Transgenic alfalfa plants expressing AtNDPK2 exhibit increased growth and tolerance to abiotic stresses

Zhi Wang a, b, 1, Hongbing Li a, c, 1, Qingbo Ke a, d, Jae Cheol Jeong a, d, Haeng-Soon Lee a, d, Bingcheng Xu c, Xi-Ping Deng c, Yong Pyo Lim b, Sang-Soo Kwak a, d, *

a Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KIRIBB), 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea
b Department of Horticulture, Chungnam National University, Daejeon, Republic of Korea
c State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Institute of Soil and Water Conservation, Chinese Academy of Science and Technology, Yangling, Shaanxi 712100, PR China
d Department of Green Chemistry and Environmental Biotechnology, Korea University of Science and Technology, Daejeon, Republic of Korea

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ABSTRACT

In this study, we generated and evaluated transgenic alfalfa plants (Medicago sativa L. cv. Xinjiang Daye) expressing the Arabidopsis nucleoside diphosphate kinase 2 (AtNDPK2) gene under the control of the oxidative stress-inducible SWAP2 promoter (referred to as SN plants) to develop plants with enhanced tolerance to various abiotic stresses. We selected two SN plants (SN4 and SN7) according to the expression levels of AtNDPK2 and the enzyme activity of NDPK in response to methyl viologen (MV)-mediated oxidative stress treatment using leaf discs for further characterization. SN plants showed enhanced tolerance to high temperature, NaCl, and drought stress on the whole-plant level. When the plants were subjected to high temperature treatment (42 °C for 24 h), the non-transgenic (NT) plants were severely wilted, whereas the SN plants were not affected because they maintained high relative water and chlorophyll contents. The SN plants also showed significantly higher tolerance to 250 mM NaCl and water stress treatment than the NT plants. In addition, the SN plants exhibited better plant growth through increased expression of auxin-related indole acetic acid (IAA) genes (MsIAA2, MsIAA5, MsIAA6, MsIAA7, and MsIAA16) under normal growth conditions compared to NT plants. The results suggest that induced overexpression of AtNDPK2 in alfalfa will be useful for increasing biomass production under various abiotic stress conditions.

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1. Introduction

In the natural environment, plants are often exposed to unfavorable conditions, such as drought, salinity, chilling, freezing, high temperature, flooding, and high light levels. These conditions are known collectively as abiotic stresses, and any of them can delay plant growth and development, restrict plant distribution, reduce productivity and, in extreme cases, cause the plant to die (Boyer, 1982). The ever-increasing human population, together with the loss of agricultural land due to urbanization, industrialization, desertification, improper management of water and soil, climatic changes, and the diminishing availability of resources pose serious challenges to world agriculture (Tester and Langridge, 2010).

Alfalfa (Medicago sativa L.) is an attractive legume forage grass known as “the queen of forage”. This crop has very high nutritional value and wide distribution in irrigated arid and semi-arid regions throughout the world (Osborn et al., 1997). The deep root system of alfalfa can help prevent soil and water loss in dry lands (Deak et al., 1986). Alfalfa is also a key component of many crop rotation systems and plays a crucial ecological function in preventing soil degradation and desertification (Osborn et al., 1997). As a perennial forage crop, alfalfa is a fairly hardy species and has a relatively high level of drought and salt-stress tolerance compared with other food crops. Although alfalfa can be grown in slightly saline-alkaline
conditions and semi-arid areas, its productivity is significantly reduced under high salinity and drought stress conditions. Recent developments in transgenic technology have provided an efficient tool for improving alfalfa, and since the first report of genetic transformation of alfalfa (Deak et al., 1986), many transgenic alfalfa plants have been obtained by gene transfer methods (Avraham et al., 2005; Zhang et al., 2005; Ramón et al., 2009; Tang et al., 2013).

Nucleoside diphosphate kinases (NDPKs, EC2.7.4.6) are a class of housekeeping enzymes. In general, the main function of NDPKs is to maintain the intracellular levels of all nucleoside triphosphates (NTPs) used in biosynthesis except for adenosine triphosphate (ATP). Recent studies involving ectopic expression of NDPKs in plants have suggested that NDPKs are multifunctional proteins that participate in many vital cellular processes, such as the regulation of plant growth and development (Yano et al., 1995) and signal transduction in response to phytochrome A (Choi et al., 1999), UV-B irradiation (Zimmermann et al., 1999), heat shock (Escobar Galvis et al., 2001), and H2O2-mediated oxidative stress (Moon et al., 2003; Yang et al., 2003). NDPKs also play a significant role in tolerance to various abiotic stresses.

Among the three isoforms of Arabidopsis NDPKs, NDPK2 is mainly associated with tolerance to abiotic stresses. Arabidopsis NDPK2 (AtNDPK2) is a positive signaling component of the phytochrome-mediated light signal transduction pathway (Choi et al., 1999; Kim et al., 2002; Shen et al., 2005) and is involved in H2O2-mediated mitogen-activated protein kinase (AtMAPK3 and AtMAPK6) signaling in plants. Transgenic Arabidopsis plants overexpressing NDPK2 have reduced levels of reactive oxygen species (ROS) and increased tolerance to several environmental stresses (Moon et al., 2003). In addition, AtNDPK2 participates in auxin-related cotyledon development and polar auxin transport, and it influences Aux/IAA-related gene expression by regulating auxin transport (Choi et al., 2005). Constitutive overexpression of AtNDPK2 in plants induces the expression of numerous genes involved in cellular signal transduction and protection (Yang et al., 2003). NDPK2 is also involved in salt-stress signaling by interacting with class 3 sucrose non-fermenting 1-related kinase (SOS2) and catalase (Verslues et al., 2007). Recently, transgenic plants expressing AtNDPK2, including potato, sweet potato, and poplar, also exhibited increased tolerance to multiple environmental stresses (Tang et al., 2008; Kim et al., 2009, 2010, 2011). These observations suggest that NDPK2 functions in stress protection mechanisms and can therefore be exploited to engineer plants with increased tolerance to multiple environmental stresses. However, improving the stress tolerance of alfalfa plants by overexpressing Arabidopsis NDPK2 has not previously been reported.

An appropriate promoter is necessary for efficient expression of foreign genes in plant cells (Kasuga et al., 1999). In general, a strong constitutive promoter such as the CaMV 35S promoter is used to express foreign genes in plants; however, constitutive expression of foreign genes may have harmful effects on plants. For example, the use of the CaMV 35S promoter to drive the expression of the DREB1A/CBF3 gene resulted in severe growth retardation under normal growing conditions (Kasuga et al., 1999), while the use of the stress-inducible RD29A promoter instead of the constitutive CaMV 35S promoter for the overexpression of this gene minimized its negative effects on plant growth in transgenic Arabidopsis and tobacco (Kasuga et al., 1999, 2004), indicating that this stress-inducible promoter can negate deleterious effects on plant growth. We previously isolated the oxidative stress-inducible SWPA2 promoter from sweet potato cell cultures and characterized its function in transgenic tobacco plants in response to environmental challenges including oxidative stress (Kim et al., 2003). The expression of the Gus reporter gene driven by the SWPA2 promoter in transgenic tobacco plants is strongly induced in response to environmental stresses including H2O2, wounding and UV treatment (Kim et al., 2003). In addition, transgenic plants expressing various stress-tolerance genes under the control of the SWPA2 promoter exhibit increased tolerance to methyl viologen (MV)-induced oxidative stress as well as salt, drought, and temperature stresses (Tang et al., 2008; Kim et al., 2009, 2010, 2011). These results suggest that the SWPA2 promoter will be useful for the development of transgenic plants with enhanced tolerance to environmental stresses.

In this study, we generated transgenic alfalfa plants expressing AtNDPK2 under the control of the SWPA2 promoter and characterized their tolerance to various abiotic stresses. Overproduction of AtNDPK2 conferred enhanced resistance to MV-mediated oxidative stress as well as high temperature, drought, and salt stress, and it stimulated vegetative growth of transgenic alfalfa plants under normal conditions.

2. Material and methods

2.1. Plant material and growth conditions

In a previous study, the alfalfa (M. sativa L) cv. Xinjiang Daye was demonstrated to have good adaptability and yield performance and was selected as a stress-tolerant cultivar among six alfalfa cultivars examined (Wang et al., 2009). Therefore, this cultivar was chosen for the production of transgenic lines ectopically expressing AtNDPK2. The alfalfa seeds (cv. Xinjiang Daye) were provided by Professor Zhang Bo from Xinjiang Agriculture University in China. The two transgenic alfalfa lines (SN4 and SN7) with the highest NDPK activity and enhanced tolerance to MV-mediated oxidative stress were subjected to further analysis. Plants were propagated under sterile conditions in Petri dishes containing MS medium (Murashige and Skoog, 1962) supplemented with 100 mg/L kanamycin. To evaluate stress tolerance, plantlets rooted on MS medium were transferred to pots filled with equal quantities of soil and grown in a growth chamber under a 16 h photoperiod with a light intensity of 100 μmol m−2 s−1 and 60% (w/v) relative humidity at 25 °C.

2.2. Construction of plant expression vector

The expression vector for the AtNDPK2 gene was generated as previously described (Kim et al., 2009). The AtNDPK2 gene (provided by Professor Yun DJ Cho et al., 1999; Moon et al., 2003) was placed under the control of the oxidative stress-inducible SWPA2 promoter from sweet potato (Kim et al., 2003) and the CaMV 35S terminator in the binary vector pCAMBIA2300. The AtNDPK2 cDNA insert was fused to the 5′-untranslated sequence of Tobacco Etch Virus (TEV) at the translation initiation codon, which provides highly efficient translational initiation. This cDNA was obtained by PCR using the forward primer (containing an NcoI site) 5′-CAC-CATGTTGAGGCGACT-3′ and the reverse primer (with an XbaI site) 5′-TCGTTCTAGACCAAGGATC-3′. To generate SWPA2 promoter-AtNDPK2 vector, the AtNDPK2 cDNA was ligated into the corresponding site of the pRTL2 vector (SWPA2p: AtNDPK2/pRTL2). These chimeric gene cassettes were inserted into the HindIII site of pCAMBIA2300.

2.3. Transformation

M. sativa L seeds (cv. Xinjiang Daye) were surface-sterilized with 0.5% sodium hypochlorite solution for 5 min, thoroughly rinsed 7–8 times with distilled water, and germinated on half-strength MS medium (pH 5.7) under a 16/8 h light/dark cycle,
with a light intensity of 350 μmol m$^{-2}$ s$^{-1}$ and a relative humidity of 65% at 25 °C. After 5 d of germination, the hypocotyls were utilized for plant transformation via Agrobacterium tumefaciens (EHA105 strain) containing the SWPA2 promoter-AtNDPK2 cassette.

The transformed cells were selected on SH medium containing 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/L kinetin, 250 mg/L cefotaxime, and 50 mg/L kanamycin. Shoots were regenerated from the calli by transferring to MS medium containing 1.0 mg/L benzylaminopurine (BAP), 0.3 mg/L 1-naphthalacetic acid (NAA), 250 mg/L cefotaxime, and 50 mg/L kanamycin. Throughout the experiments, the cultures were maintained in a culture room at 25 °C ± 2 °C under a 16 h photoperiod. Regenerated shoots were transferred to MS medium for rooting. The rooted plantlets were acclimatized in pots in the greenhouse for one week and transferred to soil.

2.4. Methyl viologen/salt/drought/heat-stress treatments

The oxidative stress-tolerance assay was conducted according to the method of Kwon et al. (2002). Six leaf discs (8 mm diameter) collected from alfalfa plants at the same position were floated on a solution containing 0.4% (w/v) sorbitol and 5 μM MV, placed in the dark for 12 h to allow diffusion of MV into the leaves, and subjected to continuous light treatment (150 μmol m$^{-2}$ s$^{-1}$) at 25 °C.

For salt-stress analysis at the whole-plant level, four-week-old alfalfa plants grown at 25 °C in a growth chamber in 10 cm diameter pots were irrigated with 250 mM NaCl solution once every 3 days for 2 weeks followed by tap water for one week. The tolerance of transgenic plants to salt stress was estimated based on the RWC and chlorophyll contents of the plants after exposure to salt-stress treatment.

For drought stress treatments, water was withheld from the plants, which were grown at 25 °C under a light level of 100 μmol photons m$^{-2}$ s$^{-1}$. Before withholding the water supply, the plants were irrigated with similar quantities of water through trays placed underneath the pots for 1 week. After 1 week without watering, the plants were rehydrated and permitted to recover from the dehydration conditions. Photographs of drought-stressed plants were taken after 3 days of recovery. The degree of drought stress was assessed by measuring the free proline content, RWC, and MDA measurements of plants after treatment.

For high temperature stress, four-week-old alfalfa plants grown at 25 °C were transferred to 42 °C for 24 h in a growth chamber. The treated plants were then returned to normal conditions (25 °C, 100 μmol m$^{-2}$ s$^{-1}$) for recovery from stress. The tolerance of transgenic plants to high temperature stress was estimated based on the RWC and chlorophyll contents of the plants after exposure to stress at 42 °C for 24 h.

2.5. PCR analysis

Genomic DNA was extracted using the protocol described by Kim and Hamada (2005). The PCR was conducted with purified genomic DNA in PCR premix (Bioneer, Korea) using two convergent primers complementary to the AtNDPK2 gene, i.e., the forward primer 5’-GTTGCCCGGATTGTGCTCTCA-3’ and the reverse primer 5’-CAACTGTCAGCTGCCCTC-3’. The amplification reactions consisted of 94 °C for 5 min (1 cycle), followed by 30 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, and a final extension cycle of 7 min at 72 °C. The PCR products were separated on an 1% agarose gel, stained with ethidium bromide, and visualized under UV light. All subsequent experiments were conducted on the T0-generation of transgenic plants.

2.6. Gene expression analysis

Leaves from four-week-old plants grown in soil were treated with 5 μM MV, 250 mM NaCl solution, withholding water for 1 week, or 42 °C heat stress to activate the SWPA2 promoter and induce AtNDPK2 expression. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) and treated extensively with RNase-free DNase I to remove any contaminating genomic DNA. For quantitative expression analysis of AtNDPK2, actin, and various IAA genes in alfalfa plants, total RNA (2 μg) was used for the generation of first-strand cDNA using moloney murine leukemia virus (MMLV) reverse transcriptase from an RT-PCR kit (Topscript™ RTD RTyMix) in accordance with the manufacturer’s instructions. Quantitative reverse transcriptase PCR (q-RT PCR) was performed in a fluorometric thermal cycler (DNA Engine Opticon 2, MJ Research, USA) using the fluorescent dye EveryGreen according to the manufacturer’s instructions. Transcript levels were calculated relative to the controls. Data represent means and standard errors of three replicates. The expression levels of AtNDPK2, actin, and various IAA genes were analyzed by q-RT PCR using the gene-specific primers listed in Table 1. The PCR product of each IAA gene was confirmed by sequencing analysis and further analyzed through alignment with other known plant IAA genes (data not shown).

2.7. Relative water content

RWC measurements were performed using the procedures described by Ahmad et al. (2008). The following formula was utilized: 

\[
RWC(\%) = \frac{[FW - DW]/(TW - DW)] \times 100, \quad \text{where} \quad FW = \text{the weight of freshly collected leaves (measured immediately after collection)}, \quad TW = \text{the turgid weight of leaves after incubation in water for 6 h at 20 °C in the light}}, \quad \text{and} \quad DW = \text{the dry weight of the same leaves after drying at 80 °C for 48 h}. \quad RWC\% \text{ was measured in the first fully expanded leaf from the top.}
\]

2.8. Chlorophyll content

Chlorophyll content was measured with a portable chlorophyll meter (SPAD-502, Konica Minolta, Japan) in intact fully expanded fifth leaves (from the top) of individual plants. Relative chlorophyll contents after stress treatments were determined compared with the chlorophyll contents under normal conditions.

2.9. Ion leakage analysis

The loss of cytoplasmic solutes following MV treatment, based on the electrical conductance of the solution, was measured with an ion conductivity meter (model 455C, Istek Co, Seoul, Korea) over a 0–48 h period and compared with the total conductivity of the solution following tissue destruction. The extent of cellular damage

<table>
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<tr>
<th>Table 1</th>
<th>Gene-specific primers used for genomic and q-RT-PCR analysis.</th>
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<tbody>
<tr>
<td>cDNA</td>
<td>Forward primer</td>
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<tr>
<td>AtNDPK2</td>
<td>GTTGCCCGGATTGTGCTCTCA</td>
</tr>
<tr>
<td>Actin</td>
<td>TCTAGGGCGTCTTCTCAGCAGG</td>
</tr>
<tr>
<td>MsIAA4</td>
<td>GCACTTCCCACTTATCCCTTCT</td>
</tr>
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<td>MsIAA5</td>
<td>TGGTTCATCCCAGACACGCTTCT</td>
</tr>
<tr>
<td>MsIAA6</td>
<td>AGGCAAGAAGAAGAACTCTTCTT</td>
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<tr>
<td>MsIAA7</td>
<td>CACACTTCTTATTTTCTTCTT</td>
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Z. Wang et al. / Plant Physiology and Biochemistry 84 (2014) 67–77
was quantified by measuring ion leakage, an indicator of membrane disruption.

2.10. MDA contents

Lipid peroxidation was estimated by measuring MDA contents according to a modified thiobarbituric acid (TBA) method (Sunarpi et al., 2005; Kim and Nam, 2013). Approximately 0.1 g of leaf tissue was ground in 10 mL of 10% trichloroacetic acid (TCA) using a pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 min. The reaction mixture (containing 2 mL of extract and 2 mL of TBA) was heated at 100 °C for 30 min, quickly cooled on ice, and centrifuged again at 10,000 rpm for 20 min. The absorbances at 450, 532, and 600 nm were determined using an ultraviolet spectrophotometer (Spectronic, Genesys™2, USA). Three biological replicates were performed.

2.11. NDPK and catalase activity assays

To investigate the activity of NDPK and CAT, total soluble protein was extracted from the third to fourth leaf from the top of each alfalfa plant treated with 5 µM MV, and the protein concentration was determined using a Bio-Rad protein assay kit (Bradford, 1976). NDPK enzyme activity was measured using the coupled reaction method with lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40) at 25 °C (Kim et al., 2009, 2011). NDPK activity was calculated based on the reduction in absorbance at 340 nm following a decrease in NADH. One unit of enzyme activity was defined as 1 µmol of ADP production per minute at 25 °C. Measurements of enzyme activity were carried out in triplicate for each independent sample. The CAT activity was assayed according to the method described by Aebi (1984). The activity was determined based on the reduction in absorbance at 240 nm for 1 min due to H₂O₂ consumption.

2.12. Measurement of free proline content

The free proline content of drought-treated NT and transgenic alfalfa plants (SN) was measured spectrophotometrically according to the method of Bates et al. (1973). Leaf tissue (0.1 g) was homogenized in 3 mL of sulfosalicylic acid (3%) and centrifuged at 10,000 g for 15 min. Then, 1 mL of the supernatant was placed into a test tube, followed by the addition of 1 mL of glacial acetic acid and 1 mL of ninhydrin reagent. The reaction mixture was boiled in a water bath at 100 °C for 30 min. After cooling, 2 mL of toluene was added to the reaction mixture, and the mixture was vortexed for 30 s, and the proline content in the upper phase was measured with a spectrophotometer (UV-2550, Shimadzu, Japan) at 520 nm using toluene as the blank. The proline content (mg/g FW) was quantified with the ninhydrin acid reagent method using proline as the standard (Bates et al., 1973).

2.13. Measurement of plant growth

Plant growth was determined by measuring the stem length from the top of the shoot apex to the base of the stem, the stem diameter, and the leaf area using 6 plants for each line.

2.14. Statistical analysis

The data were statistically analyzed with Statistical Package for the Social Sciences (SPSS 12). Means were separated using Duncan’s multiple range test at \( P = 0.05 \).

3. Results

3.1. Expression of AtNDPK2 in SN plants

Transgenic alfalfa plants overexpressing AtNDPK2 under the control of the oxidative stress-inducible SWPA2 promoter (Fig. 1A) were successfully generated via Agrobacterium-mediated transformation. We performed an initial screening of the putative transgenic alfalfa plants using polymerase chain reaction (PCR) analysis of genomic DNA with AtNDPK2-specific primers (Table 1). The expected amplification profiles were acquired from 14 transgenic lines, suggesting that the recombinant AtNDPK2 gene had been integrated into the genomes of transgenic plants in 14 independent lines, whereas no integration was detected in the non-transgenic (NT) line (Fig. 1B). Transgenic plants harboring AtNDPK2 under the control of the SWPA2 promoter were

**Fig. 1.** Molecular and biochemical characterization of transgenic alfalfa plants (SN) overexpressing AtNDPK2 and non-transgenic (NT) plants under methyl viologen (MV)-mediated oxidative stress. (A) Diagram of the oxidative stress-inducible SWPA2 promoter – AtNDPK2 construct. (B) Genomic DNA polymerase chain reaction (PCR) analysis of the AtNDPK2 gene from transgenic plants. M, size marker; PC, positive control; Numbers (1–14) represent independent transgenic lines. (C) Reverse transcriptase-PCR (RT-PCR) analysis of SN plants at 36 h after 5 µM MV treatment. (D) Specific nucleoside diphosphate kinases (NDPK) activity in SN and NT plants after MV treatment for 24 h. Data are the average of three replicates. Asterisks indicate significant differences between NT and SN plants by \( t \)-test at * \( p < 0.05 \) or ** \( p < 0.01 \).
designated “SN" plants. The 14 independent SN lines were propagated in a growth chamber.

We subjected these 14 transgenic plant lines to reverse transcriptase (RT)-PCR analysis using MV-treated leaf discs to determine the transcription levels of AtNDPK2 after the induction of MV-mediated oxidative stress (Fig. 1C). Induced expression of AtNDPK2 was detected in the leaf disc of all 14 transgenic lines, but not in the NT plants. In particular, AtNDPK2 expression was strongly induced in lines SN3, SN4, SN7, SN8, SN10, and SN13 after MV treatment. To further ascertain whether AtNDPK2 expression is correlated with NDPK enzyme activity, we measured the NDPK activities in soluble extracts from leaf discs of the SN and NT lines (Fig. 1D). SN4 and SN7 plants exhibited 3.2- and 4.6-fold higher NDPK activity than NT plants under MV treatment. According to the results of RT-PCR and NDPK2 enzyme activity analyses, we selected SN4 and SN7 for further study.

3.2. Increased plant growth and expression of indole acetic acid (IAA) genes in SN plants

We transferred similarly sized SN and NT alfalfa plantlets that had been rooted on MS medium to pots filled with equal quantities of soil in a growth chamber to compare the growth of these plants. The heights of SN and NT alfalfa plants were similar after 1 week of growth in the growth chamber (Fig. 2A, upper panel), whereas SN and NT alfalfa showed obvious differences in shoot height and biomass after 1 month and 2 months of growth in the growth chamber (Fig. 2A, middle and lower panels). We also measured the shoot height, stem diameter, and leaf area of the plants, which revealed that SN plants had significantly greater shoot heights and stem diameters than NT plants (Fig. 2B and C). The leaf area was 1.49- and 1.53-times higher in SN4 and SN7 plants than in NT plants, respectively (Fig. 2D).
To determine whether the increased growth of SN plants is related to the expression of auxin-response genes, we investigated the expression of five MslAA genes in NT and SN plants grown in soil under normal conditions. SN alfalfa plants showed increased transcript levels of auxin-responsive IAA genes, including MslAA5, MslAA6, MslAA7, and MtsIAA16 (Fig. 2E), indicating that the higher expression of auxin transport-related genes is related to the increased growth of SN plants.

3.3. Increased oxidative stress tolerance in SN plants

We evaluated the tolerance of SN and NT plants to MV-mediated oxidative stress using leaf discs. MV is a non-selective herbicide used to produce massive bursts of ROS, which disrupt membrane integrity and lead to cell death (Bowler et al., 1991). We prepared NT and SN leaf discs from fully expanded leaves of plants of similar ages and incubated them in 5 μM MV solution. Different degrees of visible damage were observed in leaf discs of NT vs. SN plants exposed to MV-induced oxidative stress (Fig. 3A). Severe necrosis was observed in NT leaf discs at 24 h following treatment with 5 μM MV, whereas SN leaf discs exhibited only partial necrosis. After exposure to MV, SN leaf discs exhibited less membrane damage than NT leaf discs (Fig. 3B). At 36 h following 5 μM MV treatment, the leaf discs of NT plants exhibited nearly complete cellular disruption (approximately 86% of maximum ion leakage), whereas those of the two SN plants had approximately 40% less membrane damage than NT leaf discs. The transcript levels of AtNDPK2 were significantly greater in transgenic lines SN4 and SN7 than in NT under MV treatment (Fig. 3C), indicating that ectopic expression of AtNDPK2 resulted in enhanced tolerance to MV-mediated oxidative stress in alfalfa plants.

3.4. Increased high temperature tolerance in SN plants

We exposed one-month-old NT and SN alfalfa plants to high temperature stress treatment, i.e., 42 °C for 24 h, followed by a return to 25 °C for recovery. Before stress treatment, SN plants exhibited better growth than NT plants. After high temperature treatment, the leaves of NT plants were severely wilted and curled, whereas the SN plants appeared healthy. For the recovery test, stress-treated plants were grown at 25 °C for 24 h. All of the NT plants died, whereas the SN alfalfa plants fully recovered (Fig. 4A). The transcript levels of AtNDPK2 were significantly greater in SN4 and SN7 than in NT under heat stress (Fig. 4B). We also analyzed the water loss in leaves by measuring relative water content (RWC), which showed that the RWC was higher in transgenic SN plants than in NT plants under heat stress (Fig. 4C). In addition, we measured the chlorophyll content in the fourth leaf (from the top) of each plant (Fig. 4D). The chlorophyll contents of SN plants were slightly reduced by heat stress, whereas those of NT plants were severely reduced. These results suggest that SN alfalfa plants are more tolerant to high temperature stress than NT plants due to the overexpression of AtNDPK2 in the transgenic plants.

3.5. Increased high salinity stress tolerance in SN plants

To test the tolerance of transgenic alfalfa lines to salt stress, SN and NT plants were irrigated with 250 mM NaCl solution for 2 weeks. Before NaCl treatment, the SN plants were taller than the NT plants and after 2 weeks of salt-stress treatment, the growth of the NT plants was significantly inhibited compared to the transgenic lines. Two transgenic lines grew well and exhibited only slight yellowing in a few leaves, whereas most of the leaves of NT plants showed severe wilting and chlorosis (Fig. 5A). These results indicate that overexpression of AtNDPK2 increases tolerance to salt stress in alfalfa.

To investigate the transcript levels of AtNDPK2 in SN alfalfa plants, we subjected leaves from NT and SN plants exposed to 24 h of salt treatment to real-time PCR analysis. The SN plants showed distinct increases in AtNDPK2 expression after salt-stress treatment, while the expression of this gene was not detected in the NT plants (Fig. 5B). We examined physiological and biochemical parameters of salt stress, including the chlorophyll and malondialdehyde (MDA) contents, after 3 days of salt treatment. The SN lines maintained high levels of chlorophyll (more than 80% of normal), whereas the chlorophyll levels in NT plants were only...
approximately 60% of normal (Fig. 5C). Although the MDA contents of transgenic lines were similar to those of NT plants before stress treatment, the MDA contents of NT plants were significantly greater than those of lines overexpressing \textit{AtNDPK2} under 250 mM NaCl treatment for 24 h (Fig. 5D). These results indicate that the degree of cell membrane damage was greater in NT plants than in SN plants under salt-stress conditions. We also investigated changes in the activities of catalase (CAT), a H$_2$O$_2$-scavenging enzyme, after 24 h of salt treatment. Before NaCl treatment, SN plants showed higher CAT activity than NT plants because in vitro-grown plants in pots are under stress conditions. After 24 h of salt treatment, before NaCl treatment, SN plants showed higher CAT activity than NT plants because in vitro-grown plants in pots are under stress conditions. After 24 h of salt treatment, SN4 and SN7 plants exhibited approximately 1.43- and 2.15-fold higher CAT activity than NT plants. The transcript levels of \textit{AtNDPK2} in transgenic lines SN4 and SN7 were significantly greater than that of NT after 24 h of salt treatment (Fig. 5E).

3.6. Enhanced tolerance of SN plants to drought stress

To determine whether \textit{AtNDPK2} is involved in the drought tolerance of transgenic alfalfa, we analyzed the phenotype, RWC, proline content, and MDA content of each plant after water-withholding treatment. Before stress treatment, SN plants were taller than NT plants (Fig. 6A, top). After 7 days of drought treatment, the two transgenic lines grew well and only some leaves turned yellow, whereas the NT plants showed severe wilting and curling (Fig. 6A, middle). When the plants were watered after drought treatment, the NT plants died because they could not be recovered, while the transgenic lines fully recovered from dehydration conditions (Fig. 6A, lower panel). In addition, the transcript levels of \textit{AtNDPK2} were significantly higher in transgenic lines SN4 and SN7 than in NT plants under drought treatment (Fig. 6B), and transgenic SN plants had higher RWC values than NT plants under drought treatment (Fig. 6C).
drought stress (Fig. 6C). These results indicate that the two transgenic lines had lower rates of water loss than NT plants during the dehydration process. Proline, a compatible osmolyte, plays a critical role in protecting plants under stress, particularly under dehydration conditions. Therefore, we examined the proline contents in transgenic SN and NT plants. Under control conditions, the free proline contents were not significantly different between NT and transgenic alfalfa lines. However, after withholding water for 5 days, the two transgenic lines accumulated greater free proline levels than the NT plants (Fig. 6D). Under normal conditions, the MDA contents of transgenic lines were similar to those of NT plants; however, the MDA contents of NT plants were greater than those of SN plants after withholding water for 5 days (Fig. 6E) and thus the degree of cell membrane damage was greater in NT plants than in plants overexpressing AtNDPK2 under dehydration treatment. These results suggest that overexpression of AtNDPK2 in alfalfa increases tolerance to drought stress.

4. Discussion

This study demonstrates that ectopic expression of the AtNDPK2 gene under the control of the oxidative stress-inducible SWPA2 promoter in alfalfa increases NDPK activity in vivo. In these transgenic plants, AtNDPK2 was up-regulated by MV, heat shock, salt, and drought, consequently leading to increased tolerance to various abiotic stresses. The overexpression of AtNDPK2 in alfalfa resulted in higher antioxidant enzyme levels and increased expression of auxin-responsive IAA genes. Increased expression of IAA genes in alfalfa also improves plant growth and promotes biomass formation, which helps confirm the possible auxin-related function of AtNDPK2. These results indicate that the AtNDPK2 gene is suitable for genetic engineering to develop stress-tolerant crops.

Many approaches have been used to engineer stress tolerance in plants, with limited success. One major problem is that engineering stress tolerance induces undesirable or unfavorable phenotypic changes, such as growth retardation and decreased yield. To date, several positive effects of AtNDPK2 expression in transgenic plants have been reported, even in plants grown under non-stress conditions (Kim et al., 2011). Under stress conditions, transgenic plants perform better than non-transformed controls, indicating the great potential of these plants for agricultural applications (Tang et al., 2008; Kim et al., 2009, 2010, 2011). The plant hormone auxin regulates a number of cellular and developmental processes, including cell division, cell growth, and differentiation (Choi et al., 2005). At the molecular level, auxin exerts its effect by regulating the expression of numerous auxin-responsive genes including AUX/IAA genes. Choi et al. (2005) reported that NDK2 may play a role in auxin-related cellular processes. In addition, Kim et al. (2011) demonstrated that SN poplar plants exhibit increased transcript levels of auxin-responsive genes, especially IAA2 and IAA6, under field conditions, and this increased IAA gene expression leads to an increase in plant growth. The current results (Fig. 2A) are consistent with the previous finding that transgenic SN poplar lines exhibit greater biomass accumulation than the control under normal

Fig. 5. Phenotypic and physiological analyses of AtNDPK2 transgenic plants under salt treatment. (A) Phenotypic changes before (upper panel) and after (lower panel) salt treatment (250 mM NaCl for 2 weeks). (B) Expression levels of AtNDPK2 gene were monitored by q-RT-PCR in non-transgenic (NT) and transgenic alfalfa plants (SN) after 1 day of 250 mM NaCl treatment. (C) Relative chlorophyll contents in leaves of NT and SN plants after 3 days of salt treatment. (D) Malondialdehyde (MDA) contents in leaves of SN and NT plants after 3 days of salt treatment. (E) Specific catalase (CAT) activity in NT and SN alfalfa plants after 1 day of salt stress. Values represent the means ± SD. Asterisks indicate a significant difference from that of NT at *p < 0.05 or **p < 0.01 by t-test.
conditions, suggesting that the presence of conserved regulatory processes of the AtNDPK2 transgene resulted in an acceleration in plant growth (Fig. 2B, C, and F). In parallel, the expression of endogenous auxin-responsive alfalfa IAA genes, including MsIAA3, MsIAA5, MsIAA6, MsIAA7, and MsIAA16, was also up-regulated at the transcriptional level under normal growth conditions (Fig. 2E), implying that the increased growth rate of the transgenic alfalfa plants under normal conditions is mainly due to increased expression of auxin-related IAA genes.

Transgenic lines harboring AtNDPK2 showed increased tolerance to a variety of abiotic stresses. As the AtNDPK2 gene was driven by a stress-inducible promoter, the induction patterns of AtNDPK2 under various stress conditions, including MV, heat shock, salt, and drought, verifies the stable integration and transcription of foreign genes in SN plants (Figs. 3C, 4B and 5B, and 6B).

NDPKs play a prominent role in plant defense mechanisms, and NDPK is involved in tolerance to various stresses, including oxidative stress. For example, Moon et al. (2003) demonstrated that Arabidopsis overexpressing NDPK2 exhibits increased tolerance to MV-mediated oxidative stress, freezing, and high salt. In transgenic poplar, potato, and sweet potato plants, overexpression of NDPK2 also increases tolerance to MV-mediated oxidative stress, temperature, and osmotic stresses (Tang et al., 2008; Kim et al., 2009, 2010, 2011). As expected, in the current study, SN transgenic alfalfa plants showed significantly increased tolerance to MV-mediated oxidative stress, along with high levels of NDPK production (Fig. 1D). Our results are consistent with the findings of previous reports because we observed increased tolerance to 5 μM MV-mediated oxidative stress in leaf discs, as exhibited by lower levels of visible damage and ion leakage under these challenging conditions (Fig. 3A and B).

Temperature fluctuations can induce the formation of ROS (Moon et al., 2003; Tang et al., 2008; Kim et al., 2009, 2010, 2011). Most alfalfa cultivars are sensitive to high temperatures and thus it was of interest to generate alfalfa plants with greater heat tolerance. Accordingly, we tested whether SN plants have an increased tolerance to heat stress, because SN potato plants exhibit this phenotype (Tang et al., 2008; Kim et al., 2010). The results revealed that NT alfalfa plants wilted after 24 h of heat stress (42 °C),
whereas SN plants appeared to remain healthy (Fig. 4A). Transgenic SN plants had higher RWC values than NT plants under heat stress (Fig. 4C), suggesting that the two transgenic lines had lower rates of water loss than NT plants during heat stress. In addition, chlorophyll content measurements showed that photosynthesis was transiently reduced in SN plants during heat stress, while that of NT plants was highly reduced (Fig. 4D). These results suggest that transgenic alfalfa plants expressing AtNDPK2 have increased tolerance to high temperatures. There is increasing evidence that there is a strong correlation between the responses to heat and oxidative stress. Phosphorylation of NDPK is affected by heat stress in sugarcane (Mois yadi et al., 1994). In pea seedlings, NDPK is involved in the heat-stress response through its interaction with a heat-shock protein (Esco bar Galvis et al., 2001).

Moreover, salt and drought stress induce ROS production, which results in the accumulation of MDA in plants due to membrane lipid peroxidation (Gill and Tuteja, 2010). Oxidative stress-induced membrane damage and cell membrane stability have been used as efficient criteria to assess the degree of salt and drought stress tolerance in plants. The current results show that the MDA content was lower in transgenic alfalfa than in NT plants under both salt and drought stress (Figs. 5D and 6E). These results imply that the degree of membrane injury was lower in transgenic plants than in NT plants, which is consistent with the increased salt and drought tolerance phenotype of transgenic alfalfa.

NDPK2 is involved in H2O2-mediated MAPK signaling, and its overexpression in Arabidopsis strongly induces the expression of various antioxidant genes, such as genes encoding peroxidase (POD), ascorbate peroxidase (APX), CAT, thioredoxin, and peroxiredoxin (Moon et al., 2003; Yang et al., 2003). In addition, overexpression of NDPK2 in potato, sweet potato, and poplar plants increases the activities of NDPK, APX, and CAT (Tang et al., 2008; Kim et al., 2009); these transgenic plants exhibit enhanced tolerance to multiple stresses (Moon et al., 2003; Tang et al., 2008; Kim et al., 2009, 2010, 2011). Consistent with these results, in this study, SN alfalfa plants maintained higher levels of CAT activity than NT plants during salt stress (Fig. 5E). CAT is a major enzyme responsible for H2O2 removal. NDPK2 positively regulates the expression of antioxidant genes under stress conditions, and it increases stress tolerance. Therefore, the higher CAT activity observed in SN plants likely results from increased expression of NDPK2-regulated antioxidant genes and is correlated with environmental stress defense mechanisms involving H2O2-regulated stress signaling.

When plants are suffering from salt and drought stress, they often accumulate compatible osmolytes, such as free proline (Liu and Zhu, 1997), which function as osmoprotectants that enable plants to tolerate stress. In this study, we examined the contents of the osmoprotectant proline. Our results showed that the content of free proline increased under drought stress conditions, and the free proline levels in AtNDPK2-overexpressing transgenic plants were greater than those in NT plants (Fig. 6D) under drought stress; thus the increased accumulation of proline likely contributes to the increased drought tolerance of transgenic alfalfa.

In conclusion, we successfully generated and characterized SN transgenic alfalfa plants with heterologous expression of AtNDPK2 under the control of the stress-inducible SWPA2 promoter. These transgenic alfalfa plants exhibited enhanced tolerance to multiple abiotic stresses such as oxidative, high temperature, salt, and drought stress. This increased stress tolerance was mediated by the induction or activation of ROS scavenging, enhanced NDPK enzyme activity, increased proline accumulation, and improved protection of membrane integrity and thus this study increased our understanding of the molecular mechanisms of NDPK gene function in plants. Further characterization of SN alfalfa under natural field conditions (including marginal lands) is currently underway. We anticipate that the transgenic alfalfa plants described in this current study will be used for farming in salt-affected as well as global arid and semi-arid marginal soil areas.

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Author contributions

S.S. Kwak and Z. Wang: conceived and designed the experiments. Z. Wang, H. Li, Q. Ke, and J.C. Jeong: performed the experiments. J.C. Jeong, H.S. Lee, B. Xu, and Y.P. Lim: analyzed the data. X. Deng and H.S. Lee: contributed reagents/materials/analysis tools. Z. Wang and S.S. Kwak: wrote the paper.

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