

RESEARCH PAPER

Manipulation of arginase expression modulates abiotic stress tolerance in *Arabidopsis*: effect on arginine metabolism and ROS accumulation

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Abstract

Arginine is an important medium for the transport and storage of nitrogen, and arginase (also known as arginine amidohydrolase, ARGAH) is responsible for catalyse of arginine into ornithine and urea in plants. In this study, the impact of *AtARGAHs* on abiotic stress response was investigated by manipulating *AtARGAHs* expression. In the knockout mutants of *AtARGAHs*, enhanced tolerances were observed to multiple abiotic stresses including water deficit, salt, and freezing stresses, while *AtARGAH1*- and *AtARGAH2*-overexpressing lines exhibited reduced abiotic stress tolerances compared to the wild type. Consistently, the enhanced tolerances were confirmed by the changes of physiological parameters including electrolyte leakage, water loss rate, stomatal aperture, and survival rate. Interestingly, the direct downstream products of arginine catabolism including polyamines and nitric oxide (NO) concentrations significantly increased in the *AtARGAHs*-knockout lines, but decreased in overexpressing lines under control conditions. Additionally, the *AtARGAHs*-overexpressing and -knockout lines displayed significantly reduced relative arginine (% of total free amino acids) relative to the wild type. Similarly, reactive oxygen species accumulation was remarkably regulated by *AtARGAHs* under abiotic stress conditions, as shown from hydrogen peroxide (H₂O₂), superoxide radical (O₂⁻) concentrations, and antioxidant enzyme activities. Taken together, this is the first report, as far as is known, to provide evidence that *AtARGAHs* negatively regulate many abiotic stress tolerances, at least partially, attribute to their roles in modulating arginine metabolism and reactive oxygen species accumulation. Biotechnological strategy based on manipulation of *AtARGAHs* expression will be valuable for future crop breeding.

Key words: Abiotic stress, arginase, arginine metabolism, nitric oxide, polyamines, reactive oxygen species.

Introduction

As one of the most expensive nutrients to supply, nitrogen has crucial roles in plant growth, development, and production (Brauc *et al.*, 2012; Ma *et al.*, 2012). Arginine is an important amino acid in plants as a medium for the transport and storage of nitrogen and a precursor for the synthesis of other amino acids and polyamines (Flores *et al.*, 2008; Brauc

et al., 2012). Three pathways have been shown to be responsible for the metabolism of arginine in higher plants: (1) via arginine to ornithine and urea by arginase (also known as arginine amidohydrolase, ARGAH); (2) via arginine decarboxylase to agmatine and then, via agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase, to putrescine;

and (3) via arginine to nitric oxide (NO) and citrulline by NO synthase (Flores *et al.*, 2008; Gupta *et al.*, 2011; Brauc *et al.*, 2012). The reaction products may be further metabolized to other amino acids and polyamines, including proline, putrescine, spermidine, and spermine, all of which play crucial roles in wide developmental processes and in biotic and abiotic stress responses (Flores *et al.*, 2008; Wang *et al.*, 2011b; Brauc *et al.*, 2012; Shi *et al.*, 2012a,b). Polyamines (mainly putrescine, spermidine, and spermine) are scavengers of reactive oxygen species (ROS), and proline is one of the most common compatible metabolites to regulate cell-membrane stability and balance osmotic pressure under various stress conditions (Wimalasekera *et al.*, 2011; Brauc *et al.*, 2012).

As an important route for arginine metabolism, arginase enzyme activity has been described in many plant species, and arginase gene expression can be enhanced by wounding, methyl jasmonate, and the phytotoxin coronatine (Chen *et al.*, 2004). *AtARGAH1* (At4g08900) and *AtARGAH2* (At4g08870) have been cloned and identified in *Arabidopsis*. T-DNA insertion mutants of *AtARGAHs* result in increased NO accumulation and the propensity to form lateral and adventitious roots under the control of auxin signalling (Krumpelman *et al.*, 1995; Flores *et al.*, 2008). Furthermore, only the expression of *AtARGAH2* could be induced by methyl jasmonate and *Botrytis cinerea* (Brownfield *et al.*, 2008; Brauc *et al.*, 2012), and overexpression of *AtARGAH2* in *Arabidopsis* confers increased tolerance to *B. cinerea* (Brauc *et al.*, 2012). Additionally, *AtARGAH2* was found to be largely induced in auxin/cytokinin-triggered cellular proliferation, and enhanced callus development was observed in an *argah2* mutant during clubroot infection (Gravot *et al.*, 2012). Recently, Ma *et al.* (2012) found that the arginase encoded by *OsARG* is not only a key enzyme in arginine catabolism, but also that it plays critical roles in panicle development and grain production in rice, especially under insufficient exogenous nitrogen conditions.

To date, the *in vivo* role of *AtARGAHs* in plant tolerance to abiotic stress remains largely unknown. The present research investigated the influence of manipulation of *AtARGAHs* expression on arginine metabolism and ROS accumulation, as well as plant responses to abiotic stress including water deficit, salt, and freezing. How manipulation of arginase expression influences abiotic stress tolerance through arginine metabolism or ROS accumulation is also discussed.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia was used in this study. The mutants of *argah1* (SALK_057987) and *argah2* (SAIL_181_C11) were obtained from the *Arabidopsis* Biological Resource Center as described previously (Flores *et al.*, 2008). The *argah1argah2* double mutant was subsequently generated by crossing *argah1* and *argah2*. *Arabidopsis* seeds were sown in plastic containers filled with soil or on Murashige and Skoog (MS) medium containing 1% sucrose (w/v) in the growth chamber after stratification at 4 °C for 3 days in darkness. The growth chamber was controlled at an irradiance of about 120–160 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ at 22–25 °C with 65% relative humidity under a 16/8 light/dark cycle. Irrigated nutrient solution was given twice a week.

To characterize the effects of abiotic stress on *AtARGAHs* expression, 21-day-old seedlings grown on MS medium were transferred to fresh MS liquid medium supplemented with water (control), 30 μM abscisic acid (ABA), and 300 mM NaCl, or were subjected to dehydration and 4 °C stress treatments for 1 and 3 h, respectively.

Plant transformation and selection

To obtain the overexpressing transgenic lines, the full-length cDNA fragments of *AtARGAH1* and *AtARGAH2* were inserted into the modified pEGAD vector under the control of CaMV 35S promoter (Cutler *et al.*, 2000). The corresponding specific primers of the above constructions are listed in Supplementary Table S1 (available at JXB online). Transgenic plants were selected with BASTA resistance and were confirmed by PCR as previously described (Shi *et al.*, 2012b).

Semi-quantitative reverse-transcription PCR and quantitative real-time PCR

Total RNA was isolated from 200 mg leaves using TRIzol reagent (Invitrogen) and treated by RQ1 RNase-free DNase (Promega) to remove genomic DNA. First-strand cDNA was synthesized using reverse transcriptase (TOYOBO) from 2 μg total RNA, and 1 μl cDNA was added to 30 μl PCR mixture for semi-quantitative reverse-transcription PCR (RT-PCR). For quantitative real-time PCR, a CFX96 Real Time System (BIO-RAD) with SYBR-green fluorescence and the comparative $\Delta\Delta\text{CT}$ method was used as previously described (Shi *et al.*, 2012b). All experiments were repeated at least three times and the expression levels were standardized with UBQ10. The specific primers of analysed genes are listed in Supplementary Table S1.

Determination of arginine concentration and total free amino acids

For amino acid analysis, 0.3 g 2-week-old plant leaves were ground and resuspended in 1 ml water as previously described (He *et al.*, 2004). After centrifugation at 15,294 g for 15 min, the supernatant was filtered with a 0.22 μm nylon filter. Arginine concentration was quantified by the formation of stable yellow species on the addition of thymol-sodium hypobromite reagent under alkaline conditions (Sastry and Tummuru, 1984). Total free amino acids were measured using Cd-nihydrin method as described previously (Fisher *et al.*, 2001).

Determination of endogenous NO and NO synthase activity

The endogenous NO content of *Arabidopsis* leaves was analysed by labelling with the NO-sensitive fluorescence dye 3-amino,4-amino-methyl-2',7'-difluorescein diacetate (DAF-FM-DA) and quantified by the haemoglobin assay as described previously (Shi *et al.*, 2012b). The fluorescence image of plant leaves labelled with NO was taken from fluorescence stereomicroscope (DP72, Olympus), and the NO content was quantified using a fluorometer (Molecular Device Company, Spectra Max M2). All fluorescences were measured at excitation of 485 nm and emission of 510 nm. For the haemoglobin assay, the NO content in leaf extracts was quantified spectrophotometrically by measuring the conversion of oxyhaemoglobin to methaemoglobin as previously described (Murphy and Noack, 1994). NO synthase activity was determined by adding assay mixture (NADPH, L-arginine, Ca^{2+} -CaM, BH_4 , FAD, FMN, oxygen) in the presence and absence of excess NO synthase inhibitor as described previously (Shi *et al.*, 2012b).

Quantification of polyamines

Polyamines in 2-week-old plant leaves were extracted and quantified by HPLC as previously described (Walter and Geuns, 1987) with minor modifications. Briefly, the column (length 15 cm, internal

diameter 4.6 mm) was filled with 5 μm reversed-phase material (HeSep C18-T, Weltech, Wuhan) and heated at 45 °C for analysis. The solvent was acetonitrile:H₂O (72:28) with flow 1.2 ml/min and the injection volume was 30 μl .

Assessment of salt, water deficit, and freezing tolerance

Wild-type, *AtARGAHs*-overexpressing lines, and *argah* mutant plants were grown in the growth chamber in soil for 2 weeks. For water deficit stress treatment, 2-week-old plants were subjected to water deficit condition by withholding water for 18 d and then rewatered for 10 d. For salt stress treatment, the NaCl concentration was increased stepwise by 50 mM every 2 d to the indicated concentration for the next 4 weeks, as previously described (Chan *et al.*, 2011). The whole-plant freezing test was analysed as described by Verslues *et al.* (2006) with some modifications. Briefly, 2-week-old seedlings were cold acclimated at 4 °C in the light for 1 week, then the plants were placed in a growth chamber set to -1 °C for 16 h. Ice chips were sprinkled on these plants before the chamber was programmed to cool at 1 °C per hour until -6 °C was reached. Plants were removed after being frozen, thawed at 4 °C for 12 h in the dark, and then transferred to a growth chamber at 22 °C. The survival rate was scored 7 d later.

Measurement of electrolyte leakage, lipid peroxidation, and leaf water status

For the determination of electrolyte leakage, about 0.1 g plant leaves were incubated in 10 ml deionized water, shaken on a gyratory shaker at room temperature for 6 h at about 150 rpm. First, the initial conductivity (C_i) was measured using a conductivity meter (Leici-DDS-307A, Shanghai, China). The samples were then boiled for 20 min to kill the leaf tissues and completely induce all electrolytes of the solution. After cooling to room temperature, the conductivity of the killed tissues (C_{max}) was determined. The relative electrolyte leakage could be calculated as $(C_i/C_{\text{max}}) \times 100\%$.

As major indicator of lipid peroxidation, malondialdehyde (MDA) content was determined using the thiobarbituric acid (TBA) method as previously described (Shi *et al.*, 2012b). Briefly, 1 g plant leaves were incubated in 5 ml TBA reagent (0.25%, w/v, TBA in 10%, w/v, trichloroacetic acid), and then heated at 100 °C for 20 min. After cooling to room temperature, the MDA content of sample was calculated by subtracting the non-specific absorption at 600 nm from the absorbance of the sample supernatant at 532 nm.

For water loss rate assay, the detached leaves were quantified initial fresh weight (FW) and placed into growth chamber. The FW was recorded every 30 min for 8 h and water loss rate was calculated from the relative decrease in FW. For stomatal aperture assay, plant leaves were incubated in stomatal opening buffer (5 mM MES, 10 mM KCl, 50 mM CaCl₂, pH 6.15) for 2 h. After treatment, stomatal aperture width was measured in stereomicroscope (DP72, Olympus) equipped with a CCD camera, and at least 100 stomata were tested for each line.

Determination of ROS accumulation and assay of antioxidant enzyme activities

Hydrogen peroxide (H₂O₂) and superoxide radicals (O₂⁻) were stained using diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively, according to Wang *et al.* (2011b). For H₂O₂ detection, the seedlings were immersed in NBT solution (1 mg ml⁻¹) in 10 mM phosphate buffer (pH 7.8) at room temperature for 8 h. For localization of v, the *Arabidopsis* seedlings were incubated in DAB solution (pH 3.8, 1 mg ml⁻¹) at room temperature for 8 h. The stained seedlings were then transferred to 70% (v/v) ethanol to remove chlorophyll and visualize the blue and brown spots for H₂O₂ and O₂⁻, respectively. The H₂O₂ content was quantified as

described previously (Shi *et al.*, 2012c) to confirm the DAB staining. Briefly, 1 ml plant extract using 50 mM sodium phosphate buffer (pH 7.8) was mixed with 1 ml of 0.1% (w/v) titanium sulphate (in 20%, v/v H₂SO₄) thoroughly for 10 min. After centrifugation at 15,294 g for 10 min, the absorbance of the supernatant was measured at 410 nm. The concentration of proteins was quantified using the Bradford method (Bradford, 1976). Catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), and peroxidase (POD, EC 1.11.1.7) activities were analysed as described previously (Shi *et al.*, 2012c).

Statistical analysis

All experiments were repeated at least three times in independent experiments, and the leaf samples of independent experiments were harvested from at least 30 seedlings per genotype. In these results, Student's t-test was used to determine the significant difference between the wild type and the different *AtARGAHs* lines at $P < 0.05$.

Results

The expression patterns of *AtARGAHs* after abiotic stress treatments

First, this study examined the expression patterns of *AtARGAHs* in response to exogenous stress treatments including abscisic acid, dehydration, NaCl, and cold. When subjected to dehydration stress for 1 and 3 h, transcript levels of *AtARGAH1* and *AtARGAH2* were largely activated, as well as significantly by abscisic acid, NaCl, and cold stresses at 1 and 3 h after treatments (Fig. 1). The induced expression of *AtARGAHs* under these stress conditions suggested the role for *AtARGAHs* in tolerance to these stresses, especially in response to dehydration stress.

Characterization of *AtARGAHs*-overexpressing and -knockout lines

To investigate the biological function of *AtARGAHs* in plants, previously described T-DNA insertion lines for *argah1* and *argah2* (Flores *et al.*, 2008) were isolated in this study and an *argah1argah2* double mutant was generated by crossing *argah1* and *argah2*. There was no detectable expression of *AtARGAH1* and *AtARGAH2* in homozygous mutants, suggesting that *argah1*, *argah2*, and *argah1argah2* are null alleles (Fig. 2A and B). To further assess the role of *AtARGAHs*, this study also generated *AtARGAH1*- and *AtARGAH2*-overexpressing transgenic lines and confirmed the overexpression of *AtARGAH1* or *AtARGAH2* at the mRNA level in these transgenic lines. All three independent overexpressing lines showed significantly higher mRNA levels compared with the wild type (Fig. 2A and B).

Effect of *AtARGAHs* expression on arginine concentration

Through quantification of arginine concentration, no significant differences were observed between the wild type and the *AtARGAHs*-knockout and -overexpressing lines when

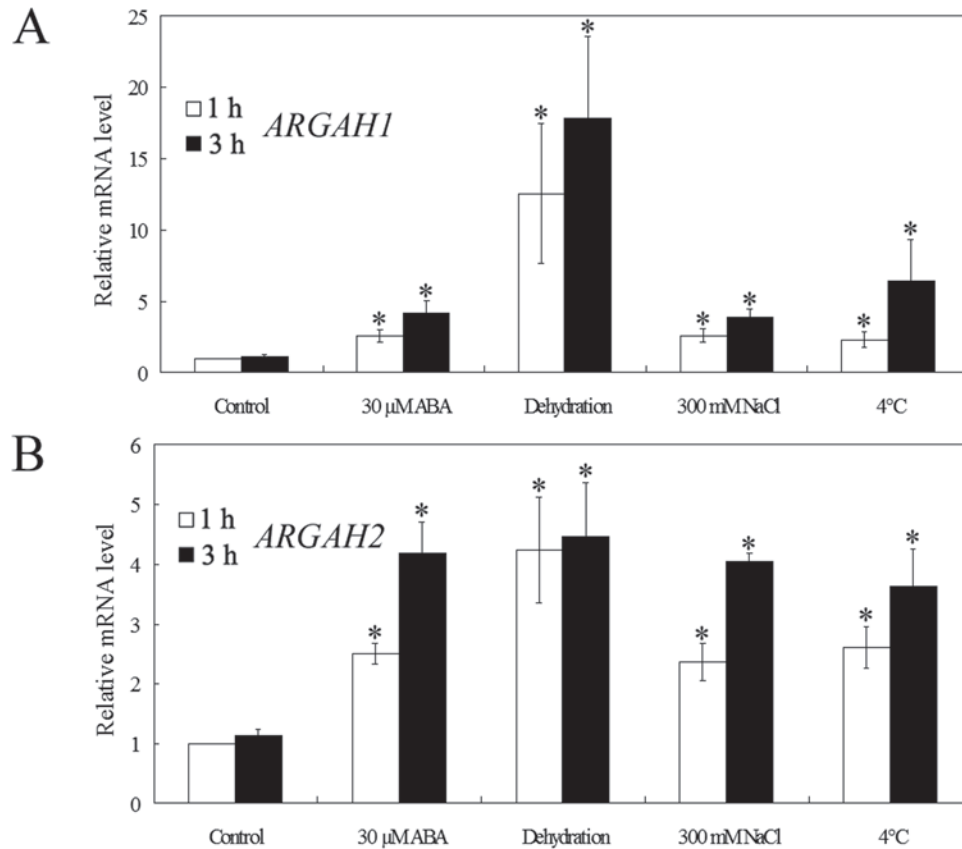


Fig. 1. Expression levels of AtARGAHs after abiotic stress treatments by quantitative real-time PCR. The expression patterns of AtARGAH1 (A) and AtARGAH2 (B) were determined after control, ABA, dehydration, NaCl, and 4 °C treatments for 1 and 3 h. The expression levels at 1 h under control conditions were normalized as 1.0. Values are mean \pm SD of three or more independent experiments.

the data was expressed as $\mu\text{M (g FW)}^{-1}$ (Supplementary Fig. S1A). However, these lines had higher concentrations of total free amino acids than the wild type (Supplementary Fig. S1B). Therefore, the relative arginine contents (% of total free amino acids) in different AtARGAHs lines were significantly lower than the wild type (Fig. 2C).

Influence of AtARGAHs expression on NO content

NO accumulation was significantly enhanced in the roots of *argah1-1* and *argah2-1* (Flores *et al.*, 2008). However, NO synthase activity changes in these mutants were still unknown. Here, the results showed that *argah1*, *argah2*, and *argah1argah2* mutants had significantly higher NO synthase activities than the wild type, while the AtARGAH1- and AtARGAH2-overexpressing lines exhibited relatively lower NO synthase activities (Fig. 3A). Both of the DAF-FM-DA and haemoglobin assays showed that *argah1*, *argah2*, and *argah1argah2* mutants had higher levels of NO emission in the leaves, with at least 40% increased NO accumulation than the wild type (Fig. 3B and C). In contrast, the AtARGAH1- and AtARGAH2-overexpressing lines only showed about 50% NO relative to the wild type (Fig. 3B and C). These results indicated that both AtARGAH1 and AtARGAH2 could negatively regulate NO synthase activity and the intracellular NO content of plant.

Influence of AtARGAHs expression on polyamine concentrations

Besides NO synthase activity and NO content, this study also examined the influence of AtARGAHs expression on gene expression of arginine decarboxylase pathway and polyamines. Quantitative real-time PCR results indicated that the AtARGAHs-knockout mutants exhibited much higher expression levels of *AIH* (agmatine iminohdrolase), *NLPI* (N-carbamoylputrescine amidohydrolase), and *ADC1* and *ADC2* (arginine decarboxylases), whereas the AtARGAH1- and AtARGAH2-overexpressing lines had significantly lower mRNA levels when compared to the wild type (Fig. 4A). Although spermidine content showed no significant differences between AtARGAH lines and the wild type, putrescine and spermine concentrations were significantly higher in the AtARGAHs-knockout mutants, and significantly lower in the AtARGAH1- and AtARGAH2-overexpressing lines when compared to the wild type (Fig. 4B and C). These observations indicated that both AtARGAH1 and AtARGAH2 could negatively regulate polyamines through arginine decarboxylase pathway.

Performance of AtARGAHs-overexpressing and -knockout lines under water deficit and salt stress

To dissect whether AtARGAHs could play roles in response to water deficit stress, a water deficit stress tolerance test was

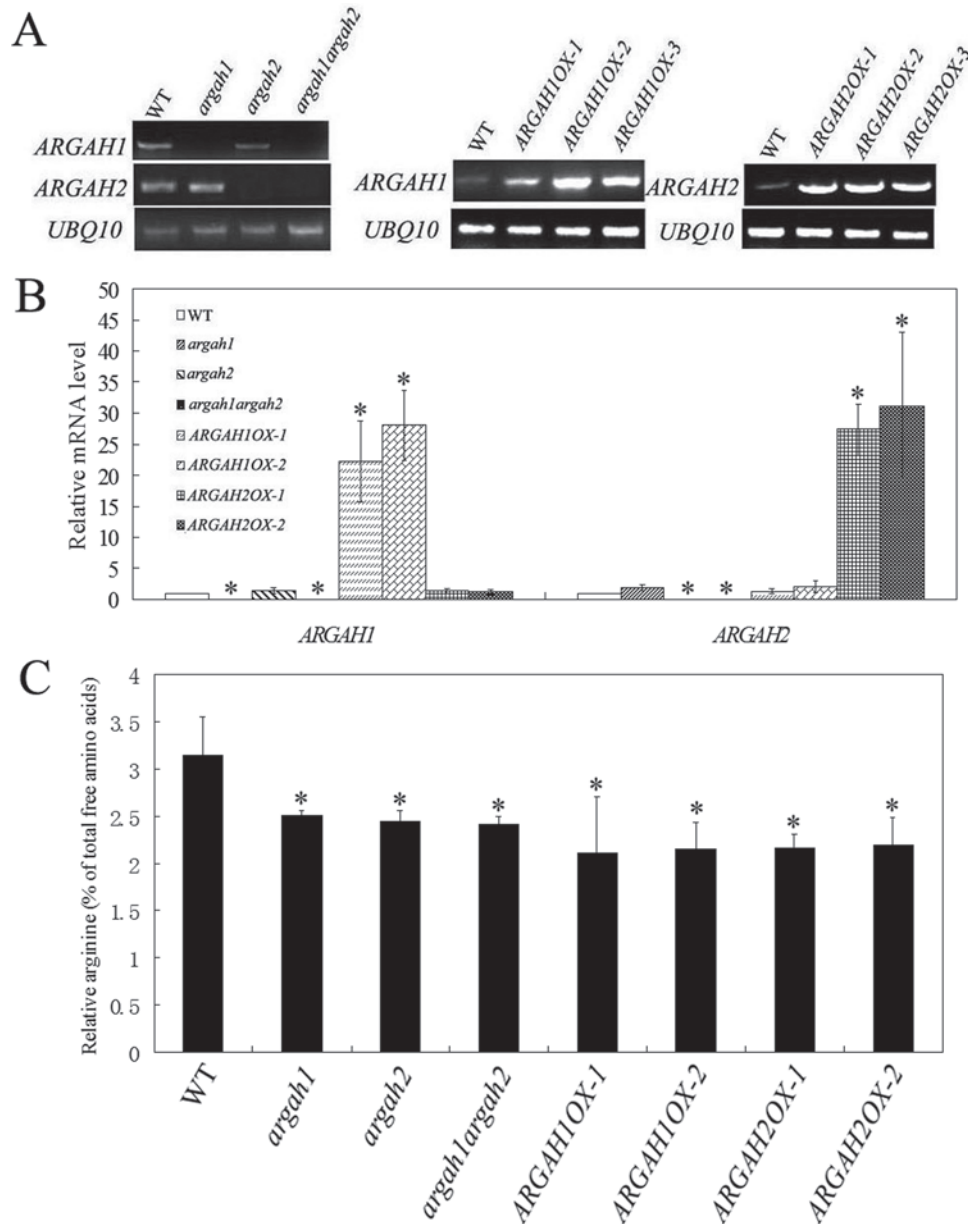


Fig. 2. Effect of *AtARGAHs* expression changes on arginine concentration. (A and B) *AtARGAHs* expression in T-DNA insertion mutants and transgenic overexpressing plants by semi-quantitative RT-PCR (A) and quantitative real-time PCR (B). *UBQ10* was used as a control. (C) Relative arginine (% of total free amino acids) in 2-week-old plant leaves. Values are mean \pm SD of three or more independent experiments.

conducted. As typical physiological indices for estimation of stress tolerance in plants (Wang *et al.*, 2011a), electrolyte leakage and survival rate were also determined to assess plasma membrane damage and stress tolerance among different plants. After water deficit stress, about 60% of the wild-type plants died, while 70% of the *AtARGAHs*-overexpressing lines died with higher level of electrolyte leakage when compared with the wild type (Fig. 5A–C). As for the *argah1*, *argah2*, and *argah1argah2* mutants, only 20–30% plants died under water deficit stress and the relative electrolyte leakages were remarkably lower than the wild type (Fig. 5A–C). Leaf water loss, another important parameter for evaluating plant

tolerance to water deficit stress, was also examined among these lines. Consistently, all *argah1*, *argah2*, and *argah1argah2* mutants had reduced water loss in comparison with the wild type, whereas the *AtARGAHs*-overexpressing lines lost more water than the wild-type plants (Fig. 5D). Furthermore, *argah1*, *argah2*, and *argah1argah2* mutants showed significantly less stomatal aperture width than the wild type, while the *AtARGAHs*-overexpressing lines did not (Fig. 5E). As the open/closed status of stomata is related to water loss, so the less stomatal aperture width might explain the lower water loss rate of the mutants, and the good water status of the mutants might reasonably contribute to the enhanced tolerance.

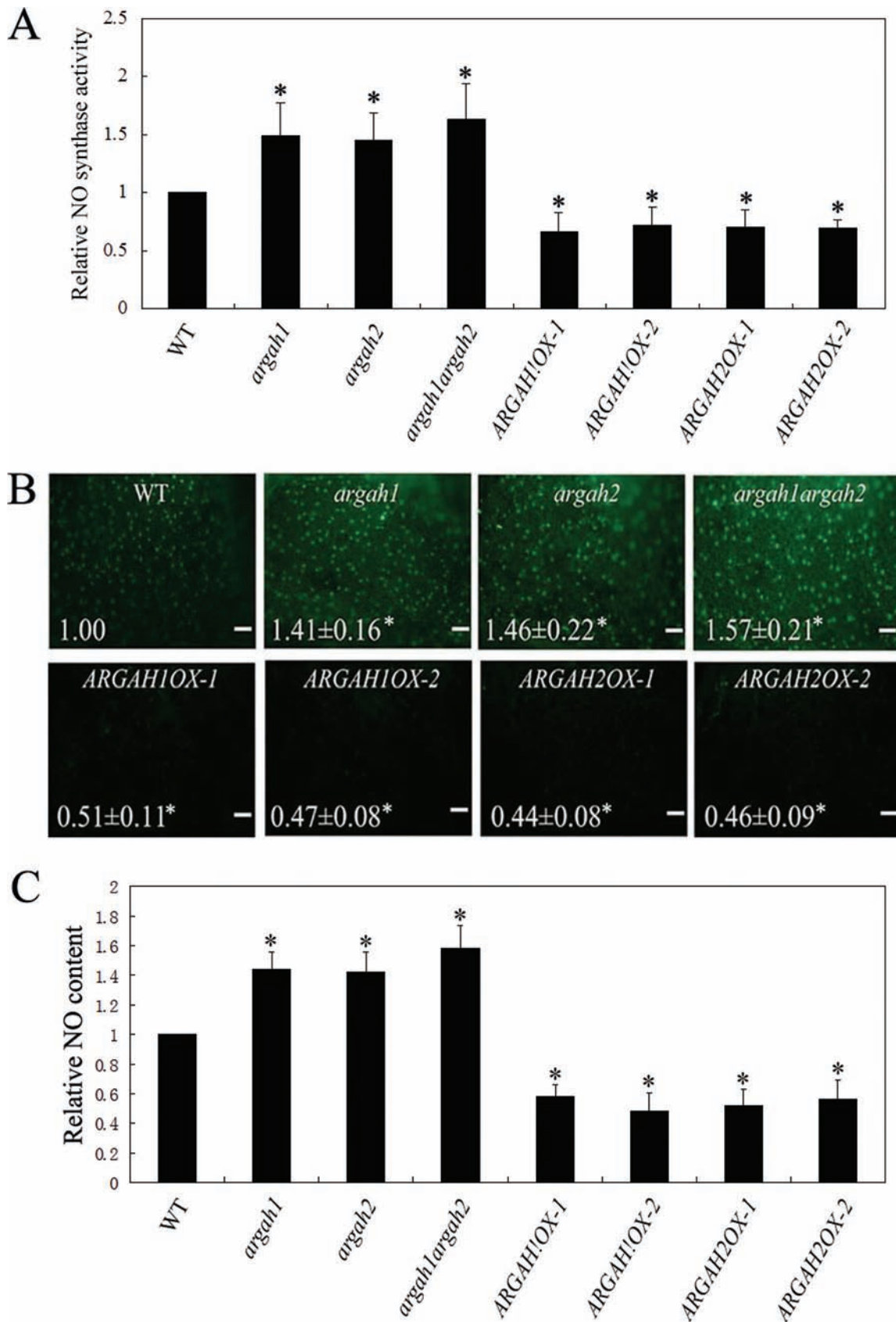


Fig. 3. Influence of AtARGAHs expression changes on NO synthase activity and NO content. (A) NO synthase activity in leaves of the wild type and the different AtARGAHs lines by the haemoglobin assay. (B) NO content in leaves of the wild type and the different AtARGAHs lines by the DAF-FM-DA assay. Bars = 50 μ m. (C) NO content in leaves by the haemoglobin assay. Values for the wild type were normalized as 1.0. Values are mean \pm SD of three or more independent experiments (this figure is available in colour at JXB online).

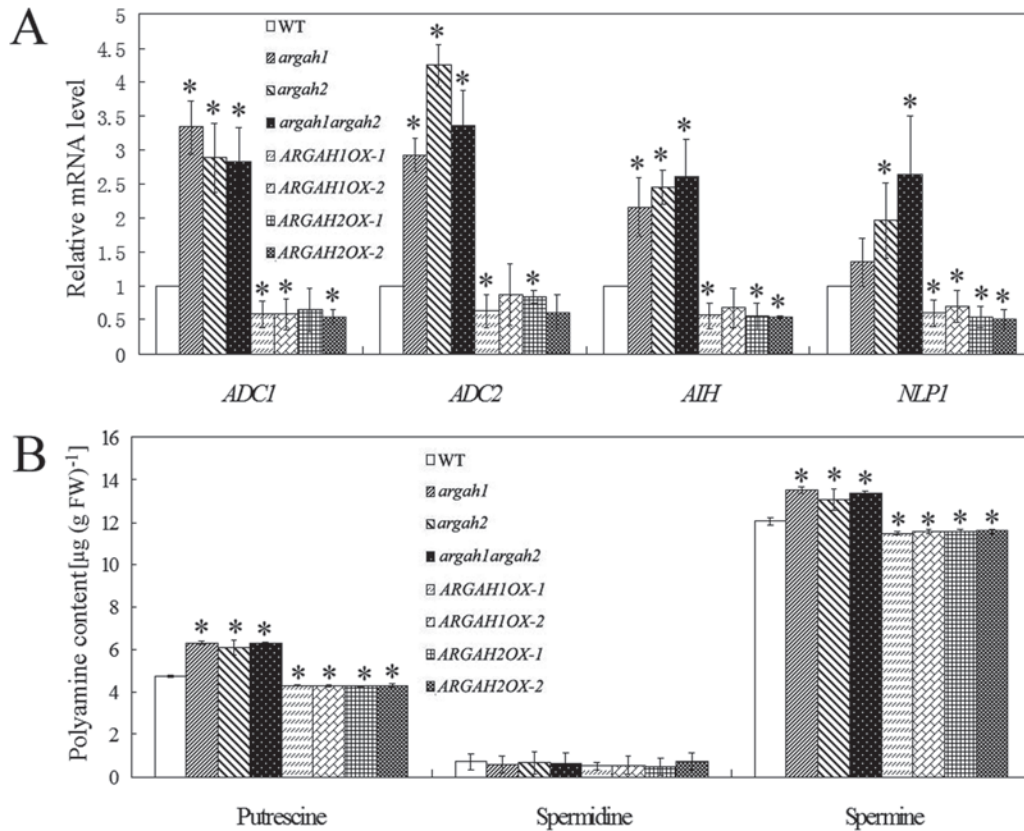


Fig. 4. Effect of *AtARGAHs* expression changes on polyamines concentrations. (A) Real-time PCR analysis of the expressions of *ADC1*, *ADC2*, *AIH*, and *NLP1* in 2-week-old plant leaves (three or more independent experiments). (B) Quantification of putrescine, spermidine, and spermine in 2-week-old plant leaves using HPLC. Values are mean \pm SD. Seeds from different lines were sown once and HPLC analysis was performed in triplicate using different leaf materials.

For salt stress treatment, 150mM NaCl was imposed on 2-week-old plants for 4 weeks. Under normal growth conditions, all *ARGAHs*-overexpressing and -knockout lines showed no significant difference in comparison to the wild-type plants in terms of the relative electrolyte leakage and survival rate (Fig. 5A–C). When 150mM NaCl was applied, nearly 70% of the wild-type plants were dead, whereas more than half of the *AtARGAHs*-knockout lines survived with relatively lower electrolyte leakage (Fig. 5A–C). On the contrary, more than 80% of the *AtARGAHs*-overexpressing lines were also dead, with higher electrolyte leakage than the wild type (Fig. 5A–C).

Additionally, at harvest, all mutant lines of *AtARGAHs* had significantly greater plant height and dry weight than the wild type under both water deficit and salt stress, whereas the overexpressing lines had much lower plant height and less dry weight compared with the wild type (Supplementary Fig. S2).

In summary, the expression repression of *AtARGAHs* displayed improved water deficit and salt tolerance, as confirmed by changes in physiological parameters including reduced electrolyte leakage, survival rate, water loss rate, and stomatal aperture. The relationship between the expression levels of *AtARGAHs* and plant tolerance to abiotic stress might be valuable for future crop breeding.

Freezing tolerance of *AtARGAHs*-overexpressing and -knockout lines

Besides water deficit and salt stresses, 2-week-old wild-type and different *AtARGAHs* plants were also used in a freezing tolerance test. After recovery from -6°C for 7 d, all *AtARGAHs* mutant lines exhibited better leaf morphology and higher survival rate than the wild-type plants, whereas the *AtARGAHs*-overexpressing lines showed the lowest survival rate (Fig. 6A and B). As the lipid peroxidation product, MDA is an important indicator of oxidative damage (Shi *et al.*, 2012b). Under freezing, MDA contents decreased in *argah* single and double mutants, but increased in the *AtARGAHs*-overexpressing lines when compared with the wild type. The changes in MDA content among the knockout mutants and overexpressing lines were correlated with the results of the freezing stress tolerance test (Fig. 6B and C).

AtARGAHs modulate ROS metabolism under stress conditions

As two major indicators of ROS accumulation that can be induced by many environmental stresses, H_2O_2 and O_2^- concentrations were determined through DAB and NBT staining, respectively. Under control conditions, no significant

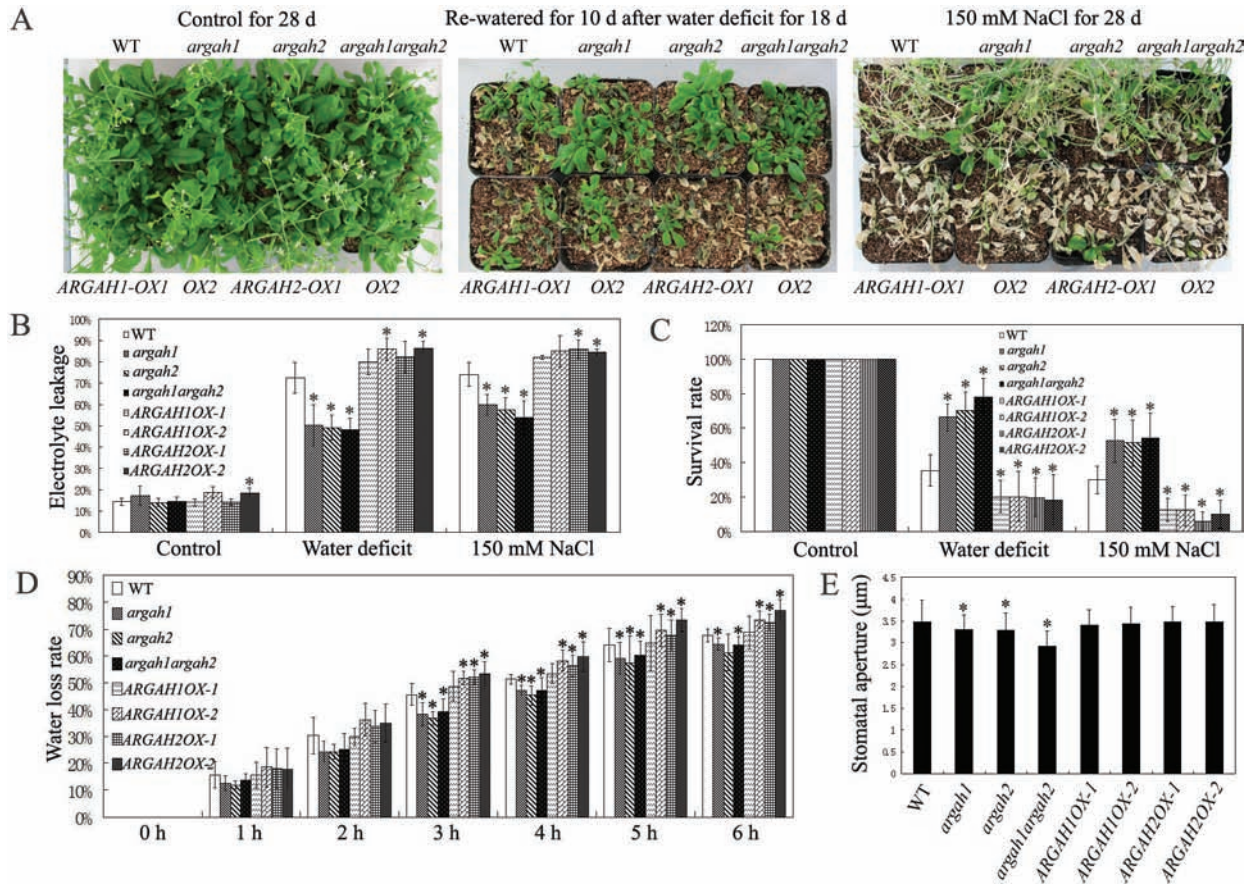


Fig. 5. Performance of the wild type and the different *AtARGAHs* lines under water deficit and salt stress conditions. (A) Two-week-old plants after 28 d under control conditions, after 10 d recovery after water deficit treatment for 18 d, and after 28 d after 150mM NaCl treatment. (B) Electrolyte leakage of plants under control, water deficit, and salt stress conditions for 12 d. (C) Survival rate of plants under control, water deficit, and salt stress conditions for 4 weeks. (D) Leaf water loss rate of the wild type and the different *AtARGAHs* lines at ambient environment. (E) Stomatal aperture width of leaves from the wild type and the different *AtARGAHs* lines. Values are mean \pm SD of three or more independent experiments (this figure is available in colour at *JXB* online).

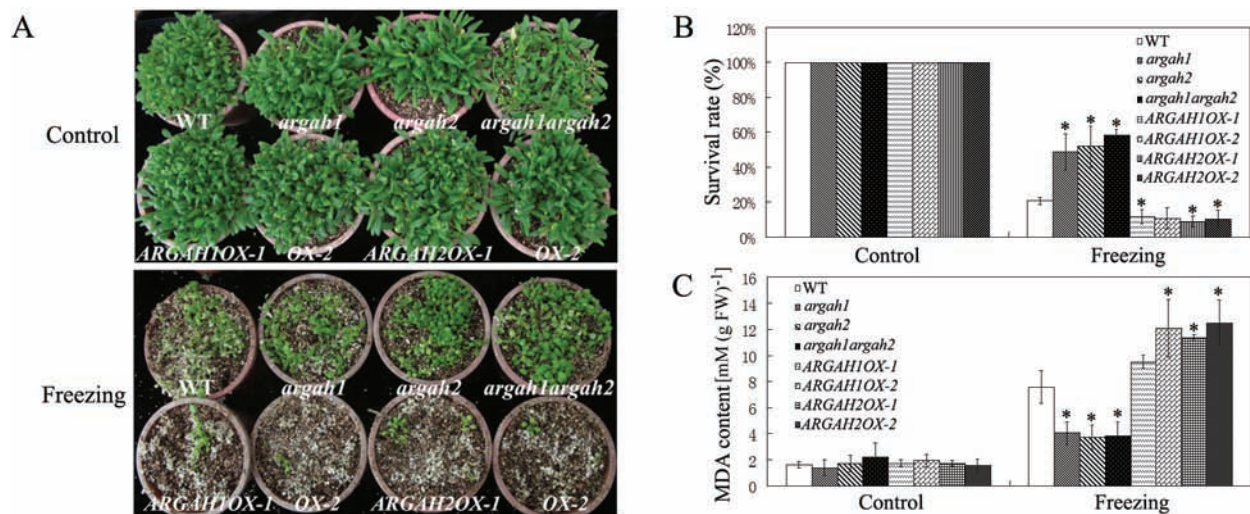


Fig. 6. Freezing tolerance of the wild type and the different *AtARGAHs* lines. (A) Phenotype of plants under control condition for 14 d and after 7 d recovery from freezing treatment. (B) Survival rate of the wild type and the different *AtARGAHs* lines under freezing stress condition. Survival rate was determined at 7 d after recovery from freezing stress test. (C) MDA content of the wild type and the different *AtARGAHs* lines under control conditions and at 7 d after 4 °C treatment. Values are mean \pm SD of three independent experiments (this figure is available in colour at *JXB* online).

difference was observed among the wild type and the different *AtARGAHs* lines (Fig. 7A). When water deficit, salt, and freezing stresses were applied, less blue and brown spots for H_2O_2 and O_2^- were stained in the seedlings of *AtARGAHs* mutant lines than the wild type, while deeper staining for H_2O_2 and O_2^- was observed in the *AtARGAHs*-overexpressing lines (Fig. 7A). Consistently, the H_2O_2 quantification assay confirmed the results of DAB staining that *argah* mutant lines accumulated less H_2O_2 while the *AtARGAHs*-overexpressing lines exhibited higher H_2O_2 content when compared with the wild type under stress conditions (Fig. 7B).

To analyse the correlation between the ROS accumulation and the activities of antioxidant enzymes in different *AtARGAHs* lines, the SOD, CAT, and POD activities were assayed under the same stress conditions. Under control conditions, no

significant differences were observed among the different *AtARGAHs* lines and the wild-type plants (Fig. 7C–E). When subjected to water deficit, salt, and cold stresses, *argah* lines showed relatively lower SOD, CAT, and POD activities when compared with the wild-type plants, while the *AtARGAHs*-overexpressing lines exhibited significantly higher SOD, CAT, and POD activities relative to the wild type (Fig. 7C–E). These results indicated that the involvement of *AtARGAHs* in ROS homeostasis through regulation of antioxidant enzymes might be indirectly responsible for abiotic stress tolerance.

Discussion

Arginine is an essential metabolite in nitrogen distribution through its catabolism, which has clearly been shown that

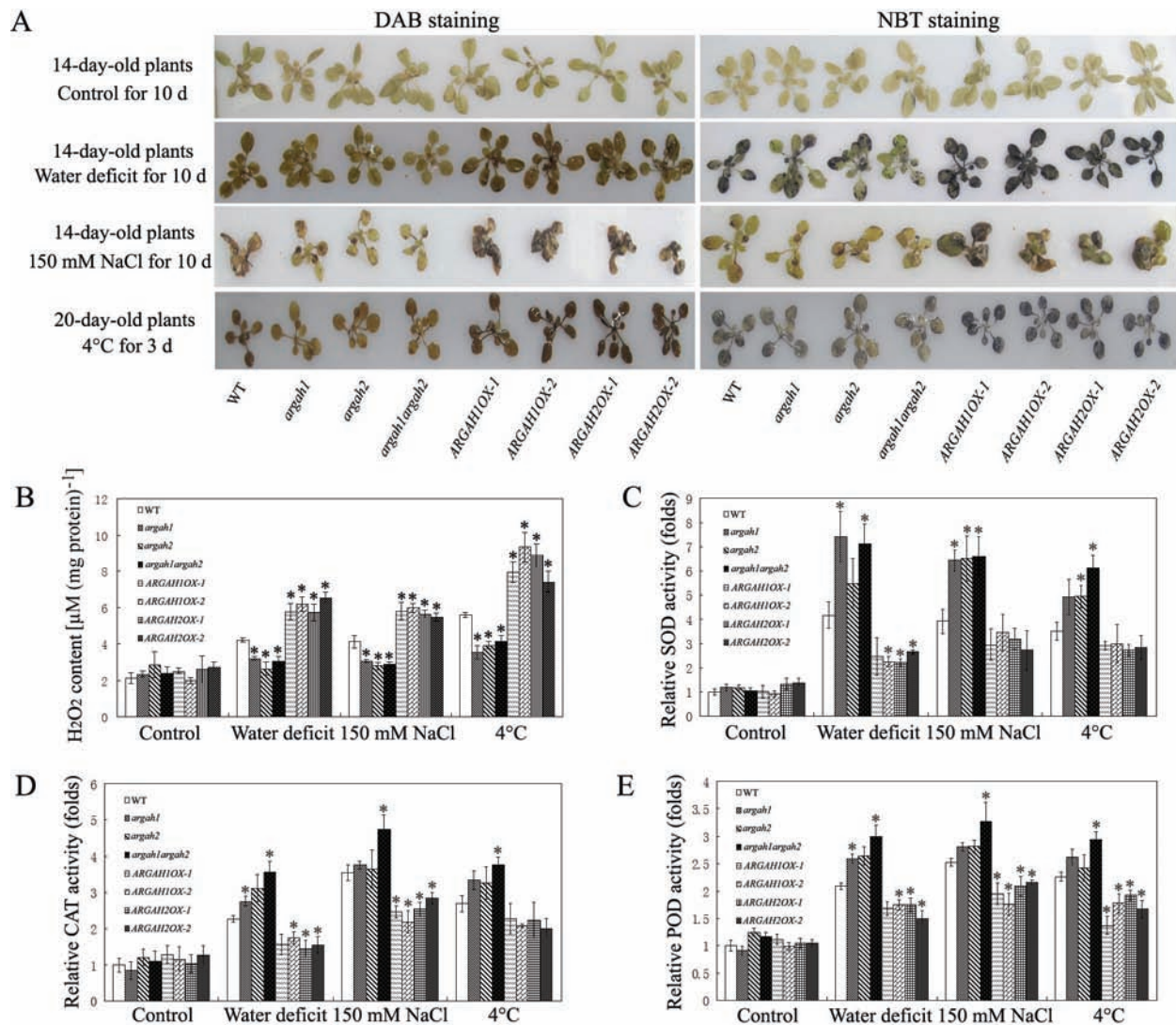


Fig. 7. ROS accumulation and antioxidant enzyme activities of the wild type and the different *AtARGAHs* lines under control and abiotic stress conditions. (A) DAB and NBT staining for H_2O_2 and O_2^- concentrations in the leaves of plants under control and stress conditions. (B) Quantification of H_2O_2 content regulated by *AtARGAHs* lines under control and abiotic stress conditions. Fourteen-day-old plants were subjected to control conditions and water deficit and 150 mM NaCl for 10 d, and 20-day-old plants were treated at 4 °C for 3 d. (C–E) Relative activities of SOD (C), CAT (D), and POD (E) quantified as fold-change relative to the wild type under control conditions. Values are mean \pm SD of four independent experiments (this figure is available in colour at JXB online).

arginase, arginine decarboxylase, and NO synthase can metabolize arginine to other amino acids such as putrescine, spermidine, spermine, and proline (Flores *et al.*, 2008; Brauc *et al.*, 2012). Although the NO synthase protein has not been identified in plants except in two strains of the green alga *Ostreococcus tauri*, NO synthase-like activity has been detected in many higher plants, and inhibitors of mammalian NO synthase have been shown to inhibit NO generation in plants (Gupta *et al.*, 2011; Shi *et al.*, 2012b). Arginine supply could induce intracellular NO content in *Arabidopsis*, and Flores *et al.* (2008) concluded a model that posits a possible NO synthase-like activity, generating NO directly from arginine. In another recent report (Brauc *et al.*, 2012), reduced arginase activity in the *Arabidopsis* transgenic lines also led to a significant increase in putrescine concentration for up to 40%. Previous research showed that polyamines could induce rapid biosynthesis of NO in root tip and primary leaves of *Arabidopsis* seedlings (Tun *et al.*, 2006; Siddiqui *et al.*, 2011; Wimalasekera *et al.*, 2011). Therefore, the mutational elimination of *AtARGAHs* can conceivably make more arginine available for the synthesis of polyamines and NO, resulting in more polyamines and NO accumulation. This study confirmed the hypothesis that *argah* lines accumulated significantly higher concentrations of polyamines (putrescine and spermine) and NO, while *AtARGAH*-overexpressing lines exhibited lower arginine, putrescine, spermine, and NO concentrations relative to the wild type (Figs. 2–4). The large changes in polyamines and NO concentrations in *AtARGAHs* transgenic lines and mutants might result in changes at the physiological, biochemical, molecular, and cellular levels.

Interestingly, the *AtARGAHs*-knockout and -overexpressing lines showed relatively less arginine accumulation than the wild type (Fig. 2C). Consistently, Flores *et al.* (2008) found that *argah1* and *argah2* mutants had lower relative arginine pools. Brauc *et al.* (2012) also reported that both *AtARGAH2*-overexpressing and -silencing lines displayed significantly reduced relative arginine relative to the wild type. The decreased relative arginine in all *AtARGAHs*-overexpressing lines indicated a direct impact of *AtARGAHs* on arginine pools. More surprising was the decreased relative arginine in the *AtARGAHs*-knockout lines; Flores *et al.* (2008) speculated that localized arginine concentrations might be affected after mutation of *AtARGAHs* and proposed that more intramitochondrial or other specific localized arginine concentrations might be available for *atargah* mutants, which were further metabolized to NO and polyamines.

Both polyamines and NO function as stress signals in plant, mediating plant development and a range of biotic and abiotic stresses (Zhao *et al.*, 2007; Neill *et al.*, 2008; Lozano-Juste and León, 2010; Wang *et al.*, 2011b). NO may be a link between polyamine-mediated stress responses and other stress mediators, and multiple abiotic stresses may use NO as a mediator where polyamines are also involved (Tun *et al.*, 2006; Neill *et al.*, 2008). Recently, the present study group found that increasing NO content in *Arabidopsis* by expressing rat neuronal NO synthase conferred enhanced multiple stress tolerances (Shi *et al.*, 2012a,b). These results together

with others (Zhao *et al.*, 2007, 2009; Corpas *et al.*, 2009) indicated that proper levels of NO might be necessary and beneficial for plants in responses to several abiotic stresses and resistance against pathogenic bacteria. To confirm this hypothesis, the *AtARGAHs*-overexpressing and -knockout lines were used for abiotic stress tolerance test in this study. The underlying mechanisms were also dissected.

As shown in Figs. 5 and 6, the results indicated that *AtARGAHs* could modulate multiple abiotic stress tolerances. The expression repression of *AtARGAHs* in *Arabidopsis* exhibited improved water deficit, salt, and freezing tolerances, while overexpression of *AtARGAHs* showed the opposite effect on stress tolerances. All these effects were quite evident in the changes of physiological parameters including cell-membrane damage (electrolyte leakage), survival rate, water loss rate, stomatal aperture, and lipid peroxidation (MDA) (Figs. 5 and 6). Consistently, pharmacological studies and genetic results have both suggested that properly increased NO content resulted in enhanced biotic and abiotic stress tolerances, whereas NO-deficiency mutants were more sensitive to salt, freezing, and disease tolerance (Zhao *et al.*, 2007, 2009; Shi *et al.*, 2012a,b). Collectively, these observations emphasize the essential role of NO in plant stress response. Plants with proper NO levels could improve the tolerance to abiotic stress, while less NO levels may result in decreased stress tolerances. To date, many previously discovered mutants with altered NO levels, such as *atnoa1*, *atnia1atnia2*, and *atnox1*, have negative effects on plant development and yield even under control conditions, which may be attributed to unspecified roles in NO synthesis (He *et al.*, 2004; Shi *et al.*, 2012b). Interestingly, in the present study, *AtARGAHs* lines did not show any apparent negative effects on plant development (plant height and dry weight) under control conditions (Supplementary Fig. S2). Therefore, this report together with previous research (Shi *et al.*, 2012a,b) may expand the possibilities for crop breeding through manipulating *ARGAHs* expression or *in vivo* NO levels.

Most of the environmental stresses such as water deficit, salt, and freezing can cause rapid and excessive accumulation of ROS, including H₂O₂, O₂⁻, and hydroxyl radical (·OH) (Zhu, 2002; Apel and Hirt, 2004; Mittler *et al.*, 2004). ROS homeostasis is very important for plant stress tolerance because over production of ROS can lead to oxidative damage and cell death. In plant, abiotic stress-induced damage can be attenuated by different ways of inhibiting ROS accumulation (Mittler, 2002; Apel and Hirt, 2004; Mittler *et al.*, 2004). Compared with the wild type, the weaker DAB and NBT staining for H₂O₂ and O₂⁻ under stress conditions were observed in *argah1*, *argah2*, and *argah1argah2* mutants (Fig. 7A and B), indicating less ROS accumulation of these mutants when subjected to abiotic stress conditions. Consistently, this study also observed that manipulation of *AtARGAHs* expression regulated the antioxidant enzyme activities including SOD, CAT, and POD under multiple abiotic stresses (Fig. 7C–E). Among these antioxidant enzymes, SOD catalyses O₂⁻ into H₂O₂ and O₂ at the first stage of the antioxidant defence system, while CAT and POD are also essential for break down of H₂O₂ (Mittler, 2002; Apel

and Hirt, 2004; Mittler *et al.*, 2004). All these antioxidant enzymes can protect plant cells from ROS injury and further help them to cope with different stresses. Thus, *AtARGAHs* may have important functions in modulating ROS accumulation and antioxidant enzyme activities, which are important components to alleviate oxidative stress-induced damages in plant responses to many environmental stresses.

As an important enzyme in arginine metabolism, arginine decarboxylase has been widely shown to be involved in abiotic stress responses (Wang *et al.*, 2011a,b; Wimalasekera *et al.*, 2011). In many plant species, including rice, tobacco, *Arabidopsis*, and tomato, overexpression of arginine decarboxylase that increases polyamine concentrations enhanced plant tolerance to water deficit, salt, and freezing stresses, partly due to its role in the regulation of ROS accumulation (Wang *et al.*, 2011a,b; Wimalasekera *et al.*, 2011). However, the role of arginase, another important enzyme involved in arginine metabolism, in abiotic stress remains largely unknown. In this study, the knockout mutants and *AtARGAHs*-overexpressing transgenic lines showed opposite effects on the accumulation of direct downstream arginine catabolism products including polyamines (putrescine and spermine) and NO (Figs. 3C and 4). These lines also showed opposite responses to water deficit, salt, and freezing stresses (Figs. 5 and 6), as well as opposite effect to maintain homeostasis of ROS in response to these stresses. Polyamines and NO may also have complex mechanisms to deal with ROS and other cellular responses derived from environmental stresses (Bright *et al.*, 2006; Siddiqui *et al.*, 2011; Wimalasekera *et al.*, 2011). Application of NO donors can alleviate oxidative stresses induced by NaCl, chilling, iron deficiency, and seed desiccation by enhancing the activities of antioxidative enzymes and accumulation of osmolytes (Bethke *et al.*, 2006; Liu *et al.*, 2006; Sun *et al.*, 2007; Bai *et al.*, 2011; Khan *et al.*, 2012).

Based on these observations, this study describes a model to depict the mechanism of *AtARGAHs* involved in abiotic stress responses (Fig. 8). As depicted, manipulating the expression of *AtARGAHs* modulates the specific localized arginine substrate for arginine decarboxylase or NO synthase and hence regulates endogenous polyamines and NO concentrations. The increased polyamines and NO modulate ROS accumulation through antioxidant enzyme activities and other adaptive responses. These in turn result in enhanced abiotic stress tolerance. Therefore, a new function of *AtARGAHs* in abiotic stress tolerance is indicated, which is, at least partly, attributed to its role in modulating polyamines, NO, and ROS concentrations. Additionally, both *ARGAH* and arginine decarboxylase are induced at the transcriptional level by abiotic stress including water deficit, salt, and freezing, supporting a role for arginine metabolism in abiotic stress responses. Interestingly, NO level is also affected by almost all abiotic stress conditions (Corpas *et al.*, 2009; Zhao *et al.*, 2009; Siddiqui *et al.*, 2011; Shi *et al.*, 2012b), indicating some links between arginine metabolism and NO level in response to abiotic stress.

Taken together, this is the first report, as far as is known, indicating possible roles of *AtARGAHs* in abiotic stress. As water deficit, salt, and freezing are worldwide problems that seriously affect crop yields, genetic engineering of crops with

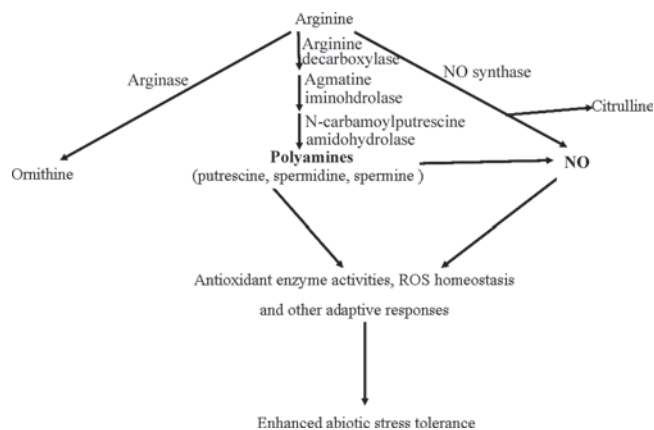


Fig. 8. Model depicting the mechanism of *AtARGAHs* involved in abiotic stress responses. Arginine can be metabolized by arginase, arginine decarboxylase, and NO synthase. Manipulating the expression of *AtARGAHs* modulates the arginine substrate for arginine decarboxylase or NO synthase, which in turn regulates endogenous polyamines and NO concentration. The increased polyamines and NO further modulate ROS accumulation through antioxidant enzyme activities and other adaptive responses, resulting in enhanced abiotic stress tolerance.

enhanced multiple environmental adaptations may be possible by manipulating arginase expression.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Quantification of arginine and total free amino acids in 2-week-old plant leaves.

Supplementary Fig. S2. Effect of *AtARGAHs* expression on plant growth after water deficit and salt treatments.

Supplementary Table S1. Primers used for plasmid construction, semi-quantitative RT-PCR, and quantitative real-time PCR.

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