NDP kinase 2 regulates expression of antioxidant genes in *Arabidopsis*

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Abstract: NDP kinases (NDPKs) are multifunctional proteins involved in cell proliferation, development, and differentiation in eukaryotes. Previously, we reported that *Arabidopsis* NDP kinase 2 (AtNDPK2) is a component of H₂O₂-activated MAPK signaling in plants and demonstrated that its over-expression in plants alters cellular redox conditions (*Proc. Natl. Acad. Sci. U. S. A.*, **100**, 358-363, 2003). To elucidate how AtNDPK2 can regulate cellular redox state, we analyzed gene expression profiles of transgenic plants over-expressing *AtNDPK2* using cDNA microarray technology. Constitutive over-expression of AtNDPK2 in plants induced numerous genes including those involved in cellular signal transduction and protection, suggesting that NDPKs are also multifunctional in plants. Among the induced genes, we further analyzed the expression of antioxidant genes by Northern blot analysis. Expression of peroxidase, catalase, thioredoxin, thioredoxin reductase, and peroxiredoxin was significantly increased in transgenic plants over-expressing AtNDPK2. Furthermore, the gene expression pattern obtained by Northern blotting was quantitatively consistent with the cDNA microarray analysis. Based on these observations, we suggest that down-regulation of cellular redox state in AtNDPK2 transgenic plants is mediated by genes involved in antioxidant and protective processes.

Key words: Nucleoside diphosphate kinase; redox regulation; cDNA microarray; gene expression; antioxidant gene.

Introduction. Redox signaling is mediated by reactive oxygen species (ROS) such as superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radical (HO[•]) and lipid hydroperoxides. A slight alteration in the homeostatic set point of intracellular ROS levels signals the cell to modulate its metabolism, gene expression, and post-translational modification of proteins,¹⁾⁻³⁾ indicating that ROS are important for regulating normal cellular functions. However, when ROS levels exceed the cellular antioxidant capacity, the cellular homeostasis is altered and this results in toxic damage to lipids, carbohydrates, proteins and DNA. In plants, environmental stress can result in excessive ROS production. For example, cold, salt, drought, heat, excessive light, osmotic shock, UV-B irradiation and pathogens can cause high levels of ROS to be generated and/or accu-

mulated.³⁾⁻¹⁰⁾ These high concentrations of ROS can damage plant tissues and, in severe cases, can lead to cell death.⁶⁾⁻¹⁰⁾

In response to oxidative stress, plants possess their own defense systems. Plants can perceive increased cellular ROS content induced by environmental stressors and activate repair and disposal systems that are mediated by intricate signaling cascades.^{11),12)} Repair systems are necessary for reversal of oxidative modifications and disposal systems for removal of damaged macromolecules. The repair systems can be divided into two types; enzymatic and non-enzymatic systems. Non-enzymatic repair systems include glutathione, ascorbic acid, thioredoxin and glutaredoxin and, in general, are involved in the removal of free radicals.^{13),14)} Catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, and thioredoxin constitute the enzymatic defense systems,^{15),16)} and these remove oxygen radicals and their products and/or repair the damage

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Table I. NDP kinase 2 regulates expression of antioxidant genes in *Arabidopsis*

Accession No.	Clone ID	Putative function/product	Microarray	Northern
			ratio	images
			Cy3/Cy5	w.t. / t.g
AAC15253		AtNDPK
O24364	APZ04h07	2-Cys peroxiredoxin BAS1	3.5
Q42547	APD18d01	Catalase 3	1.8
AAF07798	APD48b09	Putative thioredoxin	1.5
T00824	APD01c11	Thioredoxin reductase homolog	3.0
CAA67427	APD68h04	Thylakoid-bound ascorbate peroxidase	2.9
T51607	APZ36d07	Glutathione transferase	1.8

caused by oxidative stress. In addition, chaperone-like molecules and heat shock proteins also function as a repair system by mediating conformational change during oxidative stress.¹⁷⁾⁻¹⁹⁾

Nucleoside diphosphate kinases (NDPKs) are ubiquitous enzymes, catalyzing the reversible transfer of the 5'-terminal phosphates from triphosphate nucleosides (or their deoxy derivatives) to nucleoside diphosphates to generate NTPs or dNTPs. These proteins play an important role in energy conversion and in cellular house-keeping functions. In addition to this, NDPKs are involved in various signal transduction processes and their interactions with several proteins *in vivo*, including GAPDH, DnaK and heat stress proteins, modulates their activity under a wide range of stress conditions.²⁰⁾⁻²³⁾ In plants, NDPK function is associated with the phytochrome A response, UV-B signaling, heat stress and growth.^{21),24)-26)} Hence, NDPK is strongly implicated in the regulation of cellular functions, possibly through its phosphotransferase activity.^{24),27),28)}

Recently, we reported that AtNDPK2, an *Arabidopsis* NDPK isoform, is associated with MAPK-mediated H₂O₂ signaling in plants.¹¹⁾ AtNDPK2 specifically interacts with two H₂O₂-activated MAPKs, AtMPK3 and AtMPK6, and enhances the MBP phospho-

rylation activity of AtMPK3. Over-expression of AtNDPK2 in plants down-regulates the accumulation of ROS and this, in turn, enhances the tolerance of *Arabidopsis* plants to multiple abiotic stressors.¹¹⁾ In the present study, we examined how such multiple stress tolerance can arise from AtNDPK2 over-expression. To this end, we employed cDNA microarray technology and investigated the gene expression profile in response to AtNDPK2 over-expression. Here, we provide the first evidence that AtNDPK2 regulates the expression of genes involved in antioxidant and protective functions.

Materials and methods. Wild-type (ecotype WS), and *AtNDPK2*-overexpressing transgenic (T₃) *Arabidopsis thaliana* plants were used in this study. Plants were transformed with the *Agrobacterium* binary vector *pCAMBIA1300* (provided by Jefferson, CAMBIA, Australia) containing *AtNDPK2* cDNA (Accession number AF017640) inserted into the *Xba*I and *Bam*HI site under the control of the *CaMV35S* promoter. *AtNDPK2* expression in transgenic plants was confirmed by both Northern (Table I) and Western analysis.¹¹⁾ cDNA microarrays (IntelliGene™ *Arabidopsis* CHIP I Version 1.0) used in this study were purchased from Takara Shuzo Co., Ltd. The chip was arrayed and immobilized with 2,223 cDNA fragments which were iso-

lated and identified from *Arabidopsis* in the Kazusa DNA Research Institute (Japan). Detailed information on the spotted DNA is available in the Takara database (<http://www.takara.com/>).

Total RNA was isolated from 14-day old wild-type and NDPK2 over-expressing plants using an Optima™ TL Ultracentrifuge (Beckman, U. S. A.) with CsCl cushion as described previously.²⁹⁾ Probes were prepared as described^{30,31)} with several modifications. We used 100 µg of total RNA for each sample, the RNAs were mixed with 3 µg of oligo dT primer (12 - 18mer) and 1 ng of λ polyA⁺ RNA-A as an external control (Takara, Japan) in a final volume of 20.5 µl. This mix was incubated at 70 °C for 10 min, chilled on ice, and then added to 19.5 µl of labeling reaction mix containing 625 µM each of dATP, dCTP and dGTP, 250 µM dTTP, 1 mM Cy-3-dUTP/Cy 5-dUTP (Amersham Pharmacia, U. S. A.), 400 units Superscript II RT (Invitrogen, U. S. A.), 40 units RNase OUT (Invitrogen, U. S. A.), 1 mM dithiothreitol and 1 × reverse transcriptase buffer. The reaction mixture was incubated at 42 °C for 2 h, then 5 µl of 0.5 M EDTA was added and the sample was treated with 10 µl of 1 M NaOH for 10 min at 65 °C. The reaction mix was neutralized by adding 25 µl of 1 M Tris-HCl (pH 7.5). The labeled probes were purified on Sephacryl™ S-100 columns (Amersham Pharmacia, U. S. A.) and the purified probes were precipitated using 1/10 NaOAc (pH 5.2) and 2.5 volumes of ethanol (−20 °C, at least 2 h) then dried completely in the dark and dissolved in 10 µl of hybridization buffer (6X SSC, 0.2% (w/v) SDS, 5 × Denhardt's solution and 0.1 mg/ml Salmon sperm DNA). It was denatured at 95 °C for 2 min then cooled on ice for 5 min and applied to the array.

After covering the array with a 22 mm × 22 mm cover slip, the slides were placed in a humidified hybridization chamber (16 h at 60 °C). After hybridization, the slides were washed twice in 2 × SSC, 0.2% (w/v) SDS for 30 min at 58 °C then 0.05 × SSC for 5 min at room temperature and centrifuged at 800 rpm for 5 min using a table-top centrifuge.

Hybridized microarrays were scanned sequentially for Cy5- and Cy3-labeled probes with a ScanArray Lite laser scanner (GSI Lumonics, U. S. A.) at a resolution of 5 µm. Lambda DNA spots and beta-2 tubulin spots in different positions were scanned to balance the signal intensities of the two channels and total spots were normalized with the QuantArray software program (GSI Lumonics). Changes in transcript abundance ≥ 1.5-fold were calculated using the Excel software program (Microsoft).

Fig. 1. Global expression pattern of *AtNDPK2* over-expressing transgenic plants under non-stress conditions. The expression ratios of all transcripts (Cy3/Cy5) were plotted against the printed order of the ESTs (X-axis) on the microarray.

For Northern blot analysis, total RNA from control and transgenic plants was isolated as previously described¹¹⁾ and 10 µg was electrophoresed on a formaldehyde-1.5 % agarose gel. It was then transferred to a Millipore Immobilon-Ny⁺ transfer membrane, UV cross-linked and hybridized with gene-specific probes as described in the figure legends. RNA blot hybridization and membrane washing were performed as previously described.³²⁾

Results and discussion. In previous studies, we determined that the *AtNDPK2* gene appears to be involved in regulating cellular redox state.¹¹⁾ Here, we examined whether *AtNDPK2* over-expression in plants influences the expression of certain genes under non-stressed conditions using cDNA microarray technology.

To obtain a gene expression profile specific to *AtNDPK2* over-expressing plants, cDNA microarrays were hybridized with Cy3 and Cy5 fluorescent-labeled probe pairs of wild-type and transgenic plants prepared as described in the Materials and methods section. To minimize the inherent variability of the microarray assay, two microarray slides were used to analyze the transcription differences of each sample pair and dye labeling was alternated in each microarray hybridization. Dual labeling of cDNA probe pairs with Cy3-dCTP for one mRNA sample and Cy5-dCTP for the other allows simultaneous hybridization to DNA elements on microarrays and facilitates direct quantitative measurements of gene expression between two different cell types. Hybridized microarrays were scanned on two separate laser channels for Cy3 and Cy5 emissions from each DNA element. The ratio of the two fluorescent signal intensities was then used as a relative measure to determine the fold change in expression of the

Table II. Partial list of up-regulated genes from the signal transduction-related functional group

Accession No.	Clone ID	Up-regulated genes in signal transduction-related functions	Ratio
NP_192639	APD15a01	14-3-3-like protein	1.86
AAG17006	APD04f04	ARF GAP-like zinc finger-containing protein ZIGA2	2.37
T06126	APD01e03	Calcium-dependent protein kinase CPK5	2.45
AAA80652	APZ14a07	Calreticulin	2.00
AAC49697	APD60a01	Calreticulin	2.33
CAB40129	APD65g10	Cyclic nucleotide and calmodulin-regulated ion channel	1.92
NP_199563	APD33d12	GTP-binding protein-like	2.68
P18064	APD58d07	Guanine nucleotide-binding protein alpha-1 subunit	1.70
O24456	APZ05c09	Guanine nucleotide-binding protein alpha-1 subunit	1.93
T04262	APD18h08	Mitogen-activated protein kinase kinase	1.78
NP_177492	APZ09e04	MAP kinase, putative	3.16
S71170	APZ08d09	Phosphoinositide-specific phospholipase C	1.74
Q38882	APD32g05	Phospholipase D precursor	2.18
T04845	APZ24c07	Probable serine/threonine-specific protein kinase	2.27
T04432	APD57f09	Protein kinase homolog T18B16.80	1.82
NP_174427	APD20g12	Protein kinase, putative	2.13
NP_198447	APD04c11	Protein kinase-like	2.35
NP_190047	APZ08g11	Protein kinase-like protein	3.18
CAB82700	APZ28g08	Protein phosphatase 2C-like protein	1.78
NP_188334	APD19h03	Protein phosphatase-2C, putative	3.34
AAC69134	APZ04e04	Putative auxin-repressed protein	2.32
NP_172750	APD17d04	Putative calcium-binding protein, calreticulin	1.98
AAF16581	APD64g03	Putative GTPase activator protein of Rab-like small GTPases	2.77
CAB87708	APD64g07	Putative GTP-binding protein	1.81
AAD29771	APD64c12	Putative receptor-like protein kinase	2.42
BAB01126	APD20a09	Receptor protein kinase	2.50
P42818	APD56a05	Serine/threonine-protein kinase AtPK1/AtPK6	2.55
NP_195849	APD04b03	Serine/threonine-specific protein kinase NAK	1.76
NP_191981	APD17b05	Shaggy related protein kinase theta	2.53
T04863	APD04f03	Shaggy-like protein kinase eta	1.87
BAA77716	APD15h09	SNF1 related protein kinase	1.79
CAA08794	APD33d08	Wall-associated kinase 1	1.78
NP_178085	APZ07g05	Wall-associated kinase 4	2.41

sequence represented by that DNA element. In this study, differences greater than 1.5-fold between Cy3 and Cy5 fluorescent signal intensity (FSI) values of array elements in a co-hybridization were deemed to be significant. Fig. 1 shows the overall effect of AtNDPK2 on the expression of individual genes. The expression ratios of all transcripts (Cy3/Cy5) were plotted against the printed order of the ESTs (X-axis) on the microarray. In transgenic plants, approximately 741 (30 %) and 93 (0.5%) of transcripts were up- or down-regulated, respectively. However, most data points fell within two lines, which corresponded to ratios of expression between 1.5:1 and 1:1.5. This indicated that the level of expression of these genes was not significantly different in wild-type and transgenic plant cells.

Over-expression of AtNDPK2 induces expression of large number of genes which can be classified into several different functional categories, such as signal transduction, transcription, metabolism, and stress

responses (data not shown). As AtNDPK2 interacts with H_2O_2 -activated protein kinases and reduces the accumulation of ROS inside the cell,¹¹⁾ genes involved in signal transduction and stress responses were further analyzed. Our studies show that many genes involved in protein phosphorylation and dephosphorylation, calcium- and GTP-mediated signaling are specifically induced (Table II). In addition, genes involved in ROS metabolism and abiotic and biotic stress responses are also up-regulated (Table III). To corroborate the results in Tables II and III, we examined gene expression by Northern blotting. Since AtNDPK2 appears to regulate cellular redox conditions, we compared the expression of antioxidant genes in wild-type and AtNDPK2 over-expressing plants (Table I). Northern blot analysis demonstrated that 2-cysteine peroxidase, catalase, thioredoxin, thioredoxin reductase, ascorbate peroxidase and glutathione S-transferase were constitutively expressed in AtNDPK2 over-expressing plants. Furthermore, the

Table III. Partial list of up-regulated genes from the stress-related functional group

Accession No.	Clone ID	Up-regulated genes in defense related function	Ratio
BAA85108	APZ04h07	2-Cys peroxiredoxin BAS1	3.50
Q42547	APD18d01	Catalase 3	1.80
AAC83165	APD15b04	Disease resistance protein RPP8	1.84
CAB85987	APD34d12	dnaK-type molecular chaperone hsc70.1	1.83
NP_195869	APZ52b07	dnaK-type molecular chaperone hsc70.1-like	3.81
T51607	APZ36d07	Glutathione transferase	1.80
O04885	APZ20f04	Lactoylglutathione lyase	1.91
AAC97512	APZ53a10	Low temperature and salt responsive protein LTI6A	1.84
P24101	APD17e07	Neutral peroxidase C precursor	1.93
CAB61998	APZ37e04	Peroxidase	2.05
NP_195468	APZ08c10	Peroxidase, prxr2	1.96
NP_190864	APD40g05	Peroxiredoxin - like protein	2.13
AAF02131	APD60g05	Putative 2-cys peroxiredoxin	1.95
AAC04915	APD51d06	Putative leucine-rich repeat disease resistance protein	1.78
NP_181529	APD17g04	Putative nematode-resistance protein	2.12
AAF07798	APD48b09	Putative thioredoxin	1.50
AAD55637	APD61b09	Similar to part of disease resistance protein	1.87
AAB71452	APZ28g11	Strong similarity to Arabidopsis peroxidase	1.99
T00824	APD01c11	Thioredoxin reductase homolog	3.00
CAA67427	APD68h04	Thylakoid-bound ascorbate peroxidase	2.93
AAF02113	APZ09c11	Violaxanthin de-epoxidase homolog F22O13.3	1.90
T00708	APZ16c07	Violaxanthin de-epoxidase homolog F22O13.3	2.83

results obtained from the DNA microarrays were quantitatively consistent with Northern blot analysis (Table I). These results suggest that AtNDPK2 positively regulates the expression of antioxidant genes.

NDPKs are multifunctional proteins that regulate a variety of eukaryotic cellular activities, including cell proliferation, development, and differentiation. Recent studies also suggest that NDPKs play significant roles in signal transduction, which include transcriptional activation, supply of GTP to G-proteins, interaction with other signaling proteins and protein phosphorylation.²⁰⁾⁻²⁶⁾ Consistent with these reports, over-expression of AtNDPK2 induces the expression of large number of genes involved in cell signaling. The change in gene expression of many identified genes may result from indirect effect of ectopic expression of AtNDPK2 under non-stressed conditions. However, the reduction of ROS level in the transgenic plant in our previous study¹¹⁾ is consistent with up-regulation of genes encoding ROS scavenging enzymes such as peroxidases, peroxiredoxin, and thioredoxin (Tables I and III). Although the exact mechanism by which AtNDPK2 specifically regulates expression of these genes is still unclear, our cDNA microarray results provide functional clues for large numbers of genes involved in NDPK-mediated signaling and stress responses.

Conclusion. The functional and regulatory role of AtNDPK2 in relation to redox regulation was considered

here. We employed cDNA microarray technology and showed that AtNDPK2 regulates the expression of genes involved in antioxidant and protective functions. These results confirm our earlier observations¹¹⁾ and suggest that AtNDPK2 mediates multiple stress tolerance by signaling the transient expression of genes involved in antioxidant and protective functions, possibly through activation of the MAPK cascade.

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