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ORIGINAL ARTICLE

Characterization of StPPI1, a proton pump interactor from *Solanum tuberosum* L. that is up-regulated during tuber development and by abiotic stress

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Abstract Plasma membrane proton pumps (PM H^+-ATPases) are involved in several physiological processes, such as growth and development, and abiotic stress responses. The major regulators of the PM H^+-ATPases are proteins of the 14-3-3 family, which stimulate its activity. In addition, a novel interaction partner of the AHA1 PM H^+-ATPase, named PPI1 (proton pump interactor, isoform 1), was identified in *Arabidopsis thaliana*. This protein stimulates the activity of the proton pump in vitro. In this work, we report the characterization of an *A. thaliana PPI1* homolog in potato (*Solanum tuberosum* L.) named *StPPI1*. The full-length coding sequence of *StPPI1* was obtained. The open reading frame (ORF) encodes a protein of 629 amino acids showing 50% identity with *A. thaliana* PPI1 protein. The *StPPI1* ORF is divided into seven exons split by six introns. Southern blot analysis suggests that *StPPI1* belongs to a family of related genes. Recombinant StPPI1 stimulates H^+-ATPase activity in vitro. Basal levels of *StPPI1* transcripts are observed in all tissues, however, *StPPI1* expression is higher in proliferative regions (shoot apex and flower buds), flowers and leaves than in shoots and roots. *StPPI1* mRNA levels significantly increase during tuber development. *StPPI1* is induced by salt stress and cold. Drought and mechanical wounding slightly increase *StPPI1* transcript levels. In addition, the expression of *SlPPI1*, the tomato homolog of *StPPI1*, was determined under adverse environmental conditions in tomato plants. *SlPPI1* mRNA levels are increased by drought and cold, but are unaffected by salt stress. Mechanical wounding slightly increases *SlPPI1* expression.

Keywords StPPI1 · Proton pump interactor · Plasma membrane H^+-ATPase · *Solanum tuberosum* L. · Tuber development · Stress

Abbreviations

PPI	Proton pump interactor
PM	Plasma membrane
ABA	Abscisic acid

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Introduction

Plasma membrane proton pumps (PM H^+-ATPases) are P-type ATPases that generate an electrochemical proton gradient across the plasma membrane, which drives the transport of solutes and regulates cytoplasmic and apoplastic pH (Sondergaard et al. 2004; Gaxiola et al. 2007). Plants contain a large family of PM proton pump isoforms; in *Arabidopsis thaliana*, 11 genes (*AHA1-11*) predicted to encode PM H^+-ATPases have been identified (Arango et al. 2003; Baxter et al. 2003).

PM proton pumps are involved in several physiological processes. Numerous studies have shown that they play an important role in the regulation of plant growth and development (Zhao et al. 2000; Rober-Kleber et al. 2003; Gévaudant et al. 2007; Sveinsdóttir et al. 2009). In recent years, considerable effort has been devoted to the study of PM H^+-ATPase roles in abiotic stress responses, mainly

under high salinity conditions. It has been demonstrated that salt stress increases PM H^+-ATPase activity in different plant species, such as rice (*Oryza sativa*), broccoli (*Brassica oleracea*) and cucumber (*Cucumis sativus*) (Sahu and Shaw 2009; López-Pérez et al. 2009; Janicka-Russak and Klobus 2007, respectively). In tomato (*Solanum lycopersicum*), an increase in PM H^+-ATPase transcripts in response to NaCl exposure has been reported (Kalampanayil and Wimmers 2001). In *A. thaliana*, a loss-of-function mutation of *AHA4* give rise to increased salt sensitivity (Vitart et al. 2001). Salt-tolerant genotypes of barley (*Hordeum vulgare*) show intrinsically higher PM H^+-pump activity (Chen et al. 2007). In the same way, the legume *Medicago citrina* better responds to mild salinization than its close relative *Medicago arborea*, and this capacity is related to an increase in expression and activity of the PM H^+-ATPase (Sibole et al. 2005). PM proton pumps have also been involved in cellular responses to low temperature stress. PM H^+-ATPase activity increases after cold exposure in different plant species, such as sugar beet (*Beta vulgaris*) and the cryophyte *Chorispora bungeana* (Chelysheva et al. 1999; Shi et al. 2008, respectively), and during cold acclimation in many species (Hellergren et al. 1983; Mattheis and Ketchie 1990; Baruah et al. 1990; Martz et al. 2006).

The major regulators of the PM H^+-ATPase are proteins of the 14-3-3 family (Ferl 2004), which bind to the autoinhibitory domain of the ATPase in the C-terminus, relieving its effect and stimulating pump activity (Jahn et al. 1997; Fuglsang et al. 1999; Palmgren 2001). Phosphorylation of the penultimate residue of PM H^+-ATPase, a Thr, triggers the binding of regulatory 14-3-3 proteins, resulting in the formation of an activated complex (Camoni et al. 2000). This complex was recently shown to be a dodecamer of six H^+-ATPase and six 14-3-3 proteins (Ottmann et al. 2007). Activation of PM H^+-ATPase by the binding of 14-3-3 proteins is negatively controlled by phosphorylation of two residues in the H^+-ATPase 14-3-3 protein binding site (Duby et al. 2009).

Not much is known about the regulation of PM H^+-ATPase by other effectors. A novel interaction partner of the AHA1 PM H^+-ATPase, named PPI1 (proton pump interactor, isoform 1), was identified in *A. thaliana* (Morandini et al. 2002). This protein binds to the C-terminal regulatory domain of the PM H^+-ATPase, in a site different from the 14-3-3 binding site, and stimulates its activity in vitro (Viotti et al. 2005). PPI1 comprises 612 amino acids and is rich in charged and polar residues, except for the C-terminus, which is made of 24 hydrophobic amino acids, presumably representing a transmembrane domain. *PPI1* is a member of a gene family apparently consisting of six members in *A. thaliana* that share 20–50% sequence identity at the protein level and do not resemble any protein of known function (Morandini et al. 2002). The expression analysis using reporter gene constructs revealed that *PPI1* is strongly expressed in root and shoot vascular systems, particularly in meristematic and sink tissues, as well as in pollen, stigmas and siliques, but not in developing embryos (Anzi et al. 2008). The main part of PPI1 is localized at the endoplasmic reticulum, from which it might translocate to the PM for interaction with the H^+-ATPase in response to as yet unidentified signals (Bonza et al. 2009).

Two putative proton pump interactor proteins, homologous to Arabidopsis PPI1, were identified as substrates of the brassinosteroid receptor kinase BRI1 (brassinosteroid insensitive 1) in rice (*Oryza sativa*) from a direct phosphorylation screening (Hiribayashi et al. 2004). Nevertheless, there is no evidence demonstrating that these proteins interact with or activate PM H^+-ATPases.

Several expressed sequence tags (ESTs) from different plants encode proteins with significant similarity to *PPI1*; however, none of them have been characterized in any plant species other than *A. thaliana* and rice. The aim of this work is to present the characterization of an *A. thaliana PPI1* homolog from potato (*Solanum tuberosum* L.), named *StPPI1*, that is up-regulated during tuber development and under abiotic stress.

Materials and methods

Plant material, growth conditions and stress treatments

Soil grown plants: potato (*Solanum tuberosum* cv. Spunta) and tomato (*Solanum lycopersicum* cv. Castlemart) plants were cultivated in a greenhouse under a 16 h light (25°C)–8 h dark (20°C) cycle. Detached leaflets of soil grown plants with their corresponding petioles were used for some experiments. Before stress treatments, detached leaflets were placed in individual containers with water, for 48 h, to allow the wound response components induced by leaflet excision to be restored to basal levels (Capiati et al. 2006). For elicitor treatments, leaflets were placed in buffer Tris–HCl 50 mM pH 6.5, instead of water, to avoid pH decrease when elicitor solutions were added. Detached leaflets were kept in a growth chamber under a 16-h light photoperiod (4,000 lx light intensity) at 22°C.

In vitro plants: in vitro potato (*Solanum tuberosum* cv Spunta) plants were obtained by micro-propagation of virus-free single-node cuttings in commercial Murashige and Skoog medium (MS; Prod N° M519, PhytoTechnology Laboratories, Shawnee Mission, KS) containing 20 g/L sucrose solidified with 0.7% (w/v) agar. Seeds of tomato plants (*Solanum lycopersicum* cv. Castlemart) were surface sterilized with a solution of 10% (v/v) commercial bleach

(0.525% sodium hypochlorite) for 5 min and washed three times with sterile distilled water. Seeds were germinated on commercial MS medium 0.7% (w/v) agar in glass recipients. Plants were grown for 1 (potato) or 3 weeks (tomato) in a growth chamber under a 16-h light photoperiod (4,000 lx light intensity) at 22°C, and later transferred to liquid MS medium for 7 days before the corresponding stress treatments, which were carried out as follows:

Salt stress: potato detached leaflets were treated with NaCl 250 mM in water; control leaflets were kept in water. In vitro tomato plants were treated with NaCl 300 mM in MS medium (control: MS medium).

Drought stress: potato detached leaflets and tomato in vitro plants were deprived of water or MS medium, respectively. Control leaflets or plants were kept in water or MS medium, respectively.

ABA: potato detached leaflets were treated with 100 μM abscisic acid (ABA) in water. Control leaflets were kept in water.

Cold stress: in vitro grown potato and tomato plants were placed at 4°C, while control plants remained at 22°C.

Mechanical wounding: the main vein and lamina of potato detached leaflets were cut with a dented forceps. Similarly, for tomato in vitro grown plants, the main veins and lamina of apical leaflets of compound leaves were wounded. Wounding did not cause any visible alterations within the proximities of the cut, such as necrosis or loss of turgor.

Fungal elicitors: detached leaflets of potato and tomato plants were treated with polygalacturonic acid (PGA) 50 μg/ml or chitosan 100 μg/ml in buffer (Tris–HCl 50 mM pH 6.5). Control leaflets were kept in buffer. The pH of the solution was checked every 24 h during the experiment.

Molecular cloning of *StPPI1*

StPPI1 cDNA containing the full-length coding sequence was cloned by reverse transcription-PCR (RT-PCR). RNA from potato leaves exposed to salt stress was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA). M-MLV Reverse Transcriptase (Invitrogen) was used for cDNA synthesis according to the manufacturer's protocol using the specific reverse primer 5′-CCCCACCTCTTTCT ACTTTTGAAG-3′. The newly synthesized cDNA was used as template for PCR amplification with the forward primer 5′-GAAATATGGGTGTAGAGGTGGAAGC-3′ and the reverse primer mentioned above. PCR reaction was carried out using Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, FI). Primer design was based on the potato TC170935 sequence (DFCI Potato Gene Index; http://www.compbio.dfci.harvard.edu/tgi/plant.html). The purified PCR product was blunt-end-cloned into an *Eco*RV-digested pZErO-2 vector (Invitrogen) and subjected to automated sequencing.

The genomic sequence of *StPPI1* was isolated by PCR amplification with Phusion high fidelity DNA polymerase, using the primers designed for cloning the full-length cDNA sequence. A 2,680-bp genomic fragment was cloned into an *Eco*RV-digested pZErO-2 vector (Invitrogen) and subjected to automated sequencing using a primer walking strategy. To determine the exon–intron structure, genomic and cDNA sequences were aligned and acceptor, donor and branch sites were predicted by a bioinformatic approach (Hebsgaard et al. 1996). The reported nucleotide sequence data (*StPPI1*) appears in the GenBank Database under the accession number GU808087.

Southern blot analysis

Genomic DNA was isolated from young leaves of greenhouse grown plants (Murray and Thompson 1980). Aliquots of DNA (10 μg) were digested overnight at 37°C with *Hin*dIII, *Eco*RI and *Eco*RV (New England Biolabs, Beverly, MA), concentrated in a Speed Vac to 45 μl, separated on 0.8% (w/v) agarose gels, and blotted onto a nylon membrane (Hybond N+, GE Healthcare, Piscataway, NJ). A fragment of 417 bp amplified from cDNA was used as probe for Southern hybridization. This fragment involves the first exon (from base 16) and the first 67 bp of the second exon. The probe was labeled with ^{32}P using the Prime-a-Gene DNA Labeling System kit (Promega, Madison, WI). The blot was hybridized at 60°C for 16 h. Washes were carried out in two steps. Two low-stringency washes were performed: 2× SSC/0.1% SDS for 10 min and 1× SSC/0.1% SDS for 10 min, at 40°C, followed by two high-stringency washes: 1× SSC/0.1% SDS for 10 min, 0.5× SSC/0.1% SDS, for 10 min at 50°C (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate). Bands were visualized using a Storm PhosphorImager (GE Healthcare).

StPPI1 expression and purification

The full-length *StPPI1* cDNA coding sequence was cloned into the prokaryotic expression vector pET28a (Novagen, Madison, WI). The resulting plasmid was transformed into *E. coli* strain BL21(DE3) Codon plus pRIL (Stratagene, La Jolla, CA) and expression was induced in liquid cultures at 37°C starting at OD_{595} 0.6 with 1 mM IPTG. After 3-h induction, cells were cooled on ice, centrifuged and stored at −80°C. Cell pellets were lysed in the presence of 50 mM NaH_2PO_4, 300 mM NaCl, 1.5 mM imidazole, 0.5% (v/v) NP-40 and sonicated until a clear, non-viscous solution was obtained. Protein was purified by Ni-NTA column (Qiagen, Germantown, MD), following manufacturer's instructions. Eluted fractions were monitored by

SDS polyacrylamide gel electrophoresis (SDS-PAGE), pooled and dialysed for 16 h at 4°C against 1 l 30 mM Tris–HCl pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$, 10% (w/v) glycerol, with a change in dialysis solution after 8 h. The purification steps of recombinant StPPI1 were monitored by SDS-PAGE followed by colloidal Coomassie staining. The purity of the recombinant StPPI1 was estimated to be 85–90% by densitometric analysis of Coomassie-stained gel using the ImageJ software (National Institutes of Health; http://www.rsb.info.nih.gov/ij). The identity of the protein was determined by Western blot analysis using an anti-polyhistidine-tag antibody (Sigma, St. Louis, MO, USA). The concentration of purified StPPI1 was estimated by comparison with BSA standards in SDS-PAGE and confirmed by Bradford (1976) assay. Recombinant StPPI1 shows a broad diffuse band of approx. 70 kDa in SDS-PAGE. Occasionally, it appears to be composed of more than one band (as shown in Fig. 3a), although it is not possible to resolve this band into different components. The reason for this atypical migration is not known. Western blot using anti-polyhistidine tag antibody shows a wide band that seems to correspond to the broad band of the Coomassie-stained gel. None of the bands around 70 kDa are present when other two proteins (StABF and TcPARP) used as negative control are purified, suggesting that they are not bacterial contaminants.

Western blot analysis

Protein samples were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare). The membranes were blocked with TBS buffer plus 0.05% (v/v) Tween 20 complemented with 5% (w/v) non-fat dried milk and incubated with anti-polyhistidine-tag antibody (Sigma) diluted 1:3,000 in TBS-0.05% (v/v) Tween 20 buffer plus 1% (w/v) BSA for 1 h. After extensive washing procedures, the bound primary antibody was detected with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody, using the ECL technique according to the manufacturer's recommended procedures (GE Healthcare).

PM H^+-ATPase activity

The vanadate-sensitive PM H^+-ATPase activity was assayed in 5 mM ATP, 5 mM $MgCl_2$ and 10 mM PIPES pH 7.3. To reduce the background of other ATPase activities, 5 mM NaN_3, 0.1 mM sodium molybdate and 100 mM KNO_3 (mitochondrial, soluble and vacuolar ATPase inhibitors, respectively) were added. The ATPase assay was run in the presence of 0.01 mg/ml of lysophosphatidylcholine to activate the PM proton pump by displacement of the autoinhibitory C-terminal domain (Papini and De Michelis 1997) and to ensure the access of the assay mix to the vesicle interior (Schaller and DeWitt 1995). Alternatively, activity was determined in the absence of lysophosphatidylcholine, with the addition of the detergent Brij 58 (0.1 mg/ml, Sigma) that reverses PM vesicles from the right-side-out to the inside-out form, exposing the active sites of PM proton pumps. Brij 58 increases PM H^+-ATPase activity without involving the displacement of the C-terminal autoinhibitory domain (Johansson et al. 1995). Purified plasma membranes (2.5 μg protein), obtained by a two-step aqueous two-phase partitioning system as described in Olivari et al. 2000, were incubated with His6-StPPI1 fusion protein at the concentrations specified in the figure legends, in the assay medium without ATP, at 0°C for 15 min. The assay was started by the addition of ATP. The final reaction volume was 150 μl. The reaction was carried out for the indicated times at 30°C. Released Pi was measured using the malachite green method (Hohenwallner and Wimmer 1973). The PM H^+-ATPase activity was determined as the difference between total activity and that measured in the presence of 100 mM vanadate.

Northern blot analysis and semi-quantitative reverse transcription-PCR (RT-PCR)

Samples (0.1–1 g) were collected, ground in liquid nitrogen and total RNA was extracted using the TRIzol Reagent (Invitrogen). Total RNA (10–20 μg) was separated on formaldehyde 1.2% (w/v) agarose gels and blotted onto nylon membranes (Hybond N+, GE Healthcare). Northern blots were hybridized with probes labeled with ^{32}P by random priming with Prime-a-Gene DNA Labeling System kit (Promega). The 417-bp fragment described in the subsection Southern blot analysis was used as probe for *StPPI1*. Other probes used in this study were: *Le25* homolog, *ATHB7* homolog, *Chitinase A*, *Tas14*, *Pin2* and *Patatin* (País et al. 2009, 2010). After sequential stringent washes (2× SSC/0.1% SDS for 10 min, 1× SSC/0.1% SDS for 10 min, 0.5× SSC/0.1% SDS for 10 min, at 65°C), bands were visualized using a Storm PhosphorImager (GE Healthcare).

Semi-quantitative RT-PCR was performed to detect *StPPI1* and *PM H^+-ATPase* transcripts (Figs. 5a, c, 7a). RNA was isolated as described above. M-MLV Reverse Transcriptase (Invitrogen) was used for cDNA synthesis according to the manufacturer's protocol using a mix of oligo(dT)12–18 and random primers (Invitrogen). The newly synthesized cDNA was used as template for PCR amplification with the following primers: *StPPI1* forward primer 5′-GAAATATGGGTGTAGAGGTGGAAGC-3′, *StPPI1* reverse primer 5′-GGAATTTTGGAGTGCTATTG-3′ (annealing temperature 51°C, 25 cycles), *PM H^+-ATPase* forward primer 5′-CACAGGCTCTCCCAACA

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StPPI1          MGVEVEAKLVHVPVEAGSEQINLLKENGKPNHVSGITEPIKFGSHGTEEPK--KEEVSRI 58
PPI1            MGVEVVNSGGFEVAPAPFE--GKPEKNGKLDQGKGDDAPINFGSVG-ELPKNAEENNNKV 57
                *****  .  .  . *  *  .  ::*** :: .*   **:*** * * **  :*: .::

StPPI1          PVSNVPKDAVEDWPEPKQIHSFYTVKFRRFDDPKLKARIELAEKELQKKNQARSQIIEKL 118
PPI1            VNSDAPKNAAEEWPVAKQIHSFYLVKYRSYADPKIKAKLDLADKELEKLNKARTGVLDKL 117
                  *:.**:*.*:** .******* **:* : ***:**:::**:***:* *:**: :::**

StPPI1          KAKRAEKSIFIEQRKALSAENKEFWSAIDGKIKEMVPLHEALGQLRGSRNAGRERGPTVC 178
PPI1            RAKRAERSELFDLLDPLKSERKGFNTMFDEKRKEMEPLQQALGKLR-SNDGGSARGPAIC 176
                :*****:* :::  ..*.:*.* * : :* * *** **::***:** *.:.*  ***::*

StPPI1          SSEEELNHLIKGLQYRIQHESIPLNEEKQILREIKQLEGTREDVKKVAAARAQIHETMGE 238
PPI1            SSEEELNSMIYSYQYRIQHESIPLTEEKQILKEIRLLEGTRDKVIANAAMRAKIKESMGQ 236
                ******* :* . ***********.******:**: *****:.*   ** **:*:*:**:

StPPI1          KESIQNQVKLMNVGLDGVRKGQQEVKGRLKIIDDQIDAINKQIGILDEELKGVVEKRDKT 298
PPI1            KDDIQGQVKLMGAGLDGVKKERQAISARINELSEKLKATKDEITVLENELKTVSEKRDKA 296
                *:.**.*****..*****:* :* :..*:: :.::::.* :.:* :*::*** * *****:

StPPI1          YEHILELRKLREEGNSSFYQNSNVLHKVKQLADQKDVGALKELSVTEVDKFISLWCGSKS 358
PPI1            YSNIHDLRRQRDETNSEYYQNRTVLNKARDLAAQKNISELEALANAEVEKFISLWCSKKN 356
                *.:* :**: *:* **.:*** .**:*.::** **::. *: *: :**:*******..*.

StPPI1          FRDDYEKRLLQSLDIRQLSRDGRMRNPGEKPLVLPEAP----TVSRAEVPARANAKPVKE 414
PPI1            FREDYEKRILQSLDSRQLSRDGRMRNPDEKPLIAPEAAPSKATPSETEVVPKAKAKPQPK 416
                **:*****:***** ************.****: ***.    * *.:** .:*:***  :

StPPI1          DHAPVDAAPIQKEQQEKSGKQLNDAHGKNTKSTEKKAVVVDDEEIYGLDLP-KDIKPKKT 473
PPI1            E-EPVSAPKPDATVAQNTEKAKDAVKVKNVAD-------DDDDEVYGLGKPQKEEKP--- 465
                :  **.*.  :    ::: *  : .: **. .        **:*:***. * *: **

StPPI1          EVDEATLKEMKKEEEIAKNKQAMERKRKLAEKAAAKAAKKAQLEAEKKLK-EREKKAKKK 532
PPI1            -VDAATAKEMRKQEEIAKAKQAMERKKKLAEKAAAKAAIRAQKEAEKKEKKEQEKKAKKK 524
                 ** ** ***:*:***** *******:*********** :** ***** * *:*******

StPPI1          GGSSAAGQEPTEEPTETPEEIAEEENAEETVETAVAPKVKARKDNTIRHRATRARGSE-L 591
PPI1            TGG------NTETETEEVPEASEEE-IEAPVQEEKPQKEKVFKEKPIRNR-TRGRGPETI 576
                 *.       **  **   * :***  * .*:   . * *. *::.**:* **.**.* :

                                  ________________________
StPPI1          PKSILKRKKATNYWLWAAAPAALAILVLLVIGYMYLQK 629
PPI1            PRAILKRKKSTNYWVY-AAPAALVVLLLLVLGYYYVL- 612
                *::******:****:: ******.:*:***:** *:
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Fig. 1 Alignment of the deduced protein sequence of *StPPI1* with *A. thaliana PPI1*. Sequences were translated and aligned with the ClustalW program (http://www.ebi.ac.uk/clustalw). Identities are indicated by *asterisks* below the sequence. Conserved substitutions are marked with two *vertical dots* and semi-conserved substitutions are marked with a *single dot*. Unaligned residues are shown as *dashes* within the sequence line. The putative transmembrane domain is indicated by a *line*. The partially conserved stretch among PPI from other plant species is shaded in *gray*

G-3′, *PM H^+-ATPase* reverse primer 5′-TAGGTCCAAAA TCTGCTC-3′ (annealing temperature 45.5°C, 29 cycles). The primers for *PM H^+-ATPase* were designed based on the TC197371 sequence of DFCI Potato Gene Index, which corresponds to the sequence *PHA2* (GeneBank Accession number: X76535.1). Only another potato *PM H^+-ATPase* was detected in databases: *PHA1* (GeneBank Accession number: X76536.1; TC194798, DFCI Potato Gene Index). The primers were chosen to anneal to highly conserved sequences in both genes, so that they could detect at least the *PHA2* and *PHA1* isoforms. RT-PCR was also performed at an annealing temperature of 40°C obtaining the same pattern of expression as at 45.5°C (not shown). 18S-rRNA was used for normalization of mRNA expression levels (primer 18S forward 5′-GGGCATTCGTATTT CATAGTCAGAG-3′, primer 18S reverse 5′-CGGTTCT TGATTAATGAAAACATCCT-3′; annealing temperature 58°C, 20 cycles). The described conditions were chosen to ensure that the PCR reaction was terminated within the linear range of amplification.

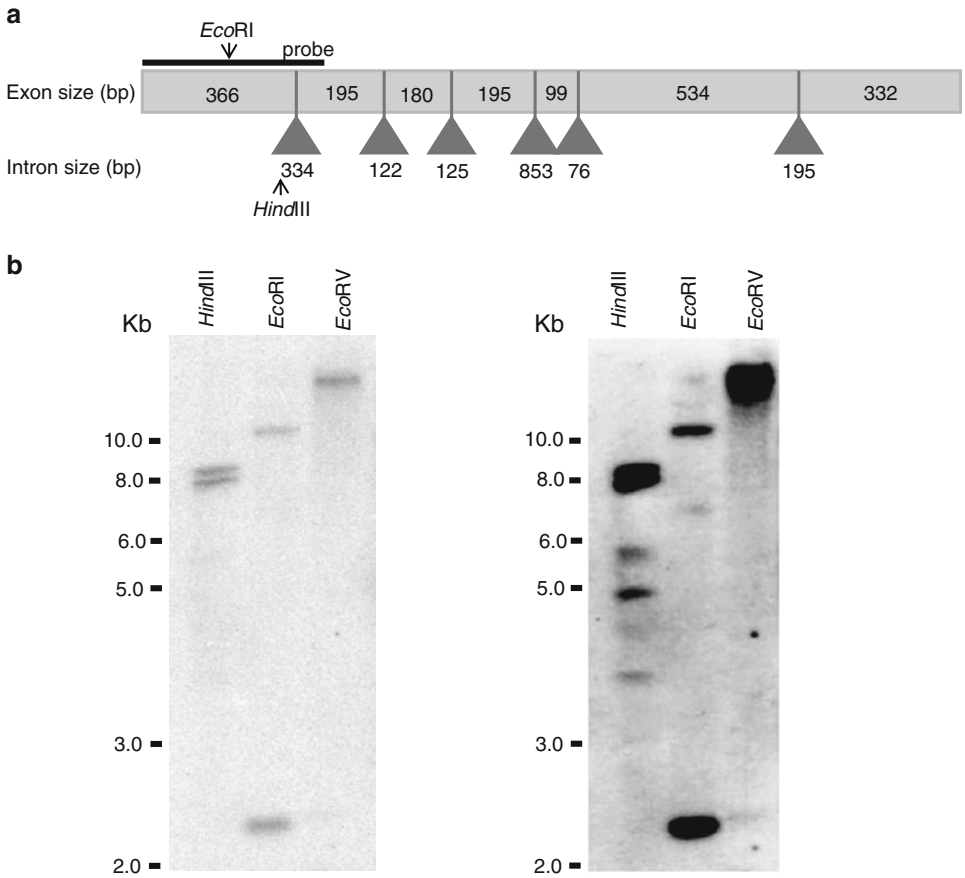

Fig. 2 Gene structure and Southern blot analysis of *StPPI1*. **a** Schematic representation of gene structure with position and size of introns and exons. The region corresponding to the probe used for Southern blot is marked with a line, and the restriction sites of the enzymes used are indicated within this region. **b** Sothern blot analysis of *S. tuberosum* genomic DNA digested with *Hin*dIII, *Eco*RI or *Eco*RV and hybridized to the *StPPI1* probe (**a**), under both high-stringency (*left*) and low-stringency (*right*) conditions. DNA size markers are indicated on the *left* of each blot

Northern blot bands of specific mRNAs were quantified relative to the ethidium bromide-stained ribosomal RNA (rRNA). RT-PCR bands were quantified relative to the internal control 18S-rRNA. Quantifications were carried out using ImageJ software. Each experiment was performed at least three times independently. Statistical analysis was carried out using the Student's *t* test. The data were considered significantly different when $p < 0.05$. In time–response experiments, controls (without stress treatment) were carried out for all the time points analyzed to ensure that control values remain unchanged throughout the experiment (only one control is shown in the blots or RT-PR gels). For each independent experiment, control samples values were averaged, and all values were calculated relative to this average which was arbitrarily set to 1.

Results

Molecular characterization of *StPPI1*

RT-PCR was used to obtain the full-length coding sequence of *StPPI1*. The open reading frame (ORF) encodes a protein of 629 amino acids with a predicted molecular mass of 71.1 kDa. The putative protein contains 400 uncharged residues and 229 charged residues (121 positive, 108 negative). StPPI1 shows 50% amino acid sequence identity with *A. thaliana* PPI1 (Fig. 1). The C-terminus presents a hydrophobic stretch of 23 amino acids probably representing a transmembrane domain highly similar to that found in *A. thaliana* PPI1. StPPI1 shows 75% identity with *A. thaliana* PPI1 within a partially conserved stretch among translated ESTs from several other plant species (residues 338–390 of StPPI1; Morandini et al. 2002).

Position and size of introns and exons were determined as described in "Materials and methods". The *StPPI1* ORF is divided into seven exons split by six introns of variable length, from 76 to 853 nucleotides (Fig. 2a). All the exons are flanked by the canonical consensus splice sites, AG at the 3′ splice site and GT at the 5′ splice site.

Southern blot analysis was performed as described in "Materials and methods". Figure 2a shows the position of the *StPPI1* probe used for hybridization. High stringency washes revealed a unique band obtained for DNA cut with *Eco*RV, while two bands were observed when DNA was digested with *Hind*III and *Eco*RI, which cut within the region recognized by the probe (Fig. 2b, left). Low stringency washes showed additional bands of lower intensity (Fig. 2b, right). These results suggest the existence of related homologs of *StPPI1* detectable by hybridization.

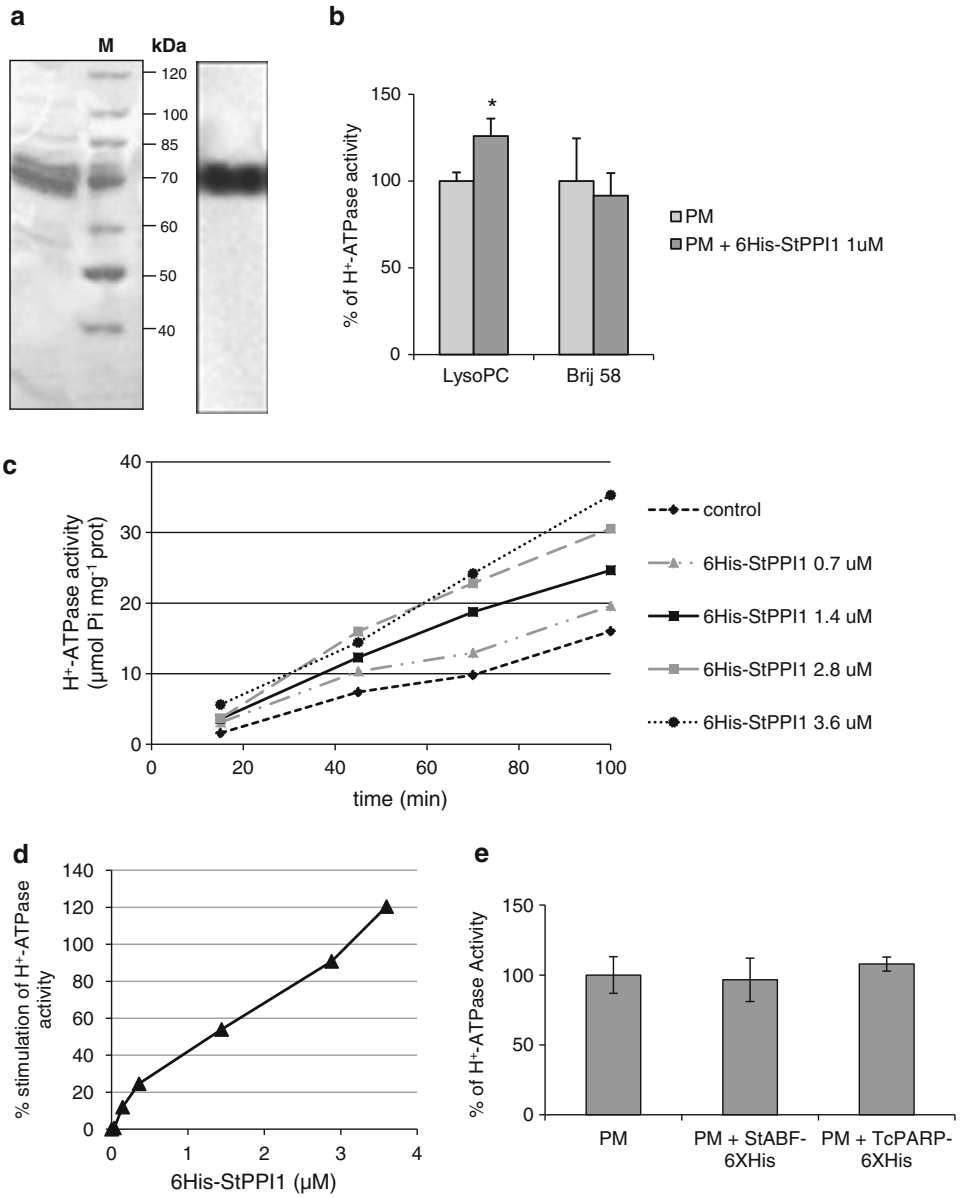

Fig. 3 Recombinant *StPPI1* stimulates *PM H*$^{+}$*-ATPase* activity. **a** *Left* Coomassie-stained SDS-PAGE gel of purified StPPI1, *M* molecular mass ladder. *Right* Western blot analysis of purified protein using the anti-polyhistidine-tag antibody. **b** Effect of recombinant StPPI1 (1 µM) on the PM H^{+}-ATPase activity measured in the presence or absence of lysophosphatidylcholine (LysoPC and Brij 58, respectively) for 30 min. Activity is expressed as percent activity with respect to the control (isolated plasma membranes, PM, arbitrarily set to 100%). Statistical analysis was carried out using the Student's *t* test. The results shown are mean ± SD (*$p < 0.05$, StPPI1 vs. control). **c** Effect of increasing concentration of recombinant StPPI1 on the PM H^{+}-ATPase activity assayed in the presence of lysophosphatidylcholine for the indicated times. **d** Activity assayed in the presence of lysophosphatidylcholine for 90 min using different concentrations of recombinant StPPI1. The activity is expressed as percent stimulation above control samples measured in the absence of *StPPI1*. Data reported in **c** and **d** show the average of one experiment run in duplicate, representative of two independent experiments. **e** Effect of unrelated His-tagged proteins (StABF-6×His and TcPARP-6×His) on PM H^{+}-ATPase activity. Both proteins were used at a final concentration of 3.6 µM. The activity is expressed as percent stimulation with respect to the control (isolated plasma membranes, PM, arbitrarily set to 100%). The results shown are mean ± SD of two independent determinations

StPPI1 stimulates PM H^{+}-ATPase activity

The full-length *StPPI1* coding region was expressed as a fusion protein with a His-tag in *E. coli*. The recombinant protein was purified as described in "Materials and methods". Western blot analysis of purified StPPI1, using an anti-polyhistidine-tag antibody, showed a band of approximately 70 kDa, as expected for the recombinant protein (Fig. 3a).

The purified recombinant protein was used in PM H^{+}-ATPase activity assays to determine whether StPPI1 could

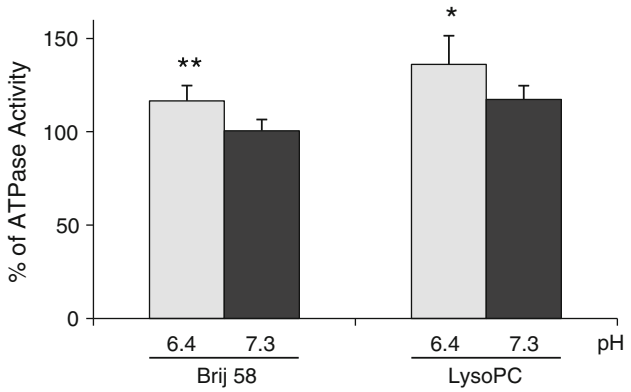

Fig. 4 Effect of recombinant *StPPI1* on the *PM H*$^+$*-ATPase* activity at different pHs. Effect of recombinant StPPI1 (0.5 μM) on the PM H$^+$-ATPase activity measured in the absence or presence of lysophosphatidylcholine (Brij 58 and LysoPC, respectively). Activity is expressed as percent activity with respect to the control (isolated plasma membranes in the absence of recombinant StPPI1, arbitrarily set to 100%). Statistical analysis was carried out using the Student's *t* test. The results shown are mean ± SD (**$p < 0.005$, *$p < 0.05$ for 7, 3 vs. 6.4)

affect the enzyme activity. It has been established that *A. thaliana* PPI1 is not able to suppress the auto-inhibitory action of the C-terminal domain, but can hyper-activate H$^+$-ATPase molecules whose C-terminus has been displaced by other factors, such as 14-3-3 proteins (Viotti et al. 2005), controlled tryptic cleavage or treatment with lysophosphatidylcholine (Papini and De Michelis 1997). Therefore, the ATPase assay was run in the presence of 0.01 mg/ml of lysophosphatidylcholine to activate the PM proton pump prior to the addition of recombinant StPPI1. Alternatively, activity was measured in the absence of lysophosphatidylcholine, using the detergent Brij 58. Assays were carried out at pH 7.3, typical of the cytoplasm of a plant cell. StPPI1 increased PM H$^+$-ATPase activity in the presence of lysophosphatidylcholine, but had no effect in the absence of the lysophospholipid (Fig. 3b), suggesting that StPPI1 is unable to suppress the auto-inhibitory effect of the proton pump C-terminus, but further enhances the activity of the PM H$^+$-ATPase whose C-terminus has been displaced by lysophosphatidylcholine. StPPI1 stimulated the PM proton pump activity in a dose-dependent manner (Fig. 3c, d).

Other recombinant proteins containing the same His-tag were used in the PM H$^+$-ATPase activity assays instead of StPPI1: StABF, a *S. tuberosum* transcription factor of 50 kDa and TcPARP, a *Trypanosoma cruzi* poly ADP-ribose polymerase of 65 kDa. Both proteins were expressed, purified and dialyzed following the same protocol used for StPPI1. None of these proteins affected the enzyme activity when used at the same concentration as StPPI1 (Fig. 3e). This result confirms that neither the His-tag motif nor the trace contaminant bacterial proteins that remain after purification were responsible for the effect on PM H$^+$-ATPase activity. In addition, assays were performed using whole cell extracts from induced and non-induced cultures of *E. coli* transformed with the StPPI1 expression vector. Induced extracts stimulated PM H$^+$-ATPase activity by 76.0 ± 36.6 ($n = 4$) above control (non-induced extracts), confirming that StPPI1 is responsible for the stimulation of PM H$^+$-ATPase.

When PM H$^+$-ATPase activity was determined in the presence of non-dialyzed TcPARP (eluted with 250 mM imidazole), activity was increased by 161 ± 31%, while dialyzed TcPARP (added at the same volume of non-dialyzed samples) showed no significant effect on PM H$^+$-ATPase activity (8 ± 5%). These results indicate that the dialysis protocol applied to the purified recombinant proteins removes the contaminants that might interfere with the activity assays.

Activation of PM H$^+$-ATPase by StPPI1 was also determined at pH 6.4 in the presence of Brij 58 or lysophosphatidylcholine. In the assays carried out with Brij 58, StPPI1 was able to increase PM H$^+$-ATPase activity at pH 6.4, while no activation occurred at pH 7.3. In the presence of lysophosphatidylcholine, the activation by StPPI1 was higher at pH 6.4 than at pH 7.3 (Fig. 4).

StPPI1 expression in different tissues and during tuber development

StPPI1 mRNA was detected in all of the tissues screened by RT-PCR (Fig. 5a). However, *StPPI1* showed higher expression levels in proliferative regions (shoot apex and flower buds), flowers and leaves than in shoots and roots.

To determine if steady-state *StPPI1* mRNA level changes during tuber formation, total RNA was extracted from progressive stages of tuber development and Northern blot analysis was performed to detect *StPPI1* transcript. The same blot was hybridized with *Patatin* probe (Fig. 5b, Yamagishi et al. 1993). Patatin is the major storage protein of potato tubers and the expression of this gene was used as a marker for tuberization. Four stages, classified according to tuber morphology and size (photograph in Fig. 5b), were analyzed. For each stage, random samples were collected from different plants and pooled to normalize the effect of variations in the biological replicates. *StPPI1* expression levels increased during tuber development and then decreased in the last stage (S4), when the tuber is already formed (Fig. 5b). *PM H*$^+$*-ATPase* mRNA levels also increased during tuber development and decreased in mature tubers (Fig. 5c).

StPPI1 expression profiles under stress conditions

Time-course expression of *StPPI1* mRNA under different stress conditions was determined. The expression of

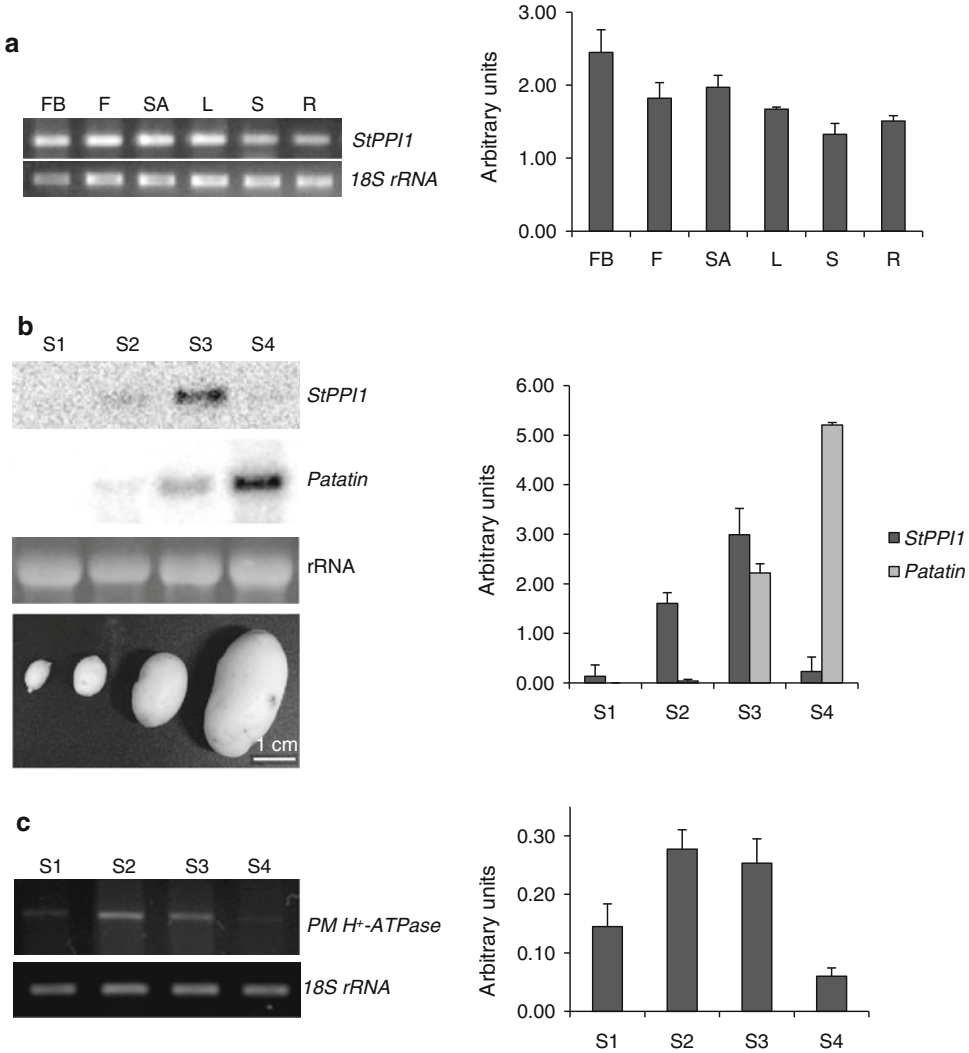

Fig. 5 Expression of *StPPI1* in different tissues and during tuber development. **a** Total RNA was isolated from different tissues of soil grown potato plants, cDNA was synthesized and semi-quantitative RT-PCR for *StPPI1* was performed. Quantitative data of RT-PCR bands (mean ± SD) are displayed in the *bar graph*. A representative example of RT-PCR analysis is shown. **b**, **c** Total RNA was extracted from progressive stages of tuber development (S1–S4). Northern blot hybridization with *StPPI1* and *Patatin* probes was performed (**b**) and semi-quantitative RT-PCR for *PM* H^+*-ATPase* was carried out (**c**). Quantitative data of Northern blots or RT-PCR are displayed in the *bar graphs* (mean ± SD). Representative blots or RT-PCR analyses are shown. *FB* Flower bud, *F* flower, *SA* shoot apex, *L* leaf, *S* stem, *R* root, *rRNA* ribosomal RNA

specific stress-induced genes was also determined to monitor stress responses.

Salt stress increased mRNA levels of *StPPI1* after a short time of exposure (2 h; Fig. 6a). A slight increase in *StPPI1* transcript levels was observed in response to drought after 8 h (Fig. 6b). The phytohormone abscisic acid (ABA), the major player in mediating the physiological adaptation of plants to water deficit, did not affect *StPPI1* expression (Fig. 6c). Low temperature up-regulated *StPPI1* mRNA levels after long-term exposure (48 h; Fig. 6d). A potato homolog of the tomato late embryogenesis abundant gene *Le25* (TC153563, DFCI Potato Gene Index) was used as marker of salt stress, dehydration and ABA responses (Fig. 6a–c, respectively). This gene was shown to be induced by salt stress in potato leaves (País et al. 2009). The potato homolog of *Arabidopsis ATHB7* (homeobox-leucine zipper protein 7, TC140026) was determined as marker of cold response in potato leaves (Rensink et al. 2005; Fig. 6d).

A slight increase in *StPPI1* transcript levels was observed in response to mechanical wounding after 0.5–1 h (Fig. 7a). No significant changes in *StPPI1* expression were observed in response to the fungal elicitor polygalacturonic acid (Fig. 7b). The same result was obtained using 100 µg/ml chitosan (not shown). The expression of *Pin2* (proteinase inhibitor 2; Sanchez-Serrano et al. 1986) and the pathogenesis related (PR) protein *chitinase A* (Büchter et al. 1997) were determined as markers of mechanical wounding and defense response against fungal pathogens, respectively. Low pH stress (from 6.3 to 4.3) did not significantly affect *StPPI1* mRNA levels after 2.5, 6, 14 or 30 h of exposure (not shown).

PPI1 expression profiles under stress conditions in tomato (*Solanum lycopersicum* L.)

Bioinformatic searches of the DFCI Potato Gene Index revealed the presence of a homolog of *StPPI1* in tomato

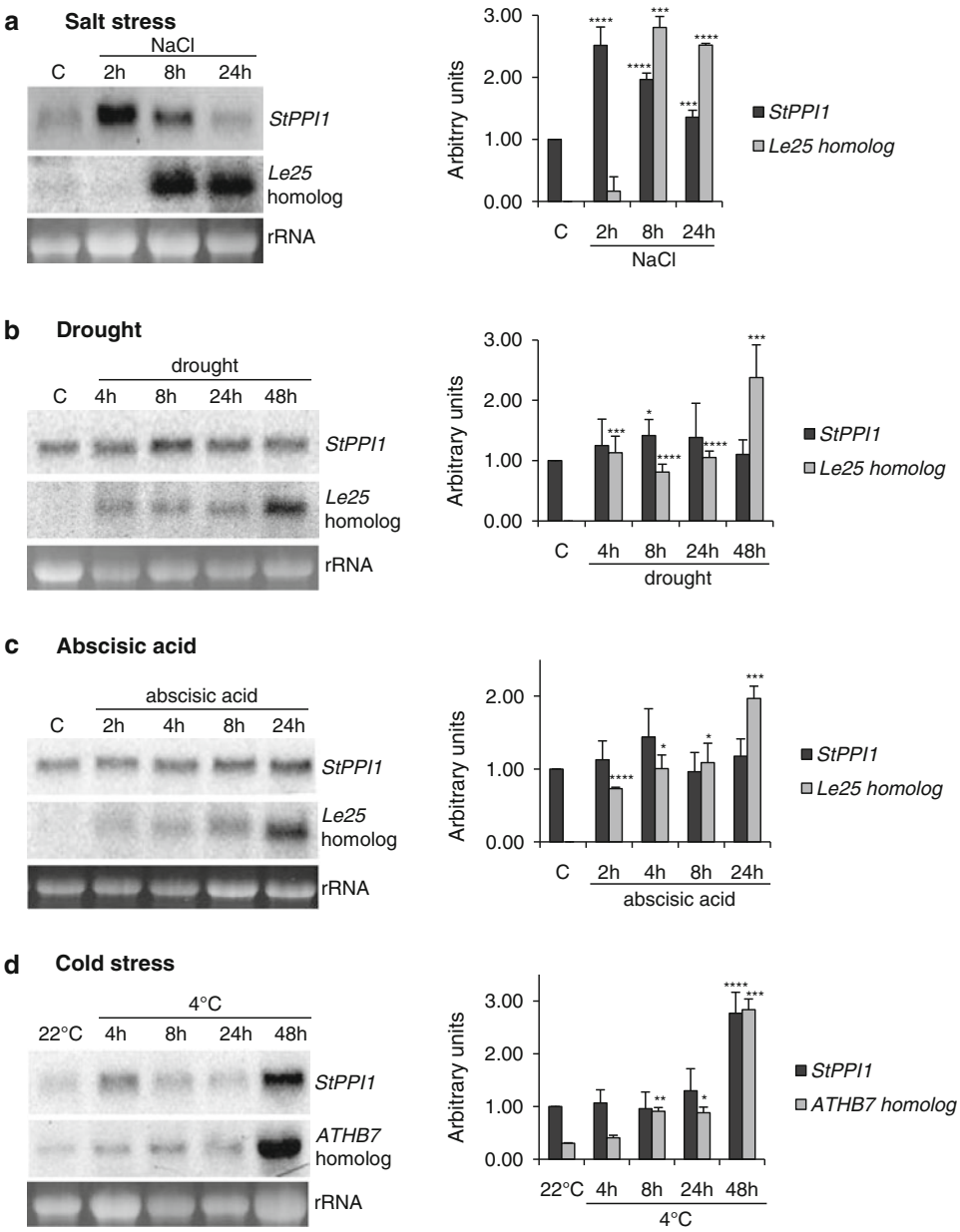

Fig. 6 *StPPI1* expression profiles under salt stress, drought and cold. **a** Detached leaflets of potato plants were treated with 250 mM NaCl for the indicated times. **b** Potato detached leaflets were deprived of water for the indicated times. **c** Potato detached leaflets were treated with 100 µM abscisic acid (ABA) for the indicated times. **d** In vitro grown potato plants were exposed to 4°C for the indicated times. Total RNA was isolated from leaflets and Northern blot hybridization was performed with *StPPI1* probe and the corresponding stress marker probes (*Le25* homolog or *ATHB7* homolog). Quantitative data of Northern blots (mean ± SD) are displayed in the *bar graphs* (arbitrary units). *StPPI1* values were calculated relative to control sample values, which were arbitrarily set to 1. The *asterisks* indicate statistical significance (****$p < 0.001$, ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$ with respect to controls, by Student's *t* test). Representative blots are shown. *C* Control, *rRNA* ribosomal RNA

(TC193097), arbitrarily named *SlPPI1*. SlPPI1 and StPPI1 share 96% sequence identity at the protein level. Since *StPPI1* probe presents a high identity (99%) with *SlPPI1* cDNA sequence, it was used to detect *SlPPI1* transcripts in tomato leaves under different stress conditions.

Salt stress did not affect *SlPPI1* expression (Fig. 8a), while drought significantly increased its transcript levels after 24–48 h of exposure (Fig. 8b). *SlPPI1* mRNA levels were up-regulated by cold showing slight increases after 2–8 h and significant increases after 24–48 h (Fig. 8c). The expression of *Tas14* was determined to monitor the response to salt, drought and cold stress. *Tas14* encodes a dehydrin induced by osmotic stress and abscisic acid treatment in tomato plants (Godoy et al. 1990). *Tas14* was also shown to be induced by drought and cold in tomato leaves (Fig. 8b, c).

A slight increase in *SlPPI1* expression was observed in response to mechanical wounding after 2 h (Fig. 8d). No significant changes were observed in response to the fungal elicitor chitosan (Fig. 8e). The same result was obtained using polygalacturonic acid 50 µg/ml (not shown). *Pin2* and *Chitinase A* were determined as markers of mechanical wounding and defense response against fungal pathogens, respectively.

Discussion

In this paper, we report the cloning of *StPPI1*, a *Solanum tuberosum* homolog of the *A. thaliana* proton pump interactor 1 (PPI1) that is up-regulated during tuber development

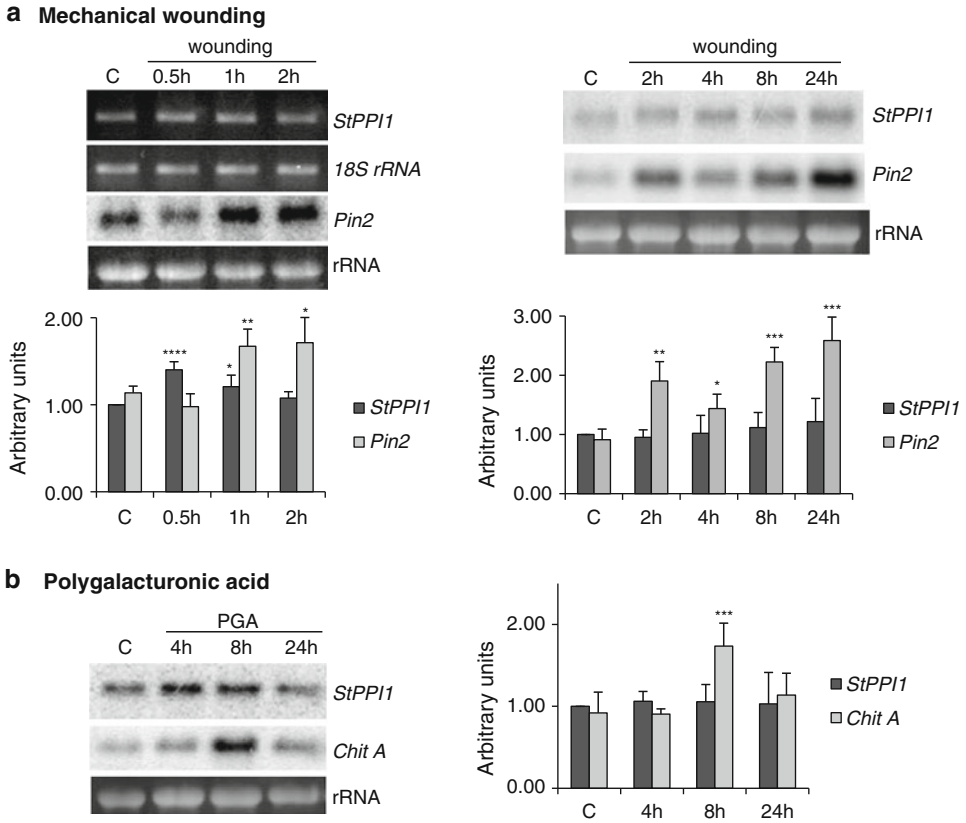

Fig. 7 Expression of *StPPI1* in response to mechanical wounding and fungal elicitors. **a** Potato detached leaflets were subjected to mechanical wounding and collected after the indicated times. **b** Potato detached leaflets were treated with polygalacturonic acid (PGA) 50 μg/ml for the indicated times. Total RNA was isolated and semi-quantitative RT-PCR for *StPPI1* or Northern blot hybridization for *StPPI1* and the corresponding stress markers (*Pin2* or *chitinase A*) was performed. Quantitative data of Northern blots or semi-quantitative RT-PCR (mean ± SD) are displayed in the *bar graphs* (arbitrary units). *StPPI1* values were calculated relative to control sample values, which were arbitrarily set to 1. The *asterisks* indicate statistical significance (****$p < 0.001$, ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$ with respect to controls, by Student's *t* test). Representative blots or RT-PCR analyses are shown. *C* Control, *rRNA* ribosomal RNA, *Chit A chitinase A*

and in response to abiotic stress in potato plants. The ORF encodes a protein of 629 amino acids showing 50% identity with *A. thaliana* PPI1 protein. Southern blot analysis suggests the existence of other genes related to *StPPI1* that share homology within the region encoding the N-terminal amino acid sequence of the protein. The N-terminal region of *A. thaliana* PPI1 is involved in the interaction with the PM H^+-ATPase (Morandini et al. 2002), however, this region is not conserved between *A. thaliana* PPI family members (PPI1-6), that show the most conserved region in the middle part of the protein-coding sequence. The results obtained suggest that other members of the PPI family, different from those found in *A. thaliana*, are encoded in the genome of *S. tuberosum*. Supporting this hypothesis, a homology search in the *Solanum phureja* genome database (http://www.potatogenome.net, database *Solanum phureja* scaffold v3) using the sequence of the first exon of *StPPI1* revealed the existence of four sequences from different scaffolds (PGSC0003DM000002385, PGSC0003DM000001642, PGSC0003DM000001344, PGSC0003DM000004352) showing more than 60% identity with the query.

Recombinant StPPI1 enhances PM H^+-ATPase activity in a dose-dependent manner. StPPI1 increases PM H^+-ATPase activity in the presence of lysophosphatidylcholine, but has no effect in the absence of the lysophospholipid. The activation of PM H^+-ATPase by StPPI1 is higher at pH 6.4 than at pH 7.3. The available evidence indicates that *A. thaliana* PPI1 is not able to suppress the auto-inhibitory action of the C-terminal domain, but can hyperactivate H^+-ATPase molecules, whose C-terminus has been displaced by other factors, such as 14-3-3 proteins, low pH and lysophospholipids (Morandini et al. 2002; Viotti et al. 2005). The results obtained in this study suggest that the modulation of PM H^+-ATPase activity by StPPI1 is consistent with the model proposed for *A. thaliana* PPI1. It would be interesting, in future studies, to confirm in vivo the activation of PM H^+-ATPase by

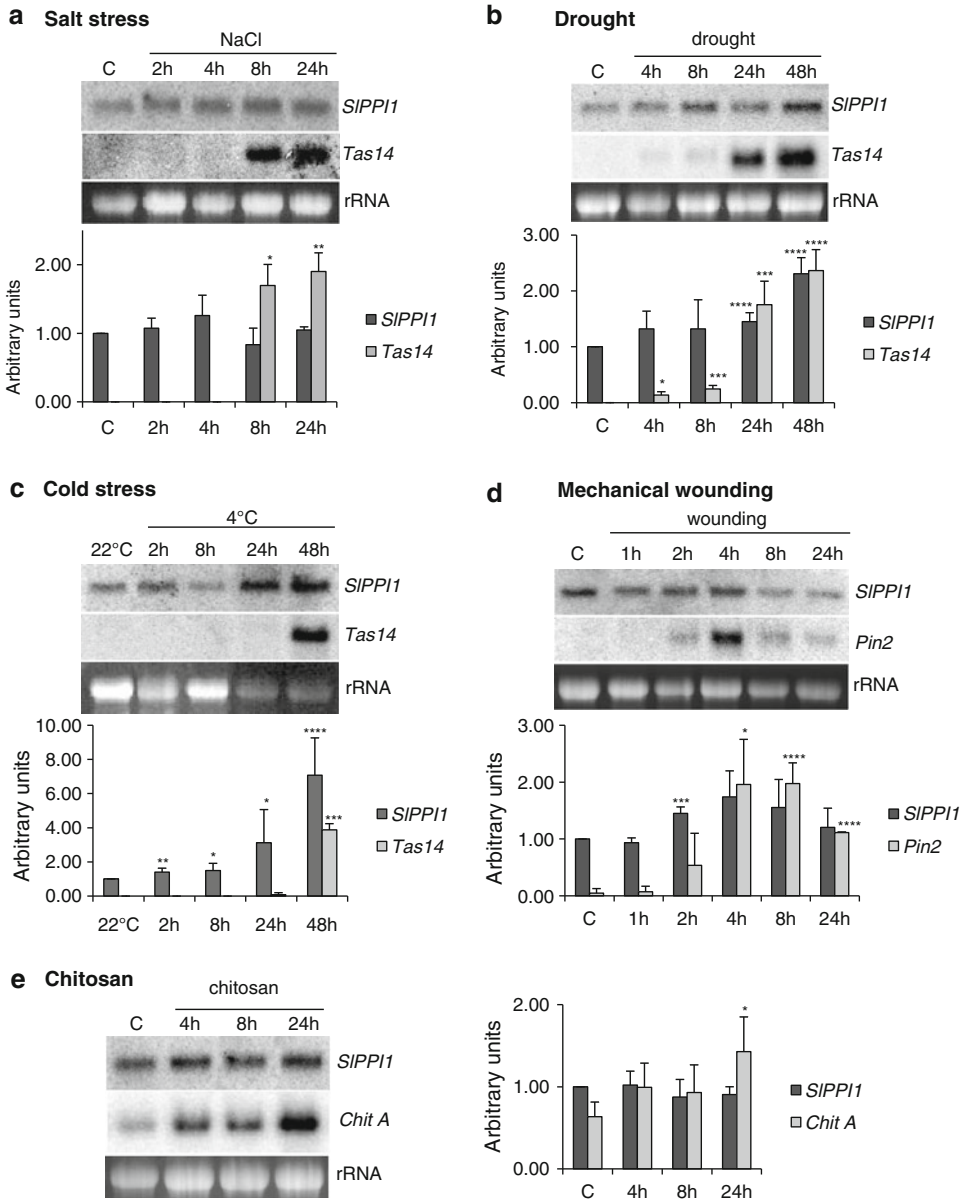

Fig. 8 Expression profiles of tomato *SlPPI1* under stress conditions. In vitro grown tomato plants were treated with 300 mM NaCl (**a**) or were deprived of water (**b**) for the indicated times. In vitro grown tomato plants were exposed to 4°C (**c**) for the indicated times, or were subjected to mechanical wounding (**d**) and collected after the indicated times. Tomato detached leaflets were treated with chitosan 100 µg/ml (**e**) for the indicated times. Total RNA was isolated from leaflets and Northern blot hybridization was performed with *StPPI1* probe and the corresponding stress marker probes (*Tas14*, *Pin2* or *chitinase A*). Quantitative data of Northern blots (mean ± SD) are displayed in the *bar graphs* (arbitrary units). *StPPI1* values were calculated relative to control sample values, which were arbitrarily set to 1. The *asterisks* indicate statistical significance (****$p < 0.001$, ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$ with respect to controls, by Student's *t* test). Representative blots are shown. *C* Control, *rRNA* ribosomal RNA, *Chit A chitinase A*

StPPI1 in potato plants to establish the significance of the in vitro results.

StPPI1 is expressed in all tissues analyzed. Its expression is higher in proliferative regions (shoot apex and flower buds), flowers and leaves than in shoots and roots. *StPPI1* RNA levels increase during tuber development, suggesting a possible role in the regulation of this process. This hypothesis is supported by the fact that at least one *PM* H^+*-ATPase* isoform expression also increases during tuber development, although more studies are necessary to corroborate this idea, especially considering the differences in the expression profile and in the magnitude of induction. The primers used to determine the expression of potato *PM* H^+*-ATPase* would detect the *PHA2* and *PHA1* isoforms, which are the only *PM* H^+*-ATPases* reported for *S. tuberosum* (Harms et al. 1994), however, the possibility that other isoforms may be amplified by the primers cannot be excluded. It would be interesting in the future to identify and characterize the isoforms that are induced during tuber development.

The analysis of expression profiles under different stress conditions revealed that *StPPI1* mRNA levels increase rapidly in response to NaCl treatment and after long-term exposure to low temperature. The differences observed in the response time to each stress probably reflect differences in the perception and transduction mechanisms of the stress signals, which have distinct features. Drought and mechanical wounding slightly increase *StPPI1* expression. No significant changes were observed in *StPPI1* expression in response to fungal elicitors or low pH stress, at least in

the time points analyzed. Publicly available microarray data from Arabidopsis (AtgenExpress website jsp.weigelworld.org/expviz/expviz.jsp) revealed that *PPI1* expression increases under salt and cold stress, similarly to *StPPI1* (Anzi et al. 2008), although there are differences in the response time between both species, probably due to differences in the stress conditions applied.

As mentioned before, it has been shown that salt and cold stress increase PM H^+-ATPase activity in different plant species, therefore, its involvement in the response to salinity or low temperatures is quite evident. However, there is not much evidence that such factors alter PM H^+-ATPase protein abundance. Studies, including the measurement of protein levels under stress revealed that the amount of PM H^+-ATPase protein remains unchanged (Sahu and Shaw 2009; Sibole et al. 2005; Wu and Seliskar 1998). In this context, regulation of PM H^+-ATPase activity under stress conditions is crucial for plant survival. The identification of the novel regulators PPIs may provide new insights into the mechanism of action of PM H^+-ATPase in stress tolerance. In future studies, we will attempt to determine if salt stress and cold increase the activity of PM H^+-ATPase in potato plants and to identify the proton pump that physically interacts with *StPPI1*. This will help in better understanding the mechanism of stress tolerance involving the regulation of plasma membrane proton pumps in *S. tuberosum*.

In the expression profiles, only *StPPI1* transcripts are detected. However, as suggested by Southern blot analysis, additional genes related to *StPPI1* may exist, and other members of this potato PPI family might be involved in a different way in development or in stress response.

In conclusion, this study presents the first characterization of StPPI1 from *S. tuberosum*, an economically important food crop of the Solanaceae family. The results obtained support the hypothesis that StPPI1 may be involved in tuber formation and adaptation responses to abiotic stress. This hypothesis of work will be further developed in future studies, due to the importance of these physiological processes to crop yield and plant productivity under adverse conditions.

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