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RESEARCH PAPER

Loss of *ACS7* confers abiotic stress tolerance by modulating ABA sensitivity and accumulation in *Arabidopsis*

Hui Dong[1], Zhiqin Zhen[1], Jinying Peng[2], Li Chang[1], Qingqiu Gong[1,*] and Ning Ning Wang[1,*]

[1] Department of Plant Biology and Ecology, Nankai University, Tianjin 300071, China
[2] College of Life Sciences, National Laboratory of Protein Engineering and Plant Genetic Engineering Center, Peking University, Beijing 100871, China

* To whom correspondence should be addressed. E-mail: wangnn@nankai.edu.cn; gongq@nankai.edu.cn

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Abstract

The phytohormones ethylene and abscisic acid (ABA) play essential roles in the abiotic stress adaptation of plants, with both cross-talk of ethylene signalling and ABA biosynthesis and signalling reported. Any reciprocal effects on each other's biosynthesis, however, remain elusive. ACC synthase (ACS) acts as the key enzyme in ethylene biosynthesis. A pilot study on changes in *ACS* promoter activities in response to abiotic stresses revealed the unique involvement in abiotic stress responses of the only type 3 ACC synthase, ACS7, among all nine ACSs of *Arabidopsis*. Hence an *acs7* mutant was characterized and its abiotic stress responses were analysed. The *acs7* mutant germinated slightly faster than the wild type and subsequently maintained a higher growth rate at the vegetative growth stage. Ethylene emission of *acs7* was merely one-third of that of the wild type. *acs7* exhibited enhanced tolerance to salt, osmotic, and heat stresses. Furthermore, *acs7* seeds were hypersensitive to both ABA and glucose during germination. Transcript analyses revealed that *acs7* had elevated transcript levels of the stress-responsive genes involved in the ABA-dependent pathway under salt stress. The ABA level was also higher in *acs7* following salt treatment. Our data suggest that *ACS7* acts as a negative regulator of ABA sensitivity and accumulation under stress and appears as a node in the cross-talk between ethylene and ABA.

Key words: Abiotic stresses, abscisic acid, *AtACS7*, cross-talk, ethylene.

Introduction

Both ethylene and abscisic acid (ABA) are known to regulate not only developmental processes but also adaptive stress responses of plants (Morgan and Drew, 1997; Ma *et al.*, 2006). For instance, ABA and ethylene have long been implicated in salt stress responses (Anderson *et al.*, 2004; Wang *et al.*, 2007; Cheng *et al.*, 2009). Both the ABA-dependent and -independent pathways are involved in the response to salt stress and the accompanying osmotic challenge. Three types of *cis*-regulatory elements have been identified in the promoters of stress-responsive genes, including Dehydration Responsive Elements (DRE/CRT), ABA Responsive Elements (ABRE), and MYB/MYC Recognition Sequences (MYCRS/MYBRS) (Zhu, 2002). The ABA-dependent pathway regulates the expressions of stress-responsive genes, such as *RD29A, RD29B, RAB18,* and *RD22* (Shinozaki *et al.*, 2003), through CBF4 (a member of the DREB subfamily), MYC/MYB, and bZIP-type transcription factors, which bind to the DRE/CRT, MYCRS/MYBRS, and ABRE promoter elements, respectively (Zhu, 2002; Chinnusamy *et al.*, 2004). Ethylene signalling has also been shown to play a role in salt tolerance (Achard *et al.*, 2006; Cao *et al.*, 2007). Upon abiotic stresses, ethylene binds to the endoplasmic reticulum (ER)-localized receptors ETR1, EIN4, and their homologues (Bleecker, 1999), resulting in the deactivation of CTR1 (the negative regulator of ethylene signalling) and activation of the positive regulator EIN2, eventually leading to a transcriptional cascade involving the EIN3/EIL and ERF transcription factors (Chen *et al.*, 2005; Lin *et al.*, 2009). The mutations of ETR1, EIN4, and EIN2 have all been shown to confer salt sensitivity (Cao *et al.*, 2007),

whereas the mutation of CTR1 confers salt tolerance (Achard *et al.*, 2006).

A number of researches have revealed the intertwining nature of ethylene and ABA signalling pathways (Beaudoin *et al.*, 2000; Benschop *et al.*, 2007; Cutler *et al.*, 2010). For instance, the expression of *9-CIS-EP-OXYCAROTENOID DIOXYGENASE 3* (*NCED3*), which encodes the key enzyme in ABA biosynthesis, is up-regulated in the *ein2-1* mutant, and *CYP707A2*, a cytochrome P450 gene which encodes the key component of ABA catabolism, is down-regulated in *etr1-1* (Cheng *et al.*, 2009), suggesting that when ethylene signalling is impaired, ABA biosynthesis may be enhanced. Disruption of *EIN2* alters the expression pattern of stress marker gene *RD29B* in response to salt stress (Wang *et al.*, 2007).

Ethylene and ABA have also been implicated in heat stress tolerance (Larkindale *et al.*, 2005; Clarke *et al.*, 2009). Facing heat stress, plants produce heat shock proteins (HSPs) and modulate hormone signalling pathways (Foyer *et al.*, 1997; Larkindale *et al.*, 2005). HSPs are molecular chaperones that can protect cellular proteins against irreversible heat-induced denaturation and help in the refolding of heat-damaged proteins (Hong and Vierling, 2000; Hong *et al.*, 2003). For instance, Hsp101, a member of the Clp/Hsp100 family, functions in re-solubilizing the protein aggregates formed under heat stress; while the Hsp70/DnaJ refolding complex helps refold the proteins to their native states (Gurley, 2000; Lee and Vierling, 2000). The expression of HSPs is known to be induced by heat shock transcription factors (HSFs). For instance, HsfA2 has been shown to sustain the transcript level of *HSP* genes and extend the duration of thermotolerance in *Arabidopsis* (Charng *et al.*, 2007). Meanwhile, many hormones, including ethylene, ABA, and SA are involved in thermotolerance (Larkindale *et al.*, 2005).

Apart from ethylene signalling, recent studies have started to reveal the importance of the ethylene biosynthesis pathway in modulating not only developmental growth but also stress adaptation (Yang and Hoffman, 1984; Tsuchisaka *et al.*, 2009). The key enzyme in the ethylene biosynthesis pathway is ACC synthase (ACS), which converts *S*-adenosylmethionine (AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman, 1984). In *Arabidopsis*, there are nine authentic *ACSs,* including *ACS1–2, ACS4–9*, and *ACS11.* Each member has a distinct spatial and temporal expression pattern in the different stages of plant growth and development, and under various stresses (Wang *et al.*, 2005). The catalytic core of ACS proteins is highly conserved, while the C-terminal regulatory domain varies. Based on the sequence of the C-terminal region, the ACS proteins are divided into three groups. Type 1 ACS proteins have the longest C-terminus with a single calcium-dependent protein kinase (CDPK) phosphorylation site and three mitogen-activated protein kinase (MAPK) phosphorylation sites, and type 2 ACS proteins have an intermediate length of the C-terminus with a single CDPK phosphorylation site. By contrast, type 3 ACS proteins have a very short C-terminus and no phosphorylation site (Chae and Kieber, 2005; Yoshida *et al.*, 2005), with their specific function or regulatory mechanism almost completely unknown.

Arabidopsis has a single type 3 ACS, ACS7, which has a broad expression pattern during plant growth and development. In 5-d-old etiolated seedlings, *ACS7* is expressed in the cotyledons, the elongation zone of the hypocotyls, and the vascular tissue in the root; in 5-d-old light-grown seedlings, *ACS7* is expressed in the cotyledons, primary leaves, the embryonic root, and in the roots except the root tip; in the mature plants, *ACS7* is expressed in the roots, younger leaves, inflorescence stem, and siliques (Tsuchisaka and Theologis, 2004). A recent survey on *acs* mutants has implicated the involvement of ACS7 in the determination of flowering time (Tsuchisaka *et al.*, 2009). Moreover, the expression of *ACS7* is regulated by several phytohormones and environmental factors, such as ethylene, ABA, GA_3, light, and salt (Wang *et al.*, 2005; Achard *et al.*, 2006).

Although many efforts have gone into the investigation of the interaction between ethylene and ABA signalling in abiotic stress adaptation, the possible involvement of any ethylene biosynthesis gene in such cross-talk has yet to be explored. In a pilot study, the unique responses of the *ACS7* promoter to various abiotic stresses were observed. Then a loss-of-function mutant of *ACS7* was characterized, and its responses to abiotic stresses including salt, osmotic, and heat stress were systematically analysed. Surprisingly, the *acs7* mutant showed enhanced tolerance to all the stresses tested. The *acs7* mutant was then discovered to be hypersensitive to exogenous ABA. Consistently, the *acs7* mutant showed hypersensitivity to high glucose, but not sucrose. Transcript analyses revealed that the *acs7* mutant had elevated transcript levels of stress-responsive genes involved in the ABA-dependent pathway under salt stress. The endogenous ABA level was also higher in the *acs7* mutant following salt stress. These results suggested that *ACS7* acts as a negative regulator of ABA sensitivity and accumulation under abiotic stresses and might function as a molecular link between ethylene biosynthesis and the ABA-mediated abiotic stress signal pathway.

Materials and methods

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* (ecotype Wassilewskija-4) was obtained from the Arabidopsis Biological Resource Center (ABRC, The Ohio State University, Columbus, OH, USA). The T-DNA insertion line of *AtACS7* (background Wassilewskija-4) was obtained from the Institut National de la Recherche Agronomique (INRA, Institut Jean Pierre Bourgin, Station Génétique et amélioration des plantes, UR254, F-78026 Versailles, France) (Samson *et al.*, 2002).

Seeds were surface-sterilized in 10% (v/v) sodium hypochlorite for 2 min, washed 10 times with sterilized water, plated on to half-strength Murashige and Skoog (1/2 MS) medium [0.8% (w/v) agar, pH 5.7, 1% (w/v) sucrose], stratified at 4 °C for 2 d in the dark, and grown in a plant growth chamber [22/19 °C, 16/8 h light/dark, with a photosynthetic photon flux density (PPFD) of 90 μE m^{-2} s^{-1}].

Verification of the acs7 *mutant*

The *acs7* mutant lines were verified by PCR as described (http://signal.salk.edu/tdnaprimers.2.html). The primers used were T-DNA-specific primer (FLAG LB4) and *ACS7* gene-specific primers (acs7LP and acs7RP).

The expression of *ACS7* was analysed by RT-PCR using *TIP41-LIKE* (At4g34270) as a control. Primers used to amplify the transcription of *TIP41-LIKE* and *ACS7* were rtTIP-F and rtTIP-R, and rtACS7-F and rtACS7-R. The sequences of primers are listed in Supplementary Table S1 at *JXB* online.

Phenotypic analyses

Four-day-old, vertically-grown wild-type and *acs7* seedlings were transferred to new plates and allowed to grow either vertically or horizontally for phenotypic analyses. The plates were then photographed daily, and additional root growth was measured with Image J (National Institutes of Health; http://rsb.info.nih.gov/ij/download.html).

To study the response of the wild-type and *acs7* to ethylene, 4-d-old seedlings were transferred to medium supplemented with a series of concentrations of ACC (ethylene precursor) for further observation.

The germination assay was performed on medium supplemented with 2 μM ABA, 150 mM NaCl or 300 mM mannitol. The percentage of seed germination (defined by the emergence of the radicle) was scored over time.

To evaluate salt stress tolerance, 5-d-old seedlings were transferred to medium supplemented with 150 mM NaCl, and the survival rate (defined by the death of the shoot apical meristem as observed with a stereoscope) was scored daily.

To evaluate heat stress tolerance, plates of 4-d-old seedlings were transferred to a dark incubator set at 43 °C for 3 h, then back to normal growth conditions, and afterwards the percentage of seedlings with cotyledon chlorosis was scored daily.

To evaluate their glucose- and sucrose-sensitivity, seeds were sown on medium supplemented with 6% glucose or sucrose, and afterwards the plates were photographed daily. After being germinated on 6% glucose for 10 d, the number of seedlings with true leaves was scored. Error bars ±SD.

50 μM Nordihydroguaiaretic acid (NDGA) (Sigma) was used as the inhibitor of ABA biosynthesis (Han *et al.*, 2004; Liu *et al.*, 2009).

All phenotypic analyses were performed at least in triplicate. The level of significance was evaluated by Student's *t* test.

Gene expression analyses

Real-time RT-PCR was used to compare the expression of genes of interest in the wild type and *acs7* following various stress treatments. For the salt-stress treatment, 9-d-old seedlings were transferred to medium supplemented with 175 mM NaCl and harvested after 3, 6, 12, and 24 h of treatment, respectively. For the heat-stress treatment, 9-day-old seedlings were transferred to a dark incubator set at 43 °C and harvested after 0.5, 1, and 2 h of treatment, respectively.

RNA extraction and cDNA synthesis were as described by Liu *et al.* (2010). Total RNA was extracted, and residual genomic DNA was digested with RNase-free DNase I. The absence of genomic DNA was confirmed. First strand cDNA was synthesized from 2.0 μg of total RNA using AMV reverse transcriptase (Promega, Madison, WI, USA). cDNA samples diluted 10-fold were used as templates.

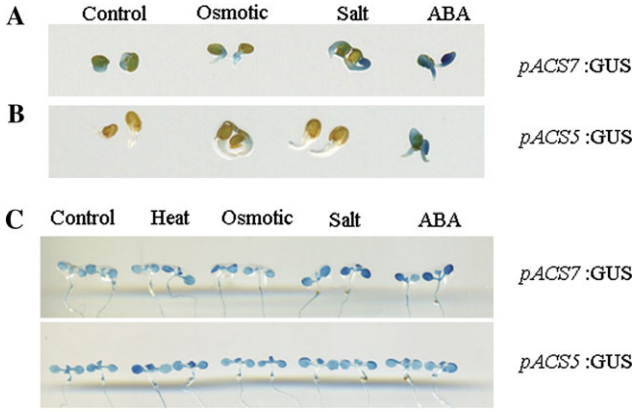

Fig. 1. The unique expression pattern of *ACS7* under various stress treatments. The seeds and 9-d-old seedlings of *pACS7*:GUS and *pACS5*:GUS lines were exposed to 43 °C, 300 mM mannitol, 150 mM NaCl, and 20 μM ABA for 3 h, respectively, before histochemical GUS staining. (A) *ACS7* was expressed in germinating seeds, and the expression was further induced by both salt and ABA. (B) By contrast, promoter activity of *ACS5* was barely detected during germination, except following the ABA treatment. (C) The expression of *ACS7* was enhanced following heat, osmotic, salt, and ABA treatments in 9-d-old seedlings, while the expression of *ACS5* was only increased following heat treatment. There were three independent replicates with at least eight seeds and seedlings each to give the typical results.

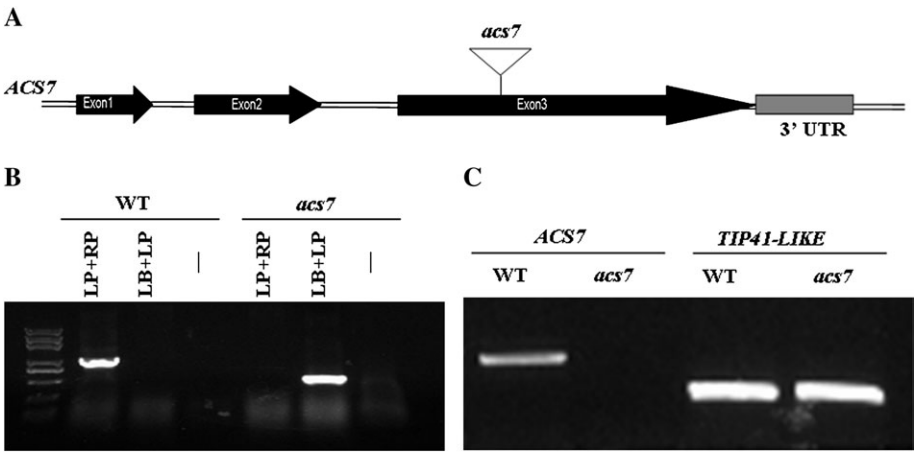

Fig. 2. Molecular characterization of the *acs7* mutant. (A) The T-DNA was inserted into the third exon of the *ACS7* gene. The black boxes represent exons and the lines represent introns. (B) Homozygous mutants were identified by PCR using primers annealing to genomic DNA (LP and RP) and left border of the T-DNA (LB). (C) The knockout of *ACS7* was confirmed in *acs7* by RT-PCR using *TIP41-LIKE* as a control.

Real-time RT-PCR analysis was performed using SYBR Green Perfect mix (TaKaRa, Dalian, China) on an iQ5 (Bio-Rad, California, USA) (Liu *et al.*, 2010). All reactions were performed under the following conditions: 95 °C for 2 min; 40 cycles of 95 °C for 10 s, and 56 °C for 30 s. All reactions were done at least in triplicate. *TIP41-LIKE* was used as an internal control. All primers used are listed in Supplementary Table S1 at *JXB* online.

Histochemical GUS staining

Histochemical GUS staining of homozygous transgenic lines harbouring *pACS*:GUS fusion genes under various stresses was performed as described previously (Wang *et al.*, 2005; Liu *et al.*, 2010). Images were recorded with a scanner (EPSON 1260). Three independent replicates with at least eight seeds and seedlings each were used to give the typical results.

Measurements of ethylene emission

Ethylene emission of the wild type and *acs7* was determined in 3-d-old etiolated seedlings by gas chromatography (Agilent 6890N) as described by Li *et al.* (2009).

ABA determination

Nine-day-old seedlings of the wild type and *acs7* were treated with 175 mM NaCl for 24 h. The seedlings, with or without salt treatment, were ground into powder in liquid nitrogen. Quantification of ABA was carried out with LC-MS at the National Center for Plant Gene Research (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) as previously described by Fu *et al.* (2011).

Measurements of endogenous proline and soluble sugar content

The leaves of 9- and 19-d-old seedlings were harvested, weighed, and ground into powder in liquid nitrogen. Proline and sugar contents were determined as described by Li *et al.* (2004).

Results

Identification of an acs7 *knockout mutant*

Previous analyses on the promoter activities of *AtACS* genes had revealed that, at any given growth stage, there is at least one *ACS* expressed (Tsuchisaka and Theologis, 2004), and that the expression of several *ACS*s can be induced by various hormones and stress conditions (Wang *et al.*, 2005). As a pilot study, the promoter activities of the three types of *ACS*s in response to various abiotic stress conditions were surveyed (data not shown), in which the uniqueness of *ACS7*, the only type 3 *ACS* in *Arabidopsis*, was revealed. *ACS7* was expressed in germinating seeds, and its promoter activity was elevated further following both salt and ABA treatment (Fig. 1A). By contrast, GUS staining was barely detected in other ACS promoter-GUS lines, such as *pACS5*:GUS during germination, except following the ABA treatment (Fig. 1B). Similarly, enhanced promoter activities were observed in 9-d-old *pACS7*:GUS seedlings following heat, osmotic, salt, and ABA treatments, while only a slightly enhanced activity was observed in *pACS5*:GUS lines following heat stress (Fig. 1C).

To explore the possible involvement of ACS7 in abiotic stress adaptation, a FLAG T-DNA insertion line (ecotype Wassilewskija-4) was obtained with a T-DNA insertion in the third exon of the *ACS7* gene (Fig. 2A). The homozygous mutant was confirmed by PCR (Fig. 2B) and RT-PCR (Fig. 2C). This line turned out to be the same *acs7* line, *acs7-1*, used before (Tsuchisaka *et al.*, 2009).

In 3-d-old etiolated seedlings, ethylene emission of *acs7* was reduced to approximately one-third compared with the wild type (Fig. 3A), suggesting that AtACS7 may have

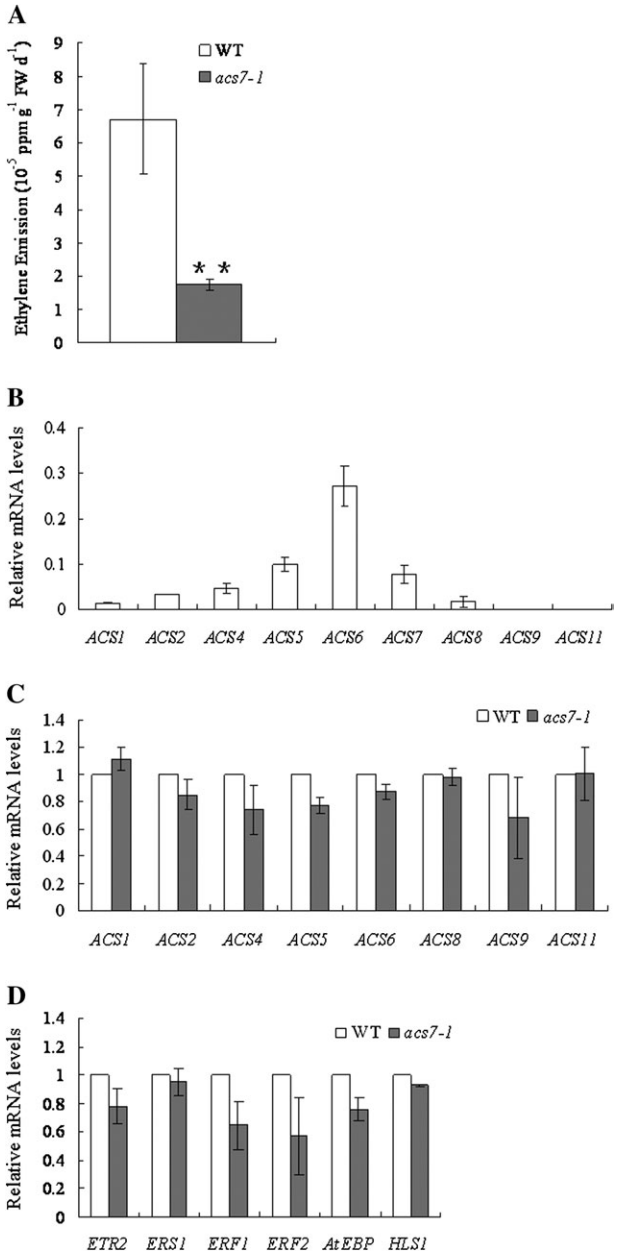

Fig. 3. The transcript levels of several *ACS*s and ethylene-responsive genes were repressed in *acs7*, with lower ethylene emission. (A) *acs7* had lower ethylene emission. Data were means (±SD) of three experiments and the level of significance was evaluated by Student's *t* test. (B) Transcript levels of *ACS*s in the wild type were normalized to the internal control, *TIP41-LIKE*. (C) Transcript levels of *ACS*s in *acs7*. (D) Transcript levels of ethylene-responsive genes in *acs7*. In (C) and (D), the transcript level of each gene was set to 1 in the wild type. There were three biological replicates with at least three technical repeats for each gene. Error bars ±SD.

a major contribution to the production of ethylene in growing etiolated seedlings.

Previously, it has been shown that the expression of several *ACS* genes is regulated by ethylene in a feed-forward fashion (Barry *et al.*, 2000; Giovannoni, 2001; Alexander and Grierson, 2002; Argueso *et al.*, 2007). The transcript levels of all other *ACS* genes in *acs7* were therefore analysed. Indeed, most ACSs, especially the ones that could form a heterodimer with ACS7, had reduced transcript levels in *acs7* (Fig. 3B, C; see Supplementary Table S2 at *JXB* online), further supporting a role for ACS7 in ethylene production. Consistently, the transcript levels of ethylene-responsive genes, including *ETR2* (*ETHYLENE RESPONSE 2*), *ERF1* (*ETHYLENE RESPONSE FACTOR 1*), *ERF2* (*ETHYLENE RESPONSE FACTOR 2*), *AtEBP* (*ETHYLENE RESPONSE FACTOR 72*), and *HLS1* (*HOOKLESS 1*) (Wang *et al.*, 2002; Christians and Larsen, 2007), were lower in *acs7* compared with that of the wild type (Fig. 3D).

Phenotypes of the acs7 *mutant under normal growth conditions*

On plates, *acs7* germinated slightly earlier than the wild type (see Supplementary Fig. S1 at *JXB* online), and had larger cotyledons and true leaves with higher chlorophyll level (Fig. 4A, B). The primary root of *acs7* also grew faster than that of the wild-type (Fig. 4C). Since ethylene is known to regulate primary root elongation negatively (Rahman *et al.*, 2001; Růzicka *et al.*, 2007), both the wild-type and *acs7* seedlings were subjected to a series of concentrations of ACC treatment, and their primary root elongation was recorded and compared with the untreated seedlings (Fig. 4C, D, E; see Supplementary Fig. S2 at *JXB* online). The difference in the primary root length between the wild type and *acs7* was reduced by the application of ACC, suggesting that the accelerated primary root elongation was indeed due to the reduced endogenous ethylene level in the roots.

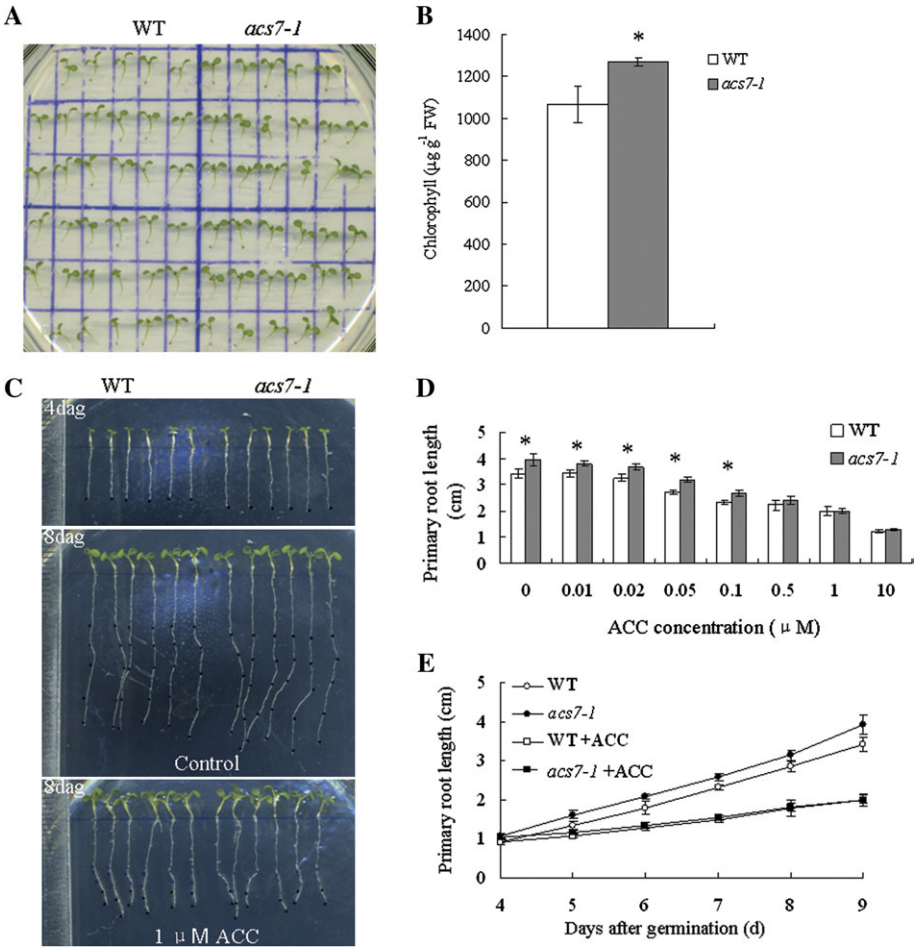

Fig. 4. Phenotypes of *acs7* seedlings. (A) Nine-day-old *acs7* seedlings had larger cotyledons and true leaves. (B) Higher level of chlorophyll content was detected in *acs7*. (C) The *acs7* seedlings had longer primary roots than the wild type, which could be restored by 1 μM ACC, the precursor of ethylene. (D) Four-day-old seedlings were treated with different concentrations of ACC for 5 d. The primary root length of the wild-type and *acs7* seedlings was quantified. (E) Quantification of the primary root length of the wild-type and *acs7* seedlings in (C). There were three independent replicates with six seedlings each. Error bars ±SD. The level of significance was evaluated by Student's *t* test.

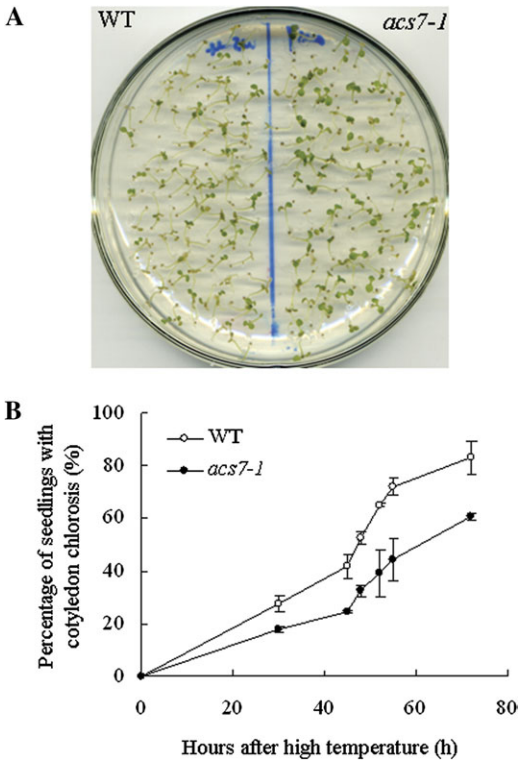

Fig. 5. Enhanced thermotolerance was observed in *acs7*. (A) Four-day-old wild-type and *acs7* seedlings were exposed to 43 °C for 3 h in the dark, and then transferred back to normal growth conditions. *acs7* exhibited enhanced tolerance to heat stress. (B) The percentage of seedlings with cotyledon chlorosis was scored over time. The *acs7* seedlings were less damaged by heat treatment. All analyses were performed at least in triplicate. Error bars ±SD.

Thermotolerance is enhanced in the acs7 *mutant*

Although ethylene has long been regarded as essential for abiotic stress responses (Morgan and Drew, 1997; Cao *et al.*, 2007), few studies have focused on the impact of ethylene synthesis in stress adaptation. Hence, the responses of *acs7* to abiotic stresses were evaluated in this study.

Previous studies have revealed the involvement of endogenous ethylene signalling in protecting plants from heat stress (Larkindale and Knight, 2002; Larkindale *et al.*, 2005). Mutants in ethylene biosynthesis, however, have not been tested for their thermotolerance. To evaluate the response of *acs7* to heat stress, 4-d-old *acs7* and wild-type seedlings were exposed to 43 °C for 3 h in the dark (to avoid light-dependent oxidative damage). After being transferred back to normal growth conditions, the *acs7* seedlings, having the lower percentage of cotyledon chlorosis (Fig 5A, B), were less damaged by heat, and were able to resume growth by producing new leaves (see Supplementary Fig. S3 at *JXB* online).

To analyse the molecular basis for the increased thermotolerance of the *acs7* mutant, the expression of a number of heat stress-related genes was examined. These include heat shock transcription factors (HSFs) and heat shock proteins (HSPs), such as *HsfA2*, *HsfB1*, *HsfB2a*, *Hsp70*, and *Hsp101* (Larkindale *et al.*, 2005). Overall, these genes were induced more quickly in *acs7* compared with the wild type (Fig. 6), indicating that the enhanced thermotolerance of *acs7* at least partly resulted from the higher and earlier induction of HSFs and HSPs. In addition, the expression of multiprotein bridging factor 1c (MBF1c), a key regulator of thermotolerance upstream of ethylene signalling (Suzuki *et al.*, 2008), was induced earlier in *acs7* (Fig. 6), which might lead to earlier induction of the expressions of heat stress-responsive genes.

The acs7 *mutant exhibits enhanced salt tolerance and accumulates more ABA under salt stress*

It has been shown that 100 μM ACC treatment could enhance salt tolerance in *Arabidopsis* (Achard *et al.*, 2006; Cao *et al.*, 2007), and that mutants defective in ethylene signalling, such as *etr1-1, ein4-1*, and *ein2-1,* showed higher sensitivity to salt (Cao *et al.*, 2007).

The salt stress tolerance of *acs7* and the wild type was then analysed in two ways. Firstly, young seedlings were treated with 150 mM NaCl for a prolonged period of time, and their survival rate was scored over time. Compared with the wild-type, *acs7* was much more tolerant (Fig. 7A, B). Secondly, germination kinetics was recorded over time for both the wild-type and mutant seeds, with or without 150 mM NaCl, or 300 mM mannitol. Although seed germination was postponed by salt and osmotic stress treatment in both the wild-type and *acs7*, the mutant still maintained higher germination rates over time (Fig. 7C, D). Combined, these data suggested that the absence of *ACS7* enhanced salt tolerance in *Arabidopsis*.

The molecular basis for the elevated salt tolerance was then explored. Since salt stress adaptation is known to be mediated by multiple pathways and at both the transcriptional and post-transcriptional levels, the expressions of a number of marker genes, which are known to be salt stress-responsive at the transcript level, were monitored in a time-course study.

NHX1 encodes a tonoplast sodium/proton antiporter which is important for salt tolerance and ion homeostasis (Zhu, 2000; Yokoi *et al.*, 2002). In unstressed seedlings, the transcript level of *NHX1* was already higher in the mutant (Fig. 8). Expression of *NHX1* in both the wild type and *acs7* was quickly and significantly induced by salt, and *acs7* maintained a high transcript level of *NHX1* for a longer time. These results suggested that the salt tolerance observed in *acs7* may be partly due to the enhanced osmotic tolerance mediated by *NHX1*.

The transcription factor DREB2A is known specifically to interact with the DRE/CRT *cis*-element to induce osmotic stress-responsive gene expression (Sakuma *et al.*, 2006). When unstressed, no difference in *DREB2A* expression was observed between the wild type and *acs7*. Nevertheless, the induction of *DREB2A* expression by salt was clearly earlier in *acs7* (Fig. 8), very likely leading to an earlier activation of salt stress-responsive genes downstream.

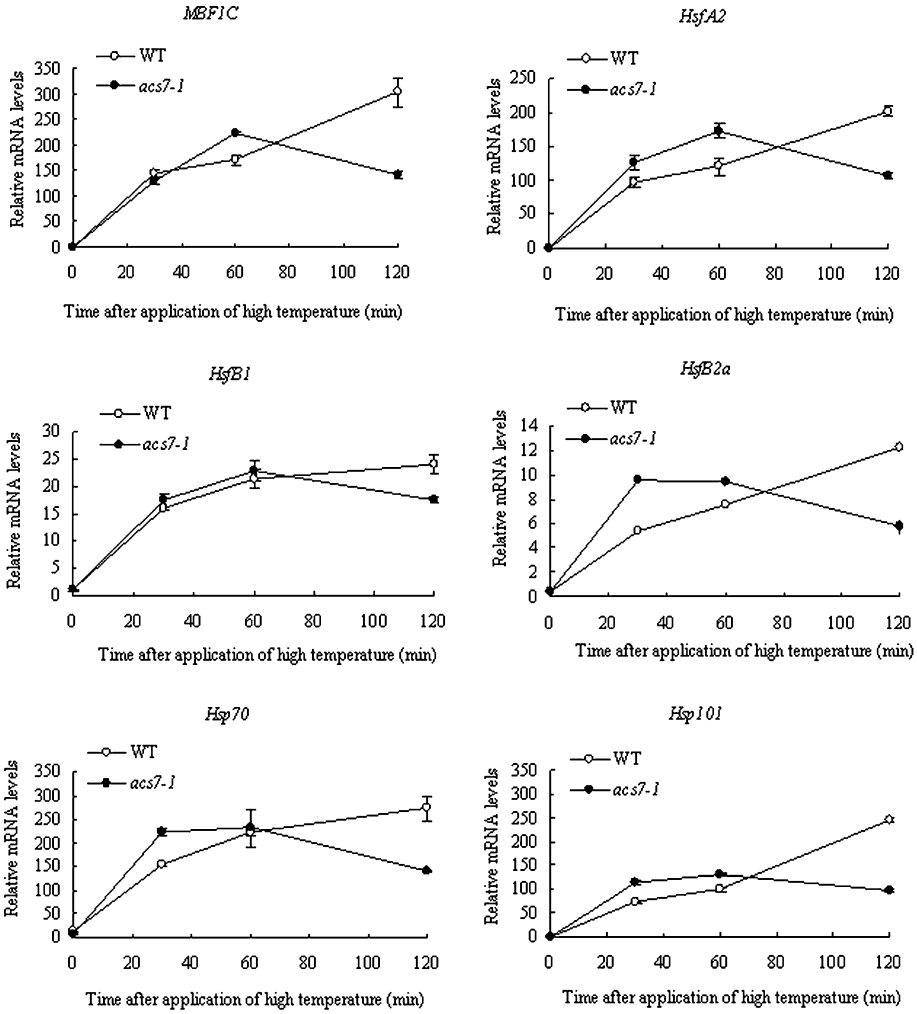

Fig. 6. The expression of heat stress-related genes, including *HsfA2*, *HsfB1*, *HsfB2a*, *Hsp70*, *Hsp101*, and *MBF1c*, were generally induced earlier and to a higher level in *acs7* following the heat treatment. There were three biological replicates with at least three technical repeats for each gene. Error bars ±SD.

The expression of *RD29A* is both ABA-dependent and ABA-independent (the latter mediated by *DREB2A*). Transcript levels of *RD29B*, *RAB18*, *MYC2*, and *RD22* are all known to be regulated mainly through ABA-dependent pathways (Zhu, 2002; Chinnusamy *et al.*, 2004). In *acs7*, expression of these genes was overall more induced by salt stress compared with the wild type (Fig. 8). The expression level of *NCED3*, which encodes a rate-limiting enzyme in ABA biosynthesis, was also higher in *acs7* following salt treatment (Fig. 8).

Moreover, in order to test whether ABA accumulation contributed to the salt tolerance of *acs7*, the endogenous ABA levels of *acs7* and the wild type at the 24 h time point following 175 mM NaCl treatment were measured with LC-MS. The ABA level in *acs7* was higher than the wild-type seedlings under salt stress (Table 1). Meanwhile, when nordihydroguaiaretic acid (NDGA), the ABA biosynthesis inhibitor (Han *et al.*, 2004; Liu *et al.*, 2009), was supplied, *acs7* seedlings became even more sensitive to salt compared with the wild type (see Supplementary Fig. S4 at *JXB* online). Since it is widely accepted that ethylene has a positive role in salinity adaptation (Achard *et al.*, 2006), such a phenotype could thus have resulted from a reduction of both endogenous ABA and endogenous ethylene in the mutant.

Combined, our results suggested that the loss of *ACS7* confers salt stress tolerance mainly by promoting the ABA-dependent stress-responsive pathway and by endogenous ABA accumulation.

The acs7 *mutant is hypersensitive to ABA*

To analyse the possible changes in ABA level and response in *acs7* further, a series of physiological measurements were carried out, including the ABA-mediated inhibition of germination, accumulation of proline and soluble sugar, and glucose sensitivity (Zhu, 2002; León and Sheen, 2003; Carrari *et al.*, 2004; Vinocur and Altman, 2005; Tuteja, 2007).

The germination sensitivity of *acs7* seeds to exogenous ABA was evaluated first. Although no difference was observed in the final germination percentage between *acs7* and the wild type, the germination kinetics was different; the *acs7* seeds germinated faster than the wild type on control plates, nevertheless, 2 μM ABA clearly halted the germination of the mutant (Fig. 9A).

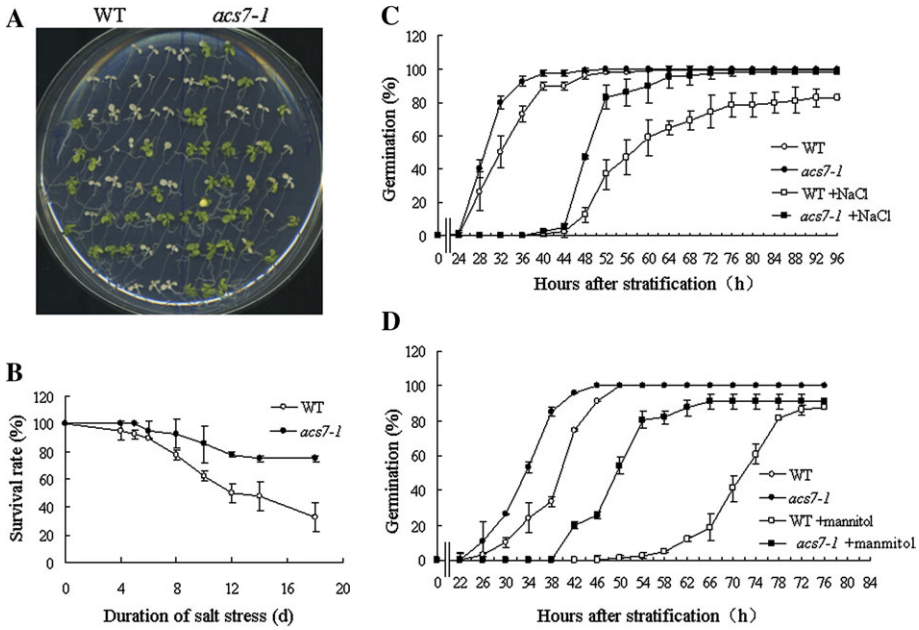

Fig. 7. *acs7* exhibited enhanced salt and osmotic stress tolerance. (A) Five-day-old wild-type and *acs7* seedlings were transferred to new medium supplemented with or without 150 mM NaCl and allowed to grow for 18 d. *acs7* showed enhanced tolerance to salt stress. (B) Quantification of the survival rate over time. The seedlings were defined to be dead with the death of the shoot apical meristem. (C) Germination kinetics of the wild type and *acs7* with or without 150 mM NaCl. (D) Germination kinetics of the wild type and *acs7* with or without 300 mM mannitol. All analyses were performed at least in triplicate. Error bars ±SD.

The 9-d-old and 19-d-old seedlings of the wild type and *acs7* under normal growth condition were collected, and measured for their proline and soluble sugar contents. Both metabolites were higher in *acs7*, especially proline (Fig. 9B, C). Consistently, the transcript level of *P5CS1*, which encodes the rate-limiting enzyme in the biosynthesis of proline, was increased in *acs7* (see Supplementary Fig. S5 at *JXB* online).

The germination assay on medium supplemented with high glucose gave consistent results. *acs7* still germinated faster, yet immediately after radicle emergence, the seedling growth rate was strongly delayed by 6% glucose (Fig. 10A, B). On day 10, the percentage of the wild-type seedlings with true leaves was significantly higher than that of *acs7* (Fig. 10C). Sucrose, however, does not have an impact on *acs7* germination (see Supplementary Fig. S6 at *JXB* online).

The expression level of *CYP707A2* was lower in the *acs7* mutant, while genes involved in ABA biosynthesis (*ABA1*, *ABA2*, *NCED3*, *ABA3*, and *AAO3*) generally had slightly higher transcript levels (see Supplementary Fig. S5 at *JXB* online). Consistently, the expression levels of two ABA-responsive genes, *RD29B* and *RAB18* (Saez *et al.*, 2008), were also higher in *acs7* (see Supplementary Fig. S5 at *JXB* online).

Discussion

ACC synthase acts as the rate-limiting enzyme in ethylene biosynthesis (Yang and Hoffman, 1984). In *Arabidopsis*, the expression of each *AtACS* gene is regulated by different developmental and environmental signals (Wang *et al.*, 2002; Tsuchisaka and Theologis, 2004; Peng *et al.*, 2005), indicating that *AtACSs* may have unique and overlapping function in the regulation of plant growth and stress response. In a recent effort to obtain a mutant with all nine *ACS*s knocked out, it was noticed that both the two octuple mutant lines were defective in seed setting, suggesting that the presence of ACS activity is required for plant survival (Tsuchisaka *et al.*, 2009). Nevertheless, to our knowledge, the specific function of any single ACS in regulating abiotic stress adaptation has not been reported. Here, by analysing the *acs7-1* mutant, the possible role of *AtACS7* in plant growth and stress tolerance was explored, mainly through physiological measurements and transcript analysis.

The most significant phenotypic difference was that *acs7* always appeared bigger than the wild type, having larger cotyledons and longer primary roots. Since ethylene has long been regarded as a negative regulator of vegetative growth, our observation was therefore explainable. Indeed, the root phenotype was successfully restored by an application of ACC to the mutant.

Although the ethylene level was lower in *acs7*, no difference was observed in the final germination percentage between *acs7* and the wild type. Interestingly, the *acs7* seeds germinated slightly faster than the wild type, and radicle emergence of *acs7* was consistently about 4 h earlier than the wild type. Considering that both the dry and germinating seeds of *acs7* were noticeably larger than the wild type (data not shown), it was inferred that the phenotype was very possibly due to the more efficient imbibitions of the mutant.

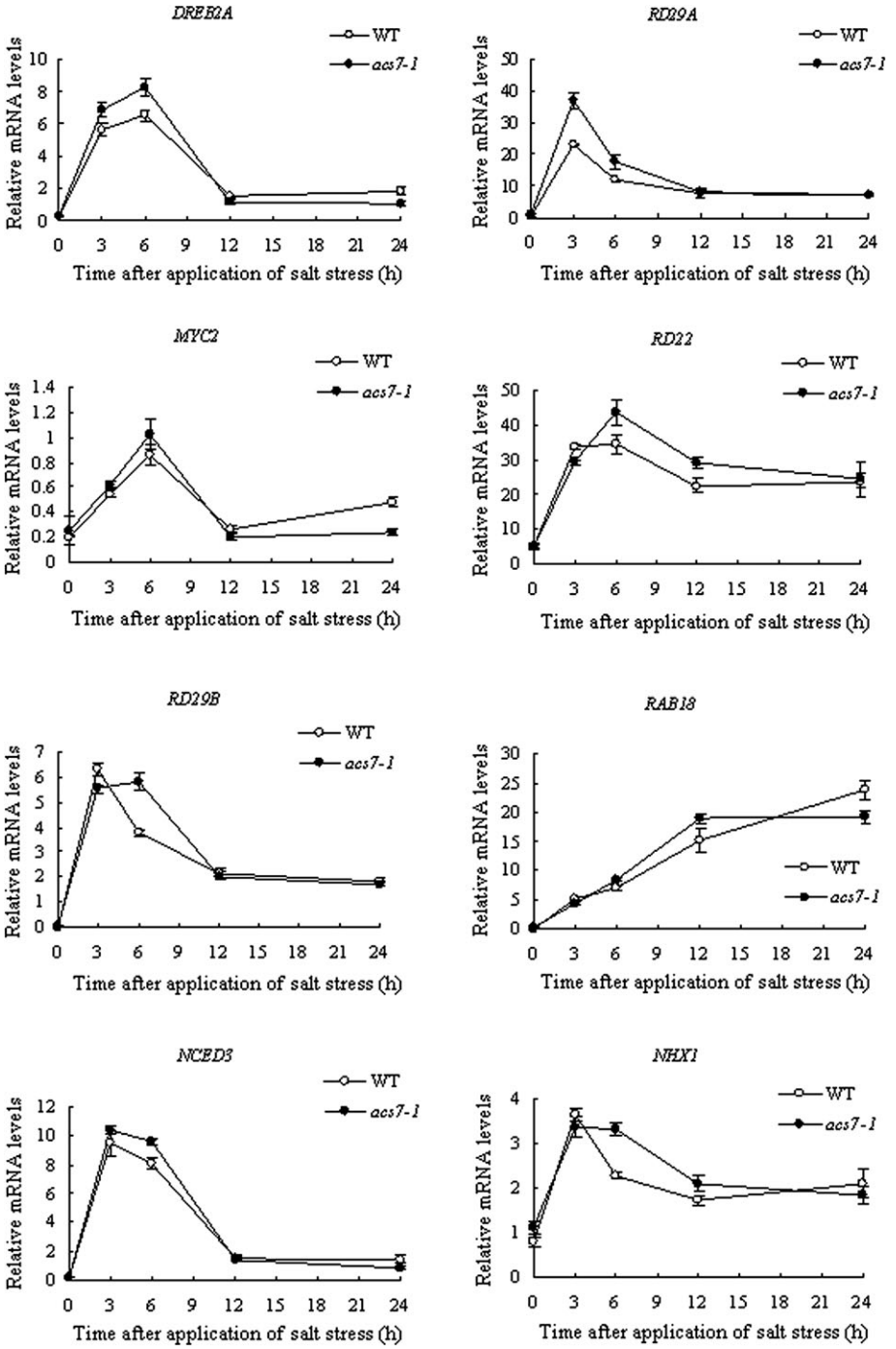

Fig. 8. The transcript levels of salt-responsive and ABA-related genes were induced earlier and to a higher level in *acs7* by 175 mM NaCl. Three biological replicates with at least three technical repeats were done for each gene. Error bars ±SD.

A previous study showed that 10-d-old *Arabidopsis* seedlings, pretreated with 100 μM ACC, had a higher survival rate after being exposed to 40 °C for 1 h (Larkindale and Knight, 2002). Furthermore, it was demonstrated that the growth of *ein2-1* and *etr1-1* was significantly compromised after a heat treatment at 45 °C for 1 h (Larkindale *et al.*, 2005). These results suggest that ethylene signalling may have a positive role in heat stress tolerance. Therefore, it was interesting to find that the *acs7* seedlings were more tolerant to heat. Since the induction of HSPs is regarded as one of the efficient (and classic) mechanisms for survival at high temperature, the expression of *HsfA2*, *HsfB1*, *HsfB2a*, *Hsp70*, and *Hsp101* in *acs7* during the heat treatment were then profiled. All these genes were induced earlier in *acs7*, consistent with the enhanced thermotolerance of *acs7*.

On the other hand, the involvement not only of ethylene but ABA as well has been implicated in heat stress tolerance. It was shown that ABA biosynthesis and, in particular, ABA signalling mutants were hypersensitive to heat stress, indicative of a positive role for ABA in thermotolerance

Table 1. ABA content was higher in *acs7* under salt stress

Nine-day-old seedlings of the wild type and *acs7* were treated with 175 mM NaCl for 24 h. The seedlings with or without salt treatment were ground into powder in liquid nitrogen and sampled for ABA determination. Data were means (±SD) of three experiments. The difference in ABA levels between *acs7* and the wild type under salt stress was significant as evaluated by Student's *t* test.

Treatment	ABA content (pg mg^{-1} FW)	
	WT	*acs7*
Control	1.16±0.03	1.08±0.06
175 mM NaCl	7.39±0.26	8.14±0.31*

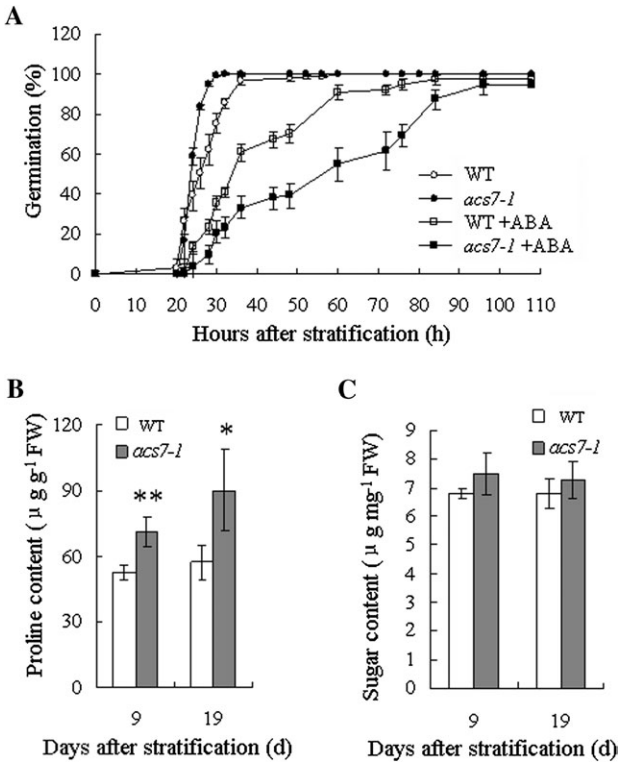

Fig. 9. ABA related phenotypes in *acs7*. (A) *acs7* was hypersensitive to exogenous ABA during germination. (B) Proline content was significantly higher in *acs7*. (C) Soluble sugar content was slightly higher in *acs7*. At least three independent repeats gave typical results. Error bars ±SD. The level of significance was evaluated by Student's *t* test.

(Larkindale *et al.*, 2005). Hence the heat stress tolerance of *acs7* might, at least partly, result from activated ABA synthesis or signalling pathways. The evidence needs to be explored.

Since previous studies have suggested a positive role of both exogenous ethylene and ethylene signalling in salt tolerance (Achard *et al.*, 2006; Cao *et al.*, 2007), it was surprising to see that the *acs7* mutant were more tolerant to salt and osmotic stresses. As a validation for our experimental system, other *ACS* knock-out mutants were assayed in parallel (data not shown). Neither type 1 nor type 2 *ACS* mutants showed the elevated salt stress tolerance that was observed in *acs7*. Hence it is concluded that *ACS7*, rather than the other *ACS* genes, or ethylene signalling genes, has a unique (negative) function in mediating the salt stress response.

Given the importance of ABA in the abiotic stress adaptation, it was tested whether the ABA content or response was altered in *acs7*. It turned out that the ABA level in *acs7* was higher than the wild type at the 24 h time point following salt treatment. The transcript analyses showed that, under salt treatment, stress-responsive genes involved in the ABA-dependent pathway were activated at the earliest time point tested (3 h after salt treatment) (Fig. 8), when changes in ABA levels might not have occurred. Meanwhile, the expression of *NCED3*, a key component of ABA biosynthesis, was not significantly altered compared with that of other stress-responsive genes. Finally, *acs7* seeds were hypersensitive to exogenous ABA. Combined, our results suggested that not only the increased ABA accumulation, but also the enhanced ABA sensitivity, may have contributed to the salt tolerance of *acs7*.

Consistently, early development of the *acs7* seedlings on glucose was strongly inhibited (Fig. 10). Genetic screens for glucose-insensitive mutants had shown them to be ABA biosynthesis mutants, such as *aba2* and *aba3*, and the glucose-oversensitive mutants were shown to be ethylene signalling mutants, such as *etr1*, *ein2*, and *ein3* (León and Sheen, 2003), therefore the germination hypersensitive phenotypes could be explained either way.

In conclusion, our data strongly suggest elevated endogenous ABA levels and enhanced ABA sensitivity in *acs7* under salt stress, which may have directly resulted from the loss of ACS7 and reduced ethylene production. Therefore, what was observed might have been mostly the long-term effects of the loss of ACS7. The immediate consequences of losing ACS7 will be explored in future. Meanwhile, why and how the loss of *ACS7*, not the other *ACS*s, could lead to such changes in ABA synthesis, signalling, and turnover, demands further attention. The unique function of *ACS7* may have arisen from its unique protein structure. Nevertheless, our study revealed the distinctive role of *ACS7*, the only type 3 *ACS*, in linking ethylene and ABA, two phytohormones essential for plant abiotic stress adaptation.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. *acs7* germinated slightly yet consistently faster than the wild type.

Supplementary Fig. S2. The primary root length of wild-type and *acs7* seedlings under a series of concentrations of ACC treatments.

Supplementary Fig. S3. *acs7* resumed vegetative growth following heat stress.

Supplementary Fig. S4. NDGA, the inhibitor of ABA biosynthesis, suppressed the salt-tolerant phenotype of *acs7*.

Supplementary Fig. S5. The expression levels of ABA-related genes in *acs7*.

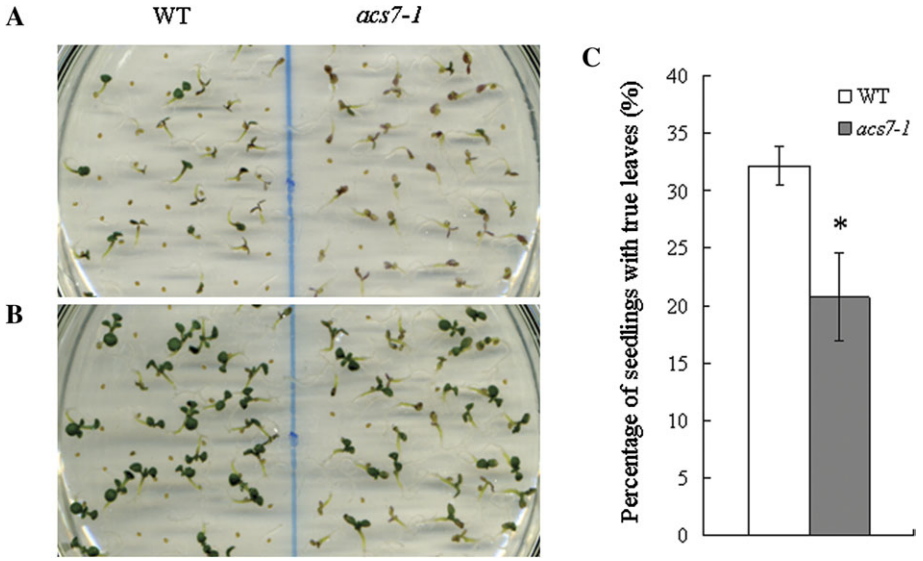

Fig. 10. *acs7* was hypersensitive to high glucose. The seeds of the wild type and *acs7* were sown on 1/2 MS medium supplemented with 6% glucose, and grown under normal growth conditions. The plates were photographed daily afterwards. (A) Accumulated anthocyanin was observed in 7-d-old *acs7* seedlings. (B) High glucose inhibited the growth of 10-d-old *acs7* seedlings. The experiment was performed in triplicate. (C) On 6% glucose, the growth rate of the wild type was higher than that of *acs7*. The number of seedlings with true leaves in (B) was scored. Error bars ±SD. The level of significance was evaluated by Student's *t* test.

Supplementary Fig. S6. Response to sucrose of *acs7* was normal.

Supplementary Table S1. Primers used for genotyping, RT-PCR, and real-time RT-PCR analyses.

Supplementary Table S2. The number of active homo- and hetero-isozymes was possibly decreased in *acs7*.

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