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# Transgenic poplar expressing *Arabidopsis NDPK2* enhances growth as well as oxidative stress tolerance

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## Summary

Nucleoside diphosphate kinase 2 (NDPK2) is known to regulate the expression of antioxidant genes in plants. Previously, we reported that overexpression of *Arabidopsis NDPK2* (*AtNDPK2*) under the control of an oxidative stress-inducible *SWPA2* promoter in transgenic potato and sweetpotato plants enhanced tolerance to various abiotic stresses. In this study, transgenic poplar (*Populus alba* × *Poplus glandulosa*) expressing the *AtNDPK2* gene under the control of a *SWPA2* promoter (referred to as SN) was generated to develop plants with enhanced tolerance to oxidative stress. The level of *AtNDPK2* expression and NDPK activity in SN plants following methyl viologen (MV) treatment was positively correlated with the plant's tolerance to MV-mediated oxidative stress. We also observed that antioxidant enzyme activities such as ascorbate peroxidase, catalase and peroxidase were increased in MV-treated leaf discs of SN plants. The growth of SN plants was substantially increased under field conditions including increased branch number and stem diameter. SN plants exhibited higher transcript levels of the auxin-response genes *IAA2* and *IAA5*. These results suggest that enhanced *AtNDPK2* expression affects oxidative stress tolerance leading to improved plant growth in transgenic poplar.

## Introduction

Environmental stress is one of the most severe agricultural problems affecting plant growth and crop yield (Toenniessen *et al.*, 2003). Recent studies have identified a large number of genetic and molecular networks underlying plant adaptations to adverse environmental growth conditions (Sreenivasulu *et al.*, 2007). All of these studies emphasize the complexity of various traits and their polygenic inheritance. The current notion is that defence mechanisms of plants against stress conditions are tightly associated with the species-specific growth habits, and hence every claim of tolerance enhancement needs to be tested on a crop-yield basis, coupled with its economic significance from an agricultural point of view (Flowers, 2004; Passioura, 2007). This consideration has only recently become generally accepted, providing the justification for a major focus on crop-specific gene transfer to enhance stress tolerance.

Trees have great values as a source of essential elements for human living. They have unique characteristics, such as perennial growth, developmental phase changes, secondary growth and metabolism, and trees also exhibit resistance systems to extreme environmental conditions (Bhalerao *et al.*, 2003; Gallardo *et al.*, 2003). They are generally exposed to recurrent cycles of injury by a variety of biotic and abiotic stresses. This is because of both the technical difficulty of transformation and the extended life cycle. Therefore, the development of techniques leading to avoidance or reduction in injuries imposed by environmental stress is important for trees. Because of the slow growth, growth enhancement of trees provides an important incentive, because growth enhancement coupled to stress tolerance are important factors not only from an environmental but also from an economical point of view. Among the various tree crops, poplar occupies a prominent place as a model system for functional genomics

studies. Poplar has many features that make the species a suitable model for forest biotechnology, such as fast growth, a relatively small genome, ease of vegetative propagation, facile transgenesis and tight coupling between physiological traits and biomass productivity (Bradshaw *et al.*, 2000; Constabel *et al.*, 2000; Taylor, 2002; Sterky *et al.*, 2004). Of particular importance and convenience is the highly efficient genetic transformation system coupled with efficient regeneration of poplar that is unsurpassed by other tree crops (Fillatti *et al.*, 1987).

Nucleoside diphosphate kinases (NDPKs, EC 2.7.4.6) are housekeeping enzymes that maintain intracellular levels of nucleoside triphosphates (NTPs), with the exception of adenosine triphosphate (ATP). In plants, they are involved in the phytochrome A (PhyA) response (Choi *et al.*, 1999), UV-B signalling (Zimmermann *et al.*, 1999), heat shock responses (Escobar Galvis *et al.*, 2001) and oxidative stress signalling (Moon *et al.*, 2003). Among the three NDPK isoforms in *Arabidopsis*, diverse functions of NDPK2 on plant development and stress responses have been reported (Moon *et al.*, 2003). It has been shown that the binding of Arabidopsis NDPK2 (AtNDPK2) to phytochrome PhyA increases the activity of NDPK2 (Choi *et al.*, 1999; Kim *et al.*, 2002; Shen *et al.*, 2005). Moreover, a mutant lacking *AtNDPK2* displays a partial defect in photomorphogenesis, including cotyledon opening and greening in response to both red and far-red light (Choi *et al.*, 1999). In addition, *AtNDPK2* appears to participate in auxin-regulated processes, partly through the modulation of auxin transport by changes of Aux/IAA-related gene expression (Choi *et al.*, 2005a). During stress conditions, *AtNDPK2* is not only involved in oxidative stress signalling by interaction with two kinds of mitogen-activated protein kinases (AtMPK3 and AtMPK6), but the enzyme also participates in salt stress signalling by interaction with class 3 sucrose-nonfermenting 1-related kinase (SOS2) and catalase (CAT) (Moon *et al.*, 2003; Verslues *et al.*, 2007). Recently, *AtNDPK2* expressing transgenic plants, such as potato and sweetpotato, also exhibited significantly enhanced tolerance to multiple environmental stresses (Tang *et al.*, 2008; Kim *et al.*, 2009). Therefore, NDPK2 appears to be a component of stress protection mechanisms that could be exploited for engineering of plant growth enhancement and stress tolerance.

A powerful expression system with an appropriate promoter is an important requisite for efficient expression of foreign genes in plant cells (Aoyama and Chua, 1997; Kasuga *et al.*, 1999). Development of stress-inducible promoters that control precisely the expression of target defence genes under particular stress conditions is very important for developing transgenic plants with an enhanced tolerance to various stresses. We have previously isolated an oxidative stress-inducible *SWPA2* promoter from cell cultures of sweetpotato and characterized its function in transgenic tobacco plants in terms of environmental stresses, including oxidative stress (Kim *et al.*, 2003). *SWPA2* had been found to encode an anionic peroxidase (POD), which was highly expressed in response to various stresses including wounding, chilling, sulphur dioxide, ozone and UV (Kim *et al.*, 1999, 2007). The *SWPA2* promoter contained several *cis*-element sequences implicated in oxidative stress such as GCN-4, AP-1, HSE and SP-1 reported in animal cells and a plant-specific G-box (Kim *et al.*, 2003). The expression of −1314 *SWPA2* promoter in transgenic tobacco plants was strongly induced in response to environmental stresses including $H_2O_2$, wounding and UV treatment and suspension cultured cells conditions (Kim *et al.*, 2003). These results indicate that the −1314 bp *SWPA2* promoter will be biotechnologically useful for the development of transgenic plants with enhanced tolerance to environmental stress.

In this study, we generated *AtNDPK2* expressing transgenic poplar lines under control of the *SWPA2* promoter, and their tolerance to oxidative stress was characterized. We demonstrate here that overproduction of this NDPK2 confers resistance to oxidative stress and stimulates vegetative growth of transgenic poplar under field conditions.

## Results

### Molecular and biochemical characterization of *AtNDPK2* expressing transgenic poplar under MV-mediated oxidative stress

Transgenic poplar plants that expressed *AtNDPK2* under the control of an oxidative stress-inducible *SWPA2* promoter (SN plants) were successfully generated by *Agrobacterium*-mediated transformation (Figure 1a). Five independent transgenic lines were established for further studies. Integration and gene copy number of the construct in the transformed plants were determined by PCR analysis with *AtNDPK2* and *NPTII* gene-specific primers (data not shown), then analysed further via Southern blot analysis. Among five transgenic lines, four lines 3, 6, 8 and 10 showed single copy insertions, whereas line four exhibited multicopy insertions (Figure 1b). The five SN transgenic lines were grown in a greenhouse for 6 months. They were used to evaluate tolerance against

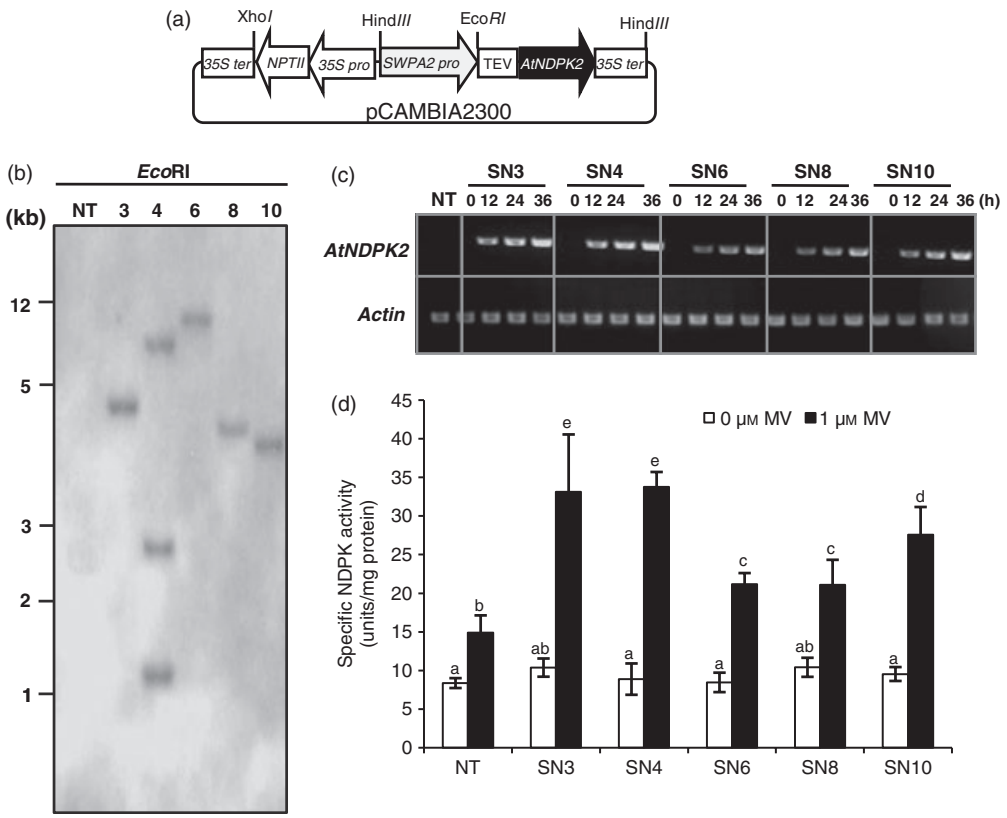


**Figure 1** Molecular and biochemical characterization of *AtNDPK2* overexpressing transgenic poplar plants (SN plants) and nontransgenic control plants (NT plants) under MV-mediated oxidative stress. (a) Diagram of the oxidative stress-inducible *SWPA2* promoter::*AtNDPK2* construct. (b) Southern blot analysis of transgenic lines. The integration and gene copy number of the construct in the transformed plants were confirmed by *AtNDPK2* gene with Eco RI digestion. (c) RT-PCR analysis of SN plants under 1 μM MV treatment for 36 h. (d) Specific NDPK activity in SN plants at 24 h after MV treatment. Data presented are the average of three replicates. Bars carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

methyl viologen (MV)-mediated oxidative stress through leaf disc assays. MV is a typical reactive oxygen species (ROS)–generating redox active compound, which has been used as a nonselective herbicide (Babbs *et al.*, 1989). To investigate the level of transgene expression in the SN plants, RT-PCR analysis was conducted using RNA from MV-treated plant leaf discs with a *AtNDPK2* gene-specific primer set (Figure 1c). After induction of MV stress, induced expression of *AtNDPK2* was detected in the leaf discs of all transgenic lines, but not in the nontransgenic (NT) plant. Particularly, prominent induction of the *AtNDPK2* expression was detected in the SN3 and SN4 plants after MV treatment. To further ascertain whether *AtNDPK2* expression is correlated with NDPK enzyme activity, we measured the NDPK activity in soluble extracts from leaf discs of the SN transgenic lines (Figure 1d). SN3 and SN4 plants exhibited 2.2 and 2.3-fold higher NDPK activity than the NT plants under MV treatment.

To investigate whether the tolerance against oxidative stress was altered in SN poplar plants, we subjected leaf discs of SN plants to MV (Figure 2). Leaf discs were incubated with 1 μM MV under illumination, and the loss of cytoplasmic solutes was determined based on the electrical conductance of the solution. The extent of cellular damage was quantified by solute leakage, which is an accepted measure of membrane disruption (Bowler *et al.*, 1991). At 24 h following MV treatment, the leaf discs of the NT plants showed nearly complete cellular disruption (about 70% of maximum solute leakage), whereas those of five SN plants showed approximately 50% less membrane damage than their NT counterparts. A comparison of leaf discs of NT and SN plants following exposure to oxidative stress in the form of MV showed that the leaf discs of the SN plants showed relatively less membrane damage (Figure 2a). SN3 and SN4 plants showed approximately 25.4 and 26% reductions in membrane damage at 24 h, respectively, when compared with the NT plants, and membrane damage of SN3 and SN4 was reduced for approximately 20.6 and 20% at 60 h, respectively, when compared with the NT plants under MV treatment

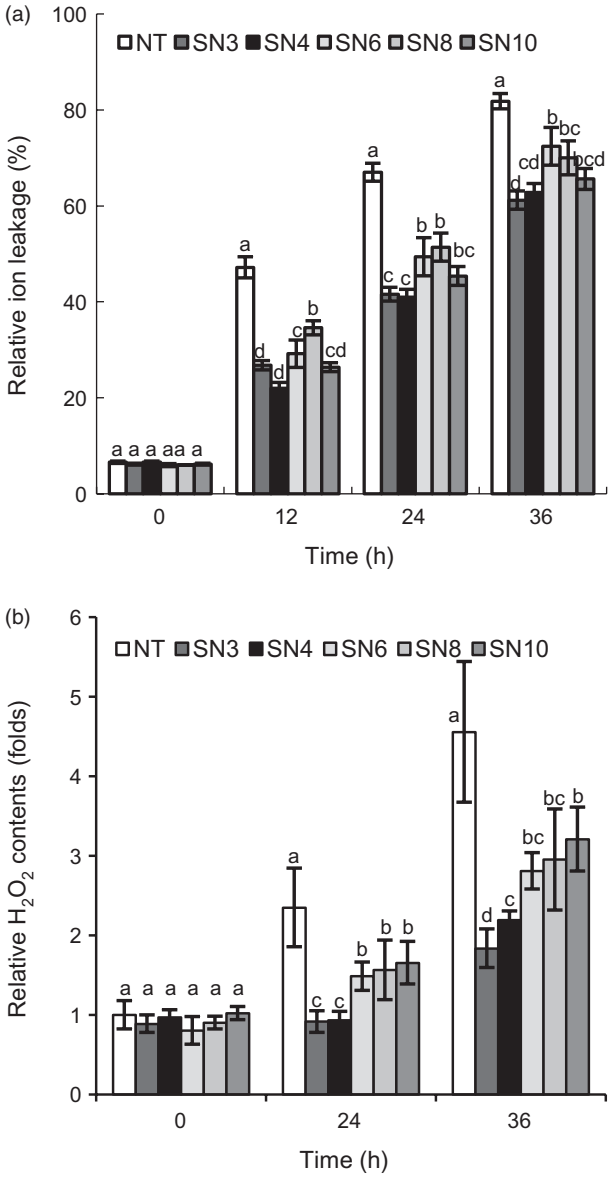


**Figure 2** Effect of MV-mediated oxidative stress treatment in NT and transgenic SN poplar plants. (a) Analysis of ion leakage in independent SN transgenic lines in response to 1 μM MV treatment for 36 h. The electrical conductivity of the MV solution was compared with the total conductivity of the solution following tissue destruction. Data presented are the average of five replicates. (b) $H_2O_2$ contents in the leaf discs of NT and SN plants treated with 1 μM MV. Leaf discs were treated with MV, and $H_2O_2$ content was determined at 0, 24 and 36 h of treatment. Data presented are the average of three replicates. Bars carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

(Figure 2a). The analysis of ion leakage in the transgenic plants subjected to MV treatment revealed that transgenic lines 3 and 4, which exhibited the highest NDPK activity (Figure 1c, d), also retained the lowest levels of ion leakage content after MV treatment. $H_2O_2$ is produced in the plants during stressful conditions; if it remains unscavenged, it can cause severe damage to the plant cells. We evaluated the relative $H_2O_2$ content of NT and SN plants after 1 μM MV treatment for 36 h. SN3 and 4 plants showed lower $H_2O_2$ content than NT and the other SN plant lines (Figure 2b).

NDPK2 participates in the activation of gene expression and activity of various antioxidant enzymes under stress conditions (Yang *et al.*, 2003; Tang *et al.*, 2008; Kim *et al.*, 2009). Therefore, to understand the mechanism of the enhanced tolerance of SN plants to MV-mediated oxidative stress, we investigated changes in the activities of $H_2O_2$-scavenging enzymes, such as ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) after MV treatment. SN3 and SN4 plants exhibited approximately 2.3- and 2.6-fold higher APX activity than NT plants (Figure 3a). CAT activity in SN3 and SN4 plants showed 2.2 and 1.8 higher than NT plants (Figure 3b). POD activity also showed approximately 2.0 and 2.1-fold higher than those of NT plants (Figure 3c). Our results suggest that activation of antioxidant enzymes by *AtNDPK2* expression plays an important role in tolerance of transgenic poplar plants to oxidative stress.

## Growth of *AtNDPK2* overexpressing transgenic poplar under field conditions

The rooted plantlets were acclimatized in pots in the greenhouse for 3 months and then transferred to the field (Figure 4f, g). In one growing season during 6 months, line 3, 4 and 10 plants grew to about 2.48, 2.65 and 2.52 m in height, which is similar level with the height of 2.22 m for NT plants (Figure 4a). During a 6 -month period, branch numbers and stem diameters were also measured at 3, 4 and 6 months. The averages of branch number were 11.9, 18.5 and 19.3 for NT, SN3 and SN4 lines at 6 months, respectively (Figure 4b). The averages of stem diameters were 25, 30.2 and 35.3 mm for NT, SN3 and SN4 lines at 6 months, respectively (Figure 4c). Therefore, average plant branch number and stem diameter in the SN3 and SN4 lines was greater than that of the NT plant line during one growing season in the field. Additionally, young leaves of SN lines and NT plants at 3 and 4 months did not show a significant difference in Fv/Fm and total chlorophyll contents (Figure 4d, e). Fv/Fm is relative chlorophyll fluorescence showing PSII photosynthetic efficiency. *Fv* is the total amount of variable fluorescence and *Fm* is the maximum fluorescence yield.

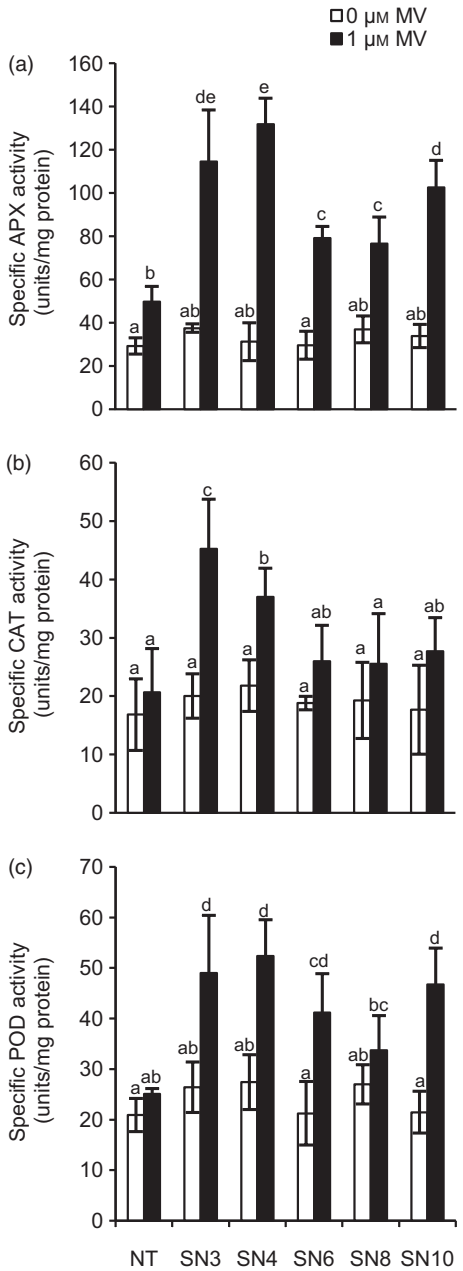


**Figure 3** Changes in the activities of $H_2O_2$-scavenging enzymes in SN plants after 1 μM MV treatment. Changes in (a) APX, (b) CAT, and (c) POD activities in SN plants at 24 h after 1 μM MV treatment. Data presented are the average of three replicates. Bars carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

### Characterization of transgenic poplar under field growth conditions

Under natural field conditions, plants typically experience more stress compared to growth chamber and greenhouse conditions. High light, variable water availability (drought) and high or low temperature can become stressful. Thus, it was important to test the molecular and biochemical effects of *AtNDPK2* on the growth of poplar in the field. The results showed increased transcript level of *AtNDPK2* in the leaves of the transgenic lines, but not in NT plants (Figure 6a). The SN3 and SN4 plant lines showed lower levels of $H_2O_2$, 42 or 57% lower, respectively, at 4 months than NT plants exposed to field conditions (Figure 5a). Clearly, *AtNDPK2* expression in the field reduced the accumulation of ROS in SN plants, suggesting that ROS-scavenging enzymes were regulated by *AtNDPK2*. Thus, we investigated whether expression of *AtNDPK2* had an effect on the activation of antioxidant enzymes under field conditions. SN3 and SN4 plants exhibited 1.6-fold higher NDPK enzyme activity than NT plants under field conditions at 4 months (Figure 5b). In addition, SN3 and SN4 plants exhibited approximately 1.45 and 1.4-fold higher APX activity than that of NT plants (Figure 5c). CAT activity in SN3 and SN4 plants was approximately 1.37 and 1.39-fold higher than NT plants at 4 months (Figure 5d). POD activity also showed a 1.29 and 1.44-fold higher activity than NT plants grown side by side (Figure 5e).

The plant hormone auxin controls many developmental effects by regulating auxin-responsive transcription factors (ARFs) that bind to auxin-responsive elements in downstream genes. Transcripts of ARFs are accumulated in response to auxin and in turn induce expression of early auxin-response genes like Aux/IAA (Leyser, 2001; Wilmoth *et al.*, 2005). In addition, previous reports showed that *AtNDPK2* appears to participate in auxin-regulated processes, partly through the modulation of auxin transport by changes in the expression of Aux/IAA genes (Choi *et al.*, 2005a). We investigated the expression of Aux/IAA genes in the transgenic poplar lines under field conditions, because the enhanced growth parameters appeared to indicate an effect of auxin metabolism. To investigate the activation of the auxin-response pathway in the transgenic poplar plants expressing *AtNDPK2*, the expressions of early auxin-response genes *IAA1, 2, 4, 5, 6* and *7* were analysed (Figure 6b). Quantitative RT-PCR analyses of poplar *IAA2* and *IAA6* clearly demonstrated that the expressions of these genes in the transgenic SN3 and SN4 are more than 3- to 3.5-fold higher than in the control plant under field conditions for 4 months (Figure 6b).

## Discussion

Over the life cycle of a plant, NDPKs perform important functions by controlling growth, development and defence

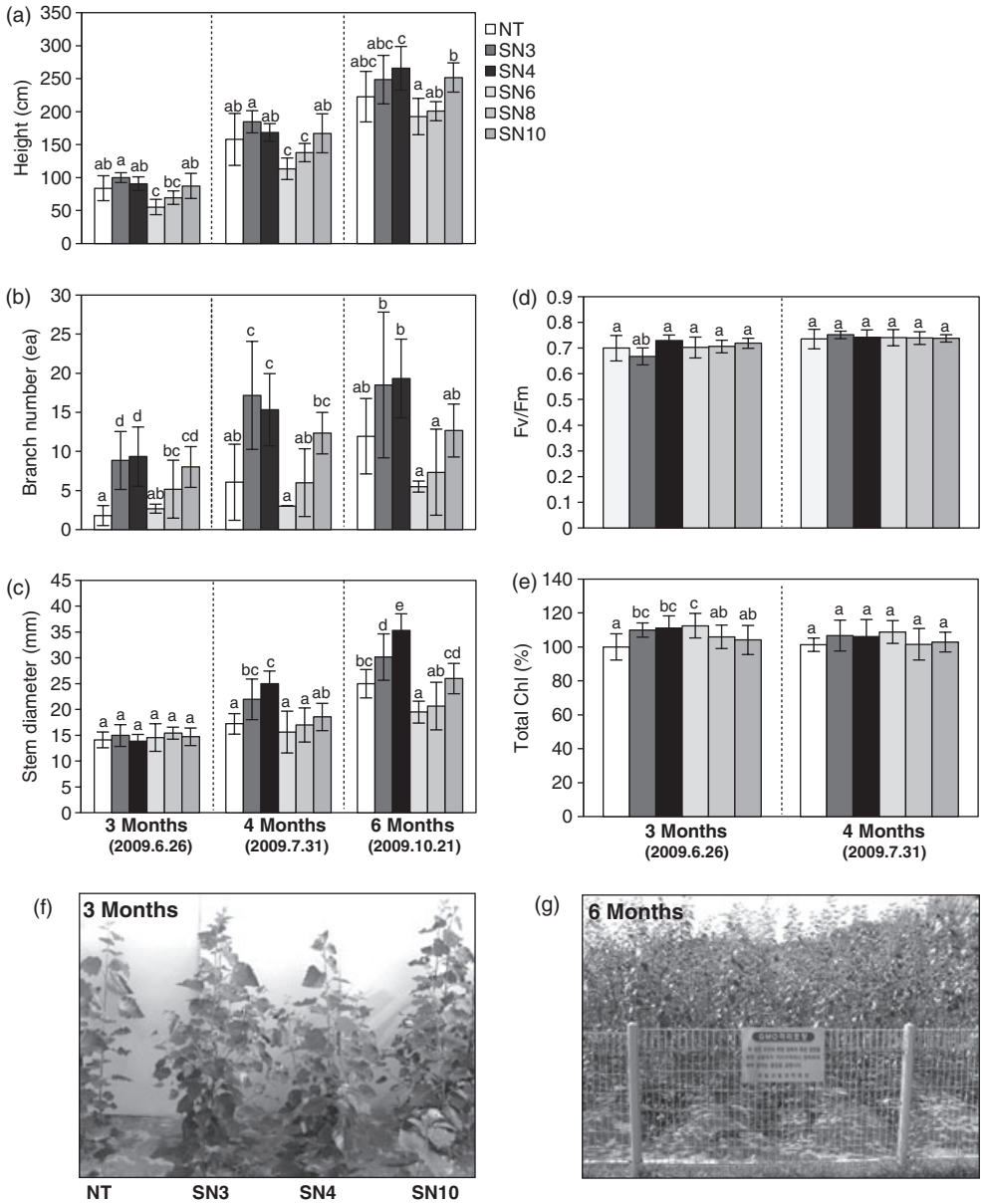


**Figure 4** Growth rate and photosynthesis-related feature of the transgenic poplar in the field conditions for 6 months. (a) Plant height of poplar plants. (b) Branch number of shoot in the poplar plants. (c) Stem diameter of shoot in the poplar plants. (d) PSII photosynthetic efficiency (Fv/Fm) in the leaves of poplar plants. (e) Total Chl content in the leaves of poplar plants. Each data point is the average of six independent plants. Phenotypes of transgenic poplars growing in the field conditions at (f) 3 months and (g) 6 months.

responses. Recently, it has been shown that NDPK2 plays a regulatory role in $H_2O_2$ or/and auxin-mediated signalling in plants, indicating that NDPK2 enzymes carry out a diverse array of biological functions (Moon *et al.*, 2003; Choi *et al.*, 2005a). In this study, we successfully developed transgenic poplar plants expressing *AtNDPK2* under control of the oxidative stress-inducible *SWPA2* POD promoter. Expression of the *AtNDPK2* gene caused activation of antioxidant enzymes in poplar, thereby conferring increased tolerance to MV-mediated oxidative stress. We also showed that the transgenic expression of *AtNDPK2* resulted in higher growth rates in transgenic poplar, confirming the possibility by auxin-related function.

NDPKs play a prominent role in plant defence mechanisms, and the involvement of NDPK is associated with various stress tolerances, including oxidative stress. For example, Moon *et al.* (2003) demonstrated that *NDPK2* overexpressing *Arabidopsis* showed enhanced tolerance to MV-mediated oxidative stress, freezing and high salt. In transgenic potato and sweetpotato plants, overexpression

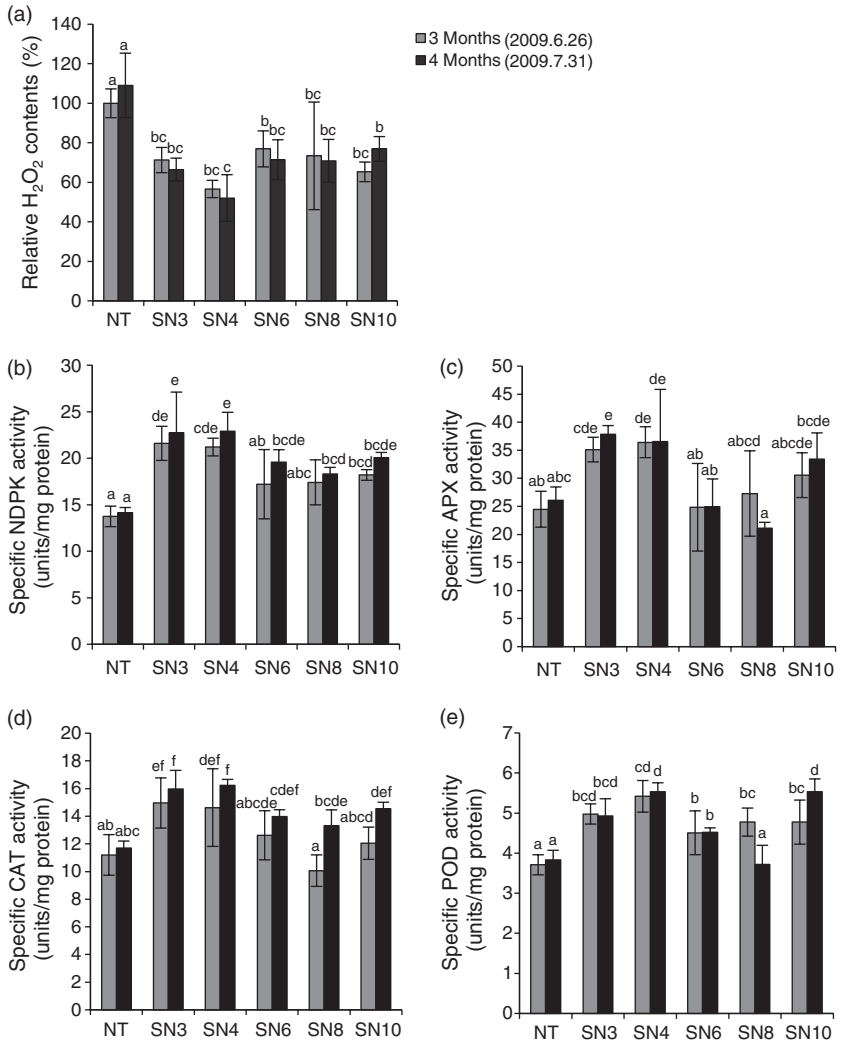


**Figure 5** $H_2O_2$ contents, NDPK and $H_2O_2$-scavenging enzymes activities in SN transgenic poplar plants under field conditions for 4 months. Changes in (a) $H_2O_2$ contents, (b) NDPK, (c) APX, (d) CAT and (e) POD activities in SN plants. Data presented are the average of three replicates. Bars carrying the same letter are not significantly different ($P$ = 0.05) according to Duncan's multiple range test.

of *NDPK2* also exhibited enhanced tolerance to MV-mediated oxidative stress, temperatures and osmotic stress (Tang *et al.*, 2008; Kim *et al.*, 2009). As expected, the SN transgenic poplar plants of our study showed a significantly enhanced tolerance to MV-mediated oxidative stress, with high levels of NDPK production (Figures 1 and 2).

*Arabidopsis NDPK2* is a component of the $H_2O_2$-activated MAPK signalling pathway, especially via AtMPK3 and AtMPK6, and overexpression of *AtNDPK2* alters cellular redox conditions in plants (Moon *et al.*, 2003). Yang *et al.* (2003) demonstrated that increased expression of numerous genes in the *NDPK2* overexpressing *Arabidopsis* including those involved in signal transduction and protection by cDNA microarray analysis. Among the induced genes, expression of various antioxidant genes, including POD, APX, CAT, was significantly increased in *NDPK2* overexpressing *Arabidopsis*. In addition, overexpression of *NDPK2* in transgenic potato and sweetpotato plants increased NDPK and $H_2O_2$-scavenging antioxidant enzyme activities (Tang *et al.*, 2008; Kim *et al.*, 2009). In this study, consistent with these results, we have demonstrated that the SN poplar plants showed increased levels of APX, CAT and POD activity under MV-mediated oxidative stress and field environment conditions (Figures 3 and 5). APX, CAT and POD are the major enzymes responsible for $H_2O_2$ scavenging during oxidative stress in plants. APX is a component of the ascorbate-glutathione pathway, which plays a key role in $H_2O_2$ scavenging in different cellular compartments such as cytosol, chloroplast, membrane and microbody and mitochondria (Mittler, 2002; Shigeoka *et al.*, 2002). CAT eliminates $H_2O_2$ by breaking it down directly to form water and oxygen in microbody. Thus, CAT does not require reducing power and has a high reaction rate, but a low affinity for $H_2O_2$, and can, thereby, only remove $H_2O_2$ when high concentrations are present (Willekens *et al.*, 1997). In addition, Plant PODs are localized in vacuoles and in the apoplast, and

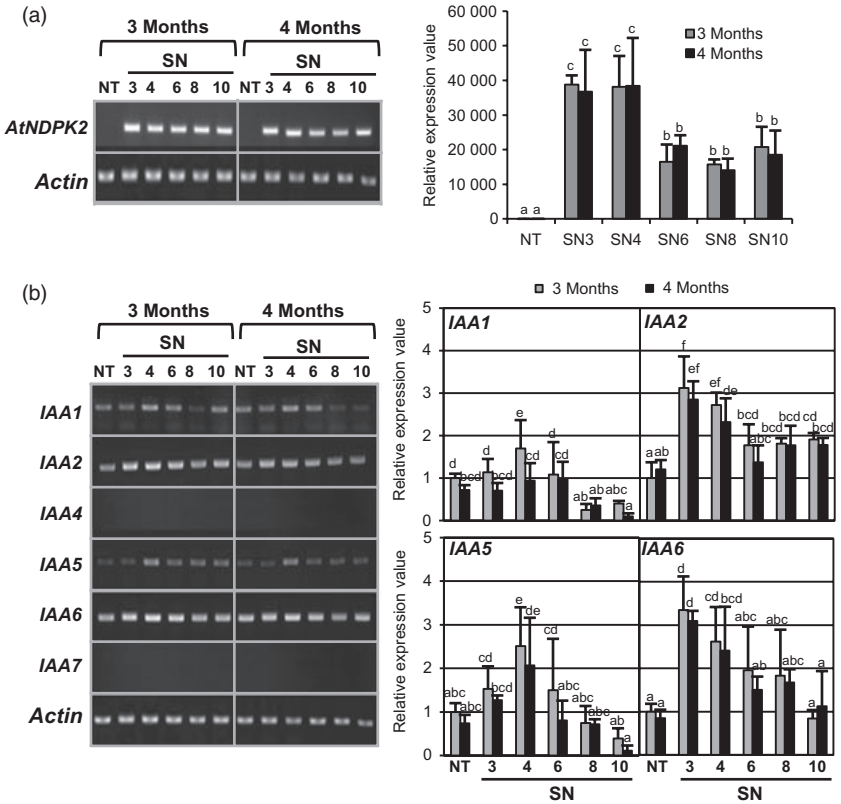


**Figure 6** Expression patterns of various *IAA* genes in the SN transgenic plants under field conditions for 4 months. (a) The expression levels of *AtNDPK2* gene in poplar plants. *AtNDPK2* transcript levels were tested using RT-PCR and measured using quantitative real-time PCR. (b) The expression levels of various *IAA* genes in poplar plants. Transcript levels of the *IAA* genes were tested using RT-PCR and measured using quantitative real-time PCR. Data represent three independent experiments.

they are involved not only in scavenging $H_2O_2$ but also in plant growth, development, lignification, suberization and cross-linking of cell wall compounds (Passardi *et al.*, 2005; Cosio and Dunand, 2009). Our data are consistent with the observations that the increase in the activities of APX, CAT and POD as the result of *NDPK2* expression in transgenic poplar plants is correlated with environmental stress defence mechanisms involving an $H_2O_2$-regulated stress response signalling pathway. While not explicitly characterized in the current experiments, the examination of monodehydroascorbate reductase (MDHR), which provides an alternative pathway for ROS detoxification and glutathione reductase (GR), which is a rate-limiting enzyme in the usual ROS-scavenging pathway, might better illuminate the interplay between these enzymes and the antioxidative function of AtNDPK2 gene in the transgenic poplar plants (Mittler, 2002). Similarly, measurements of antioxidant levels and redox state (Noctor and Foyer,1998; Mittler, 2002) might provide additional information on oxidative stress in the transgenic poplars.

It is known that cross-interaction of signal transduction pathways keep balance between growth and development of the plants and their responses to different external stimuli. Mechanical wounding induces genes related to abiotic stress and hormonal responses (Cheong *et al.*, 2002). Shukla *et al.* (2006) demonstrated that ectopic expression of a chickpea AP2 transcription factor (CAP2) showed enhanced growth rates and tolerance to dehydration and salt stress in transgenic tobacco by expressions of abiotic stress-response genes *NtERD10B*, *NtERD10C* and auxin-response genes *IAA4.2* and *IAA2.5.* The plant hormone auxin regulates a number of cellular and developmental processes, including cell division, cell growth and differentiation (Friml, 2003). At the molecular level, auxin exerts its effect by regulating expression of numerous auxin-responsive genes including AUX/IAA genes. A family of ARF binds to the auxin-response element in the promoters of auxin-inducible genes to promote auxin-mediated gene induction response (Leyser, 2001; Ljung *et al.*, 2001; Reed, 2001). In the previous study, Choi *et al.* (2005a) reported the possible role of *NDPK2* on auxin-related cellular processes. The *ndpk2* mutant of *Arabidopsis* displayed developmental defects associated with auxin, such as cotyledon development and increased sensitivity to an inhibitor of polar auxin transport. In addition, the transcript levels of specific auxin-responsive genes such as *IAA2*, *IAA4* and *IAA17* were reduced in the *ndpk2* mutant plants treated with auxin. The amount of auxin transported from the shoot apex to the shoot/root transition zone was also increased in the *ndpk2* mutant,

compared with in the wild-type plants. In this study, we demonstrated that the SN poplar plants showed increased transcript levels of auxin-responsive genes, especially *IAA2* and *IAA6*, under field environment conditions (Figure 6). Interestingly, the growth of transgenic poplar plants was substantially increased under field conditions by increases in branch number and stem diameter (Figure 4), thereby indicating that *AtNDPK2* expression may be a positive function as auxin transport-related signalling pathway. From the earlier data, we concluded that the increase in growth rates and *IAA* gene expressions resulting from the expression of *NDPK2* might be associated with developmental responses under natural field conditions. In the future, data on $CO_2$ fixation in relation to photosynthetic efficiency in transgenic plants on marginal lands will provide a useful measure of carbon sequestration and could be an important metric in ascribing carbon credits to these plants (Scarascia-mugnozza *et al.*, 1996; Boese *et al.*, 1997).

In most experiments, the transgenes are driven under the control of a strong constitutive promoter, such as the *CaMV 35S* promoter. However, a more conditional gene expression system is needed to extract greater benefits from transgenic technology (Aoyama and Chua, 1997; Kasuga *et al.*, 1999). Furthermore, development of stress-inducible promoters that control precisely the expression of target defence genes under particular stress conditions is very important for developing transgenic plants with an enhanced tolerance to various stresses. For example, Kasuga *et al.* (1999) reported that 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. Use of the stress-inducible *RD29A* promoter instead of the constitutive *CaMV 35S* promoter for the overexpression of *DREB1A/CBF3* minimized the negative effects on plant growth in transgenic *Arabidopsis* and tobacco plants (Kasuga *et al.*, 1999, 2004). *Rd29A* promoter also showed functions in gene expression in response to stress in Arabidopsis and tobacco plants (Yamaguchi-Shinozaki and Shinozaki, 1994; Kasuga *et al.*, 1999, 2004). DRE and ABRE *cis*-elements are found in the *RD29A* promoter and DRE/CRT functions in early stress signalling, whereas abscisic acid response element (ABRE) functions after the accumulation of ABA during drought and high-salinity stress response (Shinozaki and Yamaguchi-Shinozaki, 2000). In addition, our previous study also describes transgenic potato plants expressing *AtNDPK2* gene under the control of *SWPA2* (referred to SN plants) or *CaMV 35S* promoter (referred to EN plants), and their enhanced tolerance to oxidative stress induced by MV, high temperature and salt stress (Tang *et al.*, 2008). SN potato plants showed much less plant damage than NT plants after MV treatment, whereas EN plants showed an intermediate result between SN and NT plants. These data indicated that a stress-inducible *SWPA2* promoter is more efficient than *CaMV 35S* promoter for the development of stress-tolerant transgenic plants. The accumulating evidence suggested that stress-inducible *SWPA2* promoter is applicable to other plant systems for the development of stress-tolerant transgenic plants. Use of *SWPA2* promoter to conditionally induce the expression of defence genes in several plant systems made it possible to develop transgenic plants with an increased tolerance to multiple stresses. For example, in transgenic potato, an ectopically expressed various defence genes including antioxidant enzymes or osmotic protectant synthesis enzyme under the control of *SWPA2* promoter showed enhanced tolerance to salt, drought, heating and MV-mediated oxidative stresses (Tang *et al.*, 2006, 2008; Ahmad *et al.*, 2008, 2010). Transgenic sweetpotato overexpressing antioxidant defence genes such as *CuZnSOD* and *APX* or *NDPK2* also exhibited strong resistance to chilling, salt, drought and MV treatment by control of *SWPA2* promoter (Lim *et al.*, 2007; Kim *et al.*, 2009). Moreover, in monocot plants, overexpression of the antioxidant enzymes or drought tolerance proteins under the control of the *SWPA2* promoter resulted in strong stress induction of target genes, thus transgenic tall fescue and rice plants acquired higher tolerance to MV, $H_2O_2$, heavy metals and drought stress (Lian *et al.*, 2004; Wang *et al.*, 2005; Lee *et al.*, 2007). Therefore, *SWPA2* promoter can regulate the expression of target genes in both monocot and dicot plants under various stress conditions. Our present study is the first report on the use of the *SWPA2* promoter in a woody perennial species and may be one of good example of the field performance of transgenic trees. In this study, the expression of *AtNDPK2* and its activity in the transgenic poplar was induced under the control of *SWPA2* promoter by MV or natural field stress conditions (Figures 1, 5 and 6). In contrast, the *AtNDPK2* transgene was not expressed in untreated samples (Figure 1), suggesting that expression of the *AtNDPK2* gene is strictly regulated by the *SWPA2* promoter in transgenic poplar. These results suggest that the *SWPA2* promoter strictly controls expression of the *AtNDPK2* transgene in response to stress conditions in SN poplar plants.

It is reported that transgenic poplar with modified expression of genes involved in oxidative stress responses

showed tolerance to oxidative stress tolerance. For example, transgenic poplars with modified glutathione pathways or overexpressing the antioxidant enzymes showed improved stress resistance (Nicolescu *et al.*, 1996; Arisi *et al.*, 1998). Likewise, transgenic poplar plants that produce above normal levels of reduced glutathione are less susceptible to photoinhibition stress than the NT plants (Foyer *et al.*, 1995). Recently, studies also reported that overexpression of the horseradish peroxidase (*prxC1a)* gene-enhanced peroxidase activity affects plant growth rate and oxidative stress resistance in transgenic hybrid aspen (Kawaoka *et al.*, 2003). In addition, the expression of the Vitreoscilla haemoglobin (VHb)-encoding gene in white poplar resulted in the absence of positive effects on plant growth, biomass production and oxidative stress tolerance (Zelasco *et al.*, 2006). Therefore, our present study is another report on enhanced oxidative stress tolerance and growth rate using transgenic poplar and may be one of good example of the field performance of transgenic trees.

However, we also point to substantial differences in the oxidative stress resistance and growth phenotype among the five transgenic lines (Figures 2 and 4). These differences are likely attributable to differences in NDPK activity. Even though the NDPK activity of the transgenic lines 6, 8 and 10 was higher than in NT plants by approximately 1.2- to 1.4-fold (Figures 1 and 5), the lines 6, 8 and 10 plants showed a slightly higher MV tolerance and similar growth phenotype compared with NT plants (Figures 2 and 4). When considering the NDPK activity in the transgenic plants, the difference between lines 3, 4 and the other lines became even less remarkable (Figures 1 and 5). We also confirmed the low antioxidant enzyme activity and expression levels of *IAA* genes in the lines of 6, 8 and 10 of transgenic plants (Figures 3, 5 and 6). It is possible that the lack of the growth or oxidative stress tolerance phenotype in lines 6, 8 and 10 plants may be a consequence of positional effects of the gene during the tissue culture procedure and/or threshold effects of NDPK activity on plant development and stress resistance.

The deployment of elite transgenic lines such as SN3 and SN4 on marginal lands, including regions that have experienced desertification, could be a very useful remediation strategy. Examination of MDHR and GR activities, antioxidant levels, plant redox state and $CO_2$ fixation in transgenic plants grown on such marginal lands will provide important information on the utility of these plants under such circumstances.

In conclusion, we have generated transgenic poplar expressing *AtNDPK2* under the control of the oxidative stress-inducible *SWPA2* promoter by *Agrobacterium*-mediated transformation. The overexpression of the *AtNDPK2* gene in poplar plants resulted in higher antioxidant enzyme levels and enhanced auxin-responsive gene expression. Growth rates and resistance to oxidative stress of transformed plants were increased under greenhouse or field conditions. In further studies, it remains to be seen whether the transgenic lines will show the same tendencies in terms of oxidative stress protection and growth phenotypes on marginal lands including areas characterized by desertification. We suggest that the overexpression of *NDPK2* in woody plants can be an efficient strategy for producing biomass in the forestry, textile, paper industries and biomass-dependent generation of biofuels in natural environments.

## Experimental procedures

### Construction of plant expression vector

The *AtNDPK2* gene construct 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).

### Plant transformation and regeneration

A hybrid poplar clone (*Populus alba* × *P. tremular var. glandulosa*, clone Hyun 3) was used for *Agrobacterium*-mediated transformation. The protocols for plant transformation were as described by Choi *et al.* (2005b). The transformed cells were selected on an MS medium containing 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L benzylaminopurine (BAP), 0.01 mg/L 1-naphthylacetic acid (NAA) (Murashige and Skoog, 1962), 500 mg/L cefotaxime and 50 mg/L kanamycin. Shoots were regenerated from the calli by transferring to Woody Plant Medium (WPM, Lloyd and McCown, 1981) containing 1.0 mg/L zeatin, 0.1 mg/L benzyladenine (BA) and 0.01 mg/L NAA. Throughout the experiments, the cultures were maintained in a culture room at 25 ± 2 °C and provided with cool white fluorescence light (30 μmole $m^{-2}$ $s^{-1}$, 16 -h photoperiod). Regenerated shoots were transferred to MS medium containing 0.2 mg/L IBA for rooting. The rooted plantlets were then acclimated in pots in the greenhouse for 3 months before transferring them to the field.

### Southern blot analysis

For Southern hybridization, genomic DNA of poplar plants was extracted from leaves according to Kim and Hamada (2005), digested with EcoRI (Roche, Manheim, Germany), electrophoresed on 0.8% agarose gel and blotted onto Zeta-probe GT membrane

(Bio-Rad, CA, USA). The blots were hybridized to a 32P-labelled probe from the full-length *AtNDPK2* cDNA. Hybridization was carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA at 65 °C.

## Gene expression analysis

Total RNA was isolated from leaves of poplar using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated extensively with RNase-free DNase I to remove any contaminating genomic DNA. For quantitative expression analysis of *AtNDPK2*, a*ctin* and various *IAA* genes in poplar plants, total RNA (2 μg) was used for the generation of first-strand cDNA using the 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. Transcript levels were calculated relative to the controls and were expressed. Data represent means and standard errors of three replicates. The expression levels of the *AtNDPK2*, *actin* and various *IAA* genes were analysed by quantitative real-time PCR using the gene-specific primers listed in the Table 1.

## Enzyme activity assays

For analysis of the NDPK, APX, POD and CAT activities, total soluble protein was extracted from the leaves of poplar plants using an extraction buffer, and protein concentrations were determined using the Bio-Rad protein assay (Bradford, 1976). The NDPK activity was measured using the coupled reaction method with lactate dehydrogenase and pyruvate kinase (Yano *et al.*, 1995; Tang *et al.*, 2008). 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. The POD activity was assayed according to the method described by Kwak *et al.* (1995) using pyrogallol as a substrate. One unit of POD activity was defined as the amount of enzyme required to form 1 mg of purpurogallin from pyrogallol in 20 s, as measured by absorbance at 420 nm. The APX activity was assayed according to the method by Nakano and Asada (1981) using ascorbic acid as a substrate. The oxidation of ascorbate was initiated by $H_2O_2$, and the decrease in absorbance at 290 nm was monitored for 1 min 30 s. One unit of APX was defined as the amount of enzyme oxidizing 1 mol of ascorbate per minute. The CAT activity was assayed according to the method described by Aebi (1984). The activity was determined by the decrease in absorbance at 240 nm for 1 min because of $H_2O_2$ consumption.

## Methyl viologen treatment and ion leakage analysis

Methyl viologen (MV) damage was analysed using leaf discs from poplar plants. Seven leaf discs (16 mm diameter) collected from the third leaves of plants were floated on a solution containing 0.4% (w/v) sorbitol and 1 μM MV, placed in the dark for 12 h to allow diffusion of the MV into the leaf discs and then subjected to continuous light (150 μmol $m^{-2}$ $s^{-1}$) at 25 °C. Ion leakage was analysed according to the method by Bowler *et al.* (1991) with slight modifications. The loss of cytoplasmic solutes following the MV treatment, based on the electrical conductance of the solution, was measured with an ion conductivity meter (model 455C, Istek Co, Seoul, Korea) and compared with the total conductivity of the solution following tissue destruction. The extent of cellular damage was quantified by ion leakage, which is a measure of membrane disruption.

## Quantitative analysis of $H_2O_2$

The $H_2O_2$ content was assessed with xylenol orange, in which $H_2O_2$ is reduced by ferrous ions in an acidic solution that forms a ferric product–xylenol orange complex, which is detected by absorption at 560 nm (Bindschedler *et al.*, 2001). $H_2O_2$ measurements are expressed as relative values.

## Measurement of plant growth

Plant growth was determined by measuring stem length from the top of the shoot apex to the base of the stem, the diameter of the basal part of the stem and branch number.

## Analysis of photosynthetic activity and chlorophyll content

Photosynthetic activity from the leaves was estimated by chlorophyll fluorescence determination of photochemical yield (Fv/Fm), which represents the maximal yield of the photochemical reaction

**Table 1** Primer sequences used in the paper for expression analysis of *AtNDPK2* and various *IAA* genes

| cDNA | Forward primer | Reverse primer |
|---|---|---|
| *AtNDPK2* | TGTTGTTGCTTCAGCCAGGAAG | AGAGCCGAATCCCACTTGCATA |
| *IAA1* | ATCATGAAAGGGTCTGAGGCCA | TTCCAGGCTTCAAAGCTCGATG |
| *IAA2* | CCAGCTGTTCGCATGAATGTTG | CCCAAATGCAGGTCTTTGGAGA |
| *IAA4* | AAGGAGGGGGATTGGTTGATTG | TCAAGGAGAAAAGGAGACCGCA |
| *IAA5* | ATCATGAGGATGTCCGAGGCAA | AATCCAACACAAAGCCGCTGA |
| *IAA6* | GAAGACAAGGATGGCGATTGGA | AAAGAGGGAATCCAGCAGGGAA |
| *IAA7* | ATGCAAGCGGCTGCGAATAA | ATGGCTCTCGGTGCTGATGAAA |
| *Actin* | GCCATCTCTCATCGGAATGGAA | AGGGCAGTGATTTCCTTGCTCA |

on photosystem II (PSII), using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England) after 30 min of dark adaptation. Chlorophyll content after salt stress was measured by a portable chlorophyll meter (SPAD-502, Konica Minolta, Japan) from intact fully expanded fifth leaves counted from the top of individual plants.

### Statistical analysis

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

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## References

Aebi, H. (1984) Catalase *in vitro*. *Meth. Enzymol.* **105**, 121–126.

Ahmad, R., Kim, M.D., Back, K.H., Kim, H.S., Lee, H.S., Kwon, S.Y., Murata, N., Chung, W.I. and Kwak, S.S. (2008) Stress-induced expression of choline oxidase in potato plant chloroplasts confers enhanced tolerance to oxidative, salt, and drought stresses. *Plant Cell Rep.* **27**, 687–698.

Ahmad, R., Kim, Y.H., Kim, M.D., Kwon, S.Y., Cho, K., Lee, H.S. and Kwak, S.S. (2010) Simultaneous expression of choline oxidase, superoxide dismutase and ascorbate peroxidase in potato plant chloroplasts provides synergistically enhanced protection against various abiotic stresses. *Physiol. Plant.* **138**, 520–533.

Aoyama, T. and Chua, N.H. (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J.* **11**, 605–612.

Arisi, A.C.M., Cornic, G., Jouanin, L. and Foyer, C.H. (1998) Over-expression of iron superoxide dismutase in transformed poplar modifies the regulation of photosynthesis at low $CO_2$ partial pressures following exposure to the prooxidant herbicide methyl viologen. *Plant Physiol.* **117**, 565–574.

Babbs, C.F., Pham, J.A. and Coolbaugh, R.C. (1989) Lethal hydroxyl radical production in paraquat-treated plants. *Plant Physiol.* **90**, 1267–127.

Bhalerao, R., Nilsson, O. and Sandberg, G. (2003) Out of the woods: forest biotechnology enters the genomic era. *Curr. Opin. Biotech.* **14**, 1–8.

Bindschedler, L.V., Minibayeva, F., Gardner, S.L., Gerrish, C., Davies, D.R. and Bolwell, G.P. (2001) Early signaling events in the apoplastic oxidative burst in suspension cultured French bean cells involved cAMP and Ca2 $^{+}$ . *New Phytol.* **151**, 185–194.

Boese, S.R., Wolee, D.W. and Melkonian, J.J. (1997) Elevated $CO_2$ mitigates chilling-induced water stress and photosynthetic reduction during chilling. *Plant Cell Environ.* **20**, 625–632.

Bowler, C., Slooten, L., Vandenbranden, S., De-Rycke, R., Botterman, J., Sybesma, C., Van- Montagu, M. and Inze, D. (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO J.* **10**, 1723–1732.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Bradshaw, H.D., Ceulemans, J.R., Davis, J. and Stettler, R.F. (2000) Emerging model systems: poplar (*Populus*) as a model forest tree. *J. Plant Growth Regul.* **19**, 306–313.

Cheong, Y.H., Chang, H.S., Gupta, R., Wang, X., Zhu, T. and Luan, S. (2002) Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. *Plant Physiol.* **129**, 661–677.

Choi, G., Yi, H., Lee, J., Kwon, Y.K., Soh, M.S., Shin, B., Luka, Z., Hahn, T.R. and Song, P.S. (1999) Phytochrome signalling is mediated through nucleoside diphosphate kinase 2. *Nature*, **401**, 610–613.

Choi, G., Kim, J.I., Hong, S.W., Shin, B., Choi, G., Blakeslee, J.J., Murphy, A.S., Seo, Y.W., Kim, K., Koh, E.J., Song, P.S. and Lee, H. (2005a) A possible role for NDPK2 in the regulation of auxin-mediated responses for plant growth and development. *Plant Cell Physiol.* **46**, 1246–1254.

Choi, Y.I., Noh, E.W., Lee, H.S., Han, M.S., Lee, J.S. and Choi, K.S. (2005b) An efficient and novel plant-selectable marker based on organomer-curial resistance. *J. Plant Biol.* **48**, 351–355.

Constabel, C.P., Yip, L., Patton, J.J. and Christopher, M.E. (2000) Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiol.* **124**, 285–295.

Cosio, C. and Dunand, C. (2009) Specific functions of individual class III peroxidase genes. *J. Exp. Bot.* **60**, 391–408.

Escobar Galvis, M.L., Marttila, S., Hakansson, G., Forsberg, J. and Knorpp, C. (2001) Heat stress response in pea involves interaction of mitochondrial nucleoside diphosphate kinase with a novel 86-kilodalton protein. *Plant Physiol.* **126**, 69–77.

Fillatti, J.J., Sellmer, J., McCown, B., Haissig, B. and Comai, L. (1987) *Agrobacterium* mediated transformation and regeneration of *Populus*. *Mol. Gen. Genet.* **206**, 192–199.

Flowers, T.J. (2004) Improving crop salt tolerance. *J. Exp. Bot.* **55**, 307–319.

Foyer, C.H., Souriau, N., Perret, S., Lelandais, M., Kunert, K.J., Pruvost, C. and Jouanin, L. (1995) Over-expression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. *Plant Physiol.* **109**, 1047–1057.

Friml, J. (2003) Auxin transport—shaping the plant. *Curr. Opin. Plant Biol.* **6**, 7–12.

Gallardo, F., Fu, J., Jing, Z.P., Kirby, E.G. and Canovas, F.M. (2003) Genetic modification of amino acid metabolism in woody plants. *Plant Physiol. Bioch.* **41**, 587–594.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature Biotechnol.* **17**, 287–291.

Kasuga, M., Miura, S., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2004) A combination of the Arabidopsis DREB1A gene and stress-inducible rd29A promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. *Plant Cell Physiol.* **4**, 346–350.

Kawaoka, A., Matsunaga, E., Endo, S., Kondo, S., Yoshida, K., Shinmyo, A. and Ebinuma, H. (2003) Ectopic expression of a horseradish peroxidase enhances growth rate and increases oxidative stress resistance in hybrid aspen. *Plant Physiol.* **132**, 1177–1185.

Kim, S.H. and Hamada, T. (2005) Rapid and reliable method of extracting DNA and RNA from sweetpotato, *Ipomoea batatas* (L). *Lam. Biotechnol. Lett.* **27**, 1841–1845.

Kim, K.Y., Huh, G.H., Lee, H.S., Kwon, S.Y., Hur, Y. and Kwak, S.S. (1999) Molecular characterization of two anionic peroxidase cDNAs isolated from suspension cultures of sweetpotato. *Mol. Gen. Genet.* **261**, 941–947.

Kim, J.I., Kozhukh, G.V. and Song, P.S. (2002) Phytochrome-mediated signal transduction pathways in plants. *Biochem. Biophys. Res. Commun.* **298**, 457–463.

Kim, K.Y., Kwon, S.Y., Lee, H.S., Hur, Y., Bang, J.W. and Kwak, S.S. (2003) A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. *Plant Mol. Biol.* **51**, 831–838.

Kim, Y.H., Lim, S., Han, S.H., Lee, J.C., Song, W.K., Bang, J.W., Kwon, S.Y., Lee, H.S. and Kwak, S.S. (2007) Differential expression of 10 sweetpotato peroxidases in response to sulfur dioxide, ozone, and ultraviolet radiation. *Plant Physiol. Biochem.* **45**, 908–914.

Kim, Y.H., Lim, S., Yang, K.S., Kim, C.Y., Kwon, S.Y., Lee, H.S., Wang, X., Zhou, Z., Ma, D., Yun, D.J. and Kwak, S.S. (2009) Expression of Arabidopsis *NDPK2* increases antioxidant enzyme activities and enhances tolerance to multiple environmental stresses in transgenic sweetpotato plants. *Mol. Breeding* **24**, 233–244.

Kwak, S.S., Kim, S.K., Lee, M.S., Jung, K.H., Park, I.H. and Liu, J.R. (1995) Acidic peroxidase from suspension cultures of sweet potato. *Phytochemistry*, **39**, 981–984.

Lee, S.H., Ahsan, N., Lee, K.W., Kim, D.H., Lee, D.G., Kwak, S.S., Kwon, S.Y., Kim, T.H. and Lee, B.H. (2007) Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. *J. Plant Physiol.* **164**, 1626–1638.

Leyser, H.M.O. (2001) Auxin signaling: the beginning, the middle and the end. *Curr.Opin. Plant Biol.* **4**, 382–386.

Lian, H.L., Yu, X., Ye, Q., Ding, X.S., Kitagawa, Y., Kwak, S.S., Su, W.A. and Tang, Z.C. (2004) The role of aquaporin RWC3 in drought avoidance in rice. *Plant Cell Physiol.* **45**, 481–489.

Lim, S., Kim, Y.H., Kim, S.H., Kwon, S.Y., Lee, H.S., Kim, J.G., Cho, C.Y., Paek, K.Y. and Kwak, S.S. (2007) Enhanced tolerance of transgenic sweetpotato plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against methyl viologen-mediated oxidative stress. *Mol. Breeding* **19**, 227–239.

Ljung, K., Bhalerao, R.P. and Sandberg, G. (2001) Sites and homeostatic control of auxin biosynthesis in Arabidopsis during vegetative growth. *Plant J.* **28**, 465–474.

Lloyd, G. and McCown, B.H. (1981) Commercially feasible micro propagation of mountain laurel, Kalmia latifolia, by use of shoot tip culture. *Proc. Int. Plant Prop. Soc.* **30**, 421–427.

Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* **7**, 405–410.

Moon, H., Lee, B., Choi, G., Shin, D., Prasad, D.T., Lee, O., Kwak, S.S., Kim, D.H., Nam, J., Bahk, J., Hong, J.C., Lee, S.Y., Cho, M.J., Lim, C.O. and Yun, D.J. (2003) NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. *Proc. Natl Acad. Sci. USA* **100**, 358–363.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Nakano, Y. and Asada, K. (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.* **22**, 867–880.

Nicolescu, C., Sandre, C., Jouanin, L. and Chriqui, D. (1996) Genetic engineering of phenolic metabolism in poplar in relation with resistance against pathogens. *Acta Bot. Gallica* **43**, 539–546.

Noctor, G. and Foyer, C.H. (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 249–279.

Passardi, F., Cosio, C., Penel, C. and Dunand, C. (2005) Peroxidases have more functions than a Swiss army knife. *Plant Cell Rep.* **24**, 255–265.

Passioura, J. (2007) The drought environment: Physical, biological and agricultural perspectives. *J. Exp. Bot.* **58**, 113–117.

Reed, J.W. (2001) Roles and activities of Aux/IAA proteins in Arabidopsis. *Trends Plant Sci.* **6**, 420–425.

Scarascia-mugnozza, G., Angelis, P.D.E., Matteucci, G. and Valentini, R. (1996) Long-term exposure to elevated [CO2] in a natural Quercus ilex L. community: net photosynthesis and photochemical efficiency of PSII at different levels of water stress. *Plant Cell Environ* **19**, 643–654.

Shen, Y., Kim, J.I. and Song, P.S. (2005) NDPK2 as a signal transducer in the phytochrome-mediated light signaling. *J. Biol. Chem.* **280**, 5740–5749.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takedo, T., Yabuta, Y. and Yoshimura, K. (2002) Regulation and function of ascorbate peroxidase isoenzymes. *J. Exp. Bot.* **53**, 1305–1319.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Molecular response to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Curr. Opin. Plant Biol.* **3**, 217–223.

Shukla, R.K., Raha, S., Tripathi, V. and Chattopadhyay, D. (2006) Expression of CAP2, an APETALA2-family transcription factor from chickpea, enhances growth and tolerance to dehydration and salt stress in transgenic tobacco. *Plant Physiol.* **142**, 113–123.

Sreenivasulu, N., Sopory, S.K. and Kavi Kishor, P.B. (2007) Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. *Gene*, **388**, 1–13.

Sterky, F., Bhalerao, R.R., Unneberg, P., Segerman, B., Nilsson, P., Brunner, A.M., Charbonnel-Campaa, L., Lindvall, J.J., Tandre, K., Strauss, S.H., Sundberg, B., Gustafsson, P., Uhlen, M., Bhalerao, R.P., Nilsson, O., Sandberg, G., Karlsson, J., Lundeberg, J. and Jansson, S. (2004) A *Populus* EST Resource for plant functional genomics. *Proc. Natl Acad. Sci. USA* **101**, 13951–13956.

Tang, L., Kwon, S.Y., Kim, S.H., Kim, J.S., Choi, J.S., Cho, K.Y., Sung, C.K., Kwak, S.S. and Lee, H.S. (2006) Enhanced tolerance of transgenic potato plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. *Plant Cell Rep.* **25**, 1380–1386.

Tang, L., Kim, M.D., Yang, K.S., Kwon, S.Y., Kim, S.H., Kim, J.S., Yun, D.J., Kwak, S.S. and Lee, H.S. (2008) Enhanced tolerance of transgenic potato plants overexpressing nucleoside diphosphate kinase 2 against multiple environmental stresses. *Transgenic Res.* **17**, 705–715.

Taylor, G. (2002) *Populus*: Arabidopsis for forestry. Do we need a model tree?. *Annal. Bot.* **90**, 681–689.

Toenniessen, G.H., O'Tooley, J.C. and DeVriesz, J. (2003) Advances in plant biotechnology and its adoption in developing countries. *Curr. Opin. Plant Biol.* **6**, 191–198.

Verslues, P.E., Batelli, G., Grillo, S., Agius, F., Kim, Y.S., Zhu, J., Agarwal, M., Katiyar-Agarwal, S. and Zhu, J.K. (2007) Interaction of SOS2 with nucleoside diphosphate kinase 2 and catalases reveals a point of connection between salt stress and $H_2O_2$ signaling in *Arabidopsis thaliana*. *Mol. Cell. Biol.* **27**, 7771–7780.

Wang, F.Z., Wang, Q.B., Kwon, S.Y., Kwak, S.S. and Su, W.A. (2005) Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. *J. Plant Physiol.* **162**, 465–472.

Willekens, H., Chamnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Van Montagu, M., Inze, D. and Van Camp, W. (1997) Catalase is a sink for $H_2O_2$ and is indispensable for stress defence in C3 plants. *EMBO J.* **16**, 4806–4816.

Wilmoth, J.C., Wang, S., Tiwari, S.B., Joshi, A.D., Hagen, G., Guilfoyle, T.J. and Alonso, J.M. (2005) NPH4/ARF7 and ARF19 promote leaf expansion and auxin-induced lateral root formation. *Plant J.* **43**, 118–130.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low temperature, or high-salt stress. *Plant Cell*, **6**, 251–264.

Yang, K.A., Moon, H.J., Kim, G.T., Lim, C.J., Hong, J.C., Lim, C.O. and Yun, D.J. (2003) NDP kinase 2 regulates expression of antioxidant genes in Arabidopsis. *Proc. Jpn. Acad. Ser. B* **79**, 86–91.

Yano, A., Umeda, M. and Uchimiya, H. (1995) Expression of functional proteins of cDNA encoding rice nucleoside diphosphate kinase (NDK) in Escherichia coli and organrelated alteration of NDK activities during rice seed germination (*Oryza sativa* L.). *Plant Mol. Biol.* **27**, 1053–1058.

Zelasco, S., Reggi, S., Calligari, P., Balestrazzi, A., Bongiorni, C., Quattrini, E., Delia, G., Bisoffi, S., Fogher, C. and Confalonieri, M. (2006) Expression of the Vitreoscilla hemoglobin (VHb)-encoding gene in transgenic white poplar: plant growth and biomass production, biochemical characterization and cell survival under submergence, oxidative and nitrosative stress conditions. *Mol. Breeding* **17**, 201–216.

Zimmermann, S., Baumann, A., Jaekel, K., Marbach, I., Engelberg, D. and Frohnmeyer, H. (1999) UV-responsive genes of Arabidopsis revealed by similarity to the Gcn4-mediated UV response in yeast. *J. Biol. Chem.* **274**, 17017–17024.