

Expression of a High Mobility Group Protein Isolated from *Cucumis sativus* Affects the Germination of *Arabidopsis thaliana* under Abiotic Stress Conditions

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Abstract

Although high mobility group B (HMGB) proteins have been identified from a variety of plant species, their importance and functional roles in plant responses to changing environmental conditions are largely unknown. Here, we investigated the functional roles of a CsHMGB isolated from cucumber (*Cucumis sativus*) in plant responses to environmental stimuli. Under normal growth conditions or when subjected to cold stress, no differences in plant growth were found between the wild-type and transgenic *Arabidopsis thaliana* overexpressing CsHMGB. By contrast, the transgenic *Arabidopsis* plants displayed retarded germination compared to the wild-type plants when grown under high salt or dehydration stress conditions. Germination of the transgenic plants was delayed by the addition of abscisic acid (ABA), implying that CsHMGB affects germination through an ABA-dependent way. The expression of CsHMGB had impacted only the germination stage, and CsHMGB did not affect the seedling growth of the transgenic plants under the stress conditions. The transcript levels of several germination-responsive genes were modulated by the expression of CsHMGB in *Arabidopsis*. Taken together, these results suggest that ectopic expression of a CsHMGB in *Arabidopsis* modulates the expression of several germination-responsive genes, and thereby affects the germination of *Arabidopsis* plants under different stress conditions.

Key words: abiotic stress; cucumber; *Cucumis sativus*; high mobility group protein; transgenic *Arabidopsis* plant.

Introduction

High mobility group (HMG) proteins are small chromatin-associated proteins found abundantly in the nuclei of higher eukaryotes. The HMG proteins are subdivided into 3 families: the HMGA (formerly HMG-I/Y/C) family, the HMGB (formerly HMG-1/-2) family, and the HMGN (formerly HMG-14/-17) family (Bustin and Reeves 1996; Bustin 2001). The HMGB proteins have been identified from various plant species (Grasser and Felix 1991; Webster et al. 1997; Yamamoto and Minamikawa 1998; Wu et al. 2003), and it was revealed that higher plants have a family of HMGB proteins that differ in their chromatin association (Agresti and Bianchi 2003), DNA interactions (Stemmer et al. 2002), and expression pattern (Stemmer et al. 1999; Launholt et al. 2007). Although most organisms usually express only two closely related HMGB proteins, plants have a variety of HMGB proteins (Grasser 1998). The presence of various HMGB proteins in plants implies that they may have adapted to perform a variety of functions under different physiological processes, including the assembly of nucleoprotein complexes, transcription, and recombination (Thomas and Travers 2001).

Although the number of reports demonstrating the isolation of the cDNAs that encode HMGB proteins in plants is increasing, relatively little is known about the expression patterns and functional roles of HMGB proteins in the responses of plants to changing environmental conditions. In *Arabidopsis* leaves and suspension culture cells, five different *HMGB* genes are simultaneously expressed (Stemmer et al. 1997), and in maize, five HMGB proteins were expressed in significantly different quantities depending on which tissues were examined (Stemmer et al. 1999). The regulation of the expression of HMGB proteins by light or endogenous rhythm was described in *Pharbitis nil* (Zheng et al. 1993; O'Neill and Zheng 1998). However, the reports demonstrating the cellular functions of HMGB proteins in the growth, development and stress responses of plants are severely limited. Lichota et al. (2004) showed that maize HMGB1 protein affects root development of tobacco seedlings when grown under normal growth conditions. We recently reported that HMGB2, HMGB4, and HMGB5 in *Arabidopsis* play different roles during seed germination of the plants under a variety of stress conditions (Kwak et al. 2007).

To gain better insight into the functions of HMGB proteins in the responses of plants to diverse environmental stresses, it is important to isolate HMGB proteins from diverse plant species and to characterize their functional roles under different stress conditions. We recently reported the cloning of a cDNA encoding a HMGB protein in cucumber (*Cucumis sativus*), thus designated CsHMGB, and its expression pattern in response to abiotic stress treatments

(Jang et al. 2007). Here we generated transgenic *Arabidopsis* plants that express the CsHMGB isolated from the cucumber, and investigated the effect of cold, high salt or dehydration stress on germination and seedling growth of the transgenic plants. The present findings provide a novel basis for understanding the biological function of CsHMGB protein in the responses of plants to different stress conditions.

Results

Growth of the wild-type and transgenic plants under normal growth conditions

To investigate whether the expression of a cucumber HMGB in *Arabidopsis* plants influences the plant's responses to environmental stresses, we generated transgenic *Arabidopsis* plants that constitutively express the cucumber CsHMGB under control of cauliflower mosaic virus 35S promoter, and investigated the growth phenotypes of the plants under normal and stress conditions. Among 8 different transgenic lines generated, four transgenic lines were selected for further analysis, and the expression of CsHMGB was verified by RT-PCR analysis (Figure 1A). To assess the effect of CsHMGB overexpression on plant growth at favorable growth conditions, we analyzed seed germination and seedling growth of the wild-type and transgenic plants under normal growth conditions. No differences in germination and seedling growth were observed among the wild-type and overexpression lines (Figures 1B, 1C).

Effect of salt stress on germination and seedling growth of the wild-type and transgenic plants

Given that the expression of *CsHMGB* is markedly down regulated by salt, drought, or cold stress (Jang et al. 2007), we tested whether CsHMGB plays roles in plant responses to salt stress. When the seeds were germinated in MS medium supplemented with various concentrations of NaCl, germination of the wild-type and transgenic *Arabidopsis* plants differed in that the wild-type seeds completed germination at day 2 under 75 mM NaCl, while only 60-80 % of the transgenic seeds germinated at day 2 under 75 mM NaCl. When germinated on MS medium supplemented with 100 mM NaCl, approximately 95% of the wild-type seeds germinated at day 2, whereas only 30-60% of the transgenic seeds germinated by the same day (Figure 2A). We then determined whether the expression of CsHMGB protein affects the growth of *Arabidopsis* plants under salt stress conditions. The

seeds of wild-type and transgenic plants were allowed to fully germinate in normal MS medium for 4 days, and the 4-day-old seedlings were then transferred to a medium supplemented with different concentrations of NaCl, and the root length was measured. No significant differences in root growth were observed between the wild-type and transgenic plants in MS medium supplemented with up to 125 mM NaCl (Figure 2B). These observations indicate that the expression of CsHMGB has a negative effect only on seed germination of *Arabidopsis* plants under salt stress conditions.

Effect of dehydration stress on germination and seedling growth of the wild-type and transgenic plants

We next examined the effects of dehydration stress on germination and seedling growth of the wild-type and transgenic plants. When the seeds of the wild-type and transgenic plants were germinated in MS medium supplemented with various concentration of PEG, germination of the transgenic seeds delayed in that approximately 95% of the wild-type seeds germinated at day 2 in the MS medium supplemented with 25% PEG, while only 50-70% of the transgenic seeds germinated at day 2 under the same dehydration stress condition. The retarded germination of the transgenic seeds compared with the wild-type seeds was also observed when grown at 40% PEG (Figure 3). To determine whether the expression of CsHMGB protein affects the growth of *Arabidopsis* plants under dehydration stress conditions, the seeds of the wild-type and transgenic plants were allowed to fully germinate in normal growth medium for 4 days, and the 4-day-old seedlings were then transferred to a medium supplemented with various amounts of PEG. No significant differences in seedling growth were observed between the wild-type and transgenic plants in the presence of PEG (data not shown). When the plants were subjected to cold stress, no significant differences in germination and seedling growth were also observed between the wild-type and transgenic plants (data not shown). These results indicate that the expression of CsHMGB hinders seed germination of *Arabidopsis* plants under dehydration stress conditions.

Effect of abscisic acid on germination of the transgenic plants

With the observation that CsHMGB impacts negatively on the germination of *Arabidopsis* plants under salt or osmotic stress conditions, we next investigated whether this response depends on abscisic acid (ABA) that is involved in the adaptation process to salt and drought

stresses (Barrero et al. 2006). When the seeds of the wild-type and transgenic plants were germinated in the presence of 5 μM ABA, germination of the wild-type and transgenic *Arabidopsis* plants differed in that approximately 50% of the wild-type seeds germinated at day 1, while only 20-30 % of the transgenic seeds germinated at day 1 under 5 μM ABA. At day 5 under 5 μM ABA, approximately 90% of the wild-type seeds germinated, while only 40-60% of the transgenic seeds germinated (Figure 4A). The retarded germination of the transgenic seeds compared with the wild-type seeds was also apparent when the seeds were germinated in the presence of 10 to 15 μM ABA (Figure 4B). When the seeds were germinated in the presence of 15 μM ABA, approximately 50% of the wild-type seeds germinated at day 3, while only 15-20% of the transgenic seeds germinated at the same day. These results demonstrate that CsHMGB-overexpressing plants displayed retarded germination compared with the wild-type plants in the medium supplemented with ABA.

CsHMGB modulates the expression of germination-responsive genes in *Arabidopsis* plants

Because it is apparent that CsHMGB-expressing plants displayed retarded germination compared with the wild-type plants under salt or dehydration stress conditions, we next tested whether CsHMGB expression modulates the expression of germination-responsive genes. The same gene-specific primers used in our previous analysis were employed in this analysis (Kwak et al. 2007). The germination-responsive genes tested include myrosinase-binding protein, myrosinase, isocitrate lyase, SAMS, PhyB, and beta-glucosidase, the expression of which is up regulated during germination (Yamaguchi et al. 1998; Gallardo et al. 2001), and 12S seed storage protein and LEA protein in group 5, the expression of which is down regulated during germination (Gallardo et al. 2001). The transcript levels of these germination-responsive genes at day 2 were quite similar in the wild-type and transgenic plants when grown in normal MS medium (Figure 5A). However, when the seeds were germinated in MS medium supplemented with 125 mM NaCl for 2 days, it was apparent that several genes including isocitrate lyase, SAMS, and beta-glucosidase were noticeably down regulated in the transgenic plants compared to the wild-type plants (Figure 5B). By comparison, several genes including myrosinase-binding protein, myrosinase, isocitrate lyase, and beta-glucosidase were noticeably down regulated at day 2 in the transgenic plants compared to the wild-type plants in MS medium supplemented with 25% PEG (Figure 5C).

When the seeds were germinated in MS medium supplemented with 5 μ M ABA for 3 days, it was apparent that the genes encoding myrosinase-binding protein and PhyB were down regulated, whereas the transcript level of 12S seed storage protein was up regulated in the transgenic plants compared with the wild-type plants (Figure 5D). These results suggest that CsHMGB modulated the expression of these germination-responsive genes, which in turn resulted in retarded germination of *Arabidopsis* plants under salt or dehydration stress conditions.

Discussion

Although the function of HMGB proteins in the regulation of transcription and recombination has been well characterized (Singh and Dixon 1990; Bustin 1999; Thomas and Travers 2001), their biological roles in transcriptional regulation of the genes involved in the responses of plants to environmental stresses are largely unknown. The present analyses of transgenic *Arabidopsis* plants that express a cucumber CsHMGB clearly demonstrate that CsHMGB plays a role in the responses of plants to abiotic stresses. The transgenic plants displayed noticeable changes in seed germination under salt or dehydration stress conditions (Figures 2, 3). The retarded germination of CsHMGB-expressing transgenic *Arabidopsis* plants is reflected by its expression patterns, which showed a marked down regulation by salt or dehydration stress in cucumber (Jang et al. 2007). It is interesting to note that the ectopic expression of CsHMGB influences only the germination of *Arabidopsis* plants, but not the seedling growth of the plants under stress conditions. We, therefore, propose that CsHMGB impacts negatively on the seed germination of plants under salt or dehydration stress conditions. The findings that seed germination of transgenic plants is retarded compared with the wild-type plants under salt or dehydration stress conditions, and by ABA treatment suggest that CsHMGB affects seed germination through ABA-dependent way. It has been suggested that ABA is involved in the adaptation of plants to salt and drought stress, and several ABA biosynthetic genes are induced by stresses (Barrero et al. 2006). The present findings and the previous report (Lichota et al. 2004) demonstrating that the ectopic expression of maize HMGB1 in tobacco caused defects in root development only at the early stage of seedling growth suggest that a specific HMGB can influence the development of plants in a growth stage-dependent manner.

The molecular mechanisms by which HMGBs regulate the transcription of the genes involved in the responses of plants to environmental stresses are not yet understood. The

HMGB genes in mice and yeast have altered the expression levels of a variety of genes during germ cell differentiation or temperature-sensitive growth (Calogero et al. 1999; Moreira and Holmberg 2000; Ronfani et al. 2001). It has been shown that HMGB proteins could stimulate the binding of transcription factors to DNA target sites through HMGB-transcription factor interactions (Yanagisawa 1997; Krohn et al. 2002; Cavalari et al. 2003; Grasser et al. 2007). In the present analysis, it was observed that several germination-responsive genes were modulated by the expression of CsHMGB in *Arabidopsis* plants under salt or dehydration stress conditions (Figure 5). Similar results were also observed in our previous analysis on AtHMGB2-overexpressing transgenic *Arabidopsis* plants, but not on AtHMGB4-overexpressing transgenic *Arabidopsis* plants, under salt stress conditions (Kwak et al. 2007). It is noteworthy that CsHMGB has the highest sequence homology with *Arabidopsis* HMGB2; the amino acid sequence identity between CsHMGB and AtHMGB1 to AtHMGB5 is 54%, 62%, 61%, 55%, and 40%, respectively. Based on this consideration, we propose that CsHMGB and AtHMGB2 family member specifically contribute to altered germination of *Arabidopsis* plants under salt or dehydration stress conditions, and that the retarded germination is due, at least in part, to the modulation of the expression of germination-responsive genes by the specific HMGB. It is likely that CsHMGB stimulates the binding of negative regulatory protein factors to the promoter region of germination-responsive genes, thereby down regulates the expression of germination-responsive genes under stress conditions. Alternatively, it is possible that CsHMGB indirectly influences the transcript levels of these germination-responsive genes by modulating the transcription of upstream controlling factors. As mammalian HMGB1 has been demonstrated to function as a DNA chaperone facilitating the rate-limiting DNA distortion during nucleosome remodeling (Bonaldi et al. 2002), it is also likely that CsHMGB facilitates the nucleosome sliding and promotes chromatin dynamics, which is an important process for the regulation of the expression of germination-responsive genes. This hypothesis needs to be tested to completely understand the molecular mechanisms of HMGB-mediated regulation of germination under different environmental stress conditions.

In summary, analysis of the transgenic *Arabidopsis* plants indicates that CsHMGB, the expression of which is markedly down regulated by salt or dehydration stress, negatively influences seed germination of *Arabidopsis* plants under salt or dehydration stress conditions. The limited information on the functional roles and action mechanisms of plant HMGB proteins in stress responses should stimulate further investigation to search for the target genes regulated by HMGBs in plants subjected to diverse environmental stresses, which will

be valuable to further understand HMGB functions in stress adaptation process in cucumber.

Materials and Methods

Vector construction and plant transformation

Full-length cDNA corresponding to cucumber CsHMGB gene was obtained by PCR, and the PCR products were separated on 1% agarose gel, purified with a gel extraction kit (Qiagen, Valencia, CA, USA), and then cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). The transgenic *Arabidopsis* plants were generated that constitutively express a cucumber CsHMGB under control of cauliflower mosaic virus 35S promoter. To generate CsHMGB-expression constructs, the vector was digested with Xba I/Bam HI, and the DNA products were ligated into the pBI121 vector predigested with the same restriction enzymes. All DNA manipulations were performed according to standard procedures (Sambrook et al. 1989), and the CsHMGB-coding region and junction sequences were confirmed by DNA sequencing. Transformation of *Arabidopsis* was performed according to the vacuum infiltration method (Bechtold and Pelletier 1998) using *Agrobacterium tumefaciens* GV3101. Seeds were harvested and plated on the selection medium containing kanamycin ($50 \mu\text{g ml}^{-1}$) to identify homozygous T₃ or T₄ transgenic lines.

Assays for germination and seedling growth under stress conditions

Germination assays were carried out with three replicates of 50–60 seeds that were obtained from the plants grown under identical growth conditions. Seeds were sown on MS (Murashige and Skoog 1962) medium supplemented with 1.5% sucrose, and the plates were placed at 4°C for 3 days in the dark and then transferred to normal growth conditions. Plants were grown at $23 \pm 2^\circ\text{C}$ under long day conditions (16 h-light/8 h-dark cycles). The seeds were regarded as germinated when the radicles protruded the seed coat. For direct comparison of germination rates, each plate was subdivided, and seeds of all genotypes were planted on the same plate. To determine the effect of salt stress on germination, the MS medium was supplemented with 75 to 150 mM NaCl. To determine the effect of dehydration stress on germination, the MS medium was supplemented with an appropriate volume of PEG overlay solution (250 g of PEG8000 per liter) onto the top of solidified MS agar plate. To determine the effect of cold stress on germination, the MS medium containing the seeds was placed in a

growth chamber maintained at 11°C. To determine the effect of ABA on germination, the MS medium was supplemented with 5 to 15 µM ABA. To determine the effect of these stress on seedling growth, the seeds were fully germinated on normal MS medium for 4 days, and the seedlings were transferred to the MS medium supplemented with different amount of NaCl or PEG. The plates with seedlings were then placed vertically in a growth chamber, and the root length was measured daily.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from the frozen samples by using the Plant RNeasy extraction kit (Qiagen). The concentration of RNA was accurately quantified by a spectrophotometric measurement. To determine quantitatively the transcript levels of the genes, the real-time quantification of RNA target was performed with the gene-specific primers in the Rotor-Gene 2000 real-time thermal cycling system (Corbett Research, Sydney, Australia) using the QuantiTect SYBR Green RT-PCR kit (Qiagen). The reaction mixture (25 µl) contained 200 ng of total RNA, 0.5 µM of each primer, and appropriate amounts of enzymes and fluorescent dyes as recommended by the manufacturer (Qiagen). The same germination-responsive gene-specific primers used in our previous analysis (Kwak et al. 2007) were employed. All other experimental conditions were as previously described (Kim et al. 2003).

Statistical analysis

Data were square root-transformed prior to analysis, and differences in germination rate and gene expression levels between the wild-type and transgenic plants were compared by t-test ($p \leq 0.05$; SigmaPlot software; Systat Software, Inc.).

Acknowledgment

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Legends of Figures

Figure 1. Confirmation of transgenic *Arabidopsis* plants and growth of the plants under normal growth conditions. (A) The expression of *CsHMGB* in four independent transgenic *Arabidopsis* plants was confirmed by RT-PCR. Actin was used as a reference to show that equal amounts of RNA were used in the analysis. (B) The seeds of the wild-type (Col-0) and transgenic plants (T-1 to T-4) were germinated in MS medium, and germination rates were scored at indicated days. The picture was taken at day 6. (C) The root length of the wild-type and transgenic plants in MS medium was measured at indicated days. Values are means \pm SE obtained from three independent experiments.

Figure 2. Effect of salt stress on germination and root growth of the wild-type and transgenic plants. (A) The seeds of the wild-type (Col-0) and transgenic plants (T-1 to T-4) were germinated in MS medium supplemented with various concentration of NaCl, and germination rates were scored at day 2. The picture was taken 6 days after germination in 125 mM NaCl. (B) The root length of the wild-type and transgenic plants was measured at day 6 in MS medium supplemented with different concentration of NaCl. Values are means \pm SE obtained from three independent experiments.

Figure 3. Effect of dehydration stress on germination of the wild-type and transgenic plants. The seeds of the wild-type (Col-0) and transgenic plants (T-1 to T-4) were germinated in MS medium supplemented with various concentration of PEG, and germination rates were scored at day 2. The picture was taken 6 days after germination in 25% PEG. Values are means \pm SE obtained from three independent experiments. Asterisks above the columns indicate values that are statistically different from control Col-0 values ($p \leq 0.05$).

Figure 4. Effect of ABA on germination of the wild-type and transgenic plants. (A) The seeds of the wild-type (Col-0) and transgenic plants (T-1 to T-3) were germinated in MS medium supplemented with 5 μ M ABA, and germination rates were scored at the indicated day. The picture was taken 6 days after germination in 5 μ M ABA. (B) The seeds of the wild-type and transgenic plants were germinated in MS medium supplemented with various concentration of ABA, and germination rates were scored at day 3. Values are means \pm SE obtained from three independent experiments. Asterisks above the columns indicate values that are

statistically different from control Col-0 values ($p \leq 0.05$).

Figure 5. Modulation of the expression of germination-responsive genes by the expression of CsHMGB. Total RNAs were extracted from two-day-old wild-type (Col-0) and transgenic *Arabidopsis* plants (T-1 and T-2) grown in (A) MS medium or MS medium supplemented with (B) 125 mM NaCl, (C) 25% PEG, and (D) 5 μ M ABA. The expression of the target genes was analyzed by real-time RT-PCR, and the results were presented as a relative expression (fold control) of each transcript in the transgenic plants compared with the wild-type plants. Actin was used as a reference to show that equal amounts of RNA were present in the samples. Values are means \pm SE obtained from three independent experiments. Asterisks above the columns indicate values that are statistically different from control Col-0 values ($p \leq 0.05$).

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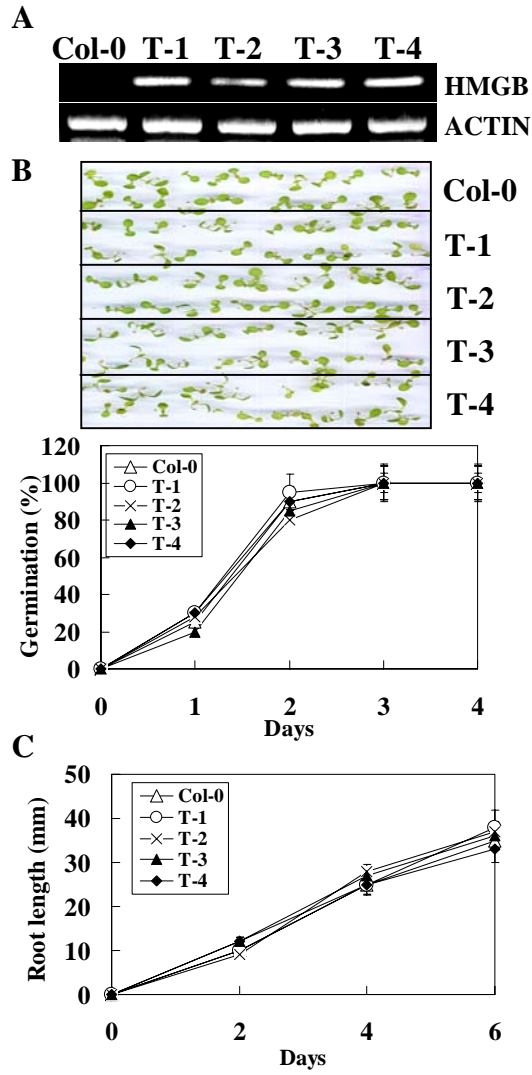


Figure 1

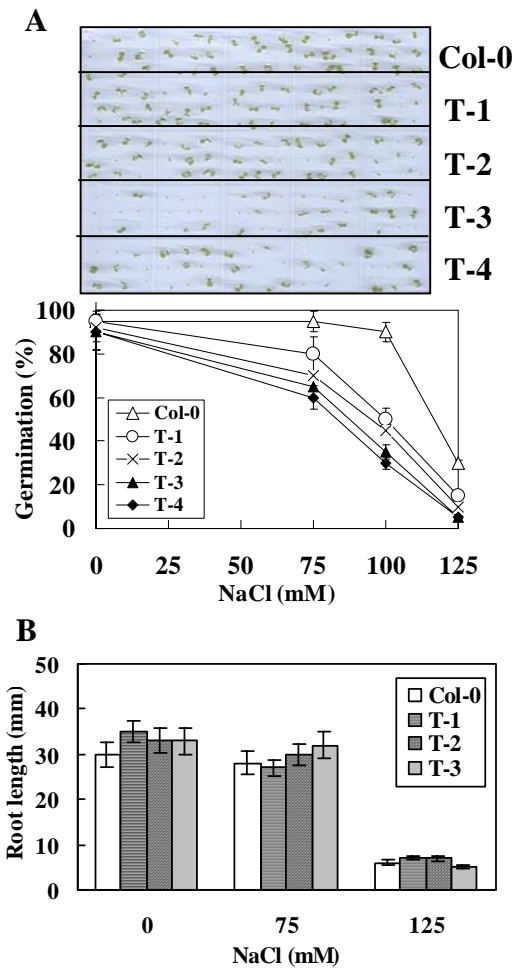


Figure 2

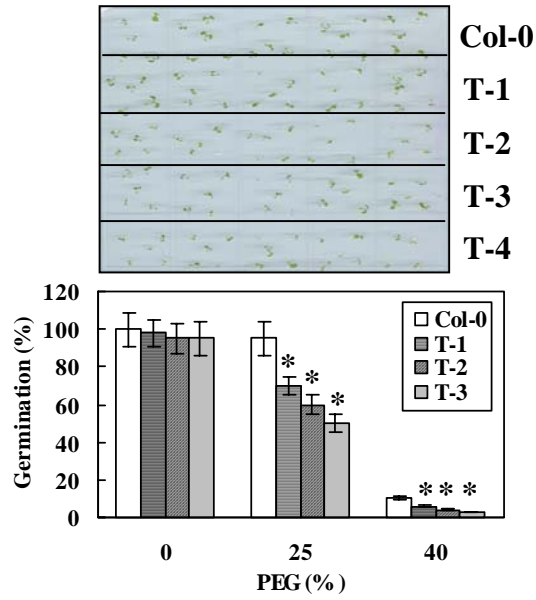


Figure 3

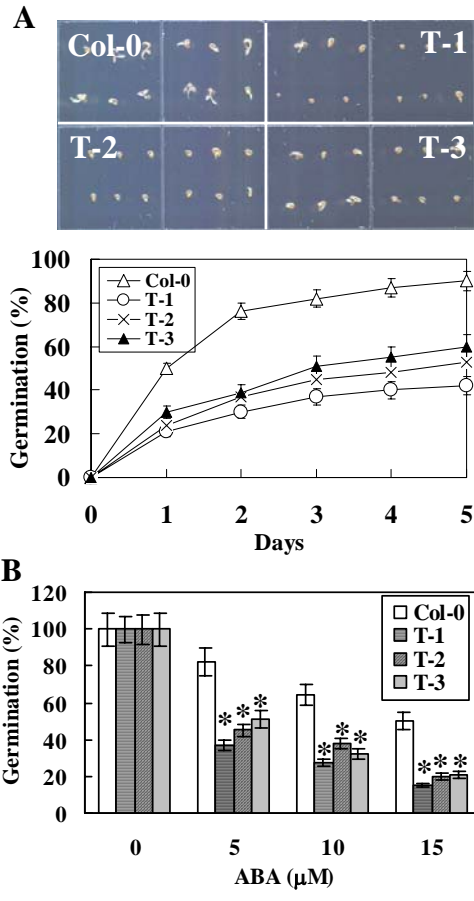


Figure 4

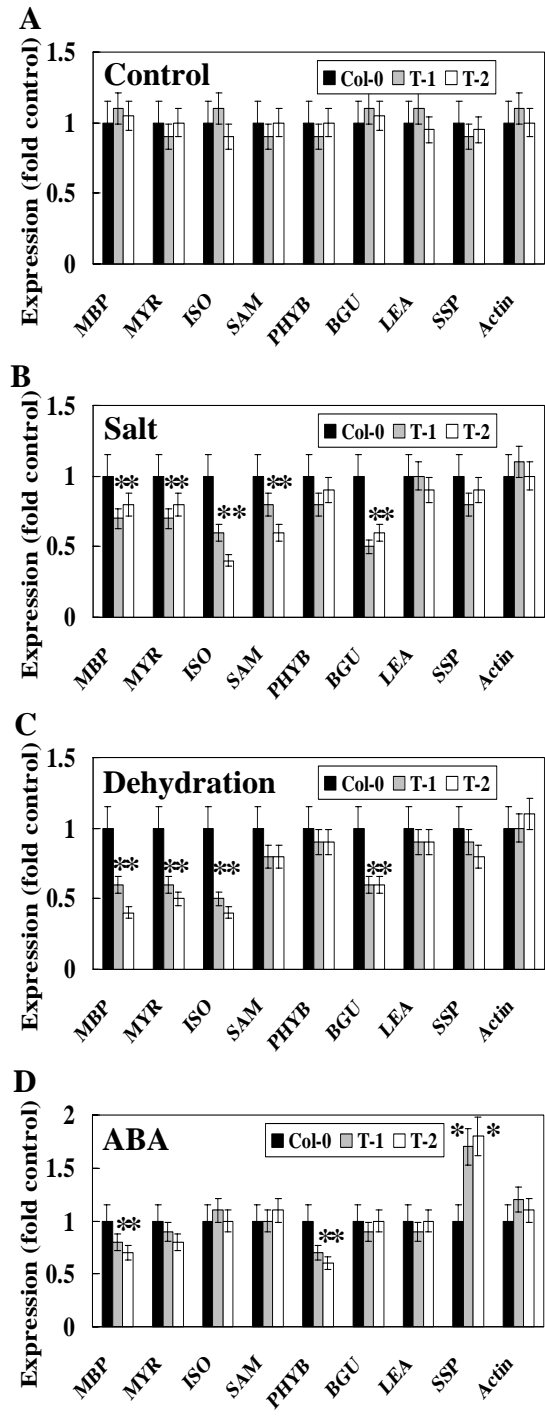


Figure 5