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ORIGINAL ARTICLE

Characterization of potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) protein phosphatases type 2A catalytic subunits and their involvement in stress responses

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Abstract Protein phosphorylation/dephosphorylation plays critical roles in stress responses in plants. This report presents a comparative characterization of the serine/threonine PP2A catalytic subunit family in *Solanum tuberosum* (potato) and *S. lycopersicum* (tomato), two important food crops of the Solanaceae family, based on the sequence analysis and expression profiles in response to environmental stress. Sequence homology analysis revealed six isoforms in potato and five in tomato clustered into two subfamilies (I and II). The data presented in this work show that the expression of different PP2Ac genes is regulated in response to environmental stresses in potato and tomato plants and suggest that, in general, mainly members of the subfamily I are involved in stress responses in both species. However, the differences found in the expression profiles between potato and tomato suggest divergent roles of PP2A in the plant defense mechanisms against stress in these closely related species.

Keywords PP2A · *Solanum lycopersicum* · *Solanum tuberosum* · Stress

Abbreviations

PP2A	Protein phosphatase type 2A
PGA	Polygalacturonic acid

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Introduction

Protein phosphorylation and dephosphorylation represent a major form of reversible post-translational modification that regulates many cellular functions (Hunter 1995). Many serine/threonine protein phosphatases (PP1, PP2A, PP2C and novel phosphatases: PP4, PP5, PP6, PP7 and protein phosphatases with kelch-repeat domains) have been identified in plants (Luan 2003; Farkas et al. 2007 and references therein). Protein phosphatase 2A (PP2A) holoenzyme consists of a 36 kDa catalytic subunit (PP2Ac) that occurs either in association with a 65 kDa regulatory A subunit or together with a third variable B subunit (Virshup 2000; Janssens and Goris 2001). The B subunits, which determine the substrate specificity and subcellular localization of PP2As, are classified into 55 kDa B, 54–74 kDa B′ and 72–130 kDa B′′ subunits families. Genes encoding PP2A subunits have been characterized in several plant species. In *Arabidopsis thaliana*, five genes encoding the catalytic subunit PP2Ac, three genes encoding the A subunit, three genes encoding the B regulatory subunit, nine genes encoding the B′ subunit and six genes encoding the B′′ subunit have been identified (Farkas et al. 2007).

Protein kinases and phosphatases play key roles in stress signaling leading to adaptive responses. In contrast to protein kinases, the understanding of the functional roles of protein phosphatases has been developed only recently. Evidence on the participation of PP2As in stress responses was found in some plant species. The *Arabidopsis RCN1* gene encoding an A subunit is a positive transducer of the response to ionic, osmotic, and oxidative stress (Blakeslee et al. 2008). In rice (*Oryza sativa*), the catalytic subunit genes *OsPP2A-1* and *OsPP2A-3* are upregulated in response to drought and high salinity, whereas heat stress induces *OsPP2A-3* and represses *OsPP2A-1* expression

(Yu et al. 2003). The expression of the *Medicago sativa MsPP2A Bβ* subunit is increased by abscisic acid indicating a specific function for this protein in the stress response (Tóth et al. 2000). The expression of wheat (*Triticum aestivum*) *TaPP2Ac-1* catalytic subunit in tobacco provides enhanced drought tolerance (Xu et al. 2007). Silencing of *NbNPP4-1* and *NbNPP4-2* catalytic subunits in *Nicotiana benthamiana* results in activation of plant defense responses and localized cell death (He et al. 2004).

Understanding the mechanisms by which plants perceive stress and transmit the signal to activate adaptive responses is of great importance to biology and vital for generating transgenic strategies to improve stress tolerance in crops. The aim of this study was to characterize the catalytic subunits of PP2A in potato (*Solanum tuberoum*) and tomato (*S. lycopersicum*), two economically important food crops of the Solanaceae family, and their involvement in the responses to diverse stress conditions. Experiments using the inhibitor okadaic acid demonstrated that serine/threonine phosphatasse have important regulatory functions in various stress responses in potato and tomato plants. Sequence homology analysis revealed six PP2Ac isoforms in potato and five in tomato clustered into two subfamilies. The expression profiles of potato and tomato isoforms were determined under different stress conditions. This analysis, together with okadaic acid experiments, yielded evidence on the involvement of PP2Ac isoforms in stress responses and revealed differences in the expression profiles between potato and tomato, suggesting divergent roles of PP2A in the plant defense mechanisms against stress in these closely related species.

Materials and methods

Plant material and stress conditions

Soil-grown plants: wild-type potato (*S. tuberosum* cv Spunta) and tomato (*S. 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. Prior to stress treatments, detached leaflets were placed in individual containers with water at 22°C in a growth chamber 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 treatment, leaflets were placed in buffer Tris–HCl 50 mM pH 6.5, instead of water, to avoid pH decrease when elicitor solutions were added.

In vitro plants

In vitro wild-type potato (*S. tuberosum* cv Spunta) plants were obtained by micropropagation of virus-free single-node cuttings in Murashige and Skoog (MS) medium containing 20 g/L sucrose solidified with 0.7% (w/v) agar. Seeds of wild-type tomato plants (*S. lycopersicum* cv Castlemart) were surface-sterilized with a solution of 10% (v/v) commercial bleach (0.525% sodium hypochlorite) for 5 min and washed thrice with sterile distilled water. Seeds were germinated on to MS medium 0.7% (w/v) agar in glass recipients. Plants were grown for 3 weeks in a growth chamber under a 16 h light photoperiod (4,000 l× light intensity) at 22°C and later transferred to liquid MS medium for 7 days prior to the corresponding stress treatments.

Stress treatments were carried out as follows:

Cold stress: detached leaflets or in vitro-grown plants were placed at 4°C, while control leaflets or plants remained at 22°C.

Salt stress: detached leaflets were treated with NaCl 200 or 300 mM in water for potato and tomato, respectively. Control leaflets were kept in water. In vitro plants were treated with NaCl 300 mM in MS medium (control: MS medium).

Mechanical wounding: the main vein and lamina of detached leaflets were cut with a dented forceps. Similarly, for 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 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.

RNA isolation and northern blot hybridization

Total RNA was isolated from leaves. Samples (0.1–1 g) were collected and ground in liquid nitrogen, and total RNA was extracted using the TRIzol Reagent (Invitrogen). Total RNA (10–20 µg) was separated on 1.2% (w/v) formaldehyde agarose gels and blotted onto nylon membranes (Hybond N+, GE Healthcare). Northern blots were hybridized with random-primed ^{32}P-labeled probes generated with Prime-a-Gene DNA Labeling System kit (Promega). Probes for PP2Ac mRNAs corresponding to the 5′ or 3′-UTRs were obtained by RT-PCR using the following primers: *StPP2Ac1*: 5′-CCGAATAC TTGAGCTGACTG-3′ and 5′-ATTACAAATCACAAT GGACC-3′; *StPP2Ac2a*: 5′-TTCCAATATCTGCGCTTG-3′ and 5′-GAACAGAATGTACCATGTTGC-3′; *StPP2Ac2b*: 5′-GAGAGTTGAGAAGAGGCACTG-3′ and 5′-CTATGG AATACCAAATATACAGAC-3′; *StPP2Ac3*: 5′-ATGTGAT GGAGAGCAATATC-3′ and 5′-CCTAACATAGGTTAGA ATATGAC-3′; *StPP2Ac4*: 5′-CCACTCGCTCACTCACTC A-3′ and 5′-GTGGGTCCAGAGGTAACTC-3′; *StPP2Ac5*: 5′-TCAGAGCTGCACAAACTTGGTG-3′ and 5′-AATGA

TACATGCATAGTGACTCC-3′; *LePP2Ac1*: 5′-ACCCTA GTTTCTTCATCAATG-3′ and 5′-TTATCCTCTACAATC ACCTGC-3′; *LePP2Ac2*: 5′-CGGCACGAGGGAAAAT GA TG-3′ and 5′-ACGCAGACCGATCAGAGAATC-3′; *LePP2Ac3*: 5′-AATTTCGAGGGCTTTCTC-3′ and 5′-TTT CGTACAACTAATCTTCAAAC-3′; *LePP2Ac4*: 5′-AGCG GATGATATGGAATG-3′ and 5′-ATGGAGCTTAGACGA TAGTG-3′; *LePP2Ac5*: 5′-TTTCCATCACCACCACTC-3′ and 5′-TTCTTCACAAAAATCACAG-3′.

Primers design was based on the sequences indicated in "Results".

After sequential stringent washes, bands were visualized using the Storm PhosphorImager (GE Healthcare) or by autoradiography.

Semi-quantitative reverse transcription-PCR (RT-PCR)

Semi-quantitative RT-PCR was performed to detect *LePP2 Ac5* transcripts. RNA was isolated using the TRIzol Reagent (Invitrogen) as described above. M-MLV reverse transcriptase (Invitrogen) was used for cDNA synthesis according to the manufacturer's protocol using the oligo(dT) 12–18 primer (Invitrogen). The newly synthesized cDNA was used as template for PCR amplification with the following primers: 5′-GATTTTATCGCATCAGTTC-3′ and 5′-GGGTGACAA ATTAATCGG-3′.

Preparation of plant extracts and Western blot analysis

Leaves from potato or tomato plants subjected to different stresses were harvested, ground in a mortar cooled with liquid nitrogen, and extracted with 50 mM Tris–HCl, pH 7.5, containing 2 mM β-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 20% (v/v) glycerol and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 µg/mL soybean trypsin inhibitor, and 25 units/mL aprotinin). The suspensions (1 mL buffer/g wet tissue) were centrifuged at 2,000 *g* for 30 min and the resulting supernatant fractions were sampled for SDS-polyacrylamide gel electrophoresis. Protein samples were separated on 12% polyacrylamide gels and transferred on 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 blotted with commercial anti-human PP2Ac 1D6 monoclonal antibody (UPSTATE) diluted 1 µg/mL 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). Equal protein loading was checked by Ponceau S staining.

The 1D6 monoclonal antibody was raised against a 15-residue peptide of the C-terminus of PP2Ac (Wei et al. 2001; Yu et al. 2001), which is highly conserved and is predicted to recognize all PP2Ac isoforms of potato and tomato. Methylesterification of the carboxy-terminal leucine residue may alter the binding of antibodies that recognize the C-terminus, therefore, controls were performed treating the samples with base (0.1 N NaOH for 5 min on ice) to remove methyl groups prior to Western blot analysis (Yu et al. 2001). No significant differences in the PP2Ac protein profiles were observed between non-treated and base-treated samples (Figs. 4c, 5c, 6c, 7c). It is possible that no changes in the methylation state occurred under the conditions studied or that 1D6 is not sensitive enough to detect those changes, in effect, among the antibodies against the C-terminus, 1D6 is the least methylation sensitive.

Quantification of blots

Northern blot bands of specific mRNAs were quantified relative to the ethidium bromide-stained ribosomal RNA (rRNA). LePP2Ac5 amplicons were quantified relative to housekeeping genes amplicons (ubiquitin or elongation factor 1α). PP2Ac bands of Western blots were quantified relative to the Ponceau S staining of the Rubisco large subunit band (potato samples) or to α-tubulin (tomato samples). Quantifications were carried out using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij). An increase equal to or greater than twofold or a decrease equal to or greater than 50% were considered significant. Significant changes are indicated with arrows (↑ for increase and ↓ for decrease) on the right side of each blot.

Rapid amplification of cDNA 3′ ends (3′-RACE) by PCR

The termination sites of mature transcripts of *StPP2Ac2b* and *LePP2Ac1* were determined by 3′-RACE PCR. cDNA was synthesized from total RNA isolated from leaves or shoot apex of soil-grown potato plants or in vitro-grown tomato plants using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer′s instructions with the polyT adapter primer 5′-GACTCGAGTCGACATCG$(T)_{17}$-3′. Specific primers for PCR amplification were 5′-CAAT GGTT GCACTAGTTG-3′ for *StPP2Ac2b* and 5′-TCCGAATACT TGAGCTGAC-3′ for *LePP2Ac1*. Nested PCRs were carried out to confirm specificity of the amplified fragments. Nested specific primers were 5′-AAGGTCTGTCATGTATATTG-3′ for *StPP2Ac2b* and 5′-GTAGCCCATCAGTATCG-3' for *LePP2Ac1*. The adapter primer 5′-GACTCGAGTCGACA TCG-3′ was used as reverse primer for each amplification. The fragments that resulted positive for nested PCR were cloned in pGEM-T Easy vector (Promega) for sequencing.

Results

Participation of serine/threonine phosphatases in stress responses

Initially, to determine if stress responses are mediated by serine/threonine phosphatases in potato and tomato plants, expression of stress-responsive genes under different stress conditions (cold, high salinity, mechanical wounding and fungal elicitors) was analyzed in the presence or absence of okadaic acid. This compound has been widely used as PP2A/PP1 inhibitor, although PP4, PP5 and PP6, other less abundant serine/threonine phosphatases, are also affected by okadaic acid when used in nanomolar concentrations (Schönthal 1998; Prickett and Brautigan 2006). Sampling times after okadaic acid treatment were chosen according to the maximal induction of the stress-responsive genes, as determined previously by time-response experiments (not shown). Treatments with okadaic acid vehicle alone (dimethyl sulphoxide**,** DMSO) carried out for different times during 24 h showed no effect on the levels of stress-responsive genes analyzed (not shown).

As marker of cold stress, levels of *LeCBF1* mRNA were determined for tomato plants. *LeCBF1* gene encodes a functional homologue of the *Arabidopsis CBF1-3* rapid cold-induced transcriptional activators (Zhang et al. 2004). Induction of *CBF1* by low temperatures was not observed in potato leaves (not shown), therefore, the levels of the potato homologue of *Arabidopsis ATHB7* (homeobox-leucine zipper protein 7; TC140026, DFCI Potato Gene Index) and the potato homologue of tomato *Tas14* (TC134741) were determined as markers of cold response in potato leaves. *ATHB7* encodes a cold-induced transcription factor. *Tas14* encodes a dehydrin induced by osmotic stress and abscisic acid treatment in tomato plants. The homologues of both genes were shown to be induced by cold in potato leaves (Rensink et al. 2005).

No changes in the degree of *HB7* or *Tas14* induction by cold were observed by okadaic acid treatment in potato leaves (Fig. 1a), indicating that okadaic acid-sensitive serine/threonine phosphatases activities are not involved in the regulation of cold stress responses leading to induction of *HB7* or *Tas14* in potato. In contrast, okadaic acid-treated tomato plants accumulated more *LeCBF1* mRNA in response to low temperatures than untreated plants (Fig. 1a), indicating that serine/threonine phosphatases might function as negative regulators of the cold response pathway leading to the *LeCBF1* induction.

Le25 and *Tas14* genes were used as markers of salt stress response. *Le25* encodes a late embryogenesis-abundant (LEA) protein and is induced by salt stress, drought and abscisic acid in tomato plants (Kahn et al. 1993). LEA proteins are thought to protect cells from stresses associated with dehydration, thus increasing stress tolerance. A potato homologue of *Le25* (TC153563) was found in the DFCI Potato Gene Index and was induced by salt stress in potato leaves (Fig. 1b). The potato homologue of tomato *Tas14* was also induced by salt stress in potato leaves (Fig. 1b). Okadaic acid did not affect the degree of induction of *Le25* by salt stress in potato or tomato plants, but diminished *Tas14* induction in potato leaves, with no changes in tomato leaves (Fig. 1b). These results indicate that okadaic acid-sensitive serine/threonine phosphatases may act as positive regulators of the salt stress response pathway leading to the induction of *Tas14* in potato, while they have no participation in tomato *Le25* or *Tas14* induction by salt.

Pin2 (proteinase inhibitor 2) mRNA expression was determined as marker of mechanical wounding. *Pin2* reduces the digestibility and nutritional quality of the leaves to help defend the plant against insect predators (Johnson et al. 1989; Orozco-Cardenas et al. 1993). It has been widely used as marker of mechanical damage (Bowles 1998) as it represents the main response to wounding in Solanaceae. Expression of *Pin2* is dependent on jasmonic acid, which is synthesized after wounding via the octadecanoic pathway (Farmer and Ryan 1990). No differences in the induction of *Pin2* by mechanical wounding were observed between okadaic acid-treated and untreated potato or tomato plants (Fig. 1c), suggesting that okadaic acid-sensitive serine/threonine phosphatases do not play an essential role in the signaling pathway that leads to *Pin2* induction in both species.

To examine the involvement of serine/threonine phosphatases in the response to fungal pathogens, the induction of *Chitinase A* mRNA was determined. *Chitinase A* is a pathogenesis related (PR) protein induced by fungal infections and elicitors in potato and tomato plants (Büchter et al. 1997; Cohen et al. 1994) that plays a defense role by degrading fungal cell walls. Okadaic acid up-regulated the expression of *Chitinase A* even in the absence of the fungal elicitors PGA or chitosan (Fig. 2), suggesting the existence of an active signaling pathway in the absence of stress signal that requires continuous serine/threonine phosphatase activity to remain inhibited.

These results indicate that serine/threonine phosphatases play key roles in various stress responses in potato and tomato plants, acting as positive or negative modulators depending on the nature of stress condition, and that there are differences in the participation of serine/threonine phosphatases in cold- and salt stress-activated pathways between both species. Next, the involvement of individual PP2Ac isoforms in stress responses was addressed.

Potato and tomato PP2Ac families

In previous studies by He et al. (2004), the existence of five isoforms of PP2Ac in tomato was reported and the

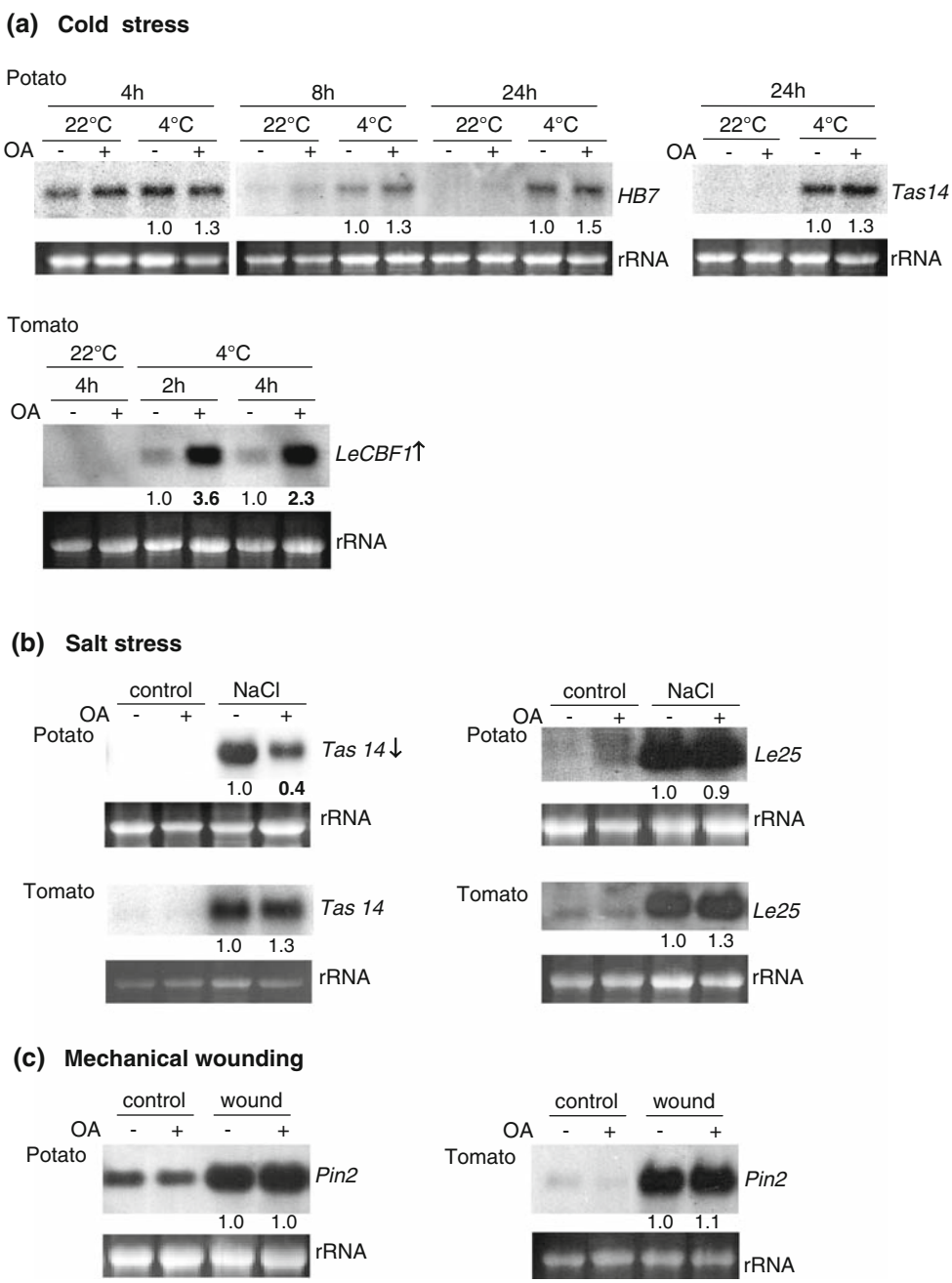

Fig. 1 Participation of serine/threonine phosphatases in cold, salt stress and wounding response. **a** Potato detached leaflets and in vitro-grown tomato plants were exposed to 4°C for the indicated times in the absence or presence of 100 nM okadaic acid (OA), which was added 1 h prior to cold stress. Total RNA was isolated from leaves and northern blot hybridization with *LeCBF1, HB7* or *Tas14* probes was performed. **b** Detached leaflets of potato and tomato plants were treated with NaCl 200 or 300 mM, respectively, for 8 h in the absence or presence of 100 nM okadaic acid (OA), which was added 1 h prior to salt stress. Total RNA was isolated and northern blot hybridization with *Le25* and *Tas14* probes was performed. **c** In vitro-grown potato and tomato plants were subjected to mechanical wounding cutting each leaflet with a dented forceps in the absence or presence of 100 nM okadaic acid (OA), which was added 1 h prior to wounding. After the indicated times, total RNA was isolated from leaves and northern blot hybridization with *Pin2* probe was performed. *Numbers* indicate quantification of blots (arbitrary units). Experiments were repeated two or three times independently. Representative blots are shown. *rRNA* ribosomal RNA

full-length cDNA of *LePP2Ac1* and *LePP2Ac2* were isolated (GeneBank accession numbers AY325817 and AY325818, respectively). In addition, in that report, a search of the DFCI Tomato Gene Index (http://compbio.dfci.harvard.edu/tgi/plant.html) revealed two other full-length cDNAs of PP2Ac isoforms: TC124303 and TC124304 that correspond to *LePP2Ac3* and *LePP2Ac4*, respectively, and one partial sequence corresponding to *LePP2Ac5* (TC121114). In this study, the full-length cDNA of *LePP2Ac5* was found by searching the nucleotide collection nr/nt database using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast) under the accession number BT013374.

Bioinformatics searches of the DFCI Potato Gene Index revealed the presence of six isoforms of PP2Ac in potato. All the potato TC and EST sequences similar to PP2Ac genes from *A. thaliana* were aligned and analyzed with the Vector NTI program (Invitrogen). This allowed the reconstruction of the cDNA sequences of the six StPP2Ac isoforms, designated according to the nomenclature established in He et al. (2004) for the homologous tomato PP2Ac genes as follows: *StPP2Ac1* (TC140898 + TC142763), *StPP2Ac2a* (TC133492 + TC148791), *StPP2Ac2b* (TC135479 + TC142684), *StPP2Ac3* (TC144550 + TC141350), *StPP2Ac4* (TC153075), *StPP2Ac5* (EST560167, partial sequence).

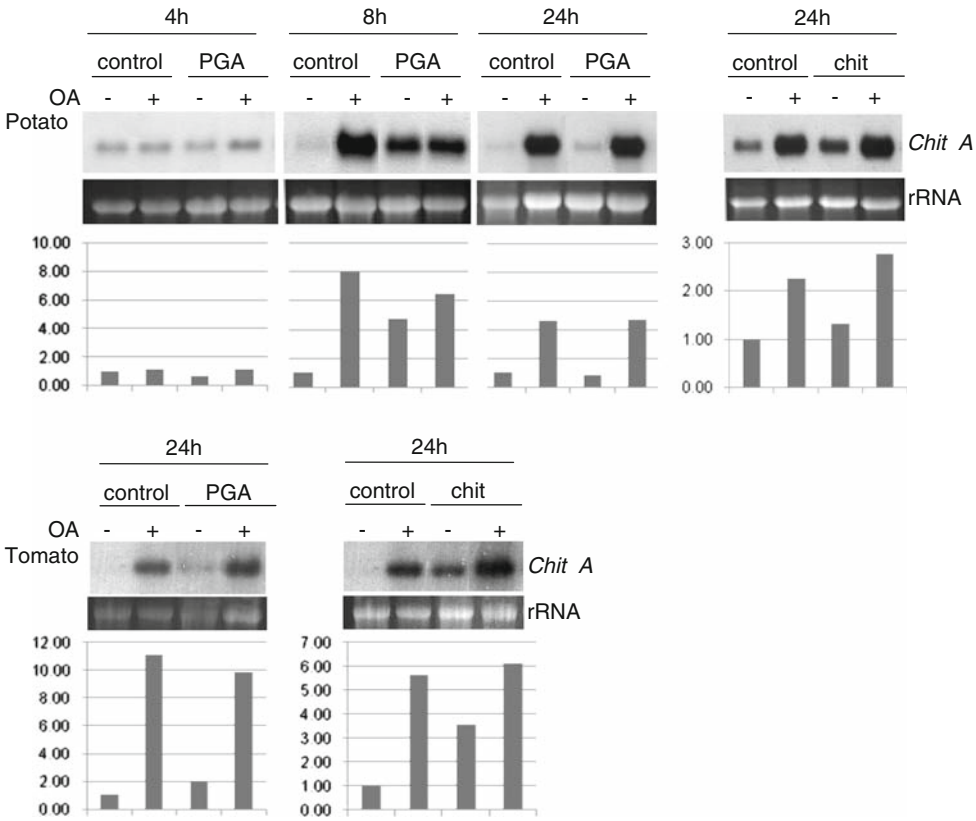

Fig. 2 Participation of serine/threonine phosphatases in fungal elicitors response. Detached leaflets of potato and tomato plants were exposed to polygalacturonic acid (PGA) 50 µg/mL or chitosan (chit) 100 µg/mL for the indicated times in the absence or presence of 100 nM okadaic acid (OA), which was added 1 h prior to elicitors. Total RNA was isolated and northern blot hybridization with *Chitinase A* (*Chit A*) probe was performed. For potato blot PGA-8 h and tomato blot chitosan-24 h, all *lanes* were from the same gel but were not originally adjacent. *Histograms* represent quantification of blots (arbitrary units). Experiments were repeated two or three times independently. Representative blots are shown. *rRNA* ribosomal RNA

With this information, a phylogenetic tree of PP2Ac proteins from potato, tomato, *A. thaliana* and rice (*O. sativa*, an important crop in which PP2Ac isoforms have been characterized) was constructed using the Maximum Parsimony method with MEGA 4.1 software (http://www.megasoftware.net/index.html) (Fig. 3). These PP2Ac proteins clustered into two distinct groups that were designated subfamily I and II according to He et al. (2004). Subfamily I contains potato StPP2Ac1, StPP2Ac2a and StPP2Ac2b as well as tomato LePP2Ac1 and LePP2Ac2, while StPP2Ac3, StPP2 Ac4 and StPP2Ac5 from potato belong to the subfamily II, the same as tomato LePP2Ac3, LePP2Ac4 and LePP2Ac5.

Multiple alignment of the deduced amino acid sequences of potato and tomato PP2Ac proteins was performed with ClustalW program (www.ebi.ac.uk/clustalw; not shown). This analysis revealed that, as previously reported for rice and *Arabidopsis* (Yu et al. 2003), clusters of subfamily-specific amino acid residues are located mainly in the C-termini while distinct amino acid residues in members of the same subfamily are found predominantly in the N-terminal region.

Further analysis of the primary structure of potato and tomato PP2Ac proteins showed that all of them contain the conserved residues of the phosphoesterase signature motif "DXH(X)*n*GDXXD(X)*n*GNHD/E" (where n = about 25) that is conserved in Ser/Thr phosphatases (Zhuo et al. 1994), the "YRCG" motif implicated in okadaic acid binding (Zhang et al. 1994) and the C-terminal "TPDYFL" motif which contains the methylesterification and tyrosine phosphorylation sites involved in the regulation of PP2Ac activity and holoenzyme assembly (Xu et al. 2006; Longin et al. 2007).

Expression profiles of potato and tomato PP2Ac isoforms under stress conditions

Changes in gene expression of signal transduction components (such as protein kinases and phosphatases) are the part of the ability of plants to respond to environmental stress (Yamaguchi-Shinozaki and Shinozaki 2005). Activation (or inhibition) of preexisting signaling components occurs rapidly after the onset of stress (within seconds or minutes) and leads to early and emergency responses, whereas changes in their expression allow slow and long-term adaptive responses (within hours or days). Therefore, to obtain information about the involvement of different PP2Ac isoforms in stress responses, time-course expression of PP2Ac mRNA isoforms under different stress conditions was determined by northern blot. As well, PP2A catalytic subunit protein was determined by Western blot using an antibody predicted to react with all isoforms of PP2Ac.

Cold did not affect the expression of any PP2Ac mRNA in potato (Fig. 4a). In contrast, *LePP2Ac1*, *LePP2Ac2* and *LePP2Ac3* mRNA level decreased in response to low

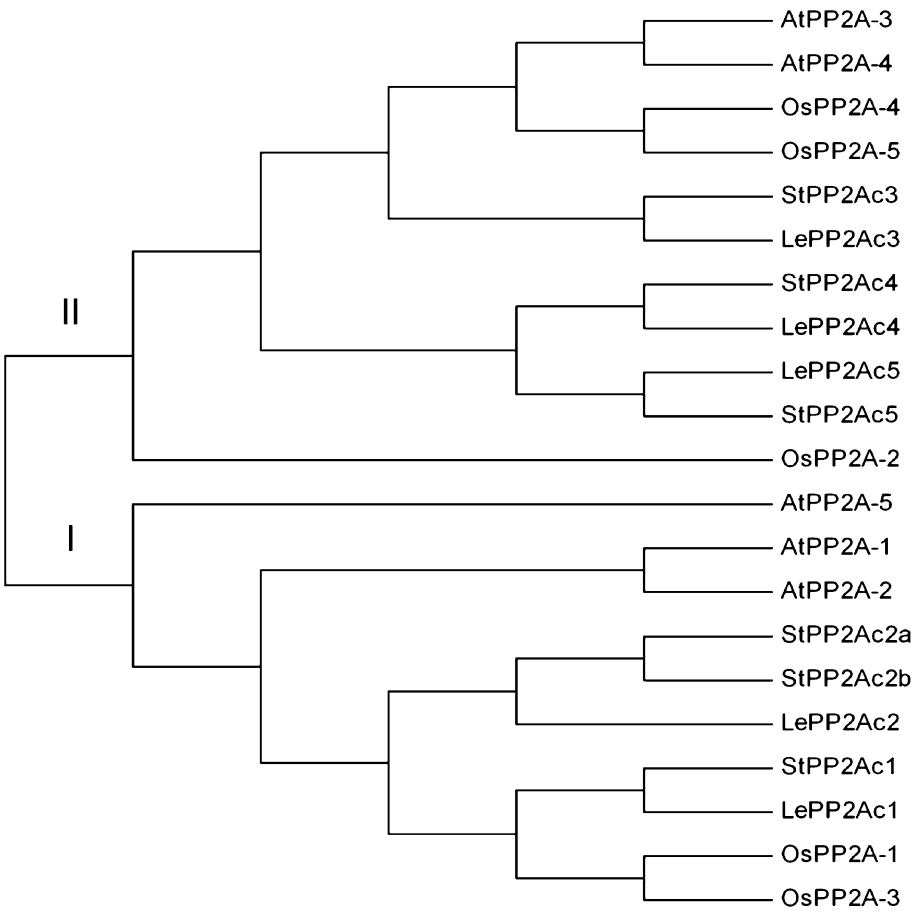

Fig. 3 The PP2Ac family of potato and tomato. The phylogenetic tree of PP2Ac proteins was constructed by the maximum parsimony method using MEGA 4.1 software. The deduced amino acid sequences of potato and tomato PP2Ac proteins were compared with PP2Ac proteins from *Arabidopsis thaliana* and rice. Distinct clusters were designated subfamily I and II according to He et al. (2004). GenBank accession numbers: AtPP2A-1 (NP_176192), AtPP2A-2 (NP_172514), AtPP2A-3 (NP_567066), AtPP2A-4 (NP_973672), AtPP2A-5 (NP_177154), OsPP2A-1 (AAC72838), OsPP2A-2 (AAD22116), OsPP2A-3 (AAD41126), OsPP2A-4 (AAD48068), OsPP2A-5 (AAF86353). *At Arabidopsis thaliana*, *Le Lycopersicon esculentum*, currently *Solanum lycopersicum*; *Os Oryza sativa*, *St Solanum tuberosum*

temperatures in tomato (Fig. 4b). It is worth noting that the expression of *LePP2Ac1* and *LePP2Ac2*, that belong to the subfamily I showed a similar behavior, declining after 8–24 h of cold exposure, while levels of *LePP2Ac3*, which belongs to the subfamily II, decreased rapidly (after 1 h of treatment). Downregulation of *LePP2Ac1* and *LePP2Ac2* appears to be a long-term response to cold stress, since their levels remain low after 48 h. According to the expression profile results, PP2Ac protein levels were unaffected in potato leaves by cold (Fig. 4c), but were downregulated in tomato leaves (Fig. 4d).

Salt stress increased mRNA levels of all members of the subfamily I (*StPP2Ac1*, *StPP2Ac2a* and *StPP2Ac2b*) and *StPP2Ac3* in potato leaves (Fig. 5a). The effect of salt stress on potato PP2Ac transcripts is rapid and transient, beginning after 2 h of treatment and returning to basal levels after 4 h. No changes in any PP2Ac mRNA of tomato were observed in response to salt stress, either using In vitro-grown plants (Fig. 5b) or detached leaflets (not shown). Correspondingly, salt stress upregulated PP2Ac protein levels in potato leaves (Fig. 5c) but not in tomato (Fig. 5d).

Mechanical wounding increased mRNA levels of potato *StPP2Ac2b* transiently and tomato *LePP2Ac1* and *LePP2Ac2* in a more sustained manner (Fig. 6a, b, respectively). In the same way, PP2Ac protein levels were increased after wounding in potato and tomato leaves (Fig. 6c, d, respectively).

The fungal elicitors PGA and chitosan upregulated *StPP2Ac1*, *StPP2Ac2a* and *StPP2Ac2b* mRNA levels in potato leaves (Fig. 7a) and *LePP2Ac1* and *LePP2Ac2* mRNA levels in tomato leaves (Fig. 7b). Noteworthy, all isoforms upregulated by fungal elicitors belong to the subfamily I. Tomato transcripts are up-regulated by elicitors in a more sustained manner than potato ones. Overall, levels of tomato PP2Ac transcripts that are increased by elicitors remain high after 24 h, while potato PP2Ac mRNA return to their basal levels after that time of treatment. According to the expression profile results, PGA and chitosan increased PP2Ac protein levels in both species (Fig. 7c, d, respectively).

LePP2Ac5 was not detected by northern blot in any experiment. Semi-quantitative RT-PCR was performed; however, in most samples it was not possible to detect the signal, probably because of its low level of expression. It was only feasible to determine *LePP2Ac5* expression complete profiles under salt stress and mechanical wounding wherein no changes were observed (Figs. 5b and 6b).

Mapping the 3′-ends of *StPP2Ac2b* and *LePP2Ac1* mRNAs

The subfamily I members *StPP2Ac2b* and *LePP2Ac1* produce at least two mature transcripts, respectively, as observed in several northern blots. To establish if the difference in

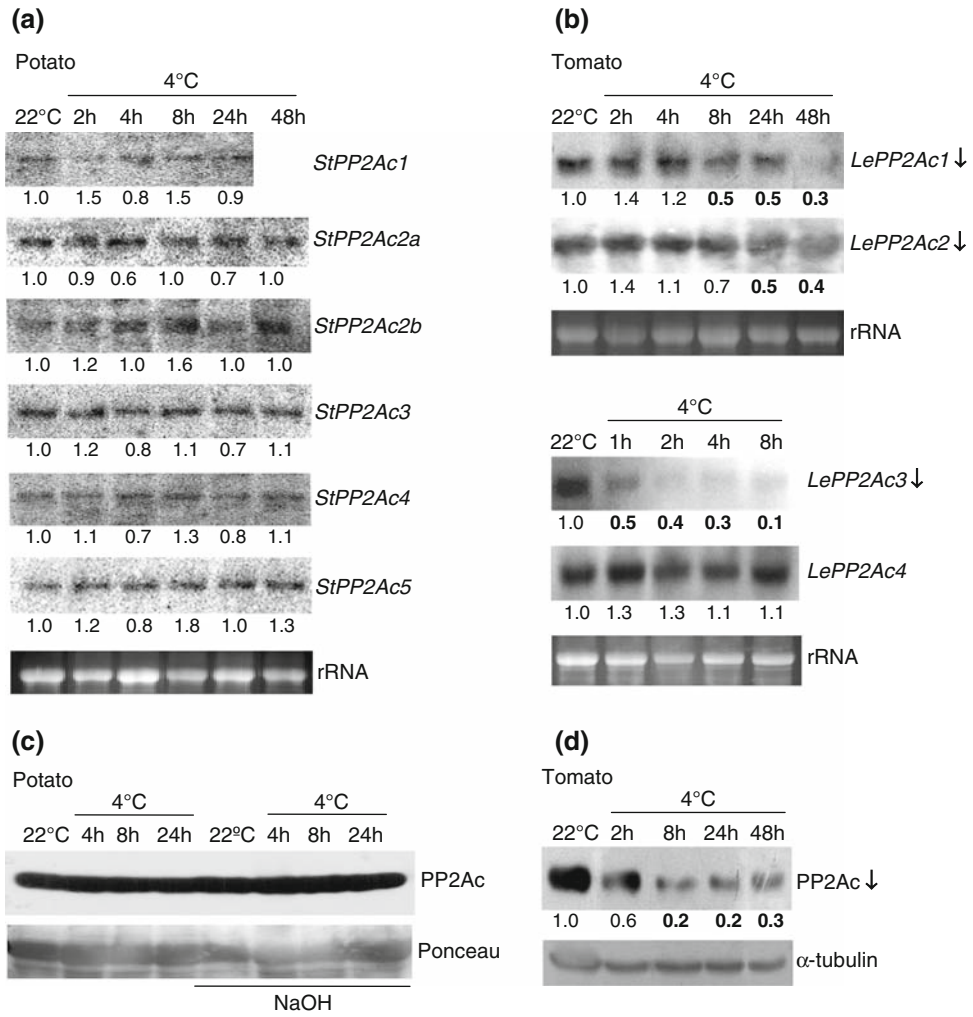

Fig. 4 Potato and tomato PP2Ac isoforms in cold stress response. **a** In vitro-grown potato plants were exposed to 4°C for the indicated times. Total RNA was isolated from leaves and northern blot analysis was carried out using specific probes for StPP2Ac isoforms. **b** In vitro-grown tomato plants were exposed to 4°C for the indicated times. Total RNA from leaves was isolated and northern blot analysis was carried out using specific probes for LePP2Ac isoforms. In vitro-grown potato (**c**) and tomato plants (**d**) were exposed to 4°C for the indicated times. Western blot analysis of leaf proteins were performed using anti-PP2Ac antibody. Potato samples were also treated with base (NaOH) prior to blotting as described in "Materials and methods". In (**d**), all *lanes* were from the same gel but were not originally adjacent. *Numbers* indicate quantification of blots. Representative blots of at least two independent experiments are shown. *rRNA* ribosomal RNA

transcripts length results from alternative polyadenylation, 3′-ends of *StPP2Ac2b* and *LePP2Ac1* mRNAs were determined by 3′-RACE PCR as described in "Materials and methods".

Sequences of 3′-RACE products from *StPP2Ac2b* revealed the presence of three different polyadenylation cleavage sites in its 3′-UTR (Fig. 8a). These sites are located at nucleotide positions +1,137, +1,189 and +1,467. Because the cDNA sequence of *StPP2Ac2b* from the DFCI database continues downstream the third cleavage site, it is possible that at least one more polyadenylation site exists in *StPP2Ac2b* mRNA.

In addition, DNA sequencing of the 3′-RACE products of *LePP2Ac1* mapped the mRNA end to three sites (Fig. 8b) at nucleotide positions +1,019, +1,092 and +1,156, respectively. The transcript ending at nucleotide +1,093 corresponds to the sequence reported previously (GeneBank accession number AY325817; He et al. 2004).

Putative polyadenylation signals type NUE (near upstream element, Loke et al. 2005) were found examining the 3′-UTR sequences of *StPP2Ac2b* and *LePP2Ac1*.

Alternative polyadenylation sites were also found in the catalytic subunit of *OsPP2A-3* from rice (Yu et al. 2003), which belongs to the subfamily I, as well as *StPP2Ac2b* and *LePP2Ac1*. The physiological meaning of this phenomenon in PP2A catalytic subunits remains unknown. However, it is thought that variation of 3′-UTR length might have a role in the regulation of transportation, stability and translation of mRNA in plant cells (Shen et al. 2008).

Discussion

This work presents the characterization of potato and tomato PP2A catalytic subunits and their involvement in stress responses.

The data achieved by PP2Ac mRNA profiles, together with the results of okadaic acid experiments, suggest that *LePP2Ac1*, *LePP2Ac2* and *LePP2Ac3* are components of the cold response in tomato plants and that *StPP2Ac1*, *StPP2Ac2a*, *StPP2Ac2b* and *StPP2Ac3* are part of the plant defense mechanism against salt stress in potato, even

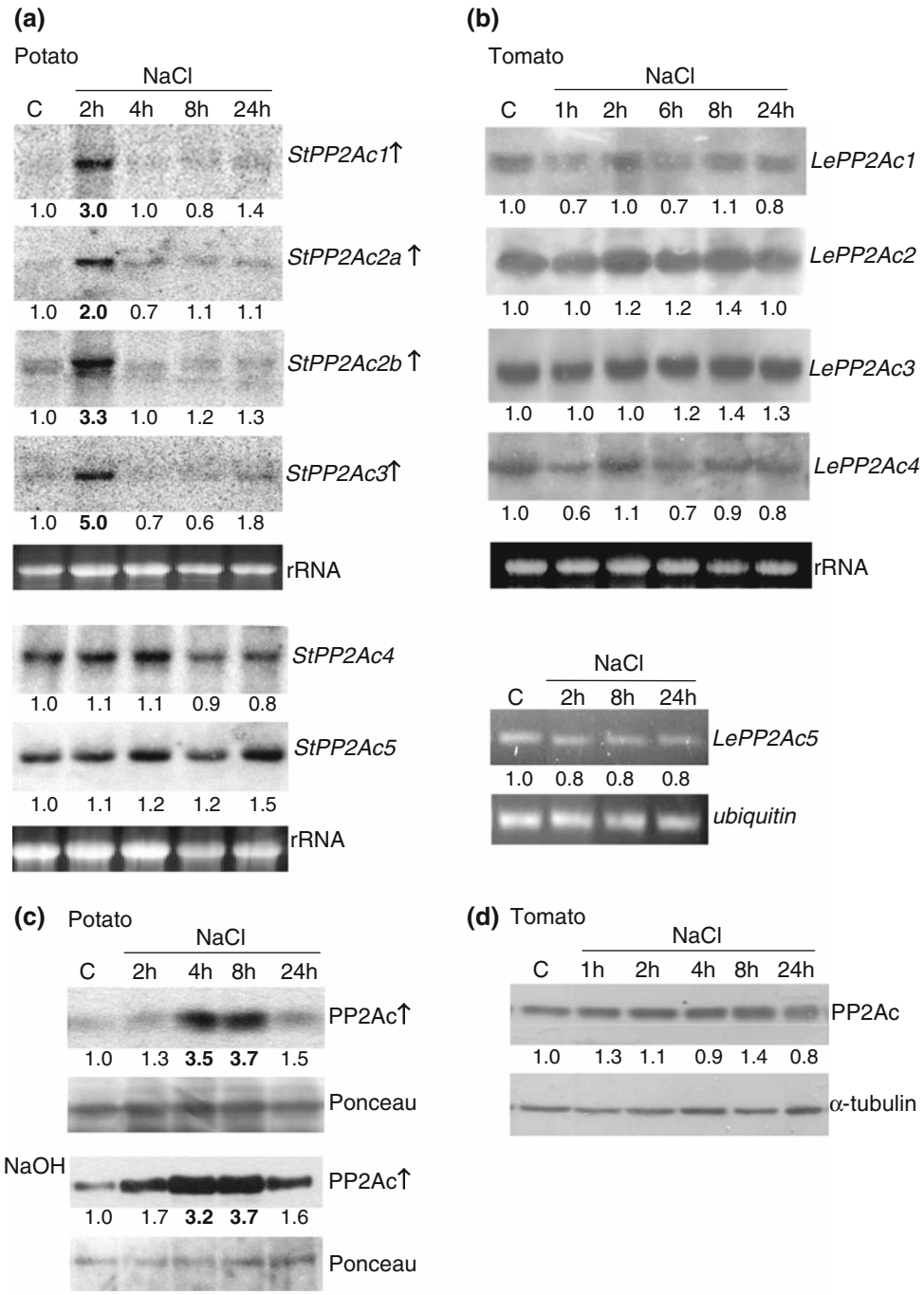

Fig. 5 Potato and tomato PP2Ac isoforms in salt stress response. **a** Detached leaflets of potato plants were treated with 200 mM NaCl for the indicated times. Total RNA was isolated and northern blot analysis was carried out using specific probes for StPP2Ac isoforms. **b**. In vitro-grown tomato plants were treated with 300 mM NaCl for the indicated times. Total RNA was isolated from leaves, northern blot analysis was carried out using specific probes for *LePP2Ac1-4* isoforms and semi-quantitative RT-PCR for *LePP2Ac5* was performed. Detached leaflets of potato plants (**c**) and in vitro-grown tomato plants (**d**) were treated with 200 or 300 mM NaCl, respectively, for the indicated times. Western blot analysis of leaf proteins were performed using anti-PP2Ac antibody. Potato samples were also treated with base (NaOH) prior to blotting as described in "Materials and methods". *Numbers* indicate quantification of blots. Representative blots of at least two independent experiments are shown. *C* control, *rRNA* ribosomal RNA

though, experiments of overexpression and/or silencing of specific isoforms are required to confirm this hypothesis.

PP2Ac profiles in response to mechanical wounding allow us to speculate that *StPP2Ac2b*, *LePP2Ac1* and *LePP2Ac2* may be involved in the wounding response. If so, considering the results of okadaic acid experiments, these PP2Ac isoforms may participate in jasmonic acid-independent signaling pathways that lead to responses different from *Pin2* induction.

The results of okadaic acid experiments indicate that serine/threonine phosphatases act as negative regulators of the defense response to pathogen elicitors. This observation agrees with previous studies by He et al. (2004), where silencing of PP2Ac subfamily I in *N. benthamiana* resulted in activation of defense responses and localized cell death. Studies carried out using phosphatase inhibitors revealed that inactivation of pathogen defense responses by phosphatases is a common feature in plants (Chandra and Low

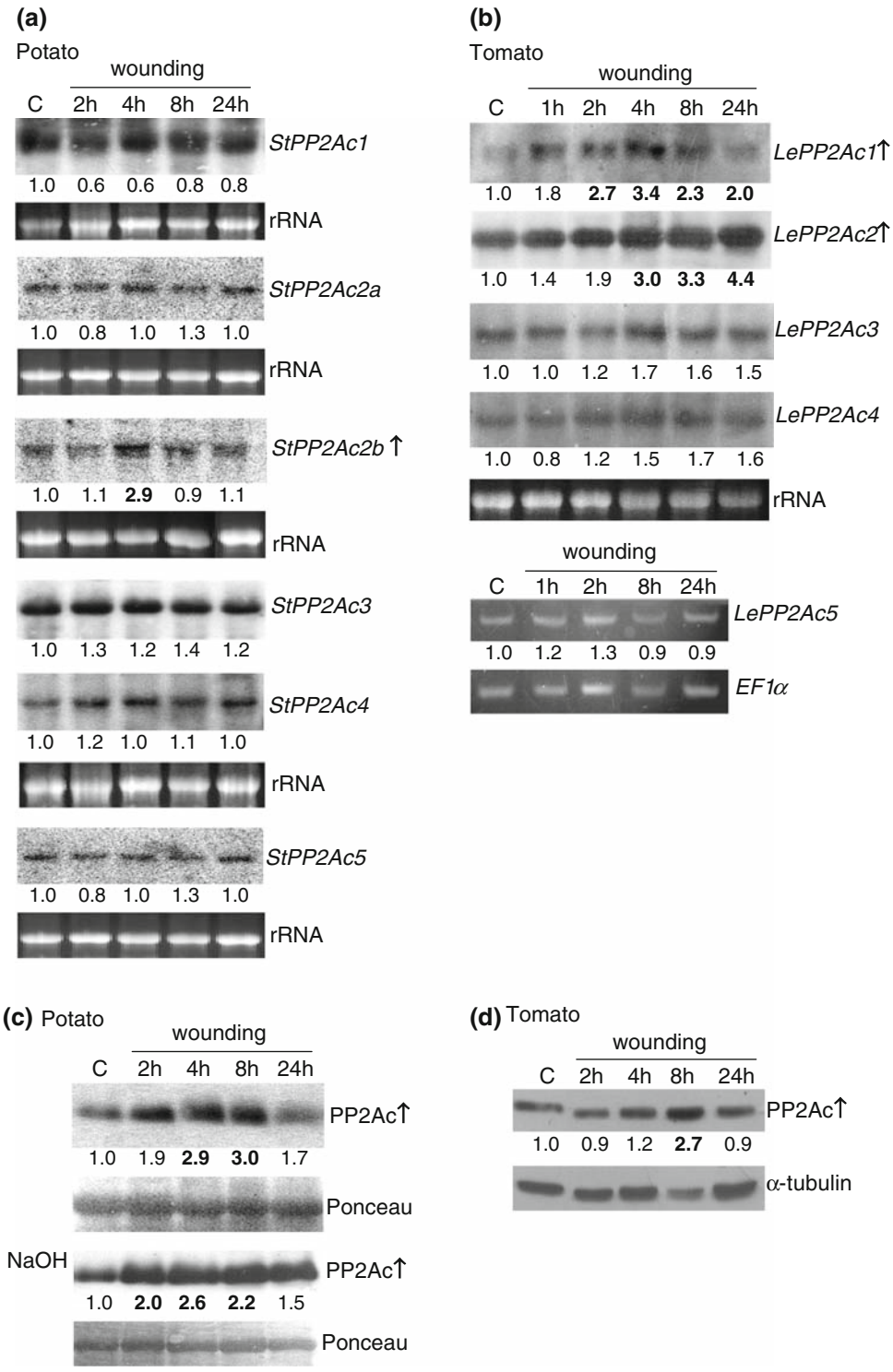

Fig. 6 Potato and tomato PP2Ac isoforms in wounding response. **a** Detached leaflets of potato plants were subjected to mechanical wounding. Total RNA was isolated and northern blot analysis was carried out using specific probes for StPP2Ac isoforms. **b** In vitro-grown tomato plants were subjected to mechanical wounding. Total RNA was isolated from leaves, northern blot analysis was carried out using specific probes for *LePP2Ac1-4* isoforms and semi-quantitative RT-PCR for *LePP2Ac5* was performed. Detached leaflets of potato plants (**c**) and in vitro-grown tomato plants (**d**) were subjected to mechanical wounding. Leaf proteins were isolated and Western blot analysis was performed using anti-PP2Ac antibody. Potato samples were also treated with base (NaOH) prior to blotting as described in "Materials and methods". *Numbers* indicate quantification of blots. Representative blots of at least two independent experiments are shown. *C* control, *rRNA* ribosomal RNA

1995; Hassa et al. 2000; Lecourieux-Ouaked et al. 2000). The upregulation of possible negative modulators by elicitors may be a protective mechanism, as plant defense response against fungal pathogens includes oxidative burst and localized cell death (de Wit 2007 and references therein), therefore, this process must be tightly regulated to prevent extensive damage to host tissues. It is tempting to speculate that this negative modulation can be ascribed to PP2Ac members of the subfamily I in potato and tomato plants, as suggested by the fact that their transcripts are

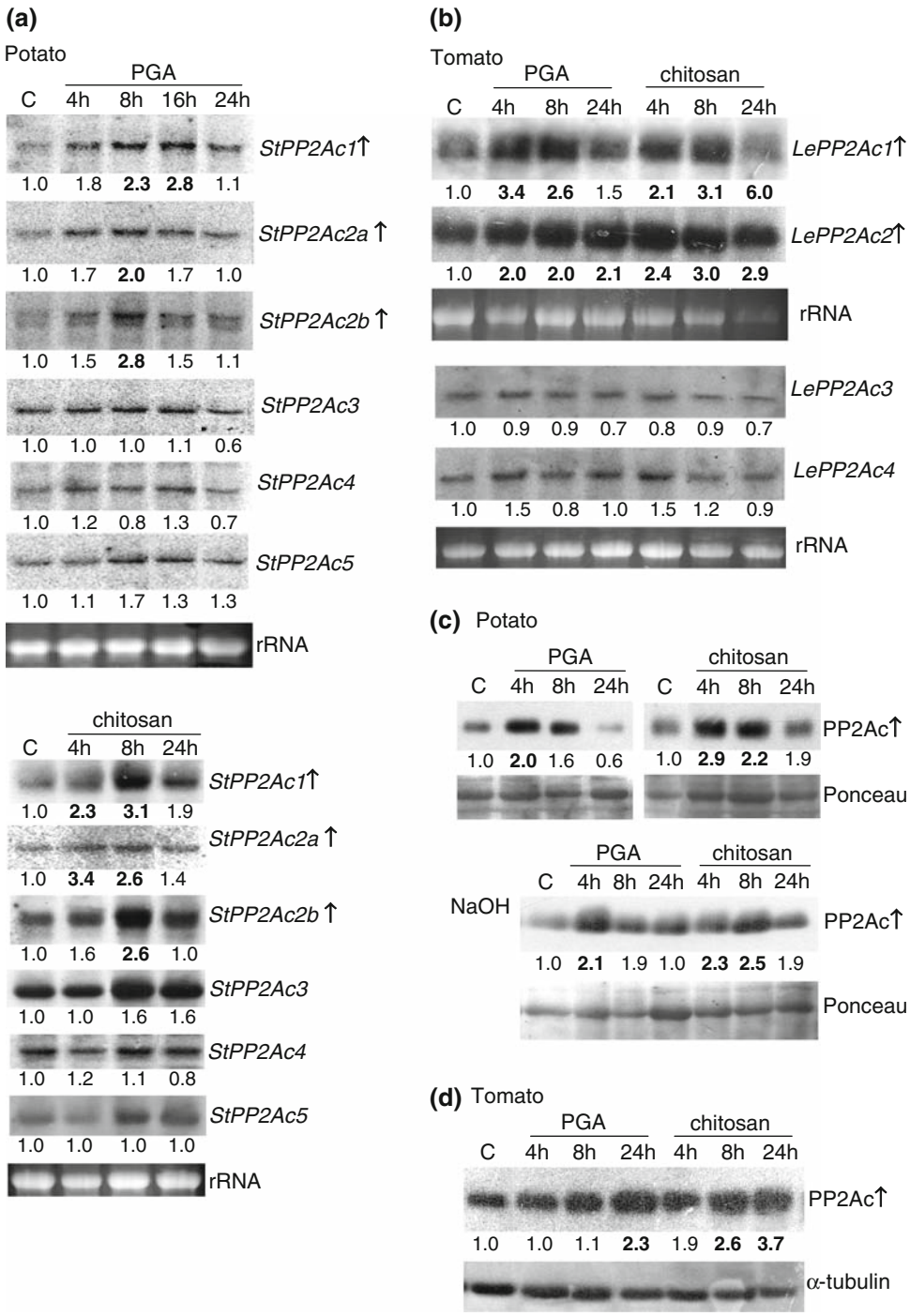

Fig. 7 Potato and tomato PP2Ac isoforms in fungal elicitors response. Detached leaflets of potato (**a**) and tomato (**b**) plants were exposed to polygalacturonic acid (PGA) 50 μg/mL or chitosan 100 μg/mL for the indicated times. Total RNA was isolated and northern blot analysis was carried out using specific probes for PP2Ac isoforms. Detached leaflets of potato (**c**) and tomato (**d**) plants were exposed to polygalacturonic acid (PGA) 50 μg/mL or chitosan 100 μg/mL for the indicated times. Western blot analysis of leaf proteins was performed using anti-PP2Ac antibody. Potato samples were also treated with base (NaOH) prior to blotting as described in "Materials and methods". In (**a**) (chitosan), all *lanes* were from the same gel but were not originally adjacent. *Numbers* indicate quantification of blots. Representative blots of at least two independent experiments are shown. *C* control, *rRNA* ribosomal RNA

upregulated by fungal elicitors, although more experiments are necessary to support this concept. Two elicitors were used for this study, PGA (derived from plant cell walls) and chitosan (a deacetylated derivative of chitin, component of fungal cell walls), however, the possibility that expression profiles of the PP2Ac isoforms were different using other fungal elicitors such as xylanase or chitin fragments cannot be excluded.

The data obtained indicate that cultivated potato and tomato exhibit notable differences in the cold and salt stress response, although they are closely related species. It is possible that these differences might be explained by the dissimilar environmental conditions in which they were domesticated. Evidence suggests that domestication of both species took place geographically far apart in the ancient Americas, with potato being domesticated in the Andes (Spooner et al. 2007), and

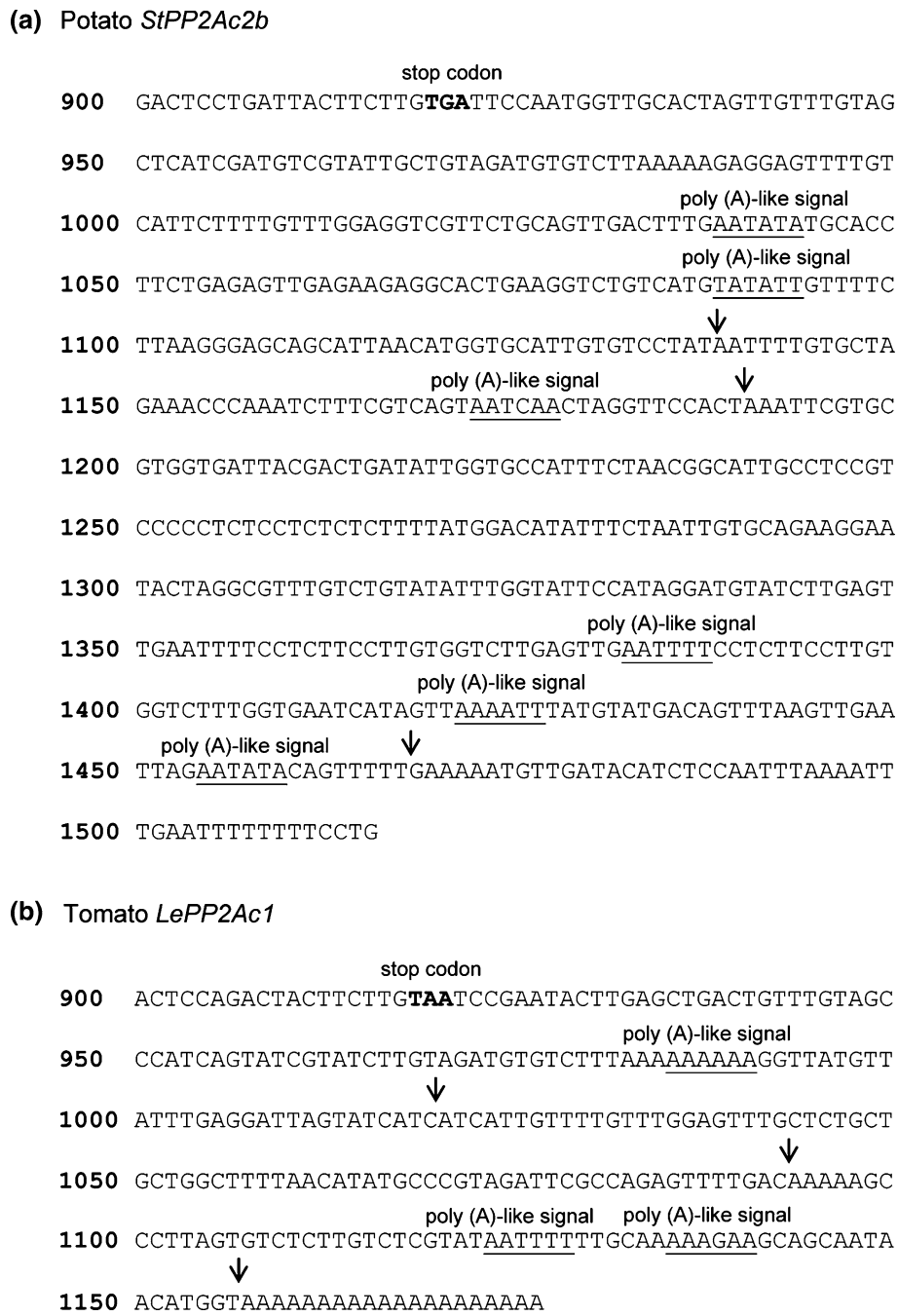

Fig. 8 Mapping the 3′-ends of StPP2A2b and LePP2Ac1 mRNAs. The polyadenylation cleavage sites of *StPP2Ac2b* (**a**) and *LePP2Ac1* (**b**) determined by 3′-RACE are indicated by *arrowheads*. Position +1 corresponds to the A nucleotide of the start codon ATG. Stop codons are in *bold* and *labeled*. The putative poly(A) signals are *underlined* and *labeled*

tomato in Central America (Bai and Lindhout 2007). Therefore, selection during domestication favored divergent phenotypic characteristics that allowed potato and tomato adaptation to very dissimilar environmental conditions.

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