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A Subset of Arabidopsis RAV Transcription Factors Modulates Drought and Salt Stress Responses Independent of ABA

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Arabidopsis RAV1, RAV1L and RAV2/TEM2 are Related to ABI3/VP1 (RAV) transcription factors that contain both plant-specific B3 and AP2 domains. RAV1 was known to be a negative regulator of growth and its transcript level was repressed by brassinolide (BL). In this study, we found that the expressions of *RAV1*, and its closest homologs *RAV1L* and *RAV2* were also regulated by other plant hormones, and especially repressed significantly by BL and abscisic acid (ABA), which mediate various abiotic stress responses in plants. Therefore, to further investigate the physiological functions of RAV1, RAV1L and RAV2 in abiotic stress responses, we isolated T-DNA insertional knockout mutants of each gene and produced transgenic plants overexpressing the *RAVs*. Under normal conditions, each single mutant showed slightly promoted growth patterns only at an early stage of development. In comparison, the *RAV1*-overexpressing plants exhibited strong growth retardation with semi-dwarfed stature. In drought conditions, *RAVs*-overexpressing transgenic plants exhibited higher transpirational water loss than the wild type. In salt conditions, seed germination of the *RAVs*-overexpressing transgenic plants was more inhibited than that of the wild type, while *ravs* mutants showed promoted seed germination. We also found that *RAVs* expressions were reduced by dryness and salt. *RAV1*-overexpressing plants showed the same patterns of increased expression as stress-inducible genes such as *RD29A*, *RD29B* and the genes encoding ABA biosynthetic enzymes, as did the wild type and *rav1* mutant. However, the *RAV1*-overexpressing transgenic plants were insensitive to ABA, regardless of the higher accumulation of ABA even in normal conditions. Taken together, these results suggest that RAVs are versatile negative regulators for growth and abiotic stresses, drought and salt, and that negative regulatory effects of RAVs on abiotic stresses are likely to be operated independently of ABA.

Keywords: ABA • Drought • Growth • RAV transcription factor • Salt • Seed germination.

Abbreviations: ABA, abscisic acid; ACC, 1-amino cyclopropane-1-carboxylic acid; BL, brassinolide; BR, brassinosteroid; GA, gibberellic acid; GUS, β-glucuronidase; IAA, indole-3-acetic acid; MS, Murashige and Skoog; NCED9, nine-*cis*-epoxycarotenoid dioxygenase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RAV, Related to ABI3/VP1; TEM, TEMPRANILLO.

Introduction

The Arabidopsis genome contains several families of transcription factors, such as MADS, bHLH, MYB and AP2/ERF (Riechmann and Ratcliffe 2000, Bailey et al. 2003, Dietz et al. 2010). These transcription factors are also found outside the plant kingdom (Magnani et al. 2004). In comparison, other classes of transcription factors, such as GRAS, NAC and B3 families, are plant specific (Riechmann et al. 2000).

Plant-specific B3 transcription factors encoded by 118 genes in Arabidopsis can be classified into four subfamilies: ARF (AUXIN RESPONSE FACTOR), LAV (LEAFY COTYLEDON 2 [LEC2]-ABSCISIC ACID INSENSITIVE 3 [ABI3]-VAL), REM (REPRODUCTIVE MERISTEM) and RAV (Related to ABI3/VP1) (Swaminathan et al. 2008). The Arabidopsis RAV subfamily consisting of 13 members was initially categorized as B3 transcription factors, as RAV1 and RAV2 were identified as homologs to maize VP1. The B3 domain of VP1 has a sequence-specific DNA-binding activity (Suzuki et al. 1997). RAV1 was first identified as a novel DNA-binding protein in Arabidopsis, possessing both an N-terminal AP2-domain and a C-terminal B3-domain, which specifically bind to bipartite sequence motifs containing consensus sequence elements, CAAC A and CACCTG, respectively (Kagaya et al. 1999). Among the 13 members, six members of the RAV subfamily contain the AP2 domain, a DNA-binding domain, as well. Therefore, they are also grouped into the AP2/ERF transcription factors. So far, 147 genes have been annotated to encode AP2/ERF (APETALA2/ethylene-response factor) in Arabidopsis (Pérez-Rodríguez et al. 2010). Most of the individual transcription factors belonging to the AP2 and ERF/DREBP subfamily are mainly involved in ABA- and ethylene-related responses (Zhu et al. 2010).

Other members of the RAV subfamily containing only the B3 domain are also called the NGA transcription factor subfamily (Swaminathan et al. 2008). The *Nga1* mutant was found in the suppressor screening of *kanadi1*, implicating that NGA1

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is involved in the control of leaf polarity (Bowman et al. 2002). A quadruple mutant between *NGA1* and three other homologs (*NGA2-4*) completely failed in style and stigma development (Alvarez et al. 2009, Trigueros et al. 2009). And overexpression of *NGA1* in flower induced ectopic expression of *STYLISH1*, an auxin biosynthetic inducer (Alvarez et al. 2009). In contrast, the expressions of the genes encoding auxin biosynthetic enzymes, such as *YUCCA2* and *YUCCA4*, were reduced in the transgenic line where an artificial miRNA targeting the four *NGA* genes was expressed (Trigueros et al. 2009).

Compared with the NGA transcription factors, six members of the RAV subfamily, containing both the B3 and AP2 domains, such as RAV1, RAV1L (RAV1-Like), RAV2/TEM2 (TEMPRANILLO 2), TEM1 and the proteins encoded by genes *At1g50680* and *At1g51120*, respectively, are not clearly designated and their molecular functions have not been understood well. Only a few functional analyses have been performed on RAV1, TEM1 and TEM2/RAV2. A transgenic plant, in which *RAV1* was constitutively overexpressed, was reported to have reduced lateral roots and rosette leaf, indicating that RAV1 acts as a negative regulator for plant development (Hu et al. 2004). The TEM transcription factors encoded by the *TEM1* and *TEM2/RAV2* genes were involved in the controlling of flowering time under photoperiodic induction. Using the constitutive overexpression of *TEM1* or *TEM2/RAV2* and *RNAi-tem* plants, it was shown that the quantitative balance between CONSTANCE (CO) and TEM determines *FLOWERING LOCUS T* (*FT*) expression. TEM1 has been reported to directly repress *FT* expression (Castillejo and Pelaz 2008). Recently, TEM proteins were reported to also repress the expression of GA_4 biosynthetic genes, resulting in growth-retarded phenotypes of the transgenic plants overexpressing the genes (Osnato et al. 2012). In addition, overexpression of *CaRAV1* enhanced resistance to biotic pathogens, including *Pseudomonas syringae* pv. tomato DC3000, and biotropic oomycete *Hyaloperonospora arabidopsidis* (Lee et al. 2010). Recently, transgenic tomato plant containing tomato *RAV2* (*SlRAV2*) was reported to have higher tolerance to *Ralstonia slonaceaerum*, which causes bacterial wilting disease, through the activation of PR genes (Li et al. 2011).

Plants, being sessile, experience various environmental stresses in their particular location over the course of their lifetime. Therefore, it is necessary for them to develop a sensitive way to perceive and respond to each stress (Zhu 2002, Shilipa and Narendra 2005). Abiotic stresses, such as drought and high salinity, trigger the production of ABA, of which the increased levels subsequently not only boost an activation of its own biosynthesis with a positive feedback loop, but also lead to the activation of the expression of a wide range of stress-inducible genes (Iuchi et al. 2000, Xiong et al. 2001, Xiong et al. 2002). More than half of the drought-inducible genes were reported to overlap with the genes that are activated by salt stress and ABA (Seki et al. 2001, 2002). Low temperature stresses also induce various gene sets that seem to be fairly specific, because only 10% of the genes induced by cold were also induced by drought. And moreover, many genes induced by cold contain the DRE (Dehydration-Responsive Element)/CRT (C-RepeaT) elements in their promoter, which are responsible for ABA-independent gene expressions (Yamaguchi-Shinozaki and Shinozaki 1994, 2005). Therefore, plants are likely to adopt both ABA-dependent and ABA-independent gene expression systems to increase the coverage for broad spectrums of stresses.

Here, we analyzed the physiological functions of Arabidopsis RAV1, RAV1L and RAV2, which show the closest homology to each other among the six members of the RAV subfamily containing AP2 and B3 domains. From the observation that the expressions of these genes were significantly repressed by ABA, we investigated whether the physiological functions of RAVs are involved with ABA-mediated abiotic stress responses, focusing on drought and salt stresses. By analyzing the T-DNA knockout mutants of each gene and *RAVs*-overexpressing transgenic plants, we demonstrated that RAVs negatively regulate growth with developmental stage-specificity. We also found that RAVs play negative roles in drought and salt tolerance in Arabidopsis in an ABA-independent manner.

Results

Expressions of a subset of RAV transcription factors are changed by plant hormones

From the sequence analyses, we found that *RAV1L* and *RAV2* are the closest homologs of *RAV1* among six genes that contain both the AP2 and B3 domains. The predicted protein products of *RAV1L* and *RAV2* share 68% and 60% identities to RAV1 protein, respectively (**Supplementary Fig. S1**). Expressional changes of the *RAV1* and *RAV1L* genes by various plant hormones have been studied in many separate experiments. *RAV1* was reported to be repressed by 1 μM of 24-epibrassinolide (epiBL) (Hu et al. 2004). *RAV1* was also down-regulated by 1 μM zeatin (Hu et al. 2004) and *RAV1L* showed reduced expression when treated with 30 μM ABA (Vogel et al. 2012). As these results were obtained from tissues at different developmental stages and with different durations of hormone treatment, it is hard to integrate the effects of each plant hormone on the expression of these genes. Therefore, it is necessary to examine the expressions of *RAV1* and its homologs *RAV1L* and *RAV2* at the same time under controlled conditions to remove the biases from different experimental systems. We performed quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses using the RNAs isolated from various hormone-treated Arabidopsis seedlings for different times. Treatments for 30 min, 1, 3, and 6 h were to monitor the early responses, and treatment for 24 h was to examine the late responses to each hormone. We observed that in most cases, the expressions of the three genes were down-regulated at the beginning of hormone treatment, and then increased until 6 h within less than two-fold ranges (**Fig. 1A** and **Supplementary Fig. S2**). Only the *RAV1L* expression was dramatically increased by indole-3-acetic acid (IAA). Even brassinolide (BL) treatment did not lead to a greatly reduced expression pattern within this duration. Compared with the

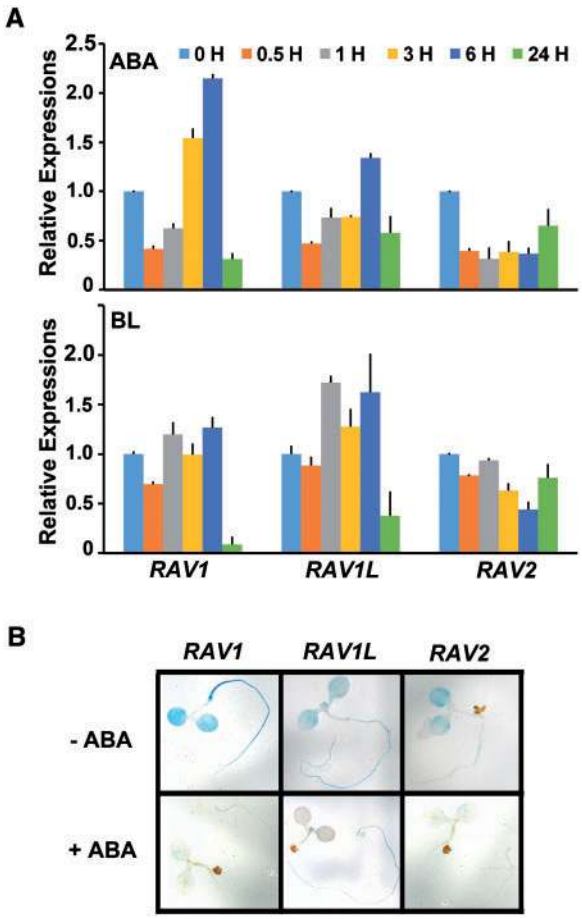

Fig. 1 Expressions of *RAV1*, *RAV1L* and *RAV2* are changed in response to ABA and BL. (**A**) Relative expressions of *RAV1*, *RAV1L* and *RAV2* in response to 20 μM of ABA, and 1 μM of BL treated for indicated hours compared with mock treatment. Quantitative RT-PCR was performed three times, and error bar denotes standard error. (**B**) *RAV1*, *RAV1L* and *RAV2* expressions are reduced by ABA determined by the *β-glucuronidase* (*GUS*)-reporter gene analyses.

early responses to hormones, when plant hormones were treated 24 hours, the effects of gibberellic acid (GA), IAA and kinetin on the expression of *RAVs* were different in each gene (**Supplementary Fig. S2**). GA resulted in the increased expression of *RAV1L* and *RAV2* after 24 h of treatment. *RAV1L* expression was increased from 1 h of treatment and remained at a higher level till 24 h when treated with kinetin. In contrast, ABA and BL led to the reduction of all three genes, notably causing a more than two-fold reduction in *RAV1* expression (**Fig. 1A**).

Regarding the ABA-induced repression of *RAVs* expression, we further confirmed this phenomenon in intact tissues. We generated transgenic plants containing β-glucuronidase (*GUS*)-reporter gene transcriptionally fused with *RAV1*, *RAV1L* or *RAV2* promoters and performed histochemical *GUS* expression analyses in each seedling. We observed *RAV1*, *RAV1L* or *RAV2* promoter-driven GUS activities in cotyledon leaves, the junction of root and hypocotyl, and vasculature of the roots of seedlings. Treatment with ABA for 24 h dramatically reduced the expression of GUS activity in tissues overall (**Fig. 1B**).

RAVs negatively regulate normal growth in Arabidopsis

To investigate the cellular functions of RAV1 and its two homologs, RAV1L and RAV2, we searched the corresponding T-DNA insertional knockout mutant lines of each gene and obtained seeds from the Arabidopsis Biological Resource Center (ABRC). When we performed genotyping from each mutant line to confirm the insertion of T-DNA and its homozygosity, we found that *rav1l* has an additional T-DNA insertion, around 200 base pairs distant from the annotated T-DNA site in the T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress) (**Supplementary Fig. S3A**). The null expression of each transcript was confirmed by RT-PCR analyses. Additionally, we examined whether the expression of any other *RAV* genes was induced by the knockout of each *RAV*. We did not detect compensational expressions of other *RAV* genes in each *rav* mutant compared with those of the wild type (**Supplementary Fig. S3B**). When grown in normal growth conditions, *rav1*, *rav1l* and *rav2* mutants did not show any morphological defects, or distinctive phenotypic changes. However, fine quantitative analyses revealed that the overall growth of *rav* mutants was slightly promoted compared with that of wild type plants, especially in the early stage of development. The petioles and leaves of mutants were quantitatively longer than those of the wild type, leading to greater rosette diameter of *rav* mutants grown for 3 weeks. However, these differences were diminished at the later stage of growth. All the growth criteria of the *rav* mutants ended up the same as those of wild type after 6 weeks of growth (**Fig. 2A**).

To further examine the role of RAVs in plant growth, we also produced transgenic plants constitutively overexpressing *RAV1*, *RAV1L* and *RAV2* under the cauliflower mosaic virus (CaMV) *35S* promoter and observed their phenotypic alterations. We confirmed the degree of overexpression from two representative transgenic lines of each *RAV* gene by qRT-PCR analyses (**Fig. 2B** left) and observed their phenotypic alterations (**Fig. 2B** right). Only the *RAV1*-overexpressing transgenic plants (*35S-RAV1OE*) showed strong growth inhibition accompanied by early senescence, while the transgenic plants overexpressing *RAV1L* (*35S-RAV1LOE*) or *RAV2* (*35S-RAV2OE*) did not show any alterations in gross morphology and growth rate compared with those of the wild type (**Fig. 2B**). To check whether the growth-retarded phenotypes of *RAV1*-overexpressing plants were caused by an ectopic overexpression due to a constitutive promoter in their constructs or not, we additionally generated *RAV1*-overexpressing transgenic plants under a native promoter of the *RAV1* gene (*RAV1-RAV1OE*). These transgenic plants also displayed growth inhibition phenotypes. At the later stage of development, the *RAV1-RAV1OE* transgenic plants were semi-dwarf and bushy due to loss of the apical dominance compared with the wild type plant, suggesting that RAV1 indeed acts as a negative regulator of growth (**Supplementary Fig. S4**). Taken together with the promoted growth in *rav* mutants, although the growth promotion was shown only in the early developmental

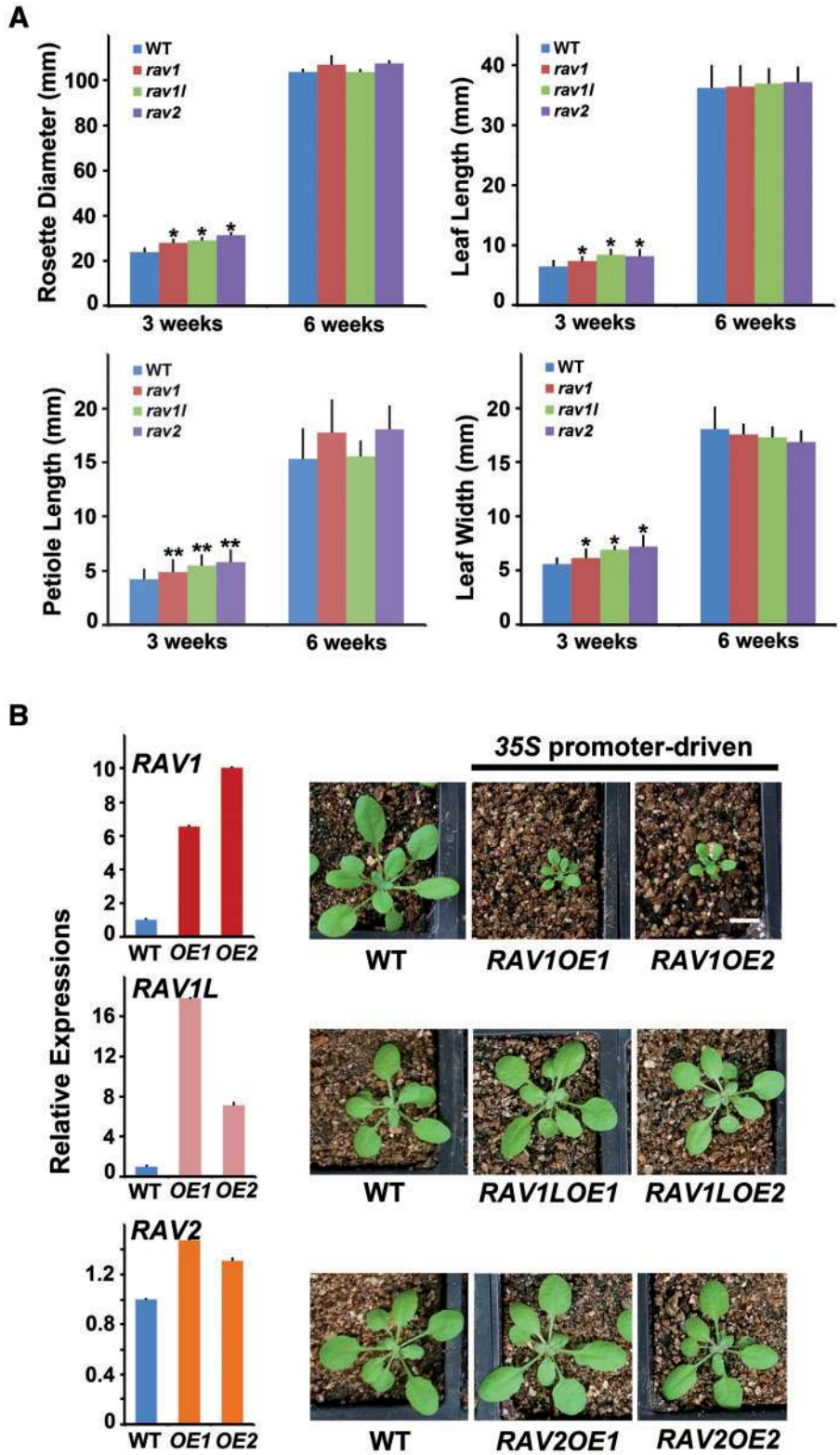

Fig. 2 RAVs negatively affect growth. (**A**) Growth in the early developmental stage is promoted by the lack of *RAV1*, *RAV1L* or *RAV2*. The rosette diameter ($n = 5$ individual plants), leaf length, petiole length and leaf width ($n = 30$ each, two longest leaves in each 15 plants) were measured from the T-DNA knockout mutants of *RAV1*, *RAV1L* and *RAV2* and the wild type plants, grown for 3 weeks or 6 weeks. This experiment was repeated three times with the whole plant set. Error bar denotes standard error (* $P \leq 0.0001$, ** $P = 0.0003$ compared with wild type). (**B**) Overexpression of *RAV1* shows negative effect of *RAV1* on growth. Two independent transgenic plants in each line overexpressing the CaMV 35 S-promoter-driven *RAV1*, *RAV1L* or *RAV2* were selected, and the degree of overexpression of each gene from the transgenic plants was confirmed by qRT-PCR analyses (left). The plant phenotypes compared with that of the wild type plant grown for 3 weeks under normal conditions were shown. White bar denotes 2 cm.

stage, these results suggest that a subset of RAVs, RAV1 and RAV1L and RAV2 regulates growth negatively stage-specifically. RAV1 may play a major role in growth repression.

RAVs negatively regulate drought and salt tolerance in Arabidopsis

In this study, as the expression of *RAV1*, *RAV1L* and *RAV2* were greatly reduced by ABA treatment for 24 h (**Fig. 1**), we further investigated their possible function in response to abiotic stresses that are known to be involved with ABA signaling (Osakabe et al. 2013) using *rav* mutants and *RAV*-overexpressing transgenic plants.

First, we examined the transpirational water loss exerted by a drought stress. To perform this, whole aerial shoot parts were cut from the 4-week-old soil-grown plants, and placed on a filter paper in an ambient temperature. Then, we weighed them to monitor water loss over time. As time went on, the weight of the plants was reduced due to loss of water in the plant tissues. The water loss of wild type and *rav1*, *rav1l* and *rav2* mutants showed similar patterns over the 3-h period. The water loss of the *35S-RAV1OE* and *35S-RAV2OE* transgenic plants showed a statistically considerable increase compared with that of the wild type plant after 2 h. The water loss rates of the *35 S-RAV1LOE* transgenic plants were much faster than those of the wild type plants, resulting in acceleration of wilt of the plants (**Fig. 3A**). Next, we examined whether the *RAVs* expressions were changed within this time period, and found that *RAVs* expressions were reduced from around 15–30 min after dryness. Reduction in *RAV2* expression was the greatest (**Fig. 3B**). These results showed that reduced expression of *RAV* genes rapidly occurred in response to dryness and that ectopic overexpression of *RAV1L* caused more water loss. We further examined whether long-term drought conditions would affect their responses, although the water loss of *rav* mutants by transpiration did not change for a short period. We grew the *rav* mutants and wild type plants in normal conditions for 4 weeks, and did not irrigate them for 9 days to expose them to drought conditions. Then, we re-watered them. On the seventh day after re-watering, we observed that more than 30% of the *rav1* and *rav1l* mutants and 12% of the *rav2* mutants recovered their growth, while less than 5% of the wild type plants resumed growth (**Fig. 3C**). Taken together, these results suggest that a subset of RAV transcription factors, here RAV1, RAV1L and RAV2, negatively acts on the tolerance to water deficient conditions, although there are certain degrees of differences.

We also investigated the effects of RAVs on plant development under salt-stressed conditions. As a salt-responsive physiological event, we examined the seed germination pattern on salt-containing media. In normal conditions, most of the wild type seeds usually completed germination in 2 days after imbibition (DAI). Mutants for *RAVs* or transgenic plants overexpressing *RAVs* showed similar germination patterns to the wild type, although they showed a little bit of fluctuation at 1 day after imbibition (**Supplementary Fig. S5**). When seeds were germinated on the salt-containing media, more than 40% of wild type seeds were not germinated at day 3, and even at day 4, germination did not reach 90%. In comparison with the wild type seeds, seed germination under salt was promoted 9% in *rav1*, 12% in *rav1l* and 3% in *rav2* mutants at 3 DAI. At 4 DAI, no difference in germination rate was observed in all *rav* mutants compared with the wild type plants. However, the *RAV1- and RAV1L*-overexpressing transgenic plants under the CaMV 35 S promoter showed considerable reduction of seed germination in response to salt compared with the wild type plants at both 3 DAI and 4 DAI. The *35 S-RAV2OE* plants also displayed reduced seed germination at 3 DAI at least (**Fig. 4A**). This result suggests that RAVs regulate salt-tolerance negatively, as they did drought stress. To further examine whether the expressions of *RAVs* are changed by salt in germination, we purified the RNAs from the seeds grown for 2 days in the absence or presence of salt and performed qRT-PCR analyses, and observed that *RAV1*, *RAV1L* and *RAV2* expressions were repressed by salt (**Fig. 4B**). These results suggest that RAVs are negative regulators for resistance to drought and salt stresses in Arabidopsis.

RAV1 inhibited germination under salt ABA-independently

We were particularly interested in the inhibited germination phenotype of the *RAV1*-and *RAV1L*-overexpressing transgenic plants in salt conditions, because it is widely known that, in response to environmental changes such as high salt, drought and elevated CO_2 concentration, ABA accumulation is one of the representative responses in plant cells, triggering ABA-mediated signaling to protect plants from possible damage (Wasilewskaa et al. 2008). It was reported that salt induces ABA accumulation and the resulting high level of ABA inhibits germination under salt conditions (Strizhov et al. 1997, Kucera et al. 2005). Therefore, we first examined the expression of *NCED9* gene encoding the nine-*cis*-epoxycarotenoid dioxygenase, a rate-limiting enzyme for ABA biosynthesis (Iuchi et al. 2001). We purified RNA from the seeds grown for 2 days in the absence or presence of salt and performed qRT-PCR analyses. The Wild type and *RAV1*-overexpressing plants showed similar basal expression levels of *NCED9* in the absence of salt. Endogenous *NCED9* expression was less in *rav1* mutant than in the wild type. Salt treatment induced the *NCED9* expression in all plants but to a much greater extent in *RAV1*-overexpressor (**Fig. 5A**). *NCED3* is also a key ABA biosynthetic gene and is known to be induced by drought (Iuchi et al. 2001, Tan et al. 2003). We also found that expression patterns of *NCED3* were similar to those of *NCED9* in the absence or presence of salt (**Supplementary Fig. S6**). These results suggest that salt-induced expressions of ABA biosynthetic genes were still operating in *RAV1*-overexpressing plants. To confirm whether the increased expressions of biosynthetic genes led to the higher levels of ABA in response to salt, we monitored the changes in ABA content in response to salt for the *rav1* mutant and *RAV1*-overexpressor compared with the wild type. The ABA level was increased in the wild type seeds grown for 2 days on

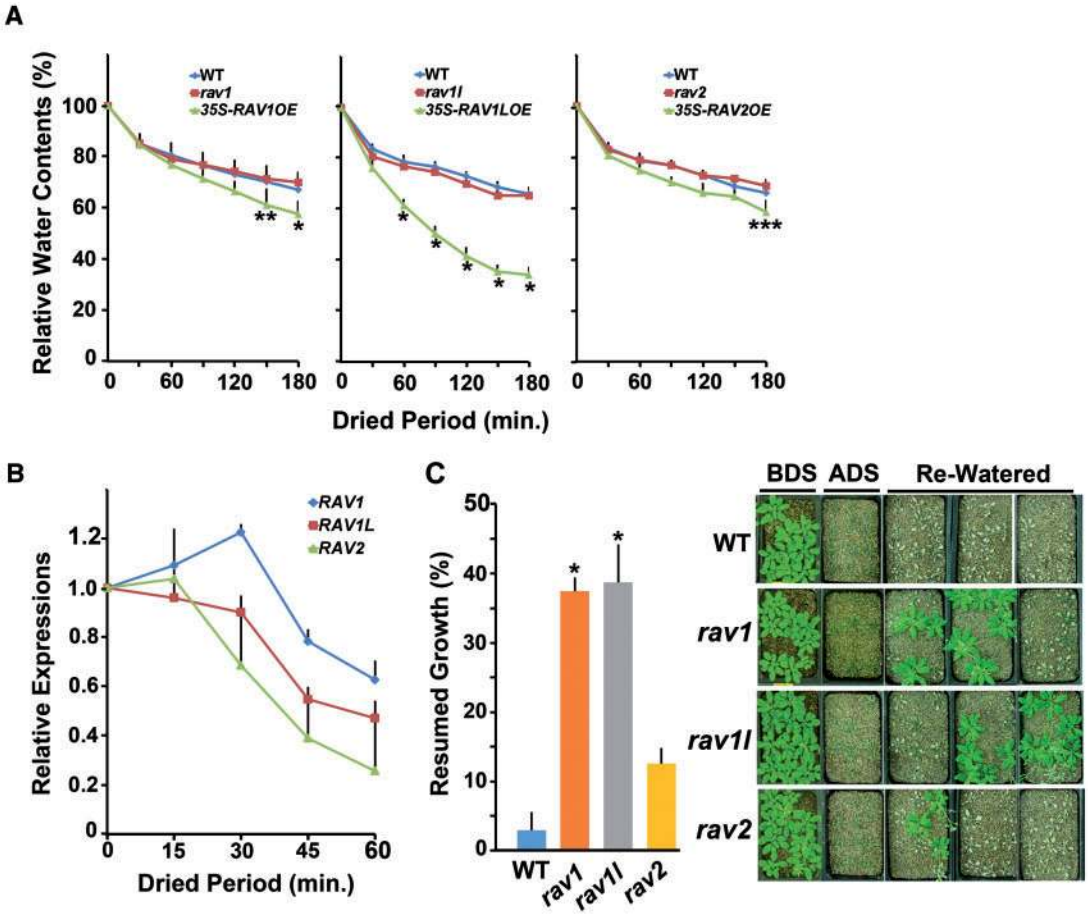

Fig. 3 RAVs play negative roles in drought resistance. (**A**) Transpirational water loss was measured from the *rav* mutants and *RAV*-overexpressing transgenic plants compared with that of the wild type (*$P \leq 0.0001$, **$P = 0.0004$, ***$P = 0.0009$ compared to the wild type). (**B**) Relative expressions of *RAV1*, *RAV1L* and *RAV2* in response to drought determined by qRT-PCR. Error bar denotes standard error. (**C**) Mutants of *rav* exhibited better resumption of growth under a long period of drought. (Left) Number of plants that recovered and resumed growth after drought stress were counted from three independent batches of experiments. Error bar denotes standard error (*$P \leq 0.001$ compared with wild type). (Right) Representative picture showing the plants re-watered. Four-week-grown plants before drought stress (BDS) were subjected to water deficiency after drought stress (ADS). Pictures of re-watered plants were taken after 7 d of re-watering.

salt-containing media compared with that of control seeds grown on normal media. A similar pattern of increased ABA contents in salt conditions was observed in the *rav1* mutant. However, interestingly, the *RAV1*-overexpressing transgenic plants contained much higher levels of ABA even in normal conditions than the wild type or the *rav1* mutant. And moreover, the degree of increase of ABA production in response to salt was not as much as shown in the wild type or *rav1* mutant (**Fig. 5B**). This result led us to check the expressional changes of *RD29A* and *RD29B*, two representative stress-inducible genes in *RAV1*-overexpressor compared with that of the wild type and *rav1* mutant. As the promoter region of *RD29A* gene contains several dehydration responsive elements (DREs), *RD29A* is known to be induced mainly ABA-independently. In comparison, *RD29B* is regulated mainly by ABA due to several ABA-responsive elements (ABREs) in its promoter (Yamaguchi-Shinozaki and Shinozaki 1994). We found that expression of both *RD29A* and *RD29B* was induced in all plant lines tested in response to salt, with a much greater increase in *RAV1*-overexpressing plants than in the wild type and *rav1* (**Fig. 5C**). As *RD29A* and *RD29B* are known to be induced by drought and salt as well as by ABA (Yamaguchi-Shinozaki et al. 1992), this increase of *RD29A* and *RD29B* expressions was likely due to both salt-stress-induced ABA and salt stress itself.

Because the *RAV1*-overexpressing transgenic plants showed a normal seed germination pattern compared with the wild type in the absence of salt, and the expression of stress-inducible genes, such as *RD29A*, *RD29B* and even *NCED3* was exhibited normally in the *RAV1*-overexpressing plants in the presence of salt, an endogenous higher ABA level under non-stressed conditions by overexpression of *RAV1* suggests that ABA level and salt-induced inhibition of germination may not be coupled in the *RAV1*-overexpressing transgenic plants. To validate this hypothesis, we directly examined a germination pattern of the *RAV1*-overexpressing transgenic plants in ABA-containing media compared with that of the wild type. Interestingly, while the germination of wild type seeds was 23% inhibited by 1 μM ABA, the germination of *RAV1*-overexpressing plant

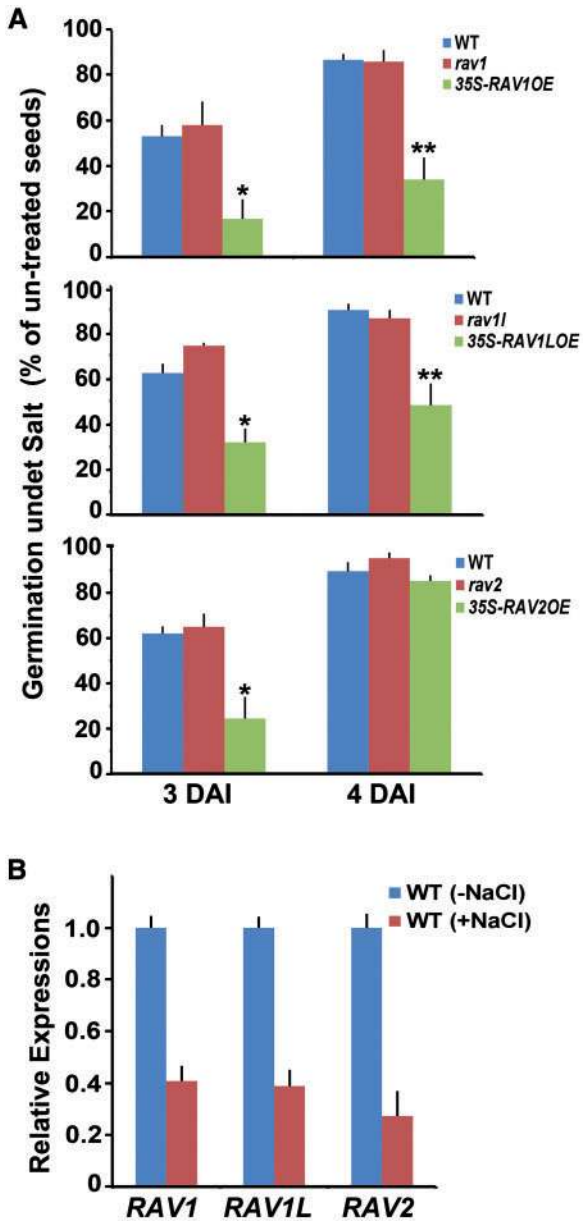

Fig. 4 RAVs play negative roles in germination under salt. (**A**) Germination of *rav* mutants and *RAV*-overexpressing plants on the 150 mM NaCl-treated 1/2 MS media compared with that of wild type at 3 DAI and 4 DAI (*$P \leq 0.001$, **$P = 0.009$ compared to wild type treated with salt). This experiment was repeated four times. More than 50 seeds from each line were plated in each experiment. Error bar denotes standard error. (**B**) Relative expressions of *RAV1*, *RAV1L* and *RAV2* in response to salt from the seeds of 2 DAI determined by qRT-PCR. Error bar denotes standard error.

seeds was only 8% inhibited at 3 DAI, suggesting that the *RAV1*-overexpressing plants are less sensitive to ABA in terms of inhibition of germination (**Fig. 5D**). Taken together, these results imply that although more ABA was accumulated by the overexpression of *RAV1*, salt-induced ABA may not be a direct factor in the inhibition of germination in the *RAV1*-overexpressing plant.

Overexpression of RAV1 resulted in the loss of ABA sensitivity

As *RAV* expression was repressed not only by ABA but also by abiotic stresses such as drought and salt (**Fig. 1**, **Fig. 3B**, and **Fig. 4B**), and *RAV1*-overexpressing transgenic plant showed a partially defective response to ABA in seed germination (**Fig. 5D**), we wanted to further examine whether overexpression of *RAVs* affects overall ABA sensitivity of the plants. Thus, we analyzed the ABA sensitivity of the *RAV1-*, *RAV1L-* and *RAV2*-overexpressing plants compared with that of their corresponding mutants and the wild type in the inhibition of root elongation in response to ABA. When exposed to 1 μM of ABA, 10-day-old seedlings of wild type and all *rav* mutant roots showed a similar reduction in root growth compared with untreated seedlings. *RAV1L-* and *RAV2*-overexpressing plants also exhibited normal responsiveness to ABA in the root growth inhibition. However, the root growth of the *RAV1*-overexpressing plant was not reduced by ABA treatment, although their initial root length was shorter, compared with that of the wild type due to the low growth potential itself (**Fig. 6A**). This result suggests that only RAV1 may be involved with the regulation of ABA sensitivity in Arabidopsis. Defective ABA sensitivity of the *RAV1*-overexpressing plant was also observed in the stomatal movement regulated by ABA. ABA-induced stomatal movement is one of the appropriate criteria to check ABA sensitivity (Kim et al. 2010). We analyzed the stomatal movement of the *RAV1*-overexpressing transgenic plant compared with that of the *rav1* mutant and wild type plant. ABA treatment applied to the epidermal tissue of the leaves that were pre-incubated in the opening solution resulted in the rapid closure of stomata in the wild type and *rav1* mutant. Compared with these plants, the stomata did not respond to ABA in the *RAV1*-overexpressing plant (**Fig. 6B**). These results clearly showed that overexpression of *RAV1* led to the ABA insensitivity.

Discussion

Expressions of genes encoding a subset of RAV transcription factors fluctuate due to plant hormones and abiotic stresses

In this study, we performed functional analyses for the three genes, *RAV1*, *RAV1L* and *RAV2*, and demonstrated that the three specific RAV transcription factors function in both growth and abiotic stresses in Arabidopsis in a redundant or distinct manner. These genes show sequence homology and overlapping expression patterns in vegetative tissues (https://www.genevestigator.ethz.ch).

As *RAV1* was known to be a representative gene that is down-regulated by BL (Hu et al. 2004), we first wanted to examine whether BL was the major plant hormone affecting the expression of *RAV1* and its homologs, although a few scattered results reported expressional changes of *RAVs* owing to other plant hormones (Alonso et al. 2003, Vogel et al. 2012). From the systemic research, in which plant

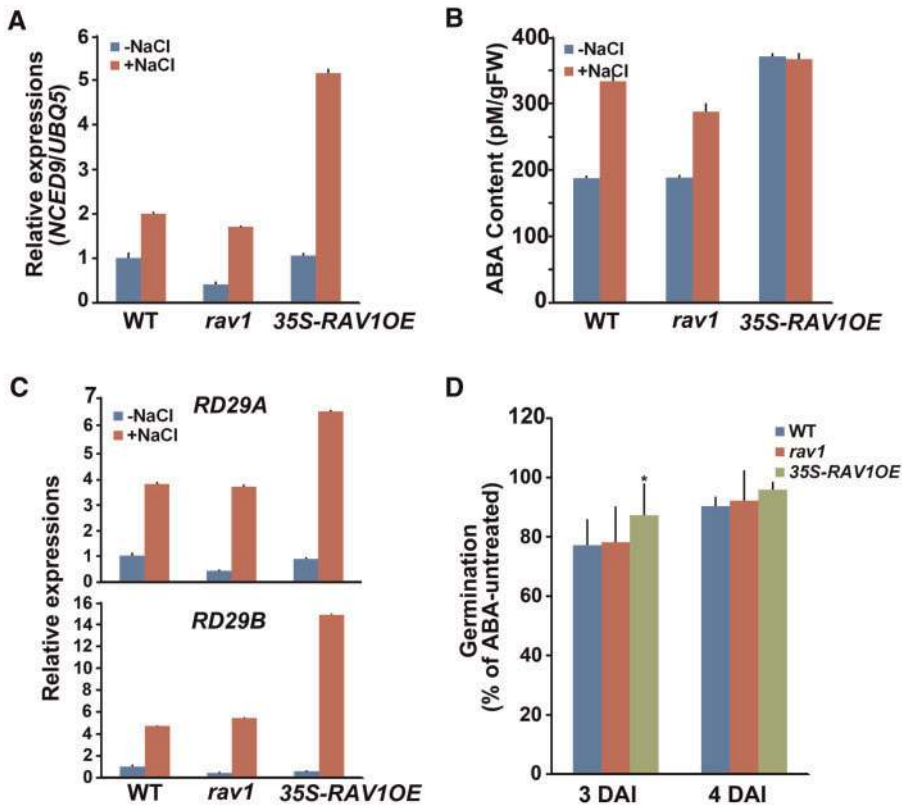

Fig. 5 RAV1 inhibits germination under salt ABA-independently. (**A**) Relative expressions of *NCED9* of the *RAV1*-overexpressing plant compared with that of *rav1* mutant and wild type plant in the presence or absence of salt determined by qRT-PCR. Error bar denotes standard error. (**B**) ABA contents were measured from the seeds grown for 2 days on the media with or without salt. (**C**) Relative expressions of stress-inducible genes, *RD29A* and *RD29B*, in response to salt determined by qRT-PCR. Error bar denotes standard error. (**D**) ABA-induced inhibition of germinations of the *RAV1*-overexpressing plant were compared with the rav1 mutant and wild type (*$P = 0.0036$ compared with the wild type treated with 1 μM of ABA).

hormones were applied to the seedlings with the same developmental status at various times, we found that not only BL, but also other plant hormones affect the expression of *RAVs*. However, we also noticed that the degree of expressional fluctuation of the *RAVs* was within 2-fold in early response periods, except *RAV1L* expression by IAA (**Fig. 1A**). The expressions of *TEM1* and *TEM2/RAV2* were shown to be under the diurnal cycle (Castillejo and Pelaz 2008, Osnato et al. 2012). Therefore, the expressional fluctuation of *RAV1* and its homologs in a day-by-plant hormone treatment may include their endogenous diurnal expression patterns. Regardless of these complex expressional changes in *RAVs* caused by plant hormones within 24 h, we repeatedly observed that their expression was reduced as a late response after 24 h of treatment with ABA or BL.

Abiotic stress conditions also affect the expressions of RAV transcription factors in various species. *BnaRAV-1* from *Brassica napus* was induced by cold, NaCl or polyethylene glycol (PEG) treatment (Zhuang et al. 2011). Even wind and gentle touch induced *RAV1* and *RAV2* expression. This induction was faster than that of *TCH3* and *TCH4*, which are typical touch-induced genes (Braam and Davis 1990, Braam 1992, Antosiewicz et al. 1995). In pepper, *CaRAV1* was shown to be induced by plant hormones and environmental stresses, such as hydrogen peroxide, wounding, NaCl and low temperature (Sohn et al. 2006). These results indicate that up-regulation of *RAV1* and *RAV2* expression is likely to be a general response to abiotic stresses, However, we showed here that at least in our conditions, *RAVs* expression was repressed by drought and salt (**Fig. 3B** and **Fig. 4B**). As endogenous *RAVs* expression may oscillate during the developmental period, as shown in *TEM1* and *TEM2/RAV2* (Osnato et al. 2012), it is possible that the time when samples were collected for analysis would affect different results. Actually, expression of *RAV1* was shown to be regulated by the internal developmental program, with an increase at the late maturation stage of green leaves, reaching the highest level at the beginning of senescence (Woo et al. 2010). Therefore, expressional changes in *RAVs* in response to various abiotic stresses may be variable, depending on the developmental stages of plants.

RAVs are versatile regulators of growth and of drought and salt abiotic stress

The inhibitory effects on growth of RAVs have been previously reported by the antisense and overexpression approaches (Hu et al. 2004, Castillejo and Pelaz 2008, Osnato et al. 2012). Here, we also showed severe growth retardation in the *RAV1*-overexpressing transgenic plants, which is consistent

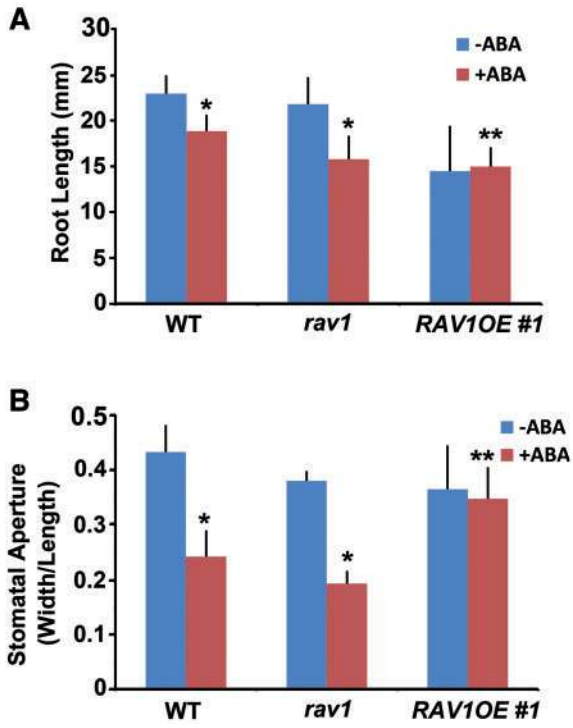

Fig. 6 ABA sensitivity is reduced in the *RAV1*-overexpressing transgenic plant compared with *rav1* or wild type. (**A**) Root growth was measured from the *rav* mutants and *RAV*-overexpressors compared with wild type plant vertically grown on the ABA-containing media for 8 d. A paired *t*-test showed significant differences from the plants without ABA treatment (*$P \leq 0.0001$). A paired *t*-test showed no statistical significance from the *RAV1*-overexpressing plants treated without ABA and treated with 1 μM ABA (**$P = 0.3942$). (**B**) ABA-induced stomatal closure was measured from the indicated plants. The stomatal aperture was the width/length ratio of the cotyledon in the absence or presence of 5 μM ABA (20–30 stomata were measured each time). Error bars indicate standard error (*$P \leq 0.0001$ compared to without ABA treatment in wild type and *rav1*, **$P = 0.3640$ showed no statistical significance from the *RAV1*-overexpressing plants treated without ABA, and treated with 1 μM ABA).

with previous reports. In addition, we observed, for the first time, slightly promoted growth in *ravs* mutants that is specific to the early stage of the growth period (**Fig. 2**). The underlying mechanisms for the developmental stage-specific effects of RAV1 on growth are currently being studied.

In addition to the negative roles of RAVs in growth, we also found that in Arabidopsis RAVs acts negatively on plants placed under drought and salt stress conditions. There was more transpirational water loss under drought conditions in the *RAVs*-overexpressing transgenic plants compared with the wild type (**Fig. 3A**) and a higher rate of growth resumption was observed in the *rav* mutants after a certain period of drought treatment (**Fig. 3C**). We did not observe the difference in response to drought in an adult stage of transgenic plants overexpressing *RAVs*. Because not only the shoot growth but also root growth was inhibited by the *RAV1* overexpression, we reason that the poor root system of *RAV1*-overexpressing transgenic plants restricted the capacity of the plant to absorb water. Their small body mass only requires a little water to sustain growth, therefore, it was possible that *RAV1*-overexpressing transgenic plants were subjected to relatively less severe drought conditions compared with the wild type and *ravs* mutants in this experimental system.

Under salt-treated conditions that also led to water deficiency, *RAV1*- or *RAV1L*-overexpressing transgenic plants exhibited reduced seed germination, whereas *rav* mutants showed promoted seed germination (**Fig. 4A**). As *RAVs* expression was repressed by drought and salt in our conditions (**Fig. 3B** and **Fig. 4B**), it is possible that tolerance to the drought- or salt stressed conditions may be obtained naturally by the reduced expression of *RAVs*. Artificial overexpression of *RAVs* may inhibit the decrease in *RAV* expressions below a certain threshold level even under the stressed conditions. In contrast to our current results, overexpression of *CaRAV1* has been previously reported to enhance plant tolerance to various stresses (Sohn et al. 2006). Compared with the wild type, *CaRAV1*-overexpressing transgenic Arabidopsis showed a higher germination ratio at 250 mM NaCl, and the seedling growth of that transgenic plant was more tolerant at 150 mM NaCl. There is no clear explanation for the discrepancies between our results and the report from *CaRAV1* at the moment. However, the possibility cannot be ruled out that heterologous expression of *CaRAV1* in Arabidopsis may not represent its physiologically natural context, as no repressive growth phenotype was observed from the transgenic Arabidopsis overexpressing *CaRAV1* (Sohn et al. 2006).

Taken together with previous reports and our current results, we propose that RAV transcription factors are versatile regulators acting negatively on growth and abiotic stresses. So far, higher seed germination in response to salt was considered to be tolerant. However, seed germination inhibited in various ways may confer evolutional advantages on the plants by preventing seeds from being germinated under salt, which is an unfavorable growth condition.

RAV1 modulates abiotic stress responses ABA-independently

It is well known that seed germination processes are regulated by the antagonistic actions of ABA and GA (Gubler et al. 2005, Seo et al. 2006). ABA inhibits seed germination, therefore, ABA contents in seeds decrease onset of germination in normal conditions. Previous reports have suggested that inhibition of seed germination under salt conditions was probably due to activation of the ABA signaling pathway (Strizhov et al. 1997, Kucera et al. 2005). ABA-insensitive (ABI) and ABA-deficient (ABA) mutants were reported to be salt-tolerant during initiation of seed germination (Yuan et al. 2011). *Abi3* and *abi4-1* mutants exhibited higher germination than the wild type under salt stress (Quesada et al. 2000, Shu et al. 2013). Expression analyses of the genes involved in ABA and GA metabolisms in *abi4* mutant compared with those of wild type plant reported that ABI4 promoted ABA biogenesis and simultaneously inhibited GA biosynthesis (Shu et al. 2013).

Moreover, expressions of the genes encoding ABA signaling transcription factors, ABI3, ABI4 and ABI5, were shown to be induced by ABA (Lopez-Molina et al. 2002). Therefore, under salt conditions, ABA contents increased by salt induce the expression of *ABI3*, *4* and *5*, resulting in repression of seed germination.

Because *RAV1*-overexpressing transgenic plants showed strong inhibition of germination under salt conditions (**Fig. 4A**), we assumed that salt-induced ABA mediated this process. However, although increased expression of *NCED9* and *NCED3* was observed under salt conditions, a subsequent greater accumulation of ABA under salt conditions did not occur in the *RAV1*-overexpressing transgenic plant (**Fig. 5A** and **B**). This may be because the much higher ABA content of the *RAV1*-overexpressing plant in normal conditions was already saturation level. This assumption was consistent with the findings that the expressions of *CYP707A1* and *CYP707A2* genes encoding catabolic enzymes for ABA (Kushiro et al. 2004, Saito et al. 2004) were reduced by *RAV1* overexpression (**Supplementary Fig. S7**).

Regardless of the fact that *RAV1*-overexpressing plants have higher levels of ABA, ABA-insensitive phenotypes of transgenic plants overexpressing *RAV1* are likely to provide additional mechanisms for plants to respond to abiotic stresses, not only in the inhibition of seed germination (**Fig. 5D**) but also in other physiological aspects, such as ABA-induced inhibition of root growth and ABA-induced stomatal movement (**Fig. 6A** and **B**). When considering that plants have developed complex mechanisms to adapt to these environmental stresses via ABA-dependent or ABA-independent signaling pathways (Zhu et al., 2010), inhibition of seed germination under salt conditions by *RAV1* overexpression seems to occur ABA-independently. Increased expression of the *RD29A* gene in response to salt in the *RAV1*-overexpressing transgenic plant also supports ABA-independency in the inhibition of seed germination in salt conditions. It was reported that induced expression of *RD29A* in response to drought and cold stresses occurred even in *aba* or *abi* mutants (Yamaguchi-Shinozaki and Shinozaki 1994) and a dehydration responsive element (DRE) in the promoter of *RD29A* was sufficient for ABA-independent stress-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki 1994, Yamaguchi-Shinozaki and Shinozaki 2005). Transcription factors belonging to the AP2/ERF family that binds to DRE/CRT elements are CBF/DREB1 and DREB2 (Yamaguchi-Shinozaki and Shinozaki 2005). The expression of CBF/DREB1 and DREB2 is rapidly induced by cold and drought stress, respectively (Liu et al. 1998). Overexpression of CBF/DREB1 was reported to lead to an increase in tolerance to freezing, drought and salt (Liu et al. 1998), and activation of DREB2 proteins by salt through post-translational modification was shown to improve drought tolerance (Sakuma et al. 2006). In addition to these DREB families, NAC and HD-ZIP transcription factors were identified as regulatinge ABA-independent stress-inducible gene expressions (Shinozaki and Yamaguchi-Shinozaki 2007). In this study we have shown that overexpression of RAV1 transcription factor reduced drought and salt tolerance. Therefore, further analyses to elucidate the underlying mechanisms of how RAV transcription factors are involved with ABA-independent signaling networks mediated by the transcription factors consisting of DRE-binding proteins (DREBs) will need to be performed. We also do not exclude the possibility that overexpression of *RAV1* negatively acts on GA biosynthesis, based on the previous report that TEM1, a homolog of TEM2/RAV2, represses growth by direct binding to the GA biosynthetic genes, *GA3OX1* and *GA3OX2*, leading to a reduction in GA content (Osnato et al. 2012). Further analyses need to be done to elucidate the underlying fine mechanisms of how the homeostasis for ABA levels, in addition to GA biosynthesis, was affected by the overexpression of *RAV1*.

RAV1, RAV1L and *RAV2* have gene-specific functions

As the three genes, *RAV1*, *RAV1L* and *RAV2*, share high sequence homology and phenotypic alterations of a single mutant of each gene are hardly detectable, it is possible to assume their physiological functions in plant growth and development are redundant. However, it is not clear whether all three genes act in the same genetic pathway or not. First, growth repressive effects were only observed during the overexpression of *RAV1* (**Fig. 2B**). Second, although each transgenic plant overexpressing *RAV1*, *RAV1L* and *RAV2* showed similar negative trends to be more susceptible to drought and salt-induced inhibition of germination (**Fig. 3** and **Fig. 4**), the degree of responsiveness to specific stresses was different for each gene. Another interesting feature we observed was that, once germinated, *rav* mutant showed better growth on the salt-containing media based on the lower proportion of the leaves exhibiting chlorosis. In comparison, Transgenic plants overexpressing RAV1L or RAV2 showed a higher ratio of dead leaves compared with the wild type. The higher remaining chlorophyll contents of *rav* mutant compared with the wild type in salt-containing media also confirmed that RAVs negatively regulate the resistance to salt stress (**Supplementary Fig. S8**). Finally, *RAV1*-overexpressing plants showed different responsiveness to ABA from *RAV1L*- or *RAV2*-overexpressing plants in the ABA-induced inhibition of root growth (**Fig. 6A**). Defective ABA sensitivity caused by *RAV1* overexpression was also observed in seed germination (**Fig. 5C**) and stomatal movement (**Fig. 6B**). These results imply that the cellular function of RAVs may be partly redundant and there may be some gene-specific functions among them.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana Columbia (Col-0) was used as the wild type plant. The T-DNA insertional mutant lines of *rav1* (Salk_021865), *rav1l* (Salk_139591) and *rav2* (Salk_070847) were obtained from the Arabidopsis Stock Center (ABRC). Seeds were sterilized with 75% ethanol containing 0.05% Tween-20, followed by washing twice with 95% ethanol, and were plated on 1/2 MS (Duchefa) plates, containing 0.8% phytoagar, supplemented with the appropriate antibiotics. When grown in soil, seeds were directly sown onto soil (Sunshine #5) top-layered with fine particles of vermiculite. After stratification at 4°C for

2 d in the dark, all plants were grown at 22°C, under long-day conditions (16 h L/8 h D).

Generation of transgenic plants

To produce the *RAV1*-overexpressing transgenic plants, wild type genomic DNA was PCR-amplified with *RAV1-Pro-F3* and *RAV1-R* primers. The resulting DNA fragment containing 2.6 Kb of *RAV1* promoter and a whole open reading frame was cloned into the *pPZP222* binary vector, cut with *Xma*I and *Sal*I restriction sites. To generate the *RAV1L*- and *RAV2*-overexpressing transgenic plants, PCR was performed with *RAV1L-F2* and *RAV1L-R* for *RAV1L*, and *RAV2-F* and *RAV2-R2* for *RAV2* to amplify the open reading frames using wild type cDNA as a template. Each resulting DNA fragment was digested with *Bam*HI/*Sal*I for *RAV1L*, and *Xma*I/*Pst*I for *RAV2* and was cloned into the *pCHF1* binary vector that harbors CaMV 35 S promoter, cut with the same set of restriction enzymes. For *GUS*-reporter gene analyses, each promoter sequence of *RAV1*, *RAV1L* and *RAV2*, which covered 2.1 kb, 1.9 kb and 1.8 kb of promoter region, respectively, was amplified using wild type genomic DNA as template. The resulting DNA fragments were cloned into the *pPZP222-GUS* binary vector (Jeong et al. 2010), cut with *Kpn*I and *Xma*I. All the primers used are listed in **Supplementary Table S1**. PCR was performed with the Phusion Hot-start Taq polymerase (Thermo) to minimize mis-incorporation of nucleotides, and PCR products were sequenced to confirm no PCR errors. The resulting constructs were transformed into the *Agrobacterium tumefaciens* (GV3101), followed by plant transformation using the floral dipping method (Clough and Bent 1998). Successful transformants that contain a single copy of transgene were selected using a gentamycin (100 μg/ml, Duchefa Biochemie, Haarlem, Netherlands), and all the experiments were performed with homozygous lines in T3 generation.

Treatments of various hormones and stresses

For plant hormones, 9-day old seedlings grown onto 1/2 MS (Murashige and Skoog) plates were treated with 20 μM each of ABA, GA_3, Kinetin, IAA and 1-amino cyclopropane-1-carboxylic acid (ACC), and with 1 μM of BL. After incubation for the indicated times under normal growth condition, samples were collected and stored for *RAVs* expression analyses. All the chemicals were purchased from Duchefa Biochemie, except IAA (Sigma Aldrich, St. Louis, USA) and BL (Synthchem. Inc., Maharashtra, India).

To load dehydration, 10-day-old seedlings grown on 1/2 MS plates were placed onto Whatman 3MM filter paper in a laminar hood for the indicated time. For drought treatment, we grew the plants for 3 weeks with watering, and then did not irrigate them for 9 d before re-watering them. Seven days after re-watering, resumption of growth was observed. To examine the effect of salt, *RAVs* expression under salt, 10-day-old seedlings grown on 1/2 MS media were treated with 150 mM NaCl for 1 h.

Histochemical *β-glucuronidase* (*GUS*) reporter gene expression

Six-day-old transgenic seedlings containing *RAV1-Pro-GUS*, *RAV1L-Pro-GUS* or *RAV2-Pro-GUS* were pre-incubated with 20 μM of ABA for 24 h, transferred into the GUS assay solution using the X-Gluc as substrate (100 mM $NaHPO_4$, 10 mM EDTA, 2.5 mM ferricyanide, 2.5 mM ferrocyanide, 30 mM X-Gluc, 0.1% Triton-X 100), and then further incubated for 1 h at 37°C. After GUS-staining, chlorophyll was removed using 100% ethanol. *GUS* signals were visualized by microscopy using a stereomicroscope (Leica $MZ12_5$, Leica, Wetzlar, Germany).

Measurement of transpirational water loss

To determine the transpirational water loss during dehydration, aerial shoot parts from 4-week-old plants were cut off and dried on 3 MM filter papers (Whatman) at room temperature. Each plant was weighed every 30 min for 3 h. This experiment was repeated six times.

Measurement of seeds germination under salt conditions or with ABA treatment

To assess the germination under salt conditions, seeds were plated onto 1/2 MS containing 150 mM NaCl or onto 1/2 MS containing 1 μM of ABA, and then grown under normal light conditions for 5 d. Each day germinated seeds with protruded radicles were counted (Kim and Nam 2010).

Determination of ABA contents

ABA was extracted from the seeds grown for 2 d on the media with or without salt in extraction buffer (80% methanol, 2% glacial acetic acid) for 24 h under darkness. After centrifugation for 10 min at 2,000 × *g*, supernatants were dried, and then partitioned between ethyl acetate and phosphate buffer [0.1 M potassium phosphate (pH 2.5)]. The ethyl acetate phase was concentrated and passed through the Sep-Pak C18 cartridges and eluted with mixtures of MeOH-water with increasing MeOH concentration. Elutes were dried and resuspended in Tris-buffered saline (Hsu and Kao, 2003). ABA was quantified by enzyme-linked immunosorbant assay (ELISA) (Phytodetek ABA kit; Agdia) according to the manufacturer's protocol.

Assessment of ABA sensitivity

To determine ABA sensitivity of the plants with the root growth inhibition assay, the sterilized seeds were placed in a line on 1/2 MS containing 0.8% phytoagar plates, supplemented with or without plant hormones. Three sets of plates were placed vertically, and grown for 8 d under normal conditions. Root lengths were measured for 20–30 seedlings in each line. All experiments were repeated three times.

To measure the ABA-induced stomatal movement, cotyledons from 10-day-old seedlings were pre-incubated in stomatal opening solution [50 mM KCl, 10 μM $CaCl_2$, 10 mM MES (pH 6.15)] for 2 h, with the light intensity set to 130 μmol/m^2/s at 22°C. ABA (5 μM) or an equal amount of distilled water was added to the opening solution, and further incubated for 2 h. The stomatal opening was evaluated by measuring the width and length of the stomata observed under the stereomicroscope (Leica, DM2500), and was calculated by the width/length ratio.

RT-PCR and quantitative RT-PCR

To monitor the expression of *RAVs*, RNA was isolated from 10-day-old seedlings subjected to various treatments using the RNAiso (TAKARA). The first-strand cDNAs were synthesized using the RNA treated with RNase-free RQ1 DNases (Promega), with the Improm-II reverse transcriptase kit (Promega) and the Oligo(dT) 15 primer. The same aliquot of first-strand cDNA (75 ng) was used as a template in a second polymerase chain reaction, which was performed for 26 to 33 cycles, with gene-specific primers. The expressions of *tubulin* and *ubiquitin 5* were used to normalize the data for RT-PCR and qRT-PCR, respectively. Quantitative RT-PCR was performed and analyzed with the Step-one Plus Real Time PCR system using the same cDNA and SYBR Green PCR Master Mix (Applied Biosystems). All the primers used are listed in **Supplementary Table S1**.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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