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Profiling of ACC synthase gene (*ACS11*) expression in *Arabidopsis* induced by abiotic stresses

Hee-Dong Eun†, Sajid Ali†, Hyeonjung Jung, Kihwan Kim and Won-Chan Kim*

Abstract

Abiotic stress induce the production of 1-aminocyclopropane-1-carboxylate (ACC), the precursor of ethylene by activating the enzyme ACC synthase. There are twelve ACC synthase genes reported in the genome of *Arabidopsis,* and the *ACC synthase 11* (*ACS11*) gene encodes a polypeptide that is functional; however, its involvement in ethylene biosynthesis in response to abiotic stresses remains unclear. We evaluated the effects of higher ACC accumulation on *A. thaliana* seedlings in response to abiotic stressors such as flooding, salinity, cold, and drought. Transgenic plants were generated with *ACS11* (*ACS11*-OX), and they demonstrated that overexpression of *ACS11* reduces both root and shoot length observed in seedlings. RT-PCR analysis revealed that abiotic stressors induce the expression of the wild type *ACS11* gene. Histochemical staining revealed that GUS activity followed the same time course as induction of wild type *ACS11* gene expression, increased ACC levels, and production of stress hormone, ethylene. One finding showed that although induction of wild type *ACS11* gene occurs under drought stress, GUS activity was highest at 6 h of drought stress and decreased to levels similar to control seedlings at 12 and 24 h. Thus, Wild type *ACS11* expression is involved in ACC production, and abiotic stressors induce the expression of *ACS11* gene. Moreover, ACC increases in response to abiotic stress lead to the production of ethylene. All of the data presented here suggest that the overexpression of *ACS11* paves the way for the production of stress hormone, ethylene, which adversely affected the growth and development of the plant.

Keywords: Abiotic stress, ACC synthase, ACS11, *Arabidopsis*, GUS assay, Ethylene

Introduction

Plants growth and development are adversely affected by unfavorable environmental conditions such as extreme temperature, salinity, drought, and flooding conditions. Salinity and drought are the major abiotic stressors that adversely affect more than 20% of arable and about 40% of irrigated land worldwide. In addition, approximately one-third of the world's irrigated land is considered to be affected by salinity and extreme temperature [1, 2]. Abiotic stresses severely affect crop yields in general, but for grains, abiotic stresses decrease survival, biomass, and grain yield by more than 50% [2–4]. When plants are continuously exposed to stressful conditions in their natural environment, they not only demonstrate stress avoidance but also develop stress tolerance mechanisms [5, 6]. In higher plants, increased levels of ethylene are produced in response to a number of different biotic and abiotic stressors. Typically, a very low concentration (0.01 µl/l) of ethylene is beneficial to the growth and development of plants; however, the 20-fold higher levels of ethylene typically produced under stressful conditions lead to physiological and anatomical damage [7–10].

Ethylene is a phytohormone that performs diverse regulatory functions in the growth and development of plants, including seed germination [11, 12] root growth [13, 14] fruit ripening [15, 16], and flower and leaf abscission [17, 18]. Additionally, ethylene plays a key role in

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biotic and abiotic stress responses [19–21] and is considered an important modulator of plant growth and development. However, stress conditions elicit increased levels of ACC synthase, which in turn increases the amount of substrate ACC available to produce higher amount of ethylene in plant tissues. In other words, when plants are subjected to stressful conditions, they respond by producing stress hormone, ethylene. Glick et al. noted that the synthesis of stress hormone, ethylene occurs in two different peaks in which the magnitude of the first ethylene peak is much smaller than that of the second peak [22, 23]. Additionally, ethylene has been shown to be a signaling molecule for different stress responses and for pathways that control plant growth and development. However, higher concentration of ethylene also has negative effects such as early flower abscission, senescence, and abnormal plant growth and development [23, 24]. The biosynthetic pathway of ethylene is well established in plants [25, 26]. Typically, the amino acid methionine is converted into *S*-adenosyl-L-methionine (SAM) by the action of SAM synthetase. Then SAM is converted into ACC by ACC synthase; however, this reaction also forms 5-methylthioadenosine (MTA), which is recycled back into the amino acid methionine by a multistep pathway also known as the Yang cycle [27]. Meanwhile, ACC is converted into ethylene by ACC oxidase.

ACC synthase performs the rate-limiting step in ethylene biosynthesis [28]. In higher plants, the regulation of the rate of ethylene biosynthesis occurs at ACC formation; therefore, ACC synthase is a key regulator of both normal and ethylene production. ACC synthase is a cytosolic protein with a short half-life and requires pyridoxal phosphate as a cofactor for its activity. The published X-ray structure of ACC synthase provides a basis for the rational design of inhibitors for agriculture applications [27–29]. Several studies report that ACC accumulation is stimulated by biotic and abiotic stress conditions. Under drought conditions, wheat leaves produce 30-fold more ethylene within 4 h, and these fluctuations in ethylene production occur in parallel with increases and decreases in substrate ACC levels [30]. Another study observed differential accumulation of *S*-adenosylmethionine synthetase (AdoMet) transcripts in response to salinity stress conditions, a relevant finding because AdoMet produces the substrate for ACC synthase in the ethylene biosynthetic pathway [31]. Similarly, a study that measured the effect of cold stress on ACC levels in cold-tolerant and cold-sensitive maize inbreeds concluded that ACC accumulation was higher in the cold-sensitive maize (Co-125) inbreed compare to the cold-tolerant one [32]. Hence, a detailed study on the role of ACC synthase gene expression and ACC accumulation will contribute to our understanding of ethylene's role in plant growth and development. Additionally, such a study can pave the way toward understanding how plants respond to different environmental stresses and how to improve sustainable agriculture. In higher plant species, almost every genome carries multiple genes that encode for ACC synthase polypeptides [33, 34]. Five ACC synthase genes are reported in rice plants [35] and *Solanum tuberosum* [36]. Eight ACC synthase genes were reported in tomato [37, 38]. Each ACC synthase gene in these multigene families are expressed throughout the plant life cycle and each is differentially regulated in response to environmental factors [33]. However, the *Arabidopsis* genome contains twelve ACC synthase genes (*ACS1-ACS12*) on five different chromosomes [34, 39]. Among the twelve *ACS* genes, *ACS2*, *ACS4*, *ACS5*, *ACS6*, *ACS7*, *ACS8*, *ACS9*, and *ACS11* encode functional ACC synthases; however, *ACS10* and *ACS12* encode aminotransferases and *ACS3* is a pseudogene [40, 41].

When ACC synthase genes are upregulated, the amount of defensive proteins increases. Higher levels of ACC synthase pave the way for increased ACC production that in turn produces stress hormone, ethylene in response to the condition that triggered the upregulation. The presence of stress hormone, ethylene is detrimental to plant growth and development and is involved in initiating undesirable processes such as chlorosis, leaf abscission, and senescence. In the present study, we tested the hypothesis that plants under different abiotic stresses (flooding, drought, salinity, and cold) would upregulate *ACS11* expression and accumulate increased levels of ACC in plant tissues, resulting in stress hormone, ethylene levels that could adversely affect the plant. Hence, the *ACS11* gene and its promoter were used to study the role of the *ACS11* gene product, a type-2 ACC synthase, and the role of ACC accumulation in the growth and development of the model plant (*A. thaliana*) under normal conditions and under conditions of abiotic stresses.

Materials and methods

Plant growth conditions

We evaluated *Arabidopsis thaliana,* ecotype Columbia (Col-0), in a growth chamber at 23 °C for 16 h of light, and at 21 °C for 8 h of dark, at 60% relative humidity, according to the growth conditions described by [42]. For phenotypic analysis of *ACS11* transgenic plants, wild type *A. thaliana* and the T2 generation of *ACS11*-OX plants were grown on soil under the same conditions. To confirm phenotypic changes in root lengths of wild type and *ACS11*-OX seedlings, all the seeds were surface sterilized with seed disinfectant (25% bleach and 0.01% Triton X-100) for 10 min, then rinsed with distilled water three times. Surface sterilized seeds were germinated and grown on filter paper by plating on 0.5 × Murashige and

Skoog (½ MS) medium (0.8% agar, 1% sucrose, 0.6 mM MES [Sigma], pH 5.7) plates at 20 °C for 4 days. After 4 days, seedlings were transferred to the same plate for measurement of their root lengths. However, some seedlings were transferred to pots filled with soil and grown at 20 °C under controlled conditions for further experiments. The plants were analyzed with a minimum of three biological replicates per line in this research.

Genomic DNA extraction and plasmid construction

For extraction of genomic DNA, the rosette leaves were ground with a pestle in a 1.5 ml micro-centrifuge tube, 500 µl of buffer was added, and then vortexed to mix thoroughly. The tube was centrifuged for 5 min at 13,000×*g*, the supernatant was transferred to a new tube, and an equivalent volume of isopropanol added to the transferred supernatant. The tube was inverted approximately 20 times to mix and then centrifuged for 10 min at 13,000×*g*. After discarding the supernatant, 500 µl of 70% ethanol was added to wash the precipitate. The tube was centrifuged for 2 min at 13,000, the supernatant was discarded, and the pellet was dried for 10–15 min. The DNA pellet was resuspended in 20 µl of DNase-free water. Plasmids were constructed by PCR amplification with specific primers. The 1383 bp coding sequence of the *A. thaliana ACS11* (*AtACS11*) gene was amplified from cDNA and the *AtACS11* promoter, including the 1076 bp immediately upstream of the 5′-untranslated region of the *AtACS11* mRNA was amplified from genomic DNA (Additional file 1: Table S1). PCR conditions for cDNA were as follows: 94 °C for 5 min; followed by 35 cycles of 94 °C for 20 s, 59 °C for 40 s, and 72 °C for 1 min 10 s; then 72 °C for 5 min. PCR conditions for genomic DNA were as follows: 94 °C for 5 min; followed by 35 cycles of 94 °C for 20 s, 55 °C for 40 s, and 72 °C for 1 min; then 72 °C for 5 min. The *AtACS11* coding sequence fragment was inserted into the pCB302-3 binary vector, and the *AtACS11* promoter fragment was inserted into the pCB308 binary vector using ligase (Promega). More information about the *AtACS11* gene and promoter regions can be found at TAIR (http://www.arabidopsis.org/).

RNA isolation and cDNA synthesis

For total RNA isolation, rosette leaf and cotyledon of *Arabidopsis* were ground in a 1.5 ml micro-centrifuge tube to a fine powder with a pestle under liquid nitrogen (LN_2). The detailed method of [43] was adopted with some modifications. By using TRIzol™ reagents the total RNAs were extracted from the crushed leaves of *Arabidopsis* (Thermo Scientific; USA). Total RNA concentration was determined by A_{260}/A_{280} ratio using a spectrophotometer (Eppendorf, Bio-Spectrophotometer®). For cDNA synthesis a total of 1 µg of total RNA was used by using SuperScript® III (Invitrogen; USA). The cDNA (1 µl) was used for quantitative real time PCR using Taq polymerase (New England BioLabs; Ipswich, MA, USA) with 30 cycles of amplification using selected primers. For sequencing of *ACS11* gene and *ACS11* promoter in the constructed vectors, a 30 µl of *E. coli* (DH5α) was inoculated in 3 ml of Luria–Bertani (LB) broth for the preparation of competent cells.

Cloning of *ACS11* gene and *ACS11* promoter

After construction, each vector was transformed into *E. coli* competent cells. A 5 µl sample of ligated vectors (10–15 ng/µl) was added to 50 µl of *E. coli* competent cells and incubated on ice for 30 min. Competent cells with ligated vectors were heated at 42 °C for 45 s and then incubated on ice for 2 min. After that, the samples were incubated at 37 °C for 50 min in 100 µl of SOC media. Transformed cells were inoculated into LB agar plates containing kanamycin antibiotic. Plates were incubated at 37 °C for 15 h, and colony PCR was performed to confirm the transformation. Confirmed colonies were isolated and incubated at 37 °C in LB broth, and plasmid DNA was prepared using EZ-Pure™ Plasmid Prep kit Ver.2 (Enzynomics). *ACS11* gene and *ACS11* promoter sequence inserted into vectors were sent to SOLGENT (Korea) for sequencing.

Arabidopsis transformation

The detail method of [44] was adopted for the transformation of Arabidopsis with some modifications. Prior to *Arabidopsis* transformation the construction of vectors with target sequences was also confirmed in *A. tumefaciens* (GV3101) competent cells on LB agar plates containing kanamycin and rifampicin antibiotics. The independent transformants were screened on ½ MS media. The seedlings were grown for 10 days at 23 °C for 16 h of light and 21 °C for 8 h of dark in 60% relative humidity. Finally, the seedlings were used for the evaluation of GUS histochemical staining under different conditions such as drought, salinity, cold and flooding.

Abiotic stress treatments

Salinity stress was evaluated on ½ MS agar plates with 100 mM NaCl. Plate composition and growth conditions were the same as mentioned above. At 4 days after sowing, wild type and transgenic seedlings were transferred to ½ MS agar plates containing NaCl (100 mM) and grown for 10 days. Seedlings were evaluated and survival rates were assessed until the end of the experiment. For flooding, drought, and cold stress, wild type and *ACS11*-OX transgenic plants were either plated on ½ MS agar plates or grown in the same pots for 3 weeks in soil. Flooding stress was evaluated by

using plastic buckets for treated plants, cold stress was evaluated at 4 °C for 12 h, and drought stress was evaluated on ½ MS agar plates containing 0.25 MPa PEG.

GUS histochemical staining

ACS11 promoter transgenic plants were grown 4 days after sowing on ½ MS agar plates and then transferred onto different ½ MS agar plates. Seedlings were grown vertically for 10 days and retransferred onto two different plates for abiotic stress assessment. For the evaluation of salinity stress, the seedlings were transferred to ½ MS agar plates with a high salt concentration (200 mM NaCl). For flooding stress, the seedlings were submerged in water. For cold stress, the plants were placed at 4 °C in a refrigerator. For drought stress, the seedlings were subjected to 0.25 MPa PEG in ½ MS agar plates. After abiotic stress treatments for 3, 6, 12, and 24 h, seedlings were vacuum infiltrated in GUS staining buffer for 3–5 min and stained at 37 °C for 12 h. Stained seedlings were assessed for chlorophyll with 70% (v/v) ethanol.

Statistical analysis

The experiments were repeated in triplicates, whereas the data collected from each treatment were compiled for further analysis. All the parameters were analyzed in triplicate and the results were expressed as the mean ± SD. Error bars represent standard deviation (SD). Asterisks were used to denote the values that were significantly different ($P < 0.01$) by *t* test.

Results

Phenotypic analysis of *ACS11*-OX transgenic seedlings

The *A. thaliana* genome contains twelve ACC synthase genes (*ACS1–ACS12*). In this study, we focused our analysis on the role of *ACS11* expression, ACC accumulation, and stress hormone, ethylene production in response to different abiotic stress conditions. To investigate how *ACS11* participates in ethylene biosynthesis and plant phenotype, we constructed transgenic *A. thaliana* plants that overexpress *ACS11* cDNA under the control of the Cauliflower Mosaic Virus (CMV) 35S promoter. The sequence alignment of *ACS* with its close isozymes in *Arabidopsis* were also revealed (Fig. 1). Where the gray shading box correspond to similar amino acid residues

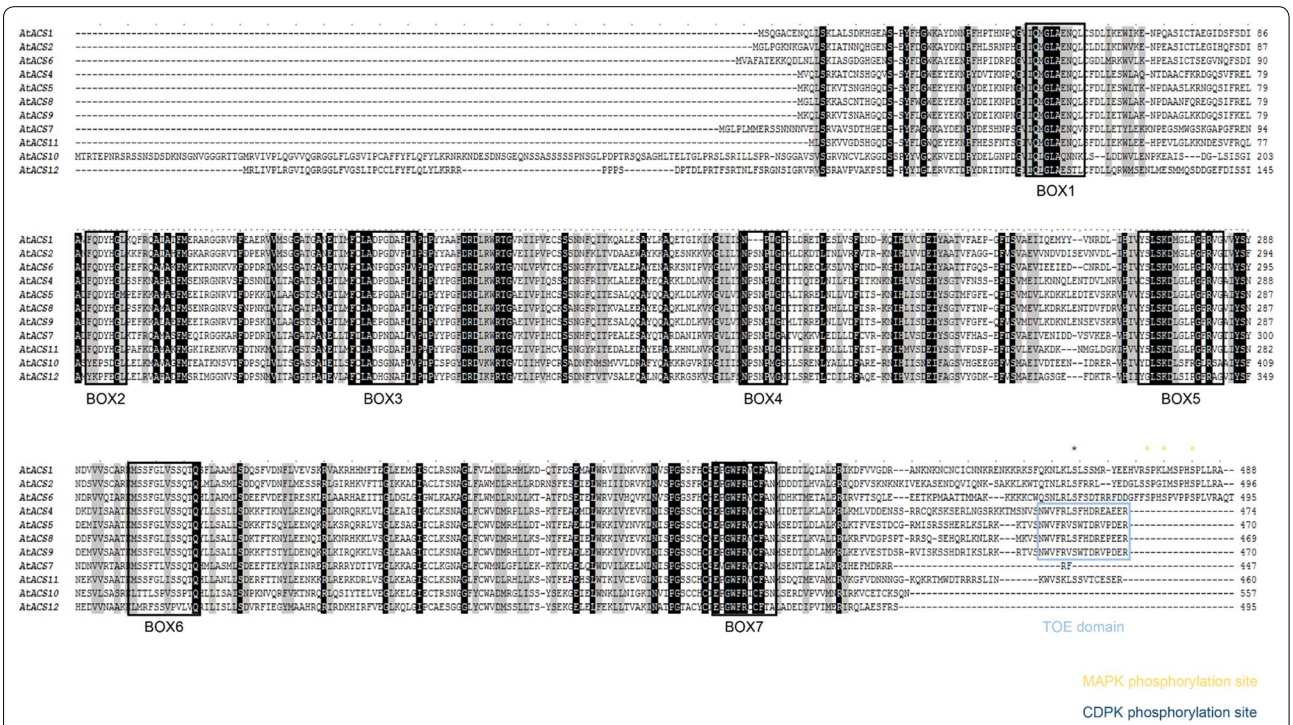

Fig. 1 Sequence alignment of *ACS* with its close isozymes in *Arabidopsis thaliana*. Alignment of the amino acid sequence from *AtACS1* (NP_191710), *AtACS2* (NP_171655), *AtACS4* (NP_179866). *AtACS5* (NP_201381), *AtACS6* (NP_192867), *AtACS7* (NP_194350), *AtACS8* (NP_195491), *AtACS9* (NP_190539), *AtACS10* (NP_564804), *AtACS11* (NP_567330), *AtACS12* (NP_199982) using ClustalW. The black shading box correspond to identical amino acid residues. The gray shading box correspond to similar amino acid residues. The seven conserved domains of the *ACS* isozymes are marked as boxes 1–7. The serine (S) residue marked with a blue and yellow *asterisk* is implicated in calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) phosphorylation site, respectively

and the conserved domains of the *ACS* isozymes were marked as boxes 1–7. By using *Agrobacterium*-mediated transformation via the floral dip method, T2 transgenic lines of *Arabidopsis* (Col-0) were assigned as *ACS11*-OX (strong) or *ACS11*-OX (weak) lines according to RT-PCR band intensity of *ACS11* compared to wild type (Fig. 2).

Overexpression of *ACS11* affected the root length of transgenic *ACS11*-OX (strong) seedlings compared to wild type seedlings at 10 days growth; whereas, the root phenotype of *ACS11*-OX (weak) seedlings was similar to wild type seedlings (Fig. 2a). Similarly, the roots of wild type and transgenic *Arabidopsis* revealed a significant difference ($P < 0.01$ by t-test) in their lengths: 5.31 ± 0.51 cm in wild type and 3.28 ± 0.38 cm in *ACS11*-OX (strong) seedlings (Fig. 2b). Therefore, it is likely that overexpression of *ACS11* in ACS11-OX (strong) lines increases ACC, the immediate precursor of stress hormone, ethylene, which adversely affects seedling growth and inhibits normal root elongation (Fig. 2c). Furthermore, we compared the rate of root growth by measuring root length on daily basis for 6 days and observed that the *ACS11*-OX (strong) seedlings had a notably lower root growth rate compared to wild type and *ACS11*-OX (weak) seedlings (Fig. 2d).

For further phenotypic analysis, wild type and *ACS11*-OX transgenic seeds were grown in soil for extended times and plant phenotype was evaluated each week. We observed a hyponastic response in 4-week-old transgenic plants. *ACS11*-OX plants also had smaller leaves and shorter petioles than wild type plants at 4 weeks, and the shoot length of transgenic plants was also shorter than wild type and similar to a dwarf phenotype after 5 weeks (Fig. 3a). Comparing the shoot length of wild type and *ACS11*-OX in 6-week-old seedlings, wild type plants measured 28.86 ± 1.36 cm tall, and *ACS11*-OX measured 22.83 ± 1.95 cm tall, a significant difference ($P < 0.01$) (Fig. 3b). The phenotype of wild type and *ACS11*-OX plants grown in soil with limited hours of daylight revealed similar results. Hence, overexpression of *ACS11* in *Arabidopsis* inhibits normal growth and development in these transgenic plants (Fig. 3c). The results presented here suggest that the overexpression of *ACS11* paves the way for the production of stress hormone, ethylene which affect the normal developmental processes of the plant.

ACS11-OX transgenic plants and abiotic stresses tolerance

Abiotic stressors are known for their prominent role in the regulation of ACC synthase gene expression. Here, we compared the effects of *ACC synthase 11* (*ACS11*) gene expression in wild type and transgenic *A. thaliana* plants that overexpress *ACS11* (*ACS11*-OX) under different abiotic stress conditions. Salinity stress tolerance was analyzed on ½ MS agar plates (Fig. 4). Four-day-old wild type and *ACS11*-OX transgenic seedlings were transferred to the ½ MS agar plates with 100 mM NaCl, and survival was recorded after 6 days (Fig. 4a). Survival rates between wild type and the *ACS11*-OX (weak) line were $90 \pm 3.33\%$ and $76.67 \pm 3.33\%$, respectively, a significant difference ($P < 0.05$) (Fig. 4b). Similarly, survival rates between wild type and the *ACS11*-OX (weak) line on the same plate also revealed survival rates: 93% and $63.33 \pm 3.33\%$, respectively (Fig. 4c).

Another finding was that the leaves of *ACS11*-OX seedlings exhibited more rapid senescence under flooding

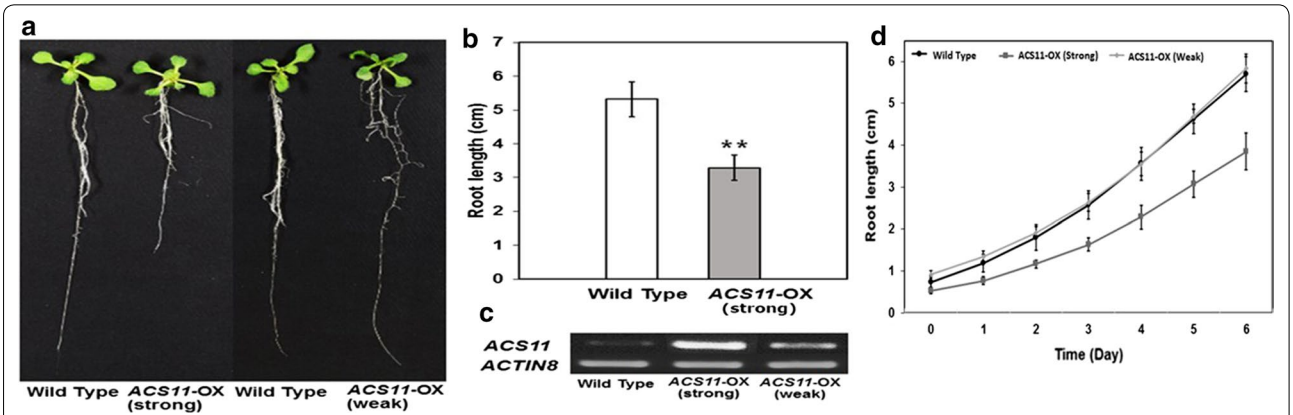

Fig. 2 Root phenotypic analysis of wild type and *ACS11*-OX transgenic seedlings on ½ MS agar plates. Seeds were grown for 4 days after sowing on ½ MS agar plates, then transferred to different ½ MS agar plates and grown for 6 days vertically. **a** Root phenotypes of wild type and *ACS11*-OX transgenic seedlings (strong, weak). **b** Root length of wild type and *ACS11*-OX (strong) seedlings. Error bars represent standard deviation (SD). Values denoted by ** were significantly different ($P < 0.01$) by t-test. **c** RT-PCR analysis of *ACS11* expression in wild type and *ACS11*-OX transgenic seedlings. *ACTIN8* was used as a control. **d** Time-course analysis of root growth rate. Four-day-old seedlings were transferred to different ½ MS agar plates, and root lengths were measured daily for 6 days. A lower root growth rate was noted in *ACS11*-OX (strong) seedlings relative to wild type seedlings; however, *ACS11*-OX (weak) seedling root growth rate was similar to that of wild type seedlings

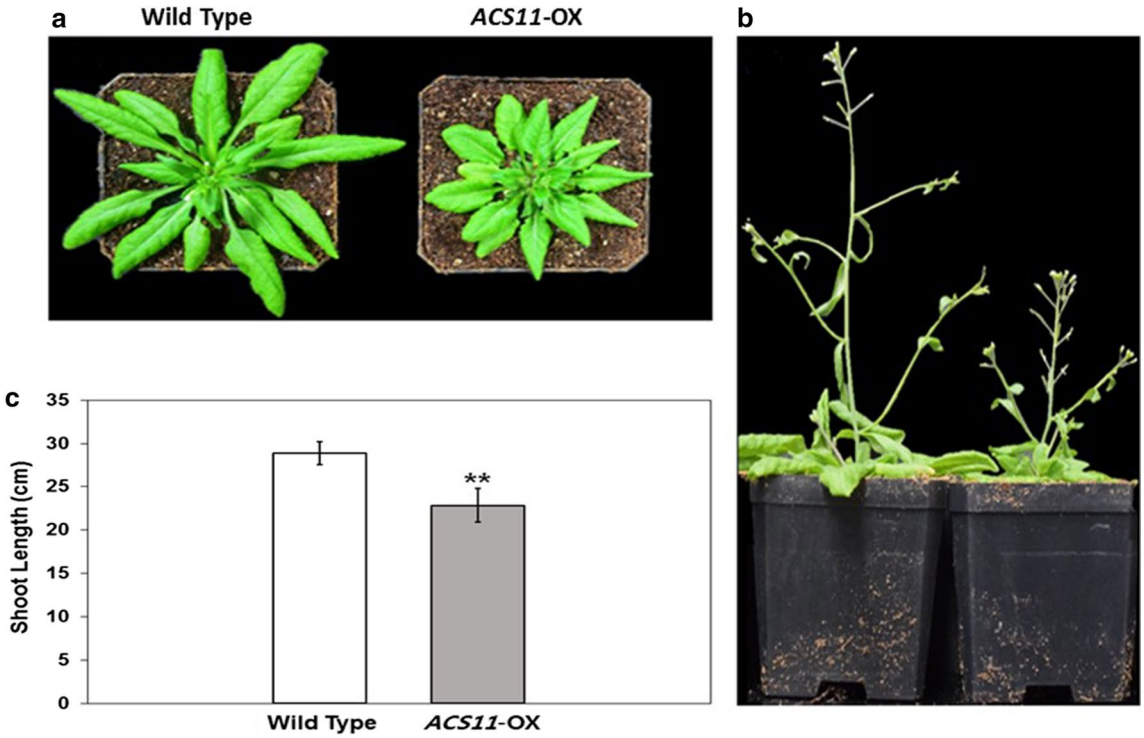

Fig. 3 Phenotypic analysis of wild type (plant on the left in each image) and *ACS11*-OX transgenic *Arabidopsis* (plant on the right in each image) seeds cultivated in soil. **a**, **b** Representative of 4-, and 5-week-old plants, respectively. **c** Measurement of shoot lengths in 6-week-old plants revealed that *ACS11*-OX shoots were significantly shorter compared to wild type plants. Error bars represent standard deviation (SD). Values denoted by ** were significantly different ($P < 0.01$) by t-test

compared to wild type seedlings. Flooding and cold stress tolerance were also analyzed by growth in soil. To induce flooding stress, 21-day-old plants were submerged in water using plastic buckets kept at 23 °C for 16 h of light and 21 °C for 8 h of dark. Changes were noted on a daily basis for both wild type and *ACS11*-OX submerged seedlings. At 3 days, *ACS11*-OX plants exhibited more leaf senescence than wild type plants, and at 5 days, *ACS11*-OX plants showed higher levels of coloring agents (anthocyanin) than wild type plants (Fig. 5).

In a similar experiment, 21-day-old plants were subjected to cold stress by alternating between 23 °C for 12 h with light and 4 °C for 12 h in the dark. There was no observable difference between wild type and *ACS11*-OX plant through 6 days of cold stress. However, *ACS11*-OX transgenic plants did show symptoms after more than 6 days of cold stress (Fig. 6). These soil growth experiments comparing wild type and *ACS11*-OX transgenic plants under normal and various stress conditions showed that the overexpression of ACC synthase cDNA in *ACS11*-OX plants produced increased levels of ACC, which was then readily available for conversion to stress hormone, ethylene. Thus, *ACS11*-OX transgenic plants were more vulnerable to salinity, flooding, and cold stress than wild type plants.

Effect of abiotic stress on the expression of *ACS11* gene

To evaluate the expression of the wild type *ACS11* gene in response to abiotic stresses, we assayed for the presence of *ACS11* mRNA by RT-PCR at different time points (0, 0.5, 1, 2, 6, and 12 h) after subjecting wild type seedlings to abiotic stressors (Fig. 7). The observed expression levels of *ACS11* under abiotic stresses revealed that, except when plants were subjected to drought stress, *ACS11* mRNA was induced at different time points. The sequence of the wild type ACS11 promoter is shown in Fig. 8.

To confirm these results in vivo as well as in vitro, the pro*ACS11*::GUS region of the pCB308 vector was introduced into wild type *A. thaliana* by using *Agrobacterium*-mediated plant transformation. The T1 generation of transgenic seeds was germinated on ½ MS agar plates and grown for 4 days and then transferred to different ½ MS agar plates. After 10 days, transgenic seedlings were transferred to appropriate ½ MS agar plates for abiotic stress treatments. Plants exposed to each treatment were

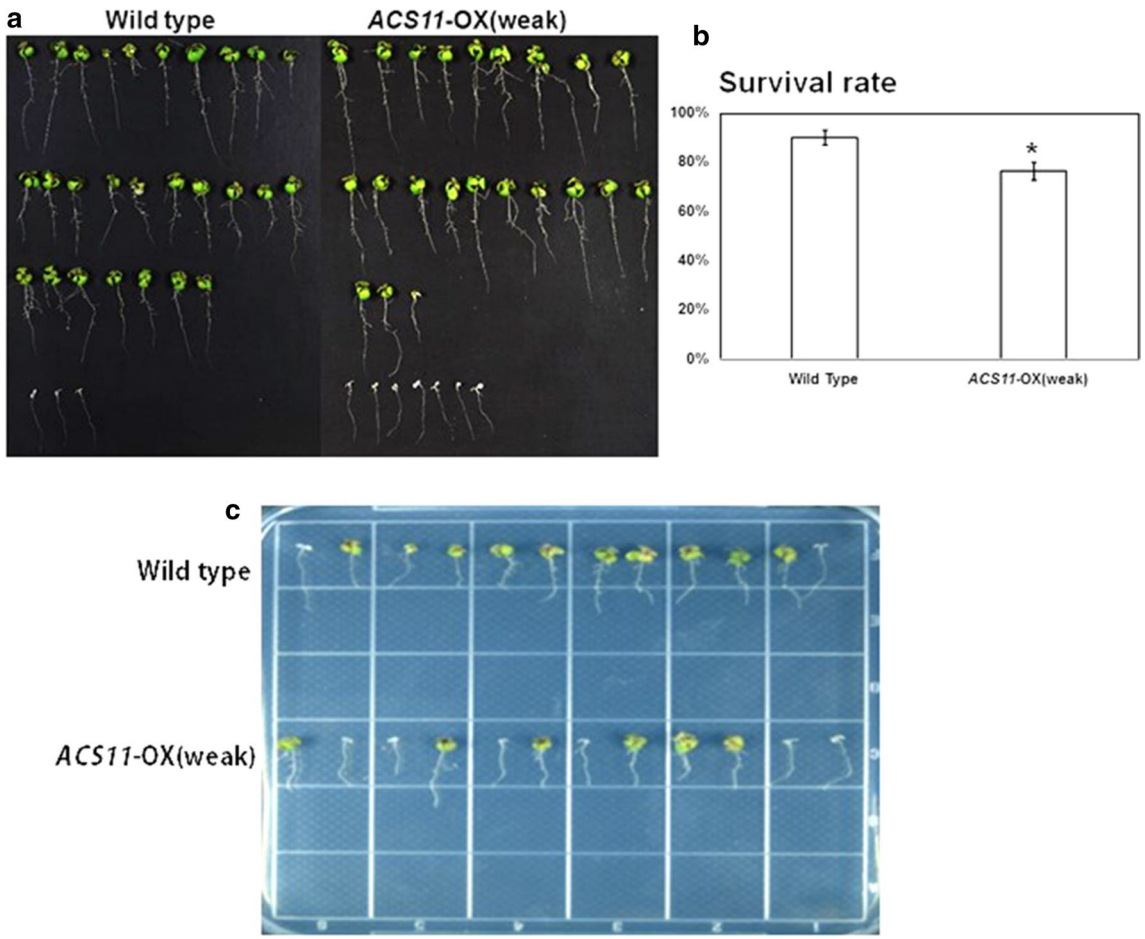

Fig. 4 Survival rates of wild type and *ACS11*-OX (weak) line on ½ MS medium under salinity stress. **a** Survival rates of 10-day-old wild type and *ACS11*-OX (weak) line on ½ MS medium under salinity stress (100 mM NaCl). **b** Percentage of survival rate of wild type and *ACS11*-OX (weak) line on ½ MS medium under salinity stress. Error bars represent standard deviation (SD). Values denoted by an * were significantly different ($P < 0.05$) by t-test. **c** A wild type and *ACS11*-OX (weak) grown on the same plate, ½ MS medium under salinity stress (100 mM NaCl)

Fig. 5 Phenotypic analysis of 21-day-old wild type (plant on the left in each image) and *ACS11*-OX (plant on the right in each image) seedlings that were subjected to flooding stress for the indicated periods of time

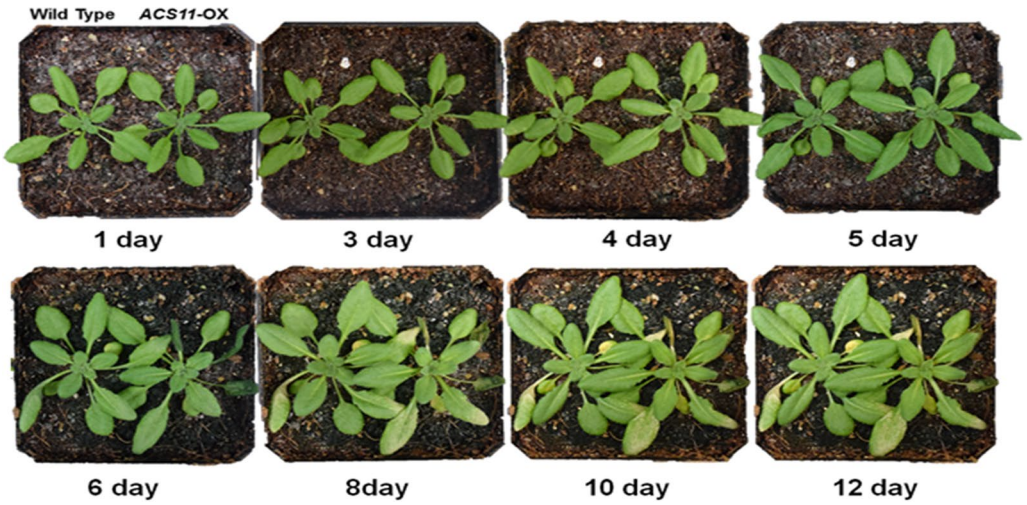

Fig. 6 Phenotypic analysis of 21-day-old wild type (plant on the left in each image) and *ACS11*-OX (plant on the right in each image) seedlings that were subjected to cold stress for the indicated periods of time

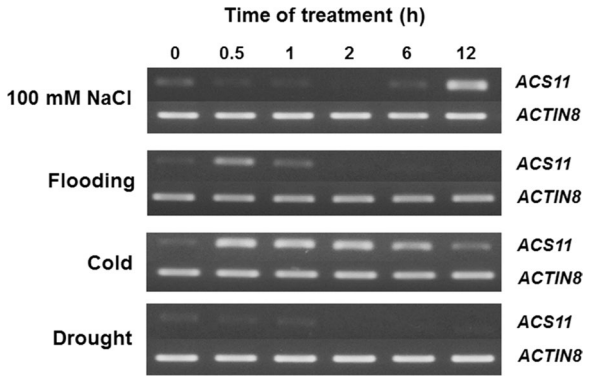

Fig. 7 Time-course of *ACS11* mRNA expression by RT-PCR analysis under abiotic stress. Wild type seedlings were subjected to abiotic stresses such as salinity (100 mM NaCl), flooding, cold, and drought for the indicated times. *ACTIN8* was used as a control. The number of PCR amplification cycles were as follows: *ACTIN8*, 27 cycles; *ACS11*, 29 cycles

subjected to histochemical staining at 0, 3, 6, 12, and 24 h.

Flooding stress was evaluated by submerging plants in pots filled with water. GUS activity in response to flooding stress was observable after 3 h and was highest at 24 h of flooding stress (Additional file 2: Fig. S1A). Similarly, seedlings were subjected to salinity stress (200 mM NaCl) in ½ MS agar plates. GUS activity was elevated at 6, 12, and 24 h of salinity stress (Additional file 2: Fig. S1B). Similarly the effect of drought stress was evaluated using 0.25 MPa PEG-infused plates. GUS activity was highest at 6 h of drought stress and decreased to levels similar to those of control seedlings at 12 and 24 h (Additional file 2: Fig. S1C). Finally, cold stress was evaluated by transferring seedlings to 4 °C in the dark. GUS activity was highest at 6 h of cold stress, decreased at 12 h, and then interestingly increased again at 24 h (Additional file 2: Fig. S1D). These results indicate that *ACS11* is induced by salinity, flooding, drought, and cold stress in vivo.

Discussion

Various abiotic stresses are deleterious to plant growth and development and negatively affect crop productivity and yield [45, 46]. However, plants have evolved robust, adaptive mechanisms to survive in adverse environmental conditions [47]. Many plants increase expression of one or more genes that encode ACC synthase, which in turn produce ACC in response to stressful environments [26]. ACC is the immediate precursor of ethylene, and fluctuations in ethylene biosynthesis affect plant growth and development. This study focused on *ACS11* expression and regulation during plant growth and development as well as in response to different abiotic stresses. Additionally, in vitro and in vivo experiments were performed to evaluate the effect of fluctuations in *ACS11* expression on the activity of ACC synthase in response to abiotic stresses (Additional file 2: Fig. S1).

Phylogenetic analysis of the *ACS* gene family has already shown that there are different *ACS* genes in *Arabidopsis* [40]. Expression of *ACS4*, *ACS5*, and *ACS7* genes has been shown to be induced by hormones and environmental stresses [34]. For this study, transgenic *Arabidopsis* plants that overexpress the *ACS11* gene,

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tcttctacttttctatctatatatctattgttgagctacaaagtgaaagtacttttacttgctatattgatcaccaagttatagcatttaaaaaaa

Fig. 8 The nucleotide sequence of the wild type *ACS11* promoter; 1076 bp

which encodes a type-2 ACC synthase, were constructed and used for phenotypic analysis by comparison with wild type plants under normal growth conditions (Figs. 2, 3, 4) and under conditions of specific abiotic stresses (Figs. 5, 6). More interestingly, the results from phenotypic variation in wild type and transgenic plants validated our hypothesis that upregulation of *ACS11* expression could adversely affect the plant because the leaves, roots, and shoots of transgenic plants were shorter than that of wild type (Figs. 2, 3, 4). Additionally, tolerance to salinity, flooding, and cold stresses was reduced in the transgenic *ACS11*-OX seedlings compared to wild type seedlings. Other studies have found that plants respond to stressful conditions by synthesizing increased amounts of the enzyme ACC synthase as well as other stress proteins. Under flooding stress, the upregulation of ACC synthase leads to an accumulation of ACC in the roots, and because ACC requires molecular oxygen to be converted into ethylene, the ACC is transported to the shoots where it has access to oxygen [23, 48, 49].

To evaluate endogenous *ACS11* expression patterns under conditions of abiotic stress, wild type plants were subjected to abiotic stresses and the time course of *ACS11* expression was measured by RT-PCR (Fig. 7). *ACS11* expression was upregulated in response to the abiotic stresses of salinity, flooding, and cold, but not drought. Previous studies showed that GUS activity in the roots of *Arabidopsis* seedlings was apparent in the hypocotyl root junction and at a lower level in the vascular tissues of the roots after 4 days [50]. Another study fused the GUS reporter gene with the promoters of *Arabidopsis ACS* genes *ACS4*, *ACS5,* and *ACS7* to make *ACS*::*GUS* transgenic plants [34]. However, in this study, we used the histochemical GUS staining experiments to investigate *ACS11* promoter activity in vivo. *ACS11* was upregulated when plants were subjected to abiotic stresses in a time course that was similar to the RT-PCR results. These findings are consistent with previously reported data; however, they provide no direct evidence on whether ACC and ethylene levels were elevated in transgenic plants by the upregulation of *ACS11* specifically. Production of stress hormone, ethylene in plants causes physiological damage such as leaf chlorosis, wilting, necrosis, and reduced biomass [51]. However, it is possible to protect plants from the effects of stress hormone, ethylene by employing different strategies such as the inhibition of ACC synthase mRNA accumulation (through salicylic acid); the use of polyamines (spermidine, putrescine), which has also revealed anti-senescence properties; and the application of ACC-deaminase-producing bacteria under different stress conditions [23, 52, 53]. A recent report [54] showed the effect of abiotic stress on *ACS8* and ethylene production. The study also reported the importance of *ACS8* in defense response and early ethylene biosynthesis induced by copper ions in *Arabidopsis* plants. Moreover, to investigate the role of *ACS2* and *ACS6* in ethylene production they also evaluated the effects of Cu^{2+} on *ACS2* and *ACS6* double knockout mutant plants. Whereas, no significant difference was observed in the ethylene of wild type, *ACS2* and *ACS6* mutant plants at early stage (2 h), however, after 24 h *ACS2* and *ACS6* induce the production of stress hormone, ethylene. Likewise, the study of Schellingen et al. [55] reported that abiotic stressors induce the production of stress hormone, ethylene in *Arabidopsis* by inducing the transcription of *ACS6* and *ACS2*. In connection to this, our results also suggest that *ACS11*-OX line affect the normal growth and development of the seedlings

because of *ACS11* induction compare to wild type. Thus, the higher amount of stress hormone, ethylene produced by *ACS11*-OX line compare to wild type seedlings under abiotic stress conditions greatly affected the viability of the plants and arrest its normal growth and development.

Additional files

Additional file 1: Table S1. List of primers used in this experiments.

Additional file 2: Fig. S1. Analysis of ACS11 promoter activity in proACS11::GUS-expressing transgenic seedlings in response to different abiotic stressors for the specific periods of time. (A) Analysis of ACS11 promoter activity in proACS11::GUS-expressing transgenic seedlings in response to flooding stress for the indicated periods of time. (B) Analysis of ACS11 promoter activity in proACS11::GUS-expressing transgenic seedlings in response to salinity stress (200 mM NaCl) for the indicated periods of time. (C) Analysis of ACS11 promoter activity in proACS11::GUS-expressing transgenic seedlings in response to drought stress for the indicated periods of time. (D) Analysis of ACS11 promoter activity in proACS11:: GUS-expressing transgenic seedlings in response to cold stress for the indicated periods of time.

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Authors' contributions
HDE, SA, HJ, and KK performed the experiments, data analysis and manuscript writing; HDE, SA and WCK designed the experiment plan and revised the manuscript together. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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