t0107600	Arginine decarboxylase	-2.97	0.00	NC		NC	
6	Os11t0116200	Nonspecific lipid-transfer protein 3	-9.16	0.00	NC		NC	
7	Os11t0427800	Nonspecific lipid-transfer protein 3	-8.40	0.00	NC		NC	
8	Os12t0115500	Nonspecific lipid-transfer protein 3	-4.40	0.01	NC		-4.29	0.01
9	Os06t0260500	3-ketoacyl-CoA synthase	-7.64	0.00	7.64	0.00	NC	
10	Os03t0181500	Beta-ketoacyl-CoA synthase	-7.23	0.00	NC		NC	
11	Os03t0830800	Pectinesterase inhibitor domain containing protein	2.31	0.00	NC		NC	
12	Os03t0845600	Glycoside hydrolase	1.93	0.05	-2.10	0.00	NC	
13	Os05t0248200	Glycoside hydrolase family 18	-1.45	0.04	4.10	0.00	NC	
14	Os08t0482300	Arabinogalactan protein 1	-1.07	0.00	NC		NC	
15	Os01t0937900	Arabinogalactan protein 2	-1.32	0.00	NC		NC	
16	Os06t0184900	Hydroxycinnamoyltransferases	-7.20	0.00	NC		NC	
17	Os12t0227500	Beta-glucosidase aggregating factor	-2.11	0.00	NC		NC	
18	Os08t0501600	Wall-associated kinase	3.20	0.03	NC		NC	
19	Os10t0103140	Wall-associated kinase	2.78	0.01	NC		NC	
20	Os09t0368200	Polyamine oxidase precursor	2.72	0.05	NC		NC	
21	Os08t0140500	Tryptophan decarboxylase	1.29	0.01	-1.27	0.01	NC	
22	XLOC_035969	Cinnamoyl-CoA reductase 1-like	1.01	0.02	NC		NC	
23	Os09t0451400	ACC oxidase	-1.30	0.00	NC		NC	
24	Os05t0210400	Lipase, GDSL domain containing protein	-1.46	0.03	1.19	0.04	NC	
25	Os09t0567800	Lipase, GDSL domain containing protein	-1.07	0.01	NC		NC	
26	Os12t0111900	GCN5-like 1 domain containing protein	1.53	0.04	NC		NC	

27	Os02t0644400	Kinesin heavy chain	1.33	0.00	-1.89	0.00	NC	
28	Os12t0154800	Germin-like protein 12-2	1.53	0.00	NC		NC	
29	Os08t0189400	Germin-like protein 8-5	1.51	0.00	NC		NC	
30	Os08t0190100	Germin-like proteins 8-11	1.46	0.00	NC		NC	
31	Os08t0189900	Germin-like proteins 8-10	1.37	0.00	NC		NC	
32	Os12t0154700	Germin-like proteins 12-1	1.27	0.00	NC		NC	
33	Os08t0189850	Germin-like protein8-9	1.18	0.00	NC		NC	
34	Os12t0155000	Germin-like protein12-4	1.01	0.00	NC		NC	
35	Os07t0476900	Thioredoxin domain 2 containing protein	1.22	0.03	NC		NC	
36	Os02t0661000	Peroxisomal biogenesis factor 19	1.07	0.00	NC		NC	
37	Os11t0189600	Cycloartenol synthase	-6.32	0.01	3.00	0.04	NC	
38	XLOC_032921	Benzothiadiazole-induced protein	-1.45	0.00	NC		NC	
39	Os09t0507300	L-ascorbate oxidase precursor	1.35	0.01	NC		NC	
40	Os07t0475700	Auxin responsive SAUR protein family protein	2.81	0.03	NC		NC	
41	Os01t0768333	Auxin responsive SAUR protein family protein	2.54	0.03	NC		NC	
42	Os02t0143300	Auxin responsive SAUR protein family protein	1.10	0.03	NC		NC	
43	Os11t0514400	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1	7.44	0.04	NC		NC	
44	Os10t0489500	Terpene synthase 2	7.38	0.00	NC		NC	
45	Os08t0168400	Sesquiterpene synthase	2.20	0.00	NC		NC	
46	Os06t0255400	Nudix hydrolase 18	1.06	0.00	NC		1.11	0.05
47	Os09t0376600	Tyrosine protein kinase	-1.64	0.01	NC	0.01	NC	
48	Os07t0692800	Eukaryotic translation initiation factor 2	3.14	0.00	NC	0.05	NC	
49	Os07t0566800	Toll-Interleukin receptor domain containing protein	6.98	0.03	NC		NC	
50	Os09t0457900	AP2/ERF transcription factor	1.97	0.02	NC		NC	
51	Os07t0674800	AP2/ERF transcription factor	1.31	0.02	NC		NC	
52	Os09t0522100	Dehydration-responsive element-binding protein 1H	1.69	0.00	NC		NC	
53	Os09t0522200	Dehydration-responsive element-binding protein 1A	1.66	0.00	NC		NC	
54	Os03t0860100	Ethylene-responsive	1.23	0.01	NC		NC	

		transcription factor 2				
55	Os04t0547600	ERF domain containing protein	1.28	0.00	NC	NC
56	Os04t0667200	Flavanone 3-hydroxylase 3	1.08	0.01	NC	NC
57	Os01t0127450	MYBL2	-2.44	0.04	NC	NC
58	Os03t0625300	OsWD40-77	-1.84	0.00	NC	NC
59	Os05t0337400	Heavy-metal-associated domain-containing protein	-1.06	0.00	NC	NC

There were three independent biological replicates for the transcriptomics analysis. The differentially expressed genes that changed more than $\log_2(fc) \geq 1$ and passed Student's *t*-test ($P \leq 0.05$) were defined as significantly different. The Al stress concentration in the solution was 30 μM, the NaHS concentration was 2 μM. CK: without Al and NaHS; Al: single Al treatment; S: single NaHS treatment; Al+S: both Al and NaHS treatment. NC: no change.

Figures

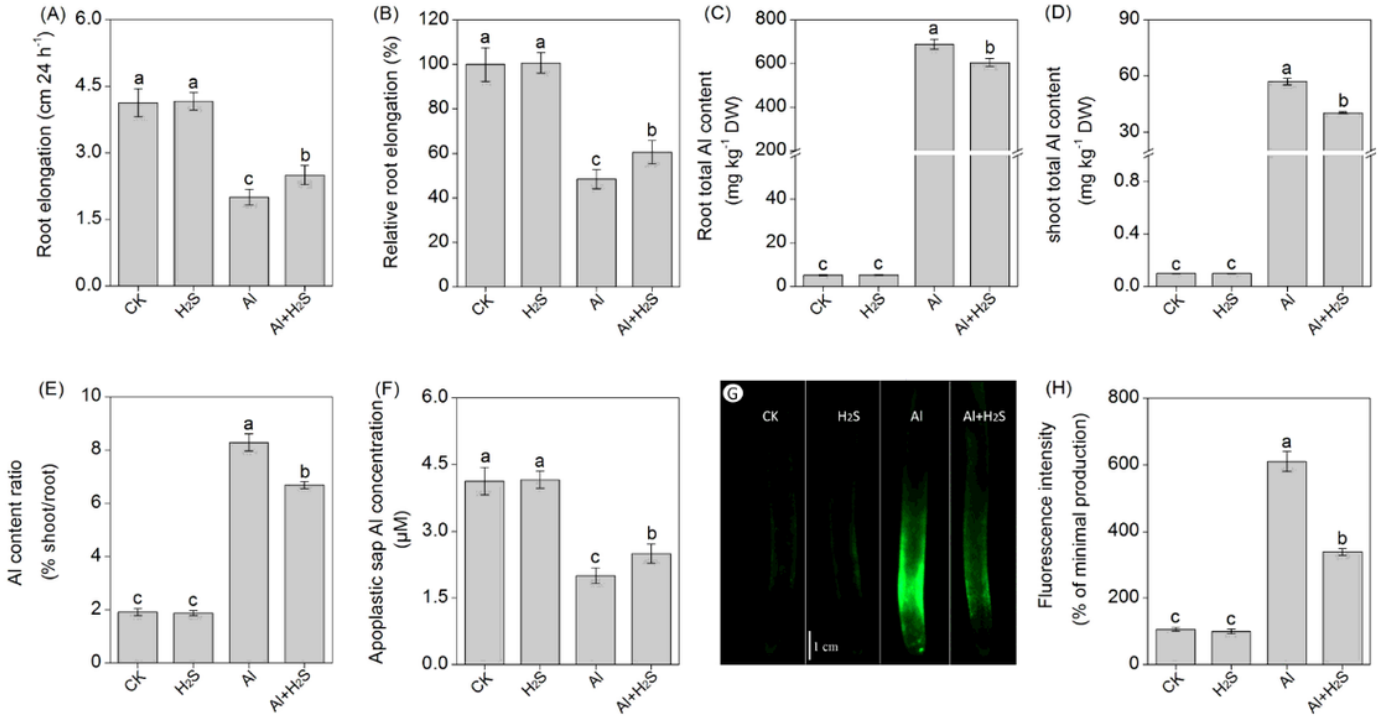

Figure 1

Effects of H2S on (A) rice root elongation, (B) relative root elongation, (C) root total Al content, (D) shoot total Al content, (E) shoot–root-Al-content ratio, (F) apoplastic sap Al concentration, (G) cytoplasm Al content (indicated by the intensity of green fluorescence after the rice roots were stained by morin regent), and (H) the fluorescence intensity. Data are means ± SD (n = 4). Columns with different letters are significantly different at P < 0.05.

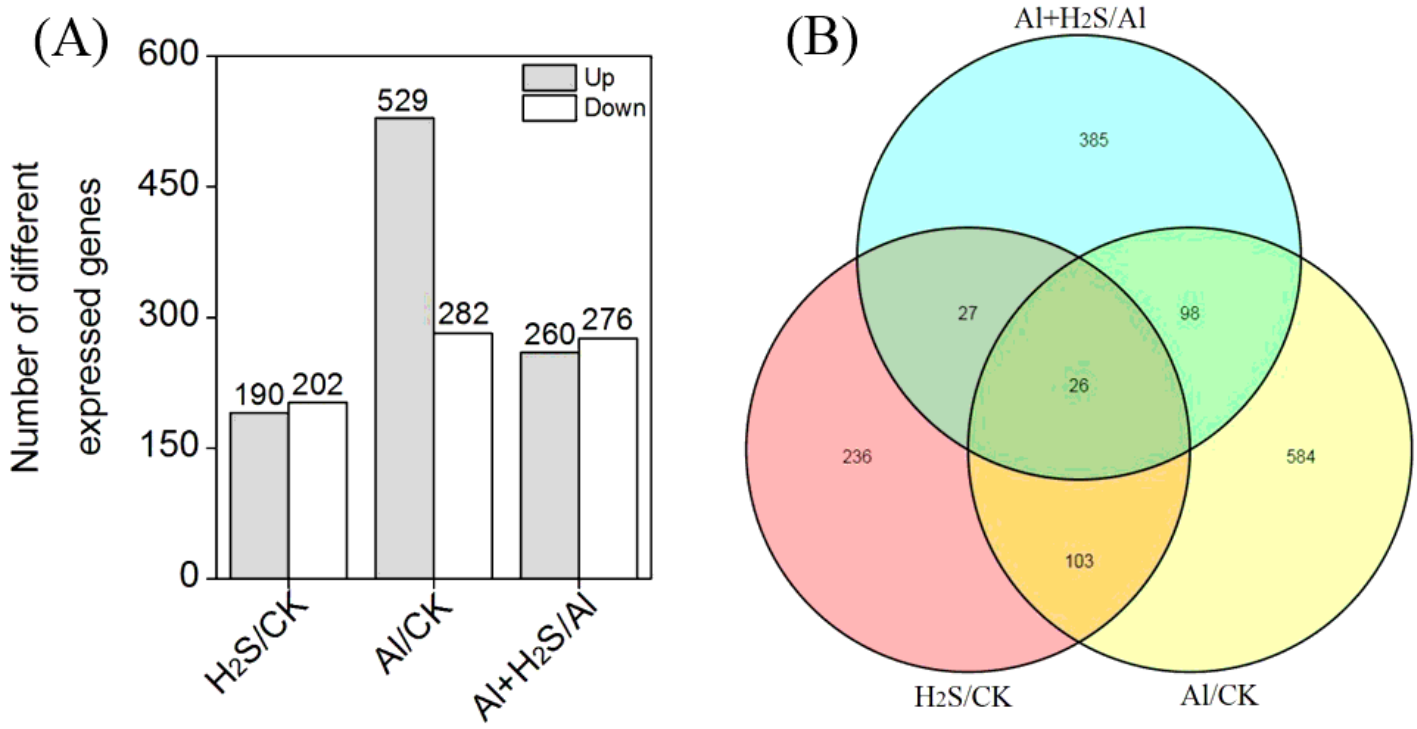

Figure 2

Number of differentially expressed genes (A) and (B) Venn diagram of all of the differentially expressed genes in the rice roots under different treatments.

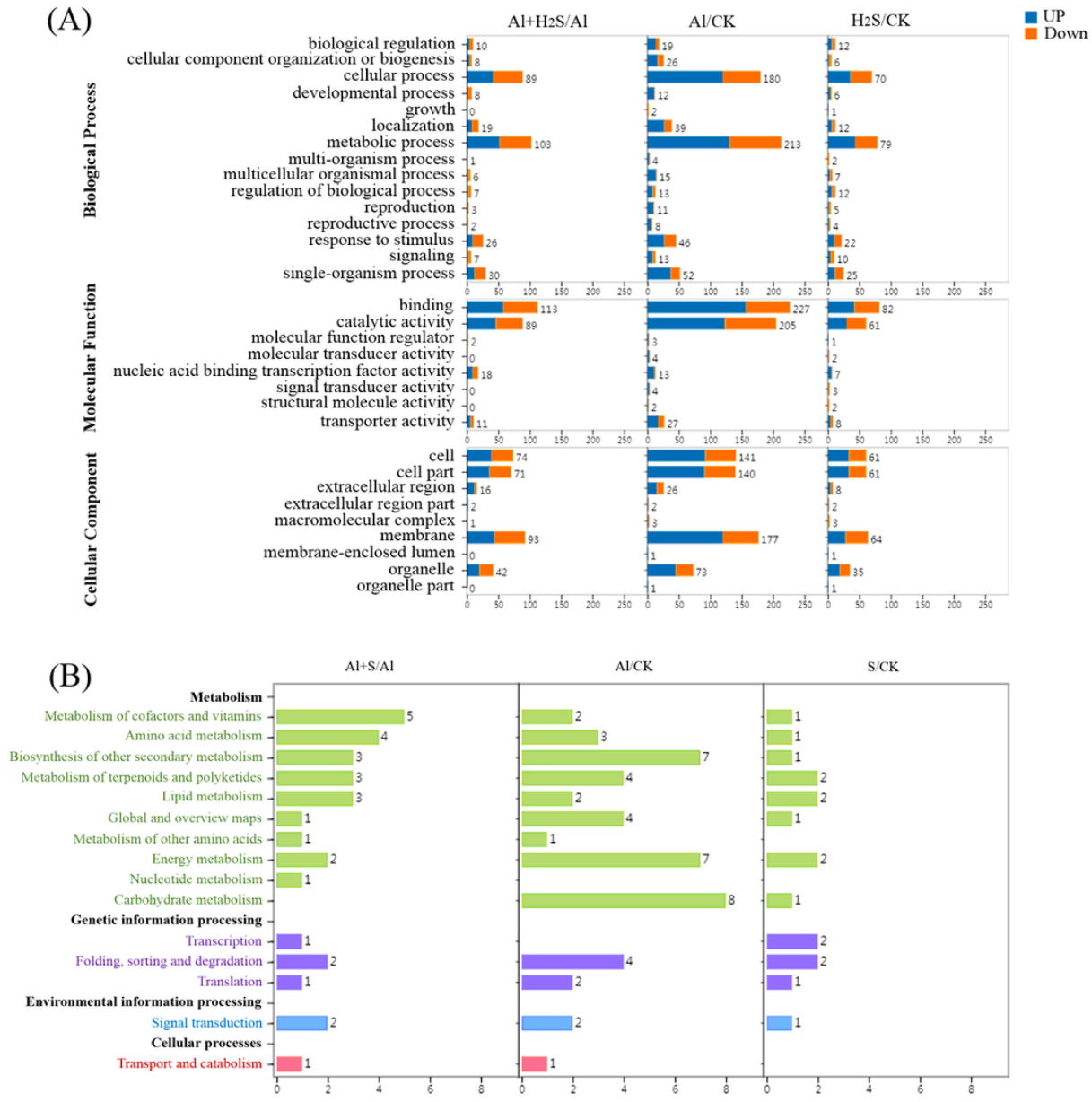

Figure 3

The Gene Ontology (GO) analysis (A) and (B) KEGG pathway annotations for all the differentially expressed genes in our study.

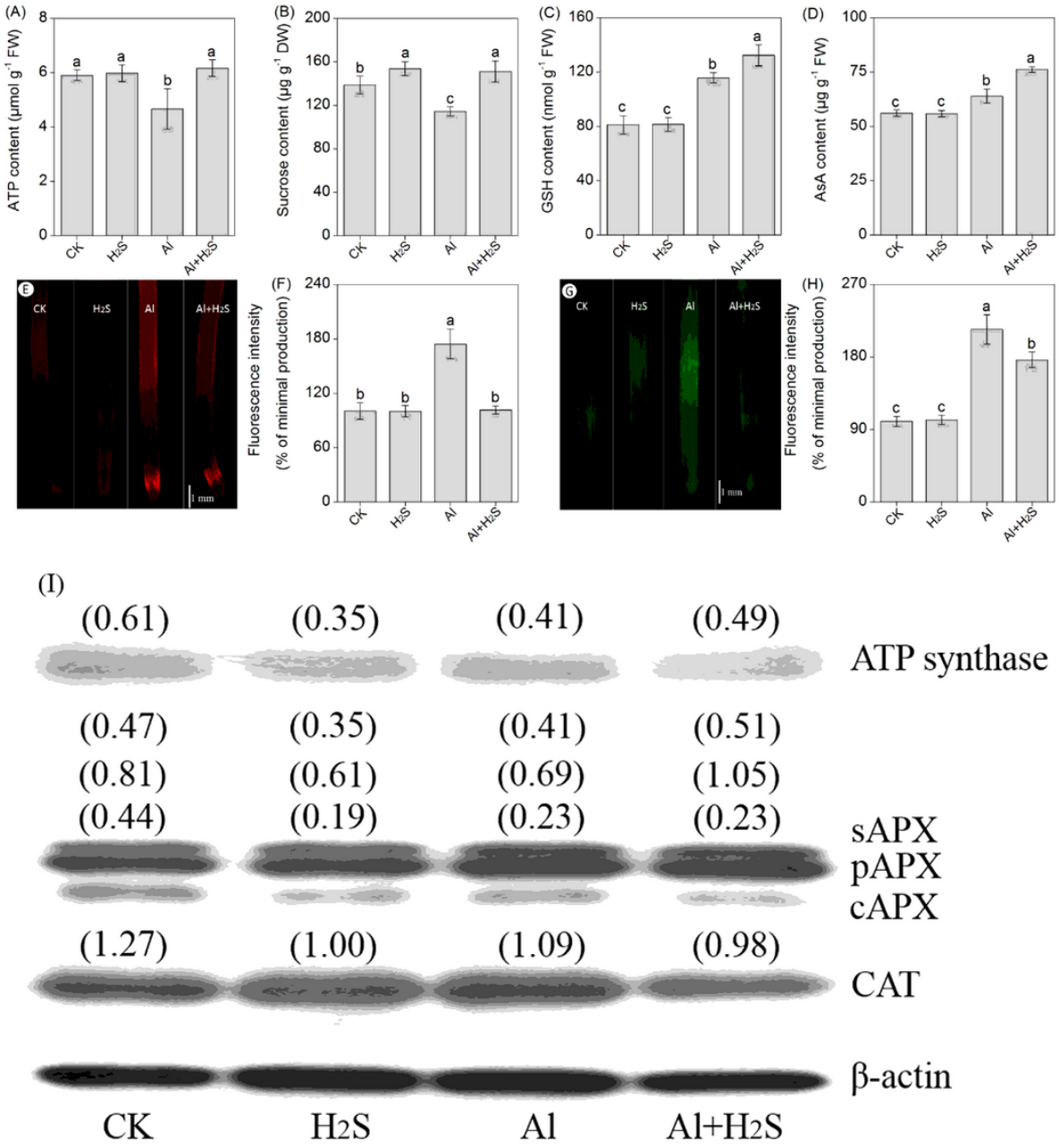

Figure 4

Effect of 2-μM NaHS on the (A) sucrose content, (B) ATP content, (C) GSH content, (D) AsA content, (E–F) superoxide anion O2·- content, (G–H) H2O2 content, and (I) protein abundance of ATP synthase, APXs, and CAT under different treatments in the rice roots. The O2·- and H2O2 contents are indicated by the intensity of the red and green fluorescence, respectively. Data are means ± SD (n = 4). Columns with different letters are significantly different at $P < 0.05$. The values in (I) were calculated based on the gray value of the target protein compared with β-actin under each treatment. sAPX: stromal APX; pAPX: peroxisomal APX; cAPX: cytoplasmic APX.

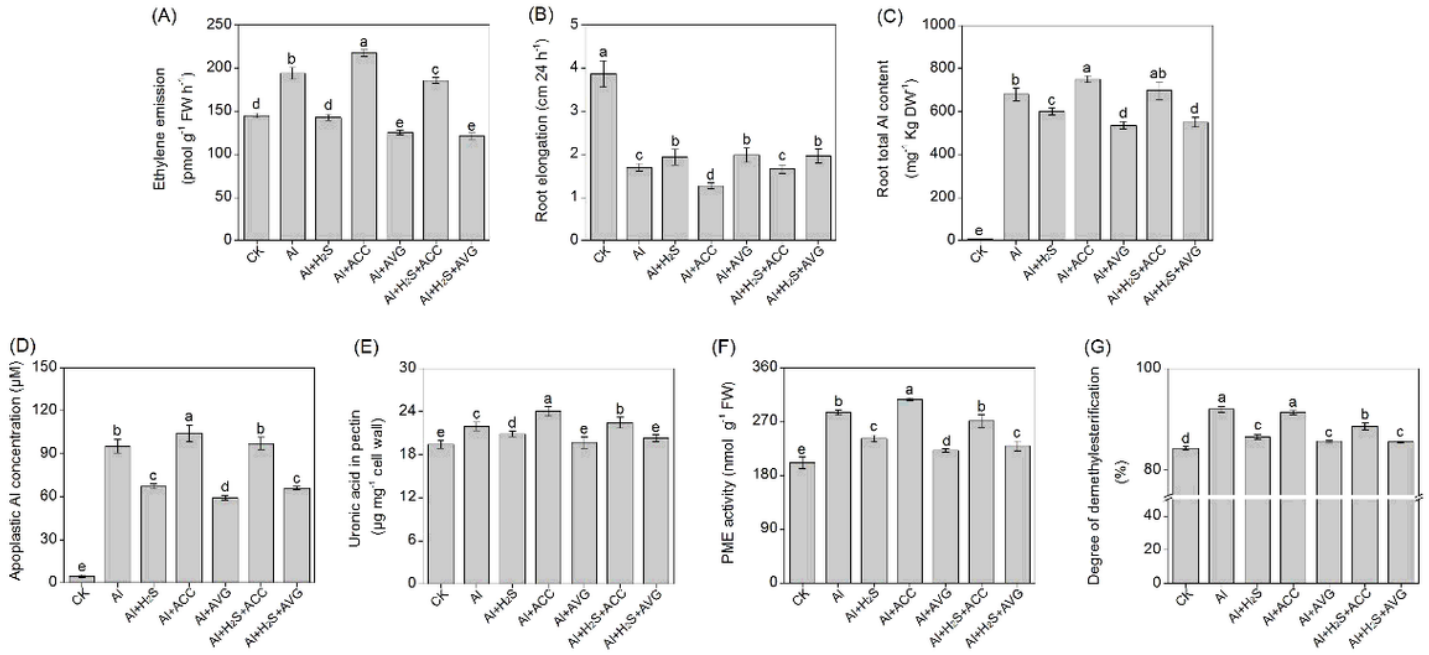

Figure 5

Effect of 2-μM NaHS on the (A) ethylene emissions, (B) root elongation, (C) root total Al content, (D) apoplastic Al concentration, (E) pectin content, (F) PME activity, and (G) pectin demethylesterification degree in the rice roots. Data are means ± SD (n = 4). Columns with different letters are significantly different at $P < 0.05$.

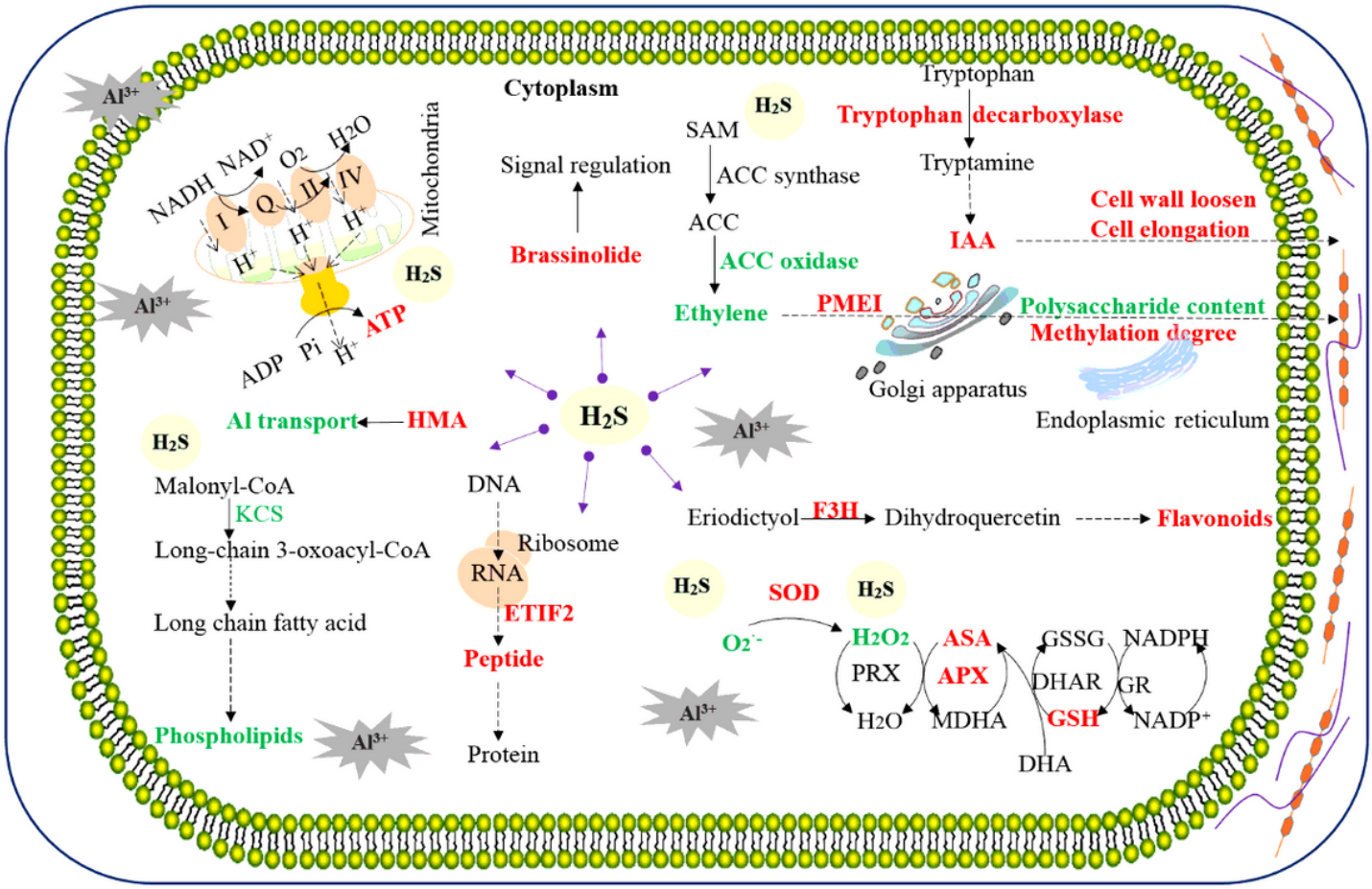

Figure 6

A schematic model for H2S-mediated improvement of root elongation under Al toxicity in rice. ACC: 1-aminocyclopropane-1-carboxylic acid; ETIF2: eukaryotic translation initiation factor 2; F3H: flavanone 3-hydroxylase 3; HMA: heavy-metal-associated domain-containing protein; KCS: ketoacyl-CoA synthase; PMEI: pectinesterase inhibitor domain containing protein.

Supplementary Files

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- Supplementarymateriials0621.docx