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Effects of exogenous 5-aminolevulinic acid on PIP1 and NIP aquaporin gene expression in seedlings of cucumber cultivars subjected to salinity stress

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ABSTRACT. Aquaporins play a direct role in plant water relation under salt stress, but the effects of 5-aminolevulinic acid (ALA) on aquaporin gene expression in salt-treated plants remain unknown. This study investigated the potential effects of exogenous ALA (50 mg/dm^3) on aquaporin expression levels under salt stress (75 mM NaCl) in the salt-sensitive (Jinchun No.4) and the relatively salt-tolerant cucumber (Jinyou No.1) seedlings. The expressions of cucumber PIP aquaporin gene (*CsPIP1:1*) and cucumber NIP aquaporin gene (*CsNIP*) were analyzed in 20-day-old seedling leaves at 2, 4, 8, 16, and 24 h after ALA treatment. After treatment with saline alone and ALA alone, *CsPIP1:1* and *CsNIP* gene expression levels in the 2 cucumber cultivars increased to maximum at 2 h. The aquaporin gene expression in salt-treated cucumber seedling leaves was considerably higher than that in leaves subjected to exogenous ALA. Further, the aquaporin expression levels in Jinchun No.4 were higher than those in Jinyou No.1, reaching 5.20-

and 2-fold induction levels, respectively. After treatment with both ALA and NaCl, the *CsNIP* gene expression was downregulated in both the cucumber cultivars, while that of *CsPIP1:1* decreased at 2 h and then increased to 3.8-fold in Jinchun No.4. In Jinyou No.1, *CsPIP1:1* gene expression gradually increased to 2.3-fold at 4 h, followed by a decline in expression. The results indicated that ALA might delay and counteract the upregulated expression of *CsPIP1:1* and *CsNIP* genes in cucumber seedlings under NaCl stress. Thus, salt tolerance of cucumber seedlings might be enhanced by ALA application.

Key words: 5-aminolevunlinic acid; NaCl stress; Aquaporin; Cucumber; Gene expression

INTRODUCTION

In recent years, facility agriculture has significantly improved the yield and quality of vegetables in China. However, under greenhouse conditions, deficiency of leaching by rainfall and over-fertilizer application increased soil salinity in facility cultivation (Stigter et al., 1998; Darwish et al., 2005); and this has become more obvious because of continuous cropping. Soil salinity is one of the most severe environmental stresses to non-halophytic plants causing severe agronomical yield losses worldwide. Cucumber is an economically important crop and has been classified as highly sensitive to salinity (Alpaslan and Gunes, 2001), especially during seed germination and in the seedling stages of development (Du et al., 2010). Application of NaCl significantly hampers the growth and development of cucumber seedlings and plants (Zhen et al., 2012). The relative water content, K^+ content, and photosynthesis rate of plant leaves are significantly decreased (Shu et al., 2012), and leaf malondialdehyde, Na^+, and Cl^- contents and superoxide dismutase (Hu et al., 2012) activities are increased.

In order to increase plant tolerance against salinity, plant growth regulators (PGRs) have been widely applied. 5-aminolevulinic acid (ALA) is one such PGR and it is known to be a key precursor in the biosynthesis of all porphyrin compounds such as chlorophyll, heme, and vitamin B12 in organisms (Hotta et al., 1997). Previous studies showed that ALA is the most effective of the 12 PGRs used to improve salt tolerance of cotton seedlings (Watanabe et al., 2000b). Application of low concentration of exogenous ALA is known to promote plant growth development and response to environment stresses (Naeem et al., 2012), for example, enhancing salt tolerance in potato (Zhang et al., 2006) and chilling tolerance in pepper seedlings (Korkmaz et al., 2010). Foliar application of ALA can also improve leaf water relations in oilseed rape (Naeem et al., 2010). Na^+ concentration of cotton seedlings under salt stress were suppressed to low concentrations with ALA (Watanabe et al., 2000a). ALA has been shown to improve plant tolerance to abiotic environmental factors such as salinity; however, the relationship between ALA and aquaporin (AQP) genes expression to improve salt tolerance of plants remains unknown.

Physiological and genetic studies have confirmed the direct role of AQPs in improving plant water relation under salt stress (Martre et al., 2002; Siefritz et al., 2002; Siefritz et al., 2004). Sequence homology patterns have led to the classification of AQPs in most plant species into 4 subgroups: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-26-like intrinsic membrane proteins (NIPs), and small basic intrinsic proteins (SIPs) (Maurel et al., 2008). Thus far, 35, 39, and 31 AQP members have been isolated from *Arabidopsis*

thaliana, *Oryza sativa*, and *Zea mays* genomes, respectively (Chaumont et al., 2001; Wallace and Roberts, 2004; Sakurai et al., 2005; Bansal and Sankararamakrishnan, 2007). During plant development and adaptation to the changing environment, AQPs significantly contribute to mediating mass flows of water into and out of the plant cell (Maurel et al., 2008). The control over plant AQP amount or activity and protein location would be particularly important during stress (Hasegawa et al., 2000). Under salt stress, some AQP genes were found to be are upregulated or downregulated in *Arabidopsis thaliana*, *Oryza sativa*, *Helianthus annuus*, and *Brassica oleracea L.* (Kawasaki et al., 2001; Tyerman et al., 2002; Martinez-Ballesta et al., 2003; Jang et al., 2004). A previous study showed *TaAQP* (a *PIP1* subgroup gene *AQP*) is upregulated under salt stress and enhances salt stress tolerance in transgenic *Nicotiana tabacum L.* (Hu et al., 2012). PIPs have been shown to be primary channels mediating water uptake in plant cells. HvPIP2 has been shown to transport water and have the ability to mediate water loss from plant cells under salt stress (Horie et al., 2011). NIPs only exist in plants and can transport different substrates, not only water, glycerol (Dean et al., 1999), and lactic acid (Choi and Roberts, 2007) but also large solutes such as urea (Wallace and Roberts, 2005), boron, silicon, and arsenic (Mitani-Ueno et al., 2011). *TaNIP* (*Triticum asetivum* L. nodulin 26-like intrinsic protein) has been reported to be associated with salt tolerance pathways in plants (Gao et al., 2010).

Since ALA mitigate the effect of salt stress in cucumber seedlings (Zhen et al., 2012), it would be interesting to determine whether the transcriptional reaction of *CsPIP1:1* and *Cs-NIP* genes to NaCl could be mediated by ALA. The purpose of this study was to compare the expression levels of AQP genes to mitigate the adverse effects of salt stress in salt-sensitive cucumber and salt-tolerant cucumber varieties. Additionally, we attempted to determine the relationship between the observed results and the possible changes in leaves and expression of AQP genes in order to better understand the ameliorative role of ALA in NaCl-stressed plants.

MATERIAL AND METHODS

The leaves of salt-sensitive cucumber (Jinchun No.4) seedlings and relatively salt-tolerant cucumber (Jinyou No.1) seedlings were used in this study. Seeds were purchased from Tianjin Kernel Cucumber Research Institute, Tianjin, China. ALA was purchased from Cosmo Oil Company (Japan).

Plant materials and stress treatments

Before sowing, the seeds of cucumber were soaked in moist gauze in 90 mm Petri dishes for 2 days in controlled growth chambers at 25°C (24 h dark). Seeds were sowed in soil (soil:vermiculite:manure, 1:1:0.2) in plastic trays (DaZhongting Ltd., China) in each replication (replicated 8 times). The seedlings of similar size with 2 true leaves were transplanted to plastic containers (8 seedlings per container) containing Hoagland solution (Hoagland and Arnon, 1950) and continuously aerated using an air pump in a greenhouse at 25°C with a photoperiod of 12 h light (600 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/12 h dark.

For salt stress and ALA treatment experiments, the seedlings were grown for 20 days in nutrient solution, which was replaced with fresh medium every 3 days. The spraying treatments (Control, distilled water; T1, 50 mg/dm^3 ALA; T2, 75mM NaCl; T3, 50 mg/dm^3 ALA + 75 mM NaCl) were initiated at dark.

After excision, the samples for stress-treated and non-stressed leaves of seedling were collected at indicated times (2, 4, 8, 16, 24 h) and frozen immediately into liquid nitrogen. The entire experiments were repeated at least 3 times.

Extraction and analysis of RNA

Frozen leaf tissue was ground to a fine powder in liquid nitrogen by using a prechilled mortar and pestle. Total RNA was extracted using the Plant RNeasy extraction Trizol kit (Qiagen, USA). The RNA was treated with an RNase-free DNase I according to manufacturer instruction (Qiagen). The concentration of RNA was accurately quantified spectrophotometrically, and the absorbance was read at 260 nm. The quality of RNA preparation was checked by calculating the ratio of A_{260}/A_{280} nm. The total RNA was checked on 1% agarose gel to monitor its integrity.

Oligonucleotide primers

The position of gene-specific primer sets was chosen in such a way that no primer shared more than 70% identity with any other genes in the whole cucumber sequence. The PCR primer sets were designed to produce PCR products of about 120-150 bp (even 300 bp) as suggested by the manufacturer (Qiagen). The PCR primer sets fulfilling these requirements were generated using the Gene Runner program. The specificity of these primer sets was checked by monitoring the reverse transcription-polymerase chain reaction (RT-PCR) products by a melting curve analysis, which can distinguish gene-specific PCR products from non-specific PCR products. Only a single band with a characteristic melting point was observed for each sample, indicating that the RT-PCR produced a product specific to the primers used for the reaction. The specific primers (Table 1) used for RT-PCR were designed on the basis of GenBank accession numbers EF202176.1 and FJ262171.1 by using the Primer Premier 5.0 software. The *β-actin* gene (GenBank: AB010922.1) was used as an internal control.

Table 1. Primers used for RT-PCR assays.

Gene	Primer pairs
CsPIP	F: CGGCGGTCACTTTTGG
	R: AGTCCTTGGCATCGTCTTGG
CsNIP	F: ATGAACTTTACCCGCCCG
	R: GGAAATCGCCGAACAGCA
Actin	F: GATGACGCAGATAATGTTTG
	R: ACCCTCATAGATGGGGAACAG

F indicates forward and R indicates reverse.

Real-time PCR

For the detection of changes in AQP expression in the leaves subjected to various stresses, quantitative real time (qRT)-PCR experiments were performed using the iCycler IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Reverse transcription PCR was performed using a one-step RT-PCR kit (TaKaRa, Dalian, China). Real-time PCR was performed using the SYBR Premix ExTaq (Takara, Dalian, China) in a final volume of 25 µL. The qRT-PCR mixture contained 1 µL cDNA template, 12.5 µL SYBR Premix ExTaq, 10.5 µL sterile water, and 0.5 µL each gene-specific primer, according to manufacturer protocol (TaKaRa, Dalian, China). The Bio-rad cycler was programmed as follows: 30 min at 50°C for reverse transcription;

15 min at 95°C for HotStarTaq DNA polymerase activation; 40 cycles of 15s at 94°C, 30s at 55°C, and 30s at 72°C. The data were collected during the extension step (30s at 72°C). For a control reaction, no RNA was added to the reaction mixture, resulting in no detectable fluorescence signal. Relative transcript levels were calculated as the ratio of AQP gene expression against *β-actin*. The data were analyzed using the $2^{-\triangle\triangle CT}$ method, and the reactions were performed in triplicate.

RESULTS

Effects of 75 mM NaCl stress on *CsPIP1:1* and *CsNIP* expression in the leaves of salt-tolerant and salt-sensitive cucumber

The results indicated that the expression levels of *CsPIPs* were altered in similar ways in the 2 cucumber varieties under salt stress. At 2 h after NaCl treatment, expression of *CsPIP1:1* was rapidly upregulated and reached the highest level in both cucumber varieties. Moreover, the relative expression level of *CsPIP1:1* in salt-sensitive cucumber was considerably higher than that in the salt-tolerant cucumber, and was 5.2 and 2 folds higher than that of the control, respectively. Subsequently, the *CsPIP1:1* expression level decreased sharply at 4 h, and then continued to decline (Figure 1A).

Under salt stress, *CsNIP* expression also increased slightly and peaked at 2 h in Jinyou No.1 seedling leaves (Figure 1B). Subsequently, the expression level gradually decreased. However, the change in *CsNIP* expression level in Jinchun No.4 was significantly different. It decreased at first after salt treatment and reached the lowest point at 4 h, followed by an increase at 8 h and 16 h after treatment. The result showed that the expression level of *CsPIP1:1* was higher than that of *CsNIP*.

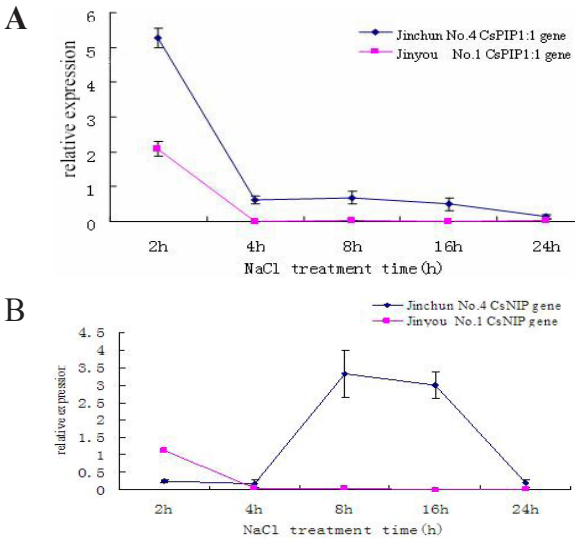

Figure 1. QRT-PCR analysis for *CsPIP1:1* genes (Figure 1A) and *CsNIP* genes (Figure 1B) in Jinchun No.4 and Jinyou No.1 leaves differently expressed under salt stress. **A.** Expression patterns of *CsPIP1:1* genes under NaCl; **B.** expression patterns of *CsNIP* genes under NaCl. All data were normalized to the actin expression level. Data represent fold change of relative quantification in Jinchun No.4 compared to Jinyou No.1. Lines represent relative quantification standard deviation calculated from three biological replicates.

Effects of low concentration ALA on *CsPIP1:1* and *CsNIP* expression in cucumber leaves

The *CsPIP1:1* expression level of the Jinyou No.1 and Jinchun No.4 cultivars was influenced differently by the application of 50 mg/dm^3 ALA to the seedling leaves. *CsPIP1:1* gene expression in salt-sensitive cucumber leaves decreased 16 h after treatment and reached the maximum level at 24 h (2.5 times higher than that of the control). Compared to the transcript level of *CsPIP1:1* in Jinchun No.4, that in Jinyou No.1 peaked at 2 h, and then rapidly decreased (Figure 2A).

Similar trends in *CsNIP* expression levels were noted in both the varieties when the cucumber seedlings were exposed to low concentration ALA. At 2 h after treatment, *CsNIP* expression level increased slightly in Jinyou No.1 compared to those in the control and Jinchun No.4. *CsNIP* expression in Jinchun No.4 was induced to 3 folds. After 2 h, the expression level rapidly decreased in both the varieties. As shown in Figure 2B, the expression level of *CsNIP* in Jinyou No.1 leaves was lower than that in Jinchun No.4 leaves.

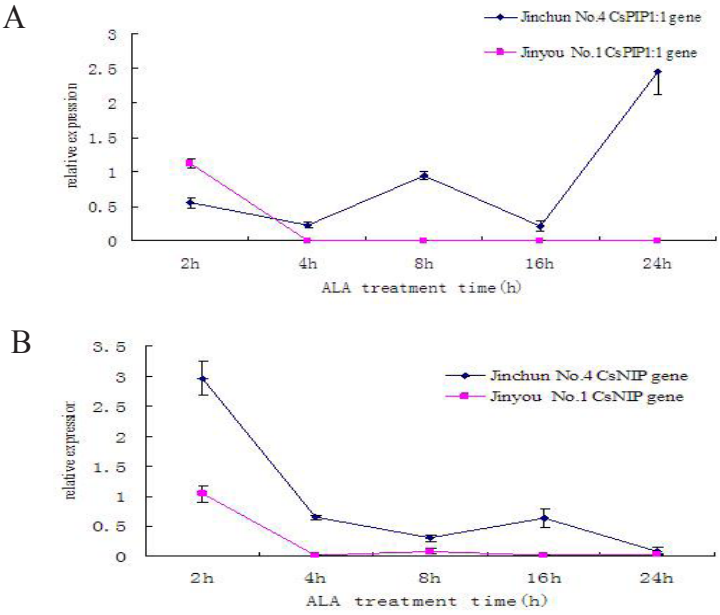

Figure 2. QRT-PCR analysis for *CsPIP1:1* genes (Figure 2A) and *CsNIP* genes (Figure 2B) in Jinchun No.4 and Jinyou No.1 leaves expressed under ALA treatment. **A.** Expression patterns of *CsPIP1:1* genes; **B.** expression patterns of *CsNIP* genes. All data were normalized to the actin expression level. Data represent fold change of relative quantification in Jinchun No.4 compared to Jinyou No.1. Lines represent relative quantification standard deviation calculated from three biological replicates.

Effects of ALA on gene expression in cucumber seedlings under salt stress

The above-mentioned results showed that not only salt stress but also ALA treatment changed the levels of *CsPIP1:1* and *CsNIP* expression (Figures 1 and 2). Hence, it would be interesting to know that changes in *CsPIP1:1* and *CsNIP* expression levels in the salt-tolerant and salt-sensitive cucumber varieties after treatment with NaCl + ALA.

Application of exogenous NaCl and ALA decreased *CsPIP1:1* expression in the salt-sensitive cucumber at 2 h. Subsequently, its expression level increased and reached the highest level, 3.8 fold to that of control, and decreased rapidly thereafter. The salt-tolerant cucumber showed only slight differences in the expression level of *CsPIP1:1*. At 2 h, *CsPIP1:1* expression level increased compared to that of the control and reached maximum (2.3 folds) at 4 h and then gradually decreased (Figure 3).

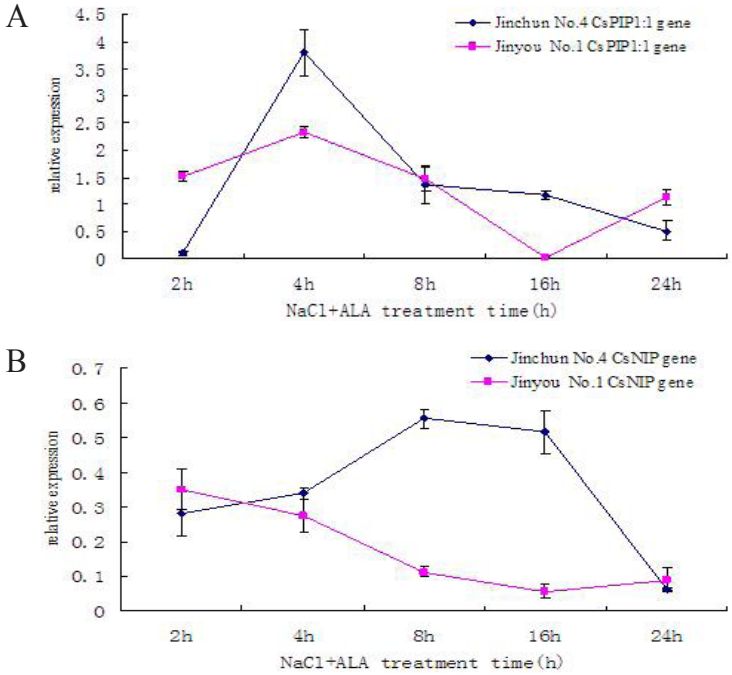

Figure 3. QRT-PCR analysis for *CsPIP1:1* genes (Figure 3A) and *CsNIP* genes (Figure 3B) in Jinchun No.4 and Jinyou No.1 leaves expressed under ALA+NaCl treatment. **A.** Expression patterns of *CsPIP1:1* genes; **B.** expression patterns of *CsNIP* genes. All data were normalized to the actin expression level. Data represent fold change of relative quantification in Jinchun No.4 compared to Jinyou No.1. Lines represent relative quantification standard deviation calculated from three biological replicates.

DISCUSSION

Soil salinity is a major threat to greenhouse vegetable production systems. Adapting plants to salt tolerance is a genetically complex procedure and depends on stress sensing, signal transduction, and specific stress-related gene and metabolite expression (Vinocur and Altman, 2005). NaCl strongly influences plant water relations (Hasegawa et al., 2000; Munns et al., 2002). Many studies have shown that AQPs are responsible for the transcellular movement of water across cell membranes; they are thought to play an important role in plant water relations (Luu and Maurel, 2005). The AQP genes are known to respond significantly and differently to environmental stress conditions. The upregulation or downregulation of *AQP* gene transcript levels by salt stress has been described in the roots and leaves of various plant species such as tobacco, *Arabidopsis*, radish, barley, and tomato.

In our analysis, CsPIP, an AQP family member, that shares high amino acid sequence homology with PIP from *Arabidopsis* was used. Studies indicated dynamic control of PIP1 AQP translation and/or degradation after exposure to salt. Under salt stress, expression of *PIPs* in *Arabidopsis* roots strongly decreased between 2 and 4 h. This is partially inconsistent with the results in this study. In the leaves of both Jinchun No.4 and Jinyou No.1 cucumber seedlings exposed to salt stress, the *CsPIP1:1* genes were significant upregulated, as revealed by mRNA accumulation at 2 h, and then remarkably downregulated. This upregulation of *CsPIP1:1* gene expression by salt stress may result in increased membrane water permeability and might promote cellular water uptake from the environment to maintain reasonable water status under salt stress. However, subsequently, the expression of *CsPIP1:1* aquaporin genes was downregulated. Previous studies have reported that the downregulation of *CsPIP1:1* gene expression may result in reduced membrane water permeability and might improve cellular water conservation during the periods of dehydration stress. Moreover, the results showed the expression level of *CsPIP1:1* in the salt-sensitive Jinchun No.4 was higher than it in the salt-tolerance Jinyou No.1. This suggested that CsPIP aquaporin proteins are highly expressed in salt-tolerant plants than in salt-sensitive ones and have an effect to alleviate the harm of plant under salt stress".

Exogenous low concentrations of ALA have been reported to alleviate the harmful effects caused by water-deficit stress to some degree, by increasing chlorophyll biosynthesis, photosynthesis, and cold and salt tolerance (Hotta et al., 1997; Hotta et al., 1998; Watanabe et al., 2000a). Recently, Zhen and colleagues found that ALA application can counteract the oxidant damage induced by salt in cucumber leaves and upregulated the expression of catalase and cytosolic ascorbate peroxidase genes in the leaves (Zhen et al., 2012). Thus far, the effects of ALA on AQP gene expression have not been described in plant species. In this study, qRT-PCR expression analysis of genes showed that the relative expression level of *CsPIP1:1*, 1.1 times (Jinyou No.1) and 2.5 times (Jinchun No.4) in cucumber seedlings with ALA treatment, was significantly lower than that in the salt stress group, 2 times (Jinyou No.1) and 5.2 times (Jinchun No.4). With ALA treatment alone, *CsPIP* expression in salt-sensitive Jinchun No.4 tended to change: first, it decreased until 16 h, and then increased and reached maximum at 24 h. Taken together, these findings suggest that changes in *CsPIP1:1* gene expression remarkably enhanced water status of the cucumber seedling leaves after ALA application.

Hence, we next treated the cucumber seedlings with both NaCl and ALA. The expression of *CsPIP1:1* in the 2 cucumber varieties increased first and then decreased. The duration of increments was longer (i.e., 4 h) when ALA + NaCl was used than that when NaCl was used alone (i.e., 2 h). The *CsPIP1:1* expression level in Jinchun No.4 after treatment with ALA + NaCl was lower than that when NaCl was used alone. However, in Jinyou No.1, the expression was almost the same as that under salt stress. The expression of *CsPIP1:1* in plants treated with ALA + NaCl was higher than that in plants treated with ALA alone. These results suggested that ALA application possibly protects cucumber seedlings from NaCl stress.

NIPs, major intrinsic proteins in plants, are typically highly regulated at the transcriptional level and respond to a diverse array of developmental- and stress-related signals (Maathuis et al., 2003; Alexandersson et al., 2005). Modulation of NIP activity and/or expression is a part of the coordinated response of plants to osmotic challenge (Wallace et al., 2006). *AtNIP1:1* was reported to be downregulated under a variety of osmotic stresses such as salinity (Weig et al., 1997). The present results showed that, under salt stress, *CsNIP*

expression decreased at 2 h and 4 h and then increased at 8 and 16 h (reaching 3.3 folds) after treatment in the salt-sensitive cucumber. However, in the salt-tolerant cucumber, the expression of *CsNIP* slightly increased at 2 h (1.1 fold) and then decreased under salt stress. Under salt stress, expression of *CsPIP* was higher, while that of *CsNIP* was considerably lower. This finding is in agreement with that reported by (Alexandersson et al., 2005). This variation in the downregulation or upregulation of AQP gene expression might play roles in limiting initial water loss during the early stage of salt stress and assisting in maintain water homeostasis by subsequent uptake of water under high cellular salt condition.

Foliar application of ALA to the plants resulted in an initial increase and then decreased expression of *CsNIP* in Jinyou No.1 and Jinchun No.4 varieties, maximum to 3 and 1.04 folds, respectively. This suggested that application ALA changed the expression level of *CsNIP* and produces an acute change in the salt-sensitive Jinchun No.1. Compared with the *CsNIP* gene expression level after treatment with salt alone, that after treatment with ALA alone or with salt and ALA in combination was downregulated in both the cultivars at all treatment times. This suggests that the expression level of *CsNIP* was probably associated with ALA treatment, irrespective of whether it was applied alone or in combination with salt. NIPs are known to have several mechanisms to enhance plant tolerance under various stresses. Maintenance of a proper water status under NaCl stress requires not only water transport via AQPs but also phosphorylation of certain serine residues that activate AQPs and their dephosphorylation to rapidly deactivate AQPs. This aquaporin inactivation can prevent water loss from cells under strong salt stress. The mechanism by which ALA induces AQPs is not clear, and further studies are needed to address this issue.

In this study, the changes in growth response to stress were obvious. Both Jinyou No.1 and Jinchun No.4 lines showed severely stunted growth under salt stress. Application of ALA improved the growth of cucumber seedlings compared to that of the control plants. Application of ALA + NaCl resulted in a considerably better growth of all seedlings than that observed under salt stress. The dry weights of the whole plants and leaves, root dry weights, and plant heights were significant different between the ALA + NaCl treatment group and the salt stress group. Moreover, the Na^+ and K^+ concentrations showed related changes (data not shown), indicating that ALA was very effective in cucumber seedling under NaCl stress.

To our knowledge, this is the first study to report the changes in the expression level of AQP genes after treatment with ALA in cucumber. In this study, we determined the relative transcript level of *CsPIP1:1* and *CsNIP* genes in response to salinity alone, ALA alone, and ALA + salt treatment in salt-sensitive and salt-tolerant cucumber. The results suggested that ALA induced changes in *CsPIP1:1* and *CsNIP* transcriptional expression. Moreover, the relative transcript level of *CsPIP1:1* and *CsNIP g*enes differed significantly, probably indicating that discrete signaling pathways might regulate the expression of these genes. However, the detailed mechanisms remain unknown and need to be elucidated in the future.

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