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Regulations of marker genes involved in biotic and abiotic stress by overexpression of the *AtNDPK2* gene in rice

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Abstract

AtNDPK2 is involved in transcriptional regulation in response to pathogen and abiotic stresses. *AtNDPK2*-expressing transgenic rice plants showed regulation of the marker genes for chilling and oxidative stresses. In the present study, we produced *AtNDPK2*-overexpressing transgenic rice lines using the co-transformation method. Morphologically, the transgenic plants, compared with the control plants, were growth retarded. We investigated how *AtNDPK2* overexpression influences the response of *rice* plants to marker genes related to chilling and ROS stress. The accumulation of transcripts of *pBC442* and *pBC601*, related to chilling stress, was induced in *AtNDPK2*-overexpressed rice plants. On further investigation, we found that *OsAPX1*-, *OsAPX2*-, and *OsSodB*-scavenging free-oxygen radicals, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), could be induced in *AtNDPK2*-overexpressed rice plants. In particular, transcripts encoding pathogenesis-related (PR) proteins OsPR2 and OsPR4, as well as oxidative stress response proteins, were confirmed to change the gene expression in the transgenic rice plants. Together, these results suggest that *AtNDPK2* plays a regulatory role in chilling and antioxidant signaling in plants.
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Keywords: AtNDPK2; Chilling stress; Oxidative stress; Pathogen-related proteins; Co-transformation

Plant productivity is severely affected by abiotic stresses resulting from high salinity, drought, high and low temperature, and heavy metals. Physiological and biochemical responses are altered and cellular aqueous and ionic equilibria are disrupted. The expression of hundreds of genes is affected by these stresses [1–3], and understanding the functions of these stress-inducible genes should help to clarify the mechanisms of stress tolerance. Inoculation of *Arabidopsis* plants with rhizobacteria enhanced protection against both *Erwinia carotovora* and dehydration stress [4]. Biotic stress studies have demonstrated significant overlaps between plant responses to osmotic stresses [5].

Aerobic organisms cannot live without oxygen, yet paradoxically, oxygen is inherently dangerous to their existence, due to the chance that oxygen is converted to reactive oxygen species (ROS) in biological pathways [6]. In fact, excessive amounts of ROS in living organisms can cause severe injury [8]. Hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), and hydroxyl radical ($HO^{\cdot}$) due are deemed ROS [7]. Dehydration, salinity, and low- as well as high-temperature stresses cause damage metabolism, leading to both the generation of ROS and the inhibition of photosynthesis [9]. At the molecular level, abiotic stress tolerance can be achieved via gene transfer, such as altering the accumulation of osmoprotectants, producing chaperones, increasing the number of superoxide radical scavenging mechanisms, or extruding or sequestering ions by means of transport and symport systems [9,10].

In animals, NDPKs play important roles in signal transduction pathways [11] as well as in processes such as transcription regulation, cell proliferation control, and protein

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phosphotransferase activity [12–14]. In plants, they are involved in the phytochrome A response, UV-B signaling, heat stress, and growth [15–18]. NDPK2 has been associated with a positive signaling component of the phytochrome-mediated light-signal-transduction pathway in *Arabidopsis* [15]. NDPK2 identified the interactions with phytochromes in a yeast two-hybrid screen, and its activity was shown to increase through binding to the red light-activated form of PhyA in vitro [15,19]. Transgenic *Arabidopsis* plants overexpressing NDPK2 had lower levels of ROS and enhanced tolerance to several environmental stresses [20]. Moreover, NDPK2 displayed an important component in the phytochrome signaling pathway from *Arabidopsis* mutant lacking NDPK2 [15].

Human efforts especially in developing tolerances to various abiotic and biotic stresses have been directed toward increasing rice production on land of limited cultivability [21]. Rice has become a model monocot system for genetic and functional genomic studies, and appropriately so, as it is the primary crop for more than half of the world's population. Additional efforts have employed biotechnological techniques in the quest to improve rice's agronomic traits [22]. However, the signal transduction pathways and response mechanisms to environmental stresses remain poorly understood. In this report, therefore, we identify genes that are changed in expression level by the overexpression of *AtNDPK2* in transgenic rice plants. We modified rice plants to overexpress *Arabidopsis thaliana NDPK2* and conducted RT-PCR experiments to identify the marker genes involved in the biotic and abiotic stresses that changed the expression levels in the genes of the transgenic rice plant leaves. In light of our results, we suggest that *AtNDPK2* plays a role in the biotic and abiotic stress response via transcriptional regulation of several marker genes.

Materials and methods

Agrobacterium-mediated transformation of rice. AtNDPK2 cDNA was cloned into the binary vector pNL under the control of the stress-inducible sweetpotato peroxidase, SWPA2 promoter [23] (Fig. 1A). The plasmid pCambia1300 contains of the hygromycin phosphotransferase gene (hpt) driven by 35S promoter (Fig. 1B). These construct was used to transform *Agrobacterium tumefaciens* strain AGL1 via the freeze-thaw method [24]. Single bacterial colony of *Agrobacterium* strain AGL1 was incubated in AB medium (AB buffer and salt, 5 g/l glucose, 15 g/l Bacto-agar, 50 mg/l spectinomycin, 10 mg/l tetracycline, pH 7.2) at 28 °C for 72 h. Bacterial cells were collected by scraping and resuspending in AAM medium (AA macro- and microsalt, amino acid stock, MS vitamins, 500 mg/l casamino acid, 68.5 g/l sucrose, and 36 g/l glucose) containing 100 mM acetosyringone and the final concentration of *Agrobacterium* was adjusted to 5×10^9 cells/ml. The *Agrobacterium* strain AGL1 was mixed at 1:1 ratio before being used for transformation. Embryogenic calli derived from the scutellum of mature rice seeds, "Dongjinbyeo" was used for transformation according to the method described by Hiei et al. with slight modification [25]. Embryogenic calli selected were mixed with *Agrobacteria* in 25 ml AAM solution containing around 5×10^9 cell/ml and incubated for 20 min at room temperature. The calli were then plated on NB3 medium (N6 4 g, casein 0.3 g, proline 2.9 g, sucrose 30 g, 2.4-D 2 ml, glutamine 0.5 g, N-B micro 1 ml, N-B vts 1 ml, myo-inositol 0.1 g, pH 5.8, gelrite 2.5 g) for co-cultivation at 25 °C in dark condition for 3 days. After washing to remove the overgrown *Agrobacteria* using cefotaxime solution (250 mg/l in distilled water), the calli were transferred to 2N6-CH medium (2N6 medium supplemented with 250 mg/l cefotaxime, 50 mg/l hygromycin B) depending on the selection scheme and cultured at 27 °C in dark condition for 3 weeks. Selection was done with hygromycin B resistance primarily during the first 5 weeks (callus proliferation stage) after transformation treatment. Selected calli were subcultured 2 weeks after the start of first selection on fresh 2N6-CH medium and further grown for 3 weeks before being transferred to MSRK5SS medium (MS 4.4 g, NAA 1 mg, Kinetine 5 mg, sucrose 20 g, sorbitol 30 g, proline 0.5 g MES 0.5 g, pH 5.8, gelrite 4 g, cefotaxium 250 mg/l, and hygromycine 50 mg/l) for second selection and regeneration for 4 weeks at 27 °C under continuous light condition. The regenerated shoots were transferred to MSO medium (MS salts and vitamins, 30 g/l sucrose, 2 g/l phytagel, pH 5.8) for root induction for 2 weeks before acclimation in a Magenta box containing 0.1% Hyponex solution (Hyponex Co., Imlay City, MI 48444) for 1 week in a culture room. The plantlets were then transplanted to a Wagner pot (1/5000a) in a greenhouse for subsequent growth.

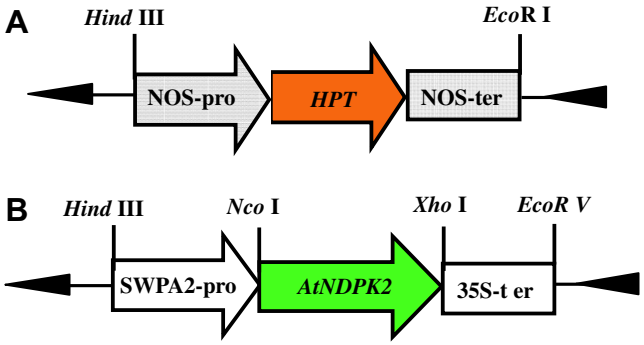

Fig. 1. Schematic representation of the T-DNA region of pCambia 1300 containing of hygromycin resistant gene (HPT) (A) and pNL containing of *Arabidopsis thaliana* NDPK2 (AtNDPK2) under SWPA2 promoter (B).

DNA analysis. All of the transgenic plants were grown under identical conditions. Transgenic plants were analyzed by PCR and Southern hybridization for the integration of the transgene genes. The processed DNA samples were used as template to amplify *AtNDPK2* gene. The primer sequences were designed for a 1.3 kb of *AtNDPK2* genes. The primer sequences used were as follows: *AtNDPK2* forward, 5′-GGCA ATTCATGCAT CCTCAACCCC-3′; and *AtNDPK2* reverse, 5′-GCTCAACACATGAGCGAAACCC-3′. PCR was carried out with *Taq* DNA polymerase in 20-μl reaction volume in a thermal cycler (Bio-red com.). The PCR cycle program consisted of initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 7 min. The PCR product was resolved by 1% agarose gel electrophoresis in 0.5× Tris–acetate–EDTA (TAE) buffer along with 1 kb DNA ladder as marker (Invitrogen). For Southern blot analysis of transgenic rice plants, 20 μg of purified plant DNA was completely digested with EcoRI restriction enzyme. DNA fragments were resolved on 0.8% agarose gels and blotted on N^+ Hybond nylon membranes. Radiolabeled probe of *AtNDPK2* gene was prepared with the ^{32}P.

Northern blot analysis. The total RNA was isolated from the transgenic rice plants [26]. Plant materials (1 g) were frozen in liquid nitrogen and homogenized in 10 ml extraction buffer [4 M guanidine isothiocyanate, 25 mM sodium citrate at pH 7.0, 0.55% (w/v) *N*-laurylsarcosine and 0.1 M 2-mercaptoethanol]. A mixture of 2 M sodium acetate (pH 4.0), water saturated phenol, and chloroform–isoamylalcohol (24:1) was added to the homogenate. After centrifugation, the pellet was suspended in 2 M LiCl solution and incubated at 4 °C for 18 h. The total RNA were determined by spectrophotometer and staining of the ribosomal RNA with ethidium bromide, respectively. Equal quantities of the total RNA (10 μg) were loaded into 1% agarose gel containing 7.4% formaldehyde. The RNA was

transferred onto nylon membranes (Hybond N^+, Amersham), then crosslinked under irradiation with UV light. Probe was used to same as Southern blot. Hybridization was performed overnight at 65 °C in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% (w/v) SDS, and 1 mM EDTA. After hybridization, the filter was washed twice with 2× SSC and 0.1% SDS for 10 min each at room temperature, and twice with 0.1× SSC and 0.1% SDS for 5 min each at 65 °C.

Conformation of differentially expressed genes by RT-PCR. Total RNA was treated with 1 U DNase for 10 min at 37 °C. From the DNase-treated total RNA (1 μg), first-strand cDNA was synthesized using the AccuPower® PCR PreMix (Bioneer, Korea) containing oligo(dT) primers, and Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen, USA).

Primers used for reverse transcriptase-PCR were as follows:

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
OsActin	ATGGCTGACGCCG AGGAT	TTAGAAGCATTTCCTGTG
pBC121	AATCTTGGTTTGC ATTCC	CGGGTACATATGGACAGC
pBC442	GGCCATATCACTG CCTGG	CACATTGGTGTCTTCAGG
pBC591	GGGGACGCTCCGT GATGC	CGGCACGTCTACACACTG
pBC601	AGAAGGAAAGAGA AGGCA	CGTCCTCGTCCTCTTGGG
OsSodA	GCTACTTCTGCCAC TTGC	GTGCTCCATGCTCCTTTC
OsSodB	GGCTCGACATGTG GACGG	GCCCAGAGACACTTCCCG
OsAPX1	ACAAAGCCCTGCT GAGTGAC	TAACAGCCCACCGAGACATT
OsAPX2	CCAAGTGACAAAG CCCTCAT	TCTTGACAGCAAATAGCTTGG
OsPR2	GTCCCGGGCGGCG GCGGC	GGTGAGCTCGGCGACCTG
OsPR4	GAAGCATCCAACG TGCGA	CCACGACCATCTGTGTCG

The PCR reaction was carried out as follows: initial 5-min denaturation at 94 °C; followed by 25 cycles of 94 °C, 30 s; 60 °C, 30 s; and 72 °C, 30 s; and a final 7 min at 72 °C. Twelve microliter samples of the reaction products were separated on 1% agarose gels and visualized after staining with ethidium bromide. All experiments were preformed in triplicate.

Results and discussion

Overexpression of rice using co-transformation method

Vector pCambia contained a single T-DNA harboring the *hpt* gene driven by the 35S promoter (Fig. 1). Vector pNL contained the *AtNDPK2* gene under the control of the SWPA2 promoter. The mixture method, which uses *Agrobacterium* strain AGL1 to deliver multiple T-DNAs to plants cells, was used to introduce pCambia 1300 and pNL binary vectors into the rice plants [27–29]. Co-transformation methods have been studied in pursuit of possible strategies for the elimination of selectable marker genes [27,30]. Negative selection tends to focus on the target gene. Each T-DNA integrated by co-transformation behaves as a Mendelian locus during inheritance [30,31]. The relative amounts of each *Agrobacterium* strain in the infection mixture were expected to influence the co-transformation efficiency [28]. We found that the co-transformation efficiency of rice was very low. By contrast, the co-transformation efficiency was dramatically highest in tobacco [32].

Regenerated shooting calli were induced from a regeneration medium, and then transferred to a shoot elongation and rooting medium (Fig. 2A). The T_0 plants were acclimated in pots containing sterilized soil. The transgenic rice plants showed an obvious growth difference compared with the control plants (Fig. 2B).

Confirmation of transformation by DNA and RNA analyses

The transformed explants were successfully regenerated and subsequently were transferred to soil. PCR was performed to confirm whether the *AtNDPK2* sequence was integrated into the genomic DNA of the T_0 transgenic plants with the primer pairs specifically targeted at the AtNDPK2 sequence to produce a 1.3 kb amplification fragment. No DNA fragment was amplified from the control plants (Fig. 3A). Transgenic rice plants were selected the 7 lines through PCR analysis. Also, the integration of the AtNDPK2 transgene into the rice genome, successfully or not, was verified by Southern blot analysis. Hybrid bands showing the copy number as the amplified DNA fragment were detected from six transgenic lines. We obtained two independent transgenic lines from the Southern blot results and then selected #1 and #4 for Northern blot or RT-PCR (Fig. 3B). These results indicated the stable integration of the AtNDPK2 sequence into the rice genome. However, the transformation frequency was extremely low (data not shown). Transformation frequency can be affected by many factors such as genotype, explants type, medium composition, and hormone combination. All transformed plants yielded the expected band. Thus, transgene expression was examined by Northern blot analysis using a 1.3 kb fragment of *AtNDPK2* as the probe. All of the tested transgenic plants were expressed as the transgene (Fig. 3C).

Altered gene expression in AtNDPK2 transgenic rice plants

Generation of ROS in plants has been implicated in abiotic and biotic stress responses, in which, as in oxidative cell death, the level of ROS is an important cellular regulator. For instance, phytochrome-mediated light signaling modulates cold-induced gene expression [33]. To elucidate the marker gene mechanism involved in chilling stress in *AtNDPK2*-overexpressed plants, the expression patterns of several chilling-related genes were monitored in both control and *AtNDPK2*-overexpressed plants. A semi-quantitative RT-PCR analysis was performed with RNA prepared from AtNDPK2-overexpressed rice leaves (Fig. 4A). Although low-temperature affects plant growth and crop productivity, the tolerance of a variety of temperate plants can be increased after a period of exposure to low, non-freezing temperatures, which process is known as cold acclimation [34]. However, plants vary in their

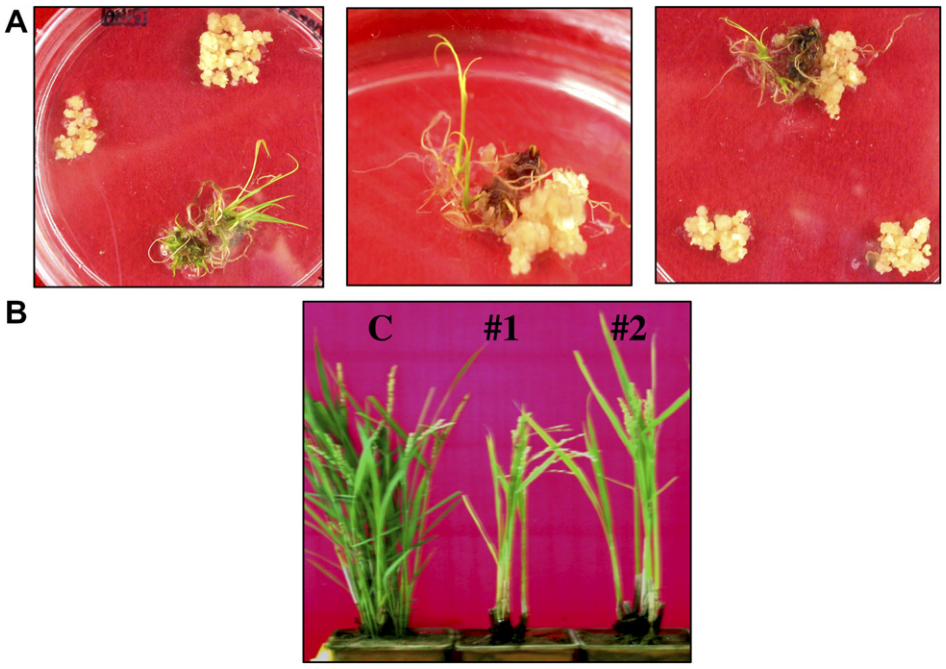

Fig. 2. Generation of transgenic plants. (A) Putative transgenic lines in selection medium. (B) T_0 transgenic plants grown in soil. Control, C and transgenic lines #1 and #4 were planted in soil in a pot.

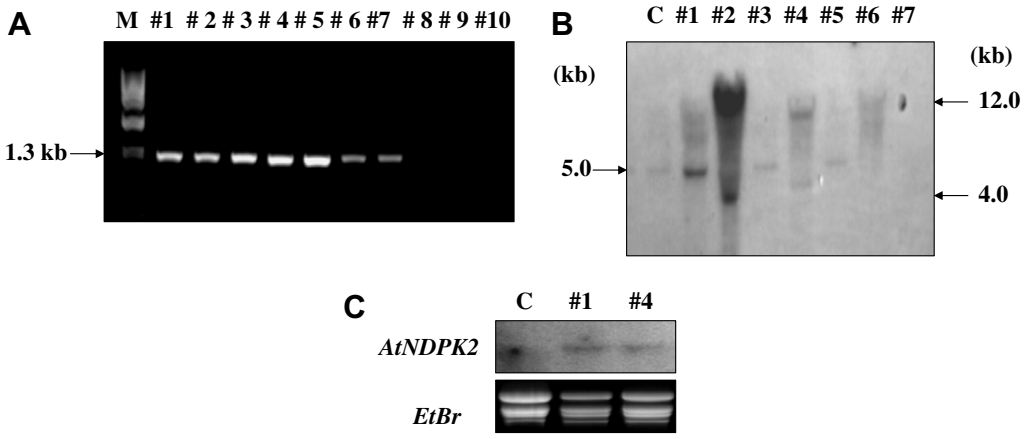

Fig. 3. Polymerase chain reaction (PCR), Southern and Northern blot analyses of transgenic rice plants. (A) PCR analysis was performed on transgenic T_0 rice plants, using *AtNDPK2* gene-specific primers. M, 1 kb DNA ladder (Invitrogen, San Diego, CA); C, control plants; seven transgenic rice plants. (B) Southern blot analysis of T_0 plants: EcoRI digest of genomic DNA (20 μg) detected the integrated AtNDPK2 gene with a 1.3 kb fragment of AtNDPK2. (C) AtNDPK2 messenger RNA expression in transgenic T_0 rice plants. Two independent transgenic lines and a control plant were subjected to RNA gel-blot analysis. Total RNA was extracted from fully expanded leaves of 3- to 4-week-old rice plants grown in green house.

ability to survive at low temperatures. The genes have been documented as chilling-related genes induced during the disease-resistance response in rice plants [35]. Rice cultivars vary widely in response to low temperatures, according to their breeding advantage or disadvantage, and thus cold tolerance has been studied in various rice cultivars [36]. For example, Kasamo [36] has found differences in the behaviors of rice cells of tonoplast and plasma-membrane ATPases during exposure to low temperatures. PSII, not PSI, is the sensitive site for light-chilling stress in chilling-sensitive rice [37]. Activation of ABA and *CBF/DREB* has an important role in the rapid responses of rice seedlings to chilling stress [38]. In the results of a cold tolerance assay, AtNDPK2 transgenic plants were 50% more tolerant to cold stress than the wild type [20]. Therefore, it can be concluded that AtNDPK2 functions as a positive regulator in the down-regulation of the cellular redox state [20]. Four clones, pBC121, pBC442, pBC591, and pBC601, contain cDNA sequences homologous to chilling-induced transcripts [39]. These genes were identified that a group of genes responsible for chilling tolerance have been isolated from rice seedlings [39]. We confirm that of those genes, pBC442 and pBC601 were regulated by AtNDPK2-overexpressed rice plants.

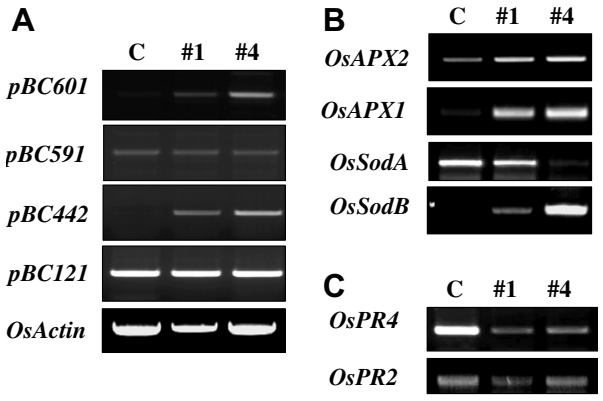

Fig. 4. RT-PCR analysis for AtNDPK2-overexpressed transgenic rice lines. The expression levels of several genes related to biotic and abiotic stress were confirmed by RT-PCR analysis. The total RNA was extracted from control plants and transgenic tomato lines #1 and #4. Expression patterns of chilling stress (A), oxidative stress (B), and defense-related (C) genes using RT-PCR analysis of AtNDPK2-overexpressed rice plants. Total RNA was extracted from leaf tissues of overexpressed plants. Genbank accession numbers of these genes are pBC121, BAA01630; pBC442, BAA01631; pBC591, BAA01632; pBC601, CAA90866; OsSodA, P28756; OsSodB, B28757; OsAPX1, BAA08264; OsAPX2, BAB20889; OsPR2, AAP92751; OsPR4, AAL11444. One microgram of total RNA was used for cDNA synthesis, and PCR was performed using the primers described in Materials and methods.

As shown in Fig. 4B, the expression of most of the genes related to the analyzed ROS-scavenging enzymes was changed in the *AtNDPK2*-overexpressed rice plants, compared with the control plants. We performed RT-PCR experiments using the primers of four genes (*OsAPX1*, *OsAPX2*, *OsSodA*, and *OsSodB*) to detect the expression levels. The experiments confirmed that *OsAPX1*, *OsAPX2*, and *OsSodA* were up-regulated in the AtNDPK2-overexpressed rice plants. However, the levels of expression of *OsSodB* were significantly decreased in the AtNDPK2-overexpressed rice plants compared with the control plants. SOD (superoxide dismutase) is considered a critical component of the biological defense against oxidative stress [40]. Such SOD activities are generally involved in specific subcellular locations such as mitochondria (Mn-SOD), plastids (Cu/Zn-SOD and/or Fe-SOD), and the cytosol (Cu/Zn-SOD), and show a differential expression pattern in response to environmental and chemical stresses [41]. The *SodA* gene encodes for another cytosolic Cu/Zn-SOD in rice [42]. However, we still do not know how transcriptional expression of the *OsSodB* gene is reduced in AtNDPK2-overexpressed rice plants. The transcript level of the cytosolic *APX* genes are known to be regulated by many environmental stresses, such as those resulting from high light, salt, wounds, pathogen infection, fruit ripening, paraquat, and H_2O_2 [43]. Differential regulation of OsAPX1/2 expressions by diverse environmental cues, including pathogens, and their developmental regulation in both the young and reproductive stages, suggests their role in stress-signaling pathways and development in rice [44]. Based on an expression analysis, it appears that OsAPX1 and OsAPX2 are more likely to be involved in plant response to oxidative stress, lending additional evidence for the involvement of AtNDPK2 in antioxidant-mediated signaling pathways. *AtNDPK2* overexpression is increased expression of a number of antioxidant genes such as peroxidase, thioredoxin reductase, peroxiredoxin, glutathione reductase, glutathione transferase, and protective genes encoding several heat-shock proteins [20].

To understand the growing complexity of the mechanism of defense/stress response, availability of defense-related marker genes, differentially regulated by components of the signaling pathways, becomes increasingly essential. The *PR4* mRNA that differentially accumulate in abundance with time during compatible and incompatible host–pathogen interaction, has been weakly detected in healthy leaves [45]. In our ongoing search for defense-/stress-related rice markers, we investigated the response to the *OsPR4* gene in AtNDPK2-overexpressed rice plants. *OsPR4* expression was reduced in all AtNDPK2-overexpressed lines compared with the control plants (Fig. 4C). In addition, the expression patterns of OsPR2 in AtNDPK2-overexpressed rice plants have no difference of transcriptional level between control and transgenic plants (Fig. 4C).

Transgenic *Arabidopsis* plants expressing AtNDPK2 had lower levels of ROS and showed tolerance to several environmental stresses, such as those resulting from cold, salt, and H_2O_2 [20]. Signaling and ROS-dependent up-regulation suggest an important role for NDPK2 in the environmental stresses associated with ROS generation [20]. A significant reduction in ion leakage has been significantly reduced in AtNDPK2 transgenic barley plants [46]. However, we have not yet characterized whether transgenic rice expressing AtNDPK2 enhanced tolerance to multiple stress. In the current study, we demonstrated that AtNDPK2 has a potential role in the chilling and oxidative stress response pathways in transgenic rice plants. Overexpressed rice plants to study the role of AtNDPK2 in plant disease resistance and in plant response to abiotic stresses will further elucidate its physiological function.

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