

# Transgenic poplar expressing *AtNDPK2* exhibits enhanced biomass in the LMO field

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**Abstract** Nucleoside diphosphate kinase 2 (NDPK2) is known to regulate the expression of antioxidant genes and auxin-responsive genes in plants. Previously, it was noted that the overexpression of *Arabidopsis NDPK2* (*AtNDPK2*) under the control of an oxidative stress-inducible *SWPA2* promoter in transgenic poplar (*Populus alba* × *P. tremular* var. *glandulosa*) plants (referred to as SN plants) enhanced tolerance to oxidative stress and improved growth (Plant Biotechnol J 9: 34–347, 2011). In this study, growth of transgenic poplar was assessed under living modified organism (LMO) field conditions in terms of biomass in the next year. The growth of transgenic poplar plants increased in comparison with non-transgenic plants. The SN3 and SN4 transgenic lines had 1.6 and 1.2 times higher dry weight in stems than non-transgenic plants at 6 months after planting, respectively. Transgenic poplar also exhibited increased transcript levels of auxin-response genes such as *IAA1*, *IAA2*, *IAA5* and *IAA6*. These results suggest that enhanced *AtNDPK2* expression increases plant biomass in transgenic poplar through the regulation of auxin-response genes.

**Keywords** Transgenic poplar, Nucleoside diphosphate kinase, Inducible promoter, Auxin-response genes, Biomass, Field conditions

## Introduction

Excess use of fossil fuel energy caused by a rapid increase in population size and industrialization has led to increased atmospheric concentrations of CO<sub>2</sub>, a major greenhouse gas. This has resulted in a variety of problems at the global scale, such as poor environmental conditions and a limited supply of energy and food (Matthews et al. 2009). Excess human activity has led to ecosystem imbalances and deterioration of the environment. Global warming, destruction of the ozone layer and expansion of desert areas worldwide have serious impacts on plant growth and crop productivity (Lehman 1998). Accordingly, increases in plant productivity and biomass through enhanced environmental stress tolerance are among the most promising technological advances for global food security and environmental sustainability.

Woody plants have long been utilized as raw materials by humans in various industrial applications, such as pulping, paper-marketing, lumber, textiles, burning for energy and many other uses (Demura and Ye 2010). Recently, the biomass of woody plants has been considered as a renewable source of bio-fuels through cellulose degradation (Solomon 2010). Woody plants also affect deposition, erosion and remediation in the soil and CO<sub>2</sub> fixation (Harfouche et al. 2011). Molecular breeding of woody plants is needed for practical use in the selection of optimal tree species and development of transgenic plants with enhanced environmental stress tolerance and biomass.

Poplar, belonging to the genus *Populus* L., is a member of the Salicaceae, which together with the Flocourtiaceae and 29 other families is widely distributed in the northern

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hemisphere. Rapid growth and adaptability are hallmarks of poplars across a wide range of climatic and soil conditions (Bradshaw et al. 2000). They play important roles in the phytoremediation of degraded sites, rehabilitation of fragile ecosystems (including deserts) and restoration of forest landscapes (Ball et al. 2005). Poplar is a particularly suitable model for genetic engineering because of its small genome size, short rotation, capacity for micro-propagation, abundant genetic information, efficient regeneration and genetic transformation system (Bradshaw et al. 2000; Jansson and Douglas 2007).

Nucleoside diphosphate kinase (NDPK, EC 2.7.4.6) is known to be a housekeeping enzyme, that catalyzing the reversible transfer from nucleoside triphosphates (NTPs) to nucleoside diphosphate to generate NTPs or dNTPs. Thus, they play an important role on energy conversion. In addition, increasing evidences suggests that NDPK also plays a significant role in signal transduction pathways in plants, such as phytochrome A (Choi et al. 1999), UV-B (Zimmermann et al. 1999), heat shock (Escobar Galvis et al. 2001) and oxidative stress (Moon et al. 2003). Recently, it has been shown that *Arabidopsis* NDPK2 (*AtNDPK2*) plays a regulatory role in H<sub>2</sub>O<sub>2</sub> and auxin-mediated signaling in plants, indicating that NDPK2 enzymes carry out diverse functions in stress and developmental signaling (Moon et al. 2003; Choi et al. 2005). Transgenic potato and sweetpotato plants expressing *AtNDPK2* under the control of the oxidative stress-inducible *SWPA2* promoter were generated and shown to possess enhanced tolerance to multiple environmental stresses such as salt, drought and extreme temperatures (Tang et al. 2008; Kim et al. 2009). Recently, it was also noted that the *AtNDPK2* expressed in transgenic poplar plants (referred to as SN poplar plants) resulted in higher antioxidant enzyme levels and enhanced auxin-responsive gene expression; accordingly, growth rates and tolerance to oxidative stress of transgenic poplar plants increased under greenhouse and field conditions in 2009 (Kim et al. 2011).

In the previous study, we did not measure the biomass of SN poplar plants on the level of dry weight. Biomass of SN poplar plants will be important for the biological applications. In addition, we are interesting in the gene expression and phenotype of asexually propagated SN poplar in the field conditions. In this study, we investigated the biomass of two selected transgenic poplar plant lines (SN3 and SN4) with good growth under field conditions in 2010. We also confirmed the expression patterns of auxin-response genes in the poplar plants under the same field conditions.

## Materials and methods

### Plant materials

Two transgenic poplar (*Populus alba* × *P. tremular* var. *glandulosa*, clone Hyun3) plant lines (SN3 and SN4) with high levels of *AtNDPK2* expression, enhanced plant growth and tolerance to oxidative stress were selected for study (Kim et al. 2011). An *AtNDPK2* gene overexpression vector was constructed using an oxidative-inducible *SWPA2* promoter of sweetpotato and a CaMV 35S terminator sequence in the pCAMBIA2300 plant expression vector, as described previously (Kim et al. 2009).

### Field cultivation and biomass measurement of transgenic poplar plants

In a previous study, SN transgenic poplar plants were transplanted to field conditions and grown for a season to produce enough cuttings for growth performance tests (Kim et al. 2011). The following spring, cuttings were prepared from the stems of transgenic plants. Two selected transgenic lines of 15 cuttings (about 20–25 cm shoot height) were planted along with a non-transgenic clone at a spacing of 70 × 70 cm at Suwon living modified organism (LMO) field of the Korea Forest Research Institute. The height growth, number of shoots per cutting and total biomass of plants were recorded for 6 months. Dry weight was measured after 7 days at 70°C in a drying oven.

### RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from leaves of poplar using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated extensively with RNase-free DNase I to remove any contaminating DNA. For quantitative expression analysis of *AtNDPK2*, *actin* and *IAA* genes in poplar plants, total RNA (2 µg) was used for the generation of first-strand cDNA using MMLV reverse transcriptase an RT-PCR kit (Promega, Madison, WI, USA), in accordance with the manufacturer's instructions. Quantitative real-time PCR was performed in a fluorometric thermal cycler (DNA Engine Opticon 2, MJ Research, USA) using the EverGreen as fluorescence dye according to the manufacturer's instructions. The inter-experimental quality control comparisons of repeated samples were assessed using CT values between the three replications. Above 1.5 of differential values was removed from data set. In addition to, quality control of the reaction by gel electrophoresis confirmed the presence of a single product

of the correct size, and multiple peaks had samples in the dissociation graph were also dismissed for exclusion of unspecific PCR reaction. Linear data were normalized to the mean CT of  $\beta$ -actin as reference gene. Primer sets of the *AtNDPK2*, *actin* and *IAA* genes were described previously (Kim et al. 2011).

#### Statistical analysis

Data were statistically analyzed with the Statistical Package for the Social Sciences (SPSS 12). Means were separated using Duncan's multiple range test at  $P = 0.05$ .

### Results and discussion

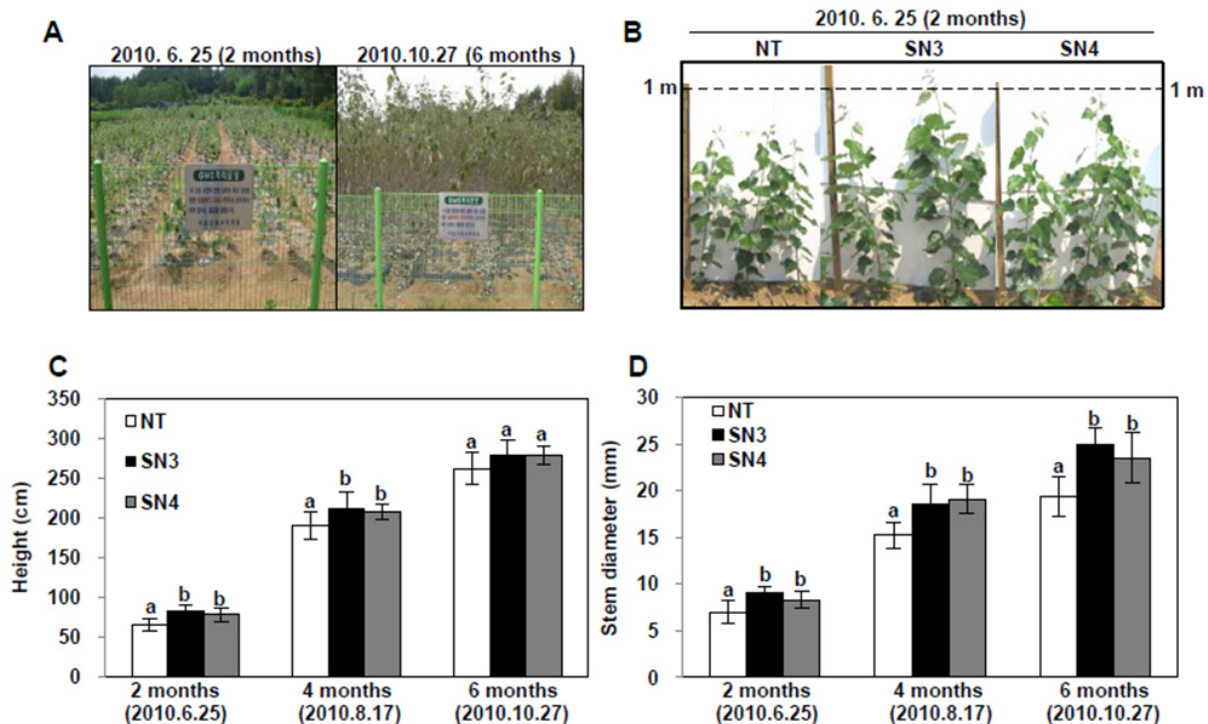
#### Growth-related features of SN transgenic poplar under field conditions

Plant height and stem diameter were analyzed in transgenic (SN3 and SN4) and non-transgenic (NT) poplar plants in the field for 6 months (Fig. 1A). SN transgenic poplar lines showed enhanced plant growth compared with NT poplar plants (Fig. 1B). At 6 months, plant height averaged 262, 279, and 278 cm for the NT, SN3, and SN4 lines, re-

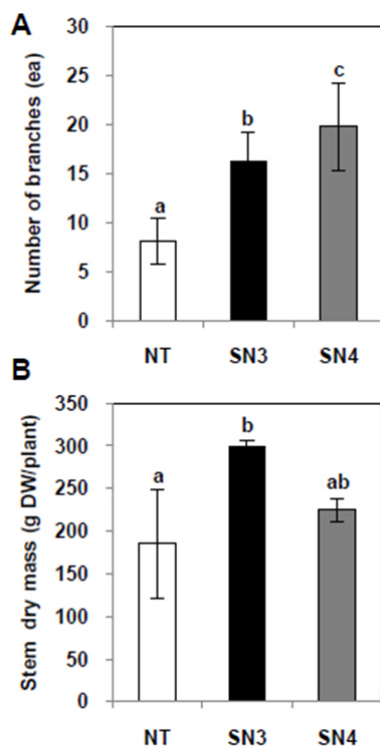
spectively (Fig. 1C). Average stem diameters were 19.5, 23.5, and 21 mm for NT, SN3, and SN4 lines at 6 months, respectively (Fig. 1D).

The plant height of the SN3 and SN4 lines was 1.3- and 1.2-fold higher than that of NT plants, respectively, until 4 months; this difference was statistically significant ( $P < 0.05$ ) (Fig. 1C). However, the height of all poplar plants was similar at 6 months under field conditions ( $P = 0.05$ ) (Fig. 1C). Plant allometric growth is usually an adaptive trait that maximizes the uptake of limiting resources and promotes whole-plant growth under environmental changes resulting from abiotic stress (Dai et al. 2009). These results suggest that competition between NT and SN transgenic plants affected plant growth at 6 months at a planting distance of  $70 \times 70$  cm under field conditions. The stem diameter of SN3 and SN4 lines was 1.3- and 1.2-fold higher than that of NT plants at 6 months, respectively, and these differences were statistically significant ( $P < 0.05$ ) (Fig. 1D). In contrast to the plant height growth, the diameter growth of transgenic poplar was less strongly affected by planting distance.

To assess the biomass of transgenic poplar lines, we analyzed the number of branches and dry weight in poplar shoots at 6 months. The average number of branches indicated that the SN3 and SN4 lines exhibited branch numbers



**Fig. 1** Growth-related features of transgenic poplar plants under field conditions for 6 months. **A:** Field conditions at 2 and 6 months. **B:** Phenotypes of transgenic poplars growing under field conditions at 2 months. **C:** Plant height of transgenic poplar plants. **D:** Stem diameter of shoots in transgenic poplar plants. Each data point is the average of 15 independent plants.



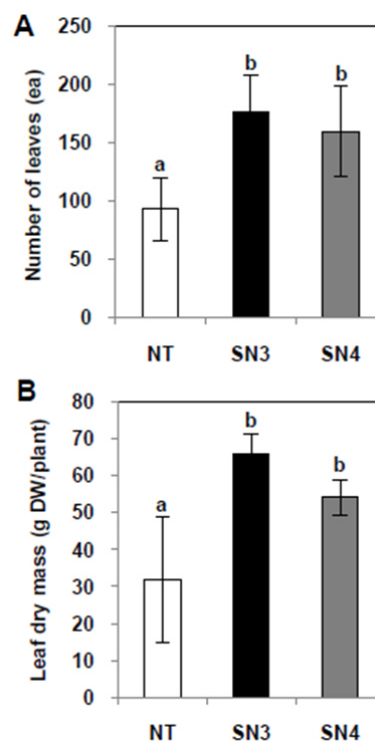
**Fig. 2** Biomass-related features of transgenic poplar plants under field conditions at 6 months. **A:** Branch number of transgenic poplar plants. Each data point is the average of 15 independent plants. **B:** Stem dry mass of transgenic poplar plants. Each data point is the average of five independent plants.

1.6 and 1.2 times greater than that of NT plants (Fig. 2A). The average dry weight of stems also indicated that SN3 and SN4 exhibited dry weights 1.6 and 1.2 times greater than that of NT plants (Fig. 2B). The average number of leaves in lines SN3 and SN4 exceeded that of NT plants by 1.8 and 1.6 times, respectively (Fig. 3A). Lines SN3 and SN4 exhibited higher dry weight, with averages exceeding that of NT plants by 2.0 and 1.7 times, respectively (Fig. 3B).

In this study of transgenic poplar lines, even though the number of branches in the SN3 lines was lower than in the SN4 lines, the SN3 lines exhibited higher shoot biomass than the SN4 lines (Fig. 1D). In the transgenic lines, high stem diameters in the SN3 lines likely led to enhanced biomass.

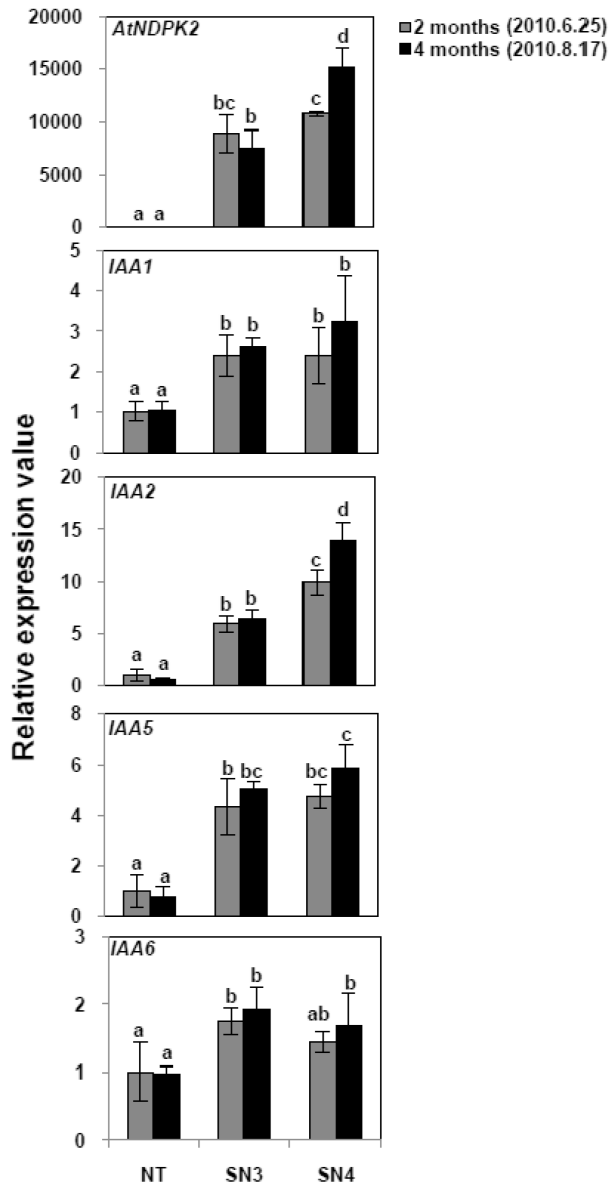
#### Expression patterns of *AtNDPK2* and auxin-related genes

It has been determined previously that SN poplar plants show increased transcript levels of auxin-responsive genes under field conditions (Kim et al. 2011). As altered plant growth and biomass were found in the transgenic lines under field conditions of the second growing season, quan-



**Fig. 3** Biomass-related features of transgenic poplar leaves under field conditions at 6 months. **A:** Leaf number of transgenic poplar plants. Each data point is the average of 15 independent plants. **B:** Leaf dry mass of transgenic poplar plants. Each data point is the average of five independent plants.

titative RT-PCR analyses were conducted to characterize the expression patterns of a variety of Aux/IAA genes in these transgenic lines (Fig. 4). Substantially increased expression of auxin-responsive genes, such as *IAA1*, *IAA2*, *IAA5* and *IAA6*, was observed in transgenic SN3 and SN4 lines. Previously SN poplar plants showed increased transcript levels of auxin-responsive genes under field environment conditions (Kim et al. 2011). Interestingly, the growth of transgenic poplar plants was enhanced under field conditions by increases in branch number and stem diameter. Choi et al. (2005) also reported on auxin-related cellular role of *NDPK2*. The *ndpk2* mutant exhibited developmental defects associated with auxin, such as cotyledon growth and sensitive phenotype to treatments with an inhibitor of polar auxin transport naphthylphthalamic acid (NPA) in *Arabidopsis*. In addition, the expression levels of auxin-responsive genes such as *IAA2*, *IAA4* and *IAA17* were decreased in the *ndpk2* mutant plants by auxin treatments. The transported auxin levels from the shoot apex to the shoot/root transition zone were also increased in the *ndpk2* mutant plants compared with in the wild-type plants. In the present study, we demonstrated that the SN poplar plants showed increased plant biomass and expression levels of auxin-responsive genes



**Fig. 4** Expression patterns of *AtNDPK2* and various *IAA* genes in SN transgenic poplar plants under field conditions at 4 months.

under field conditions of the second growing season, thereby indicating that *AtNDPK2* expression may be a positive function as auxin transport-related signaling pathway on regulation of plant growth and development.

*AtNDPK2* expression is induced by oxidative stress treatment conditions including  $H_2O_2$ , thus its induction is associated with oxidative stress defense mechanisms. For example, Kim et al. (2011) demonstrated that poplar plants overexpressing *AtNDPK2* showed enhanced tolerance to methyl viologen (MV)-mediated oxidative stress in leaf discs. In addition, transgenic *Arabidopsis*, potato and sweetpotato plants overexpressing *AtNDPK2* also exhibited enhanced tolerance to MV-mediated oxidative stress, extreme tem-

peratures and osmotic stress through increased activity and expression of the  $H_2O_2$ -scavenging antioxidant enzyme (Moon et al. 2003; Tang et al. 2008; Kim et al. 2009). For these reasons, we are currently trying to assess enhanced tolerance to environmental stress involving salt and drought in the SN transgenic poplar lines (data not shown).

Further characterization of SN poplar plants will be conducted on marginal lands and under contaminated soil conditions. It is likely that SN transgenic poplar can be used for industrial wood production, biomass enhancement and environmental planting on marginal lands such as desert areas and mine dumps.

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