Enhancement of stress tolerance in transgenic tobacco plants constitutively expressing AtIpk2β, an inositol polyphosphate 6-/3-kinase from Arabidopsis thaliana

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Received: 11 May 2007 / Accepted: 14 November 2007 / Published online: 30 December 2007 © The Author(s) 2007

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 (AtIpk2β) 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 AtIpk2β rescued the salt-, osmotic- and temperature-sensitive growth defects of a yeast mutant strain (arg82Δ) that lacks inositol polyphosphate multikinase activity encoded by the ARG82/IPK2 gene. Transgenic tobacco plants constitutively expressing AtIpk2β 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 AtIpk2β in plant stress responses.

Keywords Arabidopsis thaliana · AtIpk2β · 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 (Pattini 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...
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-tetraakisphosphate (IP4) intermediate (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). 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 arg82Δipk2 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 XbaI–SalI 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 Agrobacteria. After two days of co-cultivation, the
For yeast expression vector

| Primer name   | Primer sequence                          |
|---------------|------------------------------------------|
| pYX-F        | 5'-CTCCCATGGTAAATGCTCAAGTCCCTG-3'        |
| pYX-R        | 5'-GATTGTCGACCTAGCGCCGTTCTC-3'          |

For real-time RT-PCR

| Gene name          | Primer sequence                          |
|--------------------|------------------------------------------|
| AtIPK2β-F          | 5'-CCACGTTCTGAGGTTGGAAC-3'               |
| AtIPK2β-R          | 5'-GTGCTGACCTAGCGCCGTTCTC-3'            |
| AtActin2-F         | 5'-ACCGGGACTGAATGGAACG-3'               |
| AtActin2-R         | 5'-ACAGTCGTGAGCATTCCCAAC-3'             |

For RT-PCR

| Gene name          | Primer sequence                          |
|--------------------|------------------------------------------|
| NtActin-F          | 5'-TTACGCCCCTCTCATGCAATT-3'              |
| NtActin-R          | 5'-GGGCCACACCCCTTTGCTAC-3'               |
| AY562132-F         | 5'-ACCGGGACTGAATGGAACG-3'               |
| AY562132-R         | 5'-ACAGTCGTGAGCATTCCCAAC-3'             |
| AY554169-F         | 5'-CCACCATCATTGCTAC-3'                  |
| AY554169-R         | 5'-GGGCCACACCCCTTTGCTAC-3'              |
| AY554170-F         | 5'-ACCGGGACTGAATGGAACG-3'               |
| AY554170-R         | 5'-ACCGGGACTGAATGGAACG-3'               |
| NtERD10B-F         | 5'-ACCTTATAGTCGACCGCCG-3'               |
| NtERD10B-R         | 5'-ACCGGGACTGAATGGAACG-3'               |
| NtERD10C-F         | 5'-GGTAGCGCACAAGCTGGAAC-3'              |
| NtERD10C-R         | 5'-CTTCTACTCCACGCTGTCG-3'               |

For RT-PCR

| Gene name          | Primer sequence                          |
|--------------------|------------------------------------------|
| pYX-F              | 5'-CTCCCATGGTAAATGCTCAAGTCCCTG-3'        |
| pYX-R              | 5'-GATTGTCGACCTAGCGCCGTTCTC-3'          |

Table 1 Gene-specific primers used in this study

The samples were then boiled for 10 min, cooled down to room temperature and centrifuged at 1,200 × g. One-month-old seedlings were treated with 125 μmol m⁻² s⁻¹ under short day condition (12-h light/12-h dark). The nutrient solution was replaced weekly.

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 AtIPK2β expressing plants, 15-day-old seedlings were transferred to MS supplemented with 0, 100, 200 or 300 mM NaCl, and cultured at 25 ± 2°C with cool white fluorescent light (~125 μmol 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 ± 2°C with cool white fluorescent light (~125 μmol 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 ± 2°C with cool white fluorescent light (~125 μmol 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% trichlor acetic 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 × g. The cleared supernatant was analyzed by monitoring the difference in absorbance at 532 and 600 nm. 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 ± 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 A390 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 A550 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 \textit{AtIpk2}\textsubscript{\textit{b}}

In \textit{S. cerevisiae}, the \textit{ARG82/IPK2} gene encodes an inositol polyphosphate kinase (Odom et al. 2000). The \textit{S. cerevisiae arg82/ipk2} (\textit{arg82A}) 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 \textit{AtIpk2}\textsubscript{\textit{b}} rescues its growth defects at 37°C (Stevenson-Paulik et al. 2002; Xia et al. 2003). This result led us to postulate that \textit{AtIpk2}\textsubscript{\textit{b}} 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 \textit{arg82A} mutant was severely impaired when compared to that of the wild type (Fig. 1a). To test whether \textit{AtIpk2}\textsubscript{\textit{b}} restores the growth defect of the mutant strain under these conditions, we transformed it with the \textit{Arabidopsis AtIpk2}\textsubscript{\textit{b}} cDNA. Expression of \textit{AtIpk2}\textsubscript{\textit{b}} almost completely restored growth of the \textit{arg82A} 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 \textit{AtIpk2}\textsubscript{\textit{b}} restores growth under conditions of abiotic stress (Fig. 1b).

\textit{AtIpk2}\textsubscript{\textit{b}} is abiotic stress regulated in \textit{Arabidopsis}

We were interested to know whether different kinds of stresses such as salinity, cold and drought, or treatment with ABA would affect \textit{AtIpk2}\textsubscript{\textit{b}} transcript levels in \textit{Arabidopsis} plants. To this end we performed quantitative real-time PCR (qRT-PCR). In ten-day-old seedlings grown on MS medium, \textit{AtIpk2}\textsubscript{\textit{b}} 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 \textit{AtIpk2}\textsubscript{\textit{b}} is a stress responsive gene potentially involved in different abiotic stress- and ABA-triggered signaling pathways in plants. We further tested \textit{AtIpk2}\textsubscript{\textit{b}} protein level by Western blot analysis. Fourteen-day-old \textit{Arabidopsis} seedlings grown on MS medium were subjected to different stress treatments for 24 h. In seedlings treated with 500-mM mannitol, \textit{AtIpk2}\textsubscript{\textit{b}} 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 \textit{AtIpk2}\textsubscript{\textit{b}} in transgenic tobacco

To test whether constitutive expression of \textit{AtIpk2}\textsubscript{\textit{b}} affects abiotic stress tolerance in plants, we introduced its open reading frame (Fig. 2a) into the genome of tobacco (\textit{Nicotiana tabacum cv. SR-1}) by \textit{Agrobacterium tumefaciens}-mediated transformation. Expression of \textit{AtIpk2}\textsubscript{\textit{b}} in the transgenic plant was controlled by the \textit{CaMV} 35S promoter. Forty-six independent transgenic plants (\textit{T\textsubscript{0}} generation) were obtained; six lines were grown to produce seeds. Transgenic plants homozygous for the \textit{35S:AtIpk2}\textsubscript{\textit{b}} transgene were obtained and three lines (S13, S14 and S15) were chosen for further experiments. Constitutive expression of \textit{AtIpk2}\textsubscript{\textit{b}} 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 \textit{AtIpk2}\textsubscript{\textit{b}} protein. A 33-kDa band corresponding to \textit{AtIpk2}\textsubscript{\textit{b}} was identified in \textit{Arabidopsis} plants; similarly, 33-kDa \textit{AtIpk2}\textsubscript{\textit{b}} 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 \textit{AtIpk2}\textsubscript{\textit{b}} increases salt tolerance of transgenic tobacco plants

We first studied the salt tolerance of \textit{AtIpk2}\textsubscript{\textit{b}} 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 \textit{AtIpk2}\textsubscript{\textit{b}} 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
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

| Control (30°C) | 0.4 M NaCl | 0.8 M NaCl |
|---------------|-----------|-----------|
| WT            | arg82Δ    | arg82Δ+AtIp2β |
| arg82Δ        | arg82Δ+AtIp2β | arg82Δ+AtIp2β |
| arg82Δ+AtIp2β | arg82Δ+AtIp2β | arg82Δ+AtIp2β |

**Fig. 1** Expression of AtIp2β in *S. cerevisiae* arg82Δ. (a) AtIp2β rescues growth defects of the yeast arg82Δ 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; arg82Δ, arg82Δ mutant; arg82Δ + AtIp2β, arg82Δ mutant harbouring plasmid pYX212-AtIp2β. (b) Liquid yeast cultures. Cells were grown for three days to saturation in YPD medium (OD600 = 12.0). Five μl 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; arg82Δ, arg82Δ mutant; arg82Δ + AtIp2β, arg82Δ mutant harbouring pYX212-AtIp2β; arg82Δ + vector, arg82Δ mutant harbouring pYX212.
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 lines exposed to 200-nM 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 H₂O₂ 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.
treatment, whereas those from transgenic plants showed significant resistance to H$_2$O$_2$-caused damage (Fig. 6a).

To further examine the effect of constitutive Atipk2β 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 Atipk2β 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
dismutase (SOD) activity was largely unaffected in wild type and transgenic plants treated with 1% H₂O₂ (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 AtIpk2β 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 AtIpk2β plays an important role in regulating stress tolerance in plants. To address whether or not AtIpk2β 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).
Discussion

A number of genes encoding Ins(1,4,5)P$_3$ 3-kinase or Ins(1,4,5)P$_3$ dual specificity 6-/3-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 H$_2$O$_2$-, but not to paraquat-induced oxidative stress (Monnier et al. 2002). Further genetic studies indicated that the protective effect conferred by elevated D-IP3K1 is mainly due to a lowered IP$_3$ level and thus reduced Ca$^{2+}$ ion release from internal stores, rather than an increased IP$_4$ level.

Based on these observations, we were interested to know whether constitutive expression of AtIpk2$\beta$ confers improved resistance to abiotic stresses on higher plants. Since the Arabidopsis AtIpk2$\beta$ 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 InsP$_3$-InsP$_4$ kinase activity (Odom et al. 2000), we first produced transgenic Arabidopsis (ecotype Columbia-0) plants over-expressing AtIpk2$\beta$. 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 (AtIpk2$\alpha$ and AtIpk2$\beta$) 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 AtIpk2$\beta$ over-expresser (35S::AtIpk2$\beta$) and knock-out mutant (atipk2$\beta$, 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 AtIpk2$\beta$ 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.
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 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).

**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)
protect anti-oxidative enzyme thus alleviating the negative effects imposed by salt on transgenic AtIpk2β 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 AtIpk2β 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 \textit{AtIpk2}\textsubscript{b} 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 \textit{AtIpk2}\textsubscript{b} 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 IP\textsubscript{3} levels (DeWald et al. 2001; Takahashi et al. 2001), and the reported accumulation of IP\textsubscript{3} occurs during a time frame similar to that observed for stress induced Ca\textsuperscript{2+} mobilization. Therefore, the possibility exists that enhanced abiotic stress tolerance of transgenic \textit{AtIpk2}\textsubscript{b} plants results from changes of Ca\textsuperscript{2+} 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 Hwang 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 \textit{AtIpk2}\textsubscript{b} in plant responses to stress remains elusive, the results in this work provide direct evidence that alteration of \textit{AtIpk2}\textsubscript{b} expression can significantly modify plant tolerance to various abiotic stresses, indicating a great potential of this gene for engineering stress tolerant crops.

**Acknowledgements** We thank Professor E. Blumwald for critical reading of the manuscript and professor B. Sun for raising the antibody against \textit{AtIpk2}\textsubscript{b} protein. This work was supported by the following grants: National Natural Science Foundation of China (Grant No.: NSFC 30571196; 30471411; 30570155; 30521004; 30370142); National Basic Research Program of China (Grant No.: 2006CB101000); the National Special Key Project on Functional Genomics and Biochips of China; the Deutscher Akademischer Austauschdienst (DAAD); the Program for New Century Excellent Talents in Universities (NCET-04-0680); the Program for Changjiang Scholars and Innovative Research Teams in Universities (IRT0437). Bernd Mueller-Roeber thanks the Fonds der Chemischen Industrie (No. 0164389) and the Interdisciplinary Research Centre Advanced Protein Technologies (IZ-APT) of the University of Potsdam for financial support.

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