The Subcellular Localization of Plant Protein Phosphatase 5 Isoforms Is Determined by Alternative Splicing[^w]

Sergio de la Fuente van Bentem, Jack H. Vossen, Josephus E.M. Vermeer, Marianne J. de Vroomen, Theodorus W.J. Gadella, Jr., Michel A. Haring, and Ben J.C. Cornelissen*

Plant Pathology (S.d.I.F.v.B., J.H.V., M.J.d.V., B.J.C.C.), Molecular Cytology (J.E.M.V., T.W.J.G.), and Plant Physiology (M.A.H.), Swammerdam Institute for Life Sciences, Faculty of Science, University of Amsterdam, P.O. Box 94062, 1090 GB Amsterdam, The Netherlands

Protein serine/threonine phosphatase 5 (PP5) plays an important role in signal transduction in animal cells, but in plants, knowledge about PP5 is scarce. Here, we describe the isolation of a full-length cDNA encoding tomato (Lycopersicon esculentum) PP5 (LePP5) and its expression in Escherichia coli. Biochemical characterization showed that recombinant LePP5 has a low intrinsic protein phosphatase activity. This activity was increased 6- to 10-fold by either removal of the N-terminal tetratricopeptide repeat domain or by addition of fatty acids, indicating that biochemical features specific for PP5 homologs from other species are conserved in tomato. The single-copy LePP5 gene was cloned and shown to encode two mRNA species that arise by alternative pre-mRNA splicing. Similarly, Arabidopsis was found to express two PP5 transcripts, suggesting that alternative splicing of PP5 pre-mRNA is not specific for tomato. Alternative splicing results in a larger transcript containing an additional exon encoding two putative transmembrane domains and, hence, in a larger PP5 isoform. Subcellular fractionation studies on tomato protein lysates indicated that the majority of the 55-kD LePP5 isoform is soluble, whereas the 62-kD isoform is an integral membrane protein. Production of yellow fluorescent protein-PP5 chimeras in plant cells indicated that the 55-kD isoform is localized in both the nucleus and the cytoplasm, whereas the 62-kD isoform is targeted to the endoplasmic reticulum, including the nuclear envelope. Our findings show that alternative splicing generates two LePP5 isoforms with a different subcellular localization.

Many signal transduction processes depend on the reversible phosphorylation of proteins. Phosphorylation and dephosphorylation are catalyzed by kinases and phosphatases, respectively. Protein phosphatases dephosphorylate Tyr and/or Ser and Thr residues and are distinguished by their protein interaction and regulatory domains (Barford, 1996). Protein Ser/Thr phosphatases are divided into the protein phosphatase P (PPP) and protein phosphatase M (PPM) families. Despite the similarity between the three-dimensional structures of PPP and PPM family members, their amino acid sequences are not conserved (Barford, 1996). The PPM family mainly consists of PP2C phosphatases, such as Arabidopsis ABI1 and KAPP. The PPP family contains the well-studied protein phosphatases 1 (PP1), 2A (PP2A), and 2B (PP2B), and the more recently discovered protein phosphatase 5 (PP5) and RgdC/protein phosphatase 7 (PP7; Cohen, 1997; Andreeva and Kutuzov, 1999).

PP5 contains an N-terminal tetratricopeptide repeat (TPR) domain, a domain that serves in many proteins as interface for interaction with other proteins (Blatch and Lässle, 1999). The TPR domain of PP5 consists of three TPR motifs, each folding into two antiparallel α-helices, and a sequence that shares low homology with the TPR motif and folds into a single α-helix (Das et al., 1998). The TPR domain is connected via a short variable spacer to the phosphatase domain. The C terminus contains a signal that mediates nuclear targeting of a subset of the PP5 molecules (Borthwick et al., 2001), whereas the majority resides in the cytoplasm (Russell et al., 1999; Brown et al., 2000; Morita et al., 2001). Mammalian and cauliflower (Brassica oleracea) PP5 have a low intrinsic phosphatase activity in vitro (Chen et al., 1994; Skinner et al., 1997; Meek et al., 1999), but biochemical analysis has revealed several mechanisms that stimulate PP5 activity: (a) Mammalian PP5 is activated 10- to 40-fold upon removal of either the TPR domain or the C terminus, indicating autoinhibitory properties of these parts of the PP5 protein (Chen and Cohen, 1997; Sinclair et al., 1999; Kang et al., 2001), and removal of the TPR domain of a protozoan PP5 activates the enzyme about 3-fold (Dobson et al., 2001); (b) cauliflower, protozoan, and mammalian PP5s are stimulated between 3 and 40 times by long-chain fatty acids (Chen and Cohen, 1997; Skinner et al., 1997; Meek et al., 1999; Chaudhuri, 2001; Dobson et al., 2001; Lindenthal and Klinkert, 2002; Ramsey and Chinkers, 2002). Because fatty acids esterified to CoA, unlike free fatty acids, stimulate rat (Rattus norvegicus) PP5 at biologically different times by long-chain fatty acids, unlike free fatty acids, stimulate rat (Rattus norvegicus) PP5 at biologically different times by long-chain fatty acids.
relevant concentrations, they are potential in vivo regulators of PP5 (Ramsey and Chinkers, 2002).

Evidence for roles of PP5 in signal transduction is expanding (Chinkers, 2001). By overexpression of PP5, human (Homo sapiens) cells become independent of estrogen for proliferation (Urban et al., 2001), and overexpression of the TPR domain reduces glucocorticoid receptor (GR) function (Chen et al., 1996). Suppression of PP5 expression results in growth inhibition and causes activation of the GR and the tumor suppressor protein p53, suggesting that PP5 functions as a suppressor of GR- and p53-mediated growth inhibition (Zuo et al., 1998, 1999). In another example, PP5 overexpression reduces apoptosis mediated by the mitogen-activated protein kinase kinase kinase ASK1 by inhibiting its activity (Morita et al., 2001). Yeast (Saccharomyces cerevisiae) two-hybrid studies have revealed several protein candidates that interact with PP5, mostly, if not always, with its TPR domain. Among these are the Go-subunit of heterotrimeric G proteins, PP2A, copines, the blue-light receptor hCRY2, ASK1, the atrial natriuretic peptide receptor, and components of the anaphase-promoting complex (Chinkers, 1994; Ollendorff and Donoghue, 1997; Zhao and Sancar, 1997; Lubert et al., 2001; Morita et al., 2001; Tomsig et al., 2003). For Hsp90, PP2A, Go, and ASK1, in vivo interactions have been demonstrated by co-immunoprecipitation (Chen et al., 1996; Lubert et al., 2001; Morita et al., 2001; Yamaguchi et al., 2002).

In this study, we describe the characterization of a tomato (Lycopersicon esculentum) PP5 cDNA. Biochemical analysis of the recombinant LePP5 protein showed that the enzyme is a functional type 5 protein phosphatase. Tomato and Arabidopsis PP5 are single-copy genes and encode pre-mRNAs that are alternatively spliced, leading to the production of two protein isoforms that are differently localized in the cell.

RESULTS

Analysis of the Tomato PP5 cDNA and Gene

During the screening of a tomato cDNA library, we previously identified a clone that showed high sequence homology with protein Ser/Thr PP5. To obtain a full-length cDNA, PCR was performed on the same cDNA library using a vector-specific forward primer in combination with an insert-specific reverse primer. A full-length tomato cDNA was isolated (GenBank accession no. AY150041) that contained an open reading frame (ORF) encoding a protein of 485 amino acids with a molecular mass of 54,685 D, hereafter referred to as the 55-kD isoform. A BLAST search (Altschul et al., 1997) in the National Center for Biotechnology Information database revealed that the protein shares 88% identity with Arabidopsis PP5, 81% with rice PP5, 56% with rat and human PP5, 48% with Caenorhabditis elegans PP5, 51% with fruitfly (Drosophila melanogaster) PP5, and 39% identity with the yeast PP5 homolog Ppt1. Figure 1 shows an alignment of the 55-kD isoform of PP5 with Arabidopsis, rat, and yeast PP5. All structural features characteristic to PP5 homologs, notably three TPRs followed by an α-helical structure with low similarity to the TPR motif, a variable region, a phosphatase domain, and a putative nuclear localization signal appeared all to be conserved in the tomato protein that we tentatively called LePP5 (Fig. 1).

Biochemical Characterization of LePP5

To test whether the LePP5 protein is a functional protein phosphatase that exhibits the characteristic
enzymatic and biochemical features of other PP5 homologs as well, the LePP5 cDNA was cloned downstream of the GST (glutathione S-transferase) gene to give GST-LePP5. In addition, GST fusions of rat PP5 (Skinner et al., 1997) and of an LePP5 mutant cDNA were made and named GST-rat PP5 and GST-LePP5G340R, respectively. Rat PP5 served as a positive control for the characterization of LePP5. The mutant cDNA contained one point mutation resulting in a Gly to Arg change at position 340 (LePP5G340R, Fig. 1) and was isolated during the course of investigation. Isopropylthio-/galactoside (IPTG)-mediated induction of expression of the GST-LePP5 constructs for 4 h at 37°C resulted in the production of poorly soluble fusion proteins. However, induction at room temperature for 8 h led to the production of soluble proteins that were subsequently affinity purified as described in “Materials and Methods” (Fig. 2, lanes 1–3). Initial phosphatase activities were assessed using para-nitrophenylphosphate (pNPP) as substrate, whereas in later analysis, phosphorylated casein was used. When pNPP was used as a substrate, the activities of GST-rat PP5 and GST-LePP5 were increased by 10 mM MgCl2 (data not shown; Ramsey and Chinkers, 2002). However, using phosphorylated casein as a substrate, the addition of 10 mM MgCl2 reduced the activities of both fusion proteins by 50% (data not shown). At this point, we decided to continue the subsequent assays with phosphorylated casein, and divalent cations were omitted. The activities of the GST fusion proteins were tested at both pH 7.5, a pH value widely used for the characterization of PP5 homologs, and pH 5.0, because we had some indications that GST-LePP5 activity was highest at this pH using pNPP as substrate. GST-rat PP5 dephosphorylated phosphorylated casein with a specific activity of 81.1 and 63.6 nmol inorganic phosphate (Pi) released min mg−1 at pH 7.5 and 5.0, respectively (Fig. 3A), activities in the same order as described before (Sinclair et al., 1999). The activity of GST-LePP5 was 32.5 and

Figure 2. Purification of recombinant GST-PP5 proteins from Escherichia coli. Recombinant GST fusion proteins were isolated from E. coli as described in “Materials and Methods,” run over a 10% (w/v) acrylamide gel, and stained with Coomassie Blue. Lane 1, GST-LePP5 (81.1 kD); Lane 2, GST-LePP5G340R (81.2 kD); Lane 3, GST-rat PP5 (83.3 kD); Lane 4, GST-LePP5 ΔTPR (64.6 kD).

Figure 3. Activity of the GST-PP5 fusion proteins, stimulation of GST-LePP5 activity by fatty acids, and inhibition by okadaic acid. Protein phosphatase activity was tested toward phosphorylated casein. A, Activity of purified GST fusion proteins at pH 5.0 and 7.5. The phosphatase activities of GST-rat PP5, GST-LePP5, GST-LePP5G340R, and GST-LePP5 ΔTPR were measured at pH 5.0 and 7.5. Values are mean of an experiment performed in duplicate. Experiments were repeated with different batches of enzymes in independent experiments and gave similar results. B, Stimulation of GST-LePP5 phosphatase activity by linoleic acid (LA) and α-linolenic acid (LNA), oleic acid (OA), and arachidonic acid (AA). The first bar represents control GST-LePP5 activity. Values are mean of at least two independent experiments performed in duplicate. Experiments were repeated with different batches of enzymes in independent experiments and gave similar results. C, Inhibition of the activity of GST-LePP5 ΔTPR by okadaic acid. The activity of GST-LePP5 ΔTPR was tested in the presence of increasing concentrations of okadaic acid at pH 7.5. Values are mean of two independent experiments performed in duplicate.
Like all other homologs, the activity of GST-LePP5 full-length LePP5 to different fatty acids were tested. For example, the responses of PP5 from C. elegans, fruitfly, Arabidopsis, and human. To examine whether the observed differential splicing of PP5 is specific for tomato, we determined the presence of PP5 transcripts in Arabidopsis. RT-PCR analysis using AtPP5-specific primers that amplify nearly the complete ORF demonstrated that in this plant species, two PP5 transcripts are also present (Fig. 4C). Cloning and sequencing of both PCR products showed that the shorter transcript (GenBank accession no. AF419574) contained an insertion of 213 nucleotides at position 470 relative to the ATG. This insertion represents an additional exon in the LePP5 gene between the fourth and the fifth exons (exon 4A in Fig. 4B). The extra exon results in an insertion of 71 amino acids into the spacer that separates the TPR and the catalytic domains of PP5 (between V157 and E158, Fig. 1). Apparently, LePP5 pre-mRNA is alternatively spliced into two mRNA variants encoding two distinct isoforms. The larger PP5 isoform has a molecular mass of 61,820 D and is referred to as the 62-kD isoform.

To determine whether both transcripts corresponded to PP5 and their relative abundance, we performed an RT-PCR using LePP5-specific primers to amplify total RNA isolated from tomato roots, stems, and leaves, confirming the northern-blot results (Fig. 4C). Sometimes, a third product was observed migrating in between the two other PCR products. This product was isolated from gel, and reamplification resulted in three products with the same relative abundance as seen in Figure 4C. Northern-blot analysis revealed that PP5 is expressed in tomato roots, stems, leaves, flowers, and fruits. Surprisingly, the probe hybridized with two RNA species of slightly different sizes: one abundant, shorter RNA and one less abundant, longer RNA (data not shown). However, northern-blot analysis was not accurate enough to clearly discriminate and quantify both transcripts. The total PP5 mRNA level was essentially the same in each of the tested tissues. To determine whether both transcripts corresponded to PP5 and their relative abundance, we performed an RT-PCR using LePP5-specific primers to amplify total RNA isolated from tomato roots, stems, and leaves, confirming the northern-blot results (Fig. 4C). Sometimes, a third product was observed migrating in between the two others on a gel; the same treatment of the non-pooled fragments did not give rise to the extra band (data not shown). From this, we concluded that the third product was a heteroduplex containing one strand of each of the two PCR products. The relative abundance of the two PCR products did not significantly differ between the organs tested. Both PCR products were cloned and sequenced. The sequence of the shorter, more abundant transcript was identical to the PP5 cDNA clone described above. Compared with the shorter transcript, the longer transcript (GenBank accession no. AY182777) contained an insertion of 213 nucleotides at position 470 relative to the ATG. This insertion represents an additional exon in the LePP5 gene between the fourth and the fifth exons (exon 4A in Fig. 4B). The extra exon results in an insertion of 71 amino acids into the spacer that separates the TPR and the catalytic domains of PP5 (between V157 and E158, Fig. 1). Apparently, LePP5 pre-mRNA is alternatively spliced into two mRNA variants encoding two distinct isoforms. The larger PP5 isoform has a molecular mass of 61,820 D and is referred to as the 62-kD isoform.

To examine whether the observed differential splicing of PP5 is specific for tomato, we determined the presence of PP5 transcripts in Arabidopsis. RT-PCR analysis using AtPP5-specific primers that amplify nearly the complete ORF demonstrated that in this plant species, two PP5 transcripts are also present (Fig. 4C). Cloning and sequencing of both PCR products showed that the shorter AtPP5 transcript (GenBank accession no. AF419574) encodes a 54,667-D protein showing 88% identity with the 55-kD LePP5 isoform (Fig. 1). The longer PCR product appeared to consist of two transcripts, one (GenBank accession no. AY182779) with an insertion of 162 nucleotides.
compared with the shorter transcript and the other with an insert of 159 nucleotides. The sequence of the shorter insert is identical to that of the longer one except for the last three 3′-terminal nucleotides that are lacking in the 159-bp insert. Translation of the larger transcripts results in two AtPP5 isoforms of 60,244 and 60,188 D, respectively. In the 60,244-D isoform, the amino acid (residue 211) after the insertion is changed from Glu (E157 in the 55-kD isoform) into a Lys (Fig. 1 and 4D), due to the position of the splice junction within a codon. In the 60,188-D isoform, the amino acid (residue 210) after the insertion remains unchanged (Fig. 4D). The insertions of 54 and 53 amino acids are at the same position as the insertion of 71 amino acids in the 62-kD LePP5 isoform. Alignment of the AtPP5 transcripts with the AtPP5 gene (GenBank accession no. At2g42810) revealed that the intron-exon positions are identical to LePP5. In contrast to the LePP5 gene, the AtPP5 gene contains an upstream intron in the 5′-untranslated leader. PP5 expressed sequence tags (ESTs) are present in The Institute for Genomic Research Arabidopsis and tomato databases (release dates: Arabidopsis, October 1, 2002; and tomato, April 25, 2002). However, in accordance with their low abundance, no ESTs specific for the longer transcripts were found in these databases. Together, these results suggest that both tomato and Arabidopsis PP5 pre-mRNA are alternatively spliced, potentially resulting in two transcripts encoding two isoforms.

Subcellular Localization of Tomato PP5 Isoforms

The predicted amino acid sequences encoded by exon 4A of LePP5 and the corresponding exon of AtPP5 show less homology compared with the remainder of the proteins (Fig. 4D) and display no significant homology with other known sequences. However, hydropathy profiles of these sequences evidently show two adjacent hydrophobic peaks, suggesting that they form two membrane-spanning regions (Fig. 4E). If this is correct, then the larger PP5 isoforms may be integral membrane proteins. To test this idea, we fractionated protein extracts of tomato stems by differential centrifugation. The presence of PP5 isoforms in the fractions was determined by

Figure 4. Structure of the LePP5 gene and alternative splicing of PP5 pre-mRNA in tomato and Arabidopsis. A, Intron-exon structure of LePP5. Exons are represented by black rectangles. Exon numbers are indicated above each exon. B, Position of the alternatively spliced exon (indicated by 4A) in the LePP5 gene. C, Expression of PP5 in roots, stems, and leaves of tomato and total green tissue of Arabidopsis was analyzed by reverse transcriptase (RT)-PCR. Arrows, PCR products corresponding to the PP5 transcripts. Asterisk, Heteroduplex of the two PCR products, containing one strand of each PCR product, that is sometimes observed. D, Alignment of the amino acid sequences encoded by the alternative exons of LePP5 and AtPP5. The last three amino acids encoded by the previous exon and the first three residues encoded by the next exon are boxed. E, Hydropathy plots (Kyte-Doolittle) of the deduced amino acid sequences encoded by the alternatively spliced exons in the PP5 gene of tomato and Arabidopsis. The number shown below the plots indicates the amino acid position. The distance between the vertical bars at the top and bottom of the plots represents 10 amino acids.
western-blot analysis using antibodies raised against the TPR domain of LePP5. In total stem extract, two proteins were detected with molecular masses expected for the two PP5 isoforms (Fig. 5A, lane 1). Both proteins were also detected in extracts of roots and leaves with the same abundance, and the ratios between the isoforms in each tissue were equal (data not shown). Although most of the 55-kD isoform was present in the supernatant of 10,000g (Fig. 5A, lane 2) and 100,000g (data not shown) centrifugations, the 62-kD PP5 isoform was highly enriched in the 10,000g pellet (Fig. 5A, lane 3). A 10,000g pellet mainly consists of organelles such as nuclei, mitochondria, plastids, and large membranous and cytoskeletal structures. Next, this pellet was treated with either 0.1 M Na₂CO₃ (pH 11.5) or 6 M urea. Proteins that were not removed from the pellet by these treatments are considered to be integral membrane proteins, whereas the removed proteins are either peripheral membrane proteins or soluble (Kuhn et al., 1990). Although we cannot exclude that some of the 62-kD PP5 isoform was removed from the pellet fraction, at least a significant amount was neither by Na₂CO₃ (Fig. 5A, lane 4) nor by urea (Fig. 5A, lane 5), suggesting that this isoform is an integral membrane protein. The 55-kD isoform was removed from the pellet by both Na₂CO₃ and urea (Fig. 5A). Because the majority of this isoform was present in the 100,000g supernatant, we conclude that this represents a cytoplasmic localization, whereas a small fraction either resides inside a large organelle and/or is peripherally associated with a membrane. No bands were visible when the blot was probed with the pre-immune serum (Fig. 5A, lanes 6–10).

To elaborate further the observed difference in subcellular localization of the two PP5 isoforms, we produced both polypeptides as YFP fusions in cowpea mesophyll protoplasts. For this purpose, the two LePP5 cDNAs were cloned downstream of the YFP gene. To test whether YFP chimeras of the expected sizes were produced and localized subcellularly as the genuine LePP5 isoforms, protein extracts of protoplasts were analyzed by western blotting using anti-LePP5 TPR antibodies. As expected, most of the YFP-55-kD LePP5 fusion protein was detected in the soluble fraction rather than in the pellet (Fig. 5B, lanes 1 and 2), whereas the YFP-62-kD LePP5 chimera was preferentially found in the pellet (Fig. 5B, lanes 3 and 4). Imaging of the YFP fusion proteins in living protoplasts by confocal microscopy showed that the 55-kD isoform is localized mainly in the cytoplasm but also is present inside the nucleus although excluded from the nucleolus (Fig. 5B). In Figure 6C, the chloroplasts of the cell are shown in which the fusion protein was produced. Contrasting with this fusion protein, the YFP-62-kD LePP5 isoform was excluded from the nucleus but instead localized at the nuclear envelope (Fig. 6G). This LePP5 isoform was further localized in intracellular structures that probably are based on the fractionation studies of tomato protein lysates, endomembranous structures (Fig. 5B). To analyze whether the YFP-62-kD LePP5 chimera colocalized with the endoplasmic reticulum (ER), an ER marker (CFP-ER) that consists of CFP, containing an N-terminal chitinase signal peptide and a C-terminal HDEL motif for ER retention, was expressed in the same cell. The ER encompasses the nuclear envelope and intracellular structures surrounding the chloroplasts and extends to the plasma membrane (Fig. 6, A and F). The merged image of Figure 6, F to H, is depicted in Figure 6I. The localization of the cyan fluorescence of CFP-ER (y axis, Fig. 6I) in the cell clearly correlated with that of the yellow fluorescence of YFP-62-kD LePP5 (x axis, Fig. 6I). This strongly suggests that the 62-kD LePP5 isoform is localized to the ER (Fig. 6, F–J). As expected, there was no substantial colocalization of the YFP-55-kD LePP5 chimera with the CFP-ER marker (Fig. 6, A–E). In Figure 6E, the yellow fluorescence within the nucleus is indicated by a black arrow. Other examples of cells expressing the YFP-LePP5 chimeras are presented in Supplemental Figure 1. In conclusion, the fractionation of tomato proteins in combination with subcellular localization in plant cells indicate that the two LePP5 isoforms are targeted to different subcellular locations.

Figure 5. The two LePP5 isoforms have different subcellular localizations. A, Proteins (5 μg) were separated in a SDS-PAGE gel, blotted, and incubated with either a polyclonal antibody against the recombinant TPR domain of LePP5 (lanes 1–5) or pre-immune serum (lanes 6–10). Lanes 1 and 6, Total protein. Lanes 2 and 7, Supernatant (10,000g). Lanes 3 and 8, Pellet (10,000g). Lanes 4 and 9, Pellet (10,000g) washed with 0.1 M Na₂CO₃ (pH 11.5). Lanes 5 and 10, Pellet (10,000g) washed with 6 M urea. B, Detection of yellow fluorescent protein (YFP)-LePP5 fusion proteins in cowpea (Vigna unguiculata) protoplasts. Protein lysates from protoplasts expressing YFP-55-kD LePP5 (83.6 kD; lanes 1 and 2) or YFP-62-kD LePP5 (90.7 kD; lanes 3 and 4) were analyzed by western blotting using anti-LePP5 TPR antibodies. Centrifugation yielded 8,000g supernatants (lanes 1 and 3) and pellets (lanes 2 and 4). In each lane, eight micrograms of protein was loaded.
DISCUSSION

LePP5 Activity Is Stimulated in Vitro by Removal of the TPR Domain and by Fatty Acids

Protein Ser/Thr PP5 is a component of various signal transduction cascades in animal cells. Here, we describe the isolation of a full-length tomato PP5 cDNA and its expression in E. coli. The activity of the purified protein was elevated 6- to 10-fold by either fatty acids or removal of the TPR domain, and its activity was inhibited by okadaic acid, indicating that characteristics of PP5 homologs from other species also apply to LePP5. This suggests that the catalytic domain is shielded by the TPR domain, as has been suggested previously for animal PP5 (Chen and Cohen, 1997). When phosphocasein was used as a substrate, the level of stimulation by either fatty acids or by removal of the TPR domain was similar to that of mammalian and cauliflower PP5 (Chen and Cohen, 1997; Skinner et al., 1997; Meek et al., 1999; Sinclair et al., 1999; Ramsey and Chinkers, 2002). However, using a substrate widely used for the characterization of PP5 homologs, pNPP, we found that the basal activity of the tomato PP5 protein was very high and was stimulated only 2- to 3-fold by fatty acids or removal of the TPR domain (data not shown). In control experiments, the activity of rat PP5 was increased 20-fold toward pNPP by 200 μM different fatty acids, to a level similar to that described before (data not shown; Skinner et al., 1997). Therefore, we conclude that pNPP is not a suitable substrate for the characterization of LePP5. A Gly to Arg mutation (G340R) in the catalytic domain of LePP5 greatly reduced its enzymatic activity. Although this Gly is conserved in all PPP family members, it is not likely to be involved in catalysis itself. However, the reduction of the phosphatase activity may be explained by the fact that the mutation is next to a His residue implicated in catalysis by phosphatases belonging to the PPP family.

PP5 has been implicated to be an arachidonic acid-stimulated regulator of an ion channel in mammalian cells (Skinner et al., 1997). In plant cells, fatty acids can be precursors for structural membrane glycerolipids or for signaling molecules such as jasmonic acid. Interestingly, free fatty acids are released from structural membrane lipids by phospholipases during many different stress responses and can function as second messengers. If PP5 is regulated by free fatty acids in plant cells, one could speculate that PP5 is activated during adaptation to certain stress conditions.

The Single-Copy Tomato PP5 Gene Encodes Two Isoforms That Are Targeted to Different Subcellular Locations

PP5 is a single copy gene that is evolutionarily conserved throughout the eukaryotic kingdom. The PP5 gene of fruitfly, C. elegans, and human contain eight, nine, and 13 exons, respectively, with only a few conserved intron-exon positions. In contrast, the intron and exon positions of the PP5 gene of tomato...
and Arabidopsis are conserved, except that \textit{AtPP5} contains an additional exon in the 5' leader. Analysis of \textit{PP5} mRNA from these plant species revealed the presence of two transcripts. The shorter, more abundant transcript encodes a 55-kD protein with a similar domain composition compared with \textit{PP5} homologs from other species. The longer transcripts contain an additional sequence representing an additional exon in the \textit{PP5} gene. An \textit{AtPP5} transcript containing the additional exon has already been predicted by the Arabidopsis genome sequencing project, but the 3' splice site was predicted 15 bp upstream of the splice site that we have determined. This predicted \textit{AtPP5} transcript, however, was recently described as being wrongly annotated, and it was suggested that the additional exon was not a genuine one (Kerk et al., 2002). Our results show that the originally predicted exon is a true exon that is alternatively spliced. The longer \textit{AtPP5} and \textit{LePP5} transcripts encode isoforms that contain an insertion into the region separating the TPR and phosphatase domains. Two segments of the insertion are enriched in hydrophobic residues and likely represent two transmembrane-spanning domains (Fig. 4E). Subcellular fractionation of tomato proteins showed that the 62-kD isoform of \textit{LePP5}, but not the 55-kD isoform, is an integral membrane protein. In protein extracts of root and stem tissue of \textit{Nicotiana tabacum} and \textit{Nicotiana benthamiana}, two \textit{PP5} isoforms of different sizes also can be detected using anti-Le\textit{PP5} TPR antibodies (data not shown). Similar to tomato, in these plant species, the larger isoform was enriched in the 10,000g pellet. The \textit{PP5} genes of yeast, \textit{Schizosaccharomyces pombe}, and \textit{Plasmodium falciparum} lack introns, and those of the fruitfly, \textit{C. elegans}, and human do not contain intron sequences that could code for a transmembrane domain insertion into the spacer in between the TPR and phosphatase domains. Alternative splicing of \textit{PP5} resulting in a membrane-localized isoform, therefore, is likely to be specific for plants. The intron-exon positions in the rice \textit{PP5} gene are identical to those in the \textit{LePP5} gene. Surprisingly, a sequence encoding a transmembrane domain seems to be absent from the corresponding intron in the \textit{PP5} gene of rice, a monocot (data not shown). This might suggest that alternative splicing regulating the membrane localization of \textit{PP5} is specific for dicots. Interestingly, at least three splice variants of human \textit{PP5} are present in the human EST database at The Institute for Genomic Research. One EST is identical to the previously described \textit{HsPP5} mRNA (Chen et al., 1994), whereas the other ESTs either miss a complete exon encoding amino acids 212 to 233, which are localized in the phosphatase domain, or contain a different 3' terminus. The latter \textit{HsPP5} mRNA encodes a protein lacking the putative C-terminal NLS. Recently, an alternative splice form of rat \textit{PP5} encoding only the TPR domain has been described (Yamaguchi et al., 2002).

In plants, several examples of alternative splicing resulting in an altered localization of isoforms have been described (Mireau et al., 1996; Thorbjørnsen et al., 1996; Mano et al., 1997, 1999). Alternative splicing of pumpkin (\textit{Cucurbita pepo}) ascorbate peroxidase produces two isoforms with different C termini (Mano et al., 1997). One isoform is located in the stroma of chloroplasts, but the other, due to the presence of a membrane-spanning domain in its C terminus, is located in the chloroplast thylakoid membrane (Mano et al., 1997). In analogy, the 55- and 62-kD \textit{LePP5} isoforms are localized inside the nucleus and in the nuclear envelope, respectively. Subcellular fractionation of tomato proteins combined with subcellular localization of a YFP chimera in cowpea protoplasts indicated that a small fraction of the 55-kD \textit{LePP5} isoform was present in the nucleus, and the majority of the protein is cytoplasmic. This is similar in protozoan and animal cells, in which \textit{PP5} is localized in both the nucleus and in the cytosol (Chen et al., 1994; Russell et al., 1999; Brown et al., 2000; Borthwick et al., 2001; Morita et al., 2001). The nucleoplasmic localization of the 55-kD \textit{LePP5} isoform may be caused by the putative C-terminal nuclear localization signal (Borthwick et al., 2001). By expression in cowpea protoplasts as a YFP fusion protein, the 62-kD \textit{LePP5} isoform was shown to be present in the ER membrane and nuclear envelope, in agreement with fractionation of tomato proteins. Because this localization for \textit{PP5} has not been described for non-plant species, the alternative splicing event leading to an ER-localized \textit{PP5} may be unique for (dicot) plant \textit{PP5}. N- or C-terminal di-Lys or di-Arg motifs are responsible for targeting of many integral membrane proteins to the ER (Teasdale and Jackson, 1996). Because such motifs are absent from the N and C terminus of the 62-kD \textit{LePP5} isoform, it remains obscure what motif is responsible for localization of this protein in the ER membrane. A difference in subcellular localization of the \textit{LePP5} isoforms could indicate that they serve different functions. The 62-kD \textit{LePP5} protein putatively contains two transmembrane regions; therefore, the TPR and phosphatase domains of this isoform could face the same side of the ER membrane. Although the TPR and phosphatase domain of the 55-kD \textit{LePP5} isoform mediate binding and dephosphorylation of protein substrates, respectively, the 62-kD isoform could do the same at one side of the ER membrane. This isoform could either recruit its protein substrates to the ER membrane or target proteins associated with the ER membrane.

**MATERIALS AND METHODS**

**Isolation of the \textit{LePP5} cDNA and Gene**

An \textit{LePP5} cDNA was isolated from an expression library made from RNA from tomato (\textit{Lycopersicon esculentum}) cv GCR161 (Kroon and Elgersma, 1996).
roots and stems after infection with *Fusarium oxysporum* lycopersici. To obtain upstream sequence of a full-length cDNA, a PCR was performed on the cDNA library using the F688 (5'-taatcactacaattgatg-3') forward primer and the pACT vector in which the library was cloned and the FP162 (5'-ATGGATCTCCATAAGACTGACGTCACTGAGCG-3') reverse LePP5 primer. A PCR product was cloned in pGem-T easy (Promega, Madison, WI) and sequenced. To isolate the LePP5 gene, an EMBL-3 genomic library of tomato cv VFNB (CLONTECH Laboratories, Palo Alto, CA) was screened according to the manufacturer's protocol using the LePP5 cDNA as a probe. An approximately 11.5-kb fragment of the resulting number of colonies each containing complete LePP5 gene was subcloned in pBluescript KS+ and sequenced double-stranded by Baseclear (Leiden, The Netherlands).

**Design of Constructs for LePP5 Protein Expression in Escherichia coli**

For expression of the TPR domain of LePP5, including the helix (amino acids 1–151), a PCR fragment was generated using PuI polymerase (Stratagene, La Jolla, CA) and primers FP161 (5'-GGCGATCCGGCTGATGGAAGG-3') and FP162, digested with BamHI, and cloned into the Smal and BamHI sites of the pAS2-1 (CLONTECH Laboratories) vector. An Smal-Sall fragment was excised from this construct and cloned into the Smal and SalI sites of pGEX-KG (Guan and Dixon, 1991) in frame with GST. The PPS fragment encoding the phosphatase domain and putative nuclear localization signal (LePP5 ΔTPR; amino acids 148–485) was amplified using PuI polymerase with FP163 (5'-GGCGATCCGATCTGCTTATGAGT-3') and FP164 (5'-ATGGATCTCTAAAGACATGCGAAGGAAG-3'), digested with BamHI, and cloned into the Smal and BamHI sites of the pAS2-1 (CLONTECH Laboratories) vector. An Smal-Sall fragment was excised and cloned into the Smal and SalI sites in pGEX-KG. Smal-AlniI and AlniI-Sall fragments encoding the TPR and the phosphatase domain, respectively, were isolated from the corresponding pAS2-1 constructs and ligated into pGEX-KG to reconstitute the complete ORF (this construct was named GST-LePP5). Clones of each construct were sequenced. One full-length clone accidentally contained a G to A point mutation at position 1,018 relative to the ATG giving rise to a Gly to Arg change at position 340 in the protein. This GST-LePP5 mutant was used as a negative control in the phosphatase assays.

**Recombinant Protein Purification**

pET-21a for expression of rat (Rattus norvegicus) PPS, kindly provided by Sandra Rossie (Purdue University, West Lafayette, IN; Skinner et al., 1997), and pGEX-KG constructs for expression of GST-LePP5 ΔTPR, GST-LePP5, and GST-LePP5ΔC termini were transformed to *E. coli* BL21 (DE3). Cultures starting at A600 of 0.6 to 1.0 were induced with 1 mM IPTG for 4 h at 37°C. The GST-PP5 protein was isolated as described above. Eluted GST-PP5 protein was dialyzed to 10 mM Na2HPO4, 2 mM KH2PO4, 137 mM NaCl, and 2.7 mM KCl (pH 7.4). GST was removed from the TPR domain by thrombin digestion and subsequent incubation with glutathione beads. To generate antibodies against the LePP5 TPR domain, rabbits were immunized by three injections of 100 μg of TPR domain given at d 0, 14, and 28 at Eurogentec (Seraing, Belgium).

**Isolation of Tomato and Cowpea (Vigna unguiculata) Proteins and Subcellular Fractionation**

Tomato tissue of 25-d-old GCR161 plants was homogenized in ice-cold buffer A (20 mM Tris [pH 7.5], 80 mM NaCl, 0.33 mM Suc, 4 mM EDTA, 2 mM diithiothreitol, 1% [v/v] polyvinylpyrrolidone, and 1% Complete protease inhibitor cocktail [Boehringer Mannheim/Roche, Basel]) using a Sorvall omni-mixer. The homogenate was filtered through four layers of Miracloth and centrifuged at 500 g for 5 min to remove large cell debris to yield the “total protein” fraction. This fraction was centrifuged twice at 10,000g for 20 min. The first pellet was washed with buffer A and centrifuged at 10,000g for 15 min. The 10,000g pellet was then incubated with 100 mM Na2CO3 or 6 M guanidine hydrochloride (1 h on ice) and centrifuged for 30 min at 50,000g. To isolate proteins from cowpea cells expressing YFP fusion proteins (see below), pro plastids were lysed in buffer A 1 d after transfection by vortexing. The lysates were filtered through two layers of Miracloth and centrifuged at 8,000g for 10 min. Both tomato and cowpea protein samples were diluted in SDS sample buffer and separated on 8% (v/v) polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Blots were incubated with antisera against the LePP5 TPR domain (1:10,000 [v/v]) and subsequently treated with peroxidase-conjugated secondary antibodies (1:4,000 [v/v]; Pierce Biotechnology, Rockford, IL). Detection was performed with an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Phosphatase Assays**

Labeling of casein and phosphatase assays were performed as described before (Meskiene et al., 1998) with a few adaptations. Assays were performed in 60 mM Tris (pH 5.0 or 7.5), 1 mM EDTA, 1% (v/v) β-mercaptoethanol using at least 200,000 cpm of labeled casein (containing 0.24 mol phosphate/mol protein). Assays with fatty acids or okadaic acid and the corresponding control samples contained 2% (w/v) ethanol and were performed at pH 7.5. Twenty to 300 ng of GST fusion protein was used per assay, and reactions were incubated at 30°C for 15 min and terminated with 200 μL of 20% (w/v) trichloroacetic acid. After centrifugation for 5 min at 14,000g, 200 μL was transferred to a scintillation vial, and 3.5 mL of scintillation liquid was added. Released Pi was measured in a scintillation counter. In a assay described by Sinclair et al. (1999), one microgram of purified GST fusion proteins was tested for activity using 20 to 50 mm pNPP. Mixtures containing pNPP were prewarmed to 30°C before addition of recombinant PP5 that was incubated at 30°C for 1 min. Reactions containing lipids (Sigma, St. Louis) contained a final ethanol concentration of 2% (w/v) and were performed at pH 7.5. The 50-μL reactions were incubated for 15 min at 30°C and stopped by adding 450 μL of 0.2 M NaOH. Absorbance was measured at A500. Samples without protein were used to correct for nonenzymatic pNPP hydrolysis.

**Phosphatase Assays**

The ORFs of the two PPS transcripts were subcloned into the Smal and BamHI sites of the pEYPF/P69K6-C1 vector. Nhel-BamHI fragments containing the YFP-PP5 constructs were isolated and cloned in the XbaI and BamHI sites in between the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator of pMON999. To construct a CFP-ER marker, a fragment was amplified from pCtP-C1 by PCR using PuI polymerase using the primers TMPF (5'-CGCACATCTGTGAGCCACACGGCGCAGG-3') and FP448 (5'-CGGGATCTTTAAAGACATGCGAAGGAAG-3'), with EcoRI and BamHI and cloned into the same sites of a pMON999 vector downstream of a basic chitinase signal sequence for secretion. This

RNA isolation and RT-PCR

DNA was extracted from green parts of 4-week-old Arabidopsis ecotype Wassilewskija plants and from roots, stems, and leaves from 27-d-old tomato cv GCR161 using Trizol (Invitrogen, Carlbad, CA). DNA was synthesized in 20 μL from total RNA using Superscript II (Invitrogen) according to the supplied protocol. RT-PCR was performed on 2 μL of cDNA with FP161 and FP164 for amplification of LePP5 transcripts and FP255 (5'-ATTCGAGCTCTCTCCCCTGCGCGAGG-3') and FP448 for the 5′-end of the genes for 30 cycles using primers.

**Antibody Production**

A pGEX-KG construct for expression of GST-LePP5 TPR (GST-TPR; see above) was transformed to *E. coli* BL21 (DE3). Cultures starting at A600 of 0.6 to 1.0 were induced with 1 mM IPTG for 4 h at 37°C. The GST-TPR protein was isolated as described above. Eluted GST-TPR protein was dialyzed to 10 mM Na2HPO4, 2 mM KH2PO4, 137 mM NaCl, and 2.7 mM KCl (pH 7.4). GST was removed from the TPR domain by thrombin digestion and subsequent incubation with glutathione beads. To generate antibodies against the LePP5 TPR domain, rabbits were immunized by three injections of 100 μg of TPR domain given at d 0, 14, and 28 at Eurogentec (Seraing, Belgium).
construct fuses the C terminus of YFP to an HDEL signal for ER retention. Both pCFP and pEYFP(Q69K) cloning vectors were obtained by PCR-based cloning methods using Yellow Cameleon 3.1 as template. The Yellow Cameleon 3.1 vector was kindly provided by Dr. Roger Y. Tsien (University of California, San Diego).

Transient Expression of YFP/CFP Chimeras in Cowpea Mesophyll Protoplasts and Imaging by Live Confocal Microscopy

Cowpea protoplasts were prepared and transfected with 8 µg of plasmid DNA using the polyethylene glycol method as described (van Bokhoven et al., 1993). Fluorescence microscopy was performed using a Zeiss LSM 510 CLSM (Zeiss, Jena, Germany) implemented on an inverted microscope (Axiovert 100). Protoplasts were mounted by pipetting them into one well of an eight-well Nunc chambered coverslip (Nalge Nunc International, Naperville, IL). Excitation was provided by the 458- and 514-nm Ar laser lines controlled by an acousto optical tuneable filter. Three dichroic beam splitters were used to separate excitation from emission and to divide the fluorescence emission into the CFP, YFP, and chlorophyll channels. The HFT 458/514 dual dichroic beam splitter was used as a primary dichroic mirror reflecting excitation and transmitting fluorescence emission, an NFT 635 dichroic mirror was used as a secondary splitter, and an NFT 515 was used as a tertiary dichroic splitter. Fluorescence reflected by both the NFT 635 and NFT515 splitters was filtered through a BP 470- to 500-nm filter yielding the CFP signal. Fluorescence reflected by the NFT635 but transmitted by the NFT 515 splitter was filtered through a BP 530- to 600-nm filter yielding the YFP signal. Fluorescence transmitted by both the NFT 515 and NFT635 splitters was additionally filtered by an LP 650 filter to yield the chlorophyll image. Cross talk-free CFP and YFP images were acquired by operating the microscope in the multitracking mode, in which the 514-nm excitation was coupled to activation of the YFP detection channel, and the 458-nm excitation was coupled to activation of the CFP and chlorophyll detection channels. A Zeiss water immersion C-Apochromat 40x 1.2 numerical aperture 1.2) corrected for cover glass thickness (set at 0.16 µm for Nunc chambers) was used for scanning. The detection pinholes were set at 1 airy disc unit. Images were captured and analyzed with the Zeiss LSM510 software (version 3.0 SP3). Two-dimensional scatter plots for analyzing colocalization were generated from the raw CLSM image data using the NIH Image 1.62I software package (National Institutes of Health, Bethesda, MD).

ACKNOWLEDGMENTS

We are grateful to Irute Meksiene for helping to set up the phosphatase assay with casein, Sandra Rossie for the generous gift of the rat PP5 construct, and Frank Takken for critical reading of the manuscript.

Received May 9, 2003; returned for revision June 24, 2003; accepted July 8, 2003.

LITERATURE CITED

Chen MX, Cohen PTW (1997) Activation of protein phosphatase 5 by limited proteolysis or the binding of polysaurans to the TPR domain. FEBS Lett 400: 136–140
Kroon BAM, Elgensma DM (1993) Interaction between race 2 of Fusarium oxysporum f. sp. lycopersici and near-isogenic resistant and susceptible lines of intact plants or callus of tomato. J Phytopathol 137: 1–9
Subcellular Localization of Plant Phosphatase 5 Isoforms


