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RESEARCH ARTICLE

Knockdown of *ACS9* expression in *Arabidopsis* decreases the tolerance to salt and osmotic stress

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Abstract Based on the DNA sequence of *ACS9*, two produced fragments were subcloned into binary vector pCAMBIA1300 in antisense and sense orientations, and the generated RNA interference (RNAi) vector was then transformed into *Arabidopsis thaliana*. The stress resistance function of *ACS9* gene in *Arabidopsis thaliana* was researched by determination of stress resistance physiologic indexes, NaCl and PEG6000 resistance. The results showed that the inhibition of *ACS9* expression enhanced the sensitivity to high concentration NaCl (150 mmol/L) and PEG6000 (7%) in *Arabidopsis thaliana* seeding stage. The proline contents and water loss rates in transgenic plants were 0.68 and 1.4 times higher than those in the wild-type leaves, respectively, indicating that the inhibition of *ACS9* expression due to salt and drought resistant was reduced and suggested that *ACS9* gene played important roles in plant salt and drought tolerance.

Keywords *ACS9* gene, RNAi vector, functional analysis, stress resistance

Introduction

The gas ethylene (C_2H_4) is known as a signaling molecule that regulates a variety of developmental processes and stress responses in plants (Abeles et al., 1992). Ethylene production in plant cells is enhanced by a variety of external factors, including wound, pathogen attack, hormone treatment, chilling injury, drought, or the presence of heavy metals (Yang and Hoffman, 1984; Alonso and Stepanova, 2004; Chen et al., 2005). The biosynthetic pathway of ethylene is clarified by Adams and Yang (1979). The production of ethylene in higher plants is from S-adenosyl-L-methionine (AdoMet), which is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) and then to ethylene by ACC oxidase (Adams and Yang, 1979). The step of ACC formation is the main rate-limiting step in the ethylene biosynthesis (Alonso and Stepanova, 2004). ACS is the key enzyme that regulates the majority of cases in ethylene production responding to stress (Boller et al., 1979).

ACS is encoded by a multigene family in all examined plant species (Bleecker and Kende, 2000). In *Arabidopsis*, the *ACS* gene family encodes nine polypeptides including one nonfunctional gene *ACS1* and eight functional genes *ACS2*, *ACS4–9*, and *ACS11* (Yamagami et al., 2003). Each *ACS* gene shows a unique expression profile during *Arabidopsis* growth and development and is able to respond to different hormonal and environmental signals, whereas a single developmental or environmental stimulus can induce the coexpression of several *ACS* genes (Tsuchisaka and Theologis, 2004; Peng et al., 2005).

Expression of *ACS* gene can be induced either in such specific tissues as hypocotyl, leaf, root, tuber, petiole, flower, petal, pistil, and fruit or in responses to such biotic and/or abiotic factors as radiation, Cu^{2+}, Li^{+}, wound, protein kinase inhibitor, IAA, ethylene, chilling, and pathogens (Ge et al., 2000; Wang et al., 2005). Mechanical wound and insect attack can induce the ethylene synthesis and *ACS* gene expression (Liu et al., 1993; O'Donnell et al., 1996; Alonso and Stepanova, 2004), while the ethylene regulates the damage stimulation on plants in turn (Ronald et al., 2007). Numerous *ACS* genes have been found to be wound-inducible (Ge et al., 2000). It is found that *ACS4* and *ACS5* are responsive to wounding treatment, while *ACS7* is not induced (Peng et al., 2005). All these results show that *ACS* genes play important roles in plant stress-resistance. *ACS* functional

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analysis has future significance for understanding the regulating mode of ethylene in plant developmental processes.

RNA interference (RNAi) technology is currently a more effective method of gene suppressed expression (Chuang and Meyerowitz, 2000). In the previous study, we speculated that *ACS9* gene was involved in plant stress-resistance responses (Pan, 2009). To understand the role of *ACS9* gene in response to diverse stresses during plant development, RNAi expression vector was constructed according to its nucleotide sequence. The recombinant carried by *Agrobacterium* was introduced into *Arabidopsis* plants through the flowers dipped in bacterial cultures. When the transgenic plants were treated with salt and drought stress, we understood the gene function by analyzing their sensitivity to the stresses?

Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col) was used throughout this study. Seeds were surface sterilized for 10 min in 10% sodium hypochlorite, washed with sterilized water for five times, and germinated on the selection medium plate (1/2 Murashige-Skoog media containing 50 mg/L hygromycin, 1% agar, pH 5.7). After cold treatment at 4°C for 2 d, the plates were incubated at 22±1°C under 16 h light/8 h dark (light grow seedlings) for two weeks or under the dark (etiolated seedlings) for 3–10 days to produce *Arabidopsis* seedlings.

For seed germination assays, at least 100 seeds from transgenic lines and wild-type plants were sterilized and planted on MS triplicate plates supplemented with different concentrations of NaCl and PEG6000. The plates were cultured in a growth chamber at 22±1°C under long-day conditions (16 h light/8 h dark) after cold treatment at 4°C for 2 days. The germinated seeds (emergence of radicals) were counted and characterized after 10 days.

Construction of RNAi silencing vector

Specific fragment of *ACS9* gene were obtained by multiple sequence alignment using DNAMAN software. The degenerate primers, designed according to intron location of *ACS9* gene and multiple cloning site of vector pCAMBIA1300, were used for amplifying specific fragments of *ACS9* gene. The primers were given as follows: P1: TAAGGATCCGCTGCTGGTTCAACATCTGC(*Bam*HI); P2:TGTGAGCTCACACGAGACCGAAACTTGAC(*Sac*I); P3:CGCGGATCCGCTCAAGCTCTAATGGGTTC (*Bam*HI); P4:TGCTCTAGAACACGAGACCGAAACTTGAC(*Xba*I).

Antisense fragments and sense fragments were amplified, and two product fragments were subcloned into binary vector pCAMBIA1300 after restriction digestion analysis, and then, RNAi vector pCAMBIA1300-*ACS9*i was introduced into *Agrobacterium* GV3101.

Transformation and molecular identification of transgenic lines

The recombinant was introduced into *Arabidopsis* plants using floral dipping method (Clough and Bent, 1998). The independent transformants were screened on 1/2 MS media. The transformed seedlings of green and expanded leaves were screened out from the yellow-colored nontransformed seedlings and transferred to soil for 2 to 3 weeks.

The PCR primers used to confirm the recombinant transgenes in transgenic plants were P5: 5′-GCTGCTAAAGGAGATGAATG-3′ and P6: 5′-GAATAAGGAGGATCAACGAC-3′. Genomic DNA was isolated from transgenic plants as template for PCR amplification (Weigel and Glazebrook, 2004). *ACS9* gene expression in the transgenic lines was determined with total RNA isolated from seven-day-old etiolated seedlings treated with 50 mol/L CHX (Yamagami et al., 2003).

Characterization of mutant phenotypes

Hypocotyl-length and root-length measurements were carried out with light and dark-grown seedlings grown on normal conditions for 3–10 days. After potted seedlings grew in drought treatments for 2 weeks with constant watering, then the water was withheld, and pictures were taken after 3 weeks treatment.

Seedlings were watered in treatment with 150 mmol/L NaCl for 2 weeks when phenotypes were characterized.

Measurement of proline and water loss rate

Transgenic plants and wild-type plants were watered with 150 mmol/L NaCl for 48 d; proline contents were determined as previously described (Bates et al., 1973). Rosette leaves were detached from transgenic plants and wild-type plants growing under normal conditions for 3 weeks and weighed immediately on a piece of weighing paper at designated times. The percentage loss of fresh weight was calculated on the basis of the initial weight of the leaves (Zhizhong et al., 2005). Three replicates were made for each line.

Results

Construction of RNAi vector

After a series of molecular operations, the two produced fragments were introduced into binary vector pCAMBIA-1300 and generated RNAi vector pCAMBIA1300-*ACS9*i.

Restriction digestion analysis of pCAMBIA1300-*ACS9*i showed that it contains sense and antisense fragments of *ACS9* gene, indicating that the RNAi vector pCAMBIA1300-*ACS9*i was constructed correctly (Fig. 1).

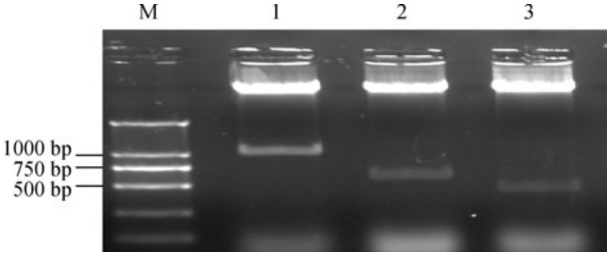

Figure 1 Restriction digestion analysis of pCAMBIA1300-*ACS9*i. Note: M is DL2000 DNA marker; 1 is *Xba*I and *Sac*I; 2 is *Xba*I and *Bam*HI, and 3 is *Bam*HI and *Sac*I.

Screening and molecular identification of transgenic plants

The independent transformants were screened on 1/2 MS media containing 50 mg/L hygromycin and 1% agar. The transformed seedlings of green and expanded leaves were screened out from the yellow-colored nontransformed seedlings.

Genomic DNA was isolated from five transgenic plants randomly selected as template for PCR amplification. The result showed that a specific purpose of bands at size of about 801 bp fragment in line for expectations from all the tested plants with the blank plasmid pCAMBIA1300 as positive control, whereas the control wild-type plants did not have amplified bands (Fig. 2).

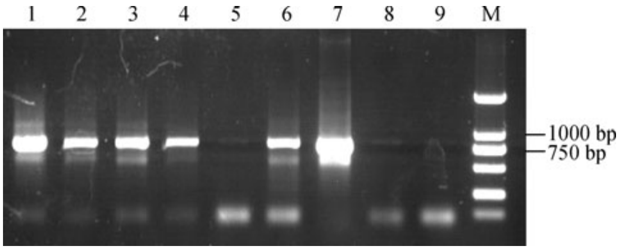

Figure 2 PCR identification of transgenic plants. Note: M is DL2000 marker; 1–4 and 6 are transgenic plants; 5 are nontransgenic plants, and 7 is pCAMBIA1300 plasmid (positive control); 8 is negative control, and 9 is water control.

Total RNA was isolated from seven-day-old etiolated seedlings treated with 50 mol/L CHX. The levels of expression of *ACS9* gene were determined in wild-type and transgenic plants by RT-PCR. Two transgenic plants with significant downexpression of *ACS9* gene were obtained, indicating that the expression of the target gene was suppressed (Fig. 3). We chose obviously the down-expression of transgenic plants (four lanes, later recorded as M) for the following study.

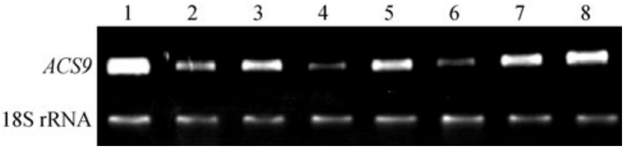

Figure 3 Expression detection of *ACS9* in transgenic plants. Note: 1 is wild-type; 2–8 are transgenic plants.

Seed growth of transgenic plants and wild-type plants

Transgenic plants could enhance the growth of hypocotyl and root of 10 d etiolated seeds better than wild-type plants whose hypocotyl length was similar to the root length of the transgenic plants (Fig. 4). However, the transgenic plants could only enhance the hypocotyl growth of 3 days light-grown seeds. In addition, phenotypes showed no apparent difference in mature transgenic plants and wild-type plants.

Tolerance of transgenic plants to osmotic stress during both germination and growth

When seeds were sown on MS medium supplemented with

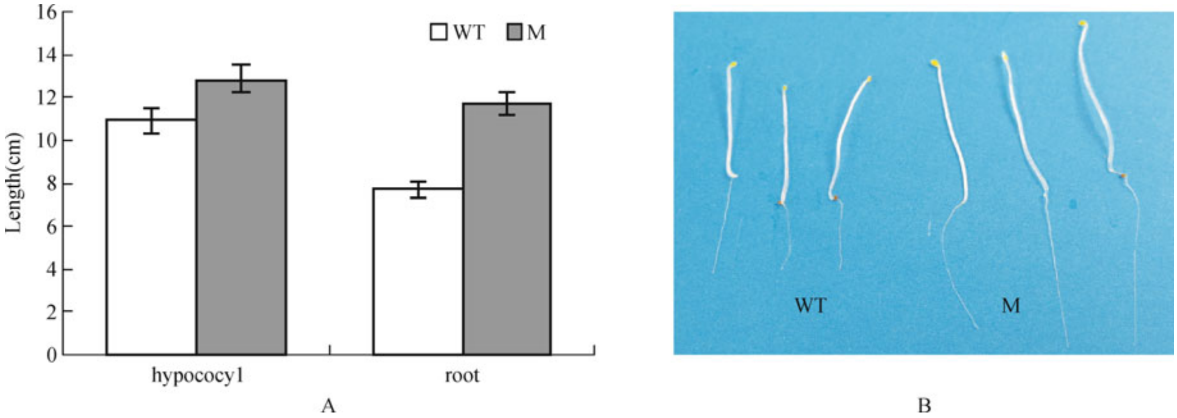

Figure 4 Seeding growth of transgenic plants and wild-type plants when etiolated for 10 days. Note: A shows the results for hypocotyl and root length, and B shows the results for growth of hypocotyl and root. WT is wild-type plants, and M is mature transgenic plants.

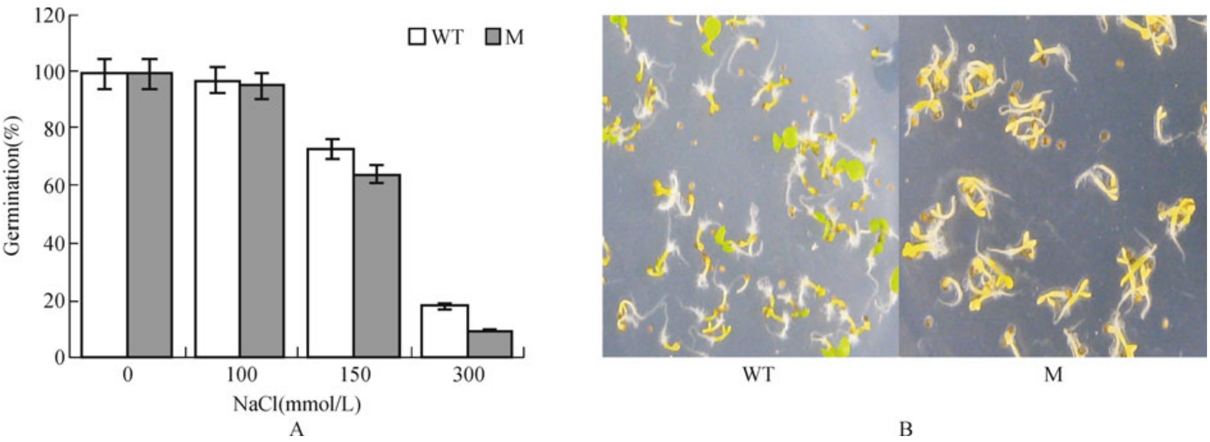

Figure 5 Germination and growth of transgenic plants and wild type plants when seeds were treated with NaCl for 10 days. Note: A shows the seed germination of transgenic plants and wild type plants; B shows the seed growth of transgenic plants and wild type plants.

different concentrations of NaCl for 10 days, the germination of transgenic plants and wild-type plants showed no apparent difference on normal condition. However, as NaCl concentrations were increased, there was a decrease in germination, while no apparent difference was found in transgenic plants and wild-type plants in the same concentrations of NaCl (Fig. 5A). The cotyledons of transgenic plants were etiolated and even died after germination, while about 40% wild-type plants grew out green cotyledons in 150 mmol/L NaCl (Fig. 5B). Wild-type plants less enhanced germination as compared with transgenic plants; however, both could hardly survive 300 mmol/L NaCl, which suggested that the suppression of *ACS9* genes affected the sensitivity of *Arabidopsis* to the NaCl and reduced its resistance to salt stress.

Seed germination of transgenic plants and wild-type plants showed no apparent difference when treated with different concentrations of PEG6000. However, the root length of 10-days-old seedlings of wild-type plants treated with 5% and 7% PEG6000 was longer than that of transgenic plants (Fig. 6). We observed that the seedlings of transgenic plants turned brown and the center parts of the seedlings blackened, while the wild-type seedlings were still green. This indicates that the *ACS9* gene was inhibited, and the tolerance of *Arabidopsis* to drought was reduced.

Detection of osmotic physiologic indexes

Proline is one of the most effective osmotic regulators, and free proline content is an important indicator of the ability of plants to be adapted to stresses. The proline content was determined when treated with 150 mmol/L NaCl solution for 48 h. The result showed that the proline content in transgenic plants was 0.82 times as high as that of wild type. When treated with 150 mmol/L NaCl solution, the significant increase in the proline content of transgenic plants appeared, but it was (0.68 times) less than that of the wild-type plants (Fig. 7).

Excised-leaf water loss rate could reflect the drought resistance of plants, and the more the drought resistance, the smaller the excised-leaf water loss rate is (Clarke and Mccaig, 1982). We found that the leaf water loss rate of transgenic plants was always higher than that of the wild-type plants in our study, indicating the greatest difference between transgenic plants and wild-type. After 4 h *in vitro*, the water loss

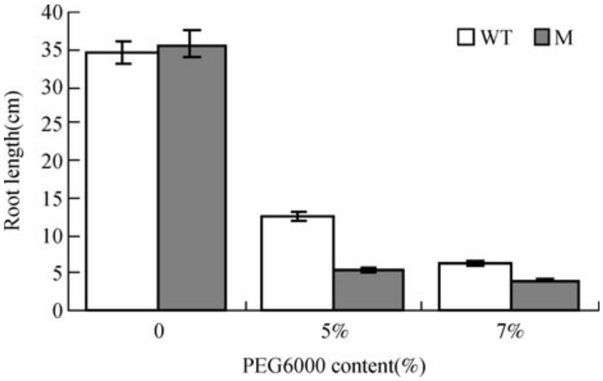

Figure 6 Root length transgenic plants and wild-type plants seeding with PEG6000.

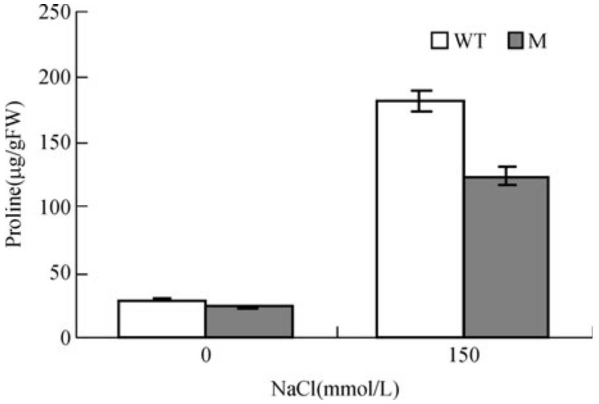

Figure 7 Proline accumulation of transgenic plants and wild-type plants with 150 mmol/L NaCl for 48 h.

rate in transgenic plants leaves was 1.4 times as high as that of wild-type leaves (Fig. 8). In conclusion, our result suggests that transgenic plants can reduce the capacity of salt and drought resistance.

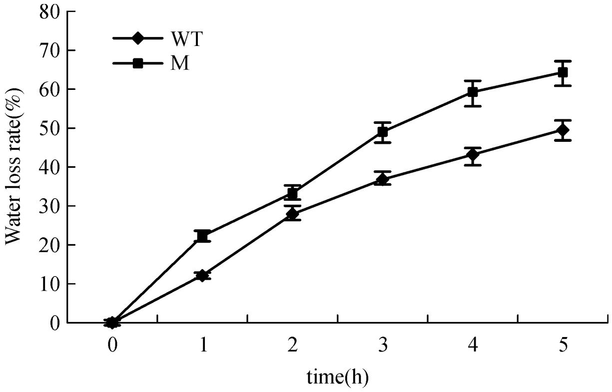

Figure 8 Leaf water loss rate transgenic plants and wild type.

Discussion

Gene expression of reduction or inactivation is often used in molecular biology studies to determine the gene functions through phenotypic observation. RNAi technology has features without a large amount of screening and separation as compared with several other technologies used for functionality missing, and it easily generates the mutation of function loss or reduction (Smith et al., 2000). In our study, we used RNAi technology in making down expression of *ACS9* gene in transgenic plants in order to further study gene functions.

In plant growth and development process, there exist a variety of unfavorable factors affecting the survival of plants as adversity stresses. Ethylene carries stress-related signal molecule regulating plant biologic stress and abiotic stress responses (Ge et al., 2000; Alonso and Stepanova, 2004). We found that ethylene as a negative regulator of plant stress resistance (Straeten et al., 1990) can inhibit root elongation and promote plant aging process under water stress, thereby reducing the drought resistance of plants. Ethylene contains a response factor to improve drought resistance capacity in tomatoes (Cornelius and James, 2006). Gómez-Cadenas et al. (1998) reported that ethylene has a moderating effect on leaf shedding by salt stress, and the ethylene insensitive mutants (*ein2*, *ein3*, *etr1*) can enhance the sensitivity of pathogens *B. cinerea* (Ferrari et al., 2003). These findings suggest that ethylene acts as an endogenetic regulator in stress responses (Pierik et al., 2006).

Recently, Tsuchisaka has reported that ethylene-mediated processes are orchestrated by a combinatorial interplay among ACS isoforms (Tsuchisaka et al., 2009). Our study showed that *ACS9* genes down-expression in transgenic *Arabidopsis* enhanced sensitivity compared with Columbia wild-type to high salt (150 mmol/L NaCl) and drought (7% PEG6000) stress, indicating *ACS9* gene may be provided protective effect for osmotic stress, directly or indirectly. Free proline content and determination of leaf water loss rate further confirmed this result. For further certify function of *ACS9* gene, knockdown of *ACS9* expression in other *Arabidopsis* lines is under study. In addition, interaction among *ACS9* gene and other family genes in response to adversity stress and the role of ethylene need to study in regulatory these processes in future.

Acknowledgements

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