

RESEARCH PAPER

A wheat PI4K gene whose product possesses threonine autophosphorylation activity confers tolerance to drought and salt in *Arabidopsis*

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Abstract

Phosphoinositides are involved in regulation of recruitment and activity of signalling proteins in cell membranes. Phosphatidylinositol (PI) 4-kinases (PI4Ks) generate PI4-phosphate the precursor of regulatory phosphoinositides. No type II PI4K research on the abiotic stress response has previously been reported in plants. A stress-inducible type II PI4K gene, named *TaPI4KIIy*, was obtained by *de novo* transcriptome sequencing of drought-treated wheat (*Triticum aestivum*). *TaPI4KIIy*, localized on the plasma membrane, underwent threonine autophosphorylation, but had no detectable lipid kinase activity. Interaction of *TaPI4KIIy* with wheat ubiquitin fusion degradation protein (TaUDF1) indicated that it might be hydrolysed by the proteinase system. Overexpression of *TaPI4KIIy* revealed that it could enhance drought and salt stress tolerance during seed germination and seedling growth. A *ubdky7* mutant, identified as an orthologue of *TaPI4KIIy* in *Arabidopsis*, was sensitive to salt, polyethylene glycol (PEG), and abscisic acid (ABA), and overexpression of *TaPI4KIIy* in the *ubdky7* mutant compensated stress sensitivity. *TaPI4KIIy* promoted root growth in *Arabidopsis*, suggesting that *TaPI4KIIy* might enhance stress resistance by improving root growth. Overexpression of *TaPI4KIIy* led to an altered expression level of stress-related genes and changes in several physiological traits that made the plants more tolerant to stress. The results provided evidence that overexpression of *TaPI4KIIy* could improve drought and salt tolerance.

Key words: Kinase activity, overexpression, PI4K, protein interaction, stress response, *Triticum aestivum*.

Introduction

Environmental stresses, such as drought and salt, limit crop productivity worldwide. Understanding plant responses to these stresses is essential for rational engineering of hardier crop plants. Recent studies revealed the important role of the phosphoinositide signalling pathway at multiple developmental stages in plants (Munnik and Vermeer, 2009; Ischebeck *et al.*, 2010). Phosphatidylinositol 4-kinases (PI4Ks) at the apex of the phosphoinositide cascade are involved in many functions, including lipid- and protein-mediated signalling transductions (Carpenter and Cantley, 1996; Balla, 2001; Abraham, 2004; Wullschleger *et al.*, 2006; Szumlanski and Nielsen, 2010). The process by which PI4Ks phosphorylate

phosphatidylinositol (PI) in yeast and mammals has been thoroughly investigated: the enzymes catalyse phosphorylation of PI to PI4-phosphate, which is the most abundant of the monophosphorylated inositol phospholipids in mammalian cells (Strahl and Thorner, 2007; D'Angelo *et al.*, 2008) and therefore represents a potentially crucial point in the regulation of phosphoinositide-dependent pathways.

Phylogenetic analysis by amino acid sequence alignment using the PI4K domain from mammals, yeast, and plant proteins revealed that PI4K-domain-containing proteins fall into major types II and III according to size and sensitivity to inhibitors. Those originally identified as type I PI4Ks were

later shown to be PI3Ks. Type II PI4K is a membrane-bound, 55 kDa enzyme that is readily renaturable after SDS–PAGE. Two forms of type III PI4K were detected in the membrane and soluble fractions of animal tissues. One is 110 kDa in size, and the other is 200–230 kDa. Type II PI4K is insensitive to wortmannin (at concentrations significantly higher than those inhibiting PI3K), whereas type III enzyme activity is inhibited (Balla, 1998).

Up to now, most of the information on PI4Ks was derived from mammals and yeast (Barylko *et al.*, 2001; Minogue *et al.*, 2001). The yeast genome contains three genes that encode PI4Ks (*Pik1*, *Stt4*, and *Lsb6*, that are functionally non-overlapping) (Han *et al.*, 2002; Strahl and Thorner, 2007). Mammalian homologues of *Pik1* and *Stt4* are called *PI4KIIIβ* and *PI4KIIIα*, respectively (Wong *et al.*, 1997; Zhao *et al.*, 2001; Strahl and Thorner, 2007). PI4Ks are not only the enzymes catalysing PI4-phosphate, but also carry out other functions, including membrane trafficking (Strahl *et al.*, 2005), cytoskeletal regulation (Audhya and Emr, 2002), and regulation of cell apoptosis (Chu *et al.*, 2012).

Although PI4K activity was found in plants many years ago, the isolation and functional analysis of a full-length plant PI4K cDNA was first reported in 1999 (Xue *et al.*, 1999). *AtPI4Kβ* was the first PI4K cloned from a plant, and its transcript levels were similar in all tissues. Treatment of seedlings with hormones, CaCl₂, or NaCl had no effect on *AtPI4Kβ* mRNA levels (Xue *et al.*, 1999). Currently, eight *Arabidopsis* putative type II PI4Ks, four *Arabidopsis* putative type III PI4Ks, and two cotton putative type II PI4Ks are reported (Mueller-Roeber and Pical, 2002; Galvão *et al.*, 2008; Liu *et al.*, 2012). *Arabidopsis* type III *PI4Kβ1* and *β2*, upstream components of the phospholipase C pathway, were induced by cold stress. Cold-responsive genes were impaired in a *PI4KIIIβ1β2* double mutant in *Arabidopsis*, whose seed germination was hypersensitive to low temperature (Delage *et al.*, 2012). Cotton type II PI4K genes *GbPI4K* and *GhPI4K* were developmentally regulated in fibres and shared a similar expression pattern of ‘from high to low’ according to the fibre development stage (Liu *et al.*, 2012). Salicylic acid (SA) addition induced a rapid decrease in the phosphoinositide pool. This decrease paralleled an increase in PI4-phosphate and PI4,5-bisphosphate. These changes were inhibited by two different inhibitors of type III PI4Ks, phenylarsine oxide and wortmannin (Krinke *et al.*, 2007). Although there has been some progress in identification and functional analyses of plant PI4Ks, limited information is available compared with that in mammals and yeast. Except for *PI4Kβ1* and *β2*, there is no report on the PI4K family that directly relates to abiotic stress responses.

Wheat (*Triticum aestivum*) is one of the most important food staples worldwide. Drought and salt affect plant growth and productivity, and reduce wheat yields worldwide (Rhoades and Loveday, 1990; Morran *et al.*, 2011). In this study, a putative type II PI4K gene named *TaPI4KIIγ* was found to be up-regulated using *de novo* transcriptome sequencing of drought-treated wheat. *TaPI4KIIγ*, localized on the plasma membrane, was identified to have threonine autophosphorylation kinase activity, but not lipid kinase

activity. *TaPI4KIIγ* conferred salt and drought tolerance to *Arabidopsis*.

Materials and methods

Plant materials

The cultivated wheat cultivar KeNong 199 was used to amplify cDNA sequences of *TaPI4KIIγ* and *TaUFD1*. *Arabidopsis* Columbia-0 (WT) was used as background for overexpressing *TaPI4KIIγ*. By amino acid sequence alignment with *Arabidopsis* genes, the gene At1g13640 was found to be the closest relative to *TaPI4KIIγ* (identity=350/611 (57%)). In this study, the At1G13640 (*UBDKγ7*) mutant (SALK_107574C) was chosen for the experiments. The *ubdkγ7* mutant is a T-DNA insertion mutant (knock out), and the insertion site is in the promoter (at about –500 bp). *UBDKγ7* is a constitutively expressed gene (Supplementary Fig. S1 available at JXB online).

Stress treatment of wheat

Wheat seedlings were grown in Hoagland’s liquid culture at 22 °C under a 16 h light/8 h dark photoperiod. Ten-day-old wheat seedlings were used for dehydration, salt, and abscisic acid (ABA) treatments. For dehydration treatment, seedlings were transferred onto filter paper, and dried at 25 °C under 60% humidity conditions. For salt and ABA treatments, seedling roots were immersed in solutions containing 200 mM NaCl and 1 μM ABA, respectively. The samples were harvested at 0, 0.5, 1, 3, 6, 12, 24, and 48 h. RNA extraction and quantitative reverse transcription–PCR (qRT–PCR) analyses were performed. The specific primers for *TaPI4KIIγ* expression profiles and the primers of the wheat *β-actin* reference gene are listed in Supplementary Table S1 at JXB online.

Cloning and sequence analysis of the *TaPI4KIIγ* and *TaUFD1* genes

The full-length opening reading frames of *TaPI4KIIγ* and *TaUFD1* were obtained from wheat cDNA. The primers for cloning *TaPI4KIIγ* were 5'-GAGACCTCGGCGGAGAATCAAC-3' and 5'-TCTCTGCTGCTCTATGGACCTAGTCA-3'. The primers for *TaUFD1* were 5'-GTTTAACTTCTTGTCCAT-3' and 5'-GATAAATACATACTCTGGCAT-3'. The PCR products were cloned into pEASY-T1 vectors (TransGen) and sequenced with an ABI 3730XL 96-capillary DNA analyzer (Lifetech).

The amino acid sequences of *TaPI4KIIγ* homologous proteins were obtained from the NCBI using Blastp (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Amino acid sequence similarity comparisons were performed using the MegAlign program in DNASTar software. The complete amino acid sequences of PI4K proteins were used to construct phylogenetic trees. Sequence alignment was performed by ClustalX using BioEdit software and adjusted manually. The Neighbor–Joining method was used to construct a phylogenetic tree by the MEGA5.1 program, and the confidence level of monophyletic groups was estimated using a bootstrap analysis of 10 000 replicates (Tamura *et al.*, 2011).

Plasmid construction for localization analysis

TaPI4KIIγ was inserted into the subcellular localization vector p16318, which contains the 35S promoter and C-terminal green fluorescent protein (GFP) (Xu *et al.*, 2007). The primers for *TaPI4KIIγ-p16318* were 5'-TTTAAGCTTATGTCCCCAACCTGGAG-3' and 5'-AAACCATGGTGAATAATTTGCAGGAGGTGC-3'. For transient expression assays, ~4 × 10⁴ mesophyll protoplasts were isolated from 14-day-old seedlings, and transfected with 10 μg of *p16318::TaPI4KIIγ*. Transfected protoplasts were incubated in darkness at 22 °C. GFP fluorescence signals were observed

with a confocal laser scanning microscope (Nikon). FM4-64 dye (Molecular Probes, Carlsbad, CA, USA) was excited at 543 nm, and its fluorescence was recorded using a 650 nm long pass filter. All transient expression experiments were repeated three times.

Protein purification and pull-down assays

TaPI4KII γ was inserted into the prokaryotic expression vector pCOLD (TaKaRa), and *TaUFD1* was inserted into pGEX-4T-1. The primers for *TaPI4KII γ -pCOLD* were 5'-TTTGGTACCATGTCCCCAACCTGGAG-3' and 5'-AAATC TAGAAAATTTGCAGGAGGTGCCAG-3'. The primers for *TaUFD1-pGEX* were 5'-TTTGGTACCATGTCCCCAACCTGGAG-3' and 5'-TTTCTCGAGTAAATACATACTCTGGCA TTCAG-3'. TF-His-TaPI4KII γ (TF refers to the trigger factor that improves the solubility of the protein) and GST-TaUFD1 were expressed in *Escherichia coli* and purified by the standard procedure using Ni and glutathione agarose beads, respectively (GE Healthcare). In brief, 100 ml of BL21 cells grown overnight and expressing the desired constructs were transferred into 500 ml of LB and grown at 37 °C for 3 h. Isopropyl- β -D-thiogalactopyranoside (IPTG; 1 mM) was then added to the LB and left overnight at 16 °C to induce protein expression. The bacterial cells were sonicated in phosphate-buffered saline (PBS) with 1% Triton X-100 and centrifuged at 10 000 g for 10 min to remove insoluble cell debris. The supernatant was incubated with PBS pre-equilibrated with Ni or glutathione agarose beads and rotated at 4 °C for 2 h. After washing with PBS five times, the glutathione *S*-transferase (GST)-tagged and His-tagged proteins were eluted using 5 mM glutathione and 125 mM imidazole, respectively (Tang *et al.*, 2008).

For GST pull-down assays, TF-His-TaPI4KII γ was incubated with GST-TaUFD1 in Z-binding buffer [25 mM HEPES (pH 7.6), 12.5 mM MgCl₂, 150 mM KCl, 0.1% NP-40, and 20% glycerol] buffer for 2 h at 4 °C. After washes in NETN buffer [100 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 20 mM TRIS (pH 8.0)], proteins were analysed on SDS-polyacrylamide gels followed by immunoblotting using anti-His antibody.

Bimolecular fluorescence complementation (BiFC) assay

TaPI4KII γ was cloned into pSPYNE, and *TaUFD1* was cloned into pSPYCE (Kerppola, 2008). The primers for *TaPI4KII γ -pSPYNE* were 5'-AAATCTAGAATGTCCCCAACCTGGAG-3' and 5'-TT TGGTACCAAATTTGCAGGAGGTGCCAG-3'. The primers for *TaUFD1-pSPYCE* were 5'-TTTTCTAGAATGAATATGTATTTCG AAGGCTAT-3' and 5'-TTCGGTACCGCCCTTCAAAGAGTAC TTCT-3'. For the BiFC assay, the *pSPYNE::TaPI4KII γ* and *pSPYCE::TaUFD1* reconstruction vectors were bombarded into onion epidermal cells by a particle gun. Plasmolysis was carried out with a filter-sterilized solution prepared by dissolving the sucrose in culture medium (50% w/v). The onion epidermal cells were treated with 50% sucrose solution for 10 min, and then monitored by confocal microscopy as previously described (Xu *et al.*, 2007).

In vitro autophosphorylation assays

TF-His-TaPI4KII γ was expressed in BL21 cells and purified using a Zi affinity gel. After elution from the beads, the protein solution was dialysed against a 1000 \times volume buffer containing 20 mM MOPS, pH 7.5, and 1 mM dithiothreitol (DTT). Autophosphorylation assays were performed by immunoblotting after transfer to polyvinylidene fluoride (PVDF) membranes using antiphosphoserine (1:500 dilution), antiphosphotyrosine (1:500), and antiphosphothreonine antibodies (1:500) (Oh *et al.*, 2009).

Generation of transgenic Arabidopsis

The coding sequence of *TaPI4KII γ -pBII21* was amplified using primers 5'-AAATCTAGAATGTCCCCAACCTGGAG-3' and 5'-TCC

CGGGCTCTGCTGCTCTATGGACCTAGTC-3', and cloned into pBII21 under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter, resulting in a *35S::TaPI4KII γ* construct. The construct was confirmed by sequencing and then transformed into wild-type (WT) plants and the *ubdk γ 7* mutant by the vacuum infiltration method (Bechtold and Pelletier, 1998). *35S::TaPI4KII γ* transgenic (WT background) and *TaPI4KII γ ::ubdk γ 7* lines (*ubdk γ 7* mutant background), with various expression levels of the *TaPI4KII γ* gene, were obtained for further analysis.

Performance of transgenic Arabidopsis under stress treatment

Homozygous T₃ seeds of the transgenic lines were used for phenotypic analysis. Seeds were surface sterilized, kept at 4 °C for 3 d, and then sown on germination medium [1/4 strength Murashige and Skoog medium with salt and vitamins (PhytoTechnology Laboratories) and further supplemented with 0.8% (w/v) agar, 30 mg l⁻¹ hygromycin B (Sigma-Aldrich), and 2% (w/v) sucrose, pH 5.7] under long-day conditions (15 h light/9 h dark) at 22 °C. For the germination assay, seeds were subjected to 100 mM or 200 mM NaCl, 5% or 10% (w/v) polyethylene glycol (PEG) 3600 (to simulate osmotic stress), and 0.5 μ M or 1 μ M ABA treatments. The percentage of germinated seeds was calculated based on the number of seedlings that reached the cotyledon stage at 2 weeks (Saleki *et al.*, 1993). For seedling phenotype analysis of the WT and the *35S::TaPI4KII γ* lines, all plants were grown in plates or soil under long-day conditions (15 h light/9 h dark) at 22 °C with 70% relative humidity. Seeds of WT and transgenic plants were plated on half-strength MS (Murashige and Skoog) agar, kept at 4 °C for 3 d, and then incubated in a growth chamber under continuous light at 23 °C. Six-day-old seedlings were transplanted in soil containing vermiculite. For salt treatment, 15-day-old seedlings were treated with 200 mM NaCl for 20 d. For water deficit treatment, watering was withheld from 15-day-old seedlings for 20 d, by which time they were fully affected by water deficit stress.

Expression analysis of *TaPI4KII γ* -regulated genes

Total RNAs from *35S::TaPI4KII γ* , *ubdk γ 7* mutant, and the WT were extracted using an RNAprep plant kit (TIANGEN), and first-strand cDNAs were synthesized using a PrimeScript First-Strand cDNA Synthesis kit (TaKaRa); cDNAs were combined with SYBR master mix for PCR (TIANGEN). qRT-PCRs were performed in triplicate with a Bio-Rad iCycler. The *Arabidopsis* β -actin gene was used as a reference. The qRT-PCRs were performed using an ABI Prism 7300 real-time PCR system (Lifetech) and repeated three times. Quantitative and data analyses were performed as previously described (Le *et al.*, 2011). The specific primers are listed in Supplementary Table S1 at JXB online.

Measurement of malondialdehyde (MDA) and free proline contents

Seven-day-old seedlings were planted in 4 cm deep rectangular plates filled with a 1:1 mixture of vermiculite and humus. After a further 7 d of growth, they were treated with 200 mM NaCl and 10% (w/v) PEG, and then grown for 7 d before harvesting and measuring. All the measurements were repeated three times.

MDA contents were assayed according to Lv *et al.* (2007). About 0.1 g of wheat leaf was used and absorbance values at 450, 532, and 600 nm were determined with a spectrometer (Perkin-Elmer Lambda 25, Boston, MA, USA). The MDA content was calculated using the formula: C (1 mol/l) = 6.45 (OD₅₃₂ - OD₆₀₀) - 0.56 OD₄₅₀.

Wheat leaf samples (~0.1 g) for the measurement of proline were treated with 3% (w/v) sulphosalicylic acid followed by boiling for 1 h. The amounts of proline were measured with ninhydrin, which was detected at 520 nm, and a proline standard liquid was used as a reference (Bates *et al.*, 1973).

Results

Phylogenetic analysis and domain organization of putative wheat PI4K

An up-regulated putative PI3/4K protein selected from the drought-treated wheat *de novo* transcriptome contained a predicted PI3/4K domain coupled with an N-terminal ubiquitin-like (UBL) domain (amino acids 53–120). The gene was cloned from wheat cDNA, and the full length was 1890 bp encoding a 69.3 kDa protein (Fig 1A). In multiple amino acid sequence alignments with 29 PI3/4K genes from human, mouse, yeast, and *Arabidopsis*, the wheat PI3/4K was classified as a member of the PI4K family (Supplementary Fig. S2 at JXB online). Currently, two major groups (types II and III) identified in the PI4K family occur in a wide range of tissues and cellular compartments in animals, yeast, and plants (Endemann *et al.*, 1987; Pike, 1992; Galvão *et al.*, 2008). Twelve *Arabidopsis* PI4K genes, comprising eight type II (gamma) and four type III PI4Ks (alpha and beta), together with the wheat PI4K, were aligned based on multiple sequence alignments (Fig. 1B). The wheat PI4K fell into the type II PI4K gamma cluster and was named *TaPI4KIIγ*.

TaPI4KIIγ was induced by dehydration, salt, and ABA

The expression pattern of *TaPI4KIIγ* was monitored by qRT-PCR. The expression of *TaPI4KIIγ* increased after 1 h treatment with salt and reached a peak (almost 6-fold) at 6 h

(Fig. 2A). *TaPI4KIIγ* was also induced by dehydration and ABA treatments. ABA initially induced the expression at an early phase (30 min after treatment), and, after a slight decline, this increased to a peak of ~40-fold at 12 h before declining again (Fig 2B). Under dehydration treatment, *TaPI4KIIγ* was not induced until 6 h, and the peak point (~11-fold) was at 6 h; followed by an abrupt decline to the normal level (Fig. 2C).

Threonine autophosphorylation activity of *TaPI4KIIγ*

The coding region of *TaPI4KIIγ* was cloned into expression vectors, and the recombinant protein was successfully produced in *E. coli* as an N-terminal TF-His fusion. Autophosphorylation assays carried out using anti-phosphoamino acid antibodies revealed that *TaPI4KIIγ* autophosphorylated threonine residues (Fig. 3). The TF-His fusion showed no detectable cross-reaction with anti-phosphoamino acid antibodies. The failure to detect autophosphorylation of TF-His was used to eliminate non-specificity of the antibodies, and served as a crucial control to validate detection of phosphoamino acid. However, TF-His-*TaPI4KIIγ* showed no *in vitro* PIK activity in the presence of PI or Type I Folch lipid fraction and [γ - 32 P]ATP (results not shown).

Subcellular localization of *TaPI4KIIγ*

To investigate the localization of *TaPI4KIIγ* in cells, the gene was inserted into a subcellular localization vector. The

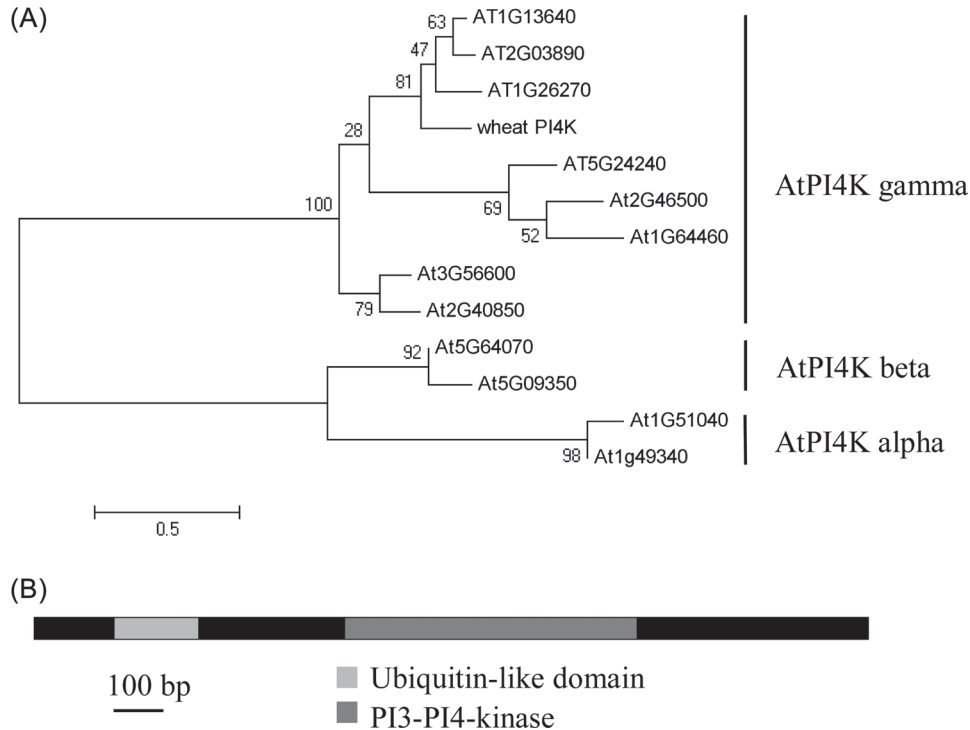


Fig. 1. Phylogenetic analysis and domain organization of *TaPI4KIIγ*. (A) Twelve *Arabidopsis* PI4K genes were divided into three subfamilies, type III PI4K alpha and beta and type II gamma. The wheat PI4K gene was classified as a type II PI4K gamma family member. The closest members to wheat PI4K were AT1G26270, AT2G03890, and AT1G13640, and were phosphoinositide 4-kinase gamma 7 types. (B) Domain organization of *TaPI4KIIγ*. Amino acid sequence alignment comparing *Arabidopsis* ubiquitin with UBL domains from *TaPI4KIIγ*.

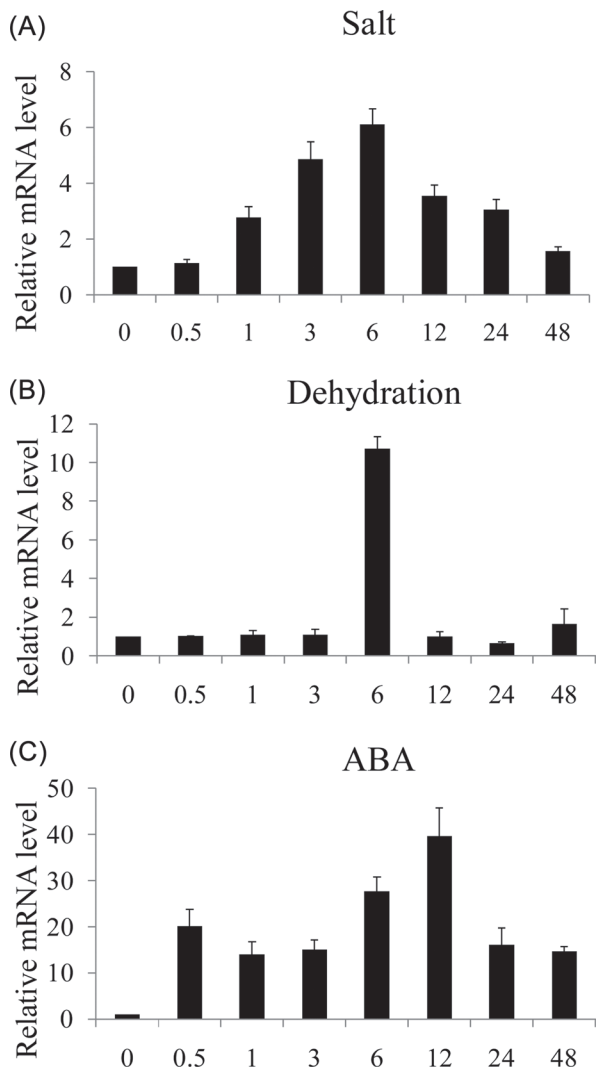


Fig. 2. *TaPI4KIIγ* is a stress-responsive gene. Expression profiles of *TaPI4KIIγ* after salt (A), dehydration (B), and ABA (C) treatments for 0, 0.5, 1, 3, 6, 12, 24, and 48 h. Ten wheat seedlings were pooled as one sample. The vertical coordinates are fold changes, and the horizontal ordinates are treatment time. The data are representative of three independent experiments.

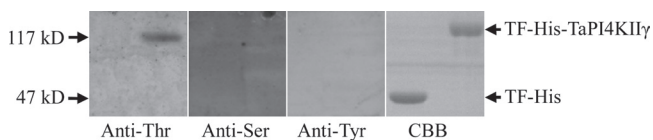


Fig. 3. Kinase activity of *TaPI4KIIγ*. Autophosphorylation activity of *TaPI4KIIγ* detected by immunoblotting with antiphosphotyrosine (Anti-Tyr), antiphosphothreonine (Anti-Thr), and antiphosphoserine (Anti-Ser) antibodies. The molecular weights of fusion proteins TF-His-TaPI4KIIγ and TF-His are marked at the left. CBB, Coomassie brilliant blue.

recombinant vector *p16318::TaPI4KIIγ* was transformed into *Arabidopsis* protoplasts and, when observed by confocal microscopy, GFP fluorescence of the *TaPI4KIIγ*-GFP fusion protein in transformed cells occurred exclusively on the

plasma membrane, whereas the GFP fluorescence of GFP-transformed cells was distributed throughout the entire cell. These data indicated that the *TaPI4KIIγ* protein is a plasma membrane-localized protein (Fig. 4).

TaPI4KIIγ interacted with a component of the ubiquitin-proteasome *TaUFD1* system

TaPI4KIIγ, as bait protein, was used to screen the wheat cDNA library by a yeast two-hybrid assay. One expressed sequence tag (EST) sequence, named *TaUFD1*, sharing the highest homology with *Arabidopsis UFD1* was obtained. UFD1 binds to some forms of ubiquitin and is known to deliver target proteins to the 26S proteasome, which is the major degradation pathway in plants (Mayor *et al.*, 2005). Interaction between *TaPI4KIIγ* and *TaUFD1* was confirmed by pull-down and BiFC assays. *TaPI4KIIγ* and *TaUFD1* were cloned into expression vectors, and the two recombinant proteins were successfully produced in *E. coli* as TF-His-TaPI4KIIγ and GST-TaUFD1, respectively. The *in vitro* pull-down assay was performed by incubating TF-His-TaPI4KIIγ and GST-TaUFD1 in combination (Fig. 5A). Interaction of the proteins was identified by BiFC. *TaPI4KIIγ* was cloned into pSPYNE, and *TaUFD1* was cloned into pSPYCE. The *TaPI4KIIγ*-YFP^N (N-terminal fragment of yellow fluorescent protein) and *TaUFD1*-YFP^C (C-terminal fragment) recombination vectors were bombarded into onion epidermis, and fluorescence signals were observed. Plasmolysis

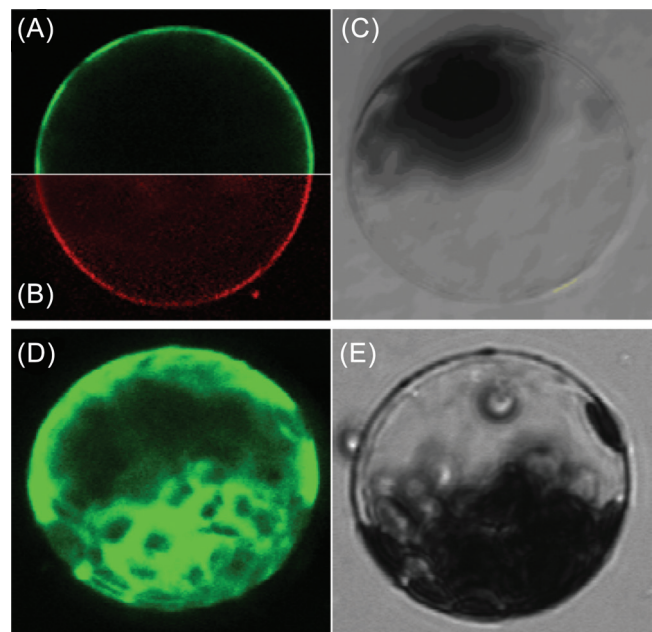


Fig. 4. Subcellular localization of *TaPI4KIIγ* in *Arabidopsis* protoplasts. The *TaPI4KIIγ*-GFP fusion proteins (A) or GFP alone (D) driven by the 35S promoter were transiently expressed in *Arabidopsis* protoplast cells and observed under a laser scanning confocal microscope. In (B), the protoplast plasma membrane was labelled with the FM4-64 steryl dye. Bright field images are shown in (C) and (E). The scale bar is 15.38 μm. (This figure is available in colour at JXB online.)

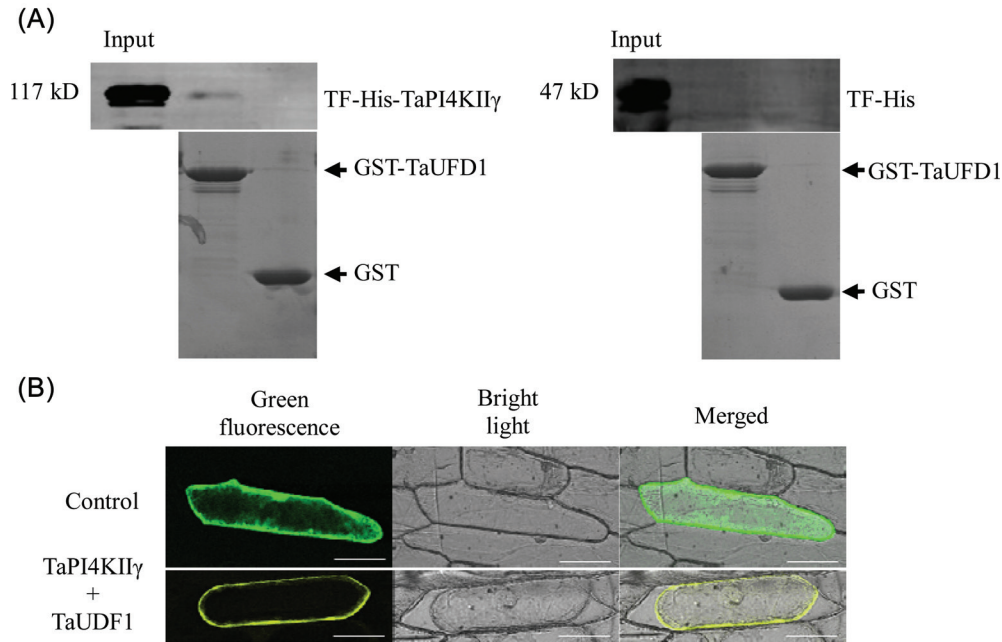


Fig. 5. Interaction of TaPI4KII γ and TaUDF1. (A) The *in vitro* pull-down assay was performed by incubating TF-His-TaPI4KII γ with GST-TaUDF1. (B) The assay for interaction between TF-His and GST-TaUDF1 was used as the control. (C) The BiFC assay of the interaction of TaPI4KII γ with TaUDF1. Fluorescence, bright field, and merged images of onion epidermal cells co-transfected with constructs encoding the indicated fusion proteins: TaPI4KII γ -YFP^N and TaUDF1-YFP^C. Plasmolysis was carried out to distinguish the plasma membrane from the cytoderm. The scale bar is 73.61 μ m. (This figure is available in colour at JXB online.)

was carried out to distinguish the plasma membrane from the cytoderm. Interaction of TaPI4KII γ -YFP^N and TaUDF1-YFP^C occurred on the plasma membrane (Fig. 5B).

TaPI4KII γ rescued the Arabidopsis *ubdky7* mutant and affected root growth

The phenotype of the *ubdky7* mutant was identified as having a shorter root length (WT 3.1 ± 0.14 mm, *ubdky7* mutant 1.56 ± 0.1 mm) (Fig. 6A). To assess whether expression of TaPI4KII γ in *Arabidopsis* could rescue the *ubdky7* mutant phenotype, TaPI4KII γ was introduced into *ubdky7* mutant plants under control of the CaMV35S promoter, and transgenic TaPI4KII γ ::*ubdky7* lines were obtained. The TaPI4KII γ ::*ubdky7* lines showed similar root lengths to the wild type (WT 3.1 ± 0.14 mm; TaPI4KII γ ::*ubdky7* lines 3.02 ± 0.17 mm and 2.9 ± 0.22 mm) after 5 ds growth (Fig. 6B).

TaPI4KII γ conferred stress tolerance to Arabidopsis

TaPI4KII γ should not only rescue root growth, but also enhance the stress tolerances of the *ubdky7* mutant and the WT (Fig. 7A). TaPI4KII γ was introduced into the WT, generating several 35S::TaPI4KII γ lines. Three lines were selected to assay for stress tolerance. Under normal conditions, nearly 100% of the seeds from the 35S::TaPI4KII γ , WT, TaPI4KII γ ::*ubdky7*, and *ubdky7* mutant lines germinated. However, when sown on MS medium containing PEG, NaCl, and ABA, these *Arabidopsis* lines displayed different germination rates (Fig. 7B). The *ubdky7* mutant treated with PEG, NaCl, and ABA exhibited obviously lower seed germination

rates than the WT (~50%). Overexpression of TaPI4KII γ in the *ubdky7* mutant to some extent rescued the resistance to stress treatments (the average germination rates of TaPI4KII γ ::*ubdky7* lines were almost 80%, and similar to the WT), and overexpressing TaPI4KII γ in the WT improved the stress tolerance (average germination rates of 35S::TaPI4KII γ lines were ~95% compared with 80% for the WT).

The three 35S::TaPI4KII γ lines displayed longer root length and larger root areas than the WT under normal growth conditions, with increases of nearly 40% in root length and a 5-fold increase in root surface area (Fig. 8A). 35S::TaPI4KII γ transgenic lines and WT seedlings were exposed to water deficit and salt treatments. After 20 d of water deficit, all WT plants exhibited severe symptoms of water loss and significant wilting. In contrast, most of the 35S::TaPI4KII γ transgenic lines were green, and only slight wilting was evident in some of the 35S::TaPI4KII γ transgenic leaves (Fig. 8B). After 20 d of salt treatment, the 35S::TaPI4KII γ lines displayed a better performance than the WT based on rosette size (nearly 2-fold larger) (Fig. 8C). Improved ABA tolerance was identified by germination rates of *Arabidopsis* on MS medium containing ABA (Fig. 8D). The germination rates of 35S::TaPI4KII γ lines were higher than those of the 35S::TaPI4KII γ lines and the WT (nearly 4-fold) (Fig. 8E).

The improved stress tolerance of 35S::TaPI4KII γ transgenic plants was correlated with changes in proline and MDA contents. Under normal growing conditions, the proline and MDA contents of 35S::TaPI4KII γ and the WT were similar. However, the MDA contents of 35S::TaPI4KII γ lines were lower (50% and 42%, respectively) than that of the WT after NaCl and PEG treatments (Fig. 9F), and the proline contents

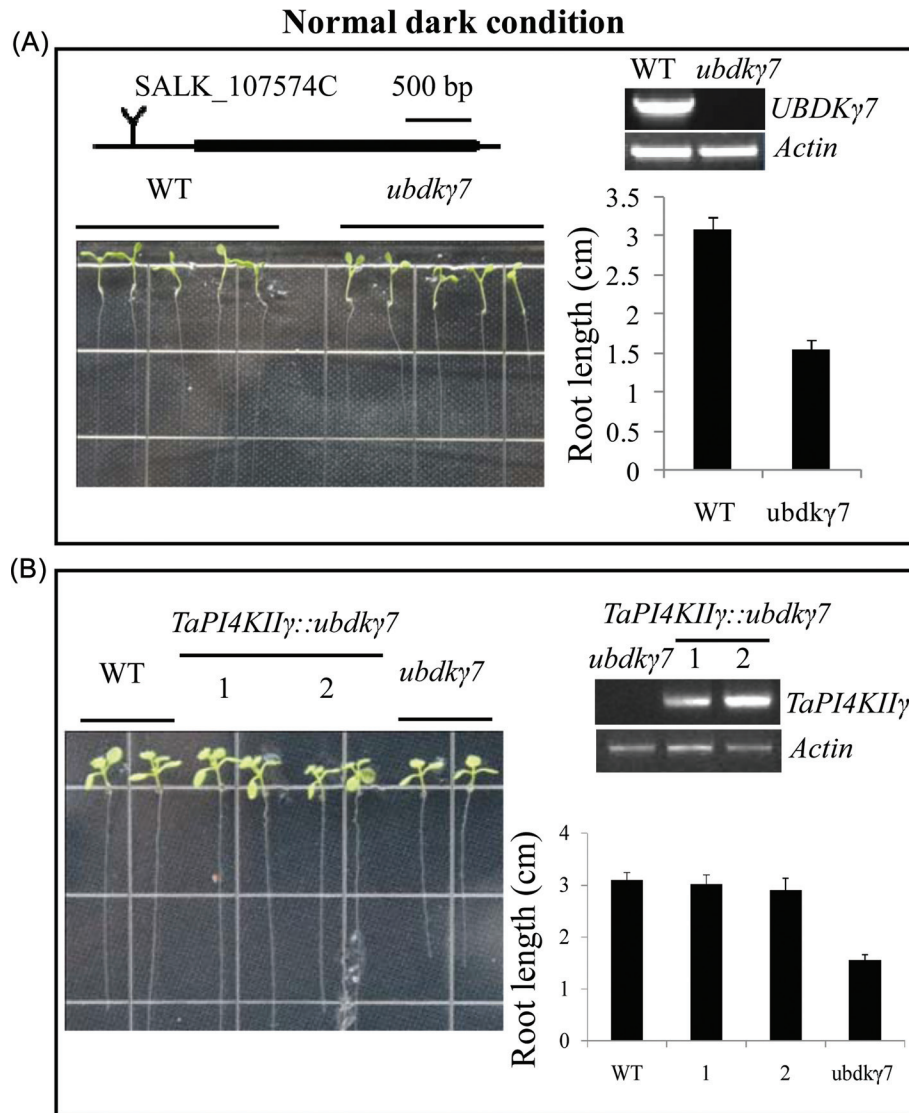


Fig. 6. *TaPI4KII γ* rescued the root length of the *ubdk γ 7* mutant. (A) The *ubdk γ 7* mutant displayed a shorter root length than the WT. The average root lengths of at least 30 seedlings are shown at the right. The *ubdk γ 7* mutant is a T-DNA insertion line, and the insertion site is in the promoter (about -500 bp). The *ubdk γ 7* mutant is a knock out line. (B) Overexpression of *TaPI4KII γ* in the *ubdk γ 7* mutant partially rescued the shorter root length phenotype. Gel electrophoretic map shows *TaPI4KII γ* expression in the WT and the *TaPI4KII γ ::UbDK γ 7* lines. The histogram on the right shows average root lengths of at least 30 seedlings. The numbers 1 and 2 represent different *TaPI4KII γ ::ubdk γ 7* lines. (This figure is available in colour at JXB online.)

of *35S::TaPI4KII γ* lines were much higher (70% and 38%, respectively) than those of the WT (Fig. 8G).

TaPI4KII γ altered expression of stress-related genes in transgenic Arabidopsis

To elucidate the possible molecular mechanisms of *TaPI4KII γ* in stress response, the expression of seven stress-response genes, namely *DREB1B/CBF1*, *DREB1C/CBF2*, *DREB1A/CBF3*, *ZAT12*, *COR15A*, *COR47*, and *LTI78*, was investigated in *ubdk γ 7* mutant, *35S::TaPI4KII γ* , and WT plants under normal growing conditions. A 2-fold change in expression was arbitrarily considered an expression induction. In three independent qRT-PCR analyses, five genes (*DREB1A/CBF3*, *ZAT12*, *COR15A*, *COR47*, and *LTI78*) showed altered expression in the

transgenic plants and the mutant (Fig. 9). The expression of the five genes increased in the *35S::TaPI4KII γ* transformant under normal conditions, but was decreased in the *ubdk γ 7* mutant. The other two genes (*DREB1B/CBF1* and *DREB1C/CBF2*) were not differentially expressed in the WT compared with the transgenic and mutant plants (data not shown).

Discussion

TaPI4KII γ generated threonine autophosphorylation, but not lipid kinase activity

PI4K was necessary for the generation of two secondary messengers from PI4,5-bisphosphate and caused a series of cellular reactions (Jung *et al.*, 2002). A plasma

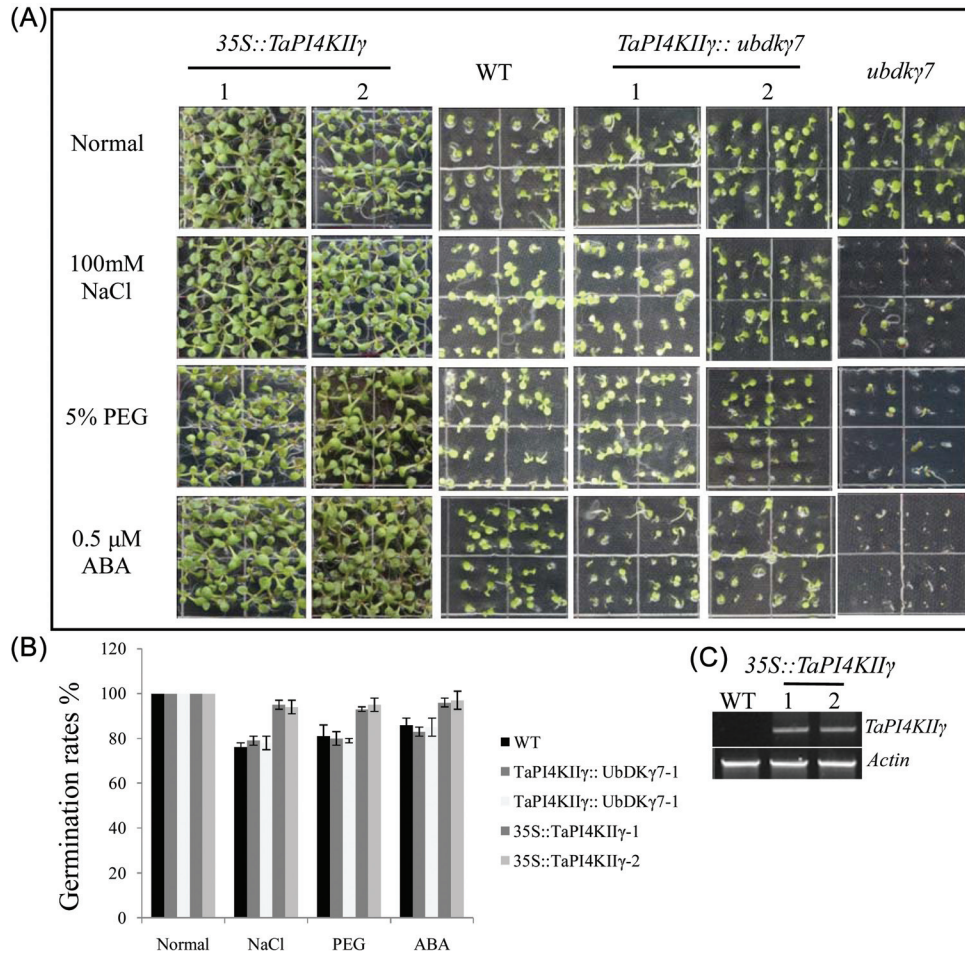


Fig. 7. Overexpression of *TaPI4KIIγ* improved seed germination under NaCl, PEG, and ABA. (A) The *ubdkγ7* mutant was sensitive to NaCl, PEG, and ABA treatments. The *TaPI4KIIγ::ubdkγ7* transformants showed a similar germination rate to the WT, and 35S::*TaPI4KIIγ* lines displayed higher germination rates than the WT. (B) The seed germination rates of different lines. Each data point is the mean of three experiments, and each experiment comprised 30 plants. (C) Gel electrophoresis image detection of *TaPI4KIIγ* in the WT and 35S::*TaPI4KIIγ* lines. (This figure is available in colour at JXB online.)

membrane-localized gene, *TaPI4KIIγ*, was identified to be up-regulated in drought-treated wheat *de novo* transcriptome sequencing. Known mammalian and yeast PI4K genes, such as *Pik1*, *Stt4*, *Lsb6*, *PI4KIIα*, *PI4KIIβ*, *PI4KIIIα*, and *PI4KIIIβ*, are responsible for catalysing PI to PI4-phosphate and PI4,5-bisphosphate, which possess lipid kinase activity (D'Angelo *et al.*, 2008). However, in this study, *TaPI4KIIγ* generated threonine autophosphorylation, but not lipid kinase activity (Fig. 3). Several PI3/4K family members show similar kinase activities (Abraham, 2004; Galvão *et al.*, 2008; Lovejoy and Cortez, 2009). As reported previously, phosphoinositide 3-kinase-related kinases (PIKKs) usually transduce cell signalling via phosphorylation of serine or threonine protein residues, such as ATM, ATR, mTOR, DNA-PK, and hSMG-1, and function as protein serine/threonine kinases (Lovejoy and Cortez, 2009). For instance, the protein kinase ATM (ataxia telangiectasia mutated) was homodimerized, and dissociation to monomers was stimulated by autophosphorylation, which activated ATM kinase activity (Bakkenist and Kastan, 2003). *Arabidopsis* type II PI4Ks UbDKγ4 and

UbDKγ7 displayed the same kinase activity as *TaPI4KIIγ*, possessing protein kinase activity, but not lipid kinase activity (Galvão *et al.*, 2008). It is proposed that *TaPI4KIIγ* is a member of a new group of protein kinases belonging to the PIKK family of atypical protein kinases (Manning *et al.*, 2002). It is somewhat confusing in that PI4Ks are usually considered part of the phosphoinositide signalling pathway and function as key enzymes that catalyse changes of PI to PI4-phosphate. Further investigation is needed to determine how *TaPI4KIIγ* functions in the phosphoinositide signalling pathway.

UFD1, which was first found in a yeast mutant defective in degrading short-lived ubiquitin fusion proteins (Johnson *et al.*, 1995), is involved in regulation of protein degradation as a 'vehicle', that binds some forms of ubiquitin and target proteins and delivers the complex to 26S proteasomes (Hartmann-Petersen and Gordon, 2004; Madura, 2004). In this study, *TaUFD1* was identified to interact with *TaPI4KIIγ* (Fig. 5), suggesting a possible degradation pathway: *TaPI4KIIγ* interacts with *TaUFD1* which is already bound to ubiquitin, and then the protein complex is transported to the

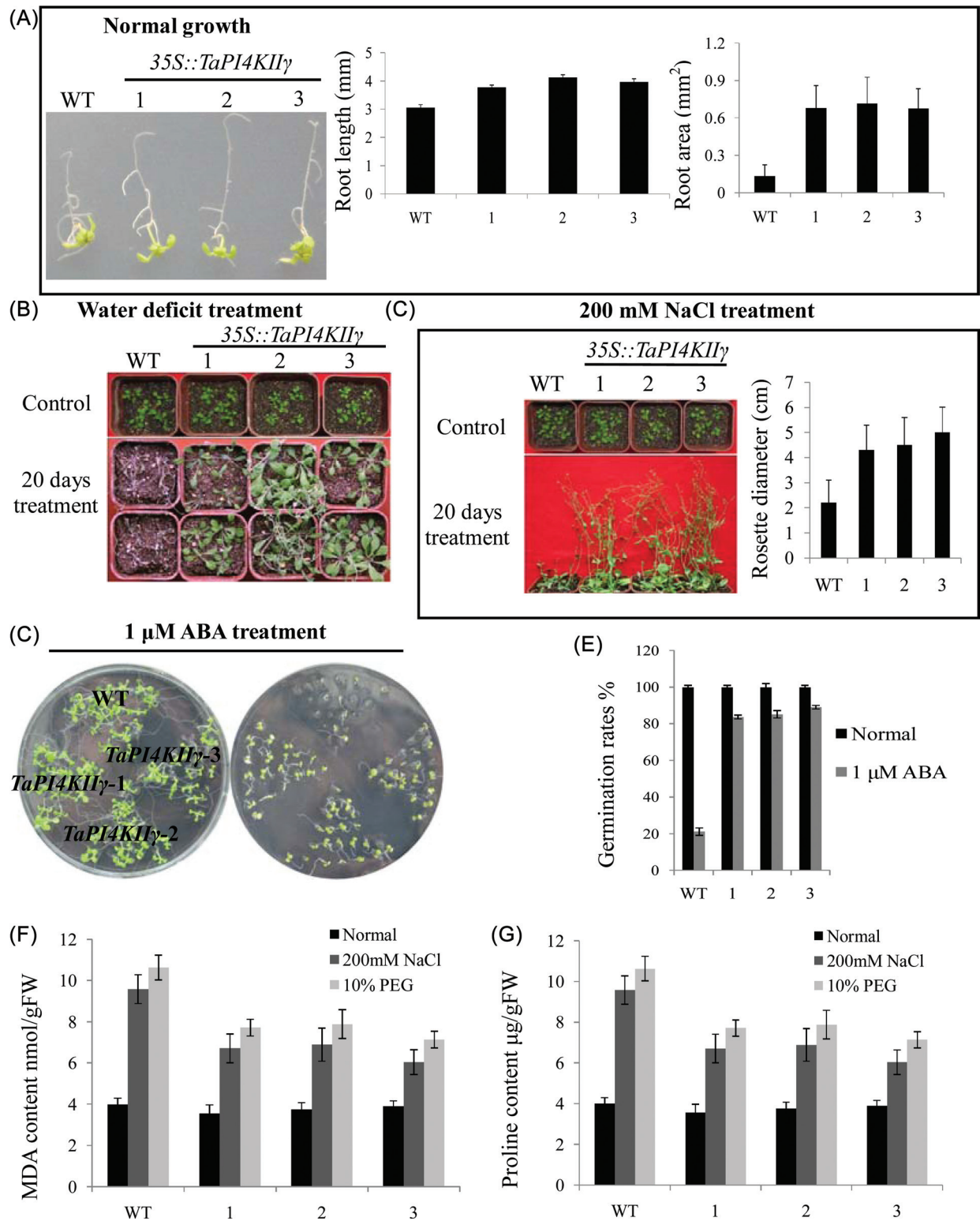


Fig. 8. Overexpression of *TaPI4KIIγ* in wild-type *Arabidopsis* improved stress tolerance. (A) *TaPI4KIIγ* affected the root growth of *Arabidopsis*. The left image shows growth of the WT compared with the *35S::TaPI4KIIγ* lines. The histograms show root length and root surface area. (B) Transgenic *Arabidopsis* plants overexpressing *TaPI4KIIγ* under water deficit treatment. The seedlings were not watered for 20 d. (C) Performance of transgenic *Arabidopsis* plants overexpressing *TaPI4KIIγ* under salt stress. The plants were treated with 200 mM NaCl for 20 d. Rosette diameters of transgenic plants and the WT under salt stress are shown on the right. Each bar is the mean of 25 seedlings. (D) *Arabidopsis* grown on MS medium containing 1 μM ABA. Germination rates are shown in (E). (F) MDA contents in seedlings treated with 200 mM NaCl and 10% PEG compared with normal growth conditions. (G) Proline contents in seedlings treated with 200 mM NaCl and 10% PEG compared with seedlings grown under normal conditions. The numbers 1, 2, and 3 represent three *35S::TaPI4KIIγ* lines, respectively. (This figure is available in colour at *JXB* online.)

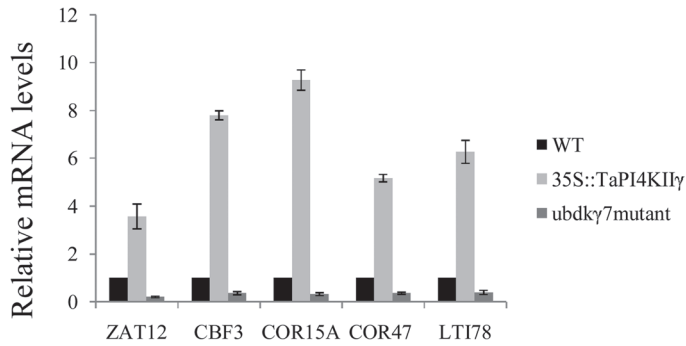


Fig. 9. Expression levels of stress-responsive genes under regulation of *TaPI4KII γ* . Gene-specific primers were used for detection of relative transcript levels of stress-responsive genes. The vertical coordinates are fold changes, and the horizontal ordinates are gene names. The data are means of three replicates.

26S proteasome degradation system. The ubiquitin–26S proteasome pathway is involved in various vital processes, such as cell cycle control, programmed cell death, and signalling transduction (Vierstra, 2009). Recently, it was found that the ubiquitin–26S proteasome pathway plays significant roles in regulation of abiotic and biotic responses in plants (Dielen *et al.*, 2010). Although there are a few reports about UFD1 regulation of stress response in plants, it was demonstrated that silencing UFD1 in *Nicotiana benthamiana* resulted in significantly improved biotic stress tolerance (Lai *et al.*, 2012). These results indicated that UFD1 might act as a negative regulator in stress response by causing the degradation of interacting proteins, and in this study the function of *TaPI4KII γ* in stress response might be negatively controlled by *TaUFD1* via the 26S proteasome degradation system.

TaPI4KII responded to multiple stresses

Phytohormones, such as SA, jasmonic acid (JA), ethylene (ET), and ABA, primarily regulate protective responses of plants to both biotic and abiotic stresses via synergistic or antagonistic actions (Fujita *et al.*, 2006). ABA is commonly considered an abiotic resistance-related phytohormone. *Arabidopsis* ABA mutants *aba1*, *aba2*, and *aba3* grow relatively normally compared with the WT, but are very sensitive to drought and high salt conditions (Zhu, 2002). Many drought-inducible genes are also activated by ABA (Abe *et al.*, 2003; Ding *et al.*, 2009). In this study, *TaPI4KII γ* was rapidly induced by ABA, and was also up-regulated by dehydration and salt (Fig. 2). However, compared with the rapid response to ABA, the *TaPI4KII γ* activity induced by dehydration and salt was delayed. The different expression profiles of *TaPI4KII γ* under ABA, dehydration, and salt implied that *TaPI4KII γ* might function via different mechanisms under different stresses.

SA is associated with biotrophic pathogen resistance (Pieterse *et al.*, 2009), and ABA is generally considered to be a negative regulator of SA-mediated disease resistance (Fujita *et al.*, 2006). For example, the ABA-deficient tomato mutant *sitiens* had increased resistance to pathogens,

and application of exogenous ABA restored susceptibility to *sitiens* (Audenaert *et al.*, 2002). In the present work, *TaPI4KII γ* , induced by ABA, was negatively regulated by SA, and showed decreased expression under SA treatment (Supplementary Fig. S3 at *JXB* online). This suggested that the ABA and SA signalling pathways affect expression of *TaPI4KII γ* in antagonistic ways.

TaPI4KII enhanced stress tolerance

Overexpression of stress-inducible genes in plants is an effective strategy for improving abiotic stress tolerance (Rudrabhatla and Rajasekharan, 2002; Flowers, 2004; Cattivelli *et al.*, 2008; Xu *et al.*, 2008, 2011). To date, there had been no report on the abiotic stress response of type II PI4Ks in plants. Overexpression of *TaPI4KII γ* in the WT background led to obviously enhanced tolerance to water deficit, salt, and ABA (Figs 7, 8). One significant piece of evidence was that the *35S::TaPI4KII γ* lines exhibited high seed germination rates following salt, PEG, and ABA treatments (Fig. 7), and the seedlings exhibited higher survival rates and better growth than the WT under water deficit and salt stresses (Fig. 8B, C). In contrast, the *ubdky7* mutant was sensitive to salt, PEG, and ABA treatments, as identified by germination rates on the culture medium (Fig. 7). These results suggested that *TaPI4KII γ* may be involved in regulating plant responses to abiotic stresses. Introduction of *TaPI4KII γ* into the *ubdky7* mutant resulted in the recovery of the normal phenotype, with near-normal root growth and stress tolerance. It was reported that PI4K was required for root tip growth in *Arabidopsis*. Plants lacking members of the PI4K family displayed defective growth and morphology of root hairs (Szumlanski and Nielsen, 2010). Root length and surface area are phenotyping traits for measuring plant drought stress tolerance (Manavalan *et al.*, 2009). In this study, the roots of *35S::TaPI4KII γ* lines were obviously longer and possessed a larger surface area than the WT, whereas the root length of the *ubdky7* mutant was shorter. Thus *TaPI4KII γ* might enhance stress tolerance by improving root growth.

Water deficit and salt stresses induce various physiological responses in plants. Proline, which functions as an osmotic regulator, prevents water loss from cells when plants are subject to water deficit and salt stresses (Bais and Ravishankar, 2002; Urano *et al.*, 2009). Increased proline content in *35S::TaPI4KII γ* transformants under PEG and salt stresses could be taken as evidence for stress resistance. Moreover, stresses such as water deficit and salt are accompanied by production of MDA, which damages the membrane and results in cell death (Levine *et al.*, 1994). The lower content of MDA in *35S::TaPI4KII γ* when subjected to PEG and salt stresses could be utilized as a sign of tolerance to stress.

Possible mechanisms of TaPI4KII in stress response

Many transcription factors and other genes responding to stress have been identified, providing the insight that plants have developed flexible molecular and cellular mechanisms

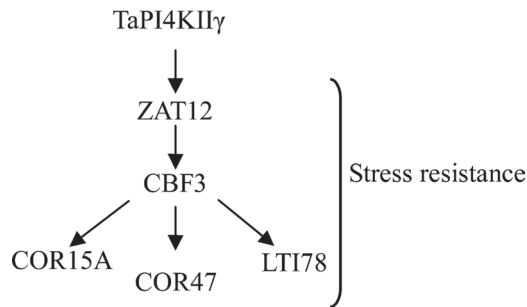


Fig. 10. A proposed model for *TaPI4KIIγ* improvement of stress tolerance. *TaPI4KIIγ* increases the expression of *ZAT12*, leading to activated expression of *CBF3*. Stress-responsive genes, including *COR15A*, *COR47*, and *LTI78*, were induced to enhance plant stress tolerance.

to tolerate various abiotic stresses. The CBF gene family encodes ERF/AP2 transcription factors that have been described as playing important roles in drought and salt tolerance in *Arabidopsis* (Dubouzet *et al.*, 2003; Shen *et al.*, 2003; Oraby and Ahmad, 2012). In this study, it was found that the expression of *CBF3* was activated in the *35S::TaPI4KIIγ* plants, but decreased in the *ubdky7* mutant. In *Arabidopsis*, *CBF* was demonstrated to control the accumulation of other stress response genes, such as *COR15A*, *COR47*, and *LTI78* (Novillo *et al.*, 2012). *COR15A*, *COR47*, and *LTI78*, which are CBF target genes, were reported as drought- and salt-responsive genes (Xiong *et al.*, 2002; Cheong *et al.*, 2011; Novillo *et al.*, 2012; Zhang *et al.*, 2011). The expression of *COR15A*, *COR47*, and *LTI78* was significantly increased in *35S::TaPI4KIIγ* plants, but was decreased in the *ubdky7* mutant. These results indicated that *TaPI4KIIγ* could activate the expression of *CBF3*, which increased the expression of *COR15A*, *COR47*, and *LTI78*, and ultimately improved the salt and drought tolerances of *Arabidopsis*. *ZAT12*, a salt- and drought-responsive gene (Qin *et al.*, 2012), controls the expression of DREB1/CBF genes (Gorsuch *et al.*, 2010; Hirayama and Shinozaki, 2010). The present work showed that the expression of *ZAT12* was changed in the *35S::TaPI4KIIγ* transformant and the *ubdky7* mutant, indicating that a *ZAT12* signal pathway may exist in the DREB1/CBF gene regulatory network that was stimulated by *TaPI4KIIγ* (Fig 10). The present work thus suggests that *TaPI4KIIγ* is involved in regulating plant responses to abiotic stress, and has potential for improving stress tolerance in crop plants.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Information on the *ubdky7* mutant.

Figure S2. Phylogeny of the PI3/4K domain.

Figure S3. Expression profile of *TaPI4KIIγ* under SA treatment.

Table S1. Drought- and salt-responsive genes selected from the TAIR database, and gene-specific primers used for qRT-PCR.

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