

Lei Yang | Renjie Tang | Jinqi Zhu | Hua Liu | Bernd Mueller-Roeber |
Huijun Xia | Hongxia Zhang

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Enhancement of stress tolerance in transgenic tobacco plants constitutively expressing *AtIpk2 β* , an inositol polyphosphate 6-/3-kinase from *Arabidopsis thaliana*

Lei Yang · Renjie Tang · Jinqi Zhu · Hua Liu · Bernd Mueller-Roeber · Huijun Xia · Hongxia Zhang

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Abstract Inositol phosphates (IPs) and their turnover products have been implicated to play important roles in stress signaling in eukaryotic cells. In higher plants genes encoding inositol polyphosphate kinases have been identified previously, but their physiological functions have not been fully resolved. Here we expressed *Arabidopsis* inositol polyphosphate 6-/3-kinase (*AtIpk2 β*) in two heterologous systems, i.e. the yeast *Saccharomyces cerevisiae* and in tobacco (*Nicotiana tabacum*), and tested the effect on abiotic stress tolerance. Expression of *AtIpk2 β* rescued the salt-, osmotic- and temperature-sensitive growth defects of a yeast mutant strain (*arg82 Δ*) that lacks inositol polyphosphate multikinase activity encoded by the *ARG82/IPK2* gene. Transgenic tobacco plants constitutively expressing *AtIpk2 β* under the control of the Cauliflower Mosaic Virus 35S promoter were generated and found to exhibit improved tolerance to diverse abiotic stresses when compared to wild type plants. Expression patterns of various stress responsive

genes were enhanced, and the activities of anti-oxidative enzymes were elevated in transgenic plants, suggesting a possible involvement of *AtIpk2 β* in plant stress responses.

Keywords *Arabidopsis thaliana* · *AtIpk2 β* · Inositol phosphate · IP₃ · Stress tolerance · Transgenic tobacco

Introduction

During their life plants are often challenged with various types of abiotic stresses. Proper physiological and biochemical responses to such stresses are controlled by an array of stress-dependent signal transduction pathways (Xiong et al. 2002). Several signaling molecules and second messengers including calcium ions (Ca²⁺; Knight et al. 1996; Knight et al. 1997) and inositol 1,4,5-trisphosphate (IP₃; Takahashi et al. 2001) have been demonstrated to be important elements of signaling pathways that link the perception of stress to various types of downstream responses. As one of the central molecules in cell signaling, IP₃ is subject to phosphorylation and dephosphorylation by specific inositol phosphate kinases and phosphatases, respectively, which helps to control intracellular IP₃ concentration (Pattni and Banting 2004).

In higher plants, over-expression of an inositol polyphosphate 5-phosphatase (Ins5Pase), possibly leading to intracellular degradation of IP₃, modulated stress and ABA signal transduction (Perera et al. 2002; Burnette et al. 2003). Likewise, mutation of an inositol polyphosphate 5-phosphatase gene (*CVP2*) results in increased sensitivity to ABA and vein patterning defects in cotyledons (Carland and Nelson 2004). IP₃ can also be hydrolyzed by FRY1-like inositol polyphosphate 1-phosphatases (Ins1Pases; Xiong et al. 2002). The *fry1* mutant of *Arabidopsis* accumulated

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L. Yang · R. Tang · J. Zhu · H. Liu · H. Zhang (✉)
National Key Laboratory of Plant Molecular Genetics,
Institute of Plant Physiology and Ecology, Shanghai
Institutes for Biological Sciences, Chinese Academy
of Sciences, 300 Fenglin Road, Shanghai 200032, China
e-mail: hxzhang@sippe.ac.cn

H. Xia
Key Laboratory of MOE for Plant Developmental Biology,
College of Life Sciences, Wuhan University, Wuhan 430072,
China

B. Mueller-Roeber
University of Potsdam, Karl-Liebknecht-Str. 24-25, Haus 20,
14476 Potsdam-Golm, Germany

around ten-fold more IP₃ than the corresponding wild type, exhibited hypersensitivity towards ABA, and was compromised in tolerance to freezing, drought and salt stresses (Xiong et al. 2001). These data demonstrate the importance of inositol polyphosphate phosphatases in IP₃ metabolism and IP₃-mediated stress signal transduction. However, evidence for a role of inositol polyphosphate kinases in relation to stress tolerance is currently lacking in plants.

Inositol 1,4,5-trisphosphate 3-kinase (IP3K) and more generally inositol polyphosphate kinases play an essential role in cellular signal transduction and maintenance of Ca²⁺ homeostasis by phosphorylating inositol 1,4,5-trisphosphate (IP₃) to inositol 1,3,4,5-tetrakisphosphate (IP₄; Communi et al. 1995). Both IP₃ and IP₄ are second messengers responsible for Ca²⁺ mobilization from intracellular stores (Berridge 1997). IP₄ itself is also able to regulate cytosolic Ca²⁺ concentration by promoting Ca²⁺ sequestration (Hill et al. 1988). In *Arabidopsis thaliana*, IP3K is encoded by two distinct genes, *AtIpk2α* and *AtIpk2β*, which are ubiquitously expressed in mature tissues (Stevenson-Paulik et al. 2002; Xia et al. 2003). Both of the *Arabidopsis* IP3Ks use IP₃ as a substrate and display dual-specificity inositol polyphosphate 6-/3-kinase activities that successively phosphorylate IP₃ to generate inositol 1,3,4,5,6-pentakisphosphate (IP₅) predominantly via an inositol 1,4,5,6-tetrakisphosphate (IP₄) intermediate (Stevenson-Paulik et al. 2002; Xia et al. 2003). Recently it was reported that *AtIpk2α* plays a role in pollen germination and root growth (Xu et al. 2005), while *AtIpk2β* functions in axillary shoot branching through the auxin signaling pathway (Zhang et al. 2007) and phytate synthesis (Stevenson-Paulik et al. 2005).

Although *AtIpk2β* has well been characterized at the biochemical level and has been shown to participate in plant development, it remained unclear whether it is also involved in stress signaling. In this study we demonstrate that *AtIpk2β* restores the salt-, osmotic- and temperature-sensitive growth defects of a yeast mutant strain (*arg82Δ*) deficient for the *ARG82/IPK2* gene that encodes inositol polyphosphate multikinase. We also show that constitutive expression of *AtIpk2β* in transgenic tobacco enhances its tolerance towards various abiotic stresses. Thus, inositol polyphosphate kinase encoded by *AtIpk2β* very probably plays an important role in signaling pathways controlling the cellular response to abiotic stresses.

Materials and methods

Expression of *AtIpk2β* in yeast mutant *arg82Δ*

The *S. cerevisiae arg82/Ipk2* deletion strain (derived from BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0;

YDR173c::kanMX4) was obtained from EUROSCARF (<http://www.web.uni-frankfurt.de/fb15/mikro/euroscarf/>). In this strain, the *ARG82/IPK2* allele is disrupted through insertion of the kanamycin resistance gene, *kanMX4*. The *AtIpk2β* open reading frame was cloned into yeast expression plasmid pYX212. The resulting plasmid, pYX212-*AtIpk2β*, was used to transform the yeast mutant. The wild type strain BY4741 was used as control. Ten-fold serial dilutions (starting at OD₆₀₀ = 1.0) of each strain were plated on YPD medium (1% yeast extract, 2% peptone, 2% glucose) supplemented with either NaCl (0.4 or 0.8 M) or mannitol (0.4 or 0.8 M), and incubated at 30°C for 48 h. For growth at 37°C, yeast cells were grown on media containing no extra NaCl or mannitol. Each experiment was repeated at least three times. For growth in liquid YPD medium, cells were cultured at 30°C for three days to saturation (OD₆₀₀ = 12.0). Five μl of the cell culture were used to inoculate 3 ml of synthetic defined minimal medium supplemented with different concentrations of NaCl or mannitol. Cell density was measured at A₆₀₀.

Quantitative real-time PCR analysis of *AtIpk2β* expression in *Arabidopsis*

Ten-day-old *Arabidopsis* seedlings grown on MS medium were used for different treatments as described previously (Shi and Zhu 2002). RNA was extracted with TRIZOL Reagent (Invitrogen) and reverse transcription of RNA was carried out using M-MLV RTase Synthesis Kit (TaKaRa). Quantitative real-time PCR was performed using the Rotor-Gene 3000 Series real time DNA amplification system under the following conditions: 95°C for 10 s; 40 cycles of 95°C for 5 s, 60°C for 20 s. *AtIpk2β*-specific primers were used (Table 1). Melting curve analysis was included to verify specificity of the DNA amplification. Expression of the reference gene *Actin*, which served as quantifying control, was monitored using gene-specific forward and reverse primers (Table 1). Data analysis was performed with Rotor-Gene software 6.0.

Vector construction and plant transformation

To create a Cauliflower Mosaic Virus (CaMV) 35S plant expression construct, the 903 bp long *AtIpk2β* cDNA containing the complete open reading frame was inserted via *Xba*I–*Sal*I sites into the binary vector pBinAR-HPT. The resulting construct was introduced into *Agrobacterium tumefaciens* (strain LBA4404). Tobacco (*Nicotiana tabacum* cv. SR-1) leaf disks were infected with the transformed *Agrobacterium*. After two days of co-cultivation, the

Table 1 Gene-specific primers used in this study

Primer name	Primer sequence
For yeast expression vector	
pYX-F	5'-CTCCCATGGTAATGCTCAAAGTCCCTG-3'
pYX-R	5'-GATTGTCGACCTAGCGCCCGTTCTC-3'
For real-time RT-PCR	
AtIPK2 β -F	5'-CCACGGTTTCGTTGGGGTTC-3'
AtIPK2 β -R	5'-GTTACAACCGCCATAAACCTCTG-3'
AtActin2-F	5'-CATCCTCCGTCTTGACCTTGC-3'
AtActin2-R	5'-CAAACGAGGGCTGGAACAAG-3'
For RT-PCR	
NtActin-F	5'-TTACGCCCTTCTCATGCAATT-3'
NtActin-R	5'-GGCGCCACCACCTTGATCTTC-3'
AY562132-F	5'-GTAGCATTGTTGGTGGTGGTGTG-3'
AY562132-R	5'-ACCGTGGAGCAGTCAATGGAAG-3'
AY554169-F	5'-ACAGTCGTGAGCATTCCCAAC-3'
AY554169-R	5'-CCAAACCTTCTGTGCTACCTC-3'
AY554170-F	5'-CCGGAGTGAAGGGGATGG-3'
AY554170-R	5'-CAAGCAATGTGAATGGTATGTGAG-3'
NtERD10B-F	5'-CAATTTAGTGCAGGCCAGGC-3'
NtERD10B-R	5'-GGTCCATGGTGGCCAGGAAG-3'
NtERD10C-F	5'-GGGTAGCGCAAACGTGGAG-3'
NtERD10C-R	5'-CTTTCCCTCAGCCTCGTGC-3'

explants were transferred to regeneration medium containing 1-mg/l BAP, 0.1-mg/l NAA, 50-mg/l hygromycin and 200-mg/l ampicillin. Regenerated shoots were separated from the calli and transferred onto rooting medium containing MS salts, 0.1-mg/l NAA and 50-mg/l hygromycin. Rooted shoots were transplanted into soil. Seeds were harvested and homozygous plants were screened on a 12-h light/12-h dark photoperiod.

Salt stress tolerance tests

Wild type and homozygous T₂ transgenic seeds were surface sterilized with a solution of 10% commercial bleach (0.525% sodium hypochlorite) for 10 min, and washed three times with sterile water. For germination assays, seeds were plated on MS medium supplemented with different concentrations of NaCl (0, 100 and 200 mM). For root growth measurements, 10-day-old seedlings cultured on solid MS medium were transferred to MS medium supplemented with different concentrations of NaCl. Plates were oriented vertically with seedlings kept upside down. Three replicates were performed for each experiment. Root length was recorded after seven days of treatment. To monitor stress effects on the growth of wild type and *AtIpk2 β* expressing plants, 15-day-old seedlings were transferred to MS medium supplemented with 0, 100, 200

or 300 mM NaCl, and cultured at 25 \pm 2°C with cool white fluorescent light (\sim 125 μ mol m⁻² s⁻¹) under short day condition (12-h light/12-h dark). For salt stress treatments and ion measurements, wild type and transgenic seedlings were transferred to pots containing soil. Plants were watered bi-weekly with 1/8 concentrated MS salt solution supplemented with or without 200-mM NaCl and grown at 25 \pm 2°C with cool white fluorescent light (\sim 125 μ mol m⁻² s⁻¹), 70% humidity, under long-day conditions (16-h light/8-h dark) for 6 weeks. Mature plants treated in the same way were also used to determine cellular Na⁺ concentrations. The third leaf from the top of each plant was collected and dried at 90°C for 24 h, and the dry weight of each sample was measured. The dried leaf material was immersed in 0.1-M HAc, followed by water bath incubation at 90°C for 2.5 h, and analyzed for Na⁺ content using atomic absorption spectrophotometry. For hydroponic salt tolerance experiments, wild type plants and two independent transgenic lines (T₂ generation) were grown hydroponically in Hoagland solution. Plants were grown at 25 \pm 2°C with cool white fluorescent light (\sim 125 μ mol m⁻² s⁻¹), 70% humidity, under long-day conditions (16-h light/8-h dark). The nutrient solution was replaced weekly.

Oxidative stress experiments

Malondialdehyde (MDA) concentration was determined as a biomarker for oxidative stress. To measure MDA concentration, leaf samples from plants grown at 200-mM NaCl were ground in 5 ml of 0.1% trichloroacetic acid (TCA) and mixed with 5 ml of 0.5% thiobarbituric acid. The samples were then boiled for 10 min, cooled down to room temperature and centrifuged at 1,200 \times g. The cleared supernatant was analyzed by monitoring the difference in absorbance at A₅₃₂ and A₆₀₀. For oxidative damage experiments, leaf disks of 1.5-cm diameter were excised from healthy and fully expanded leaves of two-month-old tobacco plants. The disks were floated in a 10-ml solution with different concentrations of H₂O₂ or water (control) for 72 h. Chlorophyll a and b concentrations were measured as described previously (Lichtenthaler 1987). The treatment was carried out with continuous white light at 25 \pm 2°C. The experiment was repeated at least three times.

Enzyme assays and protein determination

Enzyme assays were carried out spectrophotometrically using a nucleic acid and protein analyzer (Du 640, Beckman Coulter). One-month-old seedlings were treated with

H₂O or 1% H₂O₂ for 12 h, and ground in extraction buffer (1 mM ascorbate in 50-mM potassium phosphate buffer, pH 7.8) at a ratio of 0.1 g leaf material/1 ml ice-cold extraction buffer. The slurry was transferred to a centrifuge tube and centrifuged at 20,000 × g at 4°C for 20 min. The supernatant was used for enzyme activity assays and total protein determination as described previously (Badawi 2004; Bradford 1976). Ascorbate peroxidase (APX) activity was determined by measuring the rate of ascorbate oxidation at A₂₉₀ in a 100-μl-reaction mixture (1-mM EDTA in 50-mM potassium phosphate buffer, pH 7.8, 0.2-mM ascorbate, 20-mM H₂O₂ and 5-μl soluble protein extract). APX activity is expressed as micromoles of ascorbate oxidized per minute per milligram of protein. Catalase (CAT) activity was measured by monitoring the decrease in absorbance at A₂₄₀ caused by the disappearance of H₂O₂ in a 100-μl-reaction mixture (1-mM EDTA in 50-mM potassium phosphate buffer, pH 7.8, 20-mM H₂O₂ and 5-μl protein extract). The activity of superoxide dismutase (SOD) was measured at A₅₆₀, in a 3-ml reaction mixture (13.05-mM DL-methionine, 0.1-μM EDTA, 0.075-mM Nitro Blue tetrazolium chloride and 2-μM riboflavin in 50-mM phosphate buffered saline, pH 7.8, and 100-μl protein extract).

Whole plant drought tolerance test

Wild type and transgenic plant seeds were surface sterilized as described above. Seedlings germinated on MS medium were transplanted to soil. After two months, healthy plants of the same size and age were pooled into two groups. For the first group (12 individual plants, each grown in a 6.5-inch pot), plants were irrigated with 60 g/l PEG-6000 to simulate drought stress. After 21 days, the plants were photographed. Simultaneously, plants of the second group were subjected to drought stress by omitting watering. After three weeks, the plants were re-hydrated and observed for recovery. Photos were taken 21 days after initiation of the stress treatment. The relative humidity was maintained at ~60%. The experiments were repeated twice with three replicates in each experiment.

Freezing tolerance test

Three-week-old seedlings of wild type and transgenic plants grown on MS medium were cultured at 4°C for one day under long day condition (16-h light/8-h dark). After cold acclimation, the plants were left at -20°C for 1 or 2 h, transferred immediately to 4°C for another 12 h (overnight). Subsequently, plants were kept in the greenhouse at 25°C and observed further. Photographs were taken seven

days after initiation of the recovery growth in the greenhouse.

Determination of proline concentration

Four-week-old seedlings grown on MS medium were transferred onto filter paper in a Petri dish saturated with 100- or 200-mM NaCl, incubated at 25 ± 2°C for 24 h under continuous white light. After the treatment, proline content was determined as described previously (Bates and Waldren 1973).

Western blot analysis

Leaves from either *Arabidopsis* or tobacco plants were ground in liquid N₂ and suspended in 1.5 ml of 30 mM potassium phosphate buffer (pH 7.5) containing 40-mM NaCl and 3-mM dithiothreitol (Powell 1986). Homogenates were centrifuged for 5 min at 12,000 × g. Supernatants were transferred to new tubes, and protein concentrations were determined as described previously (Bradford 1976). A 10-μg sample of total protein was mixed with loading buffer, boiled for 3 min, and size-fractionated through electrophoresis in a 12.5% SDS polyacrylamide gel. Separated proteins were transferred to a PVDF membrane (Millipore Corporation) according to the manufacturer's instructions. A polyclonal antibody against AtIpk2β protein purified from recombinant *E. coli* was raised in rabbits. Antiserum was purified by IgG affinity purification. The anti-AtIpk2β antiserum was used at a 1:5,000 dilution in TBST. Peroxidase-labeled affinity purified secondary antibody isolated from a pool of serum from goats immunized with purified rabbit IgG was used at a 1:1,000 dilution in TBST (KPL Corporation).

Stress gene expression analysis using reverse transcription PCR

Three-week-old tobacco seedlings grown on MS agar plates were treated with 300-mM NaCl for 5 h. Total RNA was isolated with TRIZOL Reagent (Invitrogen) and used for reverse transcription (RT) to generate first-strand cDNA. RT was performed on 2-μg total RNA per reaction using ReverTra Ace (TOYOBO). A portion of the resulting cDNA was then subjected to PCR amplification using gene specific primers (Table 1). Expression level of the *Nicotiana tabacum ACTIN* gene, which served as reference, was monitored with NtActin forward and reverse primers (Table 1).

Statistical analysis

For statistical analyses, the Student's *t*-test was used to generate every *P*-value. The alpha level was 0.05. The tests were one-tailed. The data were normalized and all samples were normally distributed with homogeneity of variance.

Results

Yeast complementation with *AtIpk2β*

In *S. cerevisiae*, the *ARG82/IPK2* gene encodes an inositol polyphosphate kinase (Odom et al. 2000). The *S. cerevisiae arg82/Ipk2* (*arg82Δ*) mutant strain that lacks *Ipk2* activity has slowed growth at 30°C and is unable to grow at 37°C (Odom et al. 2000). We and others have reported previously that *AtIpk2β* rescues its growth defects at 37°C (Stevenson-Paulik et al. 2002; Xia et al. 2003). This result led us to postulate that *AtIpk2β* may also be able to rescue its growth defects when other abiotic stresses were imposed. We observed that under adverse growth conditions such as high salt concentration or upon osmotic stress treatment, growth of the *arg82Δ* mutant was severely impaired when compared to that of the wild type (Fig. 1a). To test whether *AtIpk2β* restores the growth defect of the mutant strain under these conditions, we transformed it with the *Arabidopsis AtIpk2β* cDNA. Expression of *AtIpk2β* almost completely restored growth of the *arg82Δ* mutant under both kinds of stresses (Fig. 1a). It also restored growth at 37°C (Fig. 1a), as reported earlier (Stevenson-Paulik et al. 2002; Xia et al. 2003). We also performed experiments using liquid media and observed again, that *AtIpk2β* restores growth under conditions of abiotic stress (Fig. 1b).

AtIpk2β is abiotic stress regulated in *Arabidopsis*

We were interested to know whether different kinds of stresses such as salinity, cold and drought, or treatment with ABA would affect *AtIpk2β* transcript levels in *Arabidopsis* plants. To this end we performed quantitative real-time PCR (qRT-PCR). In ten-day-old seedlings grown on MS medium, *AtIpk2β* transcript level increased slightly upon exposure to cold or drought stress, but decreased in response to salt or ABA treatment (Fig. S1A in Supplementary Material). These results showed that *AtIpk2β* is a stress responsive gene potentially involved in different abiotic stress- and ABA-triggered signaling pathways in plants. We further tested *AtIpk2β* protein level by Western blot analysis. Fourteen-day-old *Arabidopsis* seedlings grown on MS medium were subjected to different stress

treatments for 24 h. In seedlings treated with 500-mM mannitol, *AtIpk2β* protein level increased slightly, however no significant changes in protein level were detected in seedlings subjected to cold or NaCl stress (Fig. S1B in Supplementary Material).

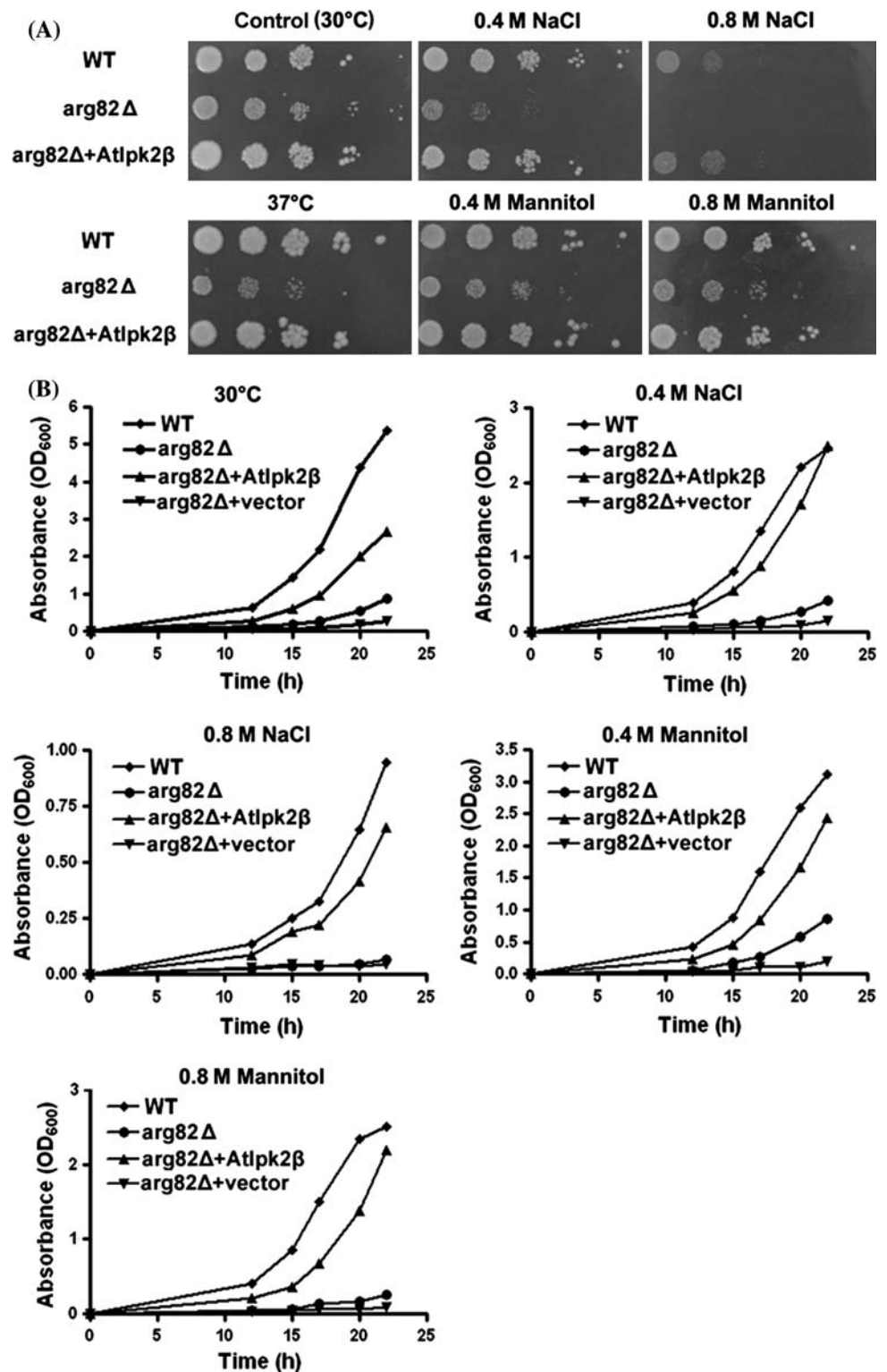
Constitutive expression of *AtIpk2β* in transgenic tobacco

To test whether constitutive expression of *AtIpk2β* affects abiotic stress tolerance in plants, we introduced its open reading frame (Fig. 2a) into the genome of tobacco (*Nicotiana tabacum* cv. SR-1) by *Agrobacterium tumefaciens*-mediated transformation. Expression of *AtIpk2β* in the transgenic plant was controlled by the Cauliflower Mosaic Virus (CaMV) 35S promoter. Forty-six independent transgenic plants (*T*₀ generation) were obtained; six lines were grown to produce seeds. Transgenic plants homozygous for the *35S:AtIpk2β* transgene were obtained and three lines (S13, S14 and S15) were chosen for further experiments. Constitutive expression of *AtIpk2β* did not affect overall plant morphology although the transgenic plants grew slightly faster than wild type plants under normal growth condition. Western blot analyses were performed to test for the presence of *AtIpk2β* protein. A 33-kDa band corresponding to *AtIpk2β* was identified in *Arabidopsis* plants; similarly, 33-kDa *AtIpk2β* protein was detected in the transgenic but not the wild type tobacco lines (Fig. 2b), indicating that it was successfully expressed in the genetically modified plants.

Expression of *AtIpk2β* increases salt tolerance of transgenic tobacco plants

We first studied the salt tolerance of *AtIpk2β* expressing plants during germination and early stages of seedling development. Seeds of wild type and the three homozygous transgenic lines S13, S14 and S15 were germinated on MS medium containing different concentrations of NaCl. Germination rates of wild type and transgenic seeds were similar when cultured on normal MS medium (Fig. 3a). However, germination of wild type seeds was significantly impaired on MS medium supplemented with 100- or 200-mM NaCl, whereas germination of transgenic seeds was less strongly affected. After eight days on high salt MS medium (100-mM NaCl), only ~28% of the wild type seeds, but more than 70% of the seeds obtained from *AtIpk2β* expressing plants germinated (Fig. 3b). When sown on MS medium supplemented with 200-mM NaCl, only ~7% of the wild type seeds, but ~30% of the transgenic seeds germinated after 18 days (Fig. 3c). At low

Fig. 1 Expression of *AtIpk2 β* in *S. cerevisiae arg82 Δ* . (a) *AtIpk2 β* rescues growth defects of the yeast *arg82 Δ* mutant under high salt or mannitol stress, and at 37°C. Ten-fold serial dilutions of the strain were plated and incubated at 30°C for 48 h on YPD medium supplemented with either 0.4- or 0.8-M NaCl, or with 0.4- or 0.8-M mannitol. Plates incubated at 37°C contained YPD medium without extra NaCl or mannitol. WT, wild type; *arg82 Δ* , *arg82 Δ* mutant; *arg82 Δ* + *AtIpk2 β* , *arg82 Δ* mutant harbouring plasmid pYX212-*AtIpk2 β* . (b) Liquid yeast cultures. Cells were grown for three days to saturation in YPD medium ($OD_{600} = 12.0$). Five μ l of cell culture were then used to inoculate 3 ml of defined synthetic minimal medium supplemented with different concentrations of NaCl or mannitol. Cell density was determined at various time points as absorbance at 600 nm. Cells were grown at 30°C. WT, wild type; *arg82 Δ* , *arg82 Δ* mutant; *arg82 Δ* + *AtIpk2 β* , *arg82 Δ* mutant harbouring pYX212-*AtIpk2 β* ; *arg82 Δ* + vector, *arg82 Δ* mutant harbouring pYX212



salt concentration (50-mM NaCl), wild type and transgenic seeds displayed similar germination rates (data now shown). At 150-mM NaCl, both wild type and transgenic seeds germinated. However, development of wild type

seedlings was impaired; plants produced smaller leaves and less root biomass than did transgenic seedlings (Fig. 3d). To further test the effect of salt on growth, 15-day-old wild type and transgenic seedlings were transferred to MS

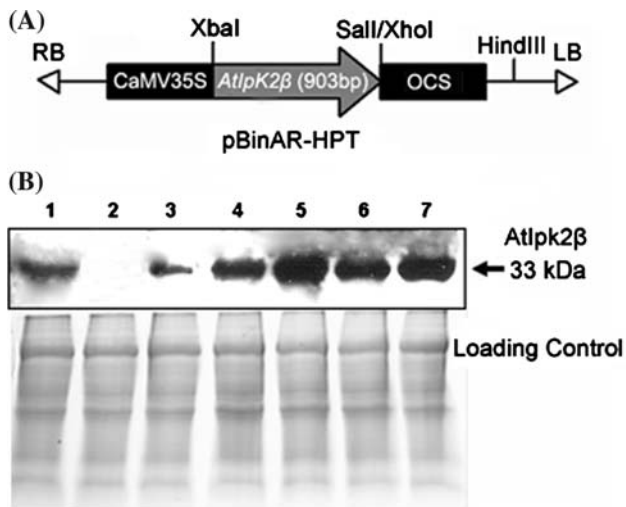


Fig. 2 Construct used for tobacco transformation and Western blot analysis of *AtIpk2β* in wild type and transgenic tobacco plants. (a) Schematic map of the binary construct used for tobacco transformation. Expression of *AtIpk2β* is driven by the Cauliflower Mosaic Virus 35S promoter. *Ocs*, *ocs* terminator; RB and LB, right and left border of T-DNA, respectively. (b) Western blot analysis. Lane 1, wild type *Arabidopsis*; lane 2, wild type tobacco; lanes 3–7, transgenic *AtIpk2β* expressing tobacco lines S11, S13, S14, S15, and S18. Coomassie Bright Blue stained gel is shown below the Western blot to demonstrate equal loading of protein

medium supplemented with NaCl at different concentrations. After two additional months, strong growth retardation was observed in wild type seedlings. Inhibition of growth was less evident in transgenic lines (Fig. 3e). A root-bending assay has previously been used to test the effect of elevated salt concentration on root growth (An et al. 2007; Zhu et al. 1998). At 50-mM NaCl on MS medium, root growth of both wild type and transgenic lines was accelerated, but it was inhibited at increasing NaCl concentrations. At 150-mM NaCl, inhibition of root growth was significantly more pronounced in wild type than in transgenic plants (Fig. 3f, g).

We also performed salt tolerance experiments with wild type and two transgenic lines grown in the greenhouse in soil. The plants were watered bi-weekly with 1/8 concentrated MS salt solution supplemented with or without 200-mM NaCl. After six weeks, distinct differences were observed between wild type and transgenic plants exposed to 200-mM NaCl (Fig. 4a). Although both sets of plants flowered under conditions of salt stress, wild type plants developed much shorter stems (Fig. 4b), produced fewer pods (Fig. 4c), and produced seeds that were not viable. We determined endogenous Na^+ levels in leaves and roots of wild type and transgenic plants grown in the absence or presence of 200-mM NaCl. Both wild type and transgenic plants exposed to salt contained elevated levels of Na^+ ions in leaves and roots (Fig. 4d), however, ion concentrations

were not significantly different between wild type and transgenic plants (the same was true for plants grown under normal conditions). We also grew tobacco plants in hydroponic culture. In the absence of salt stress, no overt morphological difference was observed between wild type and transgenic plants. However, growth of wild type tobacco was severely inhibited in the presence of 300-mM NaCl (Fig. 4e), leading to a depression of shoot height by about one third, and of plant fresh weight by about 50% in comparison to *AtIpk2β* expressing tobacco plants after a two-month stress treatment (Fig. 4f). All these results indicated that *AtIpk2β* alleviated the negative effects imposed by salt stress on plant growth.

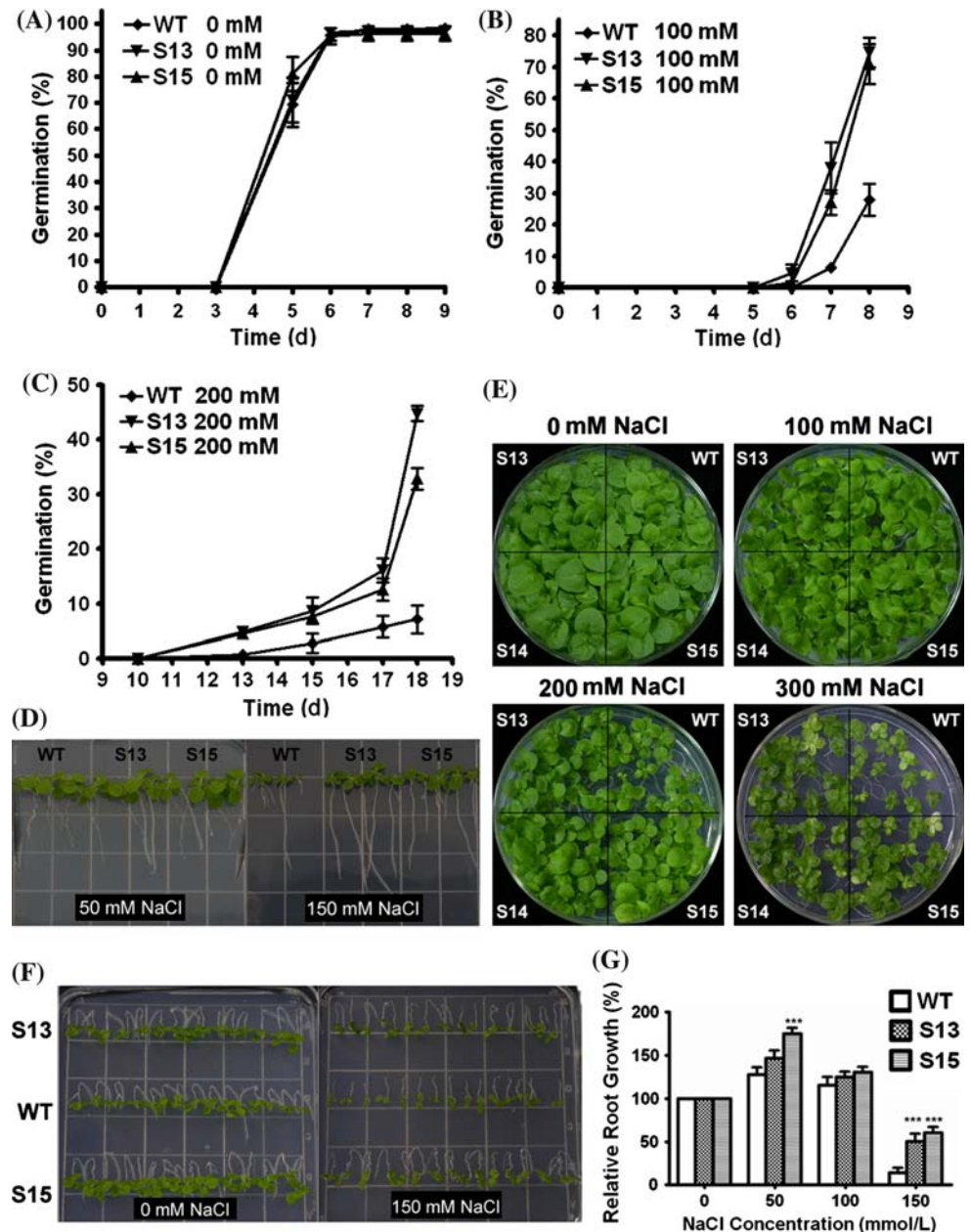
Transgenic plants showed increased tolerance to osmotic, drought, freezing temperature and oxidative stress

In order to determine whether expression of *AtIpk2β* affects osmotic stress tolerance in plants, we watered wild type and transgenic tobacco plants (lines S13 and S15) with PEG-6000 (60 g/l) as described previously (Eltayeb et al. 2006). Under such experimental conditions, wild type plants were less vigorous, flowered earlier and produced smaller leaves than *AtIpk2β* expressing plants (Fig. 5a). We also tested the effect of water shortage on plant growth. After a 21-day drought period, wild type plants completely wilted, while transgenic plants were less severely affected (Fig. 5b). At the end of the drought tolerance test, the plants were re-watered and grown further to allow seed setting. Transgenic plants produced larger shoots than wild type plants (Fig. 5c).

Proline accumulation in response to osmotic or salinity stress has been well documented in prokaryotic and eukaryotic organisms (Schobert 1997). Here, we compared the proline content of wild type and transgenic plants treated with 100- or 200-mM NaCl. Although proline accumulated in a dose-dependent manner in both wild type and transgenic plants, proline concentration was slightly higher in transgenic plants in the presence of 200-mM NaCl (Fig. 5d).

The results presented in Fig. 4 show that *AtIpk2β* expression increased the salt tolerance of transgenic tobacco plants. Since salt stress exerts osmotic stress to plant cells in addition to its ionic toxicity, we further determined if *AtIpk2β* expression confers elevated tolerance to oxidative stress on transgenic plants. Leaf disks from wild type and transgenic tobacco plants were exposed to increasing concentrations of H_2O_2 for 72 h. As shown in Fig. 6, transgenic plants exhibited a strong tolerance to oxidative stress. Leaf disks from wild type plants were almost completely bleached at the end of the stress

Fig. 3 Germination rate and early seedling development of transgenic plants. (a–c) Percentage of germinating seeds of wild type (WT) and transgenic T2 plants (S13, S15) grown on MS medium supplemented with 0-, 100- or 200-mM NaCl. Results are presented as means and standard errors from three independent experiments (≥ 100 seeds of each line were sown for each experiment). (d) Wild type (WT) and transgenic seeds (S13, S15) germinated on MS medium supplemented with 50 or 150-mM NaCl. Photo was taken three weeks after seeds were sown. (e) Wild type (WT) and transgenic lines (S13, S14, S15) grown on MS medium supplemented with different concentrations of NaCl for 2 months. (f) Root bending assay of wild type (WT) and transgenic plants (S13, S15) on MS medium supplemented with 0 or 150-mM NaCl. Representative pictures are shown. (g) Primary root lengths of wild type (WT) and transgenic plants (S13, S15) in the presence of different salt concentrations. Root growth under control condition (0-mM NaCl) was set to 100%. The results are shown as means and standard errors from three independent experiments (≥ 15 seeds of each line were sown for each experiment). *** indicates significant differences in comparison to the wild type at $P < 0.001$ (Student's *t*-test)



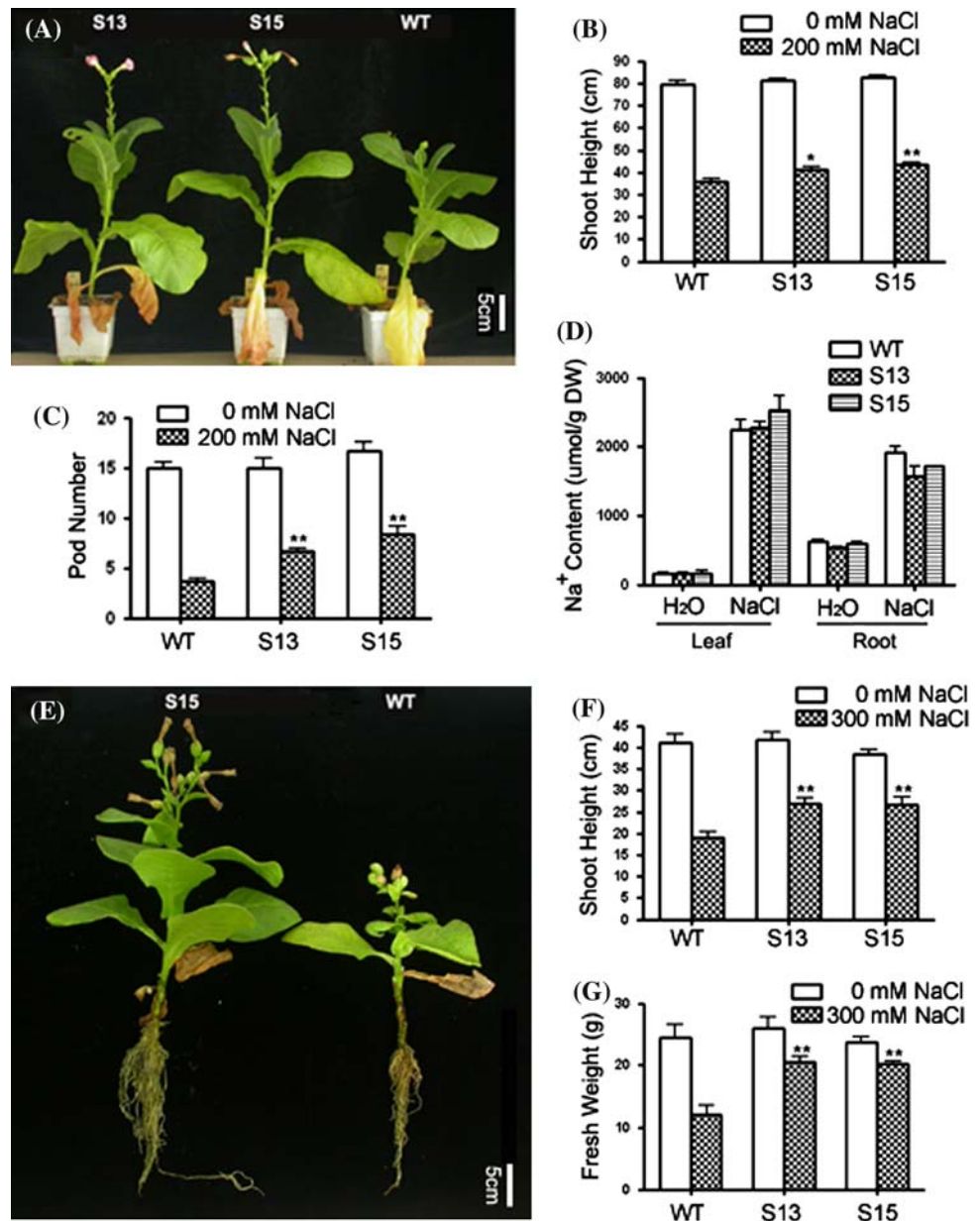
treatment, whereas those from transgenic plants showed significant resistance to H_2O_2 -caused damage (Fig. 6a).

To further examine the effect of constitutive *AtIpk2 β* expression on plant performance, the tolerance towards freezing temperature was analyzed. To this end, three-week-old seedlings were kept at -20°C for 1 or 2 h, respectively, and subsequently allowed to recover for seven days in the greenhouse. Most transgenic plants resumed normal growth after the 2 h treatment at -20°C (Fig. 6B), but only very few wild type plants did so. When treated for 1 h at -20°C , the damage was less severe, but again transgenic plants grew better than wild type plants after transfer back to the greenhouse (data not shown).

Transgenic seedlings showed a superior survival rate after short-term -20°C treatment (Fig. 6c). In the presence of oxidative stress, chlorophyll loss was significantly delayed in *AtIpk2 β* expressing plants compared to that of the wild type (Fig. 6d).

Lipid hydroperoxidation is an effective indicator of cellular oxidative damage (Yoshimura et al. 2004). Changes in the rates of lipid hydroperoxide production induced by oxidative stress were measured by determining MDA content in leaf disks. At high salinity (200-mM NaCl), MDA concentration markedly increased in wild type plants, whereas only a marginal increase was observed in the two transgenic lines (Fig. 7a). Although superoxide

Fig. 4 Salt tolerance test to compare wild type and *AtIpk2 β* expressing tobacco plants. For each experiment, twelve plants of each line were used. **(a)** Photograph of representative wild type (WT) and transgenic plants (S13, S15) watered with 200-mM NaCl for six weeks. **(b), (c)** Shoot height and pod numbers of wild type (WT) and transgenic (S13, S15) plants treated with 200-mM NaCl. * and ** indicate significant differences in comparison to the wild type at $P < 0.05$ and $P < 0.01$, respectively (Student's *t*-test). **(d)** Na^+ content in leaves and roots of wild type (WT) and transgenic plants (S13, S15) grown in soil supplied with 0 or 200-mM NaCl solution. **(e)** Photograph of a representative wild type (WT) and transgenic line S15 hydroponically grown in nutrient solution supplemented with 300-mM NaCl for two months. **(f)** Shoot height and **(g)** fresh weight of wild type (WT) and transgenic lines S13 and S15 grown for two months under control (0-mM NaCl) or high salt (300-mM NaCl) conditions. Results are presented as means and standard errors from three independent experiments. ** indicates significant differences in comparison to the wild type at $P < 0.01$ (Student's *t*-test)



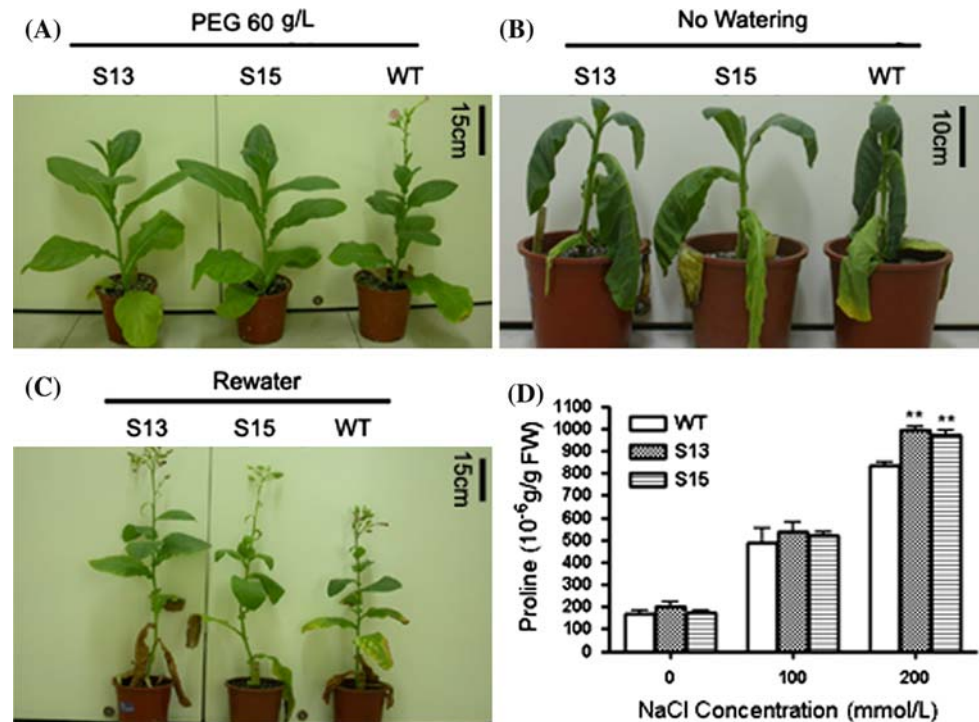
dismutase (SOD) activity was largely unaffected in wild type and transgenic plants treated with 1% H_2O_2 (Fig. 7b), a slight elevation of APX activity was observed in transgenic plants (Fig. 7c). Also, catalase (CAT) activity increased in transgenic plants (Fig. 7d). These results indicate that constitutive expression of *AtIpk2 β* enhanced tolerance to oxidative stress-induced membrane hydroperoxidation in transgenic tobacco plants.

Increased stress responsive gene expression in transgenic tobacco plants

Many genes responding to abiotic stress were cloned in recent years and used as molecular markers to monitor

the activity of stress-signaling pathways in plants (Kasuga et al. 2004). The stress experiments described above suggested that *AtIpk2 β* plays an important role in regulating stress tolerance in plants. To address whether or not *AtIpk2 β* affects the expression of stress responsive genes, we investigated the transcriptional levels of five marker genes in wild type and transgenic tobacco plants by RT-PCR. The marker genes chosen are identical or homologous to those encoding lipid transfer protein, fructose-bisphosphate aldolase, raffinose synthase, chloroplast ATPase, and group 2 LEA proteins of tobacco. As shown in Fig. 7, transcript levels of these genes were higher in transgenic plants than in the wild type under both, normal and salt stress conditions (Fig. 7).

Fig. 5 Effect of drought and osmotic stress on plant performance. For each experiment, twelve plants of wild type (WT) and each transgenic line (S13, S15) were used. (a) Wild type and transgenic plants grown in soil were watered with 60 g/l PEG-6000. Photograph was taken after 21 days of treatment. (b) Water was withheld for 21 days to impose drought stress. (c) Drought stressed plants were re-watered and grown for seed setting. (d) Proline content of wild type and transgenic plants after treatment with 100 or 200 mM NaCl for 24 h. Results are presented as means and standard errors from three independent experiments. ** indicates significant differences in comparison to the wild type at $P < 0.01$ (Student's *t*-test)



Discussion

A number of genes encoding Ins(1,4,5)P₃ 3-kinase or Ins(1,4,5)P₃ dual specificity 6-β-kinases were cloned and biochemically characterized in the past years. Examples include genes from yeast (Odom et al. 2000), animals (Bertsch et al. 1999), human (Dewaste et al. 2000), and higher plants (Stevenson-Paulik et al. 2002; Xia et al. 2003; Xu et al. 2005). It has recently been reported that in yeast (*S. cerevisiae*) inositol polyphosphate kinase (Kcs1p) is required for resistance to salt stress, cell wall integrity, and vacuolar morphogenesis (Dubois et al. 2002), and that overexpression of D-IP3K1, a *Drosophila* IP₃-kinase gene, confers resistance of flies to H₂O₂, but not to paraquat-induced oxidative stress (Monnier et al. 2002). Further genetic studies indicated that the protective effect conferred by elevated D-IP3K1 expression is mainly due to a lowered IP₃ level and thus reduced Ca²⁺ ion release from internal stores, rather than an increased IP₄ level.

Based on these observations, we were interested to know whether constitutive expression of *AtIpk2β* confers improved resistance to abiotic stresses on higher plants. Since the *Arabidopsis* *AtIpk2β* protein shares high amino acid sequence identity with the yeast IPK2 enzyme, which functions as a transcriptional regulator (also called Arg82 or ArgRIII) and displays dual-specificity InsP₃-InsP₄ kinase activity (Odom et al. 2000), we first produced transgenic *Arabidopsis* (ecotype Columbia-0) plants over-expressing *AtIpk2β*. Surprisingly, these plants did not

exhibit a significant improvement to salt or osmotic stress in our preliminary studies (data not shown). This may be due to the possible overlapping/redundant functions of the two *Arabidopsis* inositol polyphosphate kinases (*AtIpk2α* and *AtIpk2β*) in vivo, as suggested earlier by the York group (Stevenson-Paulik et al. 2002). To examine this possibility, we further compared the growth of *Arabidopsis* wild type as well as transgenic *AtIpk2β* over-expressor (*35S::AtIpk2β*) and knock-out mutant (*atipk2β*, SALK_104995, ABRC) plants. All three types of plants had similar growth phenotypes when subjected to salt or osmotic stress (data not shown). Here we introduced *AtIpk2β* into tobacco plants and found that transgenic lines developed superior tolerance to various types of abiotic stresses.

Previously, *AtNHX1*, a vacuolar Na⁺/H⁺ antiporter gene from *Arabidopsis*, was over-expressed in tomato and canola to improve the salt tolerance of transgenic plants (Zhang and Blumwald 2001). However, a comparative analysis of Na⁺ and K⁺ accumulation in leaves and roots of transgenic *AtNHX1* over-expressers and wild type plants grown under high-salt conditions was not performed because the high (200 mM) NaCl concentration used in these experiments was lethal to the wild type plants. In the study reported here, the relatively high salt tolerance of wild type tobacco plants made it possible to perform such an analysis. In the presence of 200-mM NaCl, despite a similar increase of Na⁺ concentration in wild type and transgenic plants, slightly more Na⁺ accumulated in leaves after long-term salt stress (Fig. 4d). This phenomenon is different from what has been seen in transgenic wheat

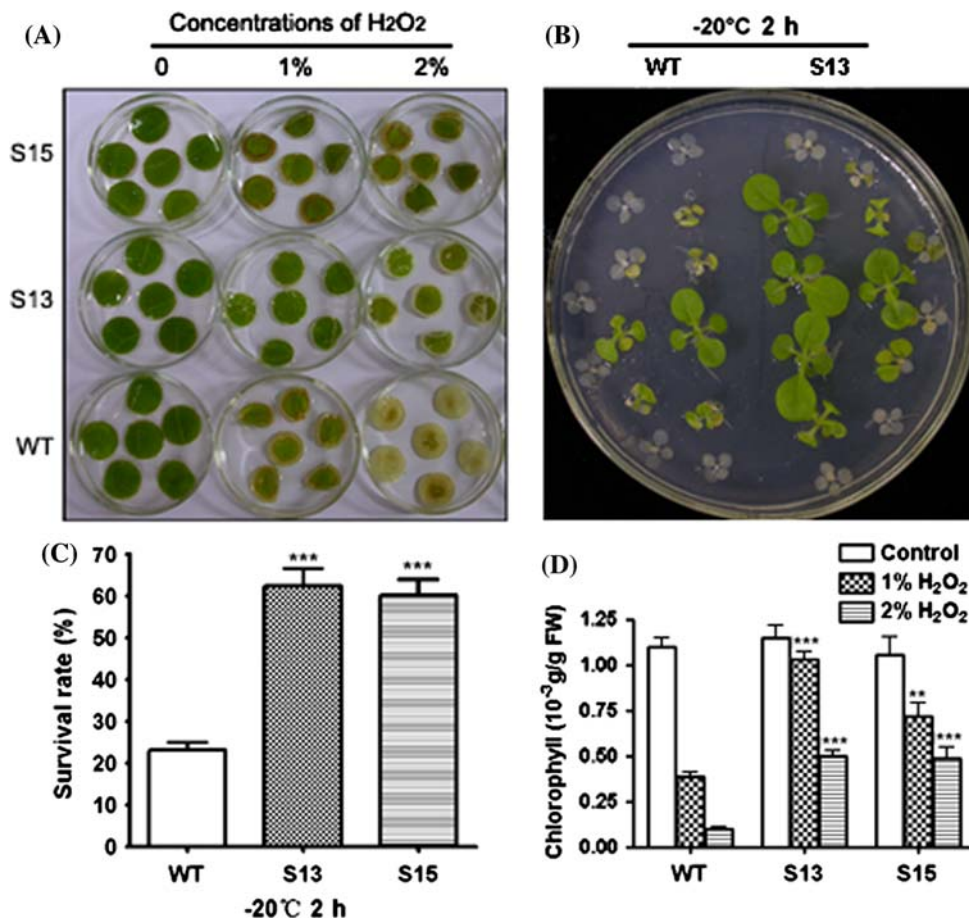


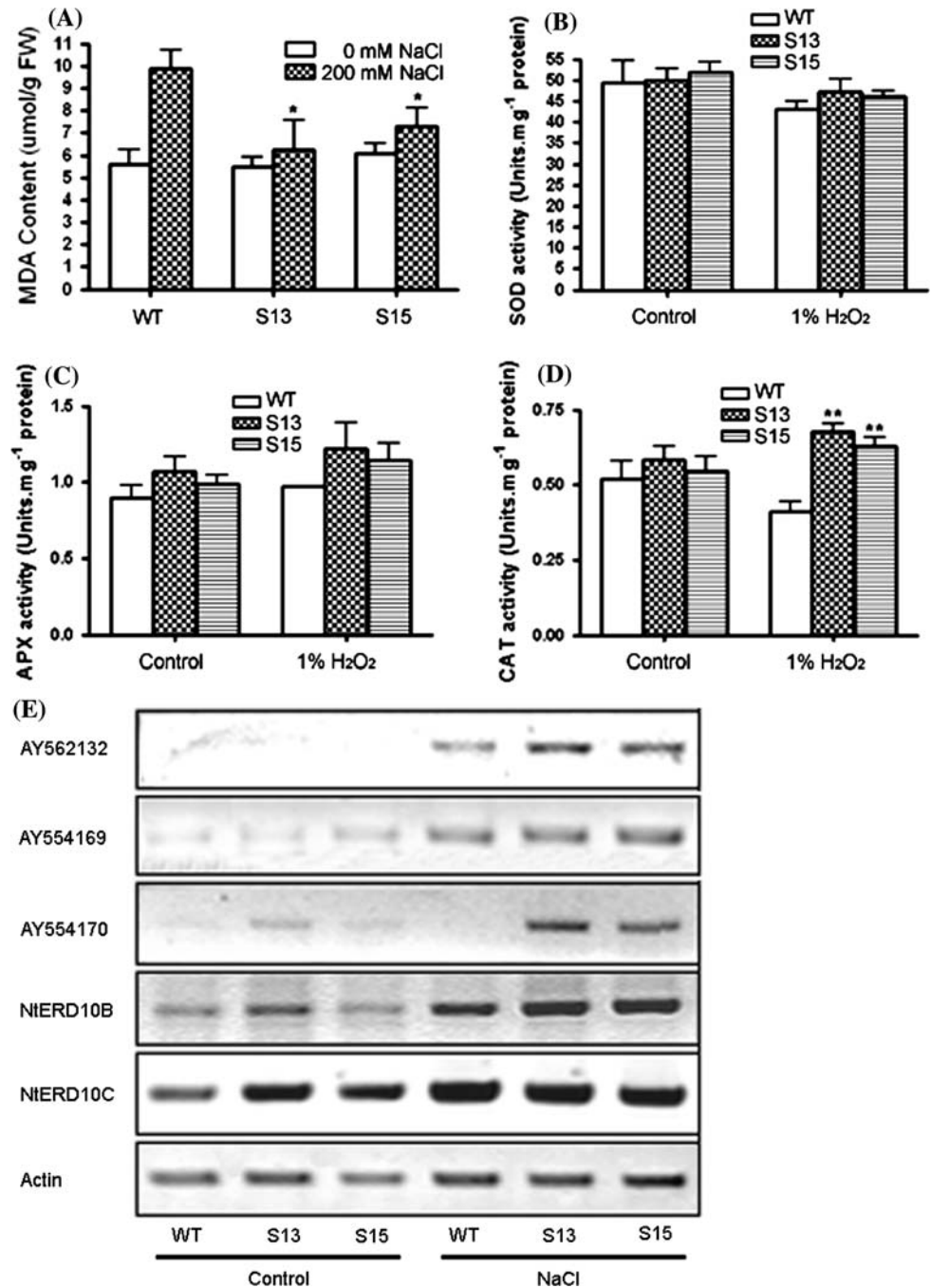
Fig. 6 Oxidative stress and short term freezing tolerance of wild type (WT) and transgenic tobacco plants (S13, S15). (a) Leaf disks were incubated in different concentration of H₂O₂ (1% and 2%, respectively) under continuous white light for 72 h. H₂O₂ induced leaf yellowing is delayed in the transgenic plants. (b) Three-week-old seedlings were stored at -20°C for 2 h, and then transferred to normal temperature for further growth. Photograph was taken seven days after the freezing treatment. (c) Quantification of the seedling survival rate (determined seven days after the freezing treatment). Results are presented as means and standard errors from three

independent experiments. For each experiment, 40 seedlings of each line were used. *** indicates significant difference in comparison to the wild type at $P < 0.001$ (Student's *t*-test). (d) Chlorophyll content was determined in leaf disks of wild type and T₂ transgenic plants kept in H₂O₂ for 72 h. Disks floated in water served as control. Results are means and standard errors from three independent experiments. ** and *** indicate significant differences in comparison to the wild type at $P < 0.01$ and $P < 0.001$, respectively (Student's *t*-test)

overexpressing *AtNHX1* (Xue et al. 2004). Salinity causes two major damages to plant cells, water deficit resulting from the relatively high solute concentrations in the soil, and ion toxicity due to altered K⁺/Na⁺ ratios as well as excessive Na⁺ and Cl⁻ contents (Apse and Blumwald 2002). The Na⁺/H⁺ exchange activity of *AtNHX1* is regulated in a Ca²⁺- and pH-dependent manner as reported earlier (Yamaguchi et al. 2005). The increased accumulation of salt in both leaves and roots suggests that unlike *AtNHX1*, which mainly works through sodium compartmentation, *AtIpk2β* may function through both sodium extrusion and compartmentation controlled by Ca²⁺-dependent signal transduction, although the precise mechanism of this still remains to be elucidated.

Proline contributes to osmotic adjustment (LeRudulier et al. 1989) and the protection of macromolecules during dehydration (Yancey et al. 1982), and as a hydroxyl radical scavenger (Hong et al. 2000). After treatment with 200-mM NaCl, but not 100-mM NaCl, proline accumulated to slightly higher levels in transgenic than in wild type tobacco plants (Fig. 5d). An increase in proline content was also observed in salt tolerant *Arabidopsis* with suppressed levels of proline degradation (Nanjo et al. 1999), transgenic salt tolerant tobacco plants with enhanced levels of proline biosynthesis (Kishor et al. 1995), and transgenic salt tolerant tomato plants overexpressing *AtNHX1* (Zhang and Blumwald 2001). So, it is possible that the elevated concentration of proline in transgenic plants helps to

Fig. 7 MDA level, anti-oxidative enzyme activity, and expression level of stress regulated genes in transgenic plants. (a) MDA levels in wild type and transgenic lines S13, S15 after treatment with 200-mM NaCl. (b–d) One-month-old seedlings were incubated for 12 h in 1% H₂O₂ or water (control) under continuous white light. SOD, APX and CAT activities were determined. Results are presented as means and standard errors from three independent experiments. * and ** indicate significant differences in comparison to the wild type at $P < 0.05$ and $P < 0.01$, respectively (Student's *t*-test). (e) Two-week-old seedlings of wild type and transgenic lines (S13, S15) were used for RNA extraction. For the salt stress experiments, seedlings were treated with 300-mM NaCl for 5 h before RNA isolation. The transcriptional levels of five stress genes were determined by RT-PCR analyses. The stress genes used for the tests are as follows: lipid transfer protein (AY562132); fructose-bisphosphate aldolase (AY554169); raffinose synthase family protein/seed imbibition protein (AY554170); group 2 LEA proteins NtERD 10B (AB049336) and NtERD 10C (AB049337)



protect anti-oxidative enzyme thus alleviating the negative effects imposed by salt on transgenic *Atlpk2β* plants.

Salt-stress-induced generation of reactive oxygen species (ROS) has been thought as one of the major causes of adverse effects of high salinity. ROS are products of chloroplast and mitochondrial metabolism produced during stress; they cause membrane damage resulting in electrolyte leakage (Apse and Blumwald 2002). At 200-mM NaCl, a relatively strong increase of MDA production was observed in wild type plants, but only a slight increase was seen in the two transgenic lines (Fig. 7a). Furthermore,

catalase (CAT) activity increased significantly in transgenic plants after treatment with 1% H₂O₂ (Fig. 7d). These results led us to hypothesize that constitutive expression of *Atlpk2β* improves cell membrane integrity. Evidence supporting this hypothesis was obtained from stress tolerance experiments with transgenic *Arabidopsis* overexpressing aldehyde dehydrogenase, or transgenic tobacco overexpressing *Chlamydomonas* glutathione peroxidase (Yoshimura et al. 2004).

The induction of stress-related genes has been taken as a hallmark of stress adaptation in plants (Thomashow 1999).

Earlier studies have shown that lipid transfer proteins are induced by osmotic stress (Smart et al. 2000). Genes encoding plastidic aldolase are induced by salt (Yamada et al. 2000). Transgenic plants overexpressing such enzymes exhibit improved tolerance to the respective stresses. Here, we observed that constitutive expression of *AtIpk2β* under the control of the CaMV 35S promoter triggered an increased expression of various stress responsive genes in transgenic tobacco. Already in the absence of salinity stress, transcript levels of genes encoding raffinose synthase family protein/seed imbibition protein (AY554170) and group 2 LEA protein NtERD 10C (AB049337) were found to be slightly elevated in the transgenic plants. At high salinity, the expression levels of genes encoding lipid transfer protein (AY562132) and raffinose synthase family protein/seed imbibition protein (AY554170) increased in the transgenic lines. These results suggest that *AtIpk2β* acts in one or more signal transduction pathway(s) thereby affecting the activity of stress-related genes and stress tolerance.

Many stresses evoke an elevation of the cytosolic calcium ion concentration, including salinity (Knight et al. 1997), drought (Knight et al. 1997), cold (Knight et al. 1996), and oxidative stress (Sanders et al. 2002). Previously, it has been shown that salinity and osmotic stress induce a rapid and transient increase of IP₃ levels (DeWald et al. 2001; Takahashi et al. 2001), and the reported accumulation of IP₃ occurs during a time frame similar to that observed for stress induced Ca²⁺ mobilization. Therefore, the possibility exists that enhanced abiotic stress tolerance of transgenic *AtIpk2β* plants results from changes of Ca²⁺ dependent signaling. Whether this is indeed the case has to be analyzed in future studies

Plant growth and productivity are severely affected by abiotic stress. Augmented resistance towards various environmental stresses has been a desirable goal for genetic improvement of plants. Stress tolerance can for example be improved by altering the expression of stress-responsive genes in transgenic plants. Representatives include genes encoding chitinase and glucanase (Hong and Hwuang 2006), betaine aldehyde dehydrogenase (Yang et al. 2005), tonoplast and plasma membrane Na⁺/H⁺ antiporters (Zhang and Blumwald 2001; Apse and Blumwald 2002), protein kinases (Xiong and Yang 2003), transcription factors (Waller et al. 2006), peroxidases (Yoshimura et al. 2004), DNA helicase (Sanan-Mishra et al. 2005), and DNA binding protein (polypeptide; Kalifa et al. 2004). Although the precise mode of action of *AtIpk2β* in plant responses to stress remains elusive, the results in this work provide direct evidence that alteration of *AtIpk2β* expression can significantly modify plant tolerance to various abiotic stresses, indicating a great potential of this gene for engineering stress tolerant crops.

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