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Molecules and Cells

AtNEK6 Interacts with ARIA and Is Involved in ABA Response during Seed Germination

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ARIA is an ARM repeat protein that is a positive regulator of ABA response. To identify ARIA-interacting proteins, we conducted yeast two-hybrid screening. One of the positive clones obtained from the screen encoded a protein kinase, AtNEK6, which belongs to the NIMA (Never In Mitosis, gene A)-related kinase family. We analyzed AtNEK6 overexpression (OX) and knockout (KO) lines to investigate its in vivo function. The AtNEK6 OX lines grew slowly, whereas the KO line germinated and grew faster than wild type plants. AtNEK6 also affected ABA and stress responses. During seed germination, AtNEK6 OX lines were hypersensitive to ABA and high osmolarity, whereas its KO line was partially insensitive to ABA and high osmolarity. Previously, AtNEK6 was shown to be involved in epidermal cell morphogenesis. Our results indicate that AtNEK6 is also involved in plant growth regulation and responses to ABA and high osmolarity during the seed germination stage.

INTRODUCTION

Plant hormone abscisic acid (ABA) plays an important role in the regulation of seed development and abiotic stress responses (Finkelstein et al., 2002; Xiong et al., 2002). In developing seeds, ABA regulates embryo development and reserve accumulation (i.e., biosynthesis of storage proteins and oils). ABA also prevents mature embryos from precocious germination. During postgermination growth, ABA mediates adaptive responses of seedlings to various abiotic stresses such as drought, high salinity and extreme temperatures.

The role of ABA in the abiotic stress response of seedlings is critical to plant survival under unfavorable environmental conditions (Xiong et al., 2002). Under water deficit conditions that occur as a result of drought and other abiotic stresses such as high salinity and freezing, ABA promotes stomatal closure, thereby reducing water loss through transpiration (Schroeder et al., 2001). ABA-induced stomatal closure is a rapid process that does not involve changes in gene expression patterns. However, many ABA-dependent processes involve changes in gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005). Genome-wide transcriptional profiling analyses have shown that approximately 20% of the Arabidopsis genes are responsive to various abiotic stresses (Kreps et al., 2002; Takahashi et al., 2004). Although not all of the stress-responsive genes are ABA-regulated, many are responsive to ABA.

Numerous genetic and biochemical studies have been conducted to identify regulatory components of ABA response (Hirayama and Shinozaki, 2007); consequently, a large number of ABA signaling components including various types of signaling intermediates such as phosphatases, kinases, lipids and inositides have been reported. In addition, ABA receptors have also recently been reported. GTG1 and GTG2, which are G-protein-coupled receptor-type G proteins, have been identified as membrane-localized ABA receptors (Pandey et al., 2009). A family of START domain proteins known as RCARs/PYR1/PYLs are soluble ABA receptors (Ma et al, 2009; Park et al., 2009). In the presence of ABA, RCARs/PYR1/PYLs interact with ABI1 and ABI2, which are type 2C protein phosphatases that negatively regulate ABA signaling by dephosphorylating SnRK2 kinases, and inhibit their phosphatase activity to activate ABA response. A number of transcription factors that regulate ABA-responsive gene expression have been identified (Yamaguchi-Shinozaki and Shinozaki, 2005), including a small subfamily of bZIP proteins known as ABFs (ABRE Binding Factors) (Choi et al., 2000) or AREBs (Uno et al., 2000), which regulate ABA response via the G-box-type (i.e., CACGTGGC) ABRE (Abscisic acid Response Element) present in many ABA-regulated promoters (Kim, 2006). A recent study conducted by Fuji et al. (2009) showed that RCAR/PYR/PYLs, ABI1, SnRK2.6, and ABF2 can reconstitute a complete ABA signaling pathway *in vitro*.

NIMA (Never in Mitosis gene A)-related kinases (NEKs) are serine/threonine kinases. NEKs are involved in cell-cycle transition in animals and fungi. For example, NIMA is essential for entry into mitosis in *Aspergillus nidulans* (Osmani and Ye, 1996), and its human homolog HsNEK2 is cell-cycle regulated and considered to influence microtubule organization (Hayward and Fry, 2005). The functions of very few plant NEKs have been reported. The tomato NEK, SAPK, plays a role in fruit development (Pnueli et al., 2001), while the poplar NEK, PNEK1, is involved in vascular development (Cloutier et al., 2005; Vigneault et al., 2007). In the Arabidopsis genome, seven NEKs (AtNek1-AtNek7) are present, one of which (AtNEK6) is

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involved in microtubule-dependent morphogenesis of epidermal cells (Motose et al., 2008; Sakai et al., 2008). However, with the exception of these NEKs, the function of plant NEKs remain unknown, and their involvement in ABA response has not yet been evaluated.

We are interested in delineating the ABA signaling pathways that lead to ABA-responsive gene expression. Toward the end, we carried out yeast two-hybrid screens employing ABFs as bait. As mentioned above, ABFs are bZIP class transcription factors that bind to ABRE (i.e., G-box type ABRE, CACGTGGC) to regulate a subset of ABA-responsive genes. We isolated several ABF-interacting proteins, one of which is an ARM repeat protein (Kim et al., 2004). *In vivo* analysis of its function revealed that the protein, ARIA (Arm repeat protein interacting with ABF), is a positive regulator of ABA response during germination and seedling growth.

To find additional upstream components of ABA signaling, we attempted to identify ARIA-interacting proteins and conducted two-hybrid screens employing ARIA as bait. Here, we report that one of the ARIA-interacting proteins is AtNEK6. To determine if AtNEK6 is involved in ABA and stress responses, we investigated the ABA sensitivity and the stress response of its overexpression and knockout lines. Our results indicate that AtNEK6 is involved in ABA and osmolarity responses during the germination stage.

MATERIALS AND METHODS

Two-hybrid screen and two-hybrid assay

To prepare a bait construct containing the full-length ARIA, the entire coding region of ARIA was amplified using the primer set, 5′-GATGGACCAACAACCGGAGAG-3′ and 5′-TTTTCCTTTTGCGGCCGCCATTCTACAACCTCAAGCTTTG-3′, and the amplified fragment was digested with Not I. The fragment was then ligated with the pPC62/LexA (Lee et al., 2009), which was pre-digested with SmaI and NotI. Reporter yeast preparation and large-scale transformation to isolate positive clones and the analysis of the positive clones were conducted as previously described (Lee et al., 2009). The full-length AtNEK6 including parts of the 5′ and the 3′ untranslated regions was isolated by PCR using the primer set 5′-CAACTTTGGAGAAGTTTCGTTGTTGG-3′ and 5′-GTTATTTTACGACAATATAAATGTTATAGAG-3′.

To prepare the prey construct containing the full-length AtNEK6, the entire coding region of AtNEK6 was amplified with the primer set 5′-ATGGAGTCACGAATGGATCAGTACGAG-3′ and 5′-TTTTCCTTTTGCGGCCGCTTTACCTTCATGAACAATTCCTGGAGC-3′. The insert fragment for the prey construct containing the kinase domain was prepared using the primer set 5′-ATGGAGTCACGAATGGATCAGTACGAG-3′ and 5′-TTTTCCTTTTGCGGCCGCCTGGAGTTATAGACGCCGCAGAAAGC-3′, and the insert fragment containing the remaining C-terminal portion was prepared using the primer set 5′-GCAGCCATATGTTGAGCAGTACCGTCC-3′ and 5′-TTTTCCTTTTGCGGCCGCTTTACCTTCATGAACAATTCCTGGAGC-3′. The amplified fragments were digested with Not1 and then ligated with pYESTrp2 (Invitrogen), which was prepared by HindIII digestion followed by a Klenow fill-in reaction and NotI digestion.

The bait construct containing the full-length ARIA was prepared by PCR using the primer set 5′-GATGGACCAACAACCGGAGAG-3′ and 5′-TTTTCCTTTTGCGGCCGCCATTCTACAACCTCAAGCTTTG-3′. The ARM repeat-containing fragment was amplified using the primers 5′-GATGGACCAACAACCGGAGAG-3′ and 5′-TTTTCCTTTTGCGGCCGCATCAACTGGAGAAAGTGCCATAG-3′, whereas the BTB/POZ domain-containing fragment was prepared by PCR using the primer set 5′-ACTTGACGGTGCAGCAGCGTTG-3′ and 5′-TTTTCCTTTTGCGGCCGCCATTCTACAACCTCAAGCTTTG-3′. The amplified fragments were treated with NotI and then ligated with pPC62/LexA, which was digested with SmaI and NotI. The intactness of the cloned fragments was confirmed by DNA sequencing.

The two-hybrid assay was carried out as previously described (Lee et al., 2009). Briefly, bait constructs were individually introduced into the reporter yeast strain L40, and the resulting transformants were then transformed with the individual prey constructs. The reporter activity (β-galactosidase activity) was measured by a liquid assay as previously described (Choi et al., 2000) using O-nitrophenyl-β-D-galactopyranoside as a substrate. For each construct, five independent transformants were assayed.

RNA isolation and expression analysis

RNA isolation and Northern analysis were conducted as previously described (Lee et al., 2009). For RT-PCR analysis, RNA was digested with DNase I to remove contaminating DNA, after which first strand cDNA was prepared using Superscript III (Invitrogen) according to the supplier's instructions. Amplification of the cDNA was conducted using gene-specific primers within a linear range.

In vitro binding assay

Construction of the GST-ARIA fusion construct and preparation of the resulting recombinant protein were described (Kim et al., 2004). The full-length and N-terminal or C-terminal partial fragments used in the GST pulldown assay were the same as those described above for the prey constructs. The fragments were cloned into pCITE (Novegen), after which recombinant proteins were prepared in the presence of ^{35}S-methionine by *in vitro* translation according to the manufacturer's instructions (Promega). Binding assays were conducted as previously described (Kim et al., 2004).

Analysis of overexpression and knockout lines

To prepare AtNEK6 overexpression lines, the coding region of AtNEK6 was amplified employing the primers, 5′-ATCTTCTTCGCATGGAAGATTTCTG-3′ and 5′-GTTATTTTACGACAATATAAATGTTATAGAG-3′. The amplified fragment was then cloned into pBI121 (Jefferson et al., 1987), which was digested with XbaI and treated with Klenow fragment to fill the 3′-overhang after removal of the GUS coding region by SacI-SmaI digestion. Arabidopsis transformation was conducted according to the method described by Bechtold and Pelletier (1998) and T3 or T4 generation homozygous plants were employed for phenotype analysis.

The AtNEK6 knockout line (SALK_000494) was obtained from the Arabidopsis stock center. Homozygous lines were recovered from the sublines segregating with a 3:1 ratio of knamycin-resistant and kanamycin-sensitive seeds. The T-DNA insertion site was confirmed by PCR of the genomic DNA and sequencing of the amplified fragment.

Arabidopsis (Ecotype Landsberg erecta, Ler, and Columbia, Col-0) was grown aseptically in Murashige and Skoog medium (1962) or in soil under long day conditions (16 h light/8 h dark cycle). A germination assay was conducted as described previously (Kang et al., 2002) using seeds collected at the same or similar times.

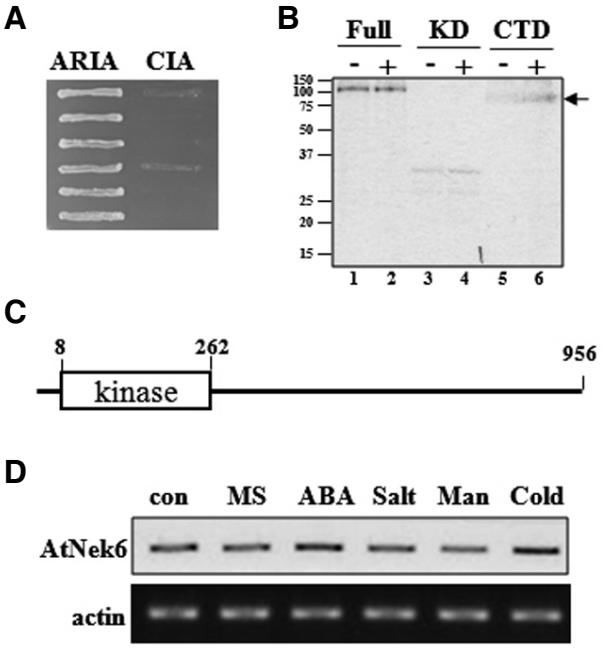

Fig. 1. Isolation of AtNEK6 as an ARIA-interacting protein. (A) Specificity of interaction. The specificity of interaction of the positive clone (#44) encoding AtNEK6 was determined using bait constructs containing ARIA or an unrelated protein, CIA, a calcium-dependent protein kinase. (B) GST pulldown assay. *In vitro* translation products of the full-length (Full), the N-terminal portion (KD) or the C-terminal portion (CTD) of AtNEK6 described in Fig. 2 were pulled down using GST-ARIA recombinant fusion protein. The numbers indicate size markers, and the position of CTD is indicated by an arrow. –, GST alone. +, GST-AtNEK6 fusion proteins. (C) Schematic presentation of AtNEK6. Kinase, the kinase domain. The numbers indicate the amino acid position. (D). Induction pattern of AtNEK6 expression was determined by semi-quantitative RT-PCR. Con, control without any treatments. 2-week-old seedlings were treated for 4 h with 1/4 MS (MS), 100 μM ABA (ABA), 250 mM NaCl (Salt) or 400 mM mannitol (Man) before RNA isolation. Cold treatment (cold) was applied for 24 h at 4°C.

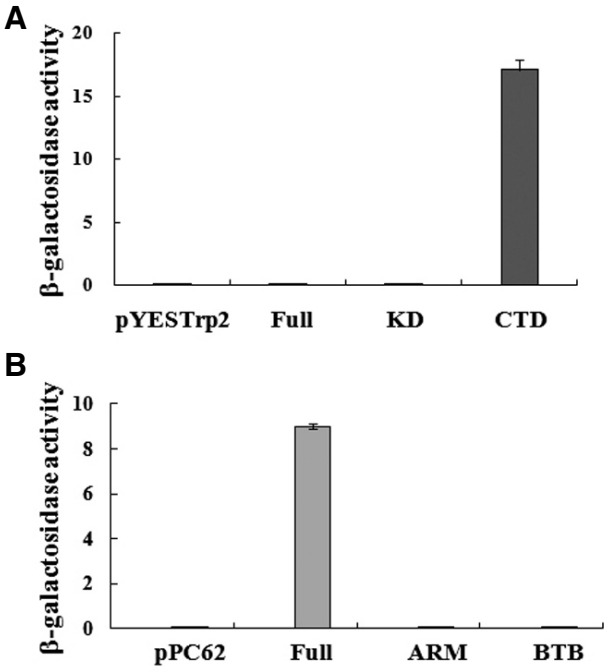

Fig. 2. Domains necessary for the interaction between ARIA and AtNEK6. Interaction domains of ARIA and AtNEK6 were determined by yeast two-hybrid assay using various deletion constructs. The graph shows the β-galactosidase reporter activity in Miller units. (A) Interactions between full-length ARIA and AtNEK deletion constructs were examined. pYESTrp2, vector alone. Full, full-length AtNEK6. KD, kinase domain (amino acids 1-281) of AtNEK6. CTD, C-terminal region (amino acids 263-956) of AtNEK6 lacking the kinase domain. (B) Interactions between the AtNEK6 kinase domain and ARIA deletion constructs were determined. pPC62, bait vector alone. Full, full-length ARIA. ARM, ARM repeat region (amino acids 1-710) of ARIA. BTB, C-terminal region (amino acids 499-710) of ARIA containing its BTB domain.

RESULTS

Isolation of AtNEK6

To isolate the genes encoding ARIA-interacting proteins, we conducted yeast two-hybrid screening. Full-length ARIA was cloned into the bait vector pPC62/LexA, after which the construct was introduced into the yeast strain L40 (Lee et al., 2009). The yeast was then transformed with cDNA library DNA, and the resulting transformants were screened for reporter activities. From the screen of 2.6 million transformants, several positive clones were isolated.

Sequence analysis of the positive clones revealed that one that specifically interacted with ARIA (Fig. 1A) encoded a protein that consisted of 643 amino acids. A database search revealed that the protein was partial and that the full-length protein (At3g44200) consisted of 956 amino acids. Thus, the positive clone was missing the N-terminal 313 amino acids. The full-length protein has a kinase domain near the N-terminus (amino acids 8-262) (Fig. 1C), indicating that it is a protein kinase.

The interaction between ARIA and the positive clone was confirmed by a GST pulldown assay. Full-length or partial fragments of the positive clone were prepared by *in vitro* translation. Their interactions with ARIA were then determined by a GST pulldown assay using a GST-ARIA fusion protein and *in vitro*-translated AtNEK6. Figure 1B shows that the full-length AtNEK6 was pulled down by both GST and GST-the full-length AtNEK6 (lanes 1, 2). Similarly, the N-terminal portion of AtNEK6 was pulled down by both GST and GST-ARIA fusion protein (lanes 3, 4). Thus, full-length AtNEK6 and its N-terminal kinase domain did not interact specifically with ARIA. However, the C-terminal portion of AtNEK6 was preferentially retained by the GST-ARIA fusion protein when compared to GST alone (lanes 5, 6), indicating that the C-terminal portion of AtNEK6 interacts with ARIA.

The ARIA-interacting protein is one of the seven Arabidopsis NEKs, AtNEK6 (Motose et al., 2008; Sakai et al., 2008; Vigneault et al., 2007), which is expressed in all organs and localized in association with cortical microtubules (Motose et al., 2008). Because our results indicated that the ARIA-interacting protein is involved in ABA and stress responses (see below), we investigated its induced expression patterns under various environmental conditions. As shown in Fig. 1D, AtNEK6 expression was slightly upregulated by ABA and cold. These findings are consistent with the expression data available in the Arabidopsis database (www.arabidopsis.org).

Interaction domains

To determine the regions necessary for the interaction between ARIA and AtNEK6, we prepared various deletion constructs of the two proteins and then investigated their interactions. First, we investigated the interactions of the full-length ARIA, which was used as bait in the two-hybrid screen, with three different constructs of AtNEK6. As shown in Fig. 2A, the full-length AtNEK6 did not interact with ARIA. Similarly, the N-terminal portion of AtNEK6 (amino acids 1-281), which includes the kinase domain, did not interact with ARIA. However, the C-terminal portion (amino acids 263-956), which lacks the kinase domain but includes the amino acids encoded by the positive clone, did interact with ARIA. These findings are consistent with the result of the GST pulldown assay described above.

Next, we determined the regions of ARIA that are necessary for its interaction with AtNEK6 using the C-terminal portion of AtNEK6 as bait. As shown in Fig. 2B, full-length ARIA interacted with the C-terminal portion of AtNEK6, thereby confirming the above results. However, the ARM repeat-containing region (amino acids 1-710) did not interact with AtNEK6. Similarly, the C-terminal portion of ARIA (amino acids 499-710) including the BTB/POZ domain did not interact with AtNEK6. Taken together, our results indicate that the C-terminal portion of AtNEK6, which lacks the kinase domain, interacts with the full-length ARIA.

Overexpression of AtNEK6 affects ABA sensitivity during germination

To investigate the in vivo function of AtNEK6, we generated overexpression (OX) lines of AtNEK6. The entire coding region of AtNEK6 was expressed under the control of CaMV 35S promoter by employing the plant transformation vector, pBI121 (Jefferson et al., 1987). We recovered more than ten T3 generation transgenic lines and, after preliminary analysis, we chose two representative lines (i.e., ST15 and ST27) with different AtNEK6 expression levels for further analysis.

The OX lines with high AtNEK6 expression levels (Fig. 3A) grew slowly when compared with wild type plants (Fig. 3B); however, they exhibited no other distinct phenotypes under normal growth conditions. We then examined the ABA response of the OX lines. As shown in Fig. 3C, the germination rates of the AtNEK6 OX lines were lower than the wild type rates in the presence of ABA. For instance, the germination rate of the wild type plants in the presence of 0.5 μM ABA was 70% of the control rate in the ABA-free medium. On the other hand, the germination rates of the AtNEK6 OX lines under the same conditions were approximately 30% of their control rates. Thus, seed germination of the AtNEK6 OX lines was hypersensitive to ABA.

To determine if AtNEK6 OX affected the ABA response at the molecular level, we examined the expression levels of several ABA-regulated genes in the OX lines. Figure 3D shows that the expression levels of RAB18, RD29B and RD29A decreased to some degree when compared with those of wild type plants, whereas the SUS1 gene expression was not altered. In addition, a very minor increase in RAB18, RD29B and RD29A expression was observed in the AtNEK6 KO line (i.e., STK3 and STK5). Thus, the expression of several ABA-responsive genes was affected by AtNEK6 overexpression or knockout, although the degree of change was not great.

AtNEK6 knockout lines are ABA-insensitive during germination

To further investigate the function of AtNEK6, we conducted phenotype analysis of an AtNEK6 knockout (KO) line. A mutant line (SALK_000494) in which T-DNA is inserted into an intron

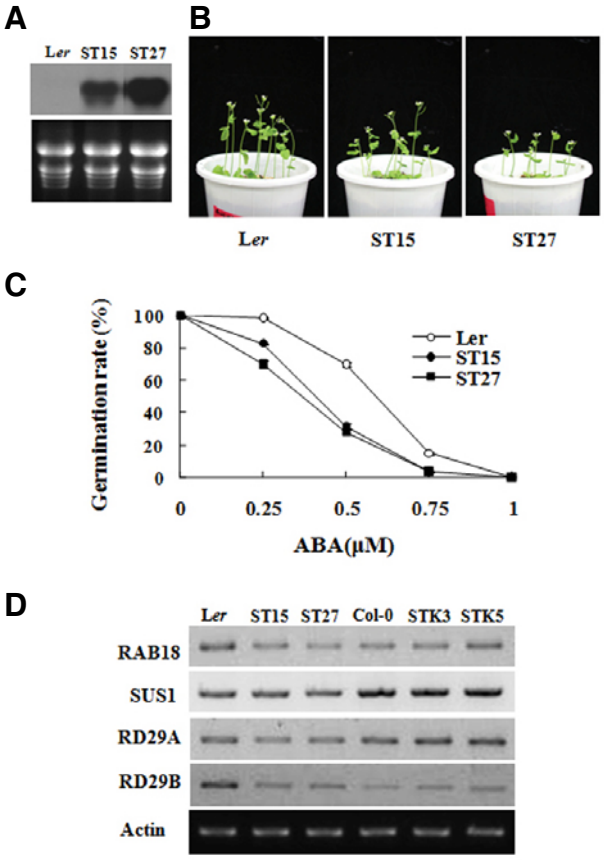

Fig. 3. Germination and growth of AtNEK6 overexpression (OX) lines. (A) AtNEK6 expression levels in the transgenic lines (ST15, ST27) determined by Northern analysis. The bottom panel shows the ethidium bromide-stained gel as a loading control. (B) Growth of the AtNEK6 OX lines in soil. Plants were grown in soil for 24 days before photographs were taken. (C) Germination rates of AtNEK6 OX lines at various concentrations of ABA. Germination (radical emergence) was scored three days after sowing. The data represents the average of three independent measurements (n = 20 each). Error bars are smaller than the symbols. (D) Expression of ABA-responsive genes in AtNEK6 OX and knockout lines were determined by semi-quantitative RT PCR.

was obtained from the Arabidopsis stock center, and after recovering homozygous sublines (STK3 and STK5), T-DNA insertion was confirmed (Fig. 4A). As expected, the expression of AtNEK6 was abolished completely in the homozygous KO line (Fig. 4A).

The AtNEK6 mutant did not exhibit distinct morphological phenotypes, except for the protuberance present on the epidermal cells of the hypocotyls and petioles (Fig. 4B, lower panel). This abnormality has also been observed in other AtNEK6 mutant alleles (Motose et al., 2008; Sakai et al., 2008). However, the AtNEK6 KO line grew faster than wild type plants in soil, as shown in Fig. 4B (upper panel). Together with the slow growth of the AtNEK6 OX lines, the result indicates that AtNEK6 affects the seedling growth rate. Because AtNEK6 overexpression resulted in slower germination, we examined the germination efficiency of the AtNEK6 KO line. Figure 4C shows that the knockout sublines germinated faster than the wild type seeds. For example, approximately 50% of the AtNEK6 knockout seeds germinated in 21 h, whereas it took ap-

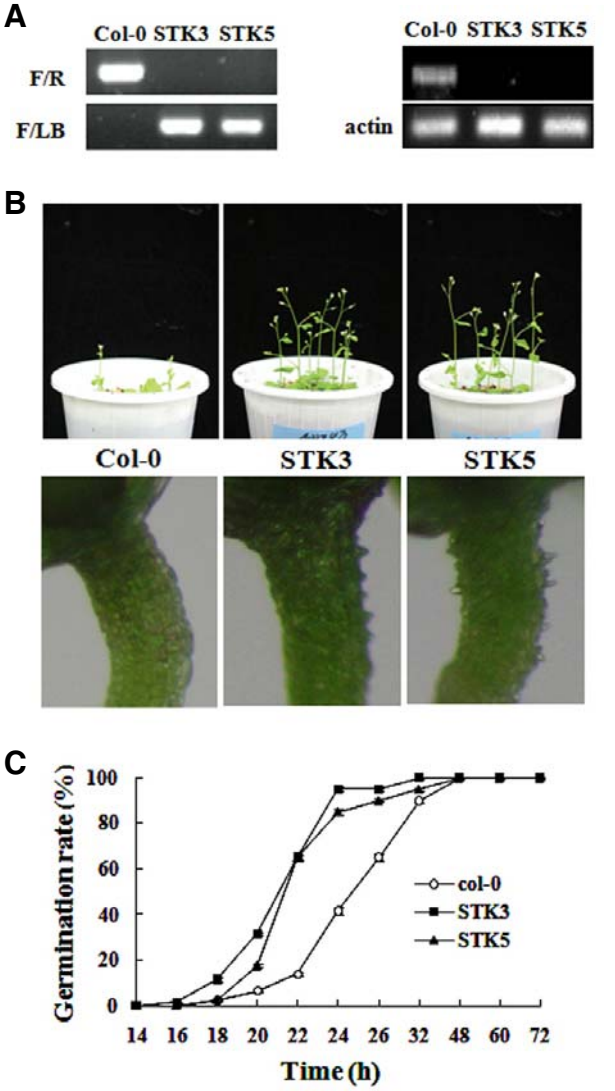

Fig. 4. Germination and growth of AtNEK6 knockout (KO) line. (A) Left panel. The insertion of T-DNA into the AtNEK6 gene in two AtNEK6 KO sublines (STK3 and STK5) was confirmed by PCR using gene-specific primers, F (forward primer) and R (reverse primer) (top panel), or the primer set, F and left border (LB) primer. Right panel. AtNEK6 expression levels in wild type (Col-0) or knockout sublines (#3, and #5) were determined by RT-PCR. (B) Top, growth of the AtNEK6 KO line in soil. Plants were grown for 24 days before photographs were taken. Bottom, the hypocotyls of the AtNEK6 KO sublines. Note the protrusions in the upper parts of the hypocotyls. (C) Germination of an AtNEK6 KO line. Germination rates of two knockout sublines in MS medium were determined. Experiments were conducted in triplicates (n = 20 each).

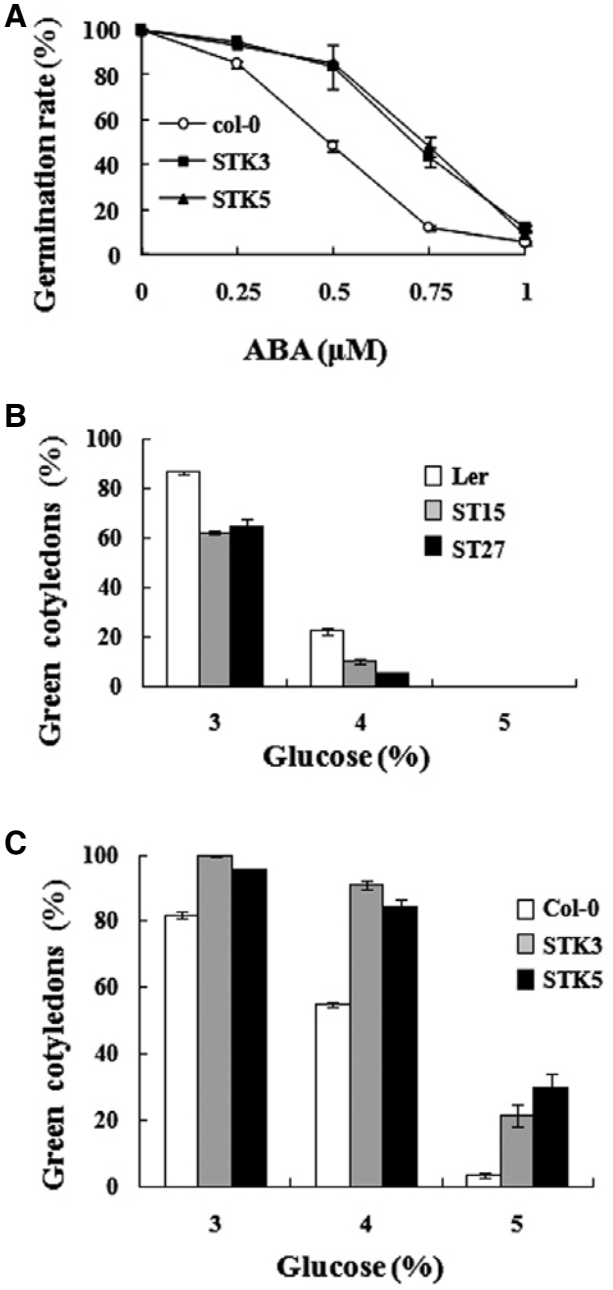

Fig. 5. ABA and glucose responses of AtNEK6 OX and KO lines. (A) ABA dose response of the germination of the AtNEK6 KO line. Germination rates were scored 3 days after sowing seeds on medium containing various concentrations of ABA. Experiments were performed in triplicate (n = 20 each), and the small bars indicate the standard errors. (B, C) Glucose responses of the AtNEK6 OX and KO lines, respectively, were determined by counting seedlings with green cotyledons five days after sowing the seeds on medium containing various concentrations of glucose. Experiments were conducted in triplicates (n = 30 each) and the small bars represent the standard errors.

proximately 25 h for 50% of the wild type seeds to germinate. Thus, both seed germination and the seedling growth rate were enhanced in the AtNEK6 KO line, indicating that AtNEK6 is a negative regulator of seed germination and seedling growth.

We next determined the ABA response of the AtNEK6 KO line during germination (Fig. 5A). The germination rates of the wild type seeds decreased from 100% to approximately 50% and 10% of the control rates in the presence of 0.5 μM and 0.75 μM ABA, respectively. However, the germination rates of the AtNEK6 KO sublines at the same ABA concentrations were 90 and 50%, respectively. Thus, seed germination of the AtNEK6 KO line was less sensitive to ABA.

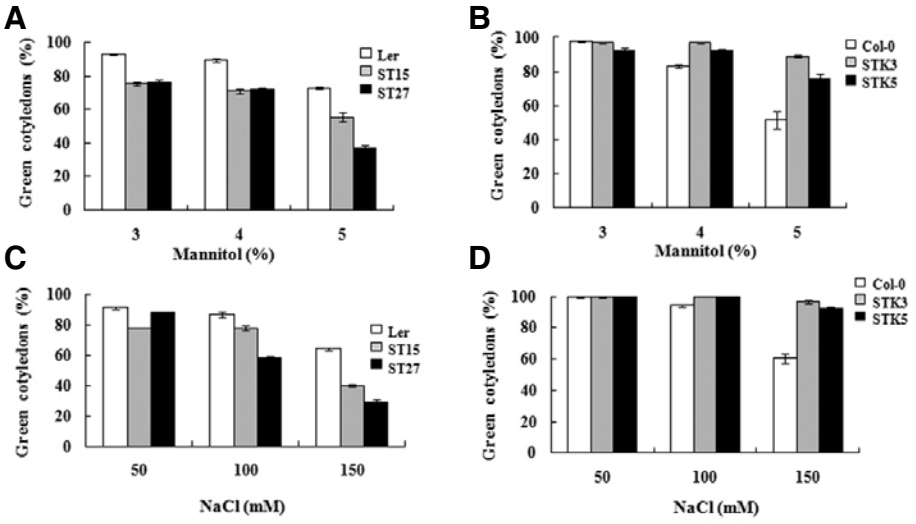

Fig. 6. Osmolarity response of AtNEK6 OX and KO lines. (A) Mannitol response of the AtNEK OX lines. (B) Mannitol response of the AtNEK6 KO line. (C) Salt response of the AtNEK6 OX lines. (D) Salt response of the AtNEK6 KO line. In each experiment, plants with fully-open green cotyledons were counted five days after sowing seeds on medium containing various concentrations of mannitol or NaCl as indicated in the graph. Experiments were conducted in triplicates (n = 30 each) and the small bars indicate the standard errors.

Germination of the AtNEK6 knockout seeds is insensitive to high osmolarity

Shoot development, i.e., cotyledon expansion/greening and true leaf development is inhibited by high concentrations of glucose (Ramon et al., 2008). This glucose-mediated process is associated with ABA, and ABA sensitivity is correlated with glucose sensitivity. Therefore, we determined the glucose sensitivity of the AtNEK OX and the AtNEK KO lines. As shown in Fig. 5B, the AtNEX6 OX lines are hypersensitive to glucose. For example, fully-open green cotyledons were observed with 85% of the wild type seeds in the presence of 3% glucose, whereas green cotyledons were observed in 60% of the AtNEK6 OX line seeds. Similarly, lower rates of cotyledon greening (20% vs 5-10%) were observed in response to 4% glucose. Thus, our results showed that the AtNEK6 OX lines are more sensitive to glucose inhibition of seedling establishment. We conducted similar experiments using the AtNEK6 KO line. The results (Fig. 5C) revealed that shoot development of the KO sublines was less sensitive to glucose than that of the wild type plants. For example, at 3% glucose, approximately 85% of the wild type cotyledons turned green, whereas 90-100% of the AtNEK6 KO cotyledons turned green at the same glucose concentration. At 4% glucose, 50% of the wild type cotyledons turned green, whereas more than 80% of the AtNEK6 KO cotyledons turned green. Higher rates of cotyledon greening were also observed at 5% glucose. Thus, our results indicate that AtNEK6 OX lines are glucose-hypersensitive and that its KO line is glucose-insensitive.

Glucose is an osmolyte; therefore, the inhibitory effect of glucose on seedling development may be due to a general osmotic effect rather than a glucose-specific effect. To distinguish between these two possibilities, we investigated the effect of high osmolarity on the germination of AtNEK6 transgenic lines using mannitol (Fig. 6A). The germination of the wild type seeds gradually decreased to 95%, 90% and 70% of the control rate in response to the addition of 3%, 4% and 5% mannitol to the medium, respectively. The AtNEK6 OX lines exhibited a similar decrease in germination efficiency with increasing amounts of mannitol in the medium. However, the degree of inhibition was greater in the OX lines. Specifically, the germination efficiency of the OX lines was 75%, 70% and 40% or 50% at the same concentrations of mannitol. Conversely, the AtNEK6 KO line was less sensitive to mannitol inhibition of germination than the wild type seedlings (Fig. 6B). For instance, fully-open green cotyledons were observed in 77 or 89% of the KO sublines, whereas green cotyledons were observed in approximately 50% of the wild type seeds. Thus, the AtNEK6 OX lines were hypersensitive to mannitol, whereas the knockout line was partially insensitive. These findings suggest that glucose inhibition of seedling establishment is not specific to glucose and may instead be due to a general osmotic effect.

To further investigate the effect of osmolarity on germination of the AtNEK6 OX and KO lines, we determined the salt effect on seed germination and seedling establishment (i.e., cotyledon greening). When NaCl was added to the growth medium at 50 mM, 100 mM and 150 mM, seedling establishment of the wild type plants was inhibited to 90%, 85% and 70%, respectively, when compared to the control (Fig. 6C). Inhibition of the seedling development by NaCl was also observed in the AtNEK6 OX lines, but the degree of inhibition was more severe than that observed with the wild type seedlings at each concentration of NaCl examined. By contrast, the AtNEK6 KO line was less sensitive to NaCl inhibition (Fig. 6D). For example, fully-open green cotyledons were observed in 50% of the wild type seeds exposed to 150 mM NaCl. However, green cotyledons were observed in 90% of the AtNEK6 KO seeds at the same concentrations of NaCl. Thus, AtNEK6 OX lines were hypersensitive to NaCl, whereas its knockout line was less sensitive to NaCl. Taken together, the results of our study demonstrate that AtNEK6 OX lines are hypersensitive to high osmolarity, whereas its KO line is less sensitive to high osmolarity. In summary, the findings presented here indicate that AtNEK6 is a positive regulator of high osmolarity response during the seed germination stage.

DISCUSSION

We previously showed that an ARM repeat protein, ARIA, interacts with one of the ABF family transcription factors that regulate ABA-responsive gene expression (Kim et al., 2004). ARIA is a positive regulator of ABA response and is also involved in high osmolarity response during germination. Therefore, seed germination of ARIA overexpression lines is hypersensitive to ABA, mannitol, glucose or NaCl. Here, we showed that a NIMA-related protein kinase, AtNEK6, interacts with ARIA and that, similar to ARIA, it is involved in ABA and high osmolarity responses during seed germination.

We isolated AtNEK6 based on its interaction with ARIA. The

results of the present study (Fig. 2) indicated that the full-length ARIA is required for its interaction with AtNEK6. By contrast, full-length AtNEK6 or the N-terminal kinase domain region did not interact with ARIA, whereas the C-terminal region of AtNEK6 did interact with ARIA (Figs. 1B and 2). These results suggest that, although AtNEK6 might associate with ARIA through its C-terminal region, ARIA itself might not be the substrate of AtNEK6. Sakai et al. (2008) and Motose et al. (2008) also observed that the C-terminal region of AtNEK6 is involved in binding to ARK1, which is an Arm repeat-containing kinesin, or microtubule.

To determine the *in planta* function of AtNEK6, we investigated the ABA- and stress-associated phenotypes of AtNEK6 OX and its knockout lines. As shown in Fig. 3, high level expression of AtNEK6 resulted in minor growth retardation. As a result, it took longer for the AtNEK6 OX lines to mature. Conversely, the AtNEK6 KO line grew faster than the wild type plants (Fig. 4). Thus, our results indicate that AtNEK6 affects the growth rate; however the AtNEK6 OX lines and the AtNEK6 KO line exhibited no other distinct morphological phenotypes at the whole plant level.

Our analysis of the AtNEK6 OX and KO phenotypes revealed that it is involved in the control of seed germination. Under normal conditions, the AtNEK6 OX lines germinated normally. However, the AtNEK6 KO line germinated several hours earlier than the wild type seeds. These results suggest that, although AtNEK6 alone is not sufficient to suppress germination, it is involved in inhibition of the germination process. Together with the regulatory role observed with plants grown in soil, these findings demonstrate that AtNEK6 is a negative regulator of both seed germination and seedling growth.

The role of AtNEK6 in seed germination was further confirmed by the enhanced response of seed germination to ABA. As shown in Fig. 3, AtNEK6 overexpression promoted the inhibition of germination by ABA. By contrast, ABA induced inhibition of germination was diminished in the AtNEK6 KO line. Thus, AtNEK6 is a positive regulator of the ABA-mediated inhibition of seed germination.

Glucose inhibits seedling establishment (i.e., cotyledon expansion and greening), and the process, which is distinct from a general osmolarity effect, is known to be mediated by ABA. Therefore, we evaluated AtNEK6 to determine if it is involved in the glucose-mediated process. The results (Fig. 5) revealed that AtNEK6 is a positive regulator of glucose response. Specifically, AtNEK6 overexpression lines were hypersensitive to glucose (Fig. 5B), whereas its knockout line was partially insensitive (Fig. 5C). However, the results of our experiments conducted to determine the possible involvement of AtNEK6 in the osmolarity response (Fig. 6) indicated that the effect was not specific to glucose. We found that the AtNEK6 OX lines were hypersensitive to mannitol and NaCl, and that their knockout lines were partially insensitive to these compounds. Together, our results demonstrated that AtNEK6 is a positive regulator of high osmolarity response during germination. We investigated other ABA- and stress-associated phenotypes but did not observe any distinct phenotypes during postgermination growth stages.

Others have reported that AtNEK6 is a regulator of epidermal cell morphogenesis (Motose et al., 2008; Sakai et al., 2008). For example, Motose et al. isolated a mutant named *ibo1*, in which local outgrowth in the middle of hypocotyls and petioles produced protuberances. They demonstrated that IBO1 gene is identical to AtNEK6, which possesses kinase activity and is associated with cortical microtubules. On the other hand, Sakai et al. (2008) identified AtNEK6 as a protein that interacts with ARM repeat kinesin1 (ARK1) and found that its mutant has pleiotropic effects on epidermal cell morphogenesis.

Our study indicated that AtNEK6 is also involved in ABA and osmolarity responses during germination. We do not know yet how AtNEK6 controls the germination process. NEKs are generally known to regulate the cell cycle in fungi and mammals, and it has been suggested that they regulate developmental processes in tomato and poplar such as shoot development and flowering (Cloutier et al., 2005; Pnueli et al., 2001). Although limited to the germination stage during plant development, our data suggest that AtNEK6 is also involved in the regulation of plant growth.

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