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SlAGO4A, a core factor of RNA-directed DNA methylation (RdDM) pathway, plays an important role under salt and drought stress in tomato

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Abstract In *Arabidopsis*, it has been clarified that AGO4 protein is implicated in a phenomenon termed RNA-directed DNA methylation (RdDM). Previously, four orthologs of *AtAGO4* were cloned in tomato, designated as *SlAGO4A–SlAGO4D*. Here, we studied the role of the *SlAGO4A* gene in regulating salt and drought tolerance in tomato. *SlAGO4A*-down-regulating (AS) transgenic tomato plants showed enhanced tolerance to salt and drought stress compared to wild-type (WT) and *SlAGO4A*-overexpressing (OE) transgenic plants, as assessed by physiological parameters such as seed germination rate, primary root length, chlorophyll/proline/MDA/soluble sugar/RWC content, and survival rate. Moreover, several genes involved in ROS scavenging and plant defense, including *CAT*, *SOD*, *GST*, *POD*, *APX*, *LOX*, and *PR1*, were up- or down-regulated consistently under salt and drought stress. Notably, expression levels of some DNA methyltransferase genes and RNAi pathway genes were significantly lower in AS plants than in WT. Taken together, our results suggest that *SlAGO4A* gene plays a negative role under salt and drought stress in tomato probably through the modulation of DNA methylation as well as the classical RNAi pathway. Hence, it may serve as a useful biotechnological tool for the genetic improvement of stress tolerance in crops.

Keywords DNA methylation · Argonaute (AGO) · Salt tolerance · Drought tolerance · Tomato

Introduction

Environmental stresses such as salt, drought and other biotic factors possess serious challenges to plant breeding. Hence, engineering crop plants to withstand these stresses without yield compromise had become imperative. Recent researches indicate the pivotal role of small RNAs in regulating gene expression during biotic and abiotic stresses in numerous crop plants (Sahu et al. 2010; Khraiwesh et al. 2012; Seo et al. 2013; Wheeler 2013). In plants, the generation of these small RNAs mainly depends on some proteins

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encoded by respective Dicer-like (DCL), Argonaute (AGO), and RNA-dependent RNA polymerases (RDR) gene families. Distinct expression patterns of DCL, AGO, and RDR gene family members in response to various stress treatments have been demonstrated in several plant species (Kapoor et al. 2008; Qian et al. 2011; Bai et al. 2012; Chandar et al. 2015), suggesting that these genes might be involved in stress response by modulating RNA silencing pathway. AGOs are groups of functionally diverse proteins that are catalytic components of both TGS and PTGS silencing complex. In *Arabidopsis*, AGO1 mainly binds miRNAs and regulates plant development and stress adaptations (Mallory and Vaucheret 2010). AGO2 is highly induced by the bacterial pathogen *Pseudomonas syringae pv.* Tomato (*Pst*), and mutation in AGO2 attenuates plant resistance to both virulent and avirulent strains of *Pst* (Zhang et al. 2011). AGO4 has been associated with antibacterial defense, but whether these activities of AGO4 involve siRNAs or RdDM is unknown, because none of the other factors involved in the RdDM pathway has effects on antibacterial immunity (Agorio and Vera 2007). In addition, overexpression of a pea *p68* (*Psp 68*) gene in rice confers salinity stress tolerance. Psp68 was proved to interact with pea Argonaute (AGO1) (Banu et al. 2015). Similarly, a salt-induced RING-type ubiquitin ligase identified in halophyte ice plant, McCPN1, also interacts with an Argonaute protein (AGO4) (Li et al. 2014). These facts provide clues about the internal connection between AGOs and stress response.

At present, a total of 10, 15, 18, and 19 AGO proteins have been identified in *Arabidopsis thaliana* (Vaucheret 2008), tomato (Xian et al. 2013), maize (Qian et al. 2011), and rice (Kapoor et al. 2008), respectively. However, the function and regulatory mechanism of most AGO members remain scarce. In an earlier report, both semi-quantitative RT-PCR and qRT-PCR results showed that *SlAGO4A* was significantly induced under salt and drought stress (Bai et al. 2012), which makes *SlAGO4A* as a good candidate gene to further explore its stress-tolerant abilities by genetic engineering methods. Besides, it was shown that a homologous gene of *AGO4* in wheat, *TaAGO4*, could be induced by cold accumulation during vernalization treatment (Meng et al. 2013). Another gene of wheat which also belongs to AGO4-9 class, *AGO802B*, might play a role in seed dormancy and preharvest sprouting (PHS) resistance through the modulation of DNA methylation (Singh et al. 2013). These two publications suggest the potential role of AGO4-RdDM regulatory networks in stress response. Thereby, it would be interesting to learn the possible involvement of stress-induced changes in gene expression, through regulating the catalytic component of TGS and PTGS machinery. DNA methylation, as one of the most important epigenetic phenomena, can regulate the function of plant genome without changing the DNA sequence via the effect of various DNA methyltransferases. DNA methylation patterns are sensitive to various environmental stimuli, which can endow plants with heritable stress adaptation. And the transgeneration effects of DNA methylation make its application in plant breeding possible.

Tomato (*Solanum lycopersicum*) is a globally important crop and a model plant for fleshy fruit development and plant defense research (Kimura and Sinha 2008). As with other plants, stress factors such as drought, high salinity and disease limit productivity and reduce yield. Previously, we found that there are four AGO4 homologs in tomato, namely *SlAGO4A–SlAGO4D*. However, the expression patterns of these four genes vary greatly (Xian et al. 2013). In the current work, we have a deeper insight into tomato *SlAGO4A* in regulating salt and drought stress. Compared with wild-type plants, down-regulation of *SlAGO4A* gene in tomato significantly improved tolerance to drought and salt stresses, whereas overexpression of *SlAGO4A* gene resulted in opposite effects to these two stressors. Moreover, the alteration of expression levels in both DNA methyltransferase genes and RNAi pathway genes indicate the internal connection between DNA methylation and drought-/salt-tolerant ability in the transgenic plants.

Materials and methods

Plant materials and growth conditions

Tomato plants (*Solanum lycopersicum* cv. Micro-Tom) were grown on soil in greenhouse with suitable conditions: 14-/10-h light/dark cycle, 25/20 °C day/night temperature and 60 % relative humidity, and the plant nutrient solution (Hoagland and Arnon 1950) were irrigated once per week. To analyze the expression pattern of *SlAGO4A*, tissues including

roots (both primary and lateral roots), stems, and leaves (5th leaf from cotyledon) were harvested from 1-month-old plants, and flowers (bud, anthesis) and fruits (immature, breaker stage, yellow fruits, and red ripening fruits) were harvested at the proper time. Floral organs, including receptacles, sepals, petals, stamens, and ovaries, were collected from anthesis flowers. All tissues were collected from six well-grown plants and thoroughly mixed, then frozen in liquid nitrogen immediately, and stored at −80 °C, and each tissue/organ was sampled for three independent times.

Promoter analysis and expression profile of *SlAGO4A* under hormone and light treatments

To study the possible function of *SlAGO4A*, in silico analysis of *SlAGO4A* promoter region was performed using the PLACE program (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?sid=&lang=en&pj=640&action=page&page=newplace) (Higo et al. 1999). Based on the promoter analysis result, we carried out hormone treatments by immersing tomato seedlings (12-day-old) in liquid MS/2 medium with 20 μM ethephon (Eth), 20 μM gibberellin (GA3), 20 μM abscisic acid (ABA), 20 μM indole acetic acid (IAA), and 20 μM salicylic acid (SA) for 3 h, respectively. Control seedlings were soaked in liquid MS/2 medium without any hormone. As for light treatments, seeds were incubated on MS/2 medium in darkness for a week and then exposed to natural light for 0, 4, 8, and 12 h. On the contrary, seeds were germinated and grown in MS/2 culture medium with continuous light for a week and then transferred to dark for 0, 1, 3, and 6 h. Different tissues and materials were collected for 3 independent times and stored at −80 °C for qRT-PCR analysis.

Plasmid construction and plant transformation

To generate *SlAGO4A*-overexpressing plants, the forward 5′-GGTGCTTCATCTGCCTCTTT-3′ and the reverse 5′-TCTTTCACATTACACCTGCTGG-3′ primers were used to amplify the 3109 bp (including 2730-bp open reading frame) of full-length *SlAGO4A*-coding sequence. The *SlAGO4A* antisense construct was made by cloning a 594-bp fragment with forward primer 5′-GGTTTCTCTGCTGATGATCTTC-3′ and reverse primer 5′-TCTTTCACATTACACCTGCTGG-3′. Then, two fragments amplified were cloned into the modified binary vector pLP100 in the sense and antisense orientation, respectively, under the CaMV 35S promoter. All transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation according to Xian et al. (2014). Transformants were first selected on kanamycin (100 mg/L) and then confirmed by both PCR and GUS (β-glucosidase) staining. All experiments were carried out using homozygous lines from T2 or later generations.

Salt and drought stress treatment

For germination assay under two stressors, a total of 30 surface-sterilized seeds from each T2 transgenic line and wild type were germinated on MS/2 medium with dedicated concentrations of sodium chloride or mannitol and then placed at a chamber with 25/20 °C (day/night) and a photoperiod of 16/8 h (day/night). Germination rates and primary root length were measured after 7 days, and photographs were taken. The experiment was repeated thrice, and identical results were observed.

To further investigate high-salinity tolerance, 1--month-old wild-type and transgenic plants grown in nutrition pots were irrigated with 200 mM NaCl solution (200 mL per plant every 48 h) in the following 30 days. Meanwhile, the drought treatment consisted of withholding water for up to 30 days, then re-watering for 7 days. As control, well-watered plants were maintained by watering plants in a normal way. Chlorophyll, proline, malondialdehyde (MDA), and soluble sugar were determined after treatment finished. Each test was repeated for three times.

Determination of chlorophyll, proline, MDA, total soluble sugar, and relative water (RWC) content

Following drought and salt treatment, leaves at similar developmental stages (5th leaf from cotyledons) from transgenic and WT plants were collected at predetermined times (30 days post-treatment). For the extraction of chlorophyll (Chl), 0.2 g of leaf tissues was decolorized using 80 % acetone under the condition of dark. Chl was quantified spectrophotometrically with the wavelength of 663 nm and 645 nm, with 80 % acetone as the control. $Ca + b\ (\mu g/mL) = 20.2 \times A645 + 8.02 \times A663$, $Chl\ (\mu g/g) = Ca + b \times V/W$, V: volume of extract liquid (mL); W: fresh weight

(g) (Lichtenthaler 1987). Proline content was determined according to the method described by Bates et al. (1973). MDA content was measured by the thiobarbituric acid (TBA) reaction method as described by Heath and Packer (1968). Total soluble sugar content was analyzed using the anthrone method with glucose as described by Fukao et al. (2006). The measurement of RWC was taken according to previous report (Pan et al. 2012).

SOD and CAT enzyme extraction and assay

Superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) activities were measured according to methods described by Mittova et al. (2000). About 0.5 g of leaves was homogenized in 2 mL of ice-cold 0.1 M phosphate buffer (pH 7.0) containing 0.1 % polyvinylpyrrolidone. The homogenates were centrifuged at 4 °C for 15 min at 12,000*g*. The supernatant was used for the determination of the activities of antioxidant enzymes. SOD activity was determined spectrophotometrically by monitoring inhibition of the cytochrome c reduction rate in the presence of the xanthine–xanthine oxidase system at 550 nm. CAT activity was determined spectrophotometrically by monitoring H_2O_2 decomposition at 240 nm.

Expression analyses by qRT-PCR

Leaves used for physiological parameters test were used for total RNA extraction (TRIzol, Invitrogen, USA) according to the manufacturer's instruction. First-strand cDNAs were synthesized from 2 μg of total RNA by using the PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRA, JAPAN) following the supplier's protocols. Real-time quantitative PCR was conducted using SsoAdvancedTM Universal SYBR Green Supermix (BIO-RAD, USA) on a CFX96 TouchTM Real-Time PCR Detection System (BIO-RAD, USA). The PCR amplification cycle was as follows: 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 58 °C for 20 s. Melting curve analysis was performed in the temperature ranging 60–95 °C to verify the specificity of the amplicon for each primer pairs. Relative fold differences were calculated based on the comparative Ct method using the $2^{-\triangle\triangle Ct}$ method with the *SlUBI* as an internal reference gene. The results were represented by three biological replicates (each with three technical replicates) for each sample. All the primers for qPCR are shown in Table S1.

Statistical analysis

All the experiments in this study were repeated at least three times, and all the data shown are the average means of three independent experiments ± SDs. Data analysis was performed using SPSS software, and significant differences were determined by Student's *t* test at significance levels of $P < 0.01$ (**) and $P < 0.05$ (*).

Results

Sequence analysis and expression profiles of *SlAGO4A*

Previously, we isolated a putative Argonaute protein SlAGO4A (SGN-U577343) based on a cDNA clone in tomato (Xian et al. 2013). SlAGO4A occupies a 2730-bp coding fragment which encodes 909 amino acid residues with an estimated molecular mass of 101.75 kDa and 8.97 as its theoretical isoelectric point. The sequence alignment showed that tomato SlAGO4A shared 72 % identity with the *Arabidopsis* AGO4 (At2g27040) protein (Fig. S1). Moreover, in silico analysis of the 2-kb promoter sequence performed using the PLACE program (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?sid=&lang=en&pj=640&action=page&page=newplace) identified several *cis*-acting elements. Indeed, many *cis*-acting regulatory elements associated with hormones and biotic/abiotic stresses were found in its promoter region (Table S2). For instance, ABREATCONSENSUS, ARFT, GAREAT, LECPLEACS2, and WBOXATNPR1 are related to ABA, IAA, GA, Eth, and SA, respectively. This was validated by qRT-PCR in the present work. CBFHV, CURECORECR, GT1GMSCAM4, MYCCONSENSUSAT, and WRKY71OS are involved in cold, oxygen, salt, dehydration, and pathogenesis response, respectively. Besides, we also found some light-related elements such as IBOXCORE in its promoter region. These findings suggest that *SlAGO4A* may participate in manifold signal pathways that have pleiotropic effects on stress response and light signaling in tomato.

Although the four AGO4-like proteins in tomato (SlAGO4A, SlAGO4B, SlAGO4C, and SlAGO4D) shared a highly identical level as much as 84.75 % (Fig. S1), our previous study by conventional PCR analysis in different organs demonstrated that their transcripts were largely different to each other (Xian et al. 2013). To further investigate the potential role of *SlAGO4A* in tomato growth and development, samples from roots, stems, leaves, floral buds, flowers, and fruits at different stages were collected to perform quantitative RT-PCR (Fig. 1a). The results showed that higher mRNA levels were detected in floral buds and flowers, and drop gradually from immature fruits to ripening fruits. Hence, we further checked the transcripts of *SlAGO4A* in different floral organs (Fig. 1b), and it was observed that receptacles, sepals, and ovaries expressed more transcripts of *SlAGO4A* than stamens and petals. These data suggest that *SlAGO4A* may have important roles in vegetative growth and reproductive development.

In plants, light and hormones are two most important factors that influence various aspects of plant growth and development. Based on the hormone- and light-related *cis*-elements observed in the promoter region of *SlAGO4A* (Table S2), it is tempting to study whether *SlAGO4A* is involved in hormone responses and light signal transduction. Thereby, WT tomato seedlings were treated with different hormones and then qRT-PCR analyses were performed. *SlAGO4A* transcripts were significantly down-regulated by all five hormones that were performed, including ABA, Ethphon (Eth), GA3, IAA, and SA (Fig. 1c). Meanwhile, to assess the light impact on *SlAGO4A* expression, we examined its transcripts patterns under light/dark condition. *SlAGO4A* abundance was induced when illuminated with light as compared to seedlings grown in darkness (Fig. 1d), by contrast, the expression of *SlAGO4A* was suppressed when placed seedling grown under continuous light into darkness (Fig. 1e). Interestingly, the expression levels displayed downward tendency over time upon both the conditions. Collectively, these results confirm the involvement of *SlAGO4A* in hormone and light signal transduction.

Identification of transgenic plants

To further estimate the function of *SlAGO4A*, we successfully constructed two different vectors and then transformed them into wild-type tomato cv. Micro-Tom to get *SlAGO4A* over/downexpression transgenic plants. More than 3 homozygous lines from independent transformation events for each construct were obtained. To evaluate the expression of *SlAGO4A* in transgenic lines, qRT-PCR was performed (Fig. S2). Compared with WT plants, the relative mRNA levels of OE L6, OE L8, and OE L12 increased by 2.34, 3.80, and 6.99 times, respectively. By contrast, three down-regulated lines, AS L1, AS L3, and AS L5, displayed 0.56-, 0.10-, and 0.14-fold change, respectively. Therefore, two most regulated transgenic lines, OE L12 and AS L3, were selected for the following physiological measurement.

Growth analysis of seedlings under salt and drought stress

To investigate the salt and drought tolerance of the transgenic plants, seeds from wild-type and homozygous lines OE L12 and AS L3 were germinated on MS medium supplemented with dedicated concentration of NaCl (75 mM) or D-mannitol (150 mM). Differences in seed germination ability were observed between wild-type and transgenic lines at 7 days post-sowing. On control mediums, no obvious difference was observed. However, the germination rates of downexpressing plants (AS) were significantly higher on the mediums containing NaCl or D-mannitol than that of the WT line, whereas decreased germination rates were observed in the overexpressing plants (OE) (Fig. 2a, c). Meanwhile, we measured the primary root length of all seedlings from the germinated seeds. In conformity with the germination rates, the primary root length was greater in AS seedlings than that in WT, while shorter roots were observed in OE seedlings (Fig. 2b, d).

Enhanced/reduced tolerance to salt and drought stress in *SlAGO4A* down-/up-regulated transgenic tomato plants

To further confirm the stress-tolerant function of *SlAGO4A* gene, 1-month-old plants of T2 homozygous transgenic lines AS L3 and OE L12 and wild type were subjected to salt stress by treating with 200 mM NaCl (200 mL every 48 h) or drought stress by withholding water for 30 days. Compared with WT plants, both the transgenic lines showed no abnormal morphological phenotype under normal growth

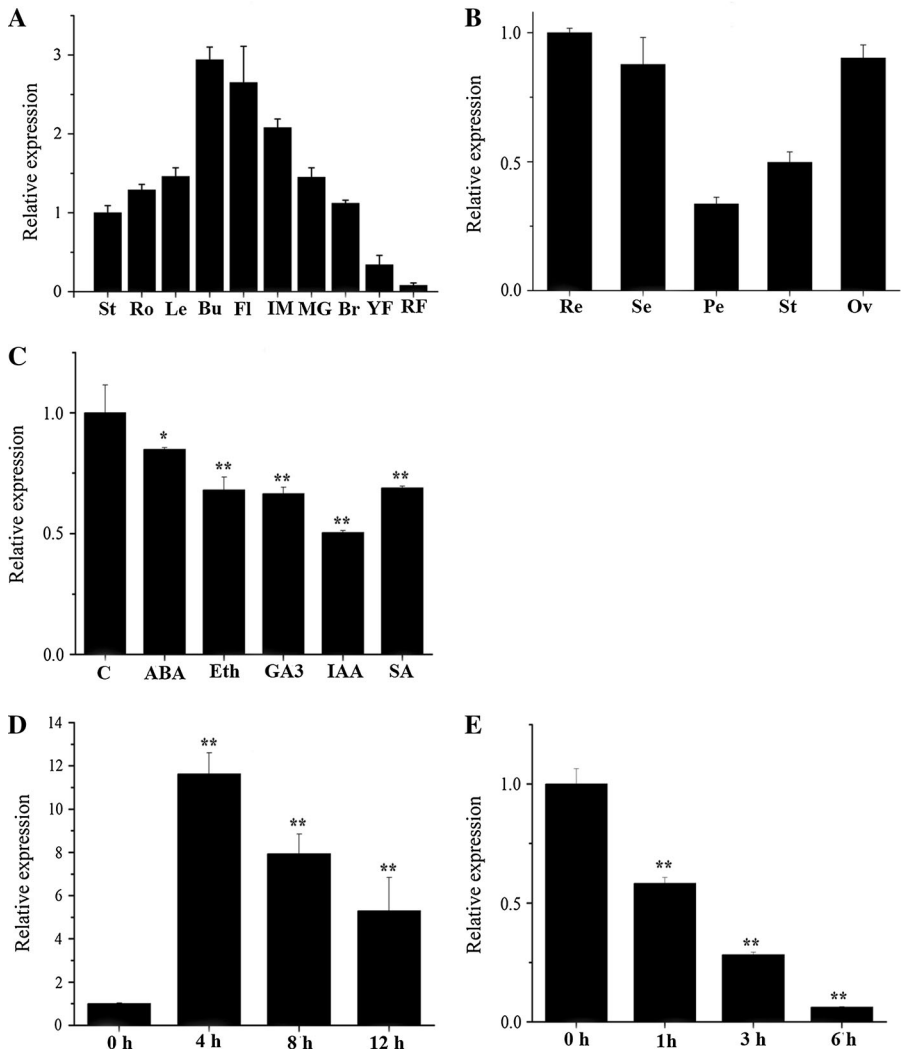


Fig. 1 Expression patterns of *SlAGO4A* in WT tomato. **a** Spatiotemporal expression of *SlAGO4A* in stem (St), root (Ro), leaf (Le), bud (Bu), flower (Fl), and fruits at four different developmental stages including immature green (IM), mature green (MG), breaker stage (Br), and red ripening fruit (RF) from tomato cv. Micro-Tom. **b** Expression of *SlAGO4A* in floral organs at anthesis. *Re* receptacle, *Se* sepal, *Pe* petal, *St* stamen, *Ov* ovary. **c** Expression of *SlAGO4A* in response to various hormones. *C* control sample, *ABA* abscisic acid, *Eth* ethephon, *GA3* gibberellin, *IAA* indole acetic acids, *SA* salicylic acid. **d** Expression of *SlAGO4A* in response to light treatment. Seedlings grown in darkness were treated with natural light for 0, 4, 8, and 12 h. **e** Expression of *SlAGO4A* in response to darkness treatment. Seedlings grown in continuous light were treated with darkness for 0, 1, 3, and 6 h. *Error bars* show the standard error between three replicates performed. For **c**, **e**, the *asterisks* indicate statistically significant differences between the untreated plants and treated plants. $*P < 0.05$, $**P < 0.01$, Student's *t* test

conditions. However, under saline or drought condition, all AS plants showed a better growth than WT plants, while a worse growth was observed in OE plants (Fig. 3a). Under salt stress, all plants exhibited reduced growth and increased chlorosis and necrosis at 20 days post-treatment, but the signs of damage showed obvious difference among three genotypes (Fig. 3a, b). For drought stress, desiccation symptoms such as wilting of lower leaves could easily be observed at 30 days post-treatment in WT and OE plants, whereas AS plants exhibited such phenotypes to only a slight extent after drought treatment (Fig. 3a, b). Four physiological parameters, including chlorophyll, MDA, proline, and soluble sugar, were

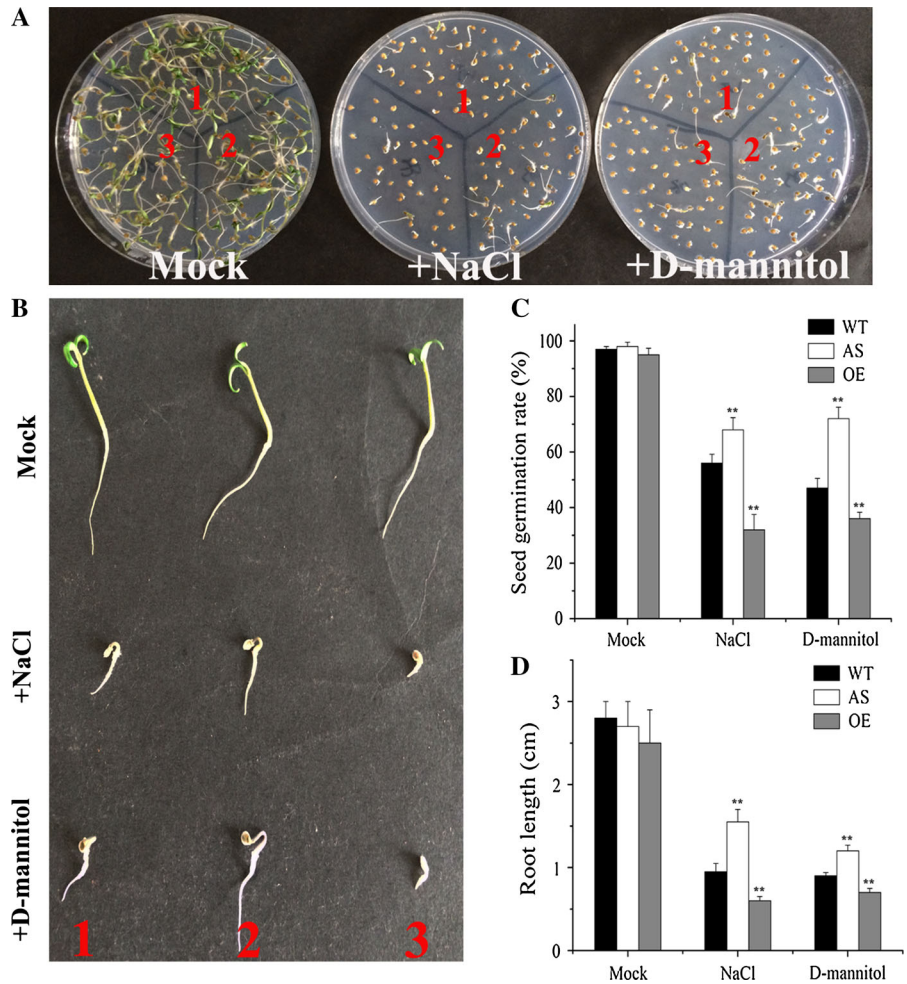
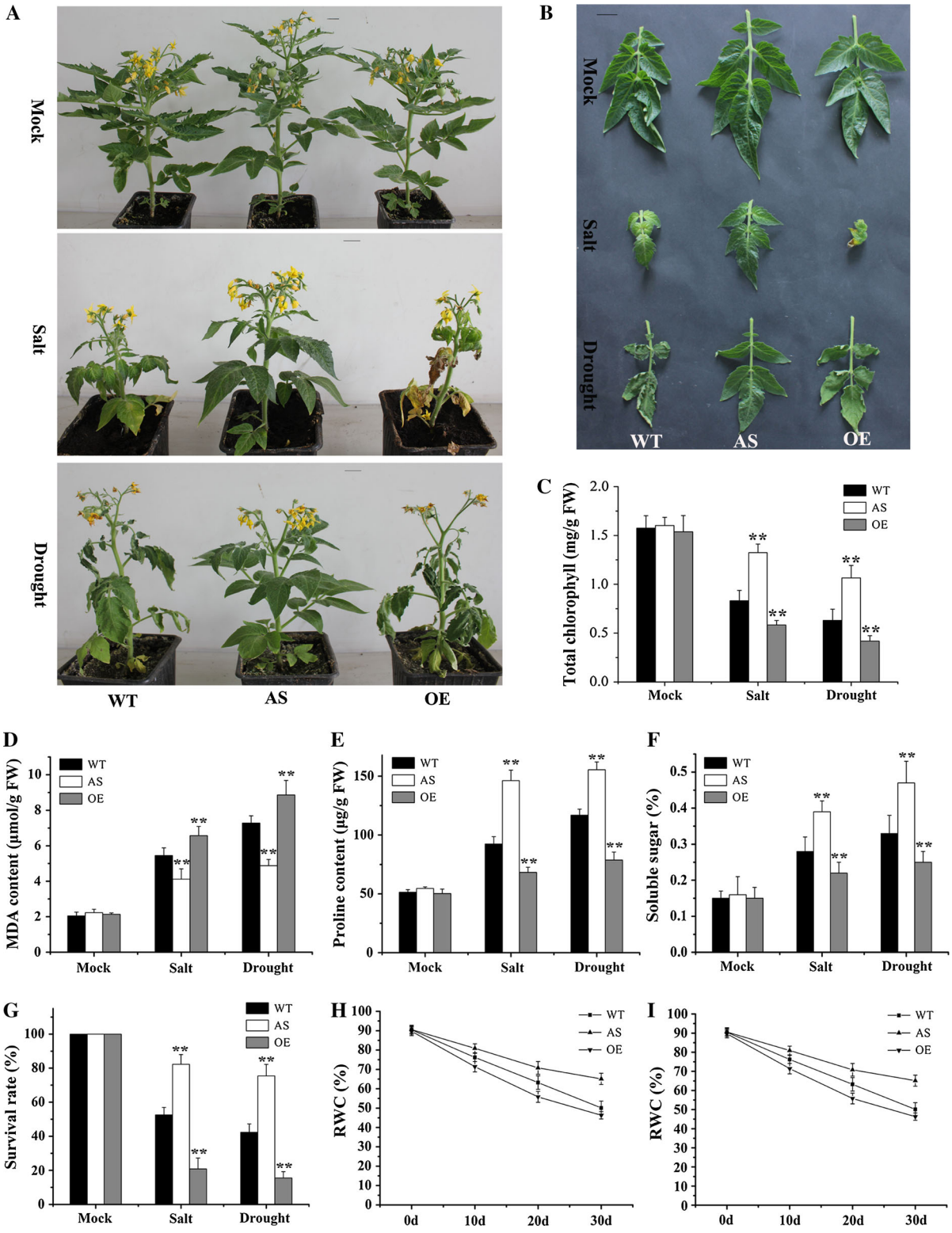


Fig. 2 Comparative analysis of transgenic lines over-/down-expressing *SlAGO4A* on salt/drought stress condition (AS L3, OE L12). **a**, **b** Seed germination of WT and transgenic lines in the medium containing 75 mM NaCl and 150 mM D-mannitol for 7 days, and then photographs were taken. *1* WT, *2* AS L3, *3* OE L12. **c** Germination rate of WT and transgenic seedlings under salt and drought treatments. **d** Primary root length of WT and transgenic seedlings under salt and drought treatments. For **c**, **d**, *error bars* show the standard error between three replicates performed. *Asterisks* indicate significant differences between transgenic lines and wild type. $*P < 0.05$, $**P < 0.01$, Student's *t* test

measured in leaves from treated plants (Fig. 3c–f). Consistently, the content of all four compounds of WT and transgenic plants showed no significant difference under control condition. Compared with control plants, all stressed plants showed decreases in total chlorophyll content, with lowest values appeared in OE plants, and then WT and AS plants, respectively (Fig. 3c). After salt and drought treatment, MDA, proline, and soluble sugar contents all showed increases in WT and transgenic plants. MDA levels increased rapidly in WT and OE plants, but AS plants suffered a lesser increase (Fig. 3d). In contrast, greater content of proline and soluble sugar was measured in AS plants than that of WT and OE plants (Fig. 3e, f). For both stress conditions, significantly higher survival rate was observed in AS plants than in WT and OE plants (Fig. 3g). During the two stresses, the leaf water loss was significantly lower in the AS transgenic lines compared with the WT and OE plants (Fig. 3h, i). Besides, to reveal the role of SlAGO4A in protecting cell damage caused by salt and drought stress, activities of two antioxidant enzymes, SOD and CAT, were assayed. Under normal conditions, no significant differences in activities of these two enzymes were found between the transgenic lines and WT (Fig. S3A and B). Both WT and transgenic plants showed a marked increase in SOD activity and a significant decrease in CAT activity, whereas the activities of SOD and CAT were significantly higher in AS plants than in WT and OE plants, indicating that AS plants have higher antioxidant activity than the WT and OE plants under salt and drought stress.


A
Mock
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Total chlorophyll (mg/g FW)
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Proline content (μg/g FW)
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◀**Fig. 3** Salt/drought stress tolerance of *SlAGO4A* transgenic plants (AS L3, OE L12). **a** Pictures show representative plants at 30 days post-treatment and control plants. **b** Phenotypes of leaves collected from plants shown in (**a**) (5th leaf from cotyledon). Comparison of total chlorophyll content (**c**), MDA content (**d**), proline content (**e**), and survival rate (**f**) of wild-type and transgenic plants under normal and stressed conditions. For **c**, **f**, *error bars* show the standard error between three replicates performed. *Asterisks* indicate significant differences between transgenic lines and wild type. $*P < 0.05$, $**P < 0.01$, Student's *t* test

Expression analysis of stress-related genes in WT and transgenic tomato plants

Since stress conditions such as salinity and drought generate reactive oxygen species (ROS), whose production affects the antioxidant system of plants, the transcript levels of several genes involved in ROS generation and scavenging, including CAT, SOD, GST, POD, LOX, and ascorbate peroxidase (APX), were monitored in WT and transgenic plants under both normal and stress conditions by qRT-PCR (Fig. 4). Under normal condition (mock), all six genes showed no significant difference between WT and transgenic plants. Expression levels of four genes, *SlCAT*, *SlSOD*, *SlGST*, and *SlPOD* (Fig. 4a–d), were activated in both WT and transgenic tomato plants under salt and drought conditions, while the other two genes, *SlLOX* and *SlAPX* (Fig. 4e, f), were inhibited. Notably, expression levels of all these six genes were higher in AS plants under two stressed conditions, whereas their transcripts were lower in OE plants. Furthermore, transcripts of a pathogenesis-related (PR) gene, *SlPR1* (Fig. 4g), was also checked. Similar induction of *SlPR1* was found when responding to two stresses, but significantly differential expression was observed in transgenic plants (AS and OE) compared to that in WT plants under normal condition.

Expression analysis of DNA methyltransferase genes and RNAi pathway genes

It has been clarified that AtAGO4 protein participates in a phenomenon termed RNA-directed DNA methylation (RdDM). To uncover the molecular mechanism of *SlAGO4A* involving in salt and drought tolerance observed in transgenic plants, expression levels of several genes encoding DNA methyltransferase were examined (Fig. 5a). The expression of *SlDRM1*, *SlDRM2*, *SlDRM7*, *SlDRM8*, and *SlKTF1* was obviously lower/higher in AS/OE plants than that in WT plants, indicating decreased/increased methylation levels. No difference was observed in *SlCMT3* and *SlKYP*, implying the presence of an alternative

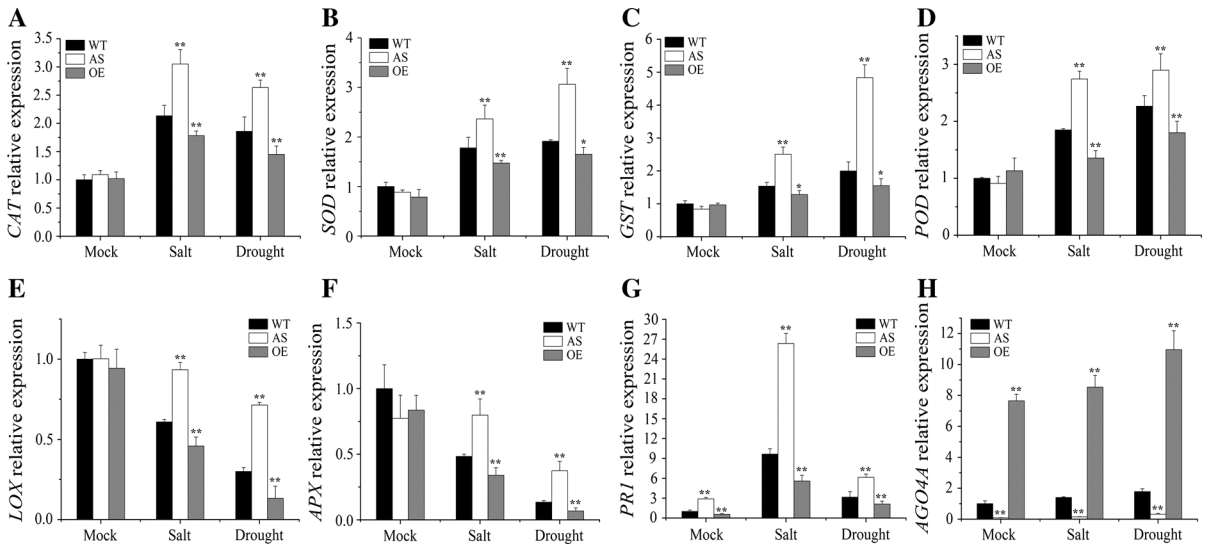

Fig. 4 Transcript levels of *CAT* (**a**), *SOD* (**b**), *GST* (**c**), *POD* (**d**), *LOX* (**e**), *APX* (**f**), *PR1* (**g**), and *AGO4A* (**h**) in wild-type and transgenic plants under normal and stressed conditions. The leaf samples collected from plants shown in Fig. 3a were used for RNA extraction. *Error bars* show the standard error between three replicates performed. *Asterisks* indicate significant differences between transgenic lines and wild type. $*P < 0.05$, $**P < 0.01$, Student's *t* test

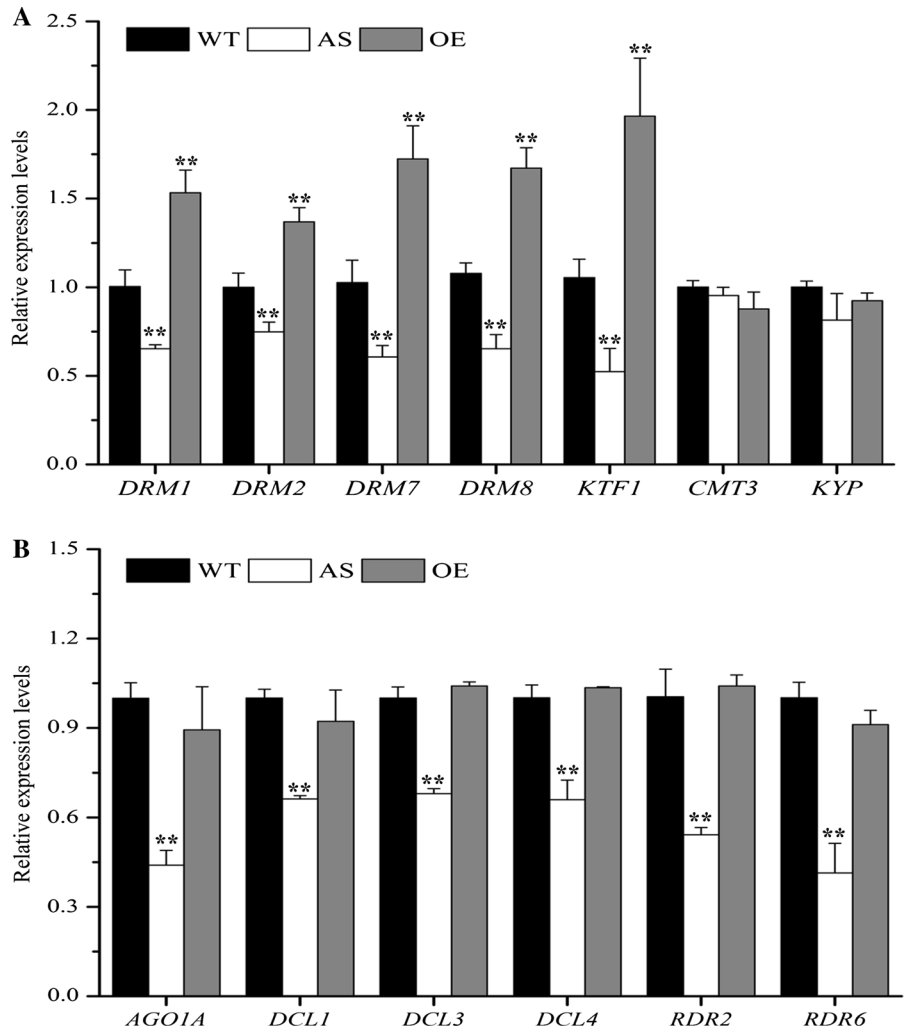

Fig. 5 Transcript levels of DNA methyltransferase genes (**a**) and RNAi pathway genes (**b**) in wild-type and transgenic plants under normal and stressed conditions. The leaf samples collected from plants shown in Fig. 3a were used for RNA extraction. *Error bars* show the standard error between three replicates performed. *Asterisks* indicate significant differences between transgenic lines and wild type. $*P < 0.05$, $**P < 0.01$, Student's *t* test

SlAGO4A-independent pathway directing DNA methylation for these two methyltransferase genes in tomato. In order to better understand other components of the RNA-induced silencing complex (RISC), we analyzed the expression levels of *SlAGO1A*, *SlDCL1*, *SlDCL3*, *SlDCL4*, *SlRDR2*, and *SlDRD6* (Fig. 5b). Interestingly, all these genes were significantly suppressed in AS plants with no change in OE plants.

Discussion

Argonaute (AGO) proteins, acting as core components of the RNA-induced silencing complex (RISC), participate in a process referred to as small RNA loading (Hutvagner and Simard 2008). The AGO-small RNA complex uses base pairing to interact with homologous RNA or DNA molecules for direct RNA cleavage, translational repression, or DNA methylation (Mallory and Vaucheret 2010).

By measuring the germination rate, primary root length, chlorophyll content, proline content, MDA content, soluble sugar content, survival rate, and relative water content (RWC) along with different phenotypic observations, we found that downexpression of *SlAGO4A* in tomato increased tolerance to salt and drought stress, whereas *SlAGO4A*-overexpressing tomato plants exhibited decreased salt and drought

tolerance. Noticeably, no significant difference in growth phenotype was observed between WT and transgenic plants under normal condition, which means that the growth of *SlAGO4A* transgenic plants could not be affected significantly by either up- or down-regulating this gene, which is important in genetic engineering approaches for crop improvement. Plants have developed sophisticated mechanisms to adapt to various stresses. It is well known that plants suffering from abiotic stress often accumulate reactive oxygen species (ROS) leading to lipid peroxidation and oxidative stress (Xiong et al. 2002; Mittler et al. 2004). Generally, ROS are generated in chloroplasts due to the inhibition of photosynthetic activity in green tissues caused by various stresses (Dat et al. 2000). In the present study, the decrease in chlorophyll level in the AS plants was significantly less compared with the WT and OE plants, suggesting that downexpression of *SlAGO4A* in tomato resulted in more photosynthetic capabilities after salt and drought treatment. Under stress conditions, MDA can serve as an indicator to evaluate the extent of lipid peroxidation (Mittler 2002), and proline can act as an antioxidant to reduce oxidative damage (Szekely et al. 2008). In this regard, the results of our study which demonstrated lower MDA and higher proline content in AS plants strongly indicated that *SlAGO4A*-downexpressing plants maintained more membrane integrity and stability as well as better cell viability. In addition, plants have developed a set of ROS scavenging system to protect cells against oxidative damage (Gill and Tuteja 2010; Choudhury et al. 2013). Therefore, the mRNA levels of several genes encoding ROS scavenging enzymes, including *CAT*, *SOD*, *GST*, *POD*, and *APX*, as well as a lipoxygenase gene (*LOX*), were investigated. Our data revealed that higher transcripts of these genes were detected in AS plants, implying that they might possess more efficient antioxidant defense machinery as compared to the WT and OE plants. Unlike other genes, *PR1* was the only gene which altered its expression levels between WT and transgenic plants under normal condition. It has been proved that *PR* genes are correlated with SA and JA (Niki et al. 1998), two hormones that are essential for regulating the systemic defense response in plants. Hence, we deduce that *SlAGO4* may play a role in regulating the defense response of tomato by modulating SA and/or JA signaling pathways.

Global changes in DNA methylation, including hyper- and hypo-methylation, in response to abiotic stress have been reported in several plant species (Boyko et al. 2010; Bilichak et al. 2012; Karan et al. 2012; Wang et al. 2014), and alterations of DNA methylation in some stress-responsive genes have also been reported. In *Nicotiana tabacum*, oxidative stress led to demethylation and activated *NtGPDL* (glycerol phosphodiesterase-like protein) (Choi and Sano 2007). Similarly, reduction of DNA methylation and activated salt-responsive transcription factors were observed in soybean after salinity stress treatment (Song et al. 2012). In *Arabidopsis*, two positive regulator genes for stomatal development, *SPCH* and *FAMA*, were reported to induce DNA methylation and suppress transcripts under low relative humidity stress (Tricker et al. 2012). The system that regulates DNA methylation also functions as a genomic defense mechanism against abiotic stresses. RNA-directed DNA methylation is required for basal tolerance against heat stress (Popova et al., 2013). Ito et al. (2011) found that the siRNA-mediated RdDM pathway restricts transcriptional activity and retrotransposition of the *copia*-type retrotransposon *ONSEN* triggered by heat stress. Argonaute4 (AGO4) is thought to be mainly involoved in the transcriptional gene silencing (TGS) process. In *Arabidopsis*, AtAGO4 is required for RNA-directed DNA methylation (RdDM) of the *SUPERMAN* (*SUP*) locus, which is linked to asymmetric DNA methylation as well as histone H3 lysine 9 (H3K9) methylation (Zilberman et al. 2003). AGO4 protein controls the *de novo* methylation in plants along with 24nt siRNAs, RDR2, DCL3, and DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE 2), and the *de novo* methylation was blocked in ago4 mutant plants (Chan et al. 2004). Generally, in RdDM, DNA methyltransferase genes directly mediate the methylation processes (Matzke et al. 2009). In our present work, the lower transcript levels of DNA methyltransferase genes, *SlDRM1*, *SlDRM2*, *SlDRM7*, and *SlDRM8*, were observed in *SlAGO4*-down-regulating plants (AS) than that of WT, whereas higher transcript levels were found in *SlAGO4A*-overexpressing (OE) plants, suggesting that *SlAGO4A* may confer stress tolerance by regulating relevant genes through modulation of DNA methylation. In plants, most RNA silencing (RNAi) pathways contain three critical stages, which are initiation, maintenance, and signal amplification (Brodersen and Vioinnet 2006). DCLs and AGOs are implicated in the initiation and maintenance stage, while RDRs are responsible for the

signal amplification stage to generate secondary small RNAs such that to give rise to a new round of RNA silencing. In maize, it was shown that distinct mechanisms, including DNA methylation, 5′-untranslated exons (5′-UTE), and microRNA-mediated feedback loops, were involved in the fine-tuned regulation of transcription and translation of ZmAGOs for different tissues and developmental stages (Zhai et al. 2014). In our results, lower expression levels of *SlAGO1A*, *SlDCL1*, *SlDCL3*, *SlDCL4*, *SlRDR2*, and *SlDRD6* were observed in AS plants, indicating that downexpression of *SlAGO4A* attenuates the signal amplification. However, none of these genes altered their expression in OE plants, which implies that the RNAi pathways could not be reinforced simply by overexpression of *SlAGO4A* gene, at least not in OE transgenic plants of the present work.

In conclusion, our study characterizes *SlAGO4A*, a tomato homolog of *AtAGO4*. Expression analysis results show that *SlAGO4A* is widely expressed in various tissues/organs and is regulated by different kinds of hormones as well as light. More importantly, downexpression of *SlAGO4A* conferred enhanced salt and drought in transgenic tomato, while *SlAGO4A*-overexpressing transgenic lines exhibited absolutely opposite effect. These results suggest that the expression level of *SlAGO4A* in tomato is negatively correlated with tolerance to salt and drought stress. The alteration of methyltransferase genes suggests a possible underlying molecular mechanism, which is modulation of methylation levels. Taken together, our work may provide a new strategy to generate stress-tolerant plants through regulating methylation process-associated genes.

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