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ORIGINAL PAPER

# *AtHSP17.8* overexpression in transgenic lettuce gives rise to dehydration and salt stress resistance phenotypes through modulation of ABA-mediated signaling

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**Abstract**
***Key message*** **Transgenic *Arabidopsis* and lettuce plants overexpressing *AtHSP17.8* showed ABA-hypersensitive but abiotic stress-resistant phenotypes. ABA treatment caused a dramatic induction of early ABA-responsive genes in *AtHSP17.8*-overexpressing transgenic lettuce.**
*Abstract* Plant small heat shock proteins function as chaperones in protein folding. In addition, they are involved in responses to various abiotic stresses, such as dehydration, heat and high salinity in *Arabidopsis*. However, it remains elusive how they play a role in the abiotic stress responses at the molecular level. In this study, we provide evidence that *Arabidopsis HSP17.8* (*AtHSP17.8*) positively regulates the abiotic stress responses by modulating abscisic acid (ABA) signaling in *Arabidopsis*, and also in lettuce, a heterologous plant when ectopically expressed. Overexpression of *AtHSP17.8* in both *Arabidopsis* and lettuce leads to hypersensitivity to ABA and enhanced resistance to dehydration and high salinity stresses. Moreover, early ABA-responsive genes, *ABI1*, *ABI5*, *NCED3*, *SNF4* and *AREB2*, were rapidly induced in *AtHSP17.8*-overexpressing transgenic *Arabidopsis* and lettuce. Based on these data, we propose that *AtHSP17.8* plays a crucial role in abiotic stress responses by positively modulating ABA-mediated signaling in both *Arabidopsis* and lettuce. Moreover, our results suggest that stress-tolerant lettuce can be engineered using the genetic and molecular resources of *Arabidopsis*.

**Keywords** Small heat shock protein · ABA-mediated signaling · Abiotic stress responses · *Arabidopsis* · Lettuce

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## Introduction

Plants, being sessile organisms, have to adapt to the ever-changing environmental conditions of the place where they are growing. Accordingly, plants evolved mechanisms to cope with environmental fluctuations and to protect them from various abiotic stresses, such as dehydration and high salt. Abscisic acid (ABA), a phytohormone, plays a pivotal role in the adaption responses to these abiotic stresses through reprogramming of the transcriptome (Cutler and Krochko 1999; Lee et al. 2006; Wilkinson and Davies 2002; Xu et al. 2013; Zeevaart 1999; Zhu 2002). ABA is also involved in other cellular processes, such as seed dormancy, germination and stomatal closure. Under abiotic stress conditions, the ABA level rapidly increases, which is then recognized by ABA receptors that localize to various subcellular compartments (Ma et al. 2009; Pandey et al. 2009; Park et al. 2009; Shen et al. 2006; Xu et al. 2013; Zeevaart 1999). Subsequently, the reprogramming of the downstream transcriptome which is mediated by ABA signaling leads to modification of various cellular components and processes, which in turn results in adjustments in growth and development of the plants adapting to the stress conditions.

Abiotic stresses have a serious effect on various cellular components and processes. One of them is the detrimental

effect on integrity of cellular proteins. The structure or assembly of cellular proteins is compromised by the high salt levels in the cytosol. In fact, it has been shown that certain molecular chaperones play critical roles in stress responses (Song et al. 2010; Sun et al. 2001, 2002; Wang et al. 2003). Ectopic expression of the genes encoding these proteins gives rise to stress tolerant plants (Song et al. 2010; Sun et al. 2001, 2002; Wang et al. 2003). These conserved proteins are involved in protein folding and assembly, and in the protection of proteins from denaturation.

Small heat shock proteins (sHSPs) with molecular mass ranging from 15 to 42 kD are found ubiquitously in all kingdoms of life and are known to have chaperone activity that can prevent heat stress-induced denatured proteins from forming non-specific aggregates (Eyles and Gierasch 2010; Kirschner et al. 2000). These proteins have an α-crystalline domain composed of nearly 90 amino acids flanked by a short C-terminal extension and an N-terminal arm of variable length (Basha et al. 2006; Kim et al. 2011; Sun et al. 2002; Sun and MacRae 2005). Generally, sHSPs are classified into multiple subfamilies based on sequence homology, immunological cross-reactivity and subcellular localization (Scharf et al. 2001; Waters et al. 1996). Plant sHSPs that are encoded by nuclear genes are divided into six classes (Basha et al. 2006; Sun et al. 2002; Sun and MacRae 2005). Three classes (class CI, CII and CIII) sHSPs are localized in the cytosol and/or nucleus and the other three are found in the endoplasmic reticulum (ER), plastids or mitochondria depending on individual classes (Basha et al. 2006; Sun et al. 2002; Sun and MacRae 2005). Except for mitochondrial-localized *Drosophila melanogaster* Hsp22, organellar sHSPs are unique to plants (Basha et al. 2006; Kim et al. 2011; Scharf et al. 2001; Sun et al. 2002; Sun and MacRae 2005).

In general, sHSPs possess a chaperone activity which prevents heat-denatured proteins from forming non-specific aggregates (Song et al. 2010; Sun et al. 2001, 2002; Wang et al. 2003). Besides its role in the heat response, plant sHSPs also function in other physiological processes including embryo development, fruit maturation, and ABA response as well as responses to various abiotic stresses, such as osmotic stress, oxidative stress, cold stress and heavy metal stress (Sun et al. 2002). In most cases, their physiological roles were inferred from their expression; two cytosolic classes CI and CII *sHSPs*, a mitochondrial *sHSP*, and a chloroplast-localized *sHSP* are induced by oxidative stress, and also several *sHSP* are induced by cold stress, heavy metal, ozone, UV-radiation and γ-irradiation (Sun et al. 2001, 2002). Consistent with these expression patterns, their expression is correlated with stress tolerance (Sun et al. 2001, 2002). For instance, in *Arabidopsis*, the ectopic expression of *AtHsp17.6A-CII* showed an increase in resistance to dehydration and salt stresses, suggesting that sHSPs function as a positive regulator in osmotic stress responses (Sun et al. 2001, 2002). In barley, dehydration shock causes a rapid induction of the barley *HSP17.8*, suggesting a possible role of sHSP in osmotic stress response (Guo et al. 2009).

The *Arabidopsis* genome encodes a large number of *sHSPs*. One of them, *Arabidopsis HSP17.8* (*AtHSP17.8*) that belongs to the class CI functions in the targeting of chloroplast outer envelope membrane protein from the cytosol to the chloroplast by acting as a cofactor of the ankyrin repeat proteins 2A (AKR2A) and AKR2B (Bae et al. 2008; Kim et al. 2011). AtHSP17.8 has a chloroplast-binding activity, and its overexpression leads to an increase in the targeting of a membrane protein to the chloroplast, suggesting that AtHSP17.8 functions as a component of the molecular machinery for chloroplast membrane protein targeting (Kim et al. 2011).

In this study, we examined the physiological role of AtSHP17.8 in other cellular processes by studying its expression pattern as well as its overexpression. In addition, in order to explore its possible application to crop plants, we examined whether the physiological role of AtHSP can be recapitulated in a heterologous system. We chose lettuce (*Lactuca sativa* L.), one of the important leafy vegetables, which is extremely vulnerable to dehydration stress. Here, we demonstrated that *AtHSP17.8* is highly induced by various abiotic stresses and also by exogenous ABA. Moreover, we provide evidence that its overexpression results in enhanced resistance to dehydration and high salinity stresses in both *Arabidopsis* and lettuce through activation of ABA-mediated signaling.

## Results

### *AtHSP17.8* is strongly induced by various abiotic stresses and exogenous ABA

To gain an insight into the physiological role of *AtHSP17.8* in plants, we examined the expression pattern of *AtHSP17.8* under various abiotic stress conditions because previous studies implicated the physiological role of sHSPs in various abiotic stress responses (Siddique et al. 2008; Sun et al. 2001). Wild-type *Arabidopsis* plants were treated with dehydration, heat, cold and exogenous ABA, and total RNAs from these plants were subject to qRT-PCR analysis for various stress-responsive genes. *HSFA3* and *RD29A* were used as positive controls for heat, dehydration, cold or ABA responses. *AtHSP17.8* transcript levels were greatly increased 1 h after treatment with heat and ABA, but marginally by the dehydration condition (Fig. 1a and b). In contrast, 3-h-cold treatment drastically reduced the

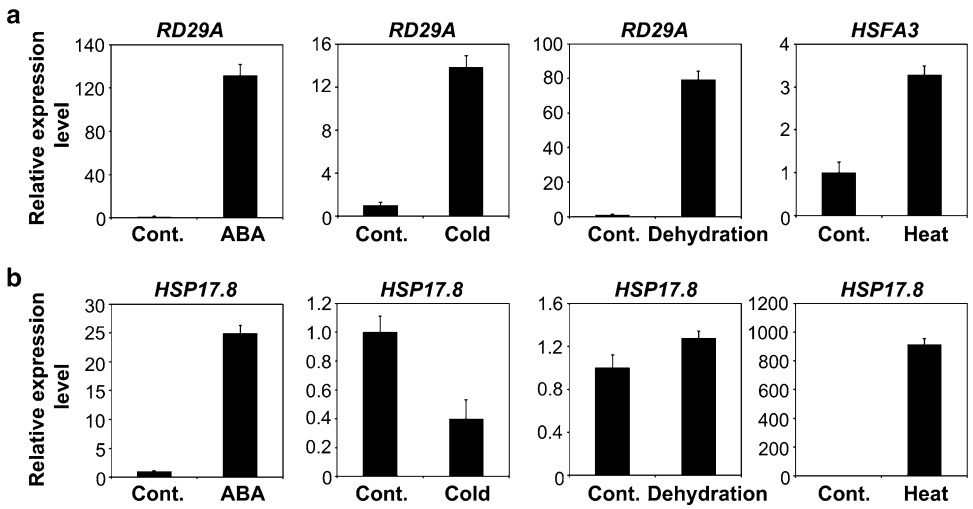


**Fig. 1** The expression pattern of *AtHSP17.8* under various abiotic stress conditions. The expression pattern of *AtHSP17.8* under various abiotic stress and ABA treatment conditions. Total RNA from wild-type plants treated with ABA (5 μM) for 1 h, dehydration stress (40 % relative humidity) for 1 h, heat stress (42 °C) for 1 h and cold stress (0 °C) for 3 h was used for real-time qRT-PCR analysis using gene-specific primers of the indicated genes. **a** *RD29A* was used as the positive control for ABA, dehydration stress and cold stress responses, and *HSFA3* as the positive control for heat stress response. **b** Transcript levels of *AtHSP17.8* were examined by qRT-PCR. *ACT2* was used as an internal control for real-time qRT-PCR. *Error bars*, standard deviation (SD) ($n = 3$). Cont., control

*AtHSP17.8* transcript levels, indicating that *AtHSP17.8* is negatively regulated by cold stress response. These results suggested that *AtHSP17.8* was positively regulated by heat and ABA, but was not, or only marginally so, by dehydration stress condition.

## *AtHSP17.8*-overexpressing plants exhibit hypersensitivity to exogenous ABA during germination and post-germination growth

To assess the role of AtHSP17.8 in abiotic stress responses in plants, we generated transgenic *Arabidopsis* plants harboring *AtHSP17.8* with a C-terminal haemagglutinin (HA) epitope under the control of the cauliflower mosaic virus 35S (*CaMV 35S*) promoter (Fig. 2a). In T3 generation, we identified two independent lines of homozygous transgenic plants overexpressing *AtHSP17.8:HA* (*AtHSP17.8:HA OX*). *AtHSP17.8:HA* transcripts and the encoded proteins were readily detected in the two independent lines, *AtHSP17.8:HA OX11-1* and *AtHSP17.8:HA OX31-1,* when examined by semi-quantitative RT-PCR and Western blot analysis using anti-HA antibody, respectively (Fig. 2b and c). However, no specific bands were detected from control transgenic plants harboring the empty vector (EV) (Fig. 2b and c), confirming the specificity. Next, to test whether this protein plays a role in osmotic stress responses, we examined at first, the sensitivity of these transgenic plants to exogenous ABA. ABA plays a crucial role in osmotic stress responses (Wang et al. 2011). The germination rate of *AtHSP17.8:HA OX* plants was greatly reduced in the presence of 0.5 μM ABA, as compared to the control EV plants (Fig. 2d and e). Their post-germination rate was also significantly reduced when examined by the primary root length in the presence of 8 μM of ABA (Fig. 2f and g), when compared with the EV plants, indicating that *AtHSP17.8* plays a positive role in ABA responses at both germination and post-germination stages of development.

In osmotic stress responses, one important role of ABA is stomatal closure under dehydration stress conditions. Accordingly, to test whether *AtHSP17.8* is involved in modulation of water relations in plants, we examined the water loss rate using the aerial part excised from the *AtHSP17.8:HA OX*s and EV plants over time (Xu et al. 2012). The water loss rate was lower in *AtHSP17.8:HA OX* plants than in the control EV plants (Fig. 2h), confirming that *AtHSP17.8* plays a critical role in water relations in plants. These results suggest that AtHSP17.8 acts as the positive regulator in ABA and dehydration stress responses.

## Generation of *AtHSP17.8:HA*-overexpressing transgenic lettuce

The positive role of *AtHSP17.8* in ABA and dehydration stress responses prompted us to test whether this evolutionary conserved sHSP plays a similar role in lettuce, an important vegetative crop that is highly sensitive to dehydration stress. To generate *AtHSP17.8:HA*-overexpressing transgenic lettuce, cotyledons of lettuce plants were excised and inoculated with *Agrobacterium* harboring *AtHSP17.8:HA*. After

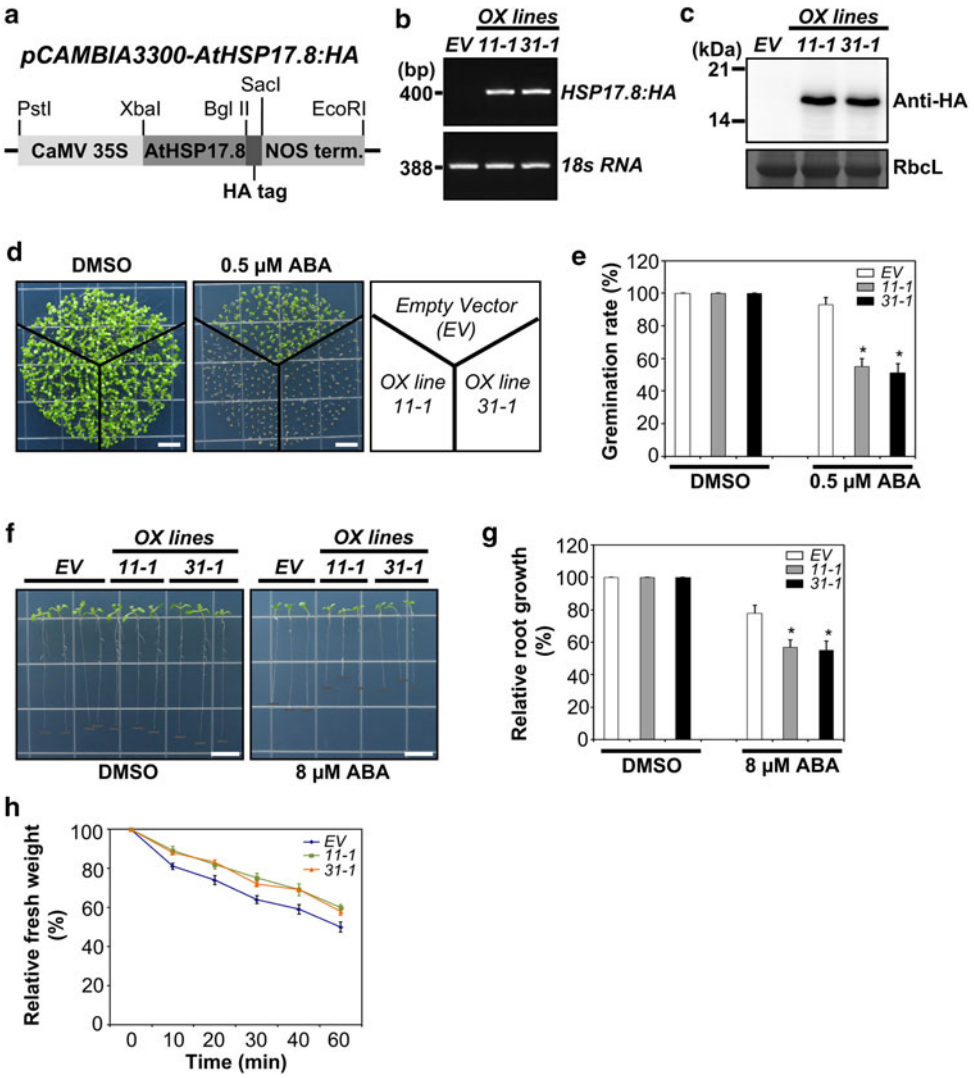


**Fig. 2** Transgenic *Arabidopsis* plants harboring *AtHSP17.8:HA* exhibit hypersensitivity to ABA and enhanced resistance to dehydration stress. **a** Schematic presentation of *AtHSP17.8:HA* used to generate transgenic *Arabidopsis* and lettuce plants. *AtHSP17.8:HA* under the *CaMV 35S* promoter was inserted into a binary vector *pCAMBIA3300*. NOS term., NOS terminator. **b**, **c** Expression of *AtHSP17.8:HA* in transgenic *Arabidopsis* plants. **b** *AtHSP17.8:HA* transcript levels were examined in two independent lines of *Arabidopsis AtHSP17.8:HA OX* and control EV plants by semi-quantitative RT-PCR using gene-specific primers. *18S rRNA* was used as an internal control. **c** AtHSP17.8:HA protein levels were examined in two independent lines by Western blot analysis using anti-HA antibody. The Rubisco large subunit (RbcL) was used as a loading control. **d**, **e** Effect of exogenous ABA on seed germination. Two independent lines of *Arabidopsis AtHSP17.8:HA OX* (line 11–1 and 31–1) and control EV seeds were planted on 1/2 MS plates supplemented with DMSO or 0.5 μM ABA and the germination rates were examined. **d** Plant images were obtained 4 days after planting. *Bar* 1 cm. **e** The germination rates were scored 4 days after planting. Three independent experiments were performed with 60 seeds per experiment. *Error bars*, SD ($n = 3$). Statistical analysis was performed between EV and *AtHSP17.8:HA OX* plants. **f**, **g** Effect of exogenous ABA on vegetative growth. Plants grown on 1/2 MS plates for 3 days were transferred onto 1/2 MS plates containing DMSO or 8 μM ABA and the plates were placed vertically for plant growth. **f** Plant images were taken 6 days after transfer. *Gray bars* indicate the root tips. **g** The primary root length was measured 6 days after transfer. Three independent experiments were performed using 20 plants in each experiment. *Error bars*, SD ($n = 3$). *Horizontal white bars*, 1 cm. Statistical analysis was performed between EV and *AtHSP17.8:HA OX* plants. **h** The water loss rate of *AtHSP17.8:HA OX* plants. The aerial parts of two independent lines of *Arabidopsis AtHSP17.8:HA OX* and EV plants grown on MS plates for 2 weeks were excised and left on the bench for 1 h. To measure the water loss rate, the fresh weight of the excised plant tissues was measured at the indicated time points. Three independent experiments were performed using 20 plants in each experiment. *Error bars*, SD ($n = 3$). The *asterisks* indicate a significant difference by Student's *t* test ($^*P < 0.01$). *EV* empty vector control, *OX AtHSP17.8:HA* overexpression

incubation on the media for callus induction, the explants were subsequently transferred onto shoot and root induction media, sequentially. Finally, the plants with well-developed roots were transferred to the greenhouse and the expression of *AtHSP17.8:HA* transcripts and proteins were examined using RT-PCR and Western blot analysis using anti-HA antibody.

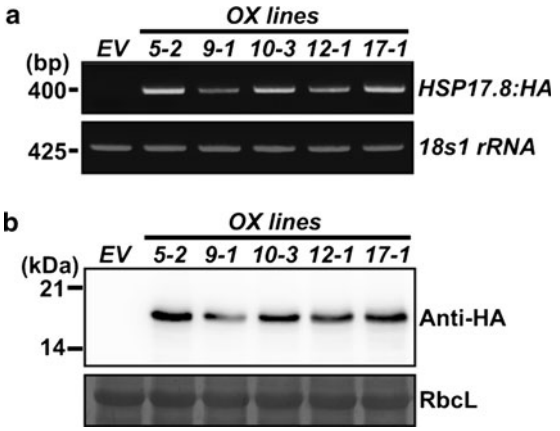


**Fig. 3** The expression of *AtHSP17.8:HA* in transgenic lettuce plants. Expression of *AtHSP17.8:HA* in *AtHSP17.8:HA OX* lettuce plants. **a** *AtHSP17.8:HA* transcript levels were examined in five independent lines of *AtHSP17.8:HA OX* and control EV lettuce plants were detected by semi-quantitative RT-PCR using gene-specific primers. Lettuce *18S1 rRNA* was used as the internal control. **b** Protein levels of AtHSP17.8:HA in five independent lines of transgenic lettuce plants were examined by Western blot analysis using anti-HA antibody. The Rubisco large subunit (RbcL) was used as the loading control. *EV* empty vector control, *OX AtHSP17.8:HA* overexpression

We tested five independent lines for the expression of AtHSP17.8:HA. Different lines displayed different expression levels of *AtHSP17.8:HA* in both the transcript and protein levels (Fig. 3a and b), confirming that transgenic lettuces were successfully generated. Among the multiple lines we generated, two homozygous lines 5–2 and 10–3 of T3 generation were used for further physiological experiments.

### Transgenic lettuce plants ectopically expressing *AtHSP17.8:HA* exhibit hypersensitivity to exogenous ABA at both germination and post-germination stages

To test whether *AtHSP17.8:HA* also acts as a positive regulator in dehydration and ABA responses in lettuce, we examined the sensitivity of transgenic lettuce plants harboring *AtHSP17.8:HA* (*AtHSP17.8:HA OX* lettuce) to exogenous ABA. Germination of lettuce seeds was less sensitive to exogenous ABA as compared to *Arabidopsis* seeds. Accordingly, we used higher concentrations of ABA to test the sensitivity of lettuce seeds to ABA in germination. *AtHSP17.8:HA OX* lettuce plants showed enhanced sensitivity to exogenous ABA as compared with the control EV plants (Fig. 4a and b) as observed with *Arabidopsis AtHSP17.8:HA OX* plants. To examine the ABA-sensitive phenotype at the post-germination stage, 4-day-old *AtHSP17.8:HA OX* lettuce seedlings grown on half strength Murashige and Skoog (1/2 MS) plate were transferred onto MS plates supplemented with DMSO or 12 μM ABA and the root growth was examined. When examined 6 days after transfer, *AtHSP17.8:HA OX* lettuce plants did not show any significant difference in the root length as compared to the EV lettuce in the presence of DMSO. However, *AtHSP17.8:HA OX* lettuce plants showed a significant retardation in the root growth as compared to the control EV plants in the presence of ABA, indicating that lettuce plants also exhibit an increase in sensitivity to ABA during germination and also post-germination growth (Fig. 4c, d). Moreover, these results indicate that *Arabidopsis HSP17.8* acts as a positive regulator in ABA-mediated responses in a heterologous system: lettuce plants.

### Ectopic expression of *AtHSP17.8:HA* in lettuce plants activates the expression of ABA-responsive genes

The results showing that *AtHSP17.8 OX* lettuce plants exhibit an enhanced sensitivity to ABA and also an increased resistance to dehydration stress raised the possibility that AtHSP17.8 acts as a positive regulator in ABA signaling in lettuce plants. To test this idea, we examined whether the expression of ABA-responsive genes was altered in *AtHSP17.8:HA OX* in lettuce plants. First, we examined by qRT-PCR the effect of exogenous ABA on various ABA-inducible genes, such as *ABI1*, *ABI5*, *NCED3*, *SNF4* and *AREB2* in the *AtHSP17.8:HA OXs* and EV lettuce plants after treating with 20 μM ABA for 0.5 h and 1 h. As a control, *GRAS-A*, a gibberellin (GA)-inducible gene, was included in the analysis (Argyris et al. 2008). The ABA-responsive genes were more rapidly induced in *AtHSP17.8:HA OX* lettuce plants as compared to the control EV lettuce plants (Fig. 5). However, the expression pattern of *GRAS-A* was not altered, confirming the specific effect of *AtHSP17.8* on ABA-inducible genes. Interestingly, besides the genes encoding ABA signaling components, an ABA biosynthetic gene *NCED3* was also more rapidly induced by exogenous ABA. Similarly, in *Arabidopsis*, *NCED3* is also induced by exogenous ABA (Endo et al. 2008). These results suggest that *AtHSP17.8* positively regulates ABA-mediated signaling in lettuce, a heterologous system.

### Ectopic expression of *AtHSP17.8:HA* in lettuce results in enhanced resistance to osmotic stress

The results showing that ABA-mediated signaling is activated in *AtHSP17.8:HA OX* lettuce prompted us to test whether *AtHSP17.8:HA OX* lettuce plants had any increase in resistance to osmotic stress as observed with *Arabidopsis*. First, we examined the water loss rate in these *AtHSP17.8:HA OX* lettuce plants. The aerial parts of *AtHSP17.8:HA OX* and control EV plants were excised and their fresh weight was measured over time. Overall, the water loss rates were slower in lettuce than in *Arabidopsis*. Interestingly, *AtHSP17.8:HA OX* lettuce plants displayed

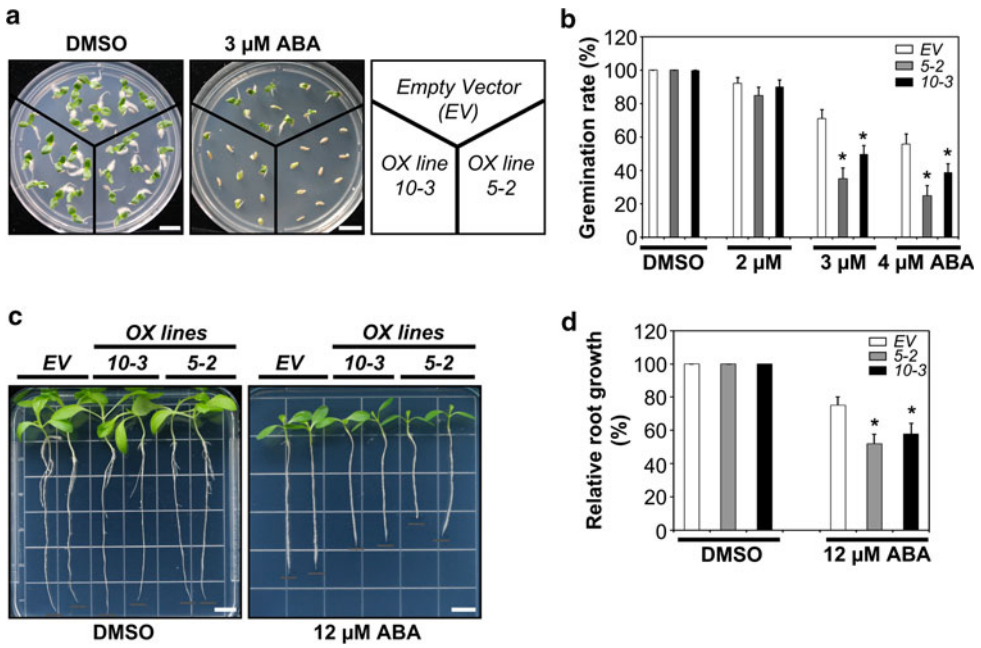


**Fig. 4** Transgenic lettuce plants harboring *AtHSP17.8:HA* are hypersensitive to ABA. **a**, **b** Effect of exogenous ABA on seed germination. Two independent lines of lettuce *AtHSP17.8:HA OX* and the control EV seeds were planted on 1/2 MS plates supplemented with DMSO or 3 μM ABA, and the germination rates were examined. **a** Plant images were obtained 5 days after planting. *Bars* 1 cm. **b** The germination rates were scored 5 days after planting. Three independent experiments were performed with 50 seeds in each experiment. *Error bars*, SD ($n = 3$). Statistical analysis was performed between EV and *AtHSP17.8:HA OX* plants. **c**, **d** Effect of exogenous ABA on vegetative growth. Lettuce seedlings grown on 1/2 MS plates for 4 days were transferred onto 1/2 MS plates containing DMSO or 12 μM ABA and the plates were positioned vertically. **c** Plant images were taken 6 days after transfer. *Gray bars* indicate the root tips. **d** The primary root length was measured 6 days after transfer. Three independent experiments were performed using 20 plants in each experiment. *Error bars*, SD ($n = 3$). *Horizontal white bars*, 1 cm. Statistical analysis was performed between EV and *AtHSP17.8:HA OXs*. The *asterisks* indicate a significant difference by Student's *t* test ($^*P < 0.01$). *EV* empty vector control, *OX AtHSP17.8:HA* overexpression

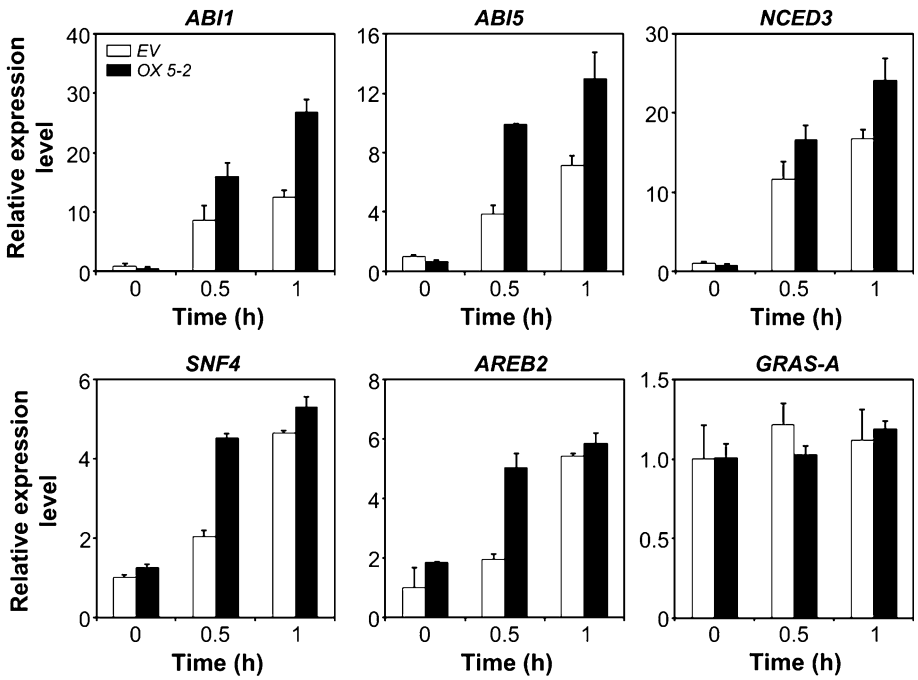


**Fig. 5** Ectopic expression of *AtHSP17.8:HA* in lettuce plants causes more rapid induction of ABA-responsive genes. Transgenic lettuce plants harboring *AtHSP17.8:HA or* the empty vector were treated with 20 μM ABA for 0.5 and 1 h, and total RNA from these plants were analyzed by qRT-PCR for the expression of ABA-inducible genes in lettuces. Lettuce *UBQ* was used as the internal control for qRT-PCR. *Error bars* SD ($n = 3$)

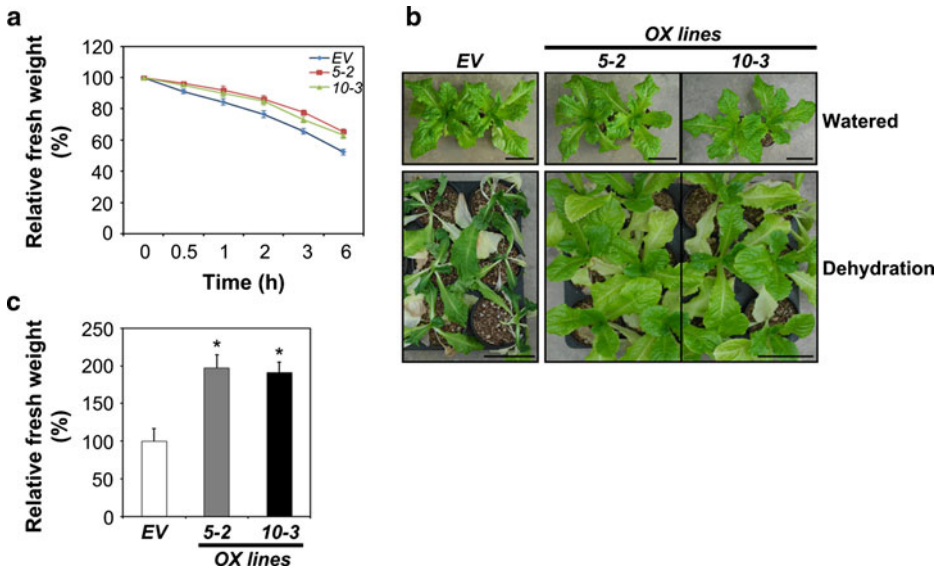


**Fig. 6** Transgenic lettuce plants expressing *AtHSP17.8:HA* show enhanced resistance to dehydration stress. **a** A reduced water loss rate in lettuce *AtHSP17.8:HA OX* plants. The aerial part of two independent lines of lettuce *AtHSP17.8:HA OX* and control EV plants grown on soil for 5 weeks were excised and left on bench for 6 h. To measure the water loss rate, the fresh weight of the excised plant tissues was measured at the indicated time points. Three independent experiments were performed using 20 plants in each experiment. *Error bars*, SD ($n = 3$). **b**, **c** Enhanced dehydration resistance of transgenic lettuce plants harboring *AtHSP17.8:HA*. **b** Two independent lines of lettuce *AtHSP17.8:HA OX* and control EV plants grown for 3 weeks on soil were kept in the greenhouse without watering for 4 weeks (drought). Images were taken 4 weeks after without watering. The control plants were kept at the normal watering conditions. *Bars* 10 cm. **c** The fresh weight represents the average value of three independent experiments with 30 plants in each experiment. *Error bar*, SD ($n = 3$). Statistical analysis was performed by Student's *t* test between EV and *AtHSP17.8:HA OX* plants. The *asterisks* indicate a significant difference by Student's *t* test ($*P < 0.01$). *EV* empty vector control, *OX AtHSP17.8:HA* overexpression

significantly less water loss than EV plants (Fig. 6a). To further support this observation *in planta*, we examined *AtHSP17.8:HA OX* lettuce and control EV plants for their dehydration stress resistance. The plants grown for 3 weeks under normal growth conditions were kept without watering for 4 weeks and their fresh weights were measured. The fresh weight of *AtHSP17.8:HA OX* lettuce plants was approximately 90 % higher than EV lettuce plants (Fig. 6b and c), indicating that *AtHSP17.8:HA OX* lettuce plants were more resistant to dehydration stress than EV plants.

Next, we examined the behavior of *AtHSP17.8:HA OX* lettuce plants under high salinity stress. The germination rate of these plants was observed in the presence of 100 or 150 mM NaCl. *AtHSP17.8:HA OX* lettuce plants showed much lower germination rates than the control EV lettuce plants in the presence of 100 or 150 mM NaCl (Fig. 7a and b), indicating that *AtHSP17.8:HA OX* lettuce plants are hypersensitive to high NaCl conditions. Next, we examined the sensitivity of these plants to high NaCl conditions during post-germination growth. *AtHSP17.8:HA OX* and EV lettuce plants grown for 5 weeks at normal growth conditions were treated with 500 mM NaCl for 10 days and their fresh weight was measured. Intriguingly, these plants showed enhanced resistance to high NaCl conditions (Fig. 7c and d). These results suggest that *AtHSP17.8* positively modulates salt stress response in lettuce plants.

## Discussion

In this study, we provide evidence that *AtHSP17.8* plays an important role in the osmotic stress response by activation of ABA-mediated signaling in both *Arabidopsis* and lettuce, a heterologous system. Previous reports have described a positive role of sHSPs in various abiotic stress responses. However, the exact mechanism of how these proteins are involved in the abiotic stress responses has remained elusive (Sun et al. 2002). In this study, we found that ectopic expression of *AtHSP17.8* in *Arabidopsis* and lettuce plants led to hypersensitivity to exogenous ABA at both germination and post-germination growth stages. Consistent with this observation, these transgenic plants also showed enhanced resistance to dehydration and high salinity stresses. These results raised the possibility that AtHSP17.8 plays a role in osmotic stress responses via ABA-mediated signaling.

The molecular mechanism underlying ABA hypersensitivity and the enhanced resistance of *Arabidopsis* and lettuce transgenic plants harboring *AtHSP17.8:HA* was

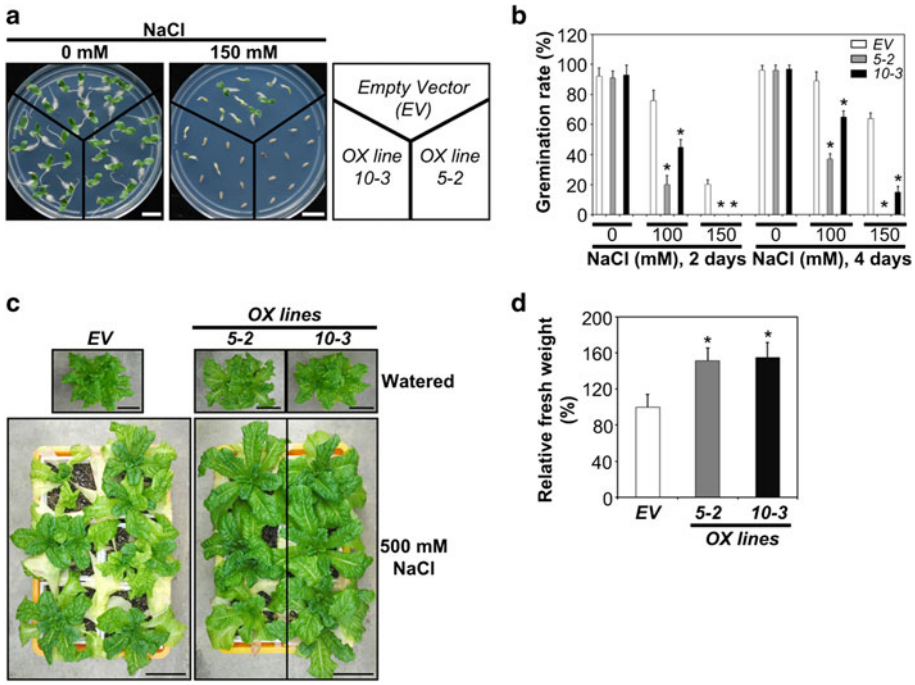


**Fig. 7** Transgenic lettuce plants expressing *AtHSP17.8:HA* shows enhanced resistance to salt stress. **a**, **b** Enhanced sensitivity of lettuce *AtHSP17.8:HA OX* plants to salt stress at the germination stage. Two independent lines of lettuce *AtHSP17.8:HA OX* and control EV seeds were planted on 1/2 MS plates supplemented with or without NaCl (100 or 150 mM) and the germination rates were measured. **a** Plant images were obtained 6 days after planting. **b** The germination rates were scored 2 or 4 days after planting. Three independent experiments were performed with 50 seeds in each experiment. *Error bars*, SD ($n = 3$). **c**, **d** Enhanced resistance of lettuce *AtHSP17.8:HA OX* plants to salt stress at the post-germination growth stage of development. **c** Two independent lines of lettuce *AtHSP17.8:HA OX* and control EV plants grown for 5 weeks on soil were treated with water (control) or 500 mM NaCl (salt stress) for 10 days. Images were taken 10 days after salt stress treatment. The control plants were kept at the normal growth condition for 10 days. *Bars* 10 cm. **d** To determine the salt stress-sensitivity of transgenic plants, the fresh weight of lettuce *AtHSP17.8:HA OX* and EV plants was measured. Three independent experiments were performed with 10 plants in each experiment. *Error bar*, SD ($n = 3$). Statistical analysis was performed between EV and *AtHSP17.8:HA OX* plants. The *asterisks* indicate a significant difference by Student's *t* test ($^*P < 0.01$). *EV* empty vector control, *OX AtHSP17.8:HA* overexpression

addressed by studying the expression of *AtHSP17.8* and representative ABA-responsive genes. The expression of *AtHSP17.8* was strongly induced in *Arabidopsis* by various abiotic stresses as well as exogenous ABA. Moreover, application of exogenous ABA causes more rapid induction of ABA-responsive genes in *AtHSP17.8 OX* lettuce plants. These results raised the possibility that AtHSP17.8 acts as a positive regulator in ABA-mediated responses. However, it is not clear how AtHSP17.8 acts as a positive regulator in ABA-mediated signaling.

In a previous study, AtHSP17.8 functions as a cofactor of AKR2A/B which is a cytosolic factor involved in targeting of membrane proteins to the chloroplast outer membrane (Kim et al. 2011). However, the physiological role of AtHSP17.8 in osmotic stress responses must be different from that in protein targeting to the chloroplast outer membrane. AtHSP17.8 is one of the sHSPs which is involved in a variety of cellular processes. In fact, one of most well-characterized activities of sHSP is the chaperone activity necessary for protein folding and assembly, and protection of proteins from denaturation (Sun et al. 2001, 2002). Thus, one possible scenario is that AtHSP17.8 protects a positive regulator(s) of ABA-mediated signaling from denaturation under dehydration stress or high salinity conditions. Consistent with this notion is that high osmotic stresses cause protein denaturation via an increase in salt concentration (Verslues and Juenger 2011). Together with its role in the targeting of proteins to the chloroplast outer envelope membrane (Kim et al. 2011), this result suggests an intriguing possibility for the role of AtHSP17.8 in plants: AtHSP17.8 is involved in at least two different cellular processes depending on the cellular conditions. Protein targeting to chloroplasts should occur under the normal growth conditions. Indeed, this gene is expressed at the normal growth condition (Kim et al. 2011). Thus, at the normal growth condition, AtHSP17.8 is involved in the protein targeting of membrane proteins to chloroplast outer membranes. By contrast, under osmotic stress conditions, AtHSP17.8 may play a role in protection of proteins from high salinity-induced denaturation. Again, a role for

AtHSP17.8 in osmotic stress is supported by its induction by high osmotic stress conditions or exogenous ABA in *Arabidopsis*. Another important feature of these sHSPs that may be crucial in determining their exact biological activity is that sHSPs can exist in multiple forms ranging from monomer to a large complex depending on cellular conditions. Indeed, AtHSP17.8 exists primarily as a dimer when it is involved in protein targeting to chloroplasts (Kim et al. 2011). By contrast, it is possible that AtHSP17.8 may exist as a higher molecular form when it is involved in osmotic stress responses. In general, sHSPs assemble into a high molecular weight form when they are involved in protein folding or protection of proteins from denaturation. In the future, additional work will be necessary to elucidate the detailed mechanism of how AtHSP17.8 acts as a positive regulator of ABA-mediated signaling.

In this study, we attempted to translate the finding on AtHSP17.8 in *Arabidopsis* into crop plants. Lettuce has a merit as a model vegetable because of its high transformation efficiency, which is a technically important feature in the genetic engineering of crop plants. Intriguingly, the physiological and molecular regulation of ABA and dehydration stress responses that resulted from overexpression of *AtHSP17.8* in *Arabidopsis* were recapitulated in transgenic lettuce plants, albeit to a slightly different extent. This finding, therefore, strongly suggests that the molecular machinery for its action in ABA-mediated signaling involved in osmotic stress responses is highly conserved in both *Arabidopsis* and lettuce and that knowledge and experiences obtained with *Arabidopsis* can be successfully translated into lettuce plants.

## Materials and methods

### Measurement of seed germination rates

To determine the germination rate, sterilized seeds were planted on 1/2 MS plate containing 1 % sucrose, 0.8 % agar, and varying concentrations of ABA or NaCl. The germination rate was scored if green cotyledons emerged. To measure post-germination growth, 4-day-old seedlings grown on 1/2 MS plate were transferred onto 1/2 MS plates supplemented with 8 μM (*Arabidopsis*) or 12 μM (lettuce) ABA. Primary root growth was measured 6 days after transplantation. For statistical analysis, at least three independent experiments were performed. *P* values were calculated using the Student's *t* test. For ABA-mediated induction of the gene expression, plants grown in MS liquid medium for 2 weeks were treated with 5 μM ABA for *Arabidopsis* and 20 μM ABA for lettuce (Xu et al. 2012).

### Plasmid DNA construction

Isolation of *AtHSP17.8* (At1g07400) and construction of *AtHSP17.8:HA* were described previously (Kim et al. 2011). To generate the *AtHSP17.8* binary construct, the *AtHSP17.8:HA* construct was digested with *Pst*I and *Eco*RI and the resulting fragment containing both the *CaMV 35S* promoter and *AtHSP17.8:HA* was ligated into *pCAMBIA3300* digested with *Pst*I and *Eco*RI.

### Generation of transgenic lettuce and *Arabidopsis* plants by *Agrobacterium*-mediated transformation

Transgenic lettuce was generated using cotyledons by the *Agrobacterium*-mediated transformation method (Park et al. 2008; Rosales–Mendoza et al. 2010). Seeds of lettuce cultivar Green Wave (Daenong Seed, Korea) were sterilized in 2 % sodium hypochlorite for 5 min and washed five times with distilled water and planted on a MS plate containing 3 % sucrose and 0.2 % phytogel (Sigma, USA) at 25 °C for 5 days under a 16/8 h light/dark cycle. Cotyledons were excised and inoculated with *A. tumefaciens* harboring *pCAMBIA3300-AtHSP17.8:HA*. The explants were dried by blotting onto a sterilized filter paper and then were transferred onto plates containing 4.43 g/L MS salts supplemented with vitamin, 30 g/L sucrose, 0.05 mg/L naphthaleneacetic acid (NAA), 0.25 mg/L kinetin, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 2 g/L phytogel, pH 7.5, at 25 °C for 2 days in the dark. After incubation, the cotyledon explants were washed with sterile deionized water and transferred to MS selection plates supplemented with 30 g/L sucrose, 0.5 mg/L kinetin, 25 mg/L DL-phosphinothricin (Duchefa, The Netherlands) and 300 mg/L cefotaxime for 4–6 weeks to induce shoots under a 16/8 h light/dark cycles. Subsequently, the regenerated shoots were excised and transferred to rooting medium (4.43 g/L MS salts supplemented with vitamin, 30 g/L sucrose, 25 mg/L DL-phosphinothricin and 100 mg/L cefotaxime). Plantlets with well-developed roots were transferred to the greenhouse. After self-pollination, T1 seeds were harvested and stored at 4 °C.

Transgenic *Arabidopsis* plants were generated by the floral dipping method (Clough and Bent 1998). Transgenic plants were screened on MS plates supplemented with 25 mg/L DL-phosphinothricin and 100 mg/L cefotaxime (Xu et al. 2012).

### SDS-PAGE and Western blot analysis

Total proteins were extracted from leaf tissues of transgenic *Arabidopsis* and lettuce plants using extraction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 % Triton X-100, protease inhibitor

cocktail [Roche, Werk Penzberg, Germany]). The plant extracts were centrifuged at 13,000 × *g* at 4 °C for 15 min to remove cell debris. The amount of proteins was measured by the Bradford protein assay (Bio-Rad, Hercule, CA, USA). Proteins (40 μg of total proteins) were separated by 12 % SDS-PAGE after boiling in the sample loading buffer and the gel was transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, USA) for Western blot analysis. For Western blot analysis, the membranes were treated with 6 % non-fat dry milk in TTBS buffer (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 % [v/v] Tween-20) for 30 min to prevent non-specific binding of antibodies and incubated with a 1:1,000 dilution of rat anti-HA antibody (Roche, Diagnostics, IN, USA) for 3 h followed by three times of washing with TTBS buffer. Subsequently, the membranes were incubated with 1:5,000 dilution of a horseradish peroxidase-conjugated anti-rat IgG (Amersham Biosciences, UK) in TTBS buffer containing 6 % non-fat dry milk for 3 h followed by three washings with TTBS. The blots were developed with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and images were obtained using the LAS3000 image-capture system (FujiFilm).

## RNA extraction, semi- and real-time quantitative RT-PCR analyses

Total RNA was isolated from whole plant tissues using an RNeasy Plant Mini Kit (Qiagen, USA) followed by the TURBO DNase (Ambion, Austin, TX, USA) treatment for 30 min (Xu et al. 2012). Total RNA (2 μg) was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). qRT-PCR was performed by a real-time PCR system (StepOne; Applied Biosystems, USA) using a SYBR Green Kit (Applied Biosystems, USA). *UBQ* (lettuce) or *ACT2* (*Arabidopsis*) was used as an internal control for qRT-PCR (Argyris et al. 2008; Huo et al. 2013; Kim et al. 2011; Xu et al. 2012). The primers used were *ABI1-F* (5′-ACCG TGGATAATACCAGTACCC-3′) and *ABI1-R* (5′-CTCA CCCACCTCGGTGTT-3′) for *ABI1*; *ABI5-F* (5′-CGGAC GGAAGCGGATAAT-3′) and *ABI5-R* (5′-TTCTAGCT CGAGACCTTGCAG-3′) for *ABI5*; *NCED3-F* (5′-AGCTC AGCTTGGTTCCCTGTTATA-3′) and *NCED3-R* (5′-CTTCACAAACTGGCTGAAAACGTAT-3′) for *NCED3*; *SNF4-F* (5′-GGATTGTATGGAGGTGTTTAGCAAA-3′) and *SNF4-R* (5′-CCGGATACGTTTTCCATGGA-3′) for *SNF4*; *AREB2-F* (5′-TCCATTTGATTTGGGGATTC-3′) and *AREB2-R* (5′-TGATTGAGTCTCTTCCGCAGT-3′) for *AREB2*, *GRAS-A-F* (5′-CCAATCAAGCCATCCTC-GAA-3′) and *GRAS-A-R* (5′-ATCCCTTGGTTCAAGCT-GAAATC-3′) for *GRAS*-A; *RD29A-F* (5′-GATATC GACAAGGATGTGCCG-3′) and *RD29A-R* (5′-GTATC CAGGTCTTCCCTTCGC-3′) for *RD29A*, *HSFA3-F* (5′-GGAGGAGCTCTTGGATTGGAA-3′) and *HSFA3-R* (5′-TGCTGGTGGTGCTTGATGA-3′) for *HSFA3*, *AtHS P17.8-F* (5′-CCAAGCTTCTTCGGCAACA-3′) and *AtHS P17.8-R* (5′-GAGTGAGAACGGGTCGAAGATG-3′) for *AtHSP17.8*; *UBQ-F* (5′-TCTTAGATCACCGTCCCATC GT-3′) and *UBQ-R* (5′-TCTGAGATTGTCCGAGGA TATGAG-3′) for *UBQ*, and *ACT2-F* (5′-TAT GAATT ACCCGATGGGCAAG-3′) and *ACT2-R* (5′-TGGAACA AGACTTCTGGGCAT-3′) for *ACT2*.

Semi-quantitative RT-PCR was performed using ExTaq polymerase (Takara). The primer sets used were as follows: *AtHSP17.8 RT-F* (5′-GTCGTCGCTCTCCGGAGAAACA-3′) and *AtHSP17.8 RT-R* (5′-AGCGTAATCTGGAAC ATCGTATGG-3′) for *AtHSP17.8*; *18S rRNA RT-F* (5′-AT GATAACTCGACGGATCGC-3′) and *18 s RNA RT-R* (5′-CCTCCAATGGATCCTCGTTA-3′) for *Arabidopsis 18S rRNA*, and *18S1 rRNA RT-F* (5′-GCATGAGTGGTGTTT GGTTTGT-3′) and *18S1 rRNA RT-R* (5′-CCAGGTAG CATTCCTCTTCGACT-3′) for lettuce *18Ss1 rRNA*.

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