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RESEARCH ARTICLE

Increased Cysteine Accumulation is Essential for Salt Stress Tolerance in *Arabidopsis Halotolerance 2-Like* (*AHL*)-Overexpressing Transgenic Plants

Tinh Van Nguyen[1,2] · Jeong-In Kim[1] · Cho-Rong Park[1] · Moon-Soo Chung[3] · Cheol Soo Kim[1]

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Abstract

Cysteine (Cys) regulates plant growth, development, and various abiotic stress responses. However, the knowledge gained from genetic and molecular studies on the role of Cys metabolism in salinity response remains limited. Here, we introduce a sulfate metabolism-related component, *Arabidopsis Halotolerance 2-like* (*AHL*), which is involved in salt stress adaptation. *AHL* expression was experimentally induced under salt stress conditions using *AHL* promoter-β-glucuronidase transgenic plants. Phenotypic analysis revealed that the survival rate of *AHL*-overexpressing (*AHL*-OE) lines was greater than that of wild-type (WT) plants under high-salinity conditions, while *ahl* mutant and *AHL*-RNAi seedlings were more sensitive than WT seedlings. Accumulation of Cys and proline was increased in *AHL*-OE transgenic lines under salinity stress conditions, but malondialdehyde and hydrogen peroxide levels were lower in *AHL*-OE than in WT, *ahl*, and *AHL*-RNAi plants. In addition, the transcription levels of genes associated with Cys biosynthesis and sulfate metabolism were higher in *AHL*-OE than *ahl* and *AHL*-RNAi seedlings after salt stress treatment. Moreover, exogenous application of Cys could rescue the salt stress-induced sensitive phenotypes of *ahl* and *AHL*-RNAi lines, and return them to the WT phenotype. Taken together, our findings indicate that AHL positively regulates salinity response in *Arabidopsis* seedlings by modulating a Cys-dependent pathway.

Keywords AHL · Cysteine · Hydrogen peroxide · Proline · Salt stress response · Sulfate metabolism

Abbreviations

Cys	Cysteine
Pro	Proline
qPCR	Quantitative real-time polymerase chain reaction
WT	Wild-type

Introduction

Salinity-induced alterations occur during plant growth and development due to the cumulative effects of excess saline on a number of physiological and biochemical properties (Iqbal et al. 2015). Some examples of the effects of salinity on plant cells include mineral ion homeostasis, osmolyte accumulation, water balance, antioxidant metabolism, nitrogen fixation, and photosynthesis (Iqbal et al. 2015). Salt stress triggers overproduction of reactive oxygen species (ROS) and affects the redox balance of plant cells, leading to damage to proteins, lipids, nucleic acids, and cell membrane integrity (Mittler 2002; Ahmad et al. 2008). Excess production of ROS causes oxidative stress, leading to cellular damage and, ultimately, cell death. To prevent or alleviate ROS-induced damage and allow the beneficial functions of ROS to continue, plants have evolved an intriguing antioxidant defense system. These defense systems aim to keep levels of reactive or active oxygen species under control. Antioxidant defense systems comprise both enzymatic as well as non-enzymatic components (Ahanger and Agarwal 2017). Plants tend to accumulate free amino acids, free proline,

✉ Cheol Soo Kim
cskim626@jnu.ac.kr

[1] Department of Applied Biology, Chonnam National University, Gwangju 61186, Republic of Korea

[2] Department of Basic Science, Buon Ma Thuot University, Buon Ma Thuot, Dak Lak 630000, Vietnam

[3] Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup, Jeollabuk 56212, Republic of Korea

non-structured carbohydrates, and quaternary ammonium compounds (betaines) in response to salt stress conditions (Ahanger and Agarwal 2017).

Sulfate metabolism is a vital nutritional metabolic process that is required for plant growth, development, and environmental response (Kopriva et al. 2012; Shin et al. 2019). In nature, sulfate is the most abundant form of sulfur (S) that can be taken up by plants. Sulfate metabolism is carried out in various subcellular compartments, such as the cytosol, chloroplast or plastids, depending on the demands of the specific plant cell (Kopriva et al. 2012). Cysteine (Cys) is the first organic compound in primary sulfate metabolism (Takahashi et al. 2011). Cys is an amino acid that participates in the process of protein synthesis. Cys is also a precursor for a large number of essential biomolecules, such as vitamins, cofactors, or Fe-S clusters (Romero et al. 2014). Moreover, Cys is involved in the formation of many important bio-components, including glutathione (GSH), methionine, polyamine, glucosinolates, and camalexin (Romero et al. 2014). Sulfur-containing metabolites are necessary for various aspects of plant growth and development, as well as responses to unfavorable environments. Some examples of such metabolites include the Cys derivatives biotin, ethylene, polyamine, and GSH, which can directly or indirectly regulate the salinity stress response (Khan et al. 2014). GSH is involved in antioxidant-regulated cellular redox homeostasis through its role in ROS product catalysis (Khan et al. 2014). *Arabidopsis HAL2-like* (*AHL*), encoding the *At1g54390* gene (Shin et al. 2019), was identified as a 3′-phosphoadenosine 5′-phosphate (PAP) phosphatase that belongs to the secondary branch of sulfate metabolism. It shares more than 40% amino acid sequence identity with the *Arabidopsis* 3′ (2′), 5′-bisphosphate nucleotidase (SAL) family (Chen et al. 2011; Shin et al. 2019). AHL has been determined to share a much higher transcript level with SAL1 than with other SAL family members (Chen et al. 2011; Shin et al. 2019). However, AHL may not directly participate in constitutive PAP accumulation under both normal and stress conditions (Chen et al. 2011; Shin et al. 2019). Suppression of PAP content in *AHL*-RNAi plants may be caused by SAL1 enzyme activity (Shin et al. 2019). Instead, AHL activity is closely involved in AMP reproduction (Shin et al. 2019; Nguyen et al. 2019). Additionally, AHL was found to participate in Cys generation through primary sulfate metabolism. In fact, the accumulation of AMP and Cys in *AHL*-overexpressing transgenic plants granted *Arabidopsis* a high degree of tolerance to osmotic stress and high glucose response (Shin et al. 2019; Nguyen et al. 2019). However, whether AHL modulates Cys production under conditions of salinity stress needs to be clarified.

Here, we wonder whether *Arabidopsis* HAL2-like (AHL) might be able to the regulation of Cys biosynthesis pathway for mediation of the salt stress response. To determine this possibility, the current study evaluated the role of AHL in salt stress by investigating sulfate metabolism of WT, *ahl* mutant, and *AHL* transgenic plants using molecular biological and physiological assays. We provided evidence indicating that gain of *AHL* function leads to an increase in salinity tolerance phenotypes. The Cys content was higher in *AHL*-overexpressing lines than in wild-type (WT) plants during salt stress treatment, but the levels of *ahl* mutant and *AHL*-RNAi lines were lower in the mutant than WT under salt stress. In addition, exogenous application of Cys ameliorates the salt stress-induced sensitive phenotype of the *ahl* and *AHL*-RNAi lines.

Materials and Methods

Plant Materials, Growth Conditions, and Salt Stress Treatment

Here, we investigated the seeds of *Arabidopsis thaliana* (Col-0), *ahl* mutant, and *AHL* transgenic plants including *AHL* pro-β-glucuronidase (GUS2-1, GUS3-3), *AHL*-RNAi (*ri2-3*, *ri5-2*), and *AHL*-overexpressing (OE2-2, OE5-1) lines previously designated by Nguyen et al. (2019) and Shin et al. (2019). To analyze *AHL* expression levels in *ahl* and *AHL* transgenic plants, reverse transcription (RT)-PCR and quantitative real-time PCR (qPCR) assays were performed. These assays reveled that two individual *AHL*-overexpressing (OE2-2 and OE5-1) lines displayed high levels of *AHL* transgene expression compared to WT, while two individual *AHL*-RNAi (*ri2-3* and *ri5-2*) lines exhibited low levels of *AHL* expression (Supplementary Fig. S1). In addition, *AHL* transcription was completely abolished in *ahl* mutant (Supplementary Fig. S1). They were selected for phenotype characterization. Plants were grown in a standard growth chamber (16 h light/8 h dark, 22 °C condition, 60% relative humidity, and 110 μmol m^{-2} s^{-1} intense light). For salt stress treatment, 10-day-old plants were submerged in 150 mM NaCl solution and collected at 0, 3, 6, 12, and 24 h.

Analysis of β-Glucuronidase (GUS) Staining

GUS staining in *AHL* pro-GUS transgenic plants was conducted as described previously (Shin et al. 2019). Whole seedlings were immersed in 100 mM sodium phosphate solution (pH 7.0) containing 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-Gluc), and then incubated for 12 h at 37 °C. After removal of chlorophyll with 70% ethanol, seedlings were photographed using a microscope.

Analysis of Salt Stress-Induced Sensitivity

For salt stress-induced sensitivity experiments, seeds were sown on one-half strength MS (for Murashige and Skoog) plates with or without 150 mM NaCl and grown in a growth chamber. Seed germination and survival rates were measured. To investigate the salt stress response to Cys, seeds were sown on 150 mM NaCl-containing one-half strength MS plates containing 40 µM Cys and grown in a growth chamber. Survival rates were measured. Data were obtained from three biological replicates, with 50 seeds per genotype per replicate.

Amino Acid and Malondialdehyde (MDA) Measurements

Proline (Pro) concentration was measured as described by Bates et al. (1973). Pro was obtained from approximately 200 mg of seedlings homogenized in 2 mL of 3% sulfosalicylic acid and reacted with 200 µL of ninhydrin reagent solution. The reaction mixture was combined with 400 µL of toluene and vortexed. The absorbance of the toluene layer was read at 520 nm with a UV/VIS spectrophotometer (JASCO, Tokyo, Japan). The Pro concentration was calculated from a standard curve.

The Cys content of the seedling samples was estimated following the method of Priya (2016). Cys was obtained from 500 mg seedling samples by homogenization in 5% cold perchloric acid solution and centrifugation at 10,000 rpm for 20 min at 4 °C. The supernatant was mixed with ninhydrin reagent and glacial acetic acid in equal volumes (1:1, mL). The reaction mixture was then heated at 95 °C for 10 min and rapidly cooled on ice. Absorbance was measured at 560 nm. The Cys concentration was calculated from a standard curve.

The MDA content of the seedling samples was measured as described by Kim et al. (2017). MDA was obtained from approximately 200 mg of seedlings by grinding in 10% trichloroacetic acid (TCA) buffer, and centrifuged at 10,000 rpm for 15 min. The supernatant (0.5 mL) was mixed with 1 mL of 0.6% thiobarbituric acid and 10% TCA. The reaction mixture was then heated at 95 °C for 15 min and rapidly cooled on ice. The absorbance values of the colored supernatants were measured at a wavelength of 532 nm and were corrected for non-specific absorbance at 450 and 600 nm.

3′-Diaminobenzidine (DAB) Staining and Hydrogen Peroxide (H_2O_2) Measurement

H_2O_2 accumulation was detected by DAB staining buffer as described by Daudi and O'Brien (2012). Ten-day-old seedlings were submerged in 150 mM NaCl solution for 12 h, then stained with DAB solution for 12 h. After removal of chlorophyll by acetic acid, ethanol, and glycerol (*v*:*v*:*v*, 1:3:1), the seedlings were photographed.

To determine the H_2O_2 concentration of the seedlings, a colorimetric technique using potassium iodide buffer was used (Junglee et al. 2014). Five hundred milligrams of 150 mM NaCl-treated frozen samples were homogenized at 4 °C in 2 mL of extraction buffer containing 0.1% TCA, 1 M potassium iodide, and 10 mM potassium phosphate buffer (pH 8.0). The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C. Absorbance of the supernatant layer was read at 350 nm in a UV/VIS spectrophotometer (JASCO, Tokyo, Japan). A calibration standard curve obtained with H_2O_2 solution prepared in 0.1% TCA was used for quantification.

Total RNA Extraction and Quantitative Real-Time PCR (qPCR) Analysis

Total RNA was isolated from the seedling samples using the Plant RNeasy extraction kit (Qiagen, Valencia, CA). Genomic DNA was removed from the total RNA preparation by treatment with RNase-free DNase I enzyme according to the manufacturer's instructions (Qiagen). The concentration of total RNA was quantified using spectrophotometric measurements, and 3 µg of total RNA was resolved on a 1.0% formaldehyde agarose gel to determine RNA concentration and visualize its integrity. qPCR was carried out using the CFX Connect quantitative PCR apparatus (Bio-Rad, Hercules, CA, USA). The IQ SYBR Green Supermix kit (Bio-Rad) was used for qPCR according to the manufacturer's instructions. *Actin 1* (*ACT1*) was used as an internal control. Quantitative analysis was carried out using the delta-delta-C_T method of Livak and Schmittgen (2001). Data were obtained from three biological replicates with ≥ 12 pooled seedlings per genotype per replicate. The reaction primers are listed in Supplementary Table S1.

Statistical Analysis

Statistical analyses were performed using SPSS 23.0 software (IBM Co, Armonk, NY), and included one-way analysis of variance (ANOVA) and Duncan's multiple-range test. Different letters indicate statistically significant differences at $P < 0.05$ by Duncan's multiple-range test.

Results

AHL is Upregulated by NaCl Treatment

AHL encodes PAP phosphatase, which participates in secondary sulfate metabolism (Chen et al. 2011). Recently, we discovered that AHL regulates the responses of young

Arabidopsis seedlings to abiotic stresses such as abscisic acid (ABA), drought, osmotic stress, oxidative stress, and high Glc treatment (Shin et al. 2019; Nguyen et al. 2019). Previously, Fatma et al. (2014) reported that excess sulfate supplementation improves photosynthetic capacity and growth in mustard plants under salt stress conditions via a reduction in glutathione content.

To investigate the function of *AHL* in response to salt stress, we initially measured the expression pattern of *AHL* in 10-day-old *Arabidopsis* seedlings subjected to 150 mM NaCl treatment. Quantitative real-time PCR (qPCR) showed that *AHL* transcript levels were significantly increased in samples within 6–12 h of salt stress treatment, but decreased thereafter (Fig. 1A). *RD29A* (*Responsive to Dehydration 29A*) was used as a positive control for salt stress treatment (Fig. 1A). The results indicate that *AHL* is up-regulated by salt treatment.

To gain further insight into the expression of *AHL* in *Arabidopsis* seedling organs, we analyzed the GUS staining of 1925-bp *AHL* promoter (pro)-GUS transgenic lines (2-1 and 3-3) following water (H_2O) or salt (150 mM NaCl) treatment. As shown in Fig. 1B, GUS staining was observed in the veins of leaves, the primary root and lateral roots under H_2O treatment conditions in two individual *AHL* pro-GUS transgenic lines. When 150 mM NaCl was applied to *AHL* pro-GUS transgenic seedlings for 12 h, GUS activity increased to a greater extent than under salt-untreated conditions (Fig. 1B). Furthermore, GUS staining was detected in the hypocotyl (arrow) after salt treatment, but not under normal conditions. Thus, AHL level is altered in response to without or with salt stress in roots, leaf veins, and hypocotyl tissues.

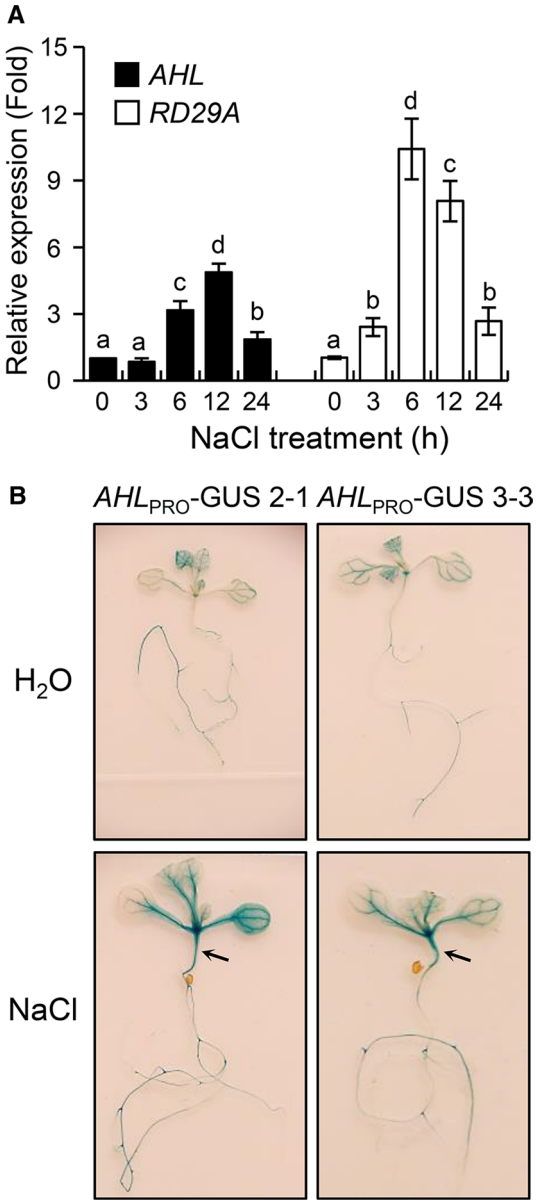

Fig. 1 *AHL* is induced by salt stress. **A** Ten-day-old seedlings were harvested for total RNA extraction at the indicated time points under 150 mM NaCl treatment, and *AHL* expression levels were quantified by qPCR. *RD29A* served as a control for salt stress treatment. The bars represent the mean values (±SD) of three biological replicates. Different letters above the bars indicate a statistically significant difference at $P < 0.05$ by Duncan's multiple-range test. **B** Histochemical GUS activity for *AHL* in response to salt stress. Ten-day-old whole seedlings of the *AHL*pro-GUS transgenic (2-1 and 3-3) lines were subjected to 150 mM NaCl. After 12 h of NaCl treatment, the seedlings were stained for GUS activity, with H_2O-treated seedlings used as a control

Overexpression of *AHL* Confers High Tolerance to Salt Stress

Since *AHL* expression is upregulated by NaCl treatment (Fig. 1), we examined whether *AHL* is required for regulation of the tolerance of *AHL* transgenic plants to salt stress by measuring seed germination and survival rates. The seeds of WT, *ahl*, *AHL*-RNAi (*ri 2-3*, *ri 5-2*), and *AHL*-overexpressing (OE 2-2, OE 5-1) plants were germinated on one-half strength MS medium containing 0 or 150 mM NaCl. The germination rates did not differ between WT, *ahl,* and *AHL* transgenic plants in the absence of 150 mM NaCl (data not shown). With exposure to 150 mM NaCl at 4 days after seed sowing, 84–92% of the two types of *AHL*-overexpressing seeds germinated, as compared to 63% of WT, while 53% and 47–49% of the *ahl* and two individual *AHL*-RNAi lines, respectively, germinated (Fig. 2A). These findings indicate that seed germination in *AHL*-overexpressing lines is more likely than that in *ahl* mutant and *AHL*-RNAi lines to be tolerant to salt stress.

High salt stress also resulted in significant differences in survival rate at 18 days after seed sowing: 65–69% of the two types of *AHL*-overexpressing seeds produced green leaves, compared with 53% of WT and 23% and 27–36%

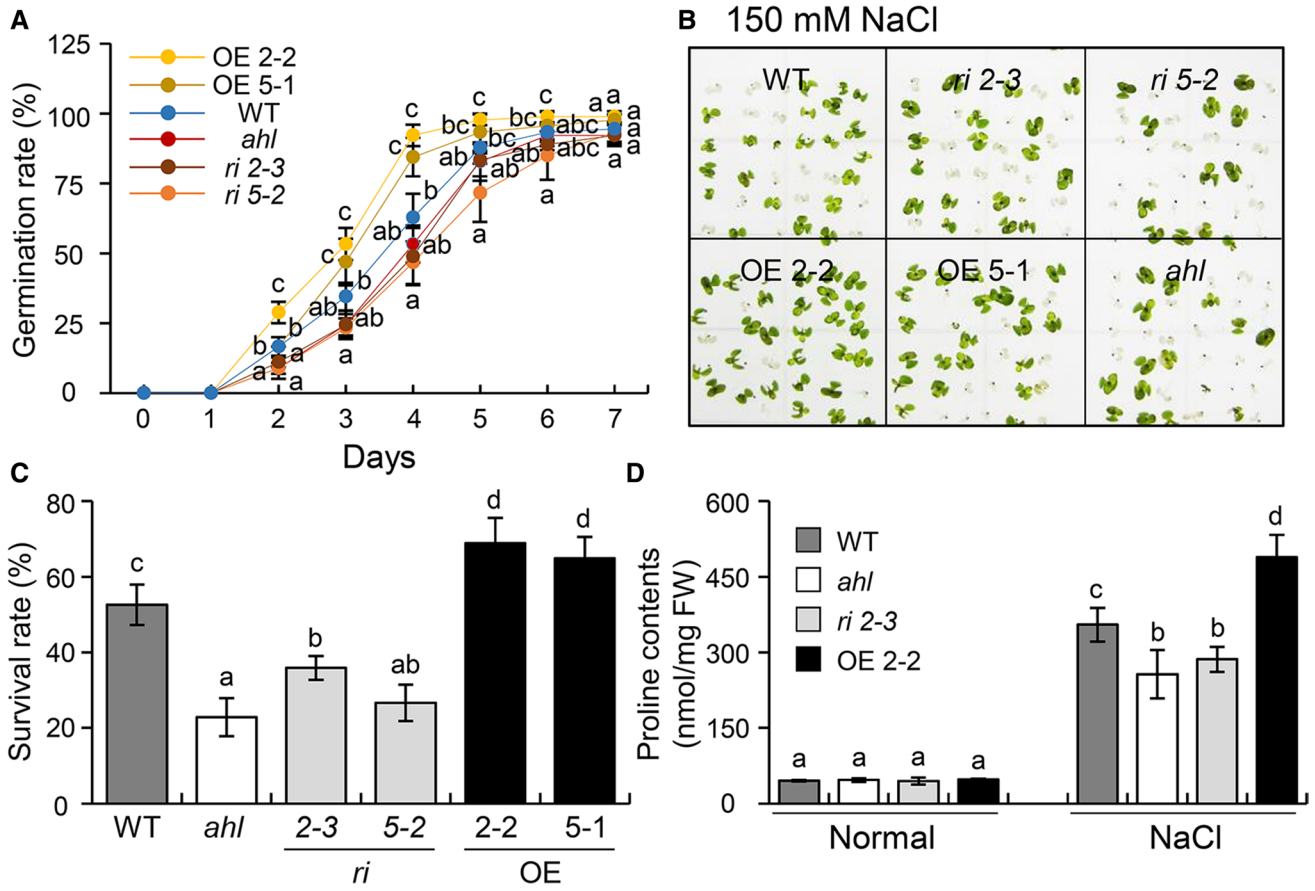

Fig. 2 Physiological responses of *ahl* mutant and *AHL* transgenic plants to salt stress. **A** Comparison of the germination rate of WT, *ahl* mutant and *AHL* transgenic plants under salt stress conditions. The seed germination rate on 150 mM NaCl is shown for samples of each genotype on different days. The points represent the mean values (± SD) of three biological replicates. Different letters above the bars indicate a statistically significant difference at $P < 0.05$ by Duncan's multiple-range test. **B** Phenotypes of WT, *ahl*, *AHL*-RNAi (*ri 2-3*, *ri 5-2*) and *AHL*-overexpressing (OE 2–2, OE 5–1) seedlings exposed to 150 mM NaCl for 18 d. **C** Quantification of the survival rates of WT, *ahl*, *AHL*-RNAi and *AHL*-OE seedlings exposed to 150 mM NaCl for 18 d. **D** Two-week-old seedlings of each sample were subjected to 0 and 150 mM NaCl for 12 h and used to measure proline concentration. The bars represent the mean values (± SD) of three biological replicates. Different letters above the bars in **C**, **D** indicate a statistically significant difference at $P < 0.05$ by Duncan's multiple-range test.

of *ahl* and the two *AHL*-RNAi lines, respectively (Fig. 2B, C). Collectively, these observations suggest that *AHL* expression is associated with early seedling growth under salt stress conditions.

A previous report demonstrated that accumulation of Pro plays a role as an adaptive mechanism that counteracts salt stress (Hasanuzzaman et al. 2013; Hayat et al. 2012). To measure Pro content in *AHL* transgenic lines under salt stress conditions, Pro content assays were applied to whole seedlings of WT, *ahl*, *AHL*-RNAi (*ri 2-3*), and *AHL*-overexpressing (OE 2-2) plants. In the absence of salt stress treatment, the Pro levels in all of these samples were similar (Fig. 2D). However, in the presence of 150 mM NaCl, the Pro content was higher in the *AHL*-OE line than in WT, *ahl,* and *AHL*-RNAi plants, while it was lower in the *ahl* and *AHL*-RNAi lines than in WT plants (Fig. 2D). This analysis reveals that *AHL* is necessary to regulate Pro accumulation under salt stress conditions.

AHL Regulates H_2O_2 Accumulation and Oxidative Damage During Salt Stress

Salt stress leads to the accumulation of ROS such as H_2O_2 and oxygen-free radicals in plant tissue (Hasanuzzaman et al. 2013; Ahanger and Agarwal 2017). To evaluate the effects of *AHL* expression on ROS production under salt stress conditions, whole seedlings of WT, *ahl,* and *AHL* transgenic samples were treated with 150 mM NaCl for 12 h, then stained with DAB solution for the H_2O_2 accumulation assay. In the absence of salt stress, H_2O_2 levels were similar among the WT, *ahl*, *AHL*-RNAi (*ri2-3*), and *AHL*-overexpressing (OE2-2) seedlings (Fig. 3A). With

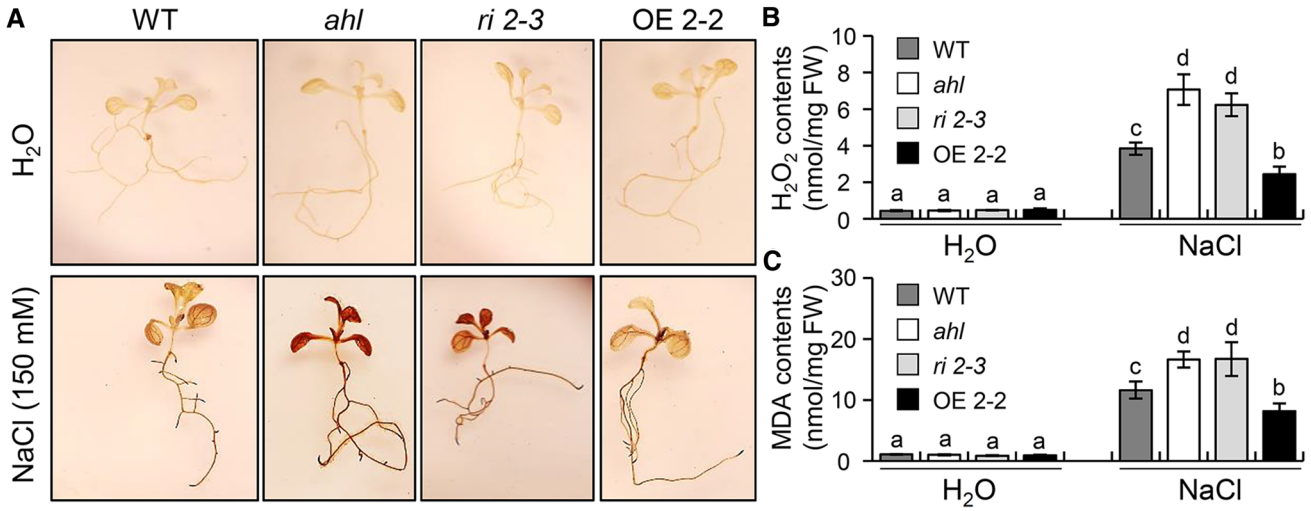

Fig. 3 H_2O_2 and MDA contents in *ahl* mutant and *AHL* transgenic plants during salt stress treatment. **A** Ten-day-old whole seedlings of each sample were treated with H_2O (control) and 150 mM NaCl for 12 h and DAB staining was conducted to assess H_2O_2 accumulation. **B**, **C** Quantification of H_2O_2 (**B**) and MDA (**C**) accumulation in 10-day-old seedlings after treatment with H_2O (control) or 150 mM NaCl for 12 h. The bars represent the mean values (±SD) of three biological replicates. Different letters above the bars indicate a statistically significant difference at $P < 0.05$ by Duncan's multiple-range test

salt stress treatment, H_2O_2 production was higher in *ahl* and *ri2-3* whole seedlings than in WT and OE2-2 seedlings (Fig. 3A). Subsequently, we measured H_2O_2 production in high salt-treated whole seedlings. As shown in Fig. 3B, H_2O_2 levels were significantly higher in the *ahl* and *AHL*-RNAi lines than in WT and *AHL*-overexpressing seedlings, while the H_2O_2 level was significantly lower in the *AHL*-overexpressing transgenic line than in WT seedlings under salt stress conditions. These observations demonstrate that AHL is involved in the regulation of hydrogen peroxide accumulation under salt stress.

In general, MDA content is considered an indicator of oxidative damage (Sadak et al. 2020). Thus, we evaluated oxidative damage in *ahl* and *AHL*-RNAi seedlings subjected to salt stress conditions by analyzing the MDA content of the seedlings. Without salt stress treatment, the basal MDA levels of all sample plants were similar (Fig. 3C). With salt stress treatment, the MDA contents of *ahl* and *AHL*-RNAi seedlings were greater than those of WT or *AHL*-overexpressing transgenic seedlings (Fig. 3C), implying that AHL regulates the salt stress response by participating in ROS-related processes.

Expression of Salt Stress-Responsive Genes in *AHL* Transgenic Lines

To examine the salt stress response in *AHL* transgenic lines at the molecular level, we analyzed the transcriptional levels of the *ABA-insensitive 5* (*ABI5*), *Arginine Decarboxylase 1* (*ADC1*), *ADC2*, *Alternative Oxidase 1a* (*AOX1a*), *ABA-Responsive Element Binding 3* (*AREB3*), *Arabidopsis thaliana Oxidation-related Zinc Finger 2* (*AtOZF2*), *Delta 1-pyrroline-5-carboxylate synthase 1* (*P5CS1*), *Response to Drought 26* (*RD26*), and *Respiratory burst oxidase homologue-D* (*RbohD*) genes, which are known to be induced by ABA, dehydration, salinity, oxidative, and biotic stresses (Lopez-Molina and Chua 2000; Li et al. 2015; Sánchez-Rangel et al. 2016; Kim et al. 2017; Zhang et al. 2017; Shin et al. 2019). qPCR analyses showed that, in comparison to WT plants, the expression levels of *ABI5*, *ADC1*, *ADC2*, *AOX1a*, *AREB3*, *AtOZF2*, *P5CS1,* and *RD26* were higher in the *AHL*-overexpressing (OE2-2) lines, whereas they were lower in the *ahl* and *AHL*-RNAi (*ri2-3*) lines under salt stress conditions (Fig. 4A–H). *RbohD* expression was higher in salt stress-treated *ahl* and *ri2-3* plants than in WT, while it was lower in salt stress-treated OE2-2 plants than in WT (Fig. 4I). These observations indicate that both *ahl* and *AHL*-RNAi lines can suppress salt stress-induced *ABI5*, *ADC1*, *ADC2*, *AOX1a*, *AREB3*, *AtOZF2*, *P5CS1,* and *RD26* or enhance salt stress-induced *RbohD* expression. Hence, it is likely that AHL can act positively or negatively depending on the signal required for modulating the type of salt stress-inducible genes under high salinity condition.

The *ahl* Mutation Reduces Cysteine Levels Under Salt Stress Conditions

Cys is a vital organic amino acid that is involved in reduced sulfur synthesis in plants. Cys is also an important precursor for essential components, such as antioxidant glutathione, vitamins, thionins, and glucosinolates (Álvarez et al. 2011; Romero et al. 2014; Wawrzynska et al. 2015). Moreover,

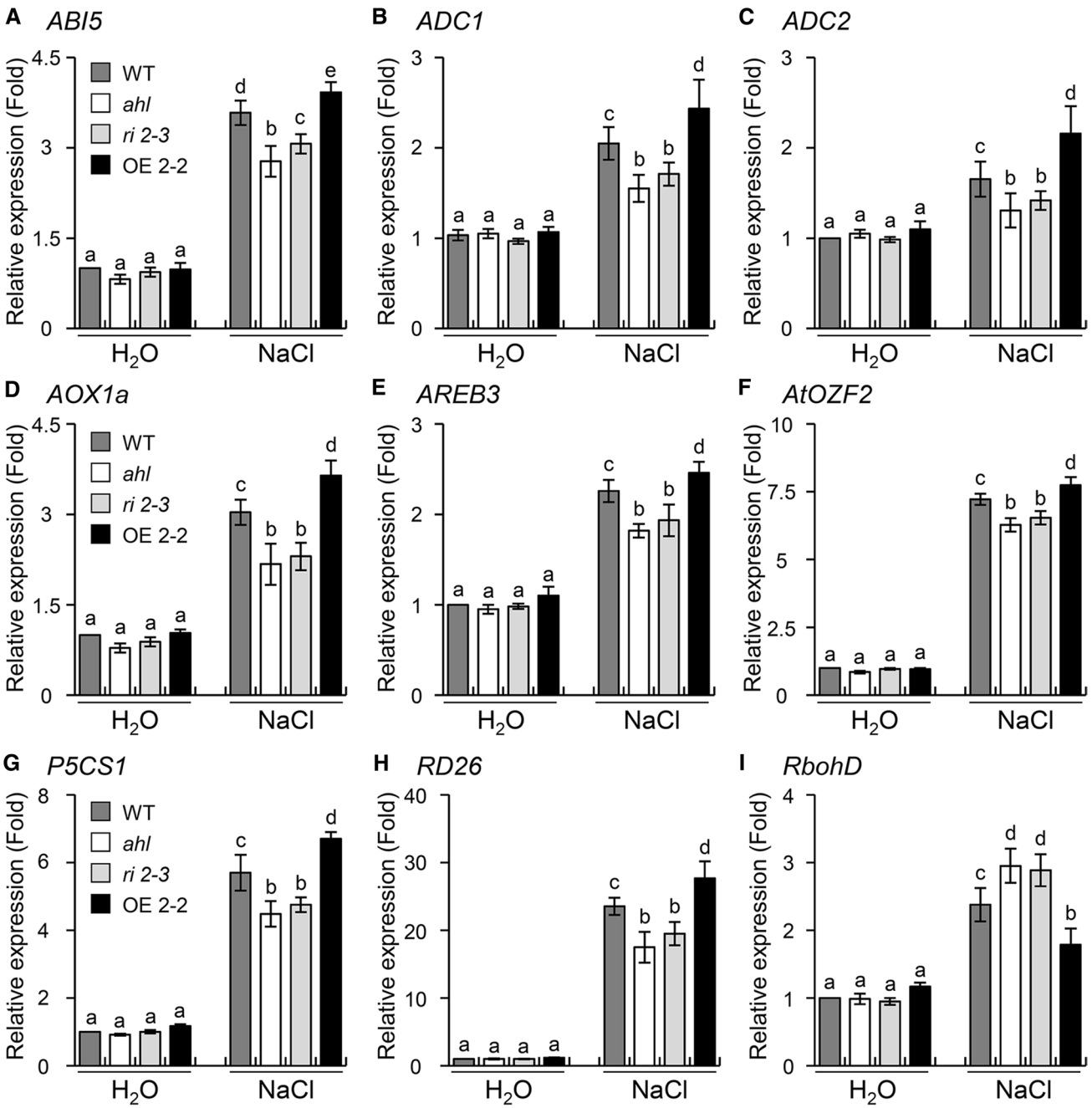

Fig. 4 qPCR analysis of salt stress-related genes in *ahl* mutant and *AHL* transgenic plants. **A**–**I** Expression of *ABI5* (**A**), *ADC1* (**B**), *ADC2* (**C**), *AOX1a* (**D**), *AREB3* (**E**), *AtOZF2* (**F**), *P5CS1* (**G**), *RD26* (**H**) and *RbohD* (**I**) in WT, *ahl*, *AHL*-RNAi (*ri 2-3*) and *AHL*-overexpressing (OE 2-2) plants under salt stress. Ten-day-old seedlings were treated with H_2O or 150 mM NaCl for 12 h. Total RNA was extracted from each type of seedling and analyzed by qPCR. The bars represent the mean values (± SD) of three biological replicates. Different letters above the bars indicate a statistically significant difference at $P < 0.05$ by Duncan's multiple-range test

Cys acts as a bio-regulator and plays an important role in promoting plant growth and productivity under high glucose, oxidative, and salt stress conditions (Genisel et al. 2015; Romero et al. 2014; Nguyen et al. 2019). To further assess the response to salt stress, the Cys contents in salt stress-treated rosette leaves of WT, *ahl,* and *AHL* transgenic plants were estimated. As shown in Fig. 5A, the Cys content was higher in the *AHL*-overexpressing line than in WT, *ahl,* and *AHL*-RNAi plants, while it was lower in the *ahl* and *AHL*-RNAi lines than in WT plants in the presence or absence of high NaCl concentration. This result indicates that AHL modulates Cys production in *A. thaliana*.

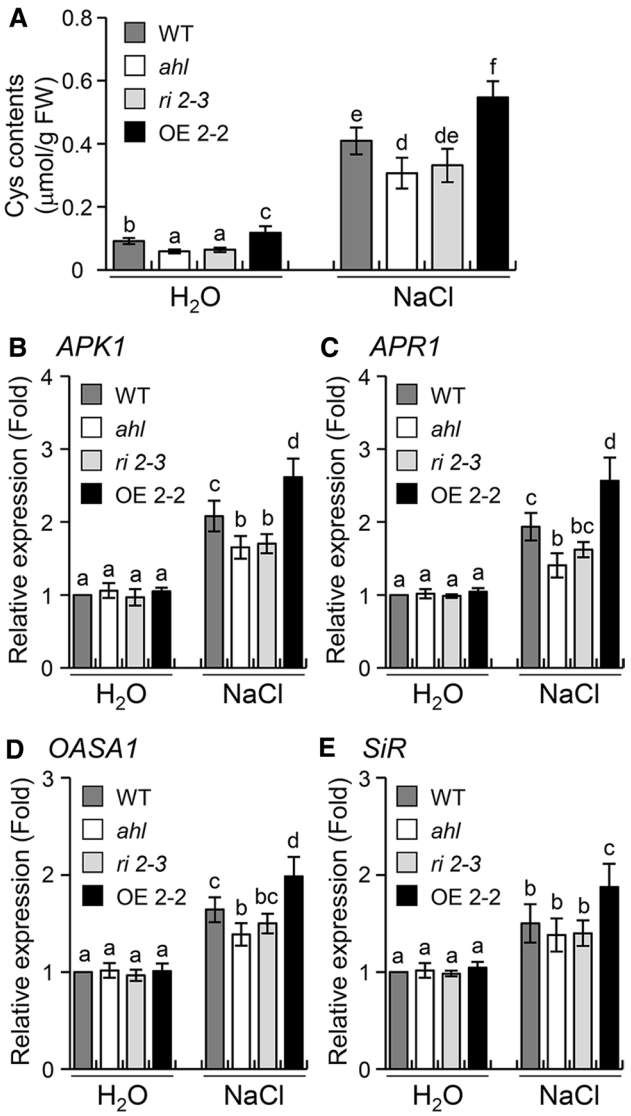

Fig. 5 Cys concentration and transcript levels of sulfate metabolism-related genes in *ahl* mutant and *AHL* transgenic plants under salt stress. **A** Cys concentration in 10-day-old seedlings after treatment with H_2O (control) or 150 mM NaCl for 12 h. The bars represent the mean values (±SD) of three biological replicates. Different letters above the bars indicate a statistically significant difference at $P < 0.05$ by Duncan's multiple-range test. **B–E** qPCR results of several genes related to sulfate metabolism in *ahl* mutant and *AHL* transgenic plants under salt stress conditions. Expression of *APK1* (**B**), *APR1* (**C**), *OASA1* (**D**) and *SiR* (**E**) in WT, *ahl*, *AHL*-RNAi (*ri 2-3*) and *AHL*-overexpressing (OE 2-2) plants under salt stress. Ten-day-old seedlings were treated with H_2O or 150 mM NaCl for 12 h. Total RNA was extracted from each type of seedling and analyzed by qPCR. The bars represent the mean values (±SD) of three biological replicates. Different letters above the bars indicate a statistically significant difference at $P < 0.05$ by Duncan's multiple-range test

The change in Cys content due to *AHL* expression prompted us to assess genes, including *Adenosin-5'-phosphosulfate kinase 1* (*APK1*), *3'-Phosphoadenosine 5'-phosphosulfate reductase 1* (*APR1*), *O-Acetylserine* (*thiol*), *Lyase 1* (*OASA1*), and *Sulfite reductase* (*SiR*), that are known to be responsible for sulfate metabolism. qPCR analyses showed that, compared to WT plants, the expression levels of *APK1*, *APR1,* and *OASA1* were higher in the *AHL*-overexpressing (OE2-2) lines and lower in salt stress-treated *ahl* and *AHL*-RNAi (*ri2-3*) lines (Fig. 5B–D). The expression level of *SiR* in salt stress-treated OE2-2 plants was greater than that in WT, *ahl,* and *ri2-3* plants, whereas transcription of *SiR* showed similar levels in WT, *ahl,* and *ri2-3* plants during salt stress treatment (Fig. 5E). These results indicate that AHL regulates the expression of these sulfate metabolism-related genes under salt stress, implying that AHL participates in the salt stress response through a sulfate metabolism-mediated pathway.

Cys Modulates Salt Stress-Induced Inhibition of Early Seedling Growth

Previous data showed that the accumulation of Cys and the expression of *OASA1* (Cys biosynthesis enzyme) were lower in the *ahl* and *AHL*-RNAi lines than in WT and *AHL*-overexpressing plants during salt stress treatment (Fig. 5A, D). In addition, the expression levels of polyamine synthesis-related genes (*ADC1* and *ADC2*) in the cysteine-methionine derivative pathway (Khan et al. 2014) were suppressed to a greater extent in *ahl* and *AHL*-RNAi lines compared to WT and *AHL*-overexpressing plants under salt stress conditions (Fig. 4B, C). Hence, we tested whether the sensitivity to salt stress of the *ahl* and *AHL*-RNAi lines could be rescued by application of exogenous Cys. To analyze the effect of Cys on the salt stress response, we estimated the survival rate of WT, *ahl*, *AHL*-RNAi (*ri2-3*, *ri5-2*), and *AHL*-overexpressing (OE2-2, OE5-1) seedlings under salt stress conditions in the presence of 40 μM Cys. Under high-NaCl conditions, all sample seedlings treated with Cys showed a higher survival rate than untreated seedlings (Fig. 6). These results indicate that Cys can modulate salt stress-induced inhibition of survival in *A. thaliana*. As shown in Fig. 6, when treated with Cys, the survival rates of WT, *ahl,* and *AHL*-RNAi seedlings were similar under high salt stress conditions, but the survival rates were lower than that of *AHL*-overexpressing transgenic lines in response to salt stress. This survival data suggests that Cys plays an important role in regulating seedling growth under salt stress conditions.

Discussion

Regulation of sulfate metabolism alleviates the adverse effects of salt stress, as sulfate metabolites modulate a wide range of plant growth processes (Khan et al. 2014). Sulfur is an essential component in plants; it is necessary for abiotic stress tolerance and an integral part of important metabolic

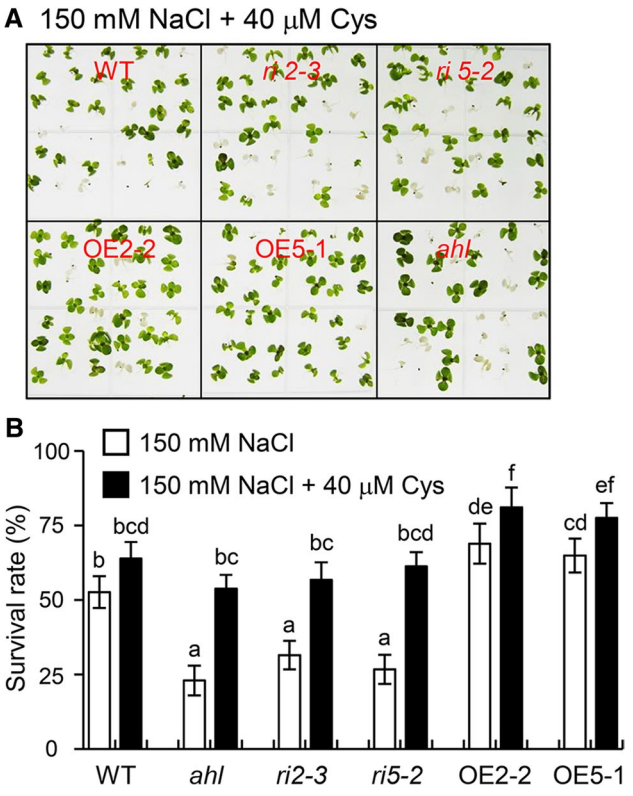

Fig. 6 Comparison of the Cys response of *ahl* mutant and *AHL* transgenic plants under salt stress. **A** Effect of exogenous Cys on growth in WT, *ahl*, *AHL*-RNAi (*ri 2-3*, *ri 5-2*) and *AHL*-overexpressing (OE 2-2, OE 5-1) seedlings under salt stress. Seedlings were grown on one-half strength MS plant medium containing 40 μM Cys with 150 mM NaCl for 18 d. **B** Survival rate of WT, *ahl* and *AHL* transgenic seedlings subjected to 150 mM NaCl or 150 mM NaCl plus 40 μM Cys for 18 d. The bars represent the mean values (±SD) of three biological replicates. Different letters above the bars indicate a statistically significant difference at $P < 0.05$ by Duncan's multiple-range test

molecules, such as sulfolipids, non-enzymatic antioxidants (glutathione), and amino acids (cysteine and methionine) (Nocito et al. 2007; Khan et al. 2014). In addition, sulfur-containing compounds are connected with the antioxidant system in plants subjected to salt stress (Khan et al. 2014). Previous studies have reported that AHL, which encodes for a component of secondary sulfate metabolism, PAP phosphatase (Chen et al. 2011), modulates sulfate metabolism. Regulated abiotic stress responses that affect sulfate metabolism include the ABA, drought, high glucose, and osmotic responses (Shin et al. 2019; Nguyen et al. 2019).

In the present study, using *AHL* transgenic lines (Shin et al. 2019), we further investigated the mechanism through which AHL underlies the sulfate metabolic pathway under high-salt conditions. A quantitative analysis of *AHL* expression under salt stress condition showed that *AHL* is up-regulated by NaCl (Fig. 1A). In addition, in the seedling stage, strong AHL activity was detected in hypocotyl tissue under salt stress conditions, but not under normal conditions, implying that AHL may perform a specific function at the spatial level in response to salt stress (Fig. 1B).

Increased expression of sulfate metabolism-responsive genes is related to improved salt tolerance in plants. The activity of *O*-acetylserine (thiol) lyase (OASTL) in *Typha* and *Phragmites* plants increases under salt stress conditions, and this increased activity fulfills the higher demand for cysteine to ensure protection and adaptation in response to salt stress (Fediuc et al. 2005). Additionally, overexpression of the *BrECS* (for *Brassica juncea Glutamylcysteine Synthetase*) gene in transgenic rice lines results in increased tolerance to high salt stress conditions by preventing membrane oxidation (Bae et al. 2013). Here, as in the salt stress assay, the germination and survival rates of the *ahl* and *AHL*-RNAi lines were lower than those of WT and *AHL*-overexpressing plants under salt stress conditions (Fig. 2A–C). In addition, the survival rate of *AHL*-overexpressing transgenic plants was higher than that of WT plants following NaCl treatment (Fig. 2B, C). These data suggest that AHL, which codes for the secondary sulfate metabolism-related enzyme PAP phosphatase, serves as a positive regulator in salt stress tolerance.

In plants, the amino acid Pro functions as an osmolyte or chaperone molecule to maintain cell turgor or stabilize the structure of a protein; it also serves as an antioxidant that regulates ROS levels (Hare et al. 1999). In addition, Pro accumulation is believed to enhance salt stress tolerance in plants (Hasanuzzaman et al. 2013; Hayat et al. 2012). Pro accumulation was higher in *AHL*-overexpressing lines than in WT, *ahl,* and *AHL*-RNAi plants after salt stress treatment (Fig. 2D). Furthermore, the Pro content of *ahl* and *AHL*-RNAi lines was lower than that of WT plants under salt stress conditions (Fig. 2D), which suggests that AHL may be responsible for the reduction in salt stress-induced sensitivity via regulation of the levels of ROS and MDA (an indicator of oxidative damage). Consequently, our data showed an apparent difference in H_2O_2 and MDA levels between *AHL*-RNAi and *AHL*-overexpressing transgenic lines during salt stress treatment (Fig. 3). The seedlings of the *ahl* and *AHL*-RNAi lines showed increased H_2O_2 and MDA induction under salt stress conditions compared with those of *AHL*-overexpressing transgenic lines (Fig. 3). Furthermore, the results shown in Fig. 4 also revealed a distinct difference in the expression levels of salt stress-associated genes between *AHL*-RNAi and *AHL*-overexpressing transgenic lines under salt stress conditions. These transcriptional profile changes imply that AHL enhances the capacity for anti-salt stress by modulating salinity-related signaling transduction. Finally, AHL activity may be a component of the oxidative stress or salinity signal that influences the adaptation mechanism.

In plants, Cys acts not only as an amino acid in protein synthesis but also as a precursor in important biological components including antioxidants, fatty acids, vitamins,

cofactors, and methionine (Khan et al. 2014; Romero et al. 2014). In addition, conservation of Cys is very important for the function and signaling processes of metabolic enzymes under environmental stressors (Meyer and Hell 2005). Several groups have established a relationship between increased Cys concentration and salt stress tolerance in plants (Barroso et al. 1999; Fediuc et al. 2005; Bae et al. 2013). Consequently, the results in Fig. 5A show an apparent difference in Cys levels between *AHL*-RNAi and *AHL*-overexpressing transgenic lines during salt stress treatment. Seedlings of the *ahl* and *AHL*-RNAi lines showed a greater reduction in Cys accumulation under salt stress conditions compared with those of *AHL*-overexpressing transgenic lines (Fig. 5A). Furthermore, the results in Fig. 5B–E also show a distinct difference in transcription of sulfate metabolism pathway-related genes between the *ahl* mutant and *AHL*-overexpressing transgenic lines under salt stress conditions. Therefore, these data imply that AHL participates in salt stress tolerance in a Cys-dependent manner or through a sulfate metabolism-mediated pathway. In addition, exogenous application of Cys can rescue the salt stress-induced sensitive phenotype of *ahl* mutant and *AHL*-RNAi lines, such that their survival rates were similar to that of WT plants (Fig. 6). These results indicate that *AHL*-induced salinity tolerance is mainly dependent on Cys concentration.

Collectively, our results demonstrate that *AHL*-overexpressing transgenic lines exhibit enhanced salt stress tolerance. Under salt stress conditions, AHL positively regulates survival by increasing the expression of salt stress tolerance- and sulfate metabolism-associated genes. Based on Figs. 2B, 3A, B, 5A, the regulation of *AHL* expression is important for Pro, H_2O_2, and Cys biosynthesis in response to salt stress. Thus, AHL is linked to the regulation of Cys concentration for mediation of the salt stress response, although its biochemical and molecular functions have not yet been fully investigated. Further functional studies of AHL in Cys metabolism are needed to elucidate salt stress response in plants.

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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