NADK3, a novel cytoplasmic source of NADPH, is required under conditions of oxidative stress and modulates abscisic acid responses in Arabidopsis

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Summary
In plants, excess reactive oxygen species are toxic molecules induced under environmental stresses, including pathogen invasions and abiotic stresses. Many anti-oxidant defense systems have been reported to require NADPH as an important reducing energy equivalent. However, the sources of NADPH and the molecular mechanisms of maintaining cytoplasmic redox balance are unclear. Here, we report the biological function of a putative cytoplasmic NAD kinase (NADK3) in several abiotic stress responses in Arabidopsis. We found that cytoplasmic NADPH is provided mostly by the product of the NADK3 gene in Arabidopsis. Expression of the NADK3 gene is responsive to abscisic acid (ABA) and abiotic stress conditions, including methyl violgen (MV), high salinity and osmotic shock. An NADK3 null mutant showed hypersensitivity to oxidative stress in both seed germination and seedling growth. Seed germination of the mutant plants also showed increased sensitivity to ABA, salt and mannitol. Furthermore, stress-related target genes were identified as upregulated in the mutant by mannitol and MV. Our study indicates that this cytoplasmic NAD kinase, a key source of the cellular reductant NADPH, is required for various abiotic stress responses.

Keywords: NADH kinase, oxidative stress, abscisic acid.

Introduction
In higher plants, the generation of reactive oxygen species (ROS), such as superoxide (\(^{•}O_2\)\(_2^-\)), hydrogen peroxide (H\(_2\)O\(_2\)) and hydroxyl radicals (\(^{•}OH\)), is exacerbated in response to environmental stress conditions, including drought, salinity and low temperature (Fedoroff, 2002; Finkelstein et al., 2002; Himmelbach et al., 2003), as well as biotic stress (Riley, 1994; Wojtaszek, 1997). Excess ROS, as toxic cellular metabolites, irreversibly damage a variety of biomolecules, such as lipids, proteins and nucleic acids, thus causing cell death and plant lethality. ROS have also been shown to be signals affecting the expression of a number of genes and signal transduction pathways (Apel and Hirt, 2004; Dat et al., 2000; Mittler, 2002; Mullineaux and Karpinski, 2002; Neill et al., 2002). Plants have evolved a set of anti-oxidant defense systems to maintain ROS homeostasis (Mittler et al., 2004). The anti-oxidant enzymes, such as thioredoxin (TRX) per-oxidases, methionine sulfoxide reductases and tripeptide glutathione (GSH) peroxidases, are dependent on the reduced forms of GSH and TRX, which are maintained through TRX and GSH reductases in an NADPH-dependent manner (Asada and Takahashi, 1987). Thus, the production of NADPH is a vital and possibly limiting step in the generation/scavenging of ROS.

NAD(H) kinases may play important roles in regulating the formation of NADP(H) by catalyzing the ATP-dependent phosphorylation of NAD(H). The activities of NAD(H) kinases have been found in all organisms investigated to date, suggesting their essential role in all organisms (Kawai et al., 2001a,b; Lerner et al., 2001; McGuinness and Butler, 1985; Zielinski, 1998). Based on their preference for the oxidized or reduced form of NAD(H), NAD(H) kinases have been classified as NAD kinases (EC 2.7.1.23) and NADH kinases (EC
2.7.1.86), respectively. Despite a prominent function in controlling NAD(H)/NADP(H) balance, genes encoding NAD(H) kinases were cloned only recently from human (Lerner et al., 2001), yeast (Kawai et al., 2001a; Outten and Culotta, 2003) and bacteria (Kawai et al., 2000, 2001b). NAD kinase activity has also been found in chloroplasts (Jarrett et al., 1982), mitochondria (Dieter and Marme, 1984) and cytoplasm (Simon et al., 1982) in many plant species. In Arabidopsis, three NAD(H) kinases, NADK1 (At3g21070; gi30686057), NADK2 (At1g16140; gi30687452) and NADK3 (At1g78590; gi30699338), have been cloned recently (Berrin et al., 2005; Chai et al., 2005; Turner et al., 2004, 2005). When expressed in Escherichia coli, both NADK1 and NADK2 were catalytically active (Turner et al., 2004). The transcript of NADK1 is induced by ionizing radiation and hydrogen peroxide. Loss of function of NADK1 causes enhanced sensitivity to oxidative stress (Berrin et al., 2005). NADK2, localized in the chloroplast, plays a vital role in chlorophyll synthesis and chloroplast protection against oxidative stress (Chai et al., 2005). Recombinant NADK3 from E. coli was able to phosphorylate NADH and NAD (Turner et al., 2005).

Although NADH kinases have been studied at the biochemical level (Turner et al., 2005) in plants, the enzyme has been poorly characterized at the molecular level. In this study, we investigated the biological roles of NADK3 in Arabidopsis. The transcript of NADK3 is induced by abiotic stress and abscisic acid (ABA). Loss of function of NADK3 reduces the NADPH content under oxidative stress, and the nadk3 mutant is sensitive to various stresses and ABA. Our study suggests that NADK3, the cytoplasmic source of NADPH, is a key enzyme in abiotic stress and the ABA response.

Results

Expression of NADK3 and localization of NADK3

Three genes encoding NAD(H) kinase have been found in Arabidopsis (Hunt et al., 2004). In this study, we focused on the biological function of one NAD(H) kinase, NADK3, a putative cytoplasmic protein. To test its organ-specific expression in Arabidopsis, RNAs were extracted from various organs including roots, stems, leaves, flowers and siliques, and the transcript levels were analyzed by RT-PCR. NADK3 was ubiquitously expressed in all organs tested, with root having the highest level of NADK3 transcripts (Figure 1a).

To determine the subcellular localization of the NADK3, the coding region of NADK3 was fused in-frame with GFP at the N-terminal region. Transient expression of this fusion protein was examined under confocal microscopy in Arabidopsis protoplasts. NADK3–GFP fluorescence was spread throughout the cytoplasm (Figure 2d–f). We found that NADK1–GFP was also localized in the cytosol (Figure 2g–i). As controls, GFP fluorescence of NADK2 was co-localized with chlorophyll autofluorescence (Chai et al., 2005) and 35S::GFP was localized ubiquitously (Figure 2a–c).

NADK3 is inducible by abiotic stress in seedlings

To investigate the effect of various stresses including cold, osmotic stress, high salinity, oxidative stress or ABA on the transcript level of NADK3, Arabidopsis seedlings were subjected to the indicated treatments, and the expression level of NADK3 was examined by quantitative real-time RT-PCR. Interestingly, the expression of NADK3 was strongly induced by cold, osmotic stress and a superoxide-generating agent methyl violgen (MV) (Figure 1b). The transcript level of NADK3 increased up to the maximum level (5–10 fold) 6 h after treatment, and then decreased afterwards. The NADK3 transcript level was slightly induced by ABA and high salinity. In contrast, the transcript levels of the other two homologs of NADK3, NADK1 and NADK2, were not affected by the indicated stresses (Figure 1c). These results indicate that NADK3 is a stress-responsive gene that is involved in abiotic stress- and ABA-mediated signaling pathways.

The NADK3-deficient mutant is hypersensitive to oxidative stress, ABA and osmotic stress

To further reveal the biological function of the NADK3 gene, we isolated a mutant line (nadk3) that harbored a T-DNA in the first intron–exon junction of NADK3 (Figure 3a). This insertion abolished NADK3 mRNA expression in nadk3 as indicated by RT-PCR analysis using NADK3-specific primers (Figure 3b). We noticed that the transcript abundance of NADK1 and NADK2 was not affected in nadk3 (data not shown). nadk3 does not exhibit any morphological or developmental abnormalities under standard growth conditions.

To assess the functional roles of NADK3 in response to abiotic and ABA stresses, seed germination and seedling growth of wild-type and nadk3 were investigated under various stresses. As shown in Figure 4, the germination and subsequent growth of nadk3 were similar to those of wild-type on MS medium only. However, a large variation in germination rate and seedling and root growth was observed in the presence of ABA, MV, high salinity and mannitol. Germination of nadk3 was delayed by about 2 days under these abiotic and ABA stresses (Figure 4b). Germination of nadk3 was significantly inhibited by MV at a concentration of 0.1 M, which did not affect the germination of wild-type plants. At 150-mM NaCl, 88% of wild-type seeds and 37% of nadk3 mutant seeds germinated on day 3 after stratification (DAS). At 1 M ABA, more than 69% of wild-type seeds germinated, whereas only about 12% of mutant seeds had germinated on day 3 after stratification.
Similarly, the germination frequencies of wild-type and nadk3 seeds were 88% and 39%, respectively, on the medium containing 200-mM mannitol. In addition to these variations in germination rate, seedling and root growth of nadk3 were also different from those of wild-type under the indicated stresses. In general, the seedlings and roots of nadk3 mutants directly germinated on media containing these stress agents grew less well than those of wild-type. When the radicles of nadk3 emerged from the seed coat, the roots of wild-type were already extended in the presence of MV, ABA or high salt. These results demonstrate that nadk3 is hypersensitive to ABA, oxidative stress, high salinity and osmotic stress during seed germination and early seedling development, which suggests that the NADK3 gene is important for plants to confer stress tolerance.

Moreover, we observed that seedlings and adult plants of nadk3 were also more sensitive to MV than wild-type after nadk3 seedlings were transferred from MS medium to MV-containing medium (Figure 6a, and data not shown). The growth of both root and leaves in nadk3 was severely inhibited at 0.2-μM MV, whereas the wild-type could grow normally under this condition. This indicates that the nadk3 mutant is more sensitive to the oxidative stress response not only at the germination stage but also later in development.

Consistent with the results for young seedlings, we found that nadk3 adult plants were more susceptible to osmotic stress.
stress, high salt and ABA than wild-type, although they were relatively tolerant to stress compared to young seedlings (data not shown).

To confirm that the altered response of nadk3 to these stresses is caused by the disruption of NADK3, we expressed NADK3 cDNA under the control of the CaMV 35S promoter in the nadk3 mutant. Transgenic plants (nadk3 35S::NADK3) were selected based on their resistance to hygromycin, and the phenotype of the T2 lines was compared to that of the wild-type under the above stress conditions. nadk3 35S::NADK3 transgenic plants and wild-type plants showed similar response to ABA, oxidative stress, osmotic stress and high salt at early seedling growth and late development (Figure 4a), indicating that the nadk3 phenotype was caused by the loss of function of NADK3.

As hyperosmotic stress and high salinity could increase endogenous ABA levels in plants (Leung and Giraudat, 1998; Seo and Koshiba, 2002), the sensitivity of nadk3 to these stresses might be a consequence of ABA accumulation. To test this possibility, we analyzed the germination of nadk3 on medium with an inhibitor of ABA biosynthesis, norflurazon (NF). As shown in Figure 5, on the MS medium, application of 100-μM NF did not affect the germination of wild-type or nadk3 seeds in a germination assay. However, on the medium containing 200-mM mannitol, NF rescued the hypersensitive phenotype of nadk3 seeds to osmotic stress (Figure 5a). About 30% of nadk3 seeds germinated on the mannitol-containing medium on day 3 after stratification; however, the germination rate was restored to 76% in the presence of 100-μM NF (Figure 5b). These results indicated that osmotic stress exerted its inhibitory role on the germination of nadk3 seeds through excess ABA.

The reducing energy equivalent in nadk3 is lower than in wild-type under oxidative stress

To further clarify the effects of oxidative stress on nadk3, we tested the levels of oxidative stress markers, including endogenous level of NADPH, chlorophyll, and the ratio of reduced and oxidized forms of glutathione (GSH/GSSG). Under normal growth conditions, no difference in the levels of NADPH, chlorophyll and GSH/GSSG was observed between nadk3 and wild-type (data not shown). However, the NADPH level was significantly reduced in nadk3 compared with wild-type in the presence of MV on MS medium (P < 0.05). When nadk3 was back-crossed to wild-type, F1 progenies had similar NADPH content to wild-type. In the F2 progeny population, a lower level of NADPH co-segregated with homozygous nadk3 mutants under oxidative stress (data not shown). The NADPH levels in nadk3 35S::NADK3 complemented lines were restored to wild-type levels (Figure 6b). The chlorophyll content in nadk3 was also reduced compared with wild-type under oxidative stress (P < 0.05). We also investigated the redox status of cells in the nadk3 mutant by measuring GSH/GSSG. Figure 6(b) shows that GSH/GSSG was dramatically decreased in nadk3 mutants under oxidative stress (P < 0.01), whereas the GSH/GSSG level in nadk3 NADK3 lines reverted to that of the wild-type. Taken together, these results demonstrate that that
decreased levels of NADPH, chlorophyll and GSH/GSSG in \textit{nadk3} were indeed because of the loss of function of NADK3 under oxidative stress.

Expression of genes in the ROS-scavenging network is increased in \textit{nadk3}

It was shown that excess H$_2$O$_2$ could induce the expression of genes associated with anti-oxidant defense, such as ZAT12, GPX1, CAT1, FER1 and RbohD (Cheong \textit{et al.}, 2002; Desikan \textit{et al.}, 2001; Levine \textit{et al.}, 1994; Mullineaux \textit{et al.}, 2000). ZAT12, GPX1, CAT1, FER and RbohD encode zinc-finger protein, peroxidase, catalase, ferredoxin and NADPH oxidase, respectively, which are involved in oxidative stress pathways.

As \textit{nadk3} is sensitive to MV, we examined the transcript abundance of oxidative stress-regulated genes in \textit{nadk3} in the presence of MV. Without oxidative stress, no significant differences were observed in steady-state mRNA levels of ZAT12, GPX1, CAT1, FER1 and RbohD between the wild-type and \textit{nadk3}. However, the expression of ZAT12, GPX1, CAT1, FER1 and RbohD was dramatically affected under oxidative stress (Figure 7a). The transcript abundance of GPX1, CAT1 and FER1 was specifically increased as early as 4 h after MV treatment and was even more upregulated subsequently in \textit{nadk3} (Figure 7a). The ZAT12 and RbohD RNA transcripts were also more abundant in \textit{nadk3} than in wild-type when treated with MV for 4 and 24 h, respectively (Figure 7a).

The expression of stress genes is enhanced in \textit{nadk3}

The germination and seedling growth assays revealed that NADK3 functions in the regulation of osmotic stress responses in plants. A number of different stress genes respond to abiotic stress stimuli at the molecular level, and are commonly used as markers for monitoring stress-response pathways in plants. They include \textit{RD29A}, \textit{KIN1}, \textit{KIN2}, \textit{RAB18}, \textit{DREB2A} and \textit{ERD10} genes. We next investigated whether NADK3 functions in transcription regulation of these stress genes. As expected, all these markers were induced by osmotic stress in wild-type plants (Figure 7b). Moreover, the transcripts of these genes in the \textit{nadk3} mutant were more abundant than in the wild-type upon stress treatments for 24 h.

Discussion

Plants may be prone to oxidative damage because of the accumulation of ROS at high levels under abiotic stress. Therefore, they have had to develop a number of anti-oxidant
defense systems to combat oxidative stress. One of these defense systems involves generation of the key reductant, NADPH, which functions in both normal metabolic activities and plant defense mechanisms in response to various environmental stresses. Moreover, the ample supply and maintenance of NADPH and the balance between NAD(H)/NADP(H) are critical for cell survival. It is logical to assume that the enzymes catalyzing the synthesis of NADPH play crucial roles in these processes. However, the source for NADPH remains elusive in plants.

In the cytosol of both yeast and mammals, NADPH is synthesized primarily by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase through the pentose phosphate pathway. These enzymes play important roles in protection from oxidative stress (Juhnke et al., 1996; Pandolfi et al., 1995; Slekar et al., 1996). POS5 and NADP-IDHm are NADH kinases that synthesize mitochondrial NADPH in yeast and mammals (Jo et al., 2001; Outten and Culotta, 2003). We demonstrate here that NADK3 is important for the generation of NADPH required for oxidative stress protection in Arabidopsis. The pools of NADPH and its functions in different organelles appear to be specific. Our previous study revealed that a chloroplast NAD kinase plays a vital role in the chloroplast NADPH supply (Chai et al., 2005). The NADH kinase in the cytosol (NADK3) provides cytosolic NADPH, which is subject to oxidative stress and ABA.

There are three Arabidopsis genes, NADK1, NADK2 and NADK3, that contain a putative NADK domain. We found in this study that NADK1 and NADK3 are localized in the cytosol, whereas NADK2 is targeted to the chloroplast (Chai et al., 2005). The null mutants of these three genes have similar and distinct morphological phenotypes. NADK1 grows almost normally under normal conditions, but is more sensitive than wild-type to gamma irradiation and paraquat-induced oxidative stress (Berrin et al., 2005). NADK2 mutants show dwarf adult plants and rosette leaves with a pale yellow color under non-stress conditions. They also display hypersensitivity to environmental stresses that provoke oxidative stress, such as UVB, drought, heat shock and salinity (Chai et al., 2005). We show here that, without stress, NADK3 displays similar growth behavior to wild-type; however, it shows enhanced sensitivity to oxidative stress, salinity and ABA. These results suggest that these genes have redundant and distinct functions in the maintenance of redox status and plant development.
NADK3 functions in oxidative stress

Mutation of nadk3 mRNA levels of these genes were clearly upregulated in increased expression of stress marker genes under osmotic stress. The ROS-scavenging network, including RbohD and RbohF, and ROS production is rate-limiting for ABA signal transduction in vivo (Kwak et al., 2003). Therefore, NADPH-associated enzymes may function as regulators mediating the ABA-signaling pathways. We showed here that NADK3 serves multiple functions in the development and stress responses in Arabidopsis. nadk3 was hypersensitive to exogenous ABA and mannnitol, showing a significant delay in germination on the corresponding media. This is consistent with the results obtained from the assay using an ABA biosynthesis inhibitor NF. Although it has been shown that NADPH is required for ABA responses in the stomatal guard cells (Allen et al., 1999), little is known about its role in seed germination in plants. So far the role of NADPH has not been addressed in ABA signaling during seed germination in dicot plants. Our results suggest that NADK3 is a negative regulator of ABA responses during seed germination in Arabidopsis. Further study of the effect of NADK3 on the regulation of NAD(H)/NADP(H) and the ABA response will shed light on the role of NADK3 on ABA signaling.

Experimental procedures

Plant materials and stress treatments

Arabidopsis thaliana seeds (ecotype Col) were treated with ethanol for 1 min and with 50% bleach for 5 min, and washed five times with sterile water. Sterilized seeds were sown on MS agar, which was supplemented with 2% w/v sucrose. The seeds were placed in the darkness at 4°C for 48 h, and thereafter transferred to a greenhouse at 22 ± 1°C under long-day conditions (16 h light/8 h dark cycle). For RNA analysis, 6-day-old seedlings grown on MS medium (Sigma, St Louis, MO, USA) were treated under various stress conditions. For ABA treatment, 1 μM S(+)ABA was sprayed on the seedlings grown on MS plates to ensure total coverage of the foliage area. The plants were incubated at room temperature under white light. In parallel experiments, water was sprayed as a control. For NaCl and mannitol treatments, 150-mm NaCl or 200-mm mannitol was added to MS plates, and the seedlings were incubated under white light. For cold treatment, seedlings were transferred to the 4°C cold room under white light. For MV treatment, 2-week-old Arabidopsis seedlings grown on MS plates were sprayed with 0.2-μM MV.

Subcellular localization of NADK1-GFP and NADK3-GFP fusion proteins

NADK1 and NADK3 full-length cDNA were amplified by PCR using primers NADK1-1F and NADK1-1R for NADK1, and NADK3-1F and NADK3-1R for NADK3.

Figure 7. Expression of oxidative stress- and ABA/stress-induced genes in nadk3.

(a) Expression of oxidative stress-related genes was determined by quantitative real-time RT-PCR under stress conditions. Relative amounts were calculated and normalized with respect to the oxidative stress-induced gene transcript levels in wide-type (100%). The ACTIN gene served as a control. ACTIN2/8; At5g15940; KIN1, At5g15960; KIN2, At5g15970; RAB18, At5g18780; RAB29A, At5g59820; FER1, At5g01600; RbohD, At5g47910; ZAT12, At5g59820.

(b) Expression of stress-regulated genes was determined by quantitative real-time RT-PCR under osmotic stress. Relative amounts were calculated and normalized with respect to the ABA-induced gene transcript levels in wide-type (100%). The data shown represent mean values and standard errors obtained from three independent experiments.

Previous studies have shown that several components of the ROS-scavenging network, including ZAT12, GPX1, CAT1, FER1 and RbohD, are elevated in Arabidopsis in response to a very large number of different abiotic and biotic stresses (Cheong et al., 2002; Desikan et al., 2001; Levine et al., 1994; Mullineaux et al., 2000). We found that the steady-state mRNA levels of these genes were clearly upregulated in nadk3 (Figure 7a) compared to the wild-type under oxidative stress. The greater expression of genes encoding the ROS-scavenging network enzymes may be attributed to the excess oxidative stress in nadk3. Furthermore, the decrease in the key reductant NADPH might be the major reason that the nadk3 mutant is hypersensitive to oxidative stress. Mutation of NADK3 also leads to early induction or increased expression of stress marker genes under osmotic stress conditions, which agrees with the hypersensitivity phenotype of nadk3 to various stresses. This suggests that NADK3 is involved in the regulation of stress gene expression in Arabidopsis.

It has been shown that the NADPH-cytochrome P450 reductase gene that is essential for oxidative catabolism of ABA in plant tissues also requires NADPH (Krochko et al., 1998; Malonek et al., 2004; Saito et al., 2004). ABA-induced ROS production is catalyzed by two NADPH oxidase genes, AtrbohD and AtrbohF, and ROS production is rate-limiting for ABA signal transduction in vivo (Kwak et al., 2003). Therefore, NADPH-associated enzymes may function as regulators mediating the ABA-signaling pathways. We showed here that NADK3 serves multiple functions in the development and stress responses in Arabidopsis. nadk3 was hypersensitive to exogenous ABA and mannnitol, showing a significant delay in germination on the corresponding media. This is consistent with the results obtained from the assay using an ABA biosynthesis inhibitor NF. Although it has been shown that NADPH is required for ABA responses in the stomatal guard cells (Allen et al., 1999), little is known about its role in seed germination in plants. So far the role of NADPH has not been addressed in ABA signaling during seed germination in dicot plants. Our results suggest that NADK3 is a negative regulator of ABA responses during seed germination in Arabidopsis. Further study of the effect of NADK3 on the regulation of NAD(H)/NADP(H) and the ABA response will shed light on the role of NADK3 on ABA signaling.
NADK3-1R for NADK3 (Table 1). They were cloned into the XbaI-BamHI and \textit{NdeI-BamHI} sites of a modified pBI221 vector (Clontech, Palo Alto, CA, USA) containing a soluble modified red-shifted GFP (SmRS-GFP) at the \textit{BamHI}-\textit{NcoI} site (Davis and Vierstra, 1998). Protoplast transformation was performed as described previously (Sheen, 2001). The autofluorescence (red) and the GFP images of chloroplasts were observed under confocal laser scanning microscopy (LSM 510 CLSM; Zeiss, Jena, Germany).

Isolation and complementation of the nadk3 T-DNA insertional mutant

The \textit{nadk3} insertion allele (SALK_079342) was obtained from the Salk T-DNA line collection (http://signal.salk.edu/cgi-bin/tdnaexpress). The insertion in the gene was identified by using the T-DNA left border primer, LB-1, and NADK3-2F and NADK3-2R primers (Table 1). The T-DNA insertion in the mutant \textit{(nadk3)} was confirmed by PCR and DNA gel-blott analysis, and its exact position was determined by sequencing. The homozygous \textit{nadk3} mutant was identified by PCR and analyzed further by DNA sequencing to confirm the insertion of the T-DNA in the gene. Plants homozygous for \textit{nadk3} were used for further analysis.

For complementation of the \textit{nadk3} mutant, a 954-bp fragment including the NADK3 coding region was amplified by PCR from Arabidopsis cDNA using NADK3-3F and NADK3-3R primers (Table 1). The PCR product was cloned into the binary vector pCAMBIA1391 (CAMBIA, Canberra, Australia) using the EcoRI and \textit{PmlI} restriction sites. The construct was transformed into \textit{Agrobacterium} strain GV3101 and introduced into \textit{nadk3} plants by the floral dip method (Clough and Bent, 1998). Transgenic seeds were plated on half-strength MS medium containing 0.8% w/v agar, 112 mg l\textsuperscript{-1} Gamborg's B5 vitamin mixture, and 15 \textmu g/ml hygromycin. Hygromycin-resistant seedlings were transplanted into soil and grown in the greenhouse. The transgenic lines (\textit{nadk3} S5S::NADK3) were used for germination assays or RNA gel-blot analyses. All of the PCR

<table>
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<td>5'-CGTGTATTACCTACATGTGAGATG-3'</td>
</tr>
<tr>
<td>RbohD-F</td>
<td>5'-GGATGAGGAGACACCATTTGGGAAGG-3'</td>
</tr>
<tr>
<td>RbohD-R</td>
<td>5'-GGAGATACGCGCCGAGCTTAC-3'</td>
</tr>
<tr>
<td>ACTIN2/8-F</td>
<td>5'-GGTAAACATTGTCAGTGATG-3'</td>
</tr>
<tr>
<td>ACTIN2/8-R</td>
<td>5'-AACACCTTAAATCTCAGTGC-3'</td>
</tr>
</tbody>
</table>

Table 1 Gene-specific primers used in this study.
procedures were performed using Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) to enhance fidelity. All constructs were verified by DNA sequencing.

**Germination assay**

Approximately 100 seeds each from the wild-type, nadk3 and nadk3 35S::NADK3 complemented lines were planted in triplicate on MS media with different concentrations of ABA, NaCl, mannitol or MV, and incubated at 4°C for 2 days before being placed at 22°C under long-day conditions. Germination (emergence of radicles) was scored daily for 6 days. Plant growth was monitored and photographed after 7 days.

**Measurement of chlorophyll content, GSH/GSSG and NADPH**

Chlorophyll was extracted with acetone at 4°C from 2-week-old seedlings. The extracts were subjected to spectrophotometric measurements at 603, 645 and 663 nm (Mochizuki et al., 2001). The relative concentrations of GSH and GSSG in wild-type and nadk3 were determined as described previously (Griffith, 1980).

Pyridine nucleotides were extracted from 30 mg of Arabidopsis leaves with 1 ml of 0.1-N NaOH. The extracts were boiled for 6 min, cooled on ice, and centrifuged at 12,000 g for 6 min, and the soluble fraction was neutralized by adding 0.1-N HCl, followed by incubation on ice for 15 min (Gogorcena et al., 1995; Hajirezaei et al., 2002). The whole neutralized mixture was then used to quantify the nucleotides by the enzyme-cycling method (Matsumura and Miyachi, 1980).

The results were subjected to statistical analysis by Student’s t test and the significance was determined at both $P < 0.05$ and $P < 0.01$.

**RT-PCR and quantitative real-time RT-PCR**

Total RNA was extracted with Tripure reagent (Roche Diagnostics, Mannheim, Germany) from 10-day-old seedlings unless stated otherwise. Total RNA (1 μg) was reverse-transcribed using SUPERSCRIPT II (Invitrogen, San Diego, CA, USA). A portion of the resulting cDNA was then subjected to PCR amplification using gene-specific primers (Table 1). For quantitative real-time PCR, the resulting cDNA was then subjected to PCR amplification using ACTIN2/8 (Invitrogen, San Diego, CA, USA) to enhance fidelity. All constructs were verified by DNA sequencing.

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**References**


