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Research Article

# Putative fructose-1,6-bisphosphate aldolase 1 (AtFBA1) affects stress tolerance in yeast and Arabidopsis

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**Abstract** Glycolysis is responsible for the conversion of glucose into pyruvate and for supplying reducing power and several metabolites. Fructose-1,6-bisphosphate aldolase (*AtFBA1*), a central enzyme in the glycolysis pathway, was isolated by functional complementation of the salt-sensitive phenotype of a calcineurin (CaN)-deficient yeast mutant. Under high salinity conditions, aldolase activity and the concentration of NADH were compromised. However, expression of *AtFBA1* maintained aldolase activity and the NADH level in yeast cells. *AtFBA1* shares a high degree of sequence identity with known class I type aldolases, and its expression was negatively regulated by stress conditions including NaCl. The fusion protein GFP-AtFBA1 was localized in the cytosol of *Arabidopsis* protoplasts. The seed germination and root elongation of *AtFBA1* knock-out plants exhibited sensitivity to ABA and salt stress. These results indicate that *AtFBA1* expression and aldolase activity is important for stress tolerance in yeast and plants.

**Keywords** Fructose-1,6-bisphosphate aldolase, NaCl, Germination assay

## Introduction

In plants, unfavorable environmental conditions such as high salinity and drought result in biochemical, molecular, and physiological changes that adversely affect plant growth and development, and ultimately compromise crop productivity (Skirycz and Inze 2010; Chew and Halliday 2011). Plants have evolved sophisticated mechanisms to adapt or survive adverse environmental conditions (Chew and Halliday 2011). The phytohormone abscisic acid (ABA) accumulates under conditions of environmental stress and activates signal transduction pathways (Chew and Halliday 2011). This leads to a variety of physiological responses including metabolic alterations and the expression of stress-related genes (Wang et al. 2011). Recently, it was reported that ABI1/PP2C along with the RCAR/PYR1/PYL family of START proteins is able to bind ABA through a gate-latch-lock mechanism in *Arabidopsis* (Melcher et al. 2009). Additionally, it was shown that ABA perception suppresses PP2C-mediated dephosphorylation of SnRKs and permits their activation (Melcher et al. 2009; Park et al. 2009). The *pyr1*/*pyl1*/*pyl2*/*pyl4* quadruple mutant shows insensitivity in ABA-induced stomatal closure and ABA-inhibited stomatal opening (Nishimura et al. 2010).

Stress conditions such as high salinity and ABA treatment restrict photosynthesis resulting in energy deprivation and growth retardation (Quan et al. 2010). This causes a disruption in the levels of metabolites resulting from normal metabolism (Rao et al. 2010). Some of these metabolites function as compatible solutes including sugars, low-complexity carbohydrates, and amino acids. Some metabolites, such as sucrose and glucose, function as signal molecules in important regulatory processes (Baier et al. 2004; Cho and Yoo 2011). In plants, glucose has emerged as a key regulator of many vital processes, including germination, seedling development, and stress responses (Jin et al. 2010; Kushwah et al. 2011). *Arabidopsis* HXK1 (AtHXK1) functions as an intracellular glucose sensor. At high glucose concentrations, nuclear AtHXK1 binds glucose and functions as a transcriptional repressor together with VHA-B1, a subunit of the peripheral V1 complex in vacuolar-type H-ATPase, and RPT5B, a subunit of the 19S regulatory particle (Moore et al. 2003; Cho et al. 2006). This interaction causes the repression of genes involved in photosynthesis (Cho et al. 2006). Jang et al. constructed transgenic plants expressing either sense or antisense constructs of two genes encoding hexokinase

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isoforms, *Arabidopsis HEXOKINASE1 (HXK1)* and *HXK2*. Plants overexpressing *HXK* genes exhibited glucose-hypersensitive characteristics (Jang et al. 1997; Moore et al. 2003). By contrast, antisense plants displayed hyposensitive phenotypes (Jang et al. 1997). High levels of exogenous glucose are believed to result in ABA accumulation (Finkelstein and Gibson 2002). This, in turn, results in delayed germination and inhibited seedling development (Finkelstein and Gibson 2002; Price et al. 2003). Arenas-Huertero et al. showed that the *glucose insensitive mutant 6* (*gin6*) mutant abolishes the glucose-induced expression of *ABI4* (Arenas-Huertero et al. 2000). In addition, the glucose-specific accumulation of ABA is compromised in the *gin5* mutant (Arenas-Huertero et al. 2000). Snf1-related protein kinases (SnRKs) function within an intricate network linking metabolic and stress signaling in plants (Halford and Hey 2009). These results suggest that ABA plays a central role in the glucose signaling network that controls postembryonic development. Fructose-bisphosphate aldolase (EC 4.1.2.13) catalyzes two reactions: the aldol cleavage of fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone-phosphate (DHAP), and the reversible aldol condensation reaction (Lorentzen et al. 2004). The enzyme functions as a constituent of both the glycolytic/gluconeogenic pathway and the pentose phosphate cycle in plants (Lorentzen et al. 2004). Fructose-bisphosphate aldolase catalyzes a readily reversible reaction, possesses no known regulatory properties, and appears irrelevant for the control of metabolism and growth. However, small changes in its activity have striking effects on photosynthesis, carbon partitioning and growth (Haake et al. 1998). Konishi *et al*. demonstrated that fructose-bisphosphate aldolase is regulated by gibberellin in rice roots. Additionally, the study suggested that aldolase associates with vacuolar $H^+$-ATPase in roots, potentially regulating the vacuolar $H^+$-ATPase-mediated control of cell elongation (Konishi et al. 2004). Nonetheless, the relationship of aldolase with glycolytic enzymes and stress responses remains to be characterized. Here, *AtFBA1* is reported to encode a putative fructose-1,6-bisphosphate aldolase in *Arabidopsis*. *AtFBA1* is able to suppress the salt-sensitive phenotype of *cnbΔ* cells and plays a role in seed germination and root growth under environmental stress conditions in plants.

## Materials and methods

### Complementation assay

The *AtFBA1* gene was subcloned into pYES2, an expression vector containing the galactose-inducible *GAL1* promoter and the URA3 selection marker. The construct was introduced into the YP9 strain (*Mata ura3 leu2 his3 trp1 ade2 lys2 cnb1::HIS3*), and transformed cells were selected on SD-Ura- medium (Shin et al. 2004). The transformants were grown in SD-Ura- medium overnight. Aliquots (2 μl) from an exponentially growing cell culture ($O.D._{600}$=0.1) were serial diluted (1:10, 1:100, 1:1000) and spotted onto YPGal plates containing NaCl (1.1 M), LiCl (100 mM), KCl (1.4 M) and sorbitol (1.5 M). Colonies were examined after 4 days of growth at 30℃.

### Enzyme assay, kinetics, and determination of NADH levels

Yeast cells were grown to a density of O.D.600=0.1 in YPD medium. *AtFBA1* expression was examined in cells that had been washed in sterile water and resuspended in YPGal medium for 18-20 hours. The cells were collected by centrifugation at 3000 rpm for 10 min. Cells were washed twice with lysis buffer, resuspended in 1/10 culture volume of lysis buffer, and ruptured with glass beads. The supernatant (50 μg protein) was precipitated with 10% TCA to estimate the aldolase activity and NADH level. An aldolase cleavage assay was performed by monitoring NADH oxidation at 340 nm and 22℃ through a coupled assay system with triose-phosphate isomerase and glycerol-3-phosphate dehydrogenase. NADH was detected using a Genesys 10uv spectrophotometer (Thermo Spectronic, USA). The reaction was initiated by adding total protein from the transformants harboring the *AtFBA1* construct or the vector alone.

### Northern blot analysis

Two-week-old *Arabidopsis* seedlings were treated with NaCl (100 mM), ABA (10 μM), or cold (4℃) for the times indicated (0, 0.5, 1, 3, 6, and 12 hours). Total RNA was isolated, and 20 μg was separated in a 1.5% formaldehyde agarose gel and transferred to a charged membrane. After transfer, the RNA was UV cross-linked to the membrane and used for northern blot analysis. Hybridization and washing was conducted according to a published protocol (Shin et al. 2011). The full-length *AtFBA1* gene was used as a probe.

### *In vivo* targeting of *AtFBA1* in Arabidopsis protoplasts

*AtFBA1* cDNA was inserted into the p326-sGFP plasmid to create chimeric GFP-fusion constructs controlled by the 35S promoter. The plasmid was introduced into *Arabidopsis* protoplasts that had been prepared from leaf tissues by

polyethylene glycol-mediated transformation (Shin et al. 2004). Expression of the fusion constructs was monitored at various time points after transformation, and images were captured using a cooled CCD camera and a Zeiss Axioplan fluorescence microscope (Carl Zeiss Co, Jena, Germany). Data were processed using Adobe Photoshop software (Adobe System, Mountain view, CA) and presented in pseudo-color format.

Plant material, germination assay and root growth measurements

*Arabidopsis thaliana* (ecotype Columbia) was grown either on MS medium or soil in a growth chamber with a 16-hour light/8-hour dark photoperiod at 22℃ and 70% relative humidity. A mutant containing a T-DNA insertion in the *AtFBA1* locus was obtained from the *Arabidopsis* Biological Resource Center at Ohio State University.

To test the germination frequency, seeds of wild-type and knock-out plants were stratified at 4℃ for 3 days. Seeds were plated on 1/2 MS medium with or without NaCl (100 mM), ABA (10 μM), or KCl (100 mM), and transferred to a growth chamber (22℃ with a 16-hour light/8-hour dark regime). Germination frequencies were determined by scoring radical emergence (n=3, ~200 seeds per experiment). To measure root length, seeds of wild-type and *atfba1-1* plants were plated on 1/2 MS medium in a vertical orientation. Five-day-old seedlings were transferred to 1/2 MS medium with or without NaCl, KCl, or mannitol. Seedlings were incubated in the growth chamber for an additional 7 days, and root lengths were measured. Three replicate plates were used for each treatment to ensure data reproducibility.

## Results

Putative fructose-1,6-bisphosphate aldolase complements the salt-sensitive phenotype of the yeast CaN null mutant (*cnbΔ*).

Several plant genes able to suppress the salt-sensitive phenotype of the CaN null mutant were identified (Shin et al., 2004). One such gene, a putative fructose-1,6-bisphosphate aldolase (*AtFBA1*, accession no. At3g52930), was further examined. The cDNA of *AtFBA1* consists of 1780 nucleotides encoding a polypeptide of 358 amino acids. A comparison of the AtFBA1 amino acid sequence with other known proteins indicates that AtFBA1 shares a high degree of sequence identity with Class I type fructose-1,6-bisphosphate aldolases from rice, maize or spinach (82.6%, 81.2%, and 78.7%, respectively; data not shown).

To validate the screening results, the full-length *AtFBA1* gene was cloned into the episomal pYES2 plasmid and introduced into the yeast *cnbΔ* strain. Transformation with AtFBA1 complemented the salt-sensitive phenotype of the *cnbΔ* cells (Fig. 1). In yeast, NaCl is known to cause ionic and non-ionic (osmotic) stress, while KCl and sorbitol specifically trigger osmotic stress. To examine whether *AtFBA1* expression plays a role in ionic stress tolerance or osmotic stress tolerance, the phenotype of yeast cells following LiCl, KCl and sorbitol treatment was assessed. As shown in Figure 1, *AtFBA1* expression increased LiCl, KCl and sorbitol tolerance in yeast. These results indicate that *AtFBA1* expression is involved in the osmotic tolerance of yeast. *AtFBA1* encodes a fructose-1,6-bisphosphate aldolase, one of the enzymes involved in glycolysis. Expression of *AtFBA1* suppressed the salt-sensitive phenotype of *cnbΔ* cells. These results suggest that aldolase metabolic activity in yeast may be affected by NaCl. To investigate this possibility, aldolase activity was examined using a coupled assay with triose-phosphate isomerase (TIM) and glycerol-3-phosphate dehydrogenase (GPDH). Under conditions of salt stress, the aldolase activity of control *cnbΔ* cells was decreased by 40%. However, when *AtFBA1* was expressed in *cnbΔ* cells, the aldolase activity increased in the presence and absence of salt treatment. When glyeraldehyde-3-phosphate (GAP) is converted into 1,3-bisphosphoglyerate (1,3-BisPGA) via GAP dehydrogenase, NADH is generated. Therefore, the concentration of NADH under high salinity conditions was examined. The NADH level of control *cnbΔ* cells was decreased by approximately 25%. By contrast, *cnbΔ* cells harboring *AtFBA1* maintained high levels of NADH following NaCl treatment (Fig. 2). These data suggest that although

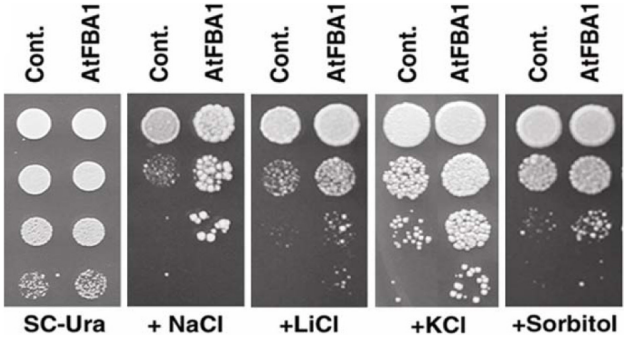


**Fig. 1** Expression of *AtFBA1* suppresses the salt sensitive phenotype of *cnbΔ*. The YP9 yeast strain was transformed with the empty vector (pYES2) containing the full-length *AtFBA1* cDNA. Transformed cells were tested for tolerance to NaCl, LiCl, KCl, and sorbitol as described in the Materials and Methods. Plates were photographed after incubation at 30℃ for 3 days

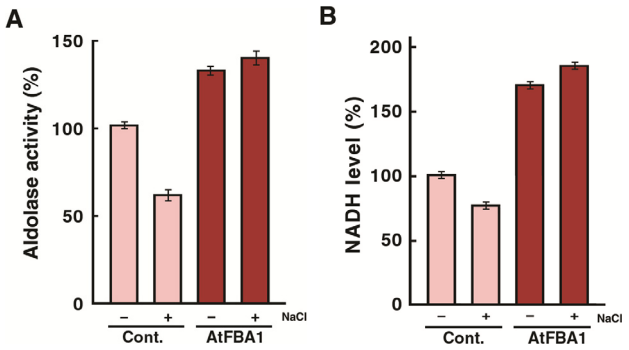


**Fig. 2** Expression of *AtFBA1* maintains aldolase activity and NADH levels under salt stress *in vivo*. Yeast were transformed with the empty vector (pYES2) containing the full-length *AtFBA1*. (A) Aldolase activity was examined in crude extracts after treatment with or without NaCl as described in the Materials and Methods. (B) NADH levels were detected from (A). The supernatant (50 μg protein) was used to estimate aldolase activity or NADH levels according to the Materials and Methods

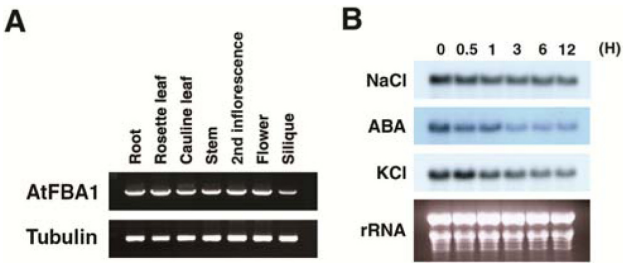


**Fig. 3** *AtFBA1* transcript levels were decreased by abiotic stress. (A) Tissue specificity of *AtFBA1*. Total RNA was prepared from each tissue and semi-quantitative reverse transcription PCR (RT-PCR) was performed using *AtFBA1* specific primers. (B) Total RNA was prepared from 2-week-old *Arabidopsis* seedlings treated with NaCl (100 mM), ABA (10 μM), or KCl (100 mM) for the indicated times. Equal loading for each lane was confirmed by staining the gels with ethidium bromide (lower). RNA blots were probed with $^{32}$P-labeled *AtFBA1*

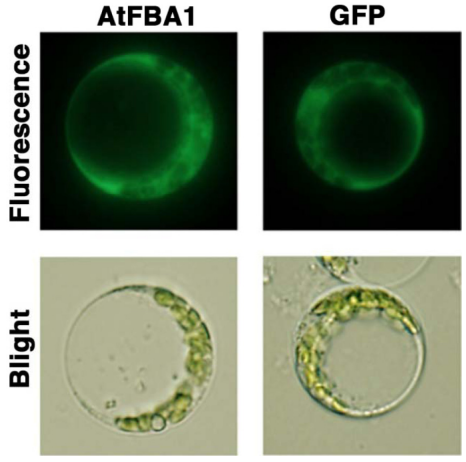


**Fig. 4** Subcellular localization of AtFBA1. Protoplasts prepared from *Arabidopsis* seedlings were transformed with *AtFBA1:GFP*. The transformed protoplasts were examined by fluorescence microscopy at 12 and 24 h after transformation

aldolase activity decreases under stress conditions, this decrease is complemented by expression of *AtFBA1* in yeast.

*AtFBA1* transcript levels are specifically reduced by abiotic stress and *AtFBA1* is cytosolic in plants

To characterize *AtFBA1* in plants, *AtFBA1* expression was monitored under various stress conditions. Total RNA was extracted from *Arabidopsis* seedlings that had been treated with NaCl, KCl, or the phytohormone ABA. Northern blot analysis was performed using the full-length *AtFBA1* as a probe. *AtFBA1* transcripts were dramatically reduced following treatment with NaCl, KCl and exogenously applied ABA (Fig. 3). To analyze the tissue-specific *AtFBA1* expression in plants, semi-quantitative RT-PCR was performed using gene specific primers. Based on the results, *AtFBA1* is expressed in all plant tissues including roots, stems, leaves, and flowers (Fig. 3).

The subcellular localization of *AtFBA1* in *Arabidopsis* was investigated using green fluorescent protein (GFP). The full-length *AtFBA1* gene was cloned into the p326GFP vector to produce a chimeric *AtFBA1:GFP*, and the construct was introduced into protoplasts isolated from wild-type *Arabidopsis* seedlings. The subcellular distribution of AtFBA1:GFP was observed as a green fluorescent signal in the cytosol (Fig. 4). Protoplasts transformed with GFP alone also produced green fluorescent signals in the cytosol. These expression pattern analyses and subcellular localization results indicate that AtFBA1 functions in all plant tissues as a glycolytic enzyme. This enzyme is present in the cytosol and may be negatively regulated in the presence of environmental stress in plants.

The *AtFBA1* knock-out mutant exhibits sensitivity to abiotic stress

To investigate the biological function of AtFBA1 in plants, a knock-out line containing a single T-DNA insertion in the *AtFBA1* locus was identified (*atfba1-1*, SALK_124383) by searching the *Arabidopsis* Biological Resource Center. Homozygous plants were screened via PCR and RT-PCR using specific primers. The T-DNA insertion occurs in the third exon of the *AtFBA1* locus, resulting in the complete loss of *AtFBA1* expression in the *atfba1-1* mutant (Fig. 5). To observe the phenotype of *atfba1-1* under stress conditions, a germination assay and root elongation assay were conducted.

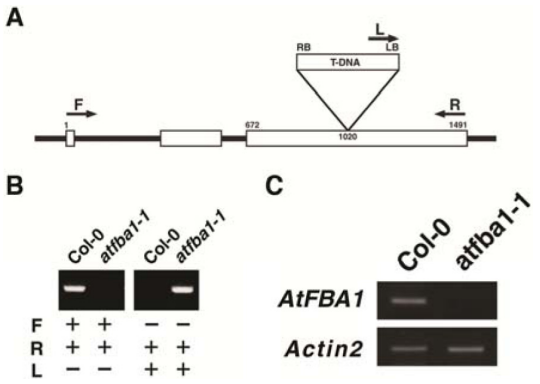


**Fig. 5** The T-DNA is inserted in the 3rd exon of the *AtFBA1* locus. (A) The diagram illustrates the location of the T-DNA insertion in *AtFBA1* (At3g52930). Exons (opened boxes) were deduced from the cDNA sequence. The open triangle indicates the T-DNA insertion site. (B) Diagnostic PCR analysis of the T-DNA insertion in wild-type (Col-0) and *atfba1-1* using forward (F), reverse (R) and LB (L) primers. (C) Transcript abundance of *AtFBA1* in wild-type and *atfba1* plants as determined by RT-PCR.

To test germination frequency, seeds of wild-type and knock-out plants were kept in a cold room (4℃) for 3 days. Seeds were then sown on 1/2 MS medium with or without ABA treatment. The germination rate of *atfba1-1* was minimally affected in comparison to wild-type seeds sown on 1/2 MS medium. More than 80% of the *atfba1-1* seeds germinated. After 2 days, the germination rate of *atfba1-1* seeds significantly decreased to approximately 40%. By contrast, more than 80% of the wild-type seeds germinated under conditions of ABA stress (Fig. 6). The germination rate of *atfba1-1* seeds was also severely delayed by NaCl and KCl treatment when compared to wild-type seeds (Fig. 6).

Next, to characterize the sensitive seedling phenotype of *atfba1-1*, a root elongation assay was performed under stress conditions. Wild-type and *atfba1-1* seeds were grown for 5 days in a vertical orientation and then transferred to 1/2 MS medium containing NaCl, KCl, or mannitol. After 7

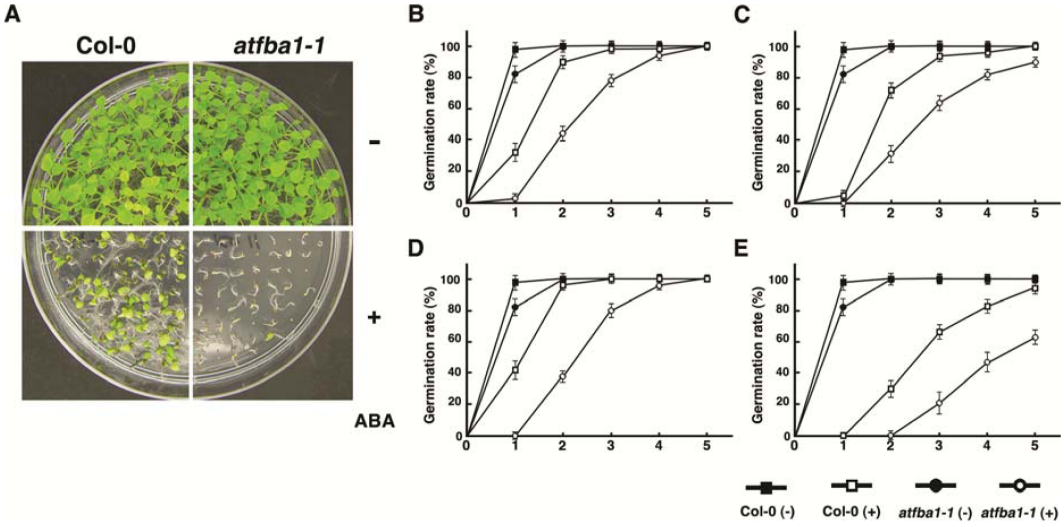


**Fig. 6** Germination of *atfba1-1* seeds was delayed by abiotic stress. (A) Seedlings of wild type (Col-0) and *atfba1-1* were grown under long day conditions in medium containing ABA. Plates were photographed after incubation in a growth chamber for 5 days. (B), (C), and (D) Wild-type (Col-0, squares) and mutant (atfba1-1, circles) seeds were sown on medium with (open) or without (filled) ABA (B), NaCl (C), and KCl (D) after 4 days of cold treatment as described in the Materials and Methods. Germination kinetics were determined by calculating the percentage of germination over time. "Germination" in this study refers to the appearance of a radicle of at least 1 mm. Data represent typical data of three independent experiments (n=100)

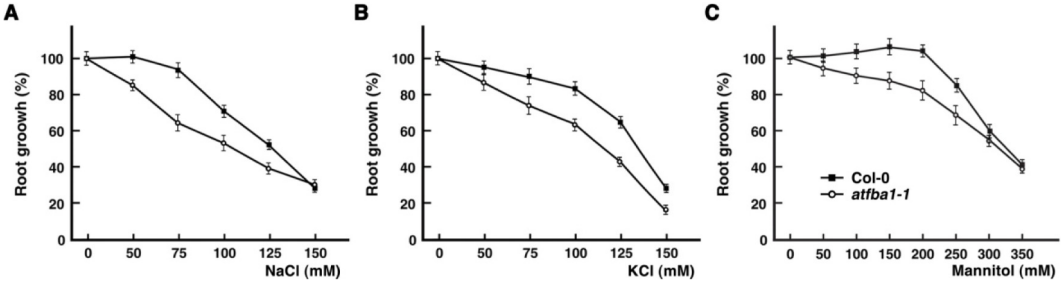


**Fig. 7** *atfba1-1* is sensitive to abiotic stress. Root growth of Col-0 and *atfba1-1* seedlings after treatment with NaCl (A), KCl (B), and mannitol (C). Seven-day-old seedlings were transferred to MS medium supplemented with or without each stress treatment. Root growth was measured after 7 days (n=12)

days, the root lengths of the wild-type and *atfba1-1* seedlings were measured. Under normal growth conditions, the root growth of the *atfba1-1* mutant was partially retarded. Wild-type root growth was not affected until the NaCl concentration reached 75 mM, and root elongation was disrupted at 100 mM NaCl. The root elongation of *atfba1-1* seedlings was inhibited at 50 mM NaCl and root lengths were reduced to approximately 50% at 100 mM NaCl treatment (Fig. 7). Under KCl and mannitol stress conditions, the *atfba1-1* seedlings exhibited similar root growth inhibition in a dosage-dependent manner (Fig. 7). These results suggest that AtFBA1 plays a role in the stress tolerance of yeast and plants.

## Discussion

Glycolysis is a ubiquitous primary metabolic pathway in plants. Several glycolytic enzymes, including hexokinase and fructose-1,6-bisphosphate aldolase, contribute to the oxidation of hexoses for the generation of reducing power and pyruvate, as well as to provide precursors for anabolism (Munoz-Bertomeu et al. 2011). However, little is known about the relationship between glycolytic proteins and stress tolerance in plants. This study presents evidence that AtFBA1 maintains cellular aldolase activity and NADH levels under abiotic stress. Based on this evidence, AtFBA1 is proposed to play a role in the stress tolerance of yeast and plants. Plant Class I fructose-1,6-bisphosphate aldolases (FBAs) are grouped into the Class-I type enzymes, forming a Schiff base with the substrate as an intermediate (Lorentzen et al., 2004). These enzymes are found in both the chloroplasts and the cytosol (Lorentzen et al. 2004). Plant chloroplast FBAs mainly function in the generation of metabolites for starch biosynthesis via the Calvin Cycle (Lorentzen et al. 2004). However, cytosolic FBA functions in glycolysis and gluconeogenesis in both source and sink tissues. *AtFBA1* shares a high degree of sequence identity with Class-I type plant FBAs from rice, maize and spinach. The purified recombinant AtFBA1 exhibits Michaelis-Menten kinetics for FBP in the catabolic (aldol cleavage) direction in a coupled assay system (data not shown). Additionally, the subcellular distribution of *AtFBA1:GFP* is cytosolic of plant cells (Fig. 4). Therefore, AtFBA1 is assumed to be a functional cytosolic Class I type aldolase in *Arabidopsis*. Metabolism is a complex non-linear pathway that interacts with many other processes. Photosynthesis is inhibited by decreased expression levels or reduced enzymatic activity of the glycolytic enzymes. Additionally, the levels of sugars and starch are altered and plant growth is compromised. Genetic analysis has revealed that the glucose response interacts with other plant hormones including ABA. The glucose responsive expression of *ABI4* and *ABI5* is absent in the glucose insensitive mutants *gin1*, *gin5*, and *gin6* (Arenas-Huertero et al. 2000). Yamada *et al*. demonstrated that *AldP2* is up-regulated and *AldP1* is down-regulated by salt treatment in the *Nicotiana* species *N. excelsior* and *N. arentsii* (Yamada et al. 2000). *Sesuvium portulacastrum SpFBA*, a gene from a seashore mangrove plant, accumulates in response to high salinity and ABA treatment (Fan et al. 2009). Recently, Gong et al. provided transcriptional profiles of drought-responsive genes in tomato. Among them, fructose-bisphosphate aldolase was down-regulated under drought stress conditions (Gong et al. 2010). In this study, the transcript abundance of *AtFBA1* was negatively regulated by ABA treatment and stress conditions (Fig. 3). Under salt stress, the aldolase activity and NADH level are decreased in yeast cells. However, when *AtFBA1* is expressed in yeast, the aldolase activity and NADH level are increased with or without salt stress (Fig. 2). These results indicate that the activity of some glycolytic enzymes, such as aldolase, are affected by stress conditions. At a minimum, these activities are regulated at the transcriptional level in yeast and plants.

GAPDH, a glycolytic enzyme, is one of the most abundant soluble proteins. GAPDH is encoded by three unlinked genes designated *TDH1*, *TDH2*, and *TDH3* in *S. cerevisiae*. Strains lacking *TDH3* (*tdh3* and *tdh1 tdh3* strains) exhibit extreme sensitivity to $H_2O_2$ compared to wild-type controls. The strain lacking both *TDH1* and *TDH3* is the most sensitive (Grant et al. 1999). Overexpression of a cyanobacterial fructose-1,6-/sedoheptulose 1,7-bisphosphatase enhances photosynthesis and growth in tobacco (Miyagawa et al. 2001). Fructose-1,6-bisphosphate aldolase (FBA1) from *S. cerevisiae* is single copy gene, and deletion mutants of this gene exhibit growth defects (Compagno et al. 1991). Inhibition of chloroplastic fructose-1,6-phosphatase in tomato leads to decreased fruit size and small changes in carbohydrate metabolism (Obiadalla-Ali et al. 2004). Konishi et al. also suggested that fructose-bisphosphate aldolase is regulated by gibberellins, and the root growth of antisense transgenic rice was repressed (Konishi et al. 2004). Haake et al. showed that decreased aldolase activity in antisense transgenic potato results in alterations in sugar and starch levels, and inhibition of plant growth (Haake et al. 1998). Expression of *AtFBA1* rescues the salt-sensitive phenotype of the *cnbΔ* yeast mutant (Fig. 1). *atfba1* shows delayed germination and root elongation under abiotic stress conditions (Fig. 6 and 7). Taken together, glycolysis and gluconeogenesis are proposed to be sensitive to abiotic stresses such as high salinity.

Furthermore, expression of *AtFBA1* maintains glycolysis flux under stress conditions.

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