Constitutive expression of the barley dehydrin gene *aba*2 enhances Arabidopsis germination in response to salt stress

Cristina Calestani,[1-3] Meena S. Moses,[1,4] Elena Maestri,[2] Nelson Marmiroli,[2] Elizabeth A. Bray[1]

[1]Department of Botany and Plant Sciences, University of California, Riverside, CA, USA; [2]Department of Biosciences, University of Parma, Italy; [3]Department of Biology, Valdosta State University, GA, USA; [4]EMD Millipore Corporation, Temecula, CA, USA

Abstract

Dehydrins (DHNs) are a sub-family of the late embryogenesis abundant proteins generally induced during development of desiccation tolerance in seeds and water deficit or salinity stress in plants. Nevertheless, a detailed understanding of the DHNs function is still lacking. In this work we investigated the possible protective role during salt stress of a *Dhn* from *Hordeum vulgare* (L.), *aba2*. The coding sequence of the *aba2* gene was constitutively expressed in transgenic lines of *Arabidopsis thaliana* (L.). During salt stress conditions germination rate, cotyledon expansion and greening were greatly improved in the transgenic lines as compared to the wild type. Between 98 and 100% of the transgenic seeds germinated after two weeks in media containing up to 250 mM NaCl, and 90% after 22 days at 300 mM NaCl. In conditions of 200 mM NaCl 93% of the transgenic cotyledons had greened after two weeks, outperforming the wild type by 45%. Our study provides further evidence that DHNs have an important role in salt stress tolerance. The production of plants constitutively expressing DHNs could be an effective strategy to improve plant breeding programs.

Introduction

Salinity stress can impact plant growth and development at all stages of the plant life cycle.

Plants respond to abiotic stress in many ways that are controlled by complex regulatory processes functioning at cellular and whole-plant levels, which are still not completely understood. A major response is the change in gene expression and synthesis of different types of proteins like those encoded by dehydrins (DHNs), a sub-family of group 2 Late Embryogenesis Abundant (LEA D-11).[1-3] DHNs are a distinct group of proteins, which may accumulate up to 1% of total soluble proteins in mature embryos and in response to stresses involving cellular dehydration.[1-3]

DHNs are conserved proteins, being identified from higher to lower plants. Presently, public databases contain over 4300 deduced amino acid sequences encoding DHNs (http://www.ncbi.nlm.nih.gov/protein/?term=dehydrin). Recently, DHNs have also been identified in animal taxa such as rotifers, tardigrades, insects and nematodes.[4] The molecular weights of DHNs vary within a broad range from 9 to 200 kDa.[5,3] DHNs have been classified into different sub-groups based on their amino acid composition and domain architecture.[1,5-8] Typically DHNs have one to 11 copies of a highly conserved domain of 15 amino acids, called the K segment.[5,3] The K segment is predicted to form an amphipathic a-helix and it is required for binding to anionic phospholipid membranes, supporting the hypothesis that DHNs stabilize cell membranes during dehydration.[5,9-11] Many DHNs have a tract of serine residues, called the S-segment,[5] which has been proposed to be a site of protein activity regulation through phosphorylation.[12-15] Another conserved domain, the Y-segment, is located near the N-terminus of several DHNs.[5,3] Previous studies suggests that the Y-segment has a role in the protection from desiccation and salt stress.[16]

Although much is known about dehydrin gene structure and expression patterns, their function has not been completely elucidated.[2,11] Data obtained through different methods of investigation support the hypothesis that DHNs protect the cell from osmotic and ionic stress. First, LEA protein accumulation correlates with the development of desiccation tolerance of seeds,[17-20] and also occurs during periods of environmental stress in vegetative parts of the plant.[21] Second, a greater DHN level is positively associated with an increased degree of stress resistance.[22-30] Enhancement of tolerance to salinity, drought and osmotic stress has also been observed in transgenic Arabidopsis plants overexpressing the maize *Rab17* or the wheat dehydrin *Dhn5*.[22,23,26] *Dhn5* and *Rab17* are both YSK$_2$ DHNs homologous to the barley *aba2*.[21] Third, several *Dhn* genes have been mapped within confidence intervals of QTLs associated with dehydration and low temperature tolerance in cereals and other plants.[6,31,32] The *Dhn* gene used in this study, *aba2*, maps on the long arm of chromosome 5H of barley within a major QTL controlling freezing tolerance,[32,33] on the 5AL chromosome of wheat within the confidence interval of a QTL associated with abscissic acid (ABA) accumulation during drought stress,[34] and with different traits determining salt tolerance.[35]

In this work we investigated the biological function of a *Dhn* from *Hordeum vulgare* (L.), *aba2* (National Center for Biotechnology Information accession number CAA66970), and specifically its possible protective role during salt stress. ABA2 was originally identified in barley coleoptiles, and its expression was induced by treatment with ABA.[36] By using a transgenic approach, we constitutively expressed *aba2* in *Arabidopsis thaliana* with the goal to unveil, if present, a role of *aba2* in protecting from the damages of salt stress. The *aba2* is the cv. Georgie allele of the barley gene

Dhn1 and it codes for a protein with a molecular mass of 22 kDa.[1,7] We tested germination, cotyledon expansion and greening, and seedling growth on medium containing NaCl concentrations that were restrictive to the Arabidopsis control plants.

When evaluating the effects of the heterologous expression of the barley *aba2* in Arabidopsis plants, it must be considered whether the observed effects result from engineering a novel gene into the Arabidopsis genome or from a change in the expression pattern of a *Dhn* with a function similar to Arabidopsis' native *Dhn* genes. For this purpose we obtained a phylogenetic tree including ABA2 and the native DHNs encoded in the Arabidopsis genome.

Materials and Methods

Construction of the plasmid vector for plant transformation

The *Eco*RI-*Nhe*I 530 bp fragment containing the *aba2* cDNA was isolated from the plasmid pGEM-3Zf+-ABA2, filled-in with DNA polymerase I Klenow fragment, and ligated into the *Spe*I site of pBlueScript II KS+ (Stratagene, now Agilent Technologies, Santa Clara, CA, USA), resulting in the plasmid pBSA2. To construct pCGN18A2, the *Bam*HI-*Xba*I fragment from pBSA2 was cloned between the *Bam*HI and *Xba*I sites of the binary vector pCGN18,[37] downstream of the constitutive promoter CaMV 35S.

Production of transgenic plants

The plasmid construct pCGN18A2 was transformed into *Agrobacterium tumefaciens*, strain ASE.[38] Transformed *A. tumefaciens* was selected on chloramphenicol (30 µg/mL[-1]), gentamicin (25 µg/mL[-1]), and kanamycin (50 µg/mL[-1]). Seeds of *Arabidopsis thaliana* (L.) ecotype Landsberg *erecta* (L*er*) were sown on a soil mixture of fine vermiculite, peat moss and coarse vermiculite (1:2:1 v v[-1]), saturated with modified Hoagland's solution (3.0 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4$, 0.5 mM KH_2PO_4, 68.0 mM Fe-EDTA, 23.0 mM H_3BO_3, 4.55 mM $MnCl_2$, 0.385 mM $ZnSO_4$, 0.16 mM $CuCO_4$, and 0.04 mM Na_2MoO_4.[39] Seeds were stratified for 3 days in the dark at 4°C. Plants were grown in 0.1 L pots with 2-3 plants per pot at 23°C, with fluorescent light at 150 mEm[-2]s[-1] and a photoperiod of 16 h. Four-week-old plants were transformed by vacuum infiltration of *A. tumefaciens* carrying the pCGN18A2 plasmid.[40] Seeds produced by the infiltrated plants (T[1] generation) were surface sterilized and sown in Petri dishes on solid Murashige and Skoog (MS) medium,[41] containing 50 µg/mL[-1] of kanamycin. Kanamycin-resistant

plants were transferred to soil and grown as described above.

Analyses of ABA2 protein synthesis in transgenic plants

ABA2 protein accumulation was analyzed in kanamycin-resistant three weeks old Arabidopsis plants of the T[2] and T[4] generation (Figure 1). Plants were grown in 0.1 L pots with 2-3 plants per pot. Total proteins were extracted from fresh leaf material (50-100 mg) by homogenizing in ice-cold 50 mM sodium borate and 50 mM citric acid buffer, pH 9, with 1 mM phenylmethanesulfonyl-fluoride (PMSF), followed by centrifugation at
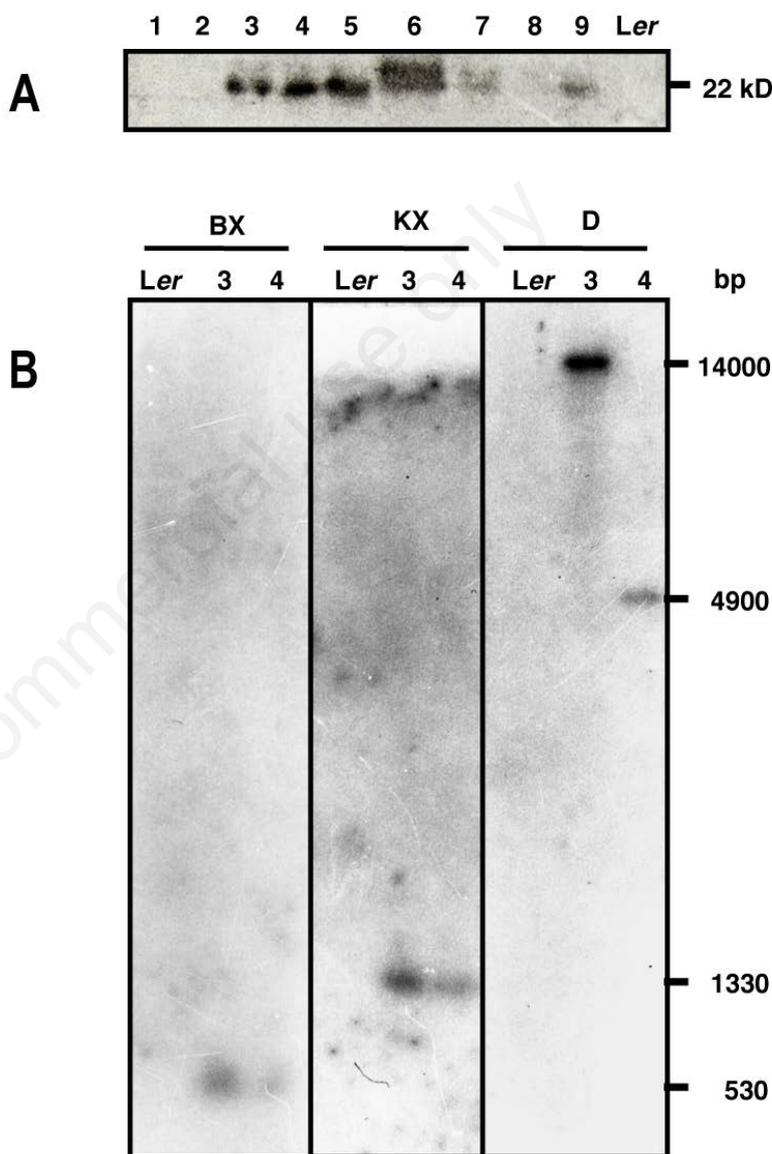
Figure 1. Characterization of ABA2 expression in Arabidopsis transgenic lines. A) Immunoblot analysis of ABA2 protein accumulation in leaves of transgenic Arabidopsis plants. Transgenic lines A2-1 through 9 (lanes 1-9) and L*er* (non-transformed plants) are shown. B) Southern blot analysis of genomic DNA isolated from transgenic plants (T3 generation) hybridized with a ^{32}P-labeled *aba2* cDNA. Genomic DNA was digested with *Bam*HI and XbaI (BX) to release a 530 bp *aba2* sequence; *Kpn*I and XbaI (KX) to release a 1330 bp fragment corresponding to the 35S CaMV promoter and the *aba2* sequence; and *Dra*I (D) which cuts once within the T-DNA sequence. The sizes of individual bands expressed in bp are indicated on the right. L*er*, non-transformed plants; 3, transgenic line A2-3; 4, transgenic line A2-4.

$12000 \times g$ for 10 min at 4°C. Proteins were also extracted from 10 mg of seedlings using the same procedure. Seedlings were extracted when the cotyledons began to expand: after 2 days on MS and after 4 days on MS supplemented with 150 mM NaCl (Figure 2). Protein concentration was determined using the Bradford Assay (Bio-Rad Laboratories). For immunoblot analyses, 10 μg of total protein extract was separated by 13% acrylamide SDS-PAGE using Mini-Protean II electrophoresis cells (Bio-Rad Laboratories) and electrophoretically transferred onto nitrocellulose membrane (Micron Separations, Westborough, MA, USA) with Mini Trans-Blot Cells (Bio-Rad Laboratories). After transfer, the nitrocellulose membrane was blocked with 3% (w v^{-1}) gelatin in Tris-Buffered Saline (TBS; 50 mM Tris-Cl, 150 mM NaCl, pH 7.5). Polyclonal antibodies raised against the carboxy terminal consensus peptide of DHN were used to recognize ABA2.[42] The secondary antibody, which binds to the primary antibody, was a goat anti-rabbit IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates, Inc., Birmingham, AL). A colorimetric detection was performed by incubating the membrane in 0.05% (w v^{-1}) 4-nitroblue tetrazolium chloride and 0.05% (w v^{-1}) 5-bromo-4-chloro-3-indoyl-phosphate (Sigma-Aldrich, St. Louis, MO, USA). In a control experiment for antibody specificity, the antiserum was incubated with the DHN carboxy terminal peptide for 30 min at 37°C prior to incubation with the nitrocellulose membrane and no bands were observed (data not shown). Proteins molecular weight were derived by using the Prestained SDS-PAGE Standards Low Range (BioRad Laboratories).

Southern blot analyses

Genomic DNA was extracted from 200-500 mg of one-week-old seedlings using a method modified from Doyle and Doyle.[43] Fresh tissue was homogenized in 300 μL extraction buffer [2.5% (w v^{-1}) sorbitol, 1% (w v^{-1}) N-lauroyl sarcosine, 0.8% (w v^{-1}) CTAB, 0.8 M NaCl, 20 mM EDTA, 1% (w v^{-1}), pH 8] and incubated for 30 min at 65°C. Samples were extracted once with phenol:chloroform (1:1 v v^{-1}) and ethanol precipitated following standard procedures.[43] For DNA blot analyses, 0.6 μg of genomic DNA of transgenic and control plants were restricted with BamHI and XbaI, with KpnI and XbaI, and with DraI. Restricted DNA was size-fractionated on a 0.8% agarose gel and transferred to nylon membrane (Hybond-N$^+$ Amersham, GE Healthcare, Piscataway, NJ, USA) using an alkaline DNA blot transfer method.[44] The aba2 cDNA was ^{32}P-labeled using the Oligolabeling Kit (Pharmacia Biotech, Piscataway, NJ, USA) and utilized for DNA hybridization.[45]

Germination and seedling growth analyses in response to salt stress

Germination was analyzed on T$_4$ seeds of transgenic lines and non-transformed Ler plants grown at the same time in the same growth chamber in approx. 0.1 L pots with 2-3 plants per pot. Seeds of each genotype were surface-sterilized with 95% ethanol for 5 min followed by 20 min in 30% bleach and sown on solid MS salt basic medium,[41] with 0.3% sucrose, supplemented with increasing concentrations of NaCl from 150 to 300 mM, at 50 mM intervals. Approximately 50 seeds of a transgenic line and 50 seeds of Ler were sown in the same Petri dish. The Petri dishes with the seeds were incubated for 3 days at 4°C in the dark and then transferred to an incubator at 23°C, with light at 150 mEm^{-2}s^{-1} and a photoperiod of 16 h. Petri dishes were kept in a vertical position. Germination percentage, measured as seeds at radicle emergence stage, and cotyledon greening was monitored at daily intervals in four replicate Petri dishes. In Figure 2 we choose to test 150 mM salt concentration because at 200mM the non transgenic seedlings would become quickly chlorotic and die. The proportions of germinating seeds determined in two independent experiments were analyzed using the Minitab Probit Analysis (Minitab Inc., State College, PA, USA; Release 13.32) in which the factor considered was cumulative germination percentage on each day (time to germination). Two independent experiments were performed. Experiment 1 had four replicates for each NaCl concentration with either transgenic A2-3 or A2-4 and the wild type plated on each plate. Experiment 2 had three replicates for each NaCl concentration. The estimated mean times to germination were analyzed as growth models. The estimated mean time to germination differences between the wild type (C) and either of the mutants (A2-3 or A2-4) were tested using a two-sample t-test assuming unequal variance.

Phylogenetic analysis

Proteins containing the conserved lysine-rich domain K were identified in the Arabidopsis genome from Pfam (http://pfam.xfam.org//family/PF00257#tab-view=tab7) and verified in TAIR (https://www.arabidopsis.org/).The nine Arabidopsis DHNs amino acid sequences, the barley ABA2/DHN1, the wheat DHN5 and the maize RAB17 were aligned using ClustalW version 2.1 (http://clustalw.ddbj.nig.ac.jp/)[46]A neighbor-joining tree was constructed using FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/).

Results

Production of transgenic Arabidopsis plants constitutively expressing aba2

The level of accumulation of the 22 kDa ABA2 protein in unstressed leaves was analyzed for nine transgenic lines (Figure 1A). Line 3, 4 and 5 showed the highest level of ABA2 accumulation; line 6,7 and 9 accumulated ABA2 to a lesser extent, with line 6 showing an additional protein of 23 kDa; line 1, 2 and 8 did not show any detectable accumulation of ABA2 (Figure 1A). Lines 3 and 4 (A2-3 and A2-4) were used for further studies. Both lines A2-3 and -4 have the aba2 gene inserted at a single locus and they were produced from inde-
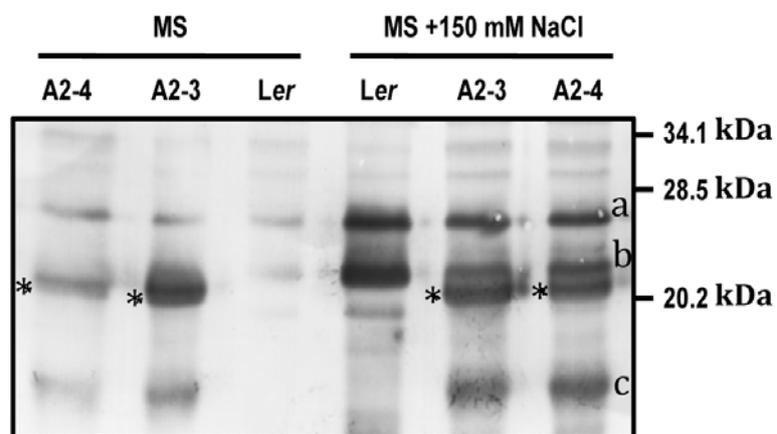

Figure 2. Immunoblot analyses of the accumulation of ABA2 and other DHNs in seedlings of transgenic lines and Ler. Seeds of control (Ler) and transgenic plants (A2-3 and A2-4) were germinated on MS or MS with 150 mM NaCl. DHNs having the same molecular mass as ABA2 are indicated (*).

pendent transformation events as verified by genomic DNA blot analyses (Figure 1B).

Constitutive expression of *aba2* enhances salt-stress resistance during seed germination

Seed germination on media containing NaCl, measured as percentage of radicle emergence, was compared between seeds from the T_4 generation of two independent transgenic lines (A2-3 and A2-4) and the wild type plants (Figure 3, Table 1). At 200, 250 and 300 mM NaCl, the final germination percentage was greater for the transgenic lines than the wild type (Figure 3B-D, Table 1). On control MS medium, the final germination percentage for the wild type L*er* was 92% after 2 days and 100% for both transgenic lines (Figure 3A). Up to 250 mM NaCl, both transgenic lines achieved between 98% to 100% germination, while the wild type a maximum of 85.2% and 70.6% on 200 and 250 mM NaCl respectively, after 12-14 days (Figure 3B,C). When seeds were imbibed and germinated on 300 mM NaCl, the transgenic line reached 90% germination after 20 days whereas after the same period of time the germination percentage for L*er* was 45% (Figure 3D).

The transgenic lines overexpressing ABA2 showed a decreased time to germination in response to salt stress. The mean time to germination, as estimated by probit analysis, was shorter for the two transgenic lines than for the wild type when seeds were exposed to 200, 250 and 300 mM NaCl (Table 1, Experiment 1). An additional replicate experiment showed similar results (germination time courses are not shown; Table 1, Experiment 2). There are no significant differences between the final germination percentages of the A2-3 and A2-4 transgenic lines.

Cotyledon emergence and greening was measured as a cumulative percentage at daily intervals (Figure 4). On MS medium, cotyledons had emerged by Day 2 in both the wild type and transgenic lines, however, 15% of the wild type cotyledons did not green (Figure 4A). Exposure to 200 mM NaCl reduced greening to 48% in the wild type seedlings whereas 91-93% of the transgenic cotyledons were greened after 2 weeks (Figure 4B,C).

To verify the accumulation of ABA2 in stressed and non-stressed seedlings, immunoblot analyses were done on proteins extracted from the transgenic and control seedlings sown on MS media or MS media with 150 mM NaCl (Figure 2). The ABA2 protein was present in the transgenic lines in stressed and non-stressed conditions while it was absent in L*er*. The immunoblot analysis also detected the native DHNs, which as expected accumulated at higher levels under salt stress conditions (Figure 2, bands a, b and c). It
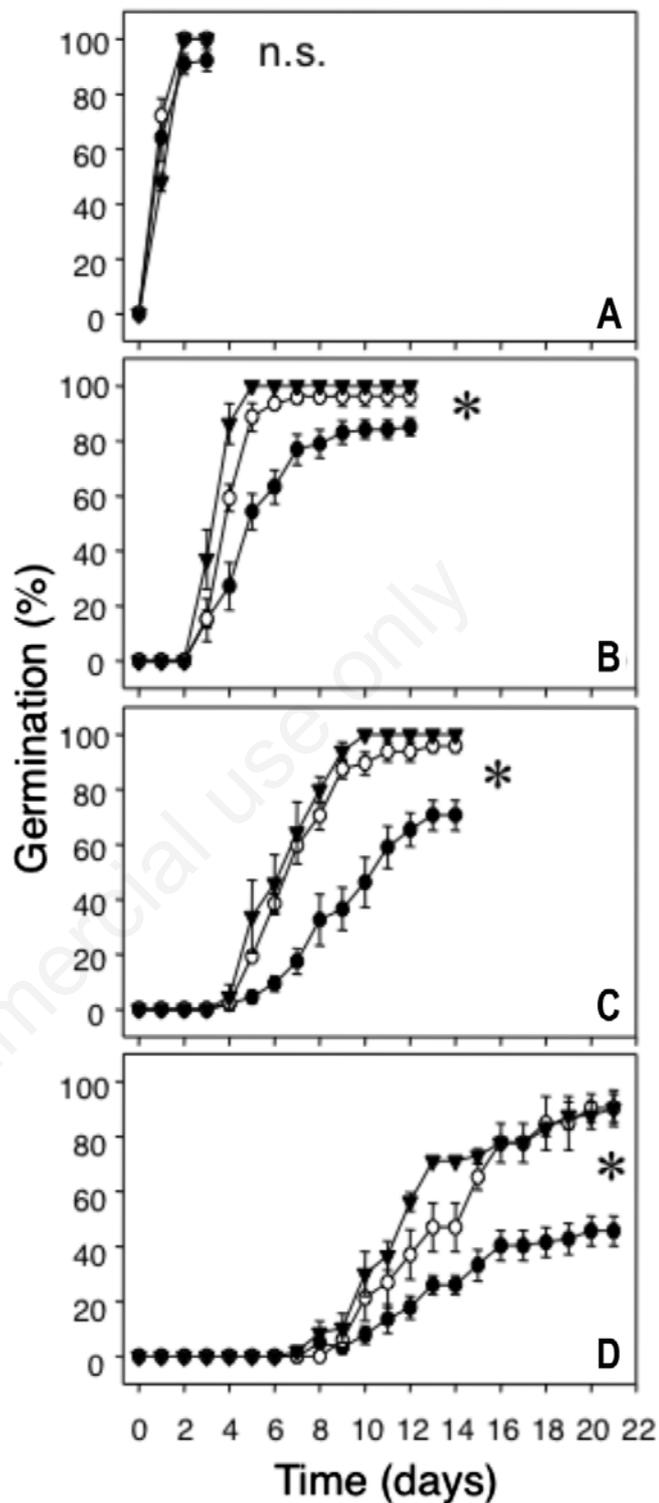

Figure 3. Germination time course of transgenic seeds constitutively expressing *ABA2* and control L*er* seeds during salt stress. Seeds of the transgenic plants (T4 generation) A2-3 (○), A2-4 (▼) and non-transformed L*er* plants (●) were plated on MS media (A; after the second day there was no change in percent germination in the control conditions, data not shown) and on MS supplemented with different concentrations of NaCl (B, 200 mM; C, 250 mM; D, 300 mM). Approximately 50 seeds of a transgenic line and L*er* were plated in the same Petri dish; three replicate plates were analyzed for each transgenic line at each NaCl concentration. Germination was measured as a cumulative percentage of radicle emergence at daily intervals for 3 weeks. Data are the average germination percentage ± s.e. Significant differences of the final germination percentages are indicated (*). A representative experiment out of five independent experiments is shown.

should be noted though that under stress some of the native DHNs appear to accumulate at lower levels in the transgenic lines as compared to Ler (Figure 2, bands a and b). Interestingly, an additional protein, smaller than 20 kDa, was present in the transgenic lines only, and it was expressed at higher levels during salt stress conditions (Figure 2, band c).

The barley ABA2 is closely related to Arabidopsis dehydrins

The barley ABA2 protein sequence was phylogenetically compared to the native DHNs encoded in the Arabidopsis genome, to the wheat DHN5 and to the maize RAB17. The Arabidopsis genome contains nine genes that code for DHN proteins, as defined by the presence of the conserved K segment.[5] To determine the phylogenetic relation between the barley gene ABA2 and the Arabidopsis genes, a neighbor-joining tree of the nine Arabidopsis was built (Figure 5). Two main clades were identified: one containing the Y_nSK_2 proteins and the other with the SK_{2-3} proteins. As predicted, ABA2 was in the clade with Y_nSK_2 proteins from Arabidopsis, DHN5 and RAB17. Arabidopsis *Dhn* genes can be grouped into three types based on the presence of previously identified amino acid domains: four YSK_2, four SK_{2-3} and one K_6. A new domain was identified in the Arabidopsis SK type genes with the consensus RGL/MFDFLXKKXEEVXE.

Discussion

In this study, we produced transgenic *A. thaliana* plants constitutively expressing the barley *aba2* gene. The transgenic lines had greater resistance to salt stress during seed germination than did the wild type. The presence of a constitutively expressed DHN in Arabidopsis correlated with an increase of the final germination percentage in salt stress conditions at 200 mM NaCl and above (Figure 3), and a decrease of the mean time to germination as compared to the wild type at all the NaCl concentrations tested (Table 1). The transgenic plants reached 90-100% final germination in salt stress conditions, while the wild type plants underperformed by 15%, 30% and 45% as the NaCl concentration in the growth media was increased (Figure 3).

According to our results, it seems unlikely that the observed effects are caused by novel functional aspects of the barley ABA2 transferred into the Arabidopsis genome. In fact, Arabidopsis has nine DHNs, four of which have the same Y_nSK_2 domain structure as ABA2 and two of which cluster into the same clade with ABA2 (Figure 5). Although this does not

ensure identical function, it does indicate that they are closely related and are likely to have the same role.

We also observed that seed lots produced at

different time varied in NaCl tolerance during germination, but this did not alter the difference between the genotypes Ler, A2-3 and A2-4. The transgenic seeds expressing the barley
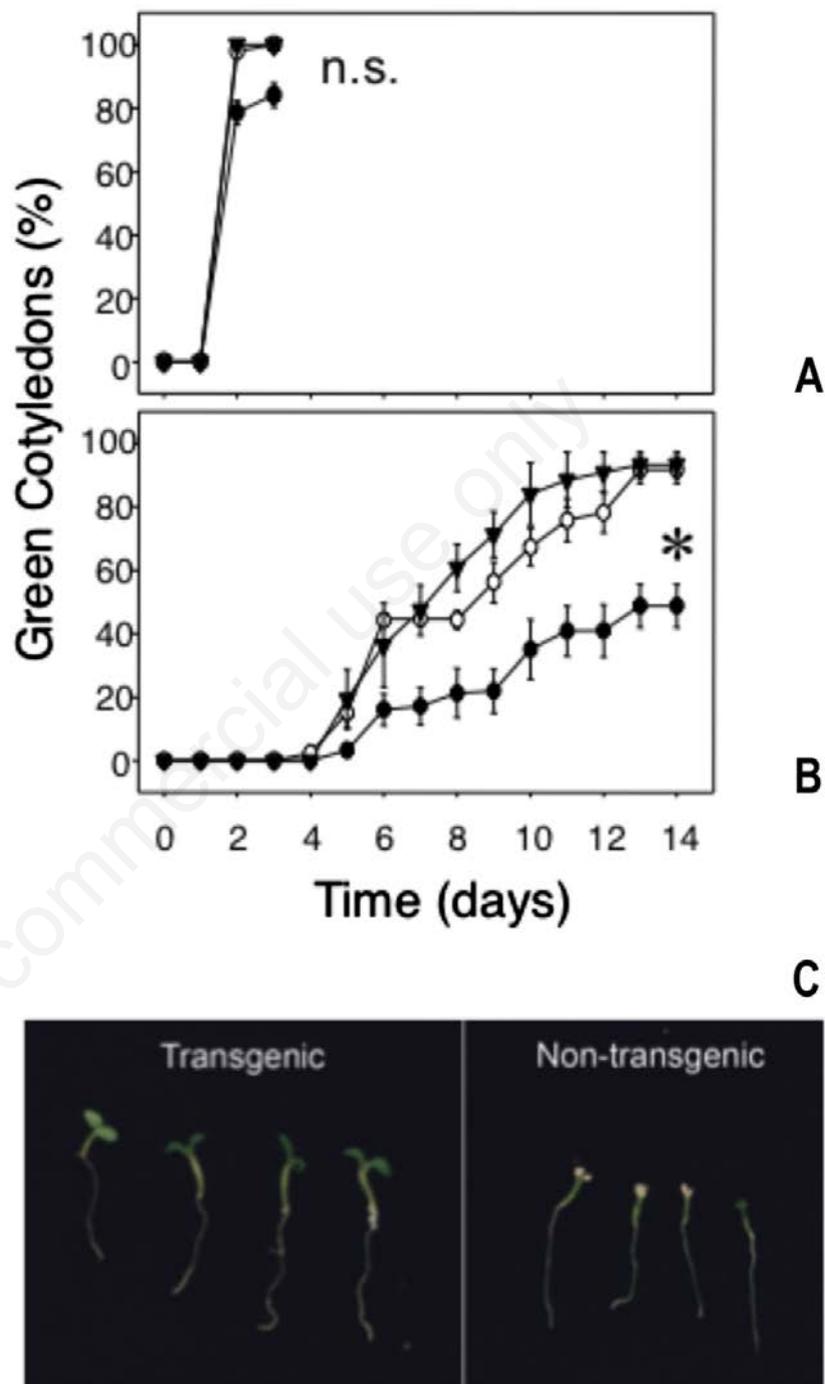

Figure 4. Cotyledon greening time course of transgenic lines constitutively expressing ABA2 and control L*er* seeds during salt stress. Cotyledon greening of germinated seeds of the transgenic plants A2-3 (○) and A2-4 (▼), and of non-transformed Ler plants (●) was recorded as a cumulative percentage at daily intervals. Seeds were plated on MS media (A; after day three there was no change in percent germination, data not shown) or MS supplemented with 200 mM NaCl (B). The average percentage ± s.e of seedlings with green cotyledons is shown. Significant differences of the final cotyledon greening percentages are indicated (*). C) A representative sample of one-week old L*er* and transgenic seedlings grown on 150 mM NaCl.

aba2 had a greater ability to germinate than wild type seeds produced at the same time in the same growth conditions in all of the seed lots tested. It has previously been reported that NaCl tolerance of Arabidopsis seeds at germination is variable. Salt tolerance of seeds appears to vary with plant nutrition as well as the length of time seeds are permitted to develop before drying.[47] Natural variation for salt tolerance at germination has also been recently described in Arabidopsis.[48]

Therefore, our data suggest that the constitutive expression of *aba2* directly or indirectly improves germination in response to salinity. Transgenic Arabidopsis plants overexpressing the maize *Rab17* or the wheat dehydrin *Dhn5* also shown an enhancement of tolerance to salinity, drought and osmotic stress.[22,23,26] In the Rab17 study salt stress was applied to 1-week-old seedlings, therefore the effects of the DHNs overexpression on germinating seeds were not tested. Whereas the wheat *Dhn5*, expressed in a Columbia ecotype background, did show a similar level of germination rate improvement as compared to our study with the barley ABA2 expressed in a L*er* ecotype.[22] Transgenic approaches employing the overexpression of a DHN have also been tested in plants other than Arabidopsis. A recent study shows that the constitutive expression of the tomato dehydrin *tas14* in tomato improves salt and drought tolerance.[30] In this case, the effects on germination were not tested. Transgenic plants over-expressing *tas14* accumulate ABA earlier and at higher levels, and counteract the osmotic stress by intracellular accumulation of K+, Na+ and sugars.[30] This suggests that the constitutive expression of Y_nSK_2 DHNs in transgenic plants may be an effective approach for the enhancement of salt tolerance in different plant species.
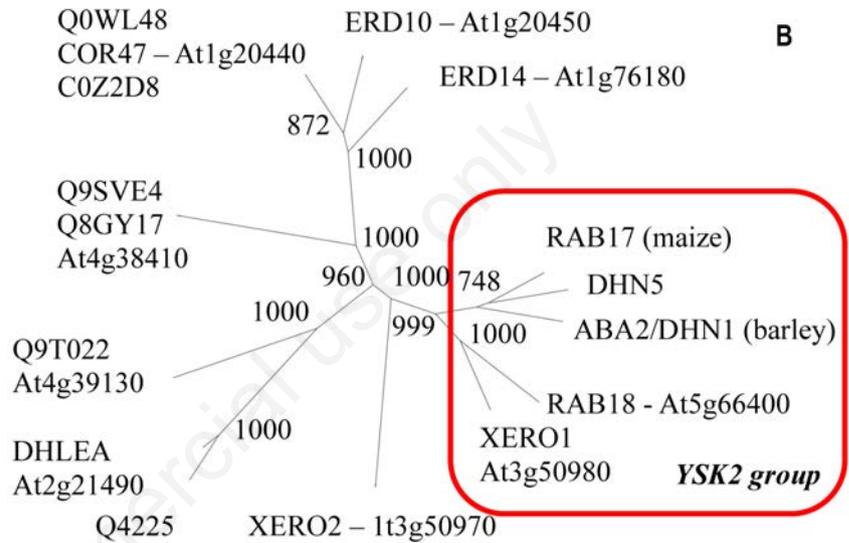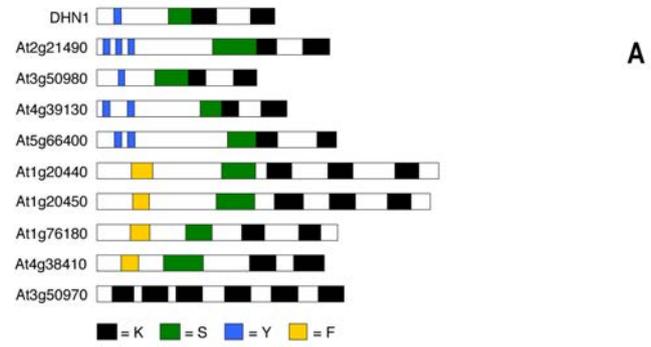

Figure 5. Phylogenetic relationships among the barley ABA2 and Arabidopsis DHNs. A) Protein conserved domains comparison of ABA2 and nine DHNs identified in the Arabidopsis genome. The Arabidopsis loci names are indicated and the DHNs conserved domains K, S, Y, and F are color-coded. B) Unrooted neighbor-joining tree of the barley ABA2 and the Arabidopsis DHNs. Bootstrap values are shown.

Table 1. Statistical analysis of the time to germination during salt stress conditions.

NaCl (mM)	Mean C (SE_C)	Mean A2-3 (SE_{A2-3})	Mean A2-4 (SE_{A2-4})	z-value A2-3[A]	P-value A2-3	z-value A2-4[A]	P-value A2-4
Experiment 1							
0	B	B	B	-	-	-	-
200	3.50 (0.61)	1.83 (0.01)	1.21 (0.16)	2.71, df=5	0.042*	3.61, df=5	0.015*
250	7.70 (0.65)	3.95 (0.14)	3.21 (0.48)	5.62, df=5	0.002*	5.54, df=6	0.001*
300	14.06 (1.4)	8.05 (0.95)	6.84 (0.18)	3.61, df=6	0.011*	5.26, df=5	0.003*
Experiment 2							
0	0.71 (0.11)	0.87 (0.24)	B	−0.61, df=1	0.653	-	-
150	3.08 (0.03)	2.43 (0.28)	2.48 (0.10)	2.26, df=2	0.152	5.57, df=3	0.011*
200	5.47 (0.23)	4.14 (0.06)	5.39 (0.51)	5.66, df=7	0.001*	0.14, df=4	0.896
250	10.60 (0.49)	8.63 (0.12)	8.68 (0.55)	3.91, df=7	0.006*	2.62, df=7	0.035*
300	27.61 (2.00)	17.39 (1.10)	17.89 (0.38)	4.49, df=9	0.002*	4.80, df=7	0.002*

The estimated mean times to germination were analyzed as growth models using the Minitab Probit Analysis. Indicated in parenthesis are the standard errors for the controls (SE_C), and the transgenic lines A2-3 or A2-4 (SE_{A2-3}, SE_{A2-4}). Significant differences of the estimated mean time to germination between the wild type (C) and either of the mutants (A2-3 or A2-4) are indicated (*P<0.05).
[A]The z-value is given by:

$$\frac{\hat{\mu}_c - \hat{\mu}_A}{\sqrt{SE_c^2 + SE_A^2}}$$

[B] The values of the estimates of mean time to germination did not converge: no tests possible for this level of salt treatment. μ_c equals the mean of the combined controls and μ_A is the mean of the transgenic line A2-3 or A2-4.

Interestingly, in addition to the constitutive expression of the barley *aba2,* the transgenic plants we produced showed the salt-stress induction of an additional protein recognized by the DHN antibody, which is not present in the wild type plants in control or salt stress conditions (Figure 2, band c). We cannot exclude that the enhancement of the salt tolerance observed in our transgenic plants is actually the results of the combined effects of the barley ABA2 and the additional stress-induced native DHN. In this regard, similar results were also observed in transgenic Arabidopsis plants overexpressing the wheat *Dhn5* or the maize *Rab17*.[23,26] The transcriptome analysis of the *Dhn-5* transgenic plants showed the induction of abiotic and biotic stress related proteins, including other LEA proteins, specifically a LEA7, the low temperature induced LTI30/XERO2, and RAB18/XERO1. Interestingly the latter is a Y_2SK2 DHN of 13 kDa, similar in size to the additional DHN induced in the transgenic plants produced in our study (Figure 2, band c). Likewise, transgenic Arabidopsis overexpressing the maize *Rab17* also showed the induction of a LEA7.[23,26] The molecular function of *Dhn5* and *Rab17* is not known but under stress conditions they are both translocated to the nucleus,[13,22] consequently they might have a role in the transcriptional regulation of other stress responsive genes. In the case of RAB17 it has been shown that nuclear targeting is mediated by a stress-induced phosphorylation of the S-segment.[49,50]

DHN and other LEA proteins may have a role in protecting the embryo during seed desiccation and rehydration.[2,17,51] It is known that DHN content increases during the late stages of seed development, is high in dormant embryos, and usually decreases upon seed imbibition and germination, together with a decline in seed desiccation tolerance.[17-19] It has been shown that re-drying (imbibition followed by dehydration) soybean seeds during the period of radicle emergence results in a decrease in germination frequency. Instead, if re-drying is accompanied by imbibition with ABA or with PEG, which results in accumulation of LEA proteins in the seeds, the seed desiccation tolerance is maintained.[17] Thus a change in the timing or location of expression of a DHN may permit increased viability during seed germination under stress conditions.

Another possible role of DHNs may be the facilitation of water uptake during seed imbibition on low osmotic potential media. It has been proposed that LEA proteins may act as a hydration buffer in the cell in the presence of sugars.[11,52] In fact, heat-soluble protein preparations in the presence of sugars absorb 2-3 times more water than a lysozyme/sugar preparation[11,52] Thus, overexpression of DHNs may alter the seeds capacity to absorb water during imbibition promoting germination on media containing a concentration of NaCl that is restrictive to wild type germination. Alternatively, DHNs may promote cellular detoxification. Experiments studying the molecular function of DHNs have pinpointed lipid binding,[10,53] Ca^{2+},[12] or metal binding.[54] The binding capacities of DHNs may in turn participate in the ability to inhibit lipid peroxidation.[24]

Conclusions

In conclusion, transgenic plants expressing the barley ABA2 showed a significant improvement of the germination process in conditions of increased salinity and low osmotic potential. Therefore the modification of *aba2* expression via transgenic approaches could greatly benefit plant improvement programs aimed at the environmental adaptation of different types of crops.

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