Expression and characterization of PP7, a novel plant protein Ser/Thr phosphatase distantly related to RdgC/PPEF and PP5

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Abstract We have recently identified an *Arabidopsis thaliana* cDNA encoding a putative protein Ser/Thr phosphatase PP7, not closely related to any protein phosphatases in animals or fungi. Here, we describe the characterization of PP7 expressed in a bacterial system. The recombinant protein was inactive unless the longest insert in its catalytic domain was cleaved, suggesting that this insert is an autoinhibitory region. PP7 was resistant to okadaic acid, calyculin and fumonisin B_1, and was stimulated by Mn^{2+} or Fe^{2+}, while Ni^{2+} and Zn^{2+} were inhibitory. Polylysine stimulated PP7 activity towards *p*-nitrophenylphosphate but inhibited activity towards the most efficient protein substrate, myelin basic protein. A tentative model of the control of PP7 activity is proposed.

Key words: Protein Ser/Thr phosphatase; PP7; Okadaic acid; Calyculin A; Fumonisin B_1; *Arabidopsis thaliana*

1. Introduction

Protein Ser/Thr phosphatases have been implicated in a great variety of cellular processes in plants (for reviews, see [1,2]). A number of plant protein phosphatases (PP) of the PPP family [3] have been cloned including PPX and multiple isoforms of catalytic subunits of PP1 and PP2A, as well as regulatory subunits of some of them [3]. All these enzymes have structural homologues in animals, although precise functions of most of them in plants have not been clarified. Phosphatases of different PPP subfamilies are characterized by different sensitivities to several phosphatase inhibitors, some of which are commonly used to ascribe observed effects to certain phosphatase types (see references in [3]).

Arabidopsis thaliana PP7 cDNA encoding a putative novel protein Ser/Thr phosphatase has recently been described [4]. While PP7 is a member of the PPP family, no closely related structures have been found so far in animals or fungi. Detailed comparison of PP7 to other PPP phosphatases indicates that it is structurally more similar to the enzymes of the rdgC/PP5 subfamily [4,5]. The unique features of PP7 include an N-terminal domain which has no significant similarity to any sequence present in the databases, as well as three inserts in the C-terminal part of its catalytic domain; the latter region is thought to interact with regulators and substrates in PPP phosphatases [6].

These structural peculiarities of PP7, as well as ample evidence accumulated about the effects of phosphatase inhibitors on various functions in the plant cells, prompted us to undertake expression of this phosphatase in a heterologous system and characterization of its enzymatic activity as a part of our ongoing work aimed at analysis of PP7 function in vivo. Here, we describe expression in *Escherichia coli* and biochemical characterization of recombinant *A. thaliana* PP7. The results demonstrate that PP7 is an active protein Ser/Thr phosphatase and provide a set of characteristics that may be helpful in analysis of PP7 function in the plant cell.

2. Materials and methods

2.1. Construction of vectors for PP7 expression in E. coli

A cDNA fragment encoding the putative catalytic domain of PP7 (Gln40 to Ser367) was obtained by PCR using the clone 122A12T7 [4]. A *Bam*HI-*Sal*I PCR fragment was cloned into respective restriction sites of the expression vector pQE9 (Qiagen).

The complete open reading frame encoding PP7 was assembled in pBluescript II SK(+) (Stratagene) as follows. A *Hin*cII-*Eco*RI fragment (including the coding sequence from Asn48 and 179 bp 3′ noncoding region) obtained using clone 122A12T7 was cloned in pBluescript II SK(+) vector to generate pAS64. To obtain the missing N-terminal sequence, the construct pAS63, carrying a 5′-RACE fragment [4], was chosen as a template. Oligonucleotides 5′-CC*GTCGA-C*ACTGTTCCACCATCTCCC-3′ and 5′-AAGATCATCGATAT-GAACGCAG-3′ were used to generate the *Sal*I (*Hin*cII) site (italicized) in front of the ACT triplet encoding Thr3 (underlined) and *Hin*cII site (GTCAAC, encoding Val47 and Asn48) as the internal part of the PCR fragment. The *Hin*cII fragment was cloned in pAS64 digested with *Hin*cII, resulting in pAS65. Correct assembly of the PP7 open reading frame in pAS65 was checked by sequencing. PP7 was recloned as a *Sal*I-*Pst*I fragment (where the *Pst*I site is a part of pBluescript II SK(+) polylinker) in pQE9 to generating the construct pQE9/PP7.

E. coli M15 cells carrying pQE9/PP7 were grown at 37°C until the absorbance at 600 nm reached 0.7–0.9. Isopropylthiogalactoside was then added to a final concentration of 25 μM, and the culture was grown for an additional 3 h (at 37°C or 25°C) or overnight at 8°C.

2.2. Purification of recombinant PP7

Cell lysis and recombinant PP7 purification under denaturing or native conditions were performed essentially according to Qiagen instructions. Ni-IDA Sepharose (Sigma) was used instead of Ni-NTA agarose. Benzamidine (1 mM), PMSF (125 μM) and pepstatin (2 μM) were added to all solutions used for purification under native conditions, and all procedures were done at 0–4°C. PP7 containing fractions from Ni-IDA Sepharose chromatography were diluted 10-fold with water containing protease inhibitors and dithiothreitol (1 mM) and applied on a DEAE Sepharose (Sigma) column equilibrated in 5 mM HEPES (pH 7.6), 1 mM dithiothreitol. Proteins were eluted by a stepwise salt gradient in the same buffer (100, 150, 200, 250, 300, 400 and 500 mM NaCl), and fractions analyzed for protein composition and phosphatase activity. The presence of the His_6 tag in the protein was determined by Western blotting with Penta·His or RGS·His monoclonal antibodies (Qiagen). Detection was performed using ECL detection kit (Amersham).

N-terminal sequencing of the protein transferred onto Immobilon P membrane (Millipore) was carried out at Alta Bioscience (Birmingham, UK).

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2.3. Phosphatase assays

Phosphorylated substrates were prepared using [γ-^{33}P]ATP (NEN) as follows. Protein substrates (except rhodopsin) and kinases (except rhodopsin kinase) were purchased from Sigma. Myelin basic protein, casein and mixed histones were phosphorylated by the catalytic subunit of protein kinase A. Reaction mixtures (100 μl) contained 40 mM HEPES (pH 7.4), 0.1 mM EGTA, 10 mM magnesium acetate, 10 mM dithiothreitol, 0.1 mM [γ-^{33}P]ATP (5–10 μCi), 5 mg/ml substrate, and catalytic subunit of protein kinase A (30 U). Reactions were incubated at 30°C for 2.5 h (myelin basic protein and histone) or 24 h (casein). Phosphorylase *b* was phosphorylated by phosphorylase kinase according to [7]. Free ATP was separated and buffer changed on NAP-5 desalting columns (Pharmacia) equilibrated in 5 mM HEPES (pH 7.4), 100 mM NaCl. Purification of bovine rod outer segments and phosphorylation of rhodopsin by endogenous rhodopsin kinase were performed as described elsewhere [8].

Protein phosphatase activity was assayed in a final volume of 25–50 μl at 25°C with substrate concentrations corresponding to 5 μM $^{33}P_i$ and appropriate amounts of PP7-containing fractions to ensure linearity of the $^{33}P_i$ release with time and PP7 concentration. When necessary, additions were used as specified in the figure legends. Okadaic acid, calyculin A and fumonisin B_1 (all from Sigma) were added in methanol; the final methanol concentration in the reaction mixtures (4%) did not affect PP7 activity.

p-Nitrophenylphosphate hydrolysis was measured by diluting appropriate amounts of PP7 (typically 15–40 μl) in a buffer containing (unless otherwise indicated in the figure legends) 20 mM HEPES (pH 7.8), 0.5 mM $MnCl_2$, 1 mM dithiothreitol and 1 mM *p*-nitrophenylphosphate (total volume 800 μl). *p*-Nitrophenol formation was followed by absorbance increase at 410 nm. *p*-Nitrophenol extinction coefficients at different pHs were determined experimentally using 40 μM *p*-nitrophenol.

Since the half-life of purified PP7 (kept either on ice or at −20°C in 50% glycerol) was ∼20 h, all phosphatase assays were done on the same or the next day after purification.

3. Results

3.1. Expression and purification of PP7

Initially, we attempted to express the putative catalytic domain of PP7 without N- and C-terminal extensions (residues 40–367) to avoid potential problems caused by the possible presence of autoinhibitory sites in the N- or C-terminal extensions, which are found in some PPP phosphatases [9–11]. This resulted in a high yield expression (20–30 mg/l of bacterial culture) of the recombinant protein which was, however, found entirely in inclusion bodies. No increased phosphatase activity could be detected in the supernatants of the cells expressing PP7 catalytic domain as compared to the control cells. The recombinant PP7 fragment contained the N-terminally attached His_6 tag and could be easily purified from inclusion bodies under denaturing conditions (not shown). Yet, neither of the approaches used to renature the protein was successful. It appeared therefore that some regions in the N- or C-terminal domains may be essential for correct protein folding.

As a next step, we attempted expression of the complete PP7 open reading frame in the same system. While significant amounts of recombinant protein of the expected molecular mass (∼50 kDa) were found in the inclusion bodies, crude lysates of the induced cultures transformed with the pQE9/PP7 construct showed 10–30-fold increased phosphatase activity towards *p*-nitrophenylphosphate as compared to the cells transformed with pQE9 plasmid carrying no insert (Fig. 1A). This phosphatase activity was completely dependent on the presence of Mn^{2+} (see below), was retained by Ni-IDA Sepharose and could be eluted at high imidazole concentrations (Fig. 1A), which indicated specific binding provided by the His_6 tag. In contrast, the phosphatase activity in the control cell lysates was Mn^{2+}-independent and did not bind to the Ni-IDA Sepharose. Formation of inclusion bodies was completely suppressed if the cells were induced at temperatures below 15°C, but this also led to reduced levels of the active phosphatase. Therefore, induction was routinely carried out at 25°C, which yielded 1–2 mg of active protein per liter of bacterial culture.

Fractions eluted from the Ni-IDA Sepharose contained a protein of the same molecular mass (∼50 kDa) as the main

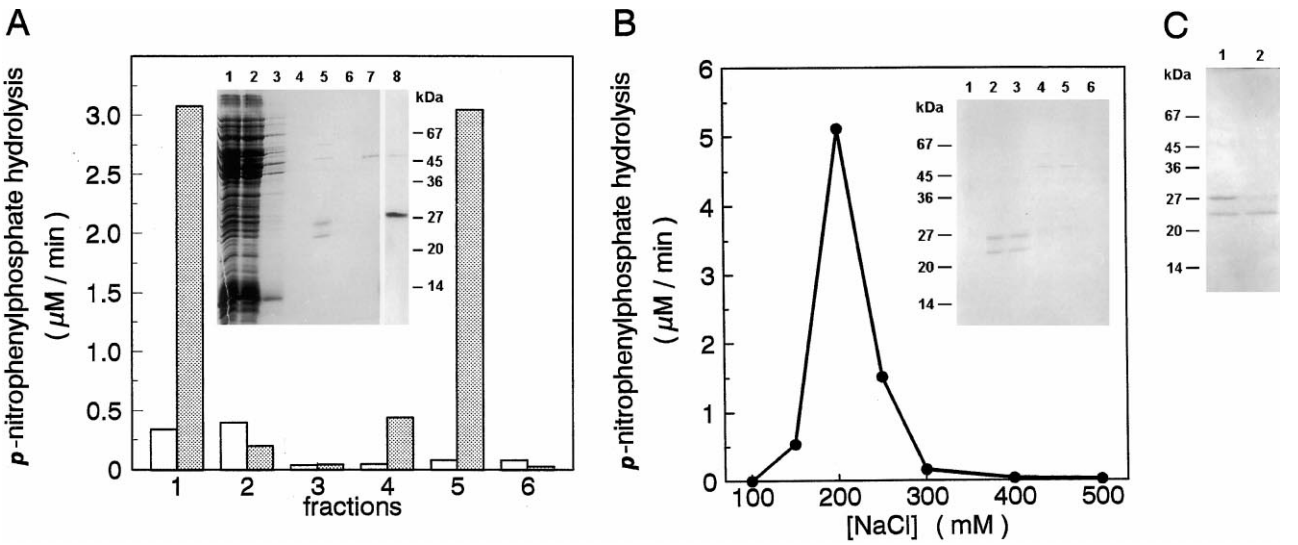

Fig. 1. Purification of PP7 expressed in *E. coli*. A: Fractions from Ni-IDA Sepharose chromatography were assayed for phosphatase activity with *p*-nitrophenylphosphate and analyzed by SDS-PAGE (inset). Empty bars, *E. coli* transformed with pQE9; stippled bars, *E. coli* transformed with pQE9/PP7. 1, crude *E. coli* extract; 2, flow-through fraction; 3, 4, 5 and 6, fractions eluted with 10, 40, 250 and 400 mM imidazole, respectively; 7, PP7 purified from inclusion bodies; 8, fraction 5 probed with Penta·His antibody. B: DEAE Sepharose chromatography of the protein eluted from Ni-IDA Sepharose with 250 mM imidazole. Lanes in the inset correspond to the fractions eluted with the following NaCl concentrations: 1, 150 mM; 2, 200 mM; 3, 250 mM; 4, 300 mM; 5, 400 mM; 6, 500 mM. C: PP7 eluted from DEAE Sepharose (fraction 2) analyzed by SDS-PAGE in the presence of 0.1 M dithiothreitol (lane 1) or in the absence of reducing agents (lane 2).

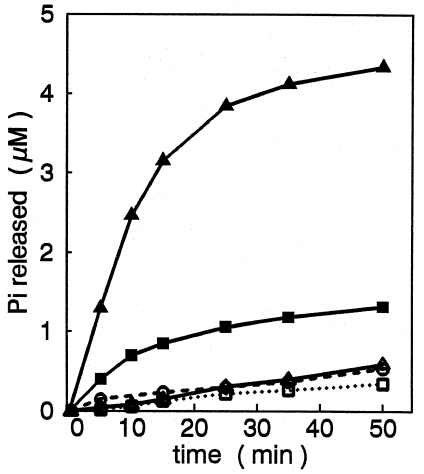

Fig. 2. Time course of dephosphorylation of myelin basic protein (▲), casein (■), phosphorylase *a* (△), rhodopsin (○) and histone IIA (□) by purified recombinant PP7 (130 nM). The reaction was carried out at pH 7.0 in the presence of $MnCl_2$ (0.5 mM).

component of the inclusion bodies (i.e. the presumable recombinant PP7), two bands of ~28 and ~25 kDa and variable amounts of contaminant proteins (Fig. 1A, inset). The 50 kDa and 28 kDa bands were recognized by monoclonal antibodies against the N-terminus of the proteins expressed in pQE9 and recognizing epitopes HHHHH (Fig. 1A, inset) or RGSHHHH (data not shown). Proteins eluted from Ni-IDA Sepharose were separated further by DEAE Sepharose chromatography, and activity of the fractions was assayed with *p*-nitrophenylphosphate. Surprisingly, phosphatase activity was not associated with the ~50 kDa band eluted at 300–400 mM NaCl, but rather with two proteins of ~28 and ~25 kDa eluted at 200–250 mM NaCl (Fig. 1B). The presence of the histidine tag in the 28 kDa protein indicated that it was likely to represent the N-terminal half of PP7. N-terminal sequencing of the 25 kDa band revealed a sequence RVVLL found in the middle of the first of the three inserts present in the PP7 catalytic domain [4]. Thus, intact recombinant PP7 appears to have no detectable phosphatase activity but can be activated by proteolytic cleavage of the insert separating the N- and C-terminal portions of the catalytic domain.

In the absence of reducing agents, the electrophoretic mobility of the 25 kDa band was not affected, while the 28 kDa protein formed a diffuse band with an apparent molecular mass of 26–27 kDa (Fig. 1C). This observation indicates that the N-terminal fragment of the recombinant PP7 contains an intramolecular disulfide bond. Since no disulfide bond formation has been described within the catalytic domains of the PPP phosphatases, the most likely candidates for the residues involved are Cys28 and Cys67 in the N-terminal domain of PP7.

Entire PP7 could be purified from inclusion bodies under denaturing conditions and refolded by two-step dialysis (urea gradients 8 M→1 M and 1 M→2 mM). This procedure yielded ≥80% soluble protein; however, renatured entire PP7 exhibited no detectable phosphatase activity (data not shown).

3.2. PP7 as a protein Ser/Thr phosphatase

The fact that PP7 shares significant sequence similarity with the protein phosphatases of the PPP family [2] suggested that this enzyme is likely to function as a protein Ser/Thr phosphatase. Indeed, purified recombinant PP7 was able to dephosphorylate, besides *p*-nitrophenylphosphate, a number of protein substrates phosphorylated at Ser and/or Thr residues (Fig. 2). Of the phosphoproteins tested, myelin basic protein phosphorylated by protein kinase A was the most efficient PP7 substrate. PP7 was inhibited by NaF (IC_{50} ~3 mM) and orthovanadate, which completely abolished phosphatase activity at 10 μM concentration (data not shown).

3.3. Effects of divalent cations, pH and phosphatase inhibitors

Of several divalent cations tested, only Mn^{2+} and Fe^{2+} were able to stimulate PP7 activity (Fig. 3). No PP7 activity could be detected with *p*-nitrophenylphosphate in the absence of added Mn^{2+}. Half-effective Mn^{2+} concentrations (Fig. 3A) were similar for protein substrates (~20 μM) and *p*-nitrophenylphosphate (~25 μM). Mn^{2+}-stimulated PP7 was strongly inhibited by Ni^{2+} and Zn^{2+}, while Mg^{2+}, Ca^{2+} and Fe^{2+} had slight or moderate inhibitory effects (Fig. 3A). An equimolar

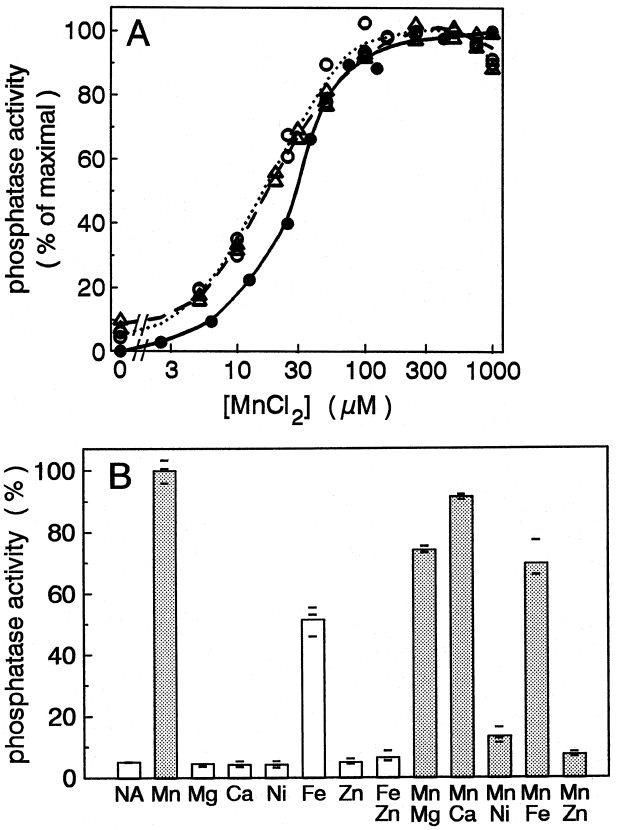

Fig. 3. Effects of divalent cations on PP7 activity. A: Effects of divalent cations on the phosphatase activity of PP7. Myelin basic protein was incubated with PP7 (180 nM) at pH 7.2 in the absence (empty bars) or in the presence (stippled bars) of $MnCl_2$ (100 μM) without or with addition of $CaCl_2$ (200 μM), $MgCl_2$ (2 mM), $NiCl_2$ (200 μM), $FeSO_4$ (200 μM), $ZnCl_2$ (200 μM) or a mixture of $FeSO_4$ and $ZnCl_2$ (100 μM each) as indicated. Results are shown as percentages of the activity in the presence of 100 μM $MnCl_2$. The bars represent the means from three aliquots (values indicated by horizontal lines). B: Stimulation of PP7 activity by Mn^{2+}. Substrates used were *p*-nitrophenylphosphate (●), myelin basic protein (△) or casein (○). Activity was assayed at pH 7.8 for *p*-nitrophenylphosphate and pH 7.2 for protein substrates in the presence of PP7 (20 nM and 200 nM, respectively) and various concentrations of $MnCl_2$. Results are shown as percentages of the maximal activity for each substrate.

mixture of Fe^{2+} and Zn^{2+}, which is effective in the activation of recombinant PP1 [12], failed to activate PP7 (Fig. 3B) and was as inhibitory for the Mn^{2+}-stimulated enzyme as Zn^{2+} alone (data not shown).

When assayed with *p*-nitrophenylphosphate, Mn^{2+}-stimulated PP7 exhibited maximal activity at pH 7.6–7.8 (data not shown). However, with protein substrates the pH optimum was shifted to 6.8 for casein and 7.2 for myelin basic protein (Fig. 4).

PP7 activity assayed with myelin basic protein or casein was resistant to inhibition by such widely used inhibitors of PPP phosphatases as okadaic acid (20 μM) and calyculin (20 μM). Fumonisin B_1, a mycotoxin recently found to inhibit, with different affinities, all tested PPP phosphatases as well as PP2C [13], slightly activated PP7 at 2 mM concentration (data not shown).

3.4. Effects of heparin and polylysine

PP7 was unaffected by heparin at a concentration of 0.5 mg/ml (data not shown). In contrast, it was efficiently inhibited by polylysine when tested with myelin basic protein and activated when tested with *p*-nitrophenylphosphate (Fig. 5). Apparent affinities of polylysine binding were similar, independently of whether this caused activation or inhibition (EC_{50} 0.15–0.2 μg/ml for 300 kDa polylysine, data not shown; EC_{50} 1.5–3 μg/ml for 1 kDa polylysine, which corresponds to 1.5–3 μM hexapeptide, Fig. 5).

4. Discussion

We have expressed in *E. coli* and characterized recombinant PP7, a novel phosphatase from *A. thaliana*. PP7 shares overall sequence similarity in its catalytic domain with the phosphatases of the PPP family but is not a close relative of any other PPP phosphatase [4]. Our preliminary observations that the highest levels of PP7 expression are found in the guard cells (Andreeva et al., in preparation), together with its unique structural features [4], make its further characterization of significant interest.

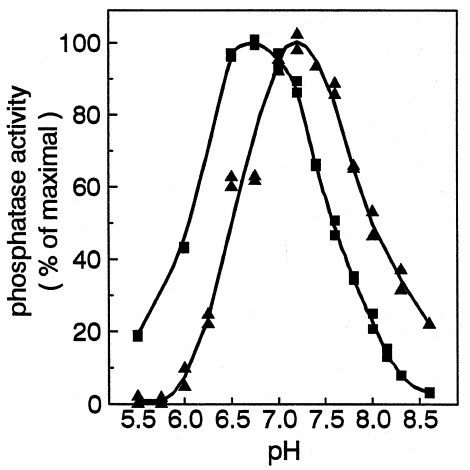

Fig. 4. pH dependence of PP7 activity. The activity was assayed with casein (■) or myelin basic protein (▲) in the presence of $MnCl_2$ (0.5 mM). PP7 concentration was 40 nM for myelin basic protein and 200 nM for casein. Buffers used (80 mM) were MES, pH 5.5–6.5; HEPES, pH 6.7–8.0; Tris-HCl, pH 8.15–8.6. Results are shown as percentages of the maximal activity for each substrate.

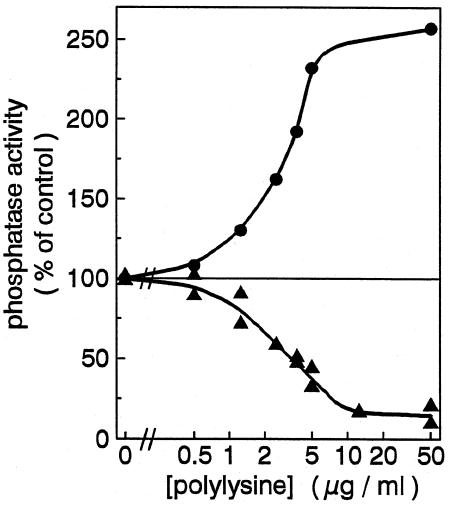

Fig. 5. Inhibition of PP7 activity by polylysine. PP7 (40 nM) was incubated with myelin basic protein (▲) at pH 7.2 or with pNPP (●) at pH 7.8 in the presence of $MnCl_2$ (0.5 mM) and various concentrations of polylysine. Results are shown as percentages of the activity in the absence of polylysine.

The fact that the recombinant PP7 is not active unless the first of the three inserts in its catalytic domain is cleaved indicates that this insert may function as an autoinhibitory region. Autoinhibitory regions which can be eliminated by limited proteolysis are known in a number of PPP phosphatases such as calcineurin [9], PPZ [10] and PP5 [11], but in all the cases described previously these sequences are located outside the catalytic domain. PP7 is the first phosphatase which appears to possess an autoinhibitory region inserted in the middle of its catalytic domain.

A working model of how PP7 activity may be controlled can be suggested on the basis of the following observations: (i) PP7 is activated by proteolytic cleavage of the first insert containing a stretch of positively charged residues; basic regions in analogous positions are conserved in the putative PP7 homologues from rice [4] and watermelon ('expressed sequence tag' AA660123); (ii) the most efficient substrate is myelin basic protein; (iii) polylysine activates hydrolysis of *p*-nitrophenylphosphate and inhibits dephosphorylation of myelin basic protein with similar affinity. These observations may be accounted for if one postulates that PP7 structure is optimized for binding basic substrates, and this binding stimulates (probably allosterically) substrate dephosphorylation. Furthermore, polylysine would bind at the same site as the basic substrates and mimic substrate binding, thus increasing the efficiency of the catalysis and activating hydrolysis of *p*-nitrophenylphosphate. In the case of myelin basic protein, polylysine binding would still stimulate catalysis but, at the same time, would prevent efficient binding of the protein substrate to the same site, thus decreasing overall efficiency of dephosphorylation. The autoinhibitory action of the first insert would rely on a similar mechanism: it seems likely that the stretch of positively charged residues found at the N-terminus of the first insert in PP7 may mimic substrate binding and allow targeting of another sequence (one candidate may be the C-terminal part of the same insert) to block the active center.

Another PPP phosphatase which prefers basic substrates is PP1, and it has been suggested to bind positively charged

substrates at the acidic groove constituted by several Asp and Glu residues [12]. Most of these residues are not conserved in PP7 (see alignment in [4]) and, therefore, the mechanisms of binding basic substrates and precise requirements for substrate structure in these two phosphatases are probably different. This is supported by the fact that histones, efficiently dephosphorylated by PP1 [14], are quite poor substrates for PP7.

While cleavage of the autoinhibitory regions/domains is known to activate a number of protein phosphatases in vitro, none of them has been shown to be proteolytically processed in vivo. The accessibility of the first insert in PP7 for proteolysis probably indicates that this region is on the surface of the folded protein and would also be easily accessible from the solvent in vivo, which might allow its cleavage by endogenous plant proteases. It can be speculated that, after such 'constitutive' activation, the phosphatase may be controlled by regulatory proteins acting as competitive inhibitors. Alternatively, regulatory proteins might bind to the entire PP7 and release the autoinhibition. Both in PP7 from *A. thaliana* and in its putative homologue from rice [4], two Thr and/or Ser are found in the positively charged N-terminal portions of the inserts. In *A. thaliana* PP7, the insert contains a potential site of phosphorylation by protein kinase A (KRTT). Although our attempts to phosphorylate entire PP7 by purified bovine protein kinase A resulted in only low substoichiometric phosphate incorporation (data not shown), these Thr residues may represent potential sites of phosphorylation by endogenous plant kinases. Phosphorylation of these residues can be expected to reduce the overall basic character of the site and possibly release phosphatase inhibition.

Human and mouse phosphatases PPEF-2 are retrieved as the highest scoring sequences when protein sequence databases are searched with the sequence of PP7 catalytic domain using the BLAST algorithm [15]. PPEF-2, like other rdgC-related phosphatases, possesses inserts of variable length in a position adjacent to the position of the first insert in plant PP7 [16]. Interestingly, despite the different lengths of the inserts in PPEF-2 and PP7, the distribution of the charged residues in these sequences is similar: basic residues are clustered at the N-termini of the inserts, while their C-terminal parts are neutral or negatively charged. Both in plant PP7 and in PPEF-2 sequence conservation between different species is lower in the inserts than in the surrounding regions of the molecule [4,16], but this characteristic charge distribution is preserved. Biochemical characterization of PPEF-2 is not yet available, and it is not known whether the inserts in these phosphatases may play any regulatory role.

The preference of PP7 for positively charged substrates may account for the different pH optima for dephosphorylation of phosphoproteins and *p*-nitrophenylphosphate. While the optimal pH for *p*-nitrophenylphosphate hydrolysis (7.6–7.8) probably reflects requirements of the catalytic center itself, the shift of the pH optimum for dephosphorylation of phosphoproteins to pH 6.8–7.2 may be due to the requirement of protonation of some substrate residues for more efficient binding to PP7. An interesting implication of such pH dependence is possible regulation of PP7 by pH in vivo. In the guard cells, pH is thought to increase in response to abscisic acid and possibly other stimuli [17–19]. These stimulus-dependent pH changes have been suggested to regulate activity of ABI1 [20], a protein Ser/Thr phosphatase related to the PPM family and essential for abscisic acid signal transduction in stomata [21]. When tested with phosphorylated casein as a substrate, ABI1 exhibits maximal activity at pH 8 and is thus expected to be activated by cytoplasm alkalinization in vivo [20]. In contrast, in the case of PP7, a pH increase would rather result in a decrease in phosphatase activity. Thus, while dephosphorylation of ABI1 substrates (which have not yet been identified) is expected to increase in response to cytoplasm alkalinization, dephosphorylation of the substrates of PP7 would rather decrease.

Unlike PP2C-related phosphatases, PP7 is not activated and is even slightly inhibited by millimolar concentrations of Mg^{2+}. The results of the experiments with divalent cations strongly suggest that the dinuclear metal center in PP7 is unlikely to be Fe^{2+}/Zn^{2+} as in PP2B [22], since the mixture of Fe^{2+} and Zn^{2+} failed to activate the phosphatase, and Zn^{2+} inhibited PP7 activated by Fe^{2+} or Mn^{2+}. The fact that only the latter two ions were efficient activators of PP7 indicates that one or both of them may be present in the catalytic center of PP7 in vivo.

PP7 is resistant to such commonly used phosphatase inhibitors as okadaic acid and calyculin, as well as fumonisin B_1, recently shown to inhibit several PPP phosphatases and PP2C. PP7 insensitivity to high concentrations of okadaic acid and calyculin clearly distinguishes it from PPP phosphatases related to PP1, PP2A and PP5 (which are inhibited by subnanomolar or nanomolar concentrations of these inhibitors [10,23–26]) and also PP2B (which is half inhibited at 5 μM okadaic acid [24]). Human PPEF-1 has been reported to be insensitive to okadaic acid and calyculin [27] but the concentration of okadaic acid used in this work (1 μM) is insufficient to compare the sensitivity of PPEF-1 with that of PP2B. Two putative novel protein Ser/Thr phosphatases from animals, which dephosphorylate rhodopsin [8] and Ca^{2+}/calmodulin-dependent protein kinase II [28], respectively, are not affected by okadaic acid concentrations up to 10 μM. However, these enzymes have not yet been identified at the molecular level. Intriguingly, Ca^{2+}/calmodulin-dependent protein kinase phosphatase is stimulated by polylysine with an apparent affinity similar to that for regulation of PP7 [28].

The characterization of the recombinant *A. thaliana* PP7 described here demonstrates that it is a functional protein Ser/Thr phosphatase. The availability of a distinctive set of biochemical characteristics, together with our ongoing study of the PP7 expression pattern in *A. thaliana* and work on production of transgenic plants, should help to clarify the role(s) PP7 plays in the plant cell. Further work will be necessary to test and refine the model of PP7 activity control proposed here and establish how this phosphatase is regulated in vivo.

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