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Research Article

Stress Inducible Overexpression of *Arabidopsis* Nucleotide Diphosphate Kinase 2 Gene Confers Enhanced Tolerance to Salt Stress in Tall Fescue Plants

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ABSTRACT

Arabidopsis nucleoside diphosphate kinase 2 (AtNDPK2) is an upstream signaling molecule that has been shown to induce stress tolerance in plants. In this study, the *AtNDPK2* gene, under the control of a stress-inducible *SWPA2* promoter, was introduced into the genome of tall fescue (*Festuca arundinacea* Schreb.) plants. The induction of the transgene expression mediated by methyl viologen (MV) and NaCl treatments were confirmed by RT-PCR and northern blot analysis, respectively. Under salt stress treatment, the transgenic tall fescue plants (SN) exhibited lower level of H_2O_2 and lipid peroxidation accumulations than the non-transgenic (NT) plants. The transgenic tall fescue plants also showed higher level of NDPK enzyme activity compared to NT plants. The SN plants were survived at 300 mM NaCl treatment, whereas the NT plants were severely affected. These results indicate that stress-inducible overexpression of *AtNDPK2* might efficiently confer the salt stress tolerance in tall fescue plants.

(**Key words** : NDPK2, salt stress, tall fescue)

Ⅰ. INTRODUCTION

Salinity is one of the most critical environmental problems that reduce plant growth and productivity (Shrivastava and Kumar, 2015). Globally, about 6% (over 800 million ha) of total land, and approximately 20% (forty-five million ha) of irrigated lands are saline affected (FAO, 2015). Soil salinization has become much more prevalent globally that restrain crop production (Vineis et al., 2011). In Asian subcontinent, salinity is being occurred mostly by soluble salt containing irrigated water, sea-level rising, tidal flooding due to the climate change, and lack of water and soil management practices (Yuan et al., 2016; Vineis et al., 2011). Plants often have to face several abiotic stresses such as cold, heat, drought, flood and high salinity (Asada, 1999). Plants exposed to salt stress provoke oxidative stress induced cellular injury that leads to the generation of reactive oxygen species (ROS) in plants (Asada, 1999; Foyer et al., 1994). High level of salt greatly reduces plant growth and productivity; results in nutritional imbalance, ionic toxicity even plants may die (Qureshi et al. 2013). Therefore, it is necessary to produce salt tolerant crop plants, and need to increase adaptable cultivars in changing climatic conditions.

Tall fescue species is a cool season grass and commonly used in pastures and lawns as forage and turf grass, and its cultivation has recently been extended to the sub-tropical climate and even to the transitional climate between the sub-tropical and the tropical (Lou et al., 2015). The expansion of its cultivation into new areas, including many that have been heavily impacted by salinization, has pushed the limits of its tolerance to abiotic stresses, including drought, salt, and extreme temperature. To increase the productivity of tall fescue

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on unfavorable lands, especially reclaimed or marginal lands, it is necessary to enhance its tolerance to abiotic stresses, and particularly to salt stress. Genetic engineering offers the ability to improve stress tolerance in a variety of plant species, and it is especially useful for plants with a complicated genome (Zhang and Blumwald 2001). Tall fescue has been previously engineered to possess several genes that promote stress tolerance, including vacuolar antiporters (Zhao et al., 2007) antioxidants (Kim et al., 2010), transcription factors (Wu et al., 2006) and heat shock proteins (Kim et al., 2012).

Nucleoside diphosphate kinases (NDPKs; EC 2.7.4.6) are housekeeping enzymes that maintain the intracellular levels of (d)NTPs used in biosynthesis, with the exception of ATP (Moon et al., 2003). Increasing evidence suggests that NDPKs play pleiotropic functions. Constitutive over-expression of an *NDPK* (*AtNDPK2*) in *Arabidopsis* resulted in an enhanced tolerance to multiple environmental stresses such as salt, cold, and methyl viologen-mediated oxidative stress (Moon et al., 2003). These effects are associated with *AtNDPK2*-induced expression of numerous genes involved in antioxidant and protective functions (Kim et al., 2009), possibly through the activation of the MAPK cascade (Yang et al., 2003). In last couple of years, it has been reported that the overexpression of *AtNDPK2* under the control of an sweet potato oxidative stress-inducible peroxidase promoter (SWPA2) showed enhanced multiple abiotic stress tolerances in sweet potato, potato, poplar, and alfalfa (Tang et al., 2008; Kim et al., 2009, 2010, 2011; Wang et al., 2014). *SWPA2* encodes and anionic peroxidase (POD) in sweet potato, and expression of -1,314 *SWPA2* promoter in tobacco transformants was strongly induced in response to abiotic stresses (Kim et al., 2003). These results indicate that inducible expression of *AtNDPK2* driven by the *SWPA2* promoter is very effective and useful for the development of plants with enhanced stress tolerance.

The aim of this study is to develop salt tolerant transgenic tall fescue plants overexpressing the *AtNDPK2* using the stress-inducible promoter *SWPA2*. Improving salt stress tolerance of tall fescue plants by overexpressing *AtNDPK2* has not been reported previously. In this study, the stress-induced expression of *AtNDPK2* conferred enhanced tolerance to salt stress in tall fescue plants.

Ⅱ. MATERIAL AND METHODS

1. Plant Material and Growth Condition

Tall fescue (*Festuca arundinacea* Schreb. cv. Kentucky-31) was chosen as host plant for this experiment. Mature seed-derived tall fescue calli were produced in petri dishes containing MS medium (Murashige and Skoog, 1962) supplemented with BAP (0.1 mg×L^{-1}), 2,4-D (6 mg×L^{-1}), casein hydrolysate (1 g×L^{-1}) and proline (500 mg×L^{-1}). Calli were propagated in growth room with dark condition at 25°C. Mainly hard and yellowish type embryogenic calli were chosen for transformation.

2. Construction of the Expression Vector

The expression vector for the *AtNDPK2* gene was generated as previously described by Kim et al. (2010). The *AtNDPK2* cDNA was obtained from Professor Yun (Moon et al., 2003), which was fused to the 5'-untranslated sequence of the tobacco etch virus (*TEV*) at the translational initiation codon, which provides highly efficient translational initiation. This construct was ligated into an oxidative stress-inducible *sweet potato peroxidase anionic 2* (*SWPA2*) promoter (Kim et al., 2003). The completed chimeric gene cassette was then inserted into *Hin*dIII site of the binary vector *pCAMBIA1300* containing *hygromycin phosphotransferase (HPT)* gene as a selectable marker, and the final construct was named *pSN-H* (Kim et al., 2010). Recombinant *pSN-H* was introduced into *A. tumefaciens* strain EHA105, which was used for genetic transformation.

3. Transformation of Tall Fescue

Mature seed-derived calli of tall fescue were used as the primary explants for the *Agrobacterium*-mediated transformation. Seed sterilization, callus induction, infection of embryogenic calli by *A. tumefaciens*, co-cultivation of infected calli and shoot regeneration were carried out following previously described procedure of Wang and Ge (2005). The hygromycin (30 mg×L^{-1}) resistant regenerated shoots were selected and moved to MS medium (Murashige and Skoog 1962) for rooting. The rooted plantlets were transferred to pots

containing medium (organic soil:perlite; 1:1), acclimatized for 7 days and grown in the greenhouse.

4. Polymerase Chain Reaction

Genomic DNA was isolated from the leaves of wild-type and transgenic tall fescue plants using CTAB. PCR amplification was carried out using primers specific for *AtNDPK2* (5'-GTTG GCCGCATTTCGTCCTCA-3' and 5'-CCACTTGCATAGCTC GCCCTCTTT-3') and *hpt* (5'-CCTGAACTCACCGCGACG-3' and 5'-AAGA-CCAATGCGGAGCATAT-3'), which resulted in 515- and 804-bp products, respectively.

5. Analysis of Gene Expression

Transgenic and nontransgenic wild-type tall fescue plants were exposed to methyl viologen (1 μM), or NaCl (300 mM) treatments to activate *SWPA2* promoter, and induction of *AtNDPK2* gene expression. Total RNA was extracted from the transgenic and wild-type tall fescue leaves using Trizol reagent (Invitrogen) and treated with RNase-free DNase I. To determine of quantitative expression of *AtNDPK2*, RT-PCR amplification was performed using an RT-PCR kit (TOPscript, RTD ryMIX), in accordance with manufacturer's protocols. For Northern blot analysis, total RNA was extracted from the salt-treated transgenic and wild-type plants using Trizol reagent (Invitrogen). Fifteen micrograms of RNA was separated by denaturing 1.2% agarose gel electrophoresis. The Northern hybridization was performed using the [α-^{32}P]-dCTP-labeled probe following the hybridization protocol (Kim et al., 2012).

6. Determination of Electrolyte Leakage (EC), H_2O_2 Concentration, and Lipid Peroxidation in Tall Fescue Leaves

Electrolyte leakage activity was performed according to the method of Kim et al. (2010). Hydrogen peroxide (H_2O_2) accumulation of transgenic and wild-type leaves was measured by spectrophotometrically as described previously by Kim et al. (2010). The level of lipid peroxidation was measured as correspondence of malondialdehyde (MDA) content. MDA accumulation was determined by the 2-thiobarbituric acid-reactive substances (TBARS) using the method of Kim et al. (2010).

7. Determination of NDPK Enzyme Activity

Total soluble protein was extracted from the tall fescue leaves that were treated with MV or NaCl for analyzing of NDPK enzyme activity. Protein concentrations were determined using the Bio-Rad protein assay kit according to Bradford (1976). The NDPK activity was measured using the coupled reaction method with lactate dehydrogenase and pyruvate kinase (Tang et al., 2008). The NDPK activity was calculated based on the loss of absorbance at 340 nm following the decrease in NADH. One unit of enzyme activity was defined as 1 μmol of ADP production per minute.

8. Determination of Salt Tolerance in Tall Fescue Plants

To examine salt stress tolerance of the transgenic plants, wild-type and transgenic plants were divided into three groups, transferred into pots (13 cm diameter) and grown in a greenhouse. Eight-week old plants were thoroughly irrigated with 300 mM NaCl solution at a 3-day interval for the next 3 weeks, as previously described (Wu et al., 2005). Morphological changes were observed during this 3 week period. Followed by salt treatments leaf samples were harvested and frozen in liquid nitrogen, stored at -80°C for anti-oxidative enzymes, hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) analyses.

9. Statistical Analysis

Data were statistically analyzed using SPSS program (version 16.0). All the results were represented as means ±SE of three independent replications. Means were separated using Duncan's multiple range test at $P = 0.05$.

Ⅲ. RESULTS and DISCUSSION

1. Expression of *ATNDPK2* and Oxidative Stress Tolerance in Transgenic Plants

In this study, we generated transgenic tall fescue that expressed *AtNDPK2* gene under the control of the stress inducible *SWPA2* promoter, and its genomic integration and inducible expression by Methyl viologen (MV)-mediated oxidative stress were confirmed by Southern blot and RT-PCR analyses, respectively (data not shown). It has been proven that oxidative stress-inducible expression of *AtNDPK2* gene effectively enhanced multiple abiotic stress tolerance in different plant species including poplar (Kim et al., 2010), and alfalfa (Wang et al., 2014). Therefore, we checked whether stress-inducible expression of *AtNDPK2* affects tolerance to MV-mediated oxidative stress. MV-mediated oxidative stress tolerance increased in SN plants than NT plants. SN leaf segments exhibited less membrane damage compared to NT plants (Fig. 1A). Following 48 h of 1 μM MV treatment, leaf segments of NT plants highly exhibited cellular disruption (proximately 87% of electrolyte leakage), whereas those five SN lines showed about 18% less membrane damage than NT plants. As a consequence, we measured H_2O_2 content of NT and SN plants, which were parallel to membrane damage activity. SN2 and SN5 plants showed lower H_2O_2 after 48 h of 1 μM MV treatment, which were 36.6% and 31.6% lower than NT plants (Fig. 1B). The NDPKs play pivotal role in plant defense system and involved in oxidative stress tolerance. According to earlier study, NDPK2 exhibits tolerance against chilling, salinity, and MV-mediated oxidative stress (Kim et al., 2009; Wang et al., 2014). In this study, we expected the SN tall fescue showed increased tolerance to oxidative stress as a consequence of expression of the *NDPK2* gene. Based on *AtNDPK2* expression and tolerance to MV-mediated oxidative stress, we have chosen SN2, SN5 and SN6 plants for further studies.

2. Increased Salt Tolerance of Transgenic Tall Fescue Plants

We checked the salt tolerance of the 3 independent transgenic tall fescue lines (SN2, SN5, and SN6) harboring *SWPA2::AtNDPK2*. No visible morphological differences were observed among the NT and SN plants in normal condition (Fig. 2; upper panel). However, significant differences were observed between the NT and SN plants following an exposure to NaCl treatment. Under the 300 mM NaCl treatment, the leaves of the NT plants became yellow and gradually wilted due to the salt-induced senescence, while the SN plants expressing *AtNDPK2* maintained their growth without any visible chlorosis (Fig. 2; lower panel). In addition, the SN plants survived up to 300 mM NaCl, whereas the NT plants were severely affected. These results clearly demonstrate that transgenic plants carrying the *AtNDPK2* gene exhibited more salt tolerance than the NT plants.

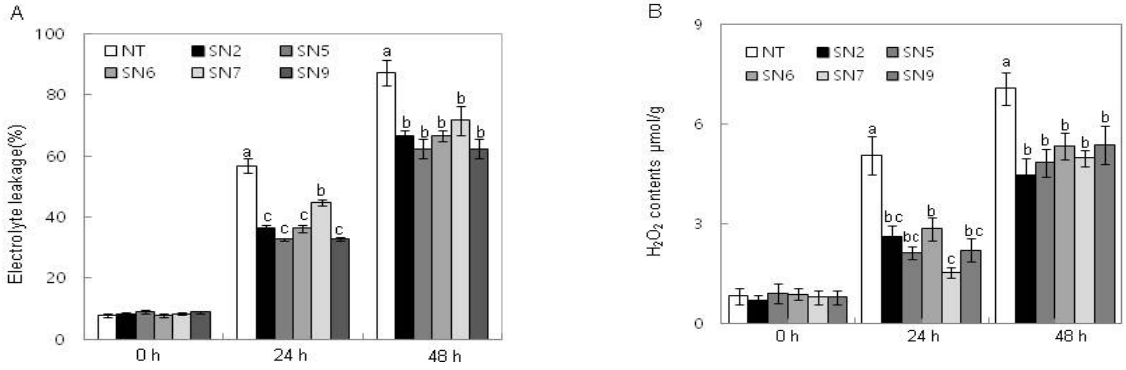

Fig. 1. Effect of MV-mediated oxidative stress treatment in NT and transgenic SN tall fescue plants. (A) Analysis of electrolyte leakage in SN plants in response to MV-treatment. (B) H_2O_2 contents in leaf segments treated with MV. The data are shown the average with SD of three independent replicates. The same letters above bars indicates data are not ignificantly different (P = 0.05) according to Duncan's multiple range test.

Moreover, salt induced ROS synthesis; as a consequence H_2O_2 and MDA contents were measured in NT and SN plants. Under normal condition, no significant variation was observed for H_2O_2 and MDA contents, while about 1.4- and 1.35-fold higher H_2O_2 and MDA contents in SN plants, respectively, were exhibited under 300 mM NaCl treatment (Fig. 3). These results indicated that a relatively lower level of ROS was accumulated in SN plants under 300 mM NaCl treatment. These results clearly suggested that the production of H_2O_2 and MDA were correlated with the generation of ROS due to the salt stress. The formation of H_2O_2 and MDA in plants exposed to adverse environmental conditions is a reliable indicator of cellular free-radical generation (Hodges et al., 1999). The low level of H_2O_2 and MDA in SN lines implies that the degree of membrane damage was lower in SN transgenic plants compared to NT plants. H_2O_2 and MDA measurements have also been routinely used in salt-treated plant samples as an indicator of cellular injury and lipid peroxidation due to the salt- induced oxidative stresses (Zhao et al., 2007).

3. Expression of *AtNDPK2* mRNA in Transgenic Plants Exposed to NaCl Stress

A Northern blot analysis was used to verify whether the stress-inducible *SWPA2* promoter could successfully drive the expression of the *AtNDPK2* gene at the mRNA level in salt-stressed transgenic plants. As shown in Fig. 4, *AtNDPK2* transcripts were undetectable in transgenic tall fescue plants under normal conditions. However, after 12 h of NaCl treatment, the *AtNDPK2* gene was induced at a low level, and

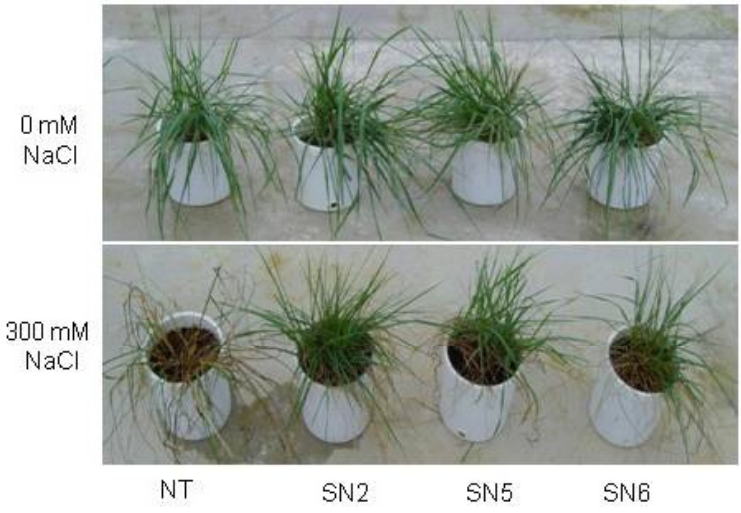

Fig. 2. Morphological changes of non-transgenic (NT) and transgenic (SN) tall fescue plants at 21 days after treatment of 300 mM NaCl.

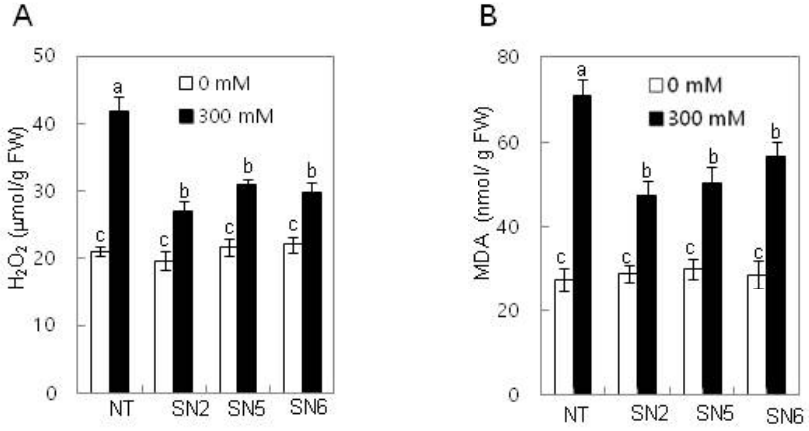

Fig. 3. Effect of salt stress treatment on H_2O_2 (A) and MDA (B) contents in NT and SN plants.

it gradually increased after 24 h and 72 h treatments. The level of *AtNDPK2* transcripts in the SN2, SN5, and SN6 lines was up-regulated with increasing the durations of NaCl treatment. This result suggests that the *SWPA2* promoter successfully drove the expression of the *AtNDPK2* gene under salt stress.

To determine whether the over-expressed *AtNDPK2* transcripts were normally translated into its functional proteins with corresponding activity, we further measured the NDPK enzyme activity in soluble extracts from salt stress-treated tall fescue leaves. The SN plants exhibited approximately 1.5-3.0 fold higher NDPK activity than NT plants after 14 days of the salt treatment (Fig. 5). Previous study documented that the *SWPA2* promoter is strongly induced by oxidative stresses such as hydrogen peroxide, wounding and UV treatment (Kim et al., 2009; Kim et al., 2011). Compared to the widely used *CaMV 35S* constitutive promoter, oxidative stress-inducible *SWPA2* promoter is more efficient for increasing stress tolerance of plants (Kim et al., 2009). We also found previously that constitutive expression of *AtNDPK2* gene driven by *CaMV 35S* promoter in transgenic tall fescue plants did not show efficient tolerance to abiotic stresses except low level of cold tolerance (Lee et al. 2009). Recently, the expression of *AtNDPK2* gene under the control of *SWPA2* promoter has been observed enhanced tolerance in transgenic alfalfa under numerous abiotic stresses (Wang et al., 2014). Moreover, the *SWPA2*-driven *AtNDPK* transgene expression showed enhanced tolerance to oxidative stress under MV, high temperature, and salt stresses (Tang et al., 2008). These reports indicate that inducible overexpression of *AtNDPK2* gene driven by the stress-inducible *SWPA2* promoter could be an effective biotechnological strategy to conferring salt tolerance in tall fescue plants.

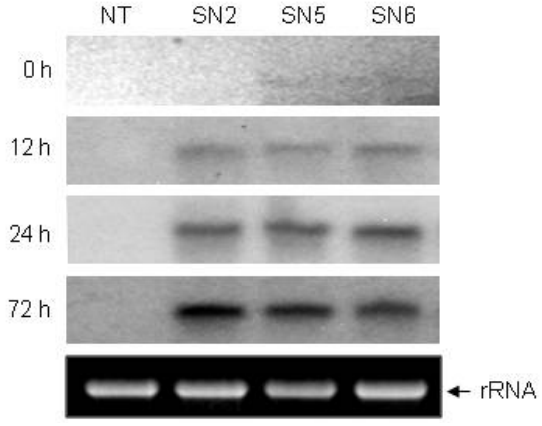

Fig. 4. Salt stress-induced *AtNDPK2* gene expression in NT and SN plants under salt stress. Samples were collected at 0 h, 12 h, 24 h and 72 h after 300 mM NaCl treatment. Total RNA from the leaves was transferred onto a nylon membrane and Northern blot analysis was carried by hybridization of *AtNDPK2*-specific probe.

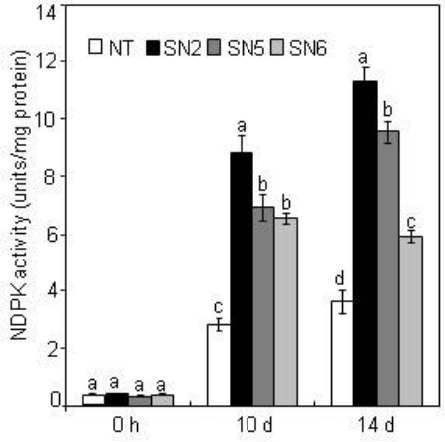

Fig. 5. NDPK enzyme activity in tall fescue plants after 300 mM NaCl treatment.

Ⅳ. CONCLUSIONS

We generated *AtNDPK2* transgenic tall fescue plants under the control of the stress-inducible *SWPA2* promoter. These transgenic fall fescue plants showed enhanced salt tolerance. We anticipate that *AtNDPK2* mediates salt stress tolerance possibly by regulation of the gene expression involved in antioxidant and defense system. Thus, the progress of studies on tall fescue with abiotic stress tolerance provides genetic information for forage and grass breeding. The transgenic tall fescue plants described in this research would be useful for farming in salt affected areas as well as marginal land.

Ⅴ. ACKNOWLEDGEMENTS

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