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A Zinc Finger-Containing Glycine-Rich RNA-Binding Protein, atRZ-1a, Has a Negative Impact on Seed Germination and Seedling Growth of *Arabidopsis thaliana* Under Salt or Drought Stress Conditions

Yeon-Ok Kim [1], SangO Pan [2], Che-Hun Jung [2] and Hunseung Kang [1,*]

[1] *Department of Plant Biotechnology, Agricultural Plant Stress Research Center and Biotechnology Research Institute, College of Agriculture and Life Sciences, Chonnam National University, Gwangju, 500-757 Korea*
[2] *Department of Chemistry, College of Life Sciences, Chonnam National University, Gwangju, 500-757 Korea*

Despite the fact that glycine-rich RNA-binding proteins (GRPs) have been implicated in the responses of plants to changing environmental conditions, the reports demonstrating their biological roles are severely limited. Here, we examined the functional roles of a zinc finger-containing GRP, designated atRZ-1a, in *Arabidopsis thaliana* under drought or salt stress conditions. Transgenic *Arabidopsis* plants overexpressing atRZ-1a displayed retarded germination and seedling growth compared with the wild-type plants under salt or dehydration stress conditions. In contrast, the loss-of-function mutants of atRZ-1a germinated earlier and grew faster than the wild-type plants under the same stress conditions. Germination of the transgenic plants and mutant lines was influenced by the addition of ABA or glucose, implying that atRZ-1a affects germination in an ABA-dependent way. H_2O_2 was accumulated at higher levels in the transgenic plants compared with the wild-type plants under stress conditions. The expression of several germination-responsive genes was modulated by atRZ-1a, and proteome analysis revealed that the expression of different classes of genes, including those involved in reactive oxygen species homeostasis and functions, was affected by atRZ-1a under dehydration or salt stress conditions. Taken together, these results suggest that atRZ-1a has a negative impact on seed germination and seedling growth of *Arabidopsis* under salt or dehydration stress conditions, and imply that atRZ-1a exerts its function by modulating the expression of several genes under stress conditions.

Keywords: Abiotic stress — Arabidopsis — Glycine-rich RNA-binding protein — Stress adaptation — Zinc finger.

Abbreviations: GRP, glycine-rich RNA-binding protein; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PEG, polyethylene glycol; RBP, RNA-binding protein; ROS, reactive oxygen species; RRM, RNA recognition motif; RT–PCR, reverse transcription–PCR.

Introduction

The regulation of gene expression at the post-transcriptional level is important for plant growth, development and stress responses. Post-transcriptional gene regulation includes pre-mRNA splicing, capping, polyadenylation, mRNA transport, stability and translation (Higgins 1991, Simpson and Filipowicz 1996). In these processes, regulation is mainly achieved either directly by RNA-binding proteins (RBPs) or indirectly, whereby RBPs modulate the function of other regulatory factors. RBPs that contain one or more RNA-recognition motifs (RRMs) at the N-terminus and a variety of auxiliary motifs at the C-terminus, such as glycine-rich, arginine-rich, acidic, SR-repeats and RD-repeats, have been identified (Kenan et al. 1991, Fukami-Kobayashi et al. 1993, Burd and Dreyfuss 1994, Albà and Pagès 1998). The genes encoding proteins that contain RRMs at the N-terminus and a glycine-rich region at the C-terminus (glycine-rich RNA-binding proteins, GRPs) were isolated from various plant species (Gómez et al. 1988, Bergeron et al. 1993, Hirose et al. 1993, van Nocker and Vierstra 1993, Carpenter et al. 1994, Dunn et al. 1996, Ferullo et al. 1997, Molina et al. 1997, Moriguchi et al. 1997, Horvath and Olson 1998). It has been shown that the expression of *GRP* genes is regulated in response to a number of external stimuli including cold, water stress, high salinity and viral infection (Sachetto-Martins et al. 2000, and references therein). An increase in the expression of the genes encoding GRPs in stress conditions has led to the hypothesis of the possible involvement of GRPs in the responses of plants to changing environmental conditions. However, information on the biological roles of GRPs under stress conditions is severely limited.

During the last several years, we have extensively investigated the biological roles of GRPs in plants under stress conditions. We reported that GRP2, one of the eight GRP family members in *Arabidopsis*, has a positive impact

*Corresponding author: E-mail, hskang@chonnam.ac.kr; Fax, +82-62-530-2047.

on seed germination and seedling growth of *Arabidopsis* plants under cold stress conditions (J. Y. Kim et al. 2007). We also reported that GRP4, one member of the *Arabidopsis* GRPs, negatively affects seed germination and seedling growth of *Arabidopsis* plants under salt or dehydration stress conditions (Kwak et al. 2005). Recently we demonstrated that GRP7, another member of the *Arabidopsis* GRPs, has an RNA chaperone activity during the cold adaptation process in *Escherichia coli* (J. S. Kim et al. 2007). The zinc finger-containing GRP, designated atRZ-1a, investigated in this study contains RRMs at the N-terminus and a glycine-rich region interspersed with CCHC-type zinc fingers at the C-terminus. We previously demonstrated that atRZ-1a confers cold and freezing tolerance in *Arabidopsis* plants (Kim et al. 2005), and that atRZ-1a exerts its roles by functioning as an RNA chaperone during the cold adaptation process (Kim and Kang 2006). To explore further the roles of atRZ-1a in plants subjected to other abiotic stress such as high salt or dehydration stress, we analyzed the seed germination and seedling growth of transgenic *Arabidopsis* plants and loss-of-function mutants under high salt or dehydration stress conditions. Here, we provide evidence that atRZ-1a has a negative impact on seed germination and seedling growth of *Arabidopsis* under salt or dehydration stress conditions.

Results

atRZ-1a has a negative impact on seed germination and seedling growth of Arabidopsis under salt stress conditions

We first examined the roles of atRZ-1a in seed germination and seedling growth of *Arabidopsis* plants in response to salt stress. The transgenic *Arabidopsis* plants (35S::atRZ-1a) and T-DNA tagged loss-of function mutants (KO1, SALK_036865; and KO2, SALK_109798) were described in detail in a previous report (Kim et al. 2005). When the wild type, mutants and 35S::atRZ-1a plants were germinated under normal growth conditions, no significant differences in germination rate and seedling growth were observed between the three genotypes (data not shown). However, as shown in Fig. 1A, when the seeds were germinated in the presence of 100 mM NaCl, approximately 55% of wild-type seeds germinated at day 3, whereas only 20–40% of 35S::atRZ-1a seeds had germinated on the same day. In contrast, approximately 80 and 95% of KO1 and KO2 lines, respectively, germinated at day 3, indicating that the mutant lines displayed much faster germination compared with the wild-type plants. Germination completed in the order of mutants, wild type and 35S::atRZ1a seeds (Fig. 1A). This retardation of germination in 35S::atRZ-1a lines was also observed in the presence of 125 or 150 mM NaCl (data not shown). These results suggest that atRZ-1a affects seed germination in a negative way under high salinity stress conditions.

We next tested whether atRZ-1a influences seedling growth under salt stress conditions. When the seeds of wild type, mutants and 35S::atRZ-1a lines were germinated and grown in the medium supplemented with 100 mM NaCl, the 35S::atRZ-1a plants displayed retarded root growth compared with the wild-type plants under salt stress conditions. In comparison, the loss-of-function mutants of atRZ-1a showed a much faster root growth compared with the wild-type plants under salt stress conditions (Fig. 1B). This response of 35S::atRZ-1a plants and mutant lines is specific to sodium chloride, because no differences in germination and seedling growth were observed between the three genotypes when germinated in MS medium supplemented with other ions such as KCl and LiCl (data not shown). These results demonstrate that atRZ-1a has a negative impact on seedling growth of *Arabidopsis* under salt stress conditions.

atRZ-1a has a negative impact on seed germination and seedling growth of Arabidopsis under dehydration stress conditions

Given that the expression of *atRZ-1a* was down-regulated by dehydration stress (Kim et al. 2005), we next examined the roles of atRZ-1a on germination and growth of *Arabidopsis* under dehydration stress conditions. When the seeds of wild-type, mutant and 35S::atRZ-1a plants were germinated in the presence of 100 mM mannitol, the mutant lines germinated earlier than the wild-type and 35S:: atRZ-1a plants (Fig. 2A). Approximately 20% of wild-type and 35S::atRZ-1a seeds germinated at day 2, whereas 40–50% of the mutant seeds germinated at day 2 in the MS medium supplemented with 100 mM mannitol. To test whether atRZ-1a plays a role in seedling growth of *Arabidopsis* under dehydration stress conditions, the seeds of the wild type, mutants and 35S::atRZ-1a plants were germinated and grown in the medium supplemented with 250 $g\,l^{-1}$ (−0.5 Mpa) polyethylene glycol (PEG). As shown in Fig. 2B, the loss-of-function mutants of atRZ-1a showed a much faster root growth compared with the wild-type plants under dehydration stress conditions. In contrast, the 35S::atRZ-1a plants displayed retarded root growth compared with the wild-type plants under dehydration stress conditions. To confirm further the responses of the plants to dehydration stress, the experiment was repeated in the medium supplemented with different concentrations of mannitol. The patterns of seedling growth between the three genotypes in the medium supplemented with mannitol were quite similar to the patterns observed in the medium supplemented with PEG (data not shown). These results demonstrate that atRZ-1a has a negative impact on

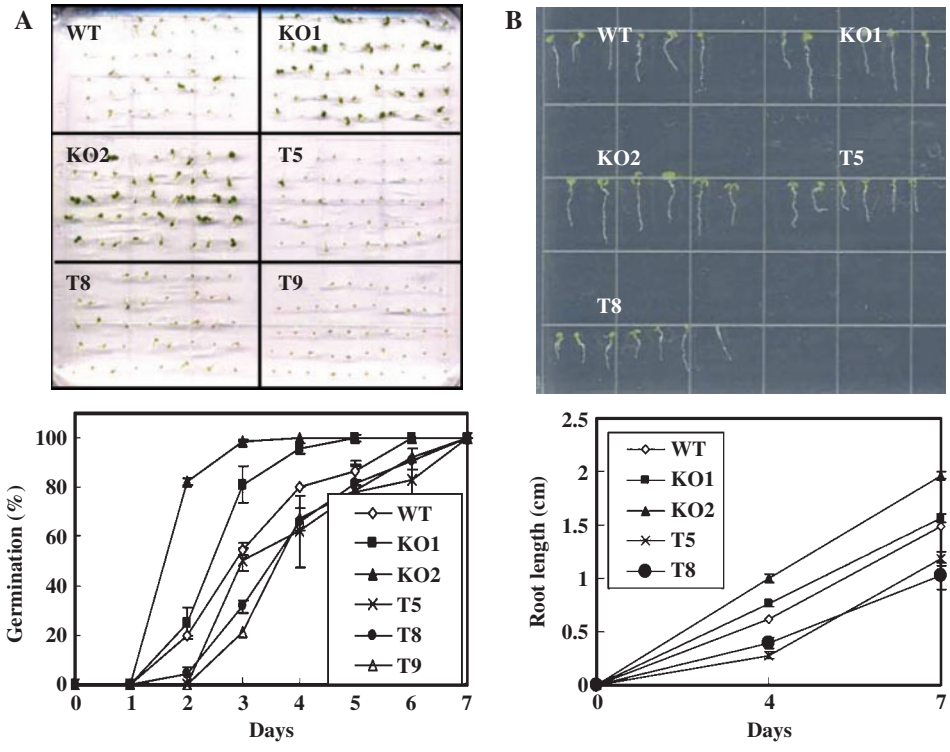

Fig. 1 Effect of salt stress on germination and seedling growth of wild type (WT), atRZ-1a knockout mutants (KO1 and KO2) and atRZ-1a overexpression lines (T5, T8 and T9). (A) Seeds were germinated in the MS medium supplemented with 100 mM NaCl, and germination rates were determined on the indicated days. (B) Seeds were sown on MS medium supplemented with 75 mM NaCl, the plates were incubated in a vertical orientation, and root length was measured on the indicated days. Experiments were performed in triplicate ($n = 40–50$). The photographs were taken on day 10.

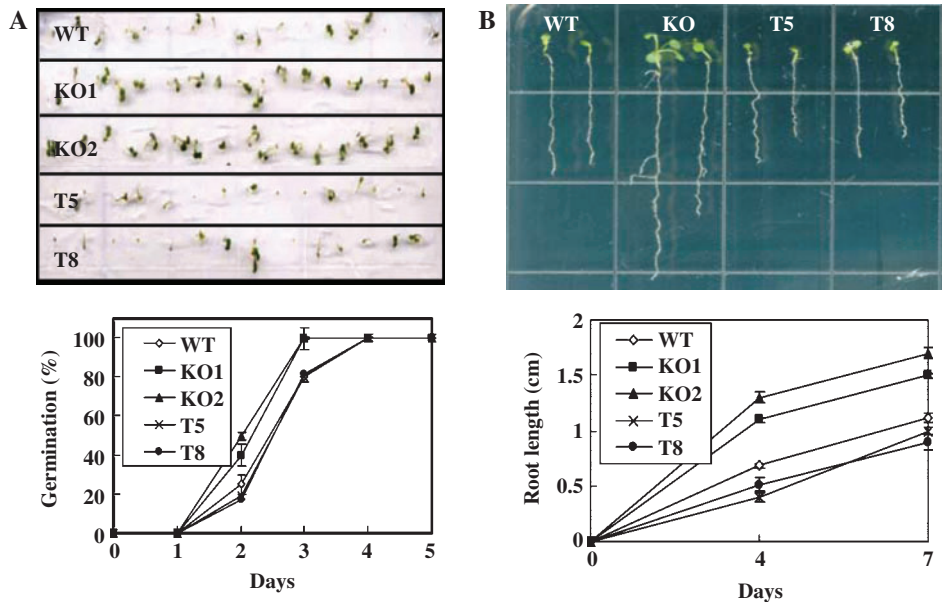

Fig. 2 Effect of dehydration stress on germination and seedling growth of wild type (WT), atRZ-1a knockout mutants (KO1 and KO2) and atRZ-1a overexpression lines (T5 and T8). (A) Seeds were germinated in MS medium supplemented with 100 mM mannitol, and germination rates were determined on the indicated days. The photograph was taken on day 7. (B) Seeds were sown on MS medium supplemented with 250 g l^{-1} (–0.5 Mpa) PEG, the plates were incubated in a vertical orientation, and root length was measured on the indicated days. Experiments were performed in triplicate ($n = 15–20$). The photographs were taken on day 10.

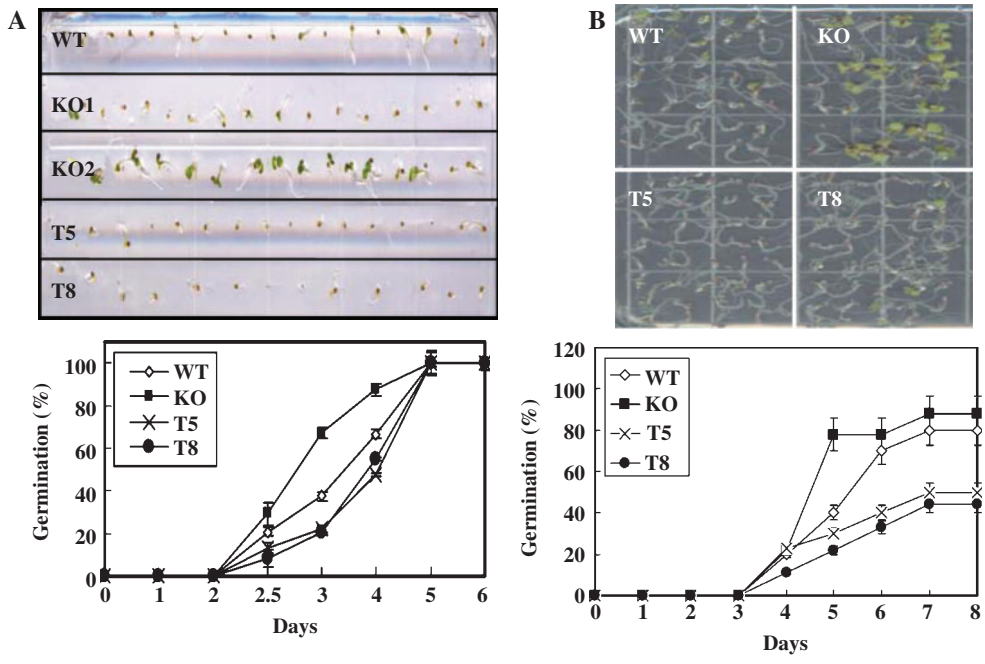

Fig. 3 Effect of ABA and glucose on germination of wild type (WT), atRZ-1a knockout mutants (KO1 and KO2) and atRZ-1a overexpression lines (T5 and T8). (A) Seeds were germinated in MS medium supplemented with (A) 1 μM ABA or (B) 6% glucose, and germination rates were determined on the indicated days. The photographs were taken on day 10.

germination and seedling growth of *Arabidopsis* under dehydration stress conditions.

Effect of ABA and glucose on germination of the transgenic plants

Because 35S::atRZ-1a plants responded to salt or osmotic stress, we next investigated the functional relationship of atRZ-1a to ABA that is involved in the adaptation process to salt and drought stress. When the seeds of the wild type, mutants and 35S::atRZ-1a plants were germinated in the presence of 1 μM ABA, the mutant lines germinated earlier than the wild type, whereas 35S:: atRZ-1a plants displayed retarded germination compared with the wild type (Fig. 3A). It was observed that, after germination, 35S::atRZ-1a plants could not develop cotyledons, whereas approximately 20% of wild-type and most of the mutant lines had opened cotyledons (Fig. 3A). Since it is believed that high levels of exogenous glucose cause ABA accumulation, which results in a delay of germination and an inhibition of seedling development (Arenas-Huertero et al. 2000), we tested the effect of glucose on germination of the seeds. Although no differences in seed germination were observed among the three genotypes when germinated in the presence of 3% glucose, the wild-type and mutant plants proceeded to the post-germination process with well-expanded green leaves but 35S::atRZ-1a plants did not develop further after germination (Fig. 3B). When the seeds were germinated in the presence of 6% glucose, germination rates for mutant, wild-type and 35S::atRZ-1a lines at day 6 were 80, 70 and 30–40%, respectively (Fig. 3B). The wild-type and mutant plants completed germination at day 11, whereas only 60% of 35S::atRZ-1a plants germinated at day 11 (data not shown). These results demonstrate that atRZ-1a-overexpressing plants displayed retarded germination in the medium supplemented with ABA or glucose.

H_2O_2 accumulates at higher levels in the transgenic plants compared with the wild-type plants under stress conditions

The production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), has been evidenced in plants during pathogen infection or under abiotic stress conditions, such as chilling, drought, salt and ozone stresses (Asada 1994). To test the possible involvement of atRZ-1a in the production of ROS in the plants subjected to salt or mannitol stress, we investigated the H_2O_2 concentration in 7-day-old wild-type and transgenic *Arabidopsis* plants under high salinity or dehydration stress conditions (Fig. 4). H_2O_2 levels increased in the wild-type, mutant and transgenic plants under salt or dehydration stress conditions. When subjected to 100 mM NaCl, H_2O_2 levels in the transgenic plants were much higher than those in the wild-type plants.

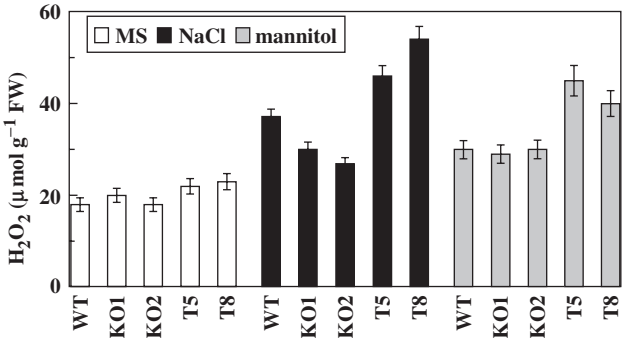

Fig. 4 Effect of salt or dehydration stress on the level of H_2O_2 in wild type (WT), atRZ-1a knockout mutants (KO1 and KO2) and atRZ-1a overexpression lines (T5 and T8). The levels of H_2O_2 were measured in 7-day-old seedlings grown in MS medium, MS medium supplemented with 100 NaCl or MS medium supplemented with 200 mm mannitol. Experiments were performed in triplicate.

In contrast, H_2O_2 levels in the mutants were lower than those in the wild-type plants. It was also observed that H_2O_2 levels in the transgenic plants were much higher than those in the wild-type plants under dehydration stress conditions. This result is consistent with the observed phenotypes in which 35S::atRZ-1a plants display retarded germination and subsequent growth compared with the wild-type plants under salt or dehydration stress conditions. It appears that atRZ-1a is involved in the regulation of H_2O_2 levels in *Arabidopsis* plants under stress conditions.

atRZ-1a modulates the expression of several germination-responsive genes under stress conditions

With the observation that atRZ-1a affected seed germination of *Arabidopsis* plants under salt stress, dehydration stress, or ABA or glucose treatment, the next important question is to determine how atRZ-1a influences germination under these conditions. Here, we investigated the changes in the transcript levels of several germination-responsive genes. The germination-responsive genes tested include myrosinase-binding protein (MBP), myrosinase (MYR), isocitrate lyase (ISO), *S*-adenosylmethionine synthetase (SAM), β-glucosidase (BGU) and phytochrome B (PHYB), the expression of which is up-regulated during germination (Yamaguchi et al. 1998, Gallardo et al. 2001), and cruciferin (CRU), 12S seed storage protein (SSP) and LEA protein in group 5 (LEAP), the expression of which is down-regulated during germination (Gallardo et al. 2001). We compared the transcript levels of germination-responsive genes in 3-day-old seedlings, because the germination rates between the three genotypes were significantly different at day 3 under stress conditions. The transcript levels of germination-responsive genes at day 3 were quite similar in the wild-type, mutant and 35S::atRZ-1a plants when grown in normal MS medium (data not shown). However, when the plants were germinated in MS medium supplemented with 100 mM NaCl, it was apparent that several genes including *MBP*, *MYR*, *SAM*, *BGU* and *PHYB* were down-regulated in 35S::atRZ-1a plants compared with the wild-type plants. In contrast, *SSP* and *LEAP* were up-regulated in 35S::atRZ-1a plants compared with the wild-type plants (Fig. 5). In the presence of 100 mM mannitol, the expression of *MBP* increased in the mutant plants, and the transcript levels of *ISO*, *SAM* and *PHYB* were decreased in 35S::atRZ-1a plants compared with the wild-type plants. When 5% glucose was added in the medium, the expression of *MBP*, *MYR*, *ISO*, *SAM* and *CRU* decreased in 35S::atRZ-1a plants compared with the wild-type plants. When the plants were germinated in MS medium supplemented with 1 μM ABA, it was apparent that several genes including *ISO*, *CRU*, *SSP* and *LEAP* were up-regulated in 35S::atRZ-1a plants compared with the wild-type plants, whereas *BGU* was up-regulated in the mutant line compared with the wild-type plants (Fig. 5). These results suggest that overexpression of atRZ-1a modulated the expression of these germination-responsive genes, which in turn resulted in altered germination of *Arabidopsis* plants under different environmental conditions.

Proteome analysis for the identification of putative target genes modulated by atRZ-1a

atRZ-1a is considered to be involved in the post-transcriptional regulation of target RNAs. It is, therefore, of critical interest to investigate and compare the expression of genes at the protein level between the transgenic and wild-type plants under salt or dehydration stress conditions. Fig. 6 shows the representative two-dimensional protein patterns of *Arabidopsis* plants subjected to salt or dehydration stress. Indicated by Arabic numbers are the protein spots that were either up- or down-regulated by >3-fold in the transgenic plants compared with the wild-type plants. These protein spots were successfully identified by MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) peptide mapping analysis (Tables 2 and 3). The level of proteins such as aconitate hydratase, ferredoxin-nitrate reductase, alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase was increased in the 35S::atRZ-1a plants compared with the wild-type plants subjected to either 100 mM NaCl or 100 mM mannitol. Phosphoglycerate mutase, chaperonin CPN60 and annexin increased, whereas glutathione *S*-transferase and thylakoid lumenal protein decreased in the 35S::atRZ-1a lines compared with the wild-type plants under salt stress conditions. When subjected to dehydration stress, the level of proteins such as glutathione reductase,

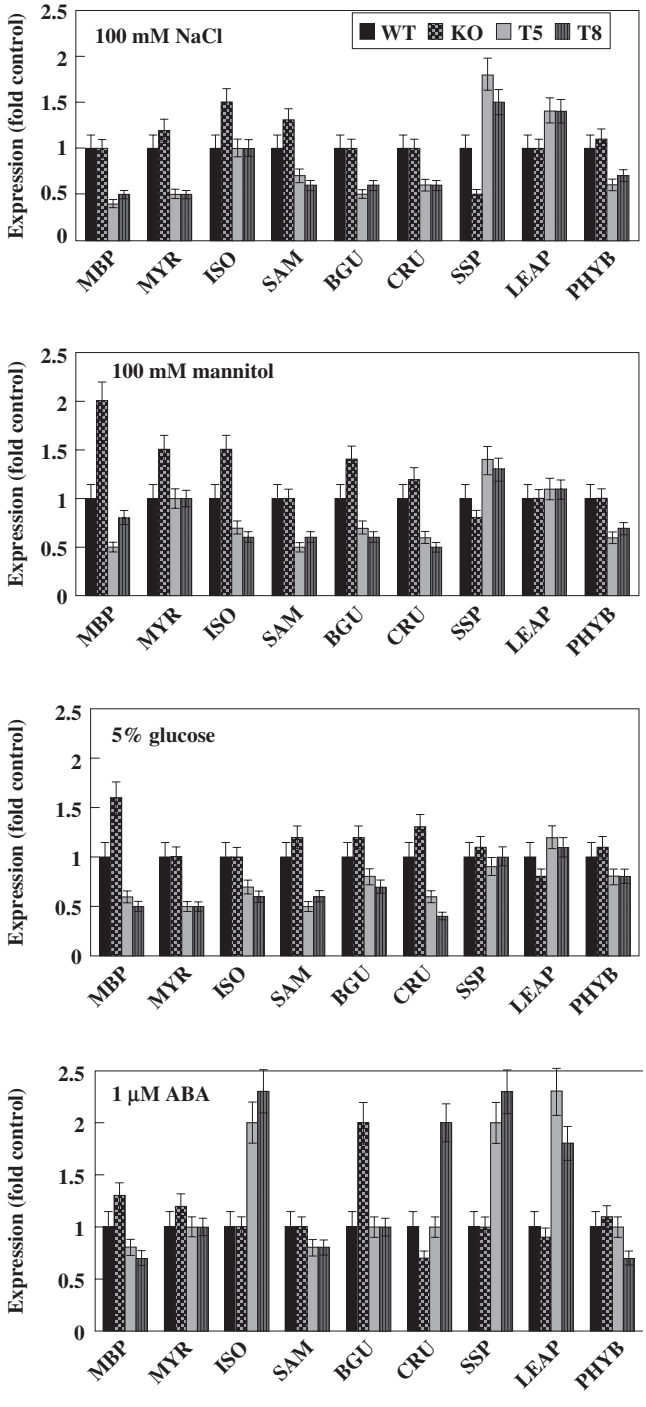

Fig. 5 Modulation of the transcript levels of germination-responsive genes in the wild type (WT), atRZ-1a knockout mutant (KO1) and atRZ-1a overexpression lines (T5 and T8). Seeds were germinated in MS medium supplemented with 100 mM NaCl, 100 mM manitol, 5% glucose or 1 μM ABA, and RNAs were extracted from 4-day-old seedlings The expression of the target genes was analyzed by real-time RT–PCR, and the results were presented as the relative expression (fold control) of each transcript in mutant and transgenic plants compared with the wild-type plants.

serine hydroxymethyltransferase, carbonic anhydrase and glutamate 1-semialdehyde aminomutase was increased in the 35S::atRZ-1a lines compared with the wild-type plants. In comparison, ribulose bisphosphate carboxylase and oxygen-evolving enhancer protein decreased in the 35S::atRZ-1a plants compared with the wild-type plants under dehydration stress conditions.

Discussion

It is apparent that atRZ-1a has a negative impact on seed germination and seedling growth of *Arabidopsis* plants under salt or dehydration stress conditions. We have previously shown that atRZ-1a, the expression of which is up-regulated by cold stress, facilitated seed germination, seedling growth and stress tolerance of *Arabidopsis* plants under cold stress conditions (Kim et al. 2005, Kim and Kang 2006). The present study demonstrates that over-expression of atRZ-1a, the expression of which is not significantly modulated by salt stress and is down-regulated by dehydration stress (Kim et al. 2005), resulted in retarded germination and seedling growth of *Arabidopsis* under salt or dehydration stress conditions (Figs. 1, 2). Given that the reports demonstrating the roles of GRPs in plant stress responses are severely limited, the present results add novel information to understand that atRZ-1a, one of the GRPs in *Arabidopsis*, affects seed germination and seedling growth of the plant under salt or dehydration stress conditions. The present report, together with our recent reports showing the roles of GRP4 on seed germination and seedling growth of *Arabidopsis* plants under salt or dehydration stress conditions (Kwak et al. 2005) and the roles of GRP2 on germination and stress tolerance of *Arabidopsis* plants under cold stress conditions (J. Y. Kim et al. 2007), clearly demonstrate that several types of GRP family members have an impact on the seed germination and seedling growth of *Arabidopsis* plants under a variety of stress conditions.

The findings that seed germination of 35S::atRZ-1a plants is retarded compared with that of the wild-type plants under salt or dehydration stress conditions and similar patterns of delayed seed germination of the transgenic plants by ABA treatment suggest that atRZ-1a affects seed germination in an 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). A cross-talk between glucose sensing and ABA signaling in germination and seedling growth has been demonstrated (Arenas-Huertero et al. 2000; Huijser et al. 2000, Laby et al. 2000); glucose acts to stimulate ABA biosynthesis in germinating seedlings by up-regulating the genes involved in ABA biosynthesis. To obtain some clues

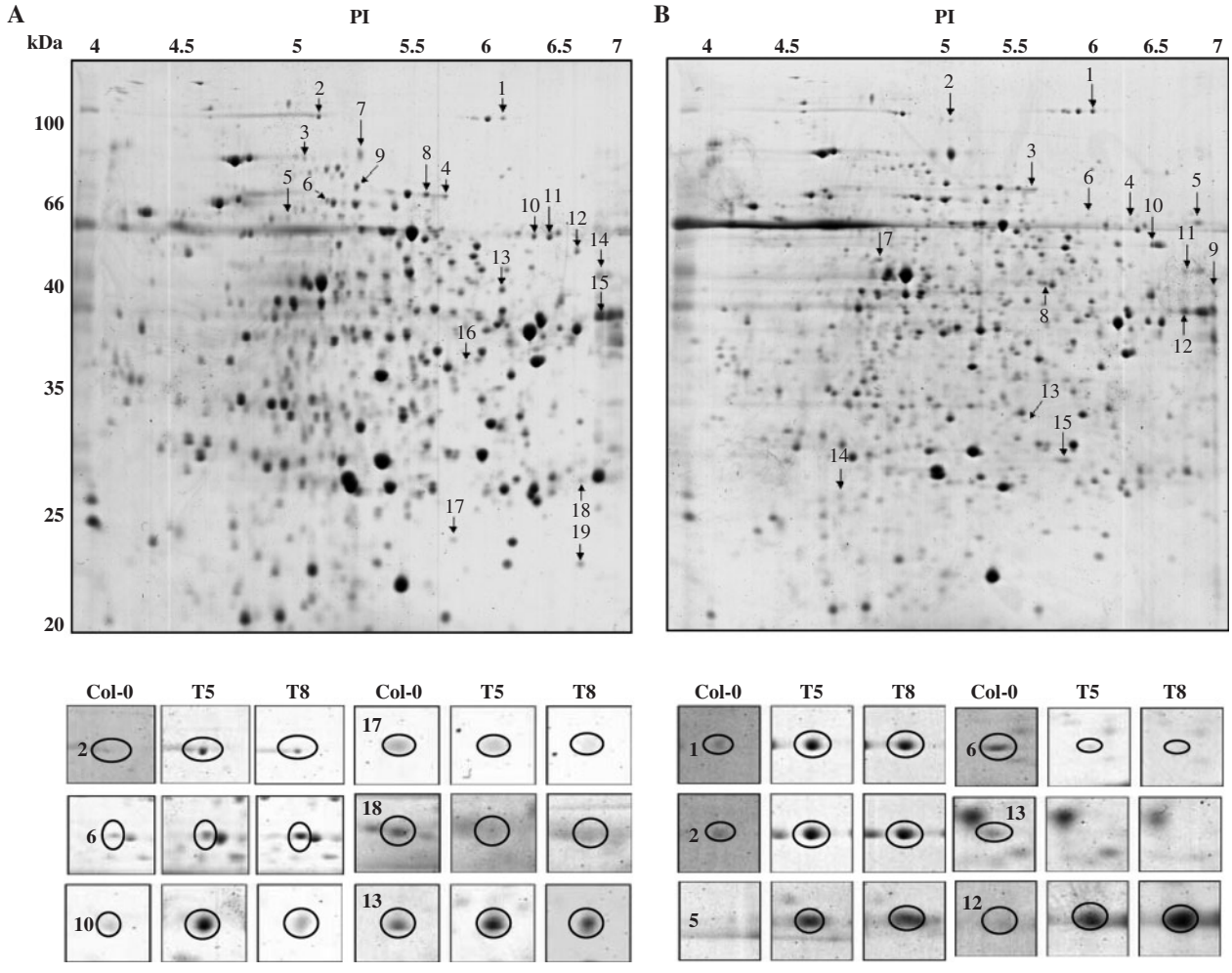

Fig. 6 Two-dimensional PAGE analysis of the protein profiles in *Arabidopsis* plants. Total proteins from 10-day-old *Arabidopsis* plants grown in MS medium supplemented with (A) 100 mM NaCl or (B) 100 mM mannitol were separated, and the protein spots whose expression increased or decreased by >3-fold in the transgenic lines compared with the wild-type plants are labeled. Examples of up- or down-regulated protein spots are shown in enlarged images. The labeled proteins are listed in Tables 2 and 3.

Table 1 Gene-specific primer pairs used in the RT–PCR experiments

Genes	Primers (5′ to 3′)	
	Forward	Reverse
MBP	ATGGGACGATGGATCGACACATG	TAGTCAACGGAGCAAAGTAAGCCC
MYR	CAAGACGGACGATCAGGATACGAG	TGAAGGATCAGGTTTCTCCAAATGG
ISO	TTGCGGAAGTGCAGACTTGGTGG	TTGCCTCCCTCTGCTTTCTGTCATG
SAM	ACCAAGGCTAACGTTGATTACGAGC	GGACACGTACAGGAACCATGGCTC
BGU	GGCTGACCAGAAGGTTGATAGTCGG	CCTAGCTGTGTACCCGTCTTGCCAC
SSP	AACAACCCACAAGGGCAGGAATG	AGCGCTAAGGCGGAGAAGTCTGAG
LEAP	ATGTGTTCGGCCATACTCAGAAAGG	CTCCAGCTAGAGCAATGACGTTGG
CRU	GATGTGTTGGTGTTTCCGTTGCTC	CATGTTCCACCTTCTGGTGCATG
PHYB	GTTTCGCAAACTCATTGTTGGAGG	ATCAGCCATTTGCTTTGAAAGTTGG
ACTIN	GGTAACATTGTGCTCAGTGGTGG	AACGACCTTAATCTTCATGCTGC

MBP, myrosinase-binding protein; MYR, myrosinase; ISO, isocitrate lyase; SAM, *S*-adenosylmethionine synthetase; BGU, β-glucosidase; SSP, 12S seed storage protein; LEAP, LEA protein in group 5; CRU, cruciferin; PHYB, phytochrome B.

about whether atRZ-1a modulates the genes involved in ABA metabolism, the transcript levels of *ABA1*, *ABA2*, *ABA3*, *ABI1* and *ABI4* were analyzed. Our preliminary analysis indicated that no differences in the transcript levels of these genes were detected between wild-type, mutant and 35S::atRZ-1a plants under salt stress, and ABA or glucose treatment (data not shown), suggesting that atRZ-1a does not influence the transcript levels of these ABA biosynthesis-related genes. However, it is premature to conclude that atRZ-1a has no effect on ABA metabolism, and determination of the amounts of ABA in wild-type and 35S::atRZ-1a plants is necessary to understand further whether the phenotypes of 35S::atRZ-1a plants observed under salt or dehydration stress are directly related to ABA.

Given that atRZ-1a affects seed germination and subsequent seedling growth of *Arabidopsis* plants under salt or dehydration stress conditions, the next important question is to understand how atRZ-1a impacts seed germination and seedling growth under stress conditions. It was apparent that atRZ-1a modulates the transcript levels of several germination-responsive genes under salt or dehydration stress conditions, and ABA or glucose treatment (Fig. 4). It is likely that atRZ-1a either binds directly to the mRNAs of these germination-responsive genes or

Table 2 Compilation of the protein spots that increased or decreased by >3-fold in the transgenic lines compared with the wild-type plants under 100 mM NaCl

Spot number	Calculated pI/M	No. of matched peptides	% Sequence coverage	Gene ID	Gene name	Modulation[a]
1	6.7/108	29	39	At2g05710	Aconitate hydratase 2, mitochondrial precursor	+
2	5.1/90	32	44	At5g03340	Cell division control protein 48 homolog E	+
3	5.1/74	20	37	At5g42020	Luminal-binding protein 2 precursor	+
4	6.0/65	18	33	At2g15620	Ferredoxin-nitrite reductase, chloroplast precursor	+
5	6.2/64	12	28	At1g55490	Rubisco large subunit-binding protein	+
6	5.7/61	17	36	At3g23990	Chaperonin CPN60, mitochondrial precursor	+
7	5.6/61	16	26	At5g26000	Myrosinase precursor	−
8	5.5/61	18	45	At3g08590	Phosphoglycerate mutase 2	+
9	5.3/61	20	45	At1g09780	Phosphoglycerate mutase 1	+
10	6.0/54	15	35	At1g16350	Probable inosine-5′-monophosphate dehydrogenase	+
11	6.2/53	17	38	At2g24270	Glyceraldehyde 3-phosphate dehydrogenase	+
12	5.7/44	18	49	At5g24040	Putative F-box/Kelch-repeat protein	+
13	5.8/41	15	44	At1g77120	Alcohol dehydrogenase	+
14	6.5/41	14	44	At5g43940	Alcohol dehydrogenase class 3	+
15	6.6/37	18	61	t3g04120	Glyceraldehyde 3-phosphate dehydrogenase	+
16	5.8/36	12	45	At5g65020	Annexin D2 (AnnAt2)	+
17	6.9/25	11	50	At3g63525	Thylakoid lumenal 19 kDa protein	−
18	6.3/24	13	69	At1g02920	Glutathione *S*-transferase 11	−
19	8.9/21	8	51	At3g06050	Peroxiredoxin-2F, mitochondrial precursor	+

Calculated pIs and M, the number of MALDI-TOF matched peptides and the percentage of sequence coverage of the protein by the matched peptides are indicated. The assigned protein of the best match is given with the GenBank accession number.
[a]Up-regulation (+) or down-regulation (–) of the expression level in the transgenic plants compared with the wild-type plants.

binds to the mRNAs of upstream protein factors that indirectly regulate the transcription of these germination-responsive genes. It is proposed that the negative effect of atRZ-1a on germination of *Arabidopsis* under salt or dehydration stress conditions arose, at least in part, from the modulation of several germination-responsive genes by atRZ-1a. Since it is believed that atRZ-1a regulates the expression of target genes at the post-transcriptional level, it is important to examine the expression at the protein level of putative genes modulated by atRZ-1a for a better understanding of the molecular mechanisms of atRZ-1a's role during stress conditions. The present proteome analysis showed that several proteins were either up- or down-regulated in the 35S::atRZ-1a lines compared with the wild-type plants under salt or dehydration stress. It was observed that several proteins such as aconitate hydratase, ferredoxin-nitrate reductase, alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase were modulated by atRZ-1a under both salt and dehydration stress conditions. In comparison, the proteins regulated by atRZ-1a under salt or dehydration stress in this work were not the same as the proteins modulated by atRZ-1a under cold stress as observed in our previous analysis (Kim and Kang 2006). These results indicate that atRZ-1a modulates the same and/or different sets of genes under salt, dehydration and cold stress, which supports the previous notion that plants respond to different stresses through both common and distinct mechanisms (Seki et al. 2003). Although we do not know presently whether all of these genes contribute to the plant's responses to salt or dehydration stress, the results imply that atRZ-1a exerts its role by modulating the expression of these putative

Table 3 Compilation of the protein spots that increased or decreased by >3-fold in the transgenic lines compared with the wild-type plants under 100 mM mannitol

Spot number	Calculated pI/M	No. of matched peptides	% Sequence coverage	Gene ID	Gene name	Modulation[a]
1	6.7/108	25	41	At2g05710	Aconitate hydratase 2, mitochondrial precursor	+
2	5.1/89	17	29	At3g09840	Cell division control protein 48 homolog A	+
3	5.9/65	22	42	At2g15620	Ferredoxin-nitrite reductase, chloroplast precursor	+
4	7.9/61	18	39	At3g54660	Glutathione reductase, chloroplast precursor	+
5	8.1/57	21	39	At4g37930	Serine hydroxymethyltransferase	+
6	5.9/53	19	32	AtCg00490	Ribulose bisphosphate carboxylase large chain	−
7	5.9/52	17	44	At2g39730	Ribulose bisphosphate carboxylase/ oxygenase	−
8	6.4/50	3	30	At5g63570	Glutamate 1-semialdehyde 2,1-aminomutase 1	+
9	8.6/44	13	38	At1g11860	Aminomethyltransferase, mitochondrial precursor	+
10	8.6/41	10	30	At2g21950	F-box/Kelch-repeat protein SKIP6	+
11	6.5/41	9	39	At5g43940	Alcohol dehydrogenase class 3	+
12	6.6/37	16	57	At3g04120	Glyceraldehyde 3-phosphate dehydrogenase	+
13	5.6/35	5	19	At5g66570	Oxygen-evolving enhancer protein 1-1	−
14	6.9/29	8	45	At3g11630	2-cys peroxiredoxin BAS1, chloroplast precursor	+
15	5.4/28	11	47	At5g14740	Carbonic anhydrase 2	+

Calculated pIs and M, the number of MALDI-TOF matched peptides and the percentage of sequence coverage of the protein by the matched peptides are indicated. The assigned protein of the best match is given with the GenBank accession number.
[a]Up-regulation (+) or down-regulation (–) of the expression level in the transgenic plants compared with the wild-type plants.

target proteins in *Arabidopsis* plant subjected to salt or dehydration stress. It was noteworthy that the proteins modulated by atRZ-1a include aconitate hydratase, glutathione S-transferase, glutathione reductase, alcohol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate mutase, serine hydroxymethyltransferase and aminomethyltransferase that have been implicated in ROS homeostasis and functions (Echave et al. 2003, Takahashi et al. 2004, Hancock et al. 2005, Moreno et al. 2005, Vanderauwera et al. 2005). It is likely that the outcome of the regulation of these putative target genes is related, at least in part, to the much higher levels of H_2O_2 in the transgenic plants compared with the wild-type plants under stress conditions (Fig. 4), which results in retarded germination and seedling growth of transgenic *Arabidopsis* plants compared with the wild-type plants under salt or dehydration stress conditions.

In conclusion, the phenotypic analyses of atRZ-1a-overexpressing transgenic plants and knockout mutants demonstrate that atRZ-1a plays a role as a negative regulator of germination and subsequent growth of *Arabidopsis* plants under salt or dehydration stress conditions. The up- or down-regulation of several germination-responsive genes and the proteins involved in control of ROS homeostasis and functions by atRZ-1a suggest that atRZ-1a exerts its roles by modulating the expression of these putative target genes. This hypothesis needs to be tested for further understanding of the action mechanism of atRZ-1a during the stress adaptation process in plants.

Materials and Methods

Plant materials

Arabidopsis thaliana ecotype Col-0 was used throughout the study. The three transgenic Arabidopsis plants (T-5, T-8 and T-9) that constitutively overexpress atRZ-1a under the control of the cauliflower mosaic virus 35S promoter were generated in our previous report (Kim et al. 2005). The transgenic plants are designated as 35S::atRZ-1a plants. The two loss-of-function *Arabidopsis* mutants of atRZ-1a (KO1, SALK_036865; and KO2, SALK_109798) were also described in our previous report (Kim et al. 2005). Plants were grown at $23 \pm 2^{\circ}C$ under long day conditions (16 h light/8 h dark cycles). Seeds harvested on the same day from individual plants grown in identical environmental conditions were used for germination and seedling growth assays.

Assays for germination and seedling growth under stress conditions

Seeds were surface sterilized with 70% ethanol for 1 min, with 2% hypochlorite for 5 min, and then rinsed with sterile deionized water. Germination assays were carried out with three replicates of 40–50 seeds. Seeds were sown on MS (Murashige and Skoog 1962) medium supplemented with 3% sucrose, and the plates were placed at $4^{\circ}C$ for 3 d in the dark and then transferred to normal growth conditions. The seeds were regarded as germinated when the radicles protruded from 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 on germination and subsequent growth, the MS medium was supplemented with 75, 100, 125 or 150 mM NaCl. To determine the effect of dehydration on germination and subsequent growth, the MS medium was supplemented with 100, 200 or 300 mM mannitol. To prepare MS medium containing PEG, an appropriate volume of PEG overlay solution (250 g of $PEG8000\,l^{-1}$) was pipetted onto the top of a solidified MS agar plate. To determine the effect of glucose on germination, the MS medium was supplemented with 3 or 6% glucose. To determine the effect of ABA on germination, the MS medium was supplemented with 1–5 μM ABA. To determine the effect of these treatments on seedling growth, the plates with seedlings were 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, Valencia, CA, USA). To remove any residual genomic DNA in the preparation, the RNA was treated with an RNase-free DNase I according to the manufacturer's instruction (Qiagen). The concentration of RNA was accurately quantified by spectrophotometric measurement, and 5 μg of total RNA was separated on a 1% formaldehyde agarose gel to check its concentration and to monitor its integrity. To determine quantitatively the transcript levels of the genes, the real-time quantification of RNA target was performed with the gene-specific primers listed in Table 1 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). All other experimental conditions were as previously described (Kim et al. 2003).

H_2O_2 measurement

The H_2O_2 level was measured as described by Jana and Choudhuri (1981). The seedlings were ground in 50 mM phosphate buffer (pH 6.8), and the homogenate was centrifuged at $6{,}000 \times g$ for 25 min. To determine the H_2O_2 level, 3 ml of the extracted solution was mixed with 1 ml of 0.1% titanium chloride in 20% (v/v) H_2SO_4, and the mixture was then centrifuged at $6{,}000 \times g$ for 15 min. The absorbance of the supernatant was measured at 410 nm, and the H_2O_2 level was calculated based on the standard curve generated using authentic H_2O_2.

Two-dimensional PAGE and proteome analysis

A 300 μg aliquot of protein sample was separated on IPG strips (13 cm, pH 4–7) and on 12% SDS–polyacrylamide gels, and other experimental procedures were essentially as described previously (Kim and Kang 2006, J. Y. Kim et al. 2007). Briefly, isoelectric focusing was performed at $20^{\circ}C$ using a Multiphor II electrophoresis unit, and PAGE was performed using a Hoefer DALT 2-D system (Amersham Biosciences, Piscataway, NJ, USA). Quantitative analysis of the images was carried out using the PDQuest software (BioRad, Hercules, CA, USA) according to the protocols provided by the manufacturer. Protein spots that deviated >3-fold in the transgenic samples compared with the control samples were selected for further analysis. The selected protein spots were digested in-gel by using modified porcine trypsin, and protein analyses were performed using an Ettan MALDI-TOF (Amersham Biosciences). The search program

ProFound developed by The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe) was used for protein identification by peptide mass fingerprinting. The spectra were calibrated with the trypsin auto-digestion ion peaks (m/z of 842.510 and 2,211.1046) as internal standards.

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References

Albà, M.M. and Pagès, M. (1998) Plant proteins containing the RNA-recognition motif. *Trends Plant Sci*. 3: 15–21.

Arenas-Huertero, F., Ayyoyo, A., Zhou, L., Sheen, J. and Leon, P. (2000) Analysis of Arabidopsis glucose insensitive mutants, gin5 and gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. *Genes Dev*. 14: 2085–2096.

Asada, K. (1994) Production and action of active oxygen species in photosynthetic tissues. *In* Causes of Photooxidative Stress and Amelioration of Defense System in Plants. Edited by Foyer, C.H. and Mulineaux, P.M. pp. 77–103. CRC Press, Boca Raton, FL.

Barrero, J.M., Rodriguez, P.L., Quesada, V., Piqueras, P., Ponce, M.R. and Micol, J.L. (2006) Both abscisic acid (ABA)-dependent and ABA-independent pathways govern the induction of *NCED3*, *AAO3* and *ABA1* in response to salt stress. *Plant Cell Environ*. 29: 2000–2008.

Bergeron, D., Beauseigle, D. and Bellemare, G. (1993) Sequence and expression of a gene encoding a protein with RNA-binding and glycine-rich domains in *Brassica napus*. *Biochim Biophys Acta* 1216: 123–125.

Burd, C.G. and Dreyfuss, G. (1994) Conserved structures and diversity of functions of RNA-binding proteins. *Science* 265: 615–621.

Carpenter, C.D., Kreps, J.A. and Simon, A.E. (1994) Genes encoding glycine-rich *Arabidopsis thaliana* proteins with RNA-binding motifs are influenced by cold treatment and an endogenous circadian rhythm. *Plant Physiol*. 104: 1015–1025.

Dunn, M.A., Brown, K., Lightowlers, R. and Hughes, M.A. (1996) A low-temperature responsive gene from barley encodes a protein with single-stranded nucleic acid-binding activity which is phosphorylated in vitro. *Plant Mol. Biol*. 30: 947–959.

Echave, P., Tamarit, J., Cabiscol, E. and Ros, J. (2003) Novel antioxidant role of alcohol dehydrogenase E from *Escherichia coli*. *J. Biol. Chem*. 278: 30193–30198.

Ferullo, J.-M., Vézina, L.-P., Rail, J., Laberge, S., Nadeau, P. and Castonguay, Y. (1997) Differential accumulation of two glycine-rich proteins during cold-acclimation of alfalfa. *Plant Mol. Biol*. 33: 625–633.

Fukami-Kobayashi, K., Tomoda, S. and Go, M. (1993) Evolutionary clustering and functional similarity of RNA-binding proteins. *FEBS Lett*. 335: 289–293.

Gallardo, K., Job, C., Groot, S.P., Puype, M., Demol, H., Vandekerckhove, J. and Job, D. (2001) Proteomic analysis of Arabidopsis seed germination and priming. *Plant Physiol*. 126: 835–848.

Gómez, J., Sánchez-Martínez, D., Stiefel, V., Rigau, J., Puigdomènech, P. and Pagès, M. (1988) A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. *Nature* 344: 262–264.

Hancock, J.T., Henson, D., Nyirenda, M., Desikan, R., Harrison, J., Lewis, M., Hughes, J. and Neill, S.J. (2005) Proteomic identification of glyceraldehyde 3-phosphate dehydrogenase as an inhibitory target of hydrogen peroxide in Arabidopsis. *Plant Physiol. Biochem*. 43: 828–835.

Higgins, C.F. (1991) Stability and degradation of mRNA. *Curr. Opin. Cell Biol*. 3: 1013–1018.

Hirose, T., Sugita, M. and Sugiura, M. (1993) cDNA structure, expression and nucleic acid-binding properties of three RNA-binding proteins in tobacco: occurrence of tissue alternative splicing. *Nucleic Acid Res*. 21: 3981–3987.

Horvath, D.P. and Olson, P.A. (1998) Cloning and characterization of cold-regulated glycine-rich RNA-binding protein genes from leafy spurge (*Euphorbia esula* L.) and comparison to heterologous genomic clones. *Plant Mol. Biol*. 38: 531–538.

Huijser, C., Kortstee, A., Pego, J., Weisbeek, P., Wisman, E. and Smeekens, S. (2000) The Arabidopsis *SUCROSE UNCOUPLED-6* gene is identical to *ABSCISIC ACID INSENSITIVE-4*: involvement of abscisic acid in sugar responses. *Plant J*. 23: 577–585.

Jana, S. and Choudhuri, M.A. (1981) Glycolate metabolism of three submerged aquatic angiosperms during aging. *Aquat. Bot*. 12: 345–354.

Kenan, D.J., Query, C.C. and Keene, J.D. (1991) RNA recognition: towards identifying determinants of specificity. *Trends Biochem. Sci*. 16: 214–220.

Kim, J.S., Kim, Y.O., Ryu, H.J., Kwak, Y.S., Lee, J.Y. and Kang, H. (2003) Isolation of stress-related genes of rubber particles and latex in fig tree (*Ficus carica*) and their expressions by abiotic stress or plant hormone treatments. *Plant Cell Physiol*. 44: 412–419.

Kim, J.S., Park, S.J., Kwak, K.J., Kim, Y.O., Kim, J.Y., Song, J., Jang, B., Jung, C.-H. and Kang, H. (2007) Cold shock domain proteins and glycine-rich RNA-binding proteins from *Arabidopsis thaliana* can promote the cold adaptation process in *E. coli*. *Nucleic Acids Res*. 35: 506–516.

Kim, J.Y., Park, S.J., Jang, B., Jung, C-H., Ahn, S.J., Goh, C-H., Cho, K., Han, O. and Kang, H. (2007) Functional characterization of a glycine-rich RNA-binding protein2 in *Arabidopsis thaliana* under abiotic stress conditions. *Plant J*. 50: 439–451.

Kim, Y.O. and Kang, H. (2006) The role of a zinc finger-containing glycine-rich RNA-binding protein during the cold adaptation process in *Arabidopsis thaliana*. *Plant Cell Physiol*. 47: 793–798.

Kim, Y.O., Kim, J.S. and Kang, H. (2005) Cold-inducible zinc finger-containing glycine-rich RNA-binding protein contributes to the enhancement of freezing tolerance in *Arabidopsis thaliana*. *Plant J*. 42: 890–900.

Kwak, K.J., Kim, Y.O. and Kang, H. (2005) Characterization of transgenic Arabidopsis plants overexpressing GR-RBP4 under high salinity, dehydration, or cold stress. *J. Exp Bot*. 56: 3007–3016.

Laby, R.J., Kincaid, M.S., Kim, D. and Gibson, S.I. (2000) The Arabidopsis sugar insensitive mutants *sis4* and *sis5* are defective in abscisic acid synthesis and response. *Plant J*. 23: 587–596.

Molina, A., Mena, M., Carbonero, P. and García-Olmedo, F. (1997) Differential expression of pathogen-responsive genes encoding two types of glycine-rich proteins in barley. *Plant Mol. Biol*. 33: 803–810.

Moreno, J.I., Martín, R. and Castresana, C. (2005) Arabidopsis SHMT1, a serine hydroxymethyltransferase that functions in the photorespiratory pathway influences resistance to biotic and abiotic stress. *Plant J*. 41: 451–463.

Moriguchi, K., Sugita, M. and Sugiura, M. (1997) Structure and subcellular localization of a small RNA-binding protein from tobacco. *Plant J*. 6: 825–834.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*. 15: 473–497.

Sachetto-Martins, G., Franco, L.O. and de Oliveira, D.E. (2000) Plant glycine-rich proteins: a family or just proteins with a common motif? *Biochim. Biophys. Acta* 1492: 1–14.

Seki, M., Kamei, A., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2003) Molecular responses to drought, salinity and frost: common and different paths for plant protection. *Curr. Opin. Biotechnol*. 14: 194–199.

Simpson, G.G. and Filipowicz, W. (1996) Splicing of precursors to mRNA in higher plants: mechanism, regulation and sub-nuclear organization of the spliceosomal machinery. *Plant Mol. Biol*. 32: 1–41.

Takahashi, S., Seki, M., Ishida, J., Satou, M., Sakurai, T., et al. (2004) Monitoring the expression profiles of genes induced by hyperosmotic, high salinity, and oxidative stress and abscisic acid treatment in

Arabidopsis cell culture using a full-length cDNA microarray. *Plant Mol Biol*. 56: 29–55.

Vanderauwera, S., Zimmermann, P., Rombauts, S., Vandenabeele, S., Langebartels, C., Gruissem, W., Inzé, D. and Van Breusegem, F. (2005) Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. *Plant Physiol*. 139: 806–821.

van Nocker, S. and Vierstra, R.D. (1993) Two cDNAs from *Arabidopsis thaliana* encode putative RNA binding proteins containing glycine-rich domains. *Plant Mol. Biol*. 21: 695–699.

Yamaguchi, S., Smith, M.W., Brown, R.G., Kamiya, Y. and Sun, T. (1998) Phytochrome regulation and differential expression of gibberellin 3 beta-hydroxylase genes in germinating Arabidopsis seeds. *Plant Cell* 10: 2115–2126.

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