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ORIGINAL PAPER

Molecular characterization of a tomato purple acid phosphatase during seed germination and seedling growth under phosphate stress

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Abstract

Key message **SlPAP1 is a phosphate starvation responsive purple acid phosphatase during tomato seed germination. Future research on its family members in tomato might improve the phosphate stress tolerance.**

Abstract Phosphate deficiency is a major constraint upon crop growth and yield. In response to phosphate deficiency, plants secrete acid phosphatases (APases) to scavenge organic phosphate from soil. In this study, we investigated the impact of Pi starvation on germination and seedling growth of tomato, and we then cloned and characterized a phosphate starvation responsive purple APase (SlPAP1) that expressed during tomato seedling growth. Our results showed that phosphate deficiency reduced germination and growth rates of tomato, and also increased intracellular and secretory APase activity in a concentration-dependent manner. An in-gel activity assay found that two APases of 50 and 75 kDa were secreted during conditions of phosphate deficiency. *SlPAP1* is a single copy gene belonging to a small gene family. It was expressed as a cDNA of approximately 1.5 kbp encoding a secreted glycoprotein of 470 amino acids. Northern blot analysis showed that *SlPAP1* was specifically expressed in root tissue during phosphate deficiency. SlPAP1 had high sequence identity (56–89 %) with other plant PAPs and contained highly conserved metal-binding residues. SlPAP1 is the first PAP to be cloned and characterized from tomato. This study provides useful information for future research on PAP family members in tomato, leading to better understanding of phosphate deficiency in this important crop plant.

Keywords Phosphate starvation · Purple acid phosphatase · Tomato · SlPAP1 · Seed germination

Abbreviations

APase	Acid phosphatase
MS	Murashige and Skoog medium
ORF	Open reading frame
PAP	Purple acid phosphatase
Pi	Phosphate
p-NPP	*p*-Nitrophenyl phosphate
RACE	Rapid amplification of cDNA ends
UTR	Untranslated region

Introduction

Phosphate (Pi) is one of the most important essential nutrients for plant growth and development. The bioavailability of phosphate (Pi) in soil is low and usually occurs as organic Pi, which plants cannot directly uptake. Clay soils, for example, virtually lack free Pi and the median

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concentrations of free Pi in most soil types are below 10 μM, which is much lower than that of other nutrients (Abel et al. 2002; Raghothama 1999). Plants have thus developed various strategies to adapt to low Pi concentrations in soil, including the production of acid phosphatases (APases) to scavenge organic Pi and Pi transporters to enhance Pi update (Abel et al. 2002; Goldstein et al. 1988; Liu et al. 1998; Raghothama 1999). APases catalyze the cleavage of Pi from phosphate esters under acidic conditions. Both intracellular and secreted APases are induced during Pi deficiency (Baldwin et al. 2001; Hur et al. 2007; Wang et al. 2014). Intracellular APases are involved in recycling and remobilizing Pi from senescent tissue, while secreted APases are involved in scavenging of Pi from organic sources in the external environment (Robinson et al. 2012a, b; Tian et al. 2012; Vance et al. 2003). Purple acid phosphatases (PAPs) form a unique group among the APases, characterized by the purple color of the purified protein in aqueous solution and an iron (III)-containing dimetal center in the catalytic site (Olczak et al. 2003; Strater et al. 1995). PAPs have been found in, and characterized from, many plant species, including Arabidopsis, rice, tobacco, sweet potato, kidney bean and soybean, as well as other crop plants (Dionisio et al. 2011; Durmus et al. 1999; Hur et al. 2007; Kaida et al. 2003; Li et al. 2002; Liang et al. 2012; Liang 1998; Schenk et al. 1999). PAPs contain five conserved amino acid motifs required for metal binding: **D**XG/G**D**XX**Y**/GN**H**[D/E]/VXX**H**/G**H**X**H** (bold letters represent the metal-binding residues) (Li et al. 2002; Schenk et al. 2000; Strater et al. 1995).

PAPs are well studied in the model plant *Arabidopsis thaliana*. There are 29 PAPs in Arabidopsis, with different spatial and temporal expression patterns (Li et al. 2002; Zhu et al. 2005). AtPAP10, AtPAP12 and AtPAP26 are three major Pi starvation-responsive secretory and intracellular APases (Robinson et al. 2012a, b; Wang et al. 2011, 2014); AtPAP15 is likely to be involved in mobilizing Pi reserves during seed and pollen germination (Kuang et al. 2009); AtPAP9 and AtPAP5 are probably involved in defense against plant pathogens (Ravichandran et al. 2013; Zamani et al. 2014); and AtPAP2, which is targeted to both plastids and mitochondria, is involved in plant growth (Sun et al. 2012). These data collectively suggest that PAPs play important roles in a wide range of physiological functions.

Tomato is an important crop plant from the family Solanaceae, a family which also contains other important crop species such as potato, eggplants and pepper. Understanding the effects of Pi deficiency on tomato plants will provide important insight into ways to tackle the reductions in yield caused by insufficient Pi in these economically important species, especially during the early stage of plant growth. Studies of tomato PAPs are still in their early stages in comparison with their well-studied orthologues in Arabidopsis. Three Pi starvation-responsive PAPs have been identified from tomato suspension cell cultures; two secreted acid phosphatases (SAP1 and SAP2), are monomeric proteins with molecular weights of 84 and 57 kDa, respectively, while the third, intracellular acid phosphatase (IAP), is an intracellular heterodimeric protein composed of 63 and 57 kDa subunits (Bozzo et al. 2002, 2004). Recently, detailed analysis found different spatial and temporal expression patterns of SAP1, SAP2 and IAP in Pi-deficient conditions (Bozzo et al. 2006). In a suspension cell system, expression of both SAP1 and SAP2 was specifically induced on the eighth day of Pi starvation, while IAP expression was induced on the sixth day of Pi starvation (Bozzo et al. 2006). In 30-day-old seedlings, SAP1 was specifically expressed in Pi-starved roots, while IAP was expressed in leaf, stem and root tissue (Bozzo et al. 2006).

Most previous studies of plant responses to Pi deficiency focus on mature plants, and studies of tomato PAPs focus on suspension cell cultures rather than on whole plants, and none of the Pi starvation-responsive PAPs in tomato have been cloned or characterized. We, therefore, investigated the responses of tomato plants to Pi starvation, especially during seed germination and early seedling growth, which are plant's first interactions with its environment. We then cloned and characterized SlPAP1, the first Pi starvation-responsive PAP known from tomato, because of the important role played by PAPs in responses to Pi starvation.

We first examined the impact of different environmental Pi concentrations (from 0 to 5 mM) on the rate of tomato seed germination and the subsequent growth rate of seedlings. The activities of intracellular and secretory APases were assessed using *p*-nitrophenyl phosphate (*p*-NPP) and in-gel activity assays. Next, we used northern blot analysis to study expression patterns of *SlPAP1* transcripts and Southern blotting to determine the presence of other members of the PAP gene family. We also cloned the *SlPAP1* cDNA and performed an in silico comparison of its sequence with those of other known plant PAPs. Our results showed the importance of Pi concentration on tomato seed germination and subsequent seedling growth, and elucidated the regulation of *SlPAP1* expression during Pi deficiency.

Results

Pi starvation reduced germination and growth rates of tomato

Most previous studies focus on the effects on gene expression pattern and growth rate in Pi-sufficient versus Pi-deficient conditions, and so little is known about the impact of different Pi concentrations on germination of tomato seeds or on

seedling growth. We examined a total of 480 seeds germinating under different Pi concentrations (0, 0.2, 1.25 and 5 mM; 120 seeds for each Pi concentration). Seed germination was defined as cotyledon emergence, which is more sensitive to the effects of nutrient deficiency compared to radicle emergence. Germination rates were lower after 15 days in Pi-deficient (0 mM) and in low Pi (0.2 mM) conditions than in Pi-sufficient (1.25 and 5 mM) conditions (Fig. 1a). In addition, low Pi concentration also reduced the growth rate of tomato seedlings, especially with respect to shoot growth, and a purplish color was observed in the stem and leaves of tomato seedlings which germinated in the complete absence (0 mM) of Pi (Fig. 1b). Decreasing Pi concentration reduced the germination rate and increased the root to shoot ratio, germination rates were 66, 76, 84 and 86 % ($p < 0.01$, comparing 5 or 1.25 mM with 0 mM Pi by Student *t* test) (Fig. 1c) and the root to shoot ratios were 1.91, 1.25, 0.79 and 0.69 (Fig. 1d) at concentrations of 0, 0.2, 1.25 and 5 mM Pi, respectively.

Pi starvation induced production of intracellular and secreted APase

Next, the levels of intracellular and secreted APase activity were determined in tomato seedlings germinating in different concentrations of Pi. Both intracellular and secreted APase activity exhibited a dose-response relationship with Pi concentration as APase activity increased as Pi concentration decreased. There was a larger induction of intracellular APase activity in root tissue in conditions of low Pi concentration than in leaf tissue. The root intracellular APase activity of plants grown in the absence of Pi (0 mM) increased by 74 and 109 % over that in plants exposed to Pi-sufficient conditions of 1.25 and 5 mM Pi, respectively, whereas leaf intracellular APase activity of plants grown without Pi (0 mM) only increased by 62 and 51 % over that in plants grown in 1.25 and 5 mM Pi, respectively (Fig. 2a). The secreted APase activity also showed a strong response to Pi deficiency, with 3.5-fold and 7.5-fold increases in activity observed in plants grown in Pi-deficient conditions over those grown in 1.25 or 5 mM Pi conditions, respectively (Fig. 2b).

In-gel staining was used to investigate secreted APase activity. Tomato seeds were germinated and grown in Pi-sufficient (1.25 mM) conditions for 21 days and transferred to Pi-deficient (0 mM) conditions for 18 days. Samples of culture media were collected and analyzed over this 18-day period. Two APases with molecular weights of around 50 and 75 kDa were differentially induced under Pi-deficient conditions, with the 50 kDa APase being induced after

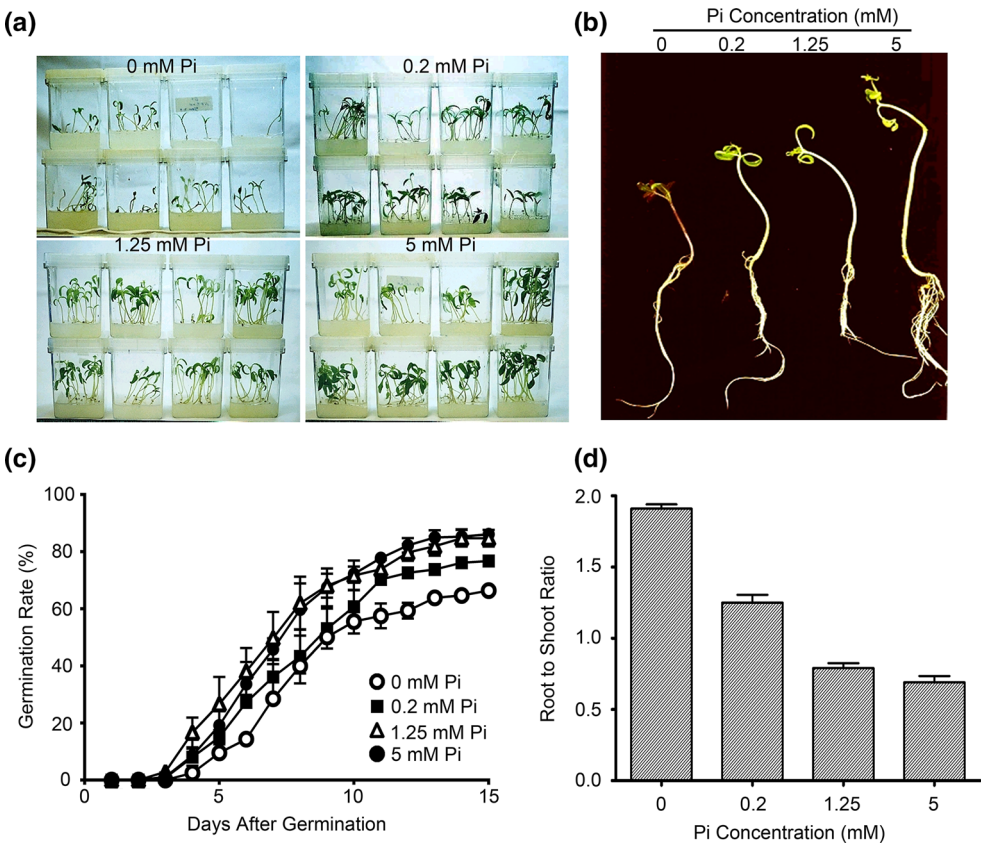

Fig. 1 Germination and growth rates of tomato under phosphate starvation. Tomato seeds were placed on MS medium containing different phosphate concentrations to determine germination rates and morphological changes over 15 days. **a** Gross overview of 15-day-old tomato seedlings. **b** Seedling growth is severely affected by low (0.2 mM) phosphate (*Pi*) concentration or Pi-deficient (0 mM) conditions. **c** Germination rate is also severely affected by low Pi and Pi-deficient conditions. **d** Phosphate starvation results in an increase in root to shoot ratio

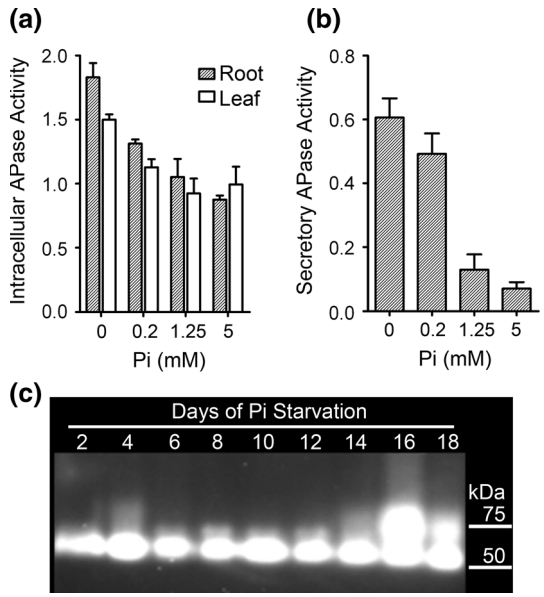

Fig. 2 Phosphate starvation induces APase production in tomato seedlings. Tissue samples were collected for APase activity assays from 15-day-old tomato seedlings germinated on MS medium containing different concentrations of Pi. **a** Pi starvation induces activity of intracellular APases in roots and leaves, with greater induction in the root. **b** Activity of secretory APase is highly enhanced by Pi starvation. **c** Two APases of approximately 50 and 75 k Da are induced after 2 and 16 days of Pi starvation, respectively. Induction of APase activity was measured using an in-gel activity assay

2 days of Pi starvation and the 75 kDa APase after 16 days of Pi starvation (Fig. 2c). The in-gel activity of APase in Pi-sufficient conditions was barely detectable (data not shown).

Expression of SlPAP1 is root specific and induced by phosphate starvation

Purple acid phosphatase plays an important role in plant responses to Pi starvation as its phosphatase activity enables scavenging of Pi from organic Pi sources (Tian et al. 2012; Wang et al. 2011, 2014). We then, therefore, investigated PAP expression induced by Pi starvation in tomato seedlings. The TPAP-500 fragment of the *SlPAP1* cDNA was cloned because it contains highly conserved regions found also in kb*PAP*, *AtPAP12*, *AoPAP32* and *IbPAP2* cDNAs. Northern blot analysis showed that *SlPAP1* expression was induced in tomato roots at low Pi (0.2 mM) concentration and was strongly expressed in Pi-deficient (0 mM) conditions. Expression of *SlPAP1* in roots was very low under Pi-sufficient (1.25 and 5 mM) conditions; however, its expression in leaf tissue was always very low, regardless of Pi concentration in the culture media (Fig. 3).

SlPAP1 is a single copy gene in the tomato genome belonging to a small gene family

Southern blot analysis was used to investigate the number of *SlPAP1* gene copies and family members in the tomato genome using high and low stringency wash conditions, respectively. Under high stringency wash conditions, only a single band was observed on a blot of tomato genomic DNA digested with *Eco*RI, *Xba*I or *Hin*dIII. The intensity of this band matched the single gene copy equivalent, indicating that only one copy of *SlPAP1* is present in the tomato genome (Fig. 4a). Under low stringency wash conditions, four bands were observed on a blot of genomic DNA digested with *Eco*RI, suggesting that there are at least four PAP family members in the tomato genome (Fig. 4b).

SlPAP1 is highly similar to other plant PAPs and shares identical metal-binding motifs

Next, the full-length *SlPAP1* cDNA and gene were cloned and analyzed. The *SlPAP1* gene, amplified from tomato genomic DNA, was 3,632 bp in length from transcription start site to 3′ end, and contained eight exons and seven introns (Supplementary Figure 1). The *SlPAP1* cDNA, reverse-transcribed and amplified from RNA extracted from Pi-starved tomato root tissue, contained an open reading frame (ORF) of 1,413 bp, encoding 470 amino acid residues (Supplementary Figure 2). Analysis of *SlPAP1* by 5′ RACE revealed a 22-bp 5′ UTR, and 3′ RACE analysis showed *SlPAP1* was alternatively polyadenylated with two different 3′ UTR of 91 and 163 bp (Fig. 5). Analysis of the predicted amino acid sequence of SlPAP1 with SignalP indicated that it contained a probable signal peptide, cleaved between amino acid residues 27 and 28, and four potential N-linked glycosylation sites. Analysis with TargetP predicted that the SlPAP1 is a secreted protein, and thus SlPAP1 may be a secretory glycoprotein. The predicted molecular mass of SlPAP1, excluding the putative signal peptide, was 50.7 kDa.

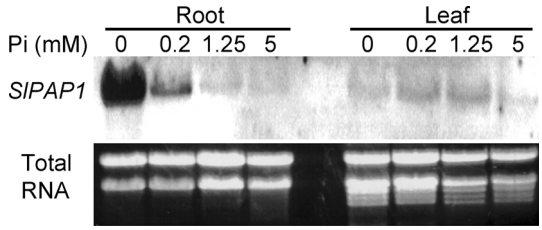

Fig. 3 Phosphate starvation induces *SlPAP1* expression. Total RNA was subjected to northern blot analysis. *SlPAP1* expression is induced in roots at low (0.2 mM) Pi concentration and increases under Pi-deficient (0 mM) conditions. *SlPAP1* expression in leaf tissue is very low regardless of Pi concentration

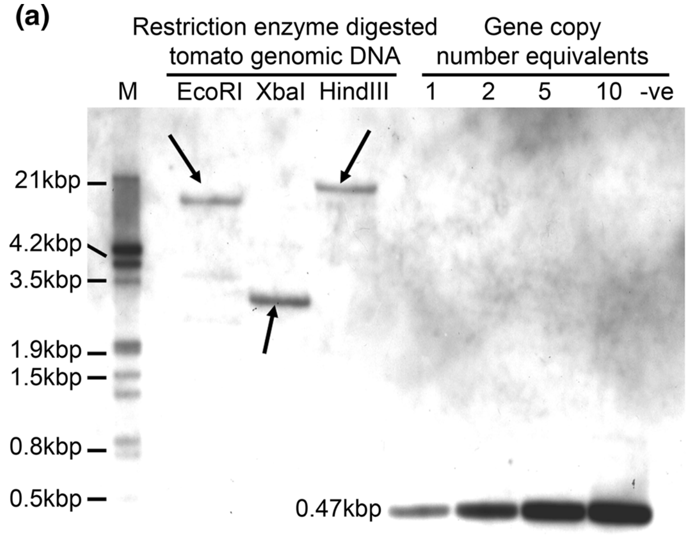

Fig. 4 *SlPAP1* is a single copy gene belonging to a small gene gamily in the tomato genome. Tomato genomic DNA was subjected to Southern blot analysis to determine numbers of *SlPAP1* gene copies and family members. **a** *SlPAP1* (indicated by *arrows*) exists as a single gene copy in the tomato genome. Each lane contains 10 µg genomic DNA digested with *Eco*RI, *Xba*I or *Hin*dIII and analyzed under high stringency conditions. Gene copy equivalents were included to determine *SlPAP1* copy number. **b** Four strong bands are detected in low stringency conditions, suggesting that SlPAP1 belongs to a small gene family in the tomato genome. *Each lane* contains 15 µg genomic DNA digested with *Eco*RI and analyzed under low stringency conditions

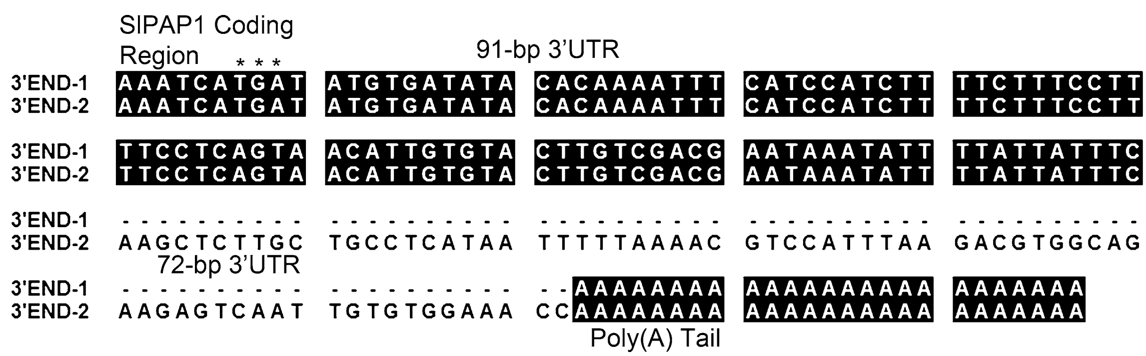

Fig. 5 Alternative polyadenylation of SlPAP1. The nucleotide sequences of the coding region and the first 91 bp 3′ UTR are identical in both versions of *SlPAP1* cDNA. The longer version, 3′ END-2, contains an additional 72 bp in the 3′ UTR not found in 3′ END-1. Identical nucleotide sequences are shaded in *black*. *Asterisks* indicate the stop codon

The nucleotide sequence of *SlPAP1* shared 63–88 % sequence identity with other plant *PAP* cDNAs, including those from tobacco, *Anchusa officinalis*, soybean, sweet potato, kidney bean and Arabidopsis. The predicted amino acid sequence showed 56–89 % identity and 71–95 % similarity with other plant PAPs (Table 1). PAPs contain conserved amino acid motifs required for metal binding in the active site (Li et al. 2002; Schenk et al. 2000; Strater et al. 1995). Sequence alignment of the putative amino acid sequence of SlPAP1 with other plant PAPs indicated that it shared these highly conserved motifs and contained identical metal-binding amino acid residues (Fig. 6a).

We used the *SlPAP1* cDNA sequence in a BLAST search against the NCBI database to identify other tomato *PAP*s. A total of four highly similar putative tomato *PAP* sequences were identified by this search, including *SlPAP1*, which has an identical nucleotide sequence to a predicted tomato acid phosphatase (GenBank accession: XM_004230858). The three other tomato *PAP*s found in the NCBI database were named according to their sequence similarity with *SlPAP1*. They shared between 48 and 67 % nucleotide sequence identity with *SlPAP1*, while their predicted amino acid sequences contained between 46 and 67 % sequence identity and between 49 and 78 % sequence homology with SlPAP1 (Table 2). As seen in other plant PAPs, the metal-binding motifs were well conserved among the tomato PAPs, although SlPAP2 had a truncated sequence (Fig. 6b).

Table 1 Sequence similarities between *SlPAP1* cDNA and its predicted amino acid sequence and other plant PAPs

Plant species	PAPs	cDNA (identity %)	Protein (identity %/similarity %)
Tobacco	NtPAP4	69	71/84
	NtPAP12	88	89/95
	NtPAP19	68	70/86
	NtPAP21	84	83/94
Anchusa officinalis	AoPAP32	72	75/87
Soybean	GmPAP	74	77/88
Sweet potato	IbPAP1	65	63/82
	IbPAP2	79	81/92
	IbPAP3	64	68/78
Arabidopsis thaliana	AtPAP10	73	75/85
	AtPAP12	64	68/79
	AtPAP26	63	56/71
Kidney bean	KBPAP	72	69/79

Both SlPAP1 cDNA and protein showed high sequence similarity to other plant PAPs, ranging from 63 to 88 % in cDNA sequence identity, from 56 to 89 % in amino acid sequence identity and from 71 to 95 % in amino acid sequence similarity

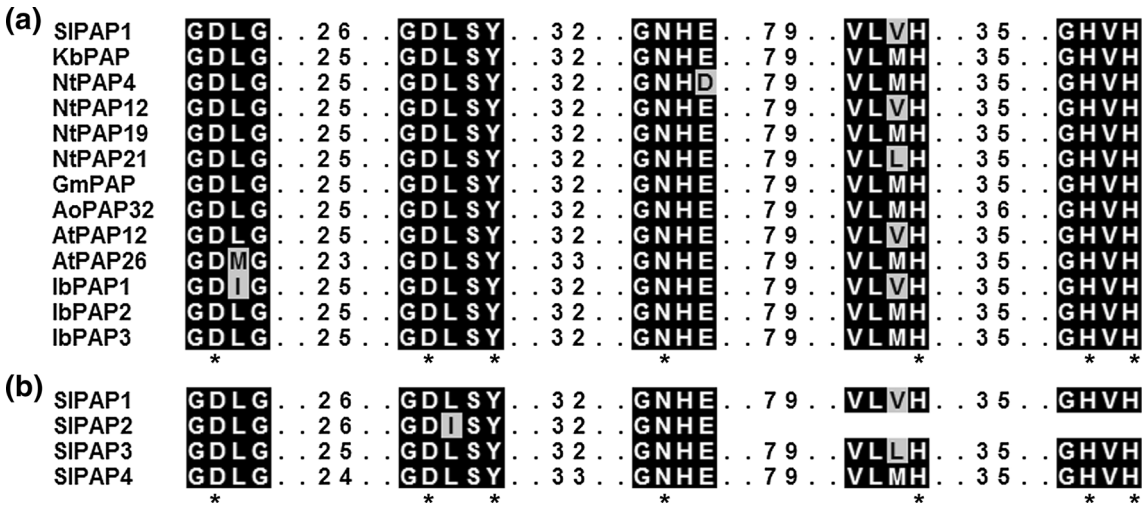

Fig. 6 SlPAP1 contains highly conserved metal-binding motifs found in other plant PAPs. The metal-binding residues of the SlPAP1 are identical to those in **a** other plant PAPs and **b** other tomato PAPs. The metal-binding motifs and their spacing are highly similar to each other, except for SlPAP2 (truncated). Similar amino acid residues are shaded in *gray* and conserved residues in *black*. *Numbers* represent the number of amino acids between motifs. *Asterisks* represent the metal-binding residues. The tyrosine residue for Fe(III) coordination is located at the GDLSY motif

Table 2 Sequence similarities between SlPAP1 cDNA and its predicted amino acid sequence and other tomato PAPs

Proposed name	cDNA (identity) (%)	Protein (identity %/similarity %)	GenBank accession
SlPAP1	Reported in this study		
SlPAP2	48	46/49	XR182419
SlPAP3	67	67/78	XM004243777
SlPAP4	59	54/70	XM004251686

The nucleotide sequences of other tomato PAPs were retrieved from the NCBI database by a BLAST search using the *SlPAP1* cDNA sequence. The *SlPAP1* cDNA shared 48–67 % nucleotide sequence identity, 46–67 % amino acid sequence identity and 49–78 % amino acid sequence similarity with other PAPs from tomato

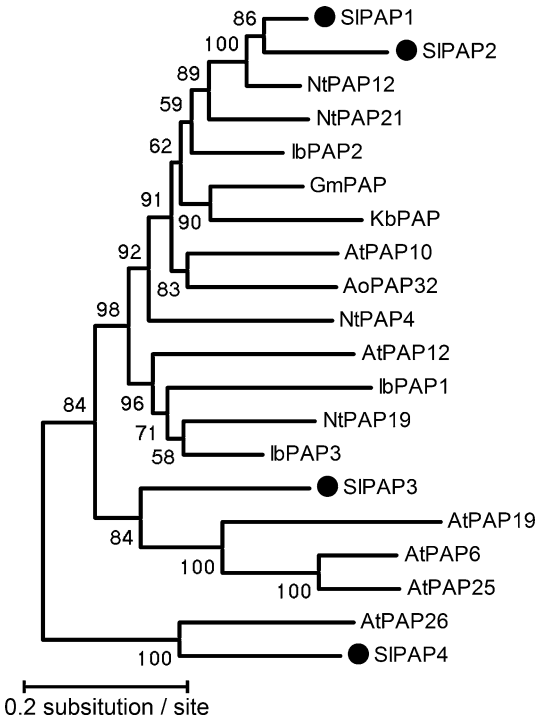

Fig. 7 Phylogenetic relationship of tomato PAPs with other plant PAPs. A phylogenetic tree of the putative amino acid sequences of SlPAP1, other putative tomato PAPs and well-known plant PAPs was constructed using the neighbor-joining method (MEGA version 6)

Phylogenetic analysis of SlPAP1 and other plant PAPs

A neighbor-joining phylogenetic tree was constructed, based on the amino acid sequences of SlPAP1 and other plant PAPs (Fig. 7). The four tomato PAPs belonged to Group Ia of PAPs (Fig. 7).

Discussion

In this study, the responses of tomato to Pi starvation during seed germination and subsequent seedling growth were investigated, and *SlPAP1*, a Pi starvation responsive gene expressed during seedling growth, was then cloned and characterized. It is well established that Pi starvation reduces tomato growth through, for example, reducing the accumulation of biomass (Bozzo et al. 2006; Goldstein et al. 1988). Little is known, however, about the impact of different Pi concentrations on seed germination rates or on seedling growth. Using tomato as a model system to study plant responses to Pi deficiency, we exposed plants to four different Pi concentrations (0, 0.2, 1,25 and 5 mM) and demonstrated a dose response between the environmental Pi concentration and seed germination rates and seedling growth, as well as other Pi starvation responses including APase activity and *SlPAP1* expression. Pi deficiency strongly reduced the germination rate, from 86 % in seeds exposed to 5 mM Pi to 66 % in seeds lacking Pi. Subsequent seedling growth was also reduced in Pi-deficient conditions, even though plants responded by increasing their root to shoot ratio, which would increase their potential capacity to uptake Pi. This implies that Pi has an important role not only in determining plant growth and yield but also during earlier stages of seed germination and seedling growth. The importance of Pi during seed germination may result from Pi enhancing the uptake of nutrients or, alternatively, reducing the need of Pi that stored in seeds (Centeno et al. 2001).

To investigate how tomato seedlings respond to Pi deficiency, intracellular APase activity in roots and leaves, as well as secreted APase activity, was analyzed in tomato seedlings grown with different Pi concentrations. As with seed germination and seedling growth, the degree of APase induction showed a dose-response relationship with environmental Pi concentration. APases are a part of the response to Pi starvation in tomato plants, and it is possible that the tomato intracellular APases are involved in remobilizing stored Pi and secreted APases in scavenging environmental Pi, as characterized in other plants (Robinson et al. 2012a, b; Tian et al. 2012; Vance et al. 2003). A more detailed analysis, using an in-gel activity assay, revealed that two secreted APases were differentially expressed by tomato seedlings grown in Pi-deficient conditions and secreted into the culture medium. A 50 kDa APase was induced early in Pi starvation, while a 75 kDa APase was induced later. However, the in-gel APase activity assay may not be a very accurate method to measure the changes in APase activity throughout the 18 days of Pi deficiency, compared with spectrophotometric analysis. As the detection of signals from the fluorescent product (4-methylumbelliferone) was amplified and saturated during the UV illumination, indeed the in-gel APase activity assay is superior to measure the molecular weight and number of APases secreted in this 18-day period. A previous study showed secretion by a tomato suspension cell culture of two APases, SAP2 (57 kDa) and SAP1 (84 kDa), after 8 days of Pi deficiency (Bozzo et al. 2002). The molecular weights of these proteins (SAP1 and SAP2) are very similar to the 50 and 75 kDa APases found in our study, and thus it is possible that SAP2 and SAP1 (Bozzo et al. 2002) are in fact the two APases we detected. The less than 10 kDa differences in molecular weight between the studies by Bozzo et al. (2002) and ours may result from a diffused band in the in-gel activity staining assay in our study, leading to a discrepancy in molecular weight prediction. It is notable that Bozzo et al. (2002) did not find differences in the temporal expression of SAP1 and SAP2. This may result from variation in Pi starvation responses between tomato suspension cells and entire plants, as Pi

deficiency does not only acts at a cellular level but also affects the physiology of the whole plant. Previous studies in Arabidopsis seedlings also revealed both short and long-term responses in gene expression during Pi starvation (Hammond et al. 2003; Wu et al. 2003). It is, therefore, possible that expression of these two secretory APases is regulated differently, but further study is required to determine the regulatory mechanism(s) behind the differences observed in their temporal expression.

Within APases, PAPs are characterized by the purple color of the purified proteins in aqueous solution and the iron (III)-containing dimetal center at the catalytic site (Olczak et al. 2003; Strater et al. 1995). Such conserved metal-binding motifs allowed easy identification, cloning and characterization of PAPs. As the tomato genome sequence was not available when we cloned *SlPAP1*, we made use of the conserved metal-binding motifs of plant PAPs to clone the gene. The *SlPAP1* cDNA contained a short 5′ UTR and an alternatively polyadenylated 3′ UTR. Alternative polyadenylation of the 3′ UTR is involved in regulating the half-life of the mRNA transcript (Tian and Manley 2013) and thus may play a role in determining the half-life of *SlPAP1* mRNA transcripts in response to Pi deficiency. *SlPAP1* expression is specifically induced in roots during Pi starvation. In addition, *SlPAP1* expression showed a dose-response relationship with Pi concentration, being induced at low concentration (0.2 mM) and strongly induced in the complete absence of Pi (0 mM). This suggested that *SlPAP1* may play an important role in Pi starvation responses, possibly act as a secreted PAP in scavenging environmental Pi, which its expression is adjusted with different environmental Pi concentrations. Most earlier studies focused on a comparison of expression of genes induced by Pi starvation under Pi-sufficient (>1 mM) versus Pi-deficient (0 mM) conditions, and thus more studies are required to determine whether expression of these genes, similar to the *SlPAP1* expression pattern as reported in this study, also has a dose-responsive relationship to differing Pi concentrations.

The sequences of SlPAP1 cDNA and protein shared a high similarity, especially at the conserved metal-binding motifs, with other well studied and characterized plant PAPs, including those from kidney bean, sweet potato and Arabidopsis. Based on its putative amino acid sequence, SlPAP1 was predicted to be a secretory glycoprotein with a molecular weight of 50.7 kDa. A previous study showed a tomato suspension cell culture secreted SAP2, a PAP with a molecular weight of 57 kDa, during Pi starvation (Bozzo et al. 2002). Our data indicate SlPAP1 may be SAP2 (Bozzo et al. 2002), although we cannot rule out the possibility that other tomato PAPs with similar biochemical properties and molecular weight are also expressed and secreted during Pi starvation. In fact, the predicted amino acid sequences of SlPAP1, SlPAP3 and SlPAP4 are all very similar to the tryptic peptide sequence of SAP2 (Supplementary Figure 3).

Southern blot analysis under high stringency wash condition showed that SlPAP1 exists as a single copy gene in the tomato genome. Later, a BLAST search with the *SlPAP1* cDNA sequence identified four close homologies of *SlPAP1* in the tomato genome. The similarity between their predicted amino acid sequences and SlPAP1 varied from 49 % (SlPAP2) to 78 % (SlPAP3). Three of these tomato PAPs contained the conserved metal-binding motifs; except that SlPAP2 is a truncated protein. *SlPAP1* shared high cDNA sequence identity with *SlPAP2*, *SlPAP3* and *SlPAP4*, especially around the metal-binding motifs located by the TPAP-500 and TPAP-1000 probes (data not shown), and these may account for the four bands observed in Southern blot analysis under low stringency wash conditions. Phylogenetically, SlPAP1 clustered with group Ia of the plant PAP family (Li et al. 2002), along with SlPAP2, SlPAP3 and SlPAP4. Also in this group are Arabidopsis AtPAP10, AtPAP12 and AtPAP26, which are well characterized as the major PAPs secreted during Pi starvation to scavenge organic Pi from the environment (Robinson et al. 2012a, b; Wang et al. 2011, 2014). Although SlPAP2 is highly homologous to SlPAP1, it is a truncated protein without all the metal-binding motifs and it is possible that it cannot function as a PAP. It remains to be elucidated whether expression of *SlPAP2*, *SlPAP3* and *SlPAP4* is also induced by Pi starvation. It is possible that tomato PAPs have different spatial and temporal expression patterns and physiological functions, and thus further studies are required to determine their expression patterns and functional roles, especially during Pi starvation.

Tomato was chosen as the model plant for this study because of its economic importance. We found that seed germination, seedling growth and stress responses all displayed a dose-response relationship with environmental Pi concentration. Future studies on the role of tomato PAP family members during seed germination and seedling growth may shed light of improving Pi stress tolerance during early stage of plant growth.

Materials and methods

Plant material and growth conditions

Seeds of tomato (*Solanum lycopersicum* L. ecotype *Nian Feng*) were germinated and grown on Murashige and Skoog (MS) culture medium supplemented with four different concentrations (0, 0.2, 1.25 and 5 mM) of phosphate (Pi). The Pi concentration of normal MS culture medium is 1.25 mM and is considered sufficient for plant growth.

Seeds were surface sterilized by washing with 70 % ethanol for 30 s, rinsed with sterile water, soaking in 15 % bleach (0.79 % hypochlorite) for 20 min and rinsed again with sterile water. Seeds were then germinated and plants grown in a growth chamber at 26 °C and a light dark cycle of 16 h light and 8 h dark.

Effects of phosphate concentration on germination rates and seedling morphology

To determine the germination rate, 120 seeds were placed on MS agar medium at each Pi concentration (0, 0.2, 1.25 and 5 mM) and the number of germinating seeds was counted each day over a period of 15 days. To determine the root to shoot ratio, 50 seeds were germinated in liquid MS medium containing each different Pi concentration and root and shoot lengths measured on day 15 post-germination.

Activities of intracellular and secreted APases

APase activity was assayed using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate and quantified at 405 nm in a spectrophotometer. The activity of APase secreted from roots was determined using 300 µl MS liquid medium collected from 15-day-old seedling cultures. Samples were mixed with 200 µl citrate buffer (0.1 M citrate, pH 4.8) and 100 µl substrate solution (1 mg/ml *p*-NPP in 0.09 M citrate buffer, pH 4.8) and incubated at 37 °C for 1 h. The reaction was stopped with 200 µl NaOH and followed by measurements of OD405 nm. For intracellular APase, leaf and root tissue from 15-day-old seedling was grounded into fine power under liquid nitrogen and mixed with protein extraction buffer (50 mM Na_3PO_4, pH7.0, 10 mM EDTA, 0.1 % Triton X-100, 1 % SDS, 10 mM β-mercaptoethanol). Supernatant containing soluble protein was collected by centrifugation at 14,000 rpm for 15 min at 4 °C. Intracellular APase activity in leaf and root tissue was determined using 200 µg protein extracts. Total protein concentration was determined using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA) and an equal amount of protein was used in each assay.

Gel activity staining of secreted APase

Tomato seeds were germinated and seedlings grown for 21 days in Pi-sufficient (1.25 mM) liquid MS medium before transfer to Pi-deficient (0 mM) liquid MS medium for up to 18 days to induce secretion of APase. Samples of culture media containing secreted proteins were collected every 2 days, lyophilized and desalted. Proteins were separated using SDS-PAGE using a methodology that preserved APase activity (Haran et al. 2000). Secreted APase was reactivated by incubation with casein/EDTA, as described previously (McGrew and Green 1990), followed by incubation in sodium acetate buffer. The substrate 4-methylumbelliferyl-phosphate (Sigma–Aldrich, St. Louis, MO, USA) was mixed with low-melting point agarose and overlaid on the polyacrylamide gel. In-gel APase activity was visualized using UV illumination following incubation for 10 min.

Extraction of genomic DNA

Total genomic DNA was extracted from tomato leaves using the CTAB DNA extraction method. Tomato leaf tissue was ground into fine powder under liquid nitrogen and mixed with CTAB extraction buffer [2 % CTAB (w/v), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0, 0.2 % β-mercaptoethanol (v/v)]. DNA was extracted from the samples by treating twice with an equal volume of chloroform/isoamyl alcohol solution (24:1, v/v), and then a 2/3 volume of isopropanol was added to the aqueous phase. Following precipitation at room temperature for 1 h, the DNA pellet was collected by centrifugation, washed with 10 mM ammonium acetate in 76 % ethanol and centrifuged again. The air-dried DNA pellet was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH8.0) and treated with RNase (1 mg/ml) at 37 °C for 30 min. DNA was then purified with three rounds of phenol/chloroform (1:1, v/v) extractions and precipitated in 1/10 volume of 3 M ammonium acetate (pH 5.2) with two volumes of cold ethanol. Finally, the DNA pellet was washed with 70 % ethanol, air-dried and dissolved in TE buffer (pH 8.0). The amount and quality of the extracted genomic DNA were checked using gel electrophoresis.

Extraction of total RNA

Total RNA was extracted, using the LiCl method (Altenbach et al. 1989), from leaf and root tissue samples collected from 15-day-old tomato seedlings grown from germination in liquid MS medium containing different Pi concentrations. Tissue samples were ground into fine powder under liquid nitrogen. Extraction buffer (0.05 M LiCl, 0.05 M Tris–HCl, pH 8.0, 0.05 M EDTA, 0.5 % SDS) and phenol (1:1, v/v) was added to the powdered tissue. RNA was extracted twice using an equal volume of chloroform/isoamyl alcohol solution (24:1, v/v). An equal volume of 4 M LiCl was added to the aqueous phase and RNA was precipitated overnight at 4 °C, collected by centrifugation and resuspended in DEPC-treated water. Total RNA was precipitated with 3 M sodium acetate (pH 5.0) and ethanol, and washed with 70 % ethanol. The amount and quality of the extracted RNA were determined using gel electrophoresis.

Cloning of *SlPAP1* full-length cDNA and gene

Two primers, based on the PAP cDNA sequences of kidney bean (*Phaseolus vulgaris*; kb*PAP*), *A. thaliana* (*AtPAP12*, At2g27190), sweet potato (*Ipomoea batatas*; *IbPAP2*) and *Anchusa officinalis* (*AoPAP32*), were used to clone the partial PAP cDNA from tomato. The primers, PAP01 (5′-CGGAATTCAATGCTCCTCAACAGGTTC-3′) and PAP02 (5′-CGGAATTCCCCAAGTATCCCATCT-3′) were designed to amplify the region containing the highly conserved metal-binding motif and to add *Eco*RI sites (underlined) to the 5′ and 3′ ends of the amplified sequence.

Total RNA extracted from root tissue samples from 15-day-old seedlings germinated and grown in Pi-deficient (0 mM Pi) liquid MS medium was used as a template. First-strand cDNA was synthesized from 1 μg RNA using MMLV-RT (Promega, Madison, WI, USA) and oligoDT, and amplified by PCR with the PAP01 and PAP02 primers. The 476 bp fragment amplified from tomato PAP (TPAP-500) was cloned into the pGEM-T vector (pGEM-T/TPAP-500) (Promega). Subsequent sequencing of the TPAP-500 fragment showed that it comprised base pairs 175 to 670 of the *SlPAP1* cDNA. The nucleotide and deduced amino acid sequences of TPAP-500 had high similarity to the four previously known PAPs (*kbPAP, AtPAP12, IbPAP2 and AoPAP32*) and contained identical metal-binding residues; therefore, the putative PAP cDNA cloned from tomato was named *SlPAP1*.

To determine the 5′ untranslated region (5′ UTR) and 3′ UTR of the *SlPAP1* transcript, Rapid Amplification of cDNA Ends (RACE) (Clontech Laboratories Inc., Mountain View, CA, USA) was performed using PAP01 and PAP02 as gene specific primers for 3′ RACE and 5′ RACE, respectively. The 5′ and 3′ RACE products were cloned into the pGEM-T vector and sequenced. Full-length cDNA and genomic sequences for *SlPAP1* were amplified from total root RNA and leaf genomic DNA, respectively, using the primers PAP03 (5′-CGGGATCCATGAGTGTGTCAACAATGACTT-3′) and PAP04 (5′-CGAGCTCGAAATAATAAAATATTTATTCGTCG-3′); these primers added restriction sites (underlined) for *Bam*HI and *Sac*I to the 5′ and 3′ ends, respectively, of the cDNA. The *SlPAP1* cDNA and genomic sequences were cloned into the pGEM-T vector and sequenced.

Northern blot analysis of *SlPAP1* expression

Total RNA was extracted from samples of leaf and root tissue from 15-day-old tomato seedlings and 15 μg RNA was subjected to electrophoresis on a 1 % formaldehyde gel, blotted onto nitrocellulose membrane and hybridized to a DIG-labeled antisense *SlPAP1* probe (TPAP-500, Supplementary Figure 1) at 42 °C for 16 h. The membrane was washed twice in low stringency buffer (2 × SSC, 0.1 % SDS) at room temperature for 15 min, and then twice in high stringency buffer (0.5 × SSC, 0.1 % SDS) at 68 °C for 15 min. Signals were detected using the chemiluminescence substrate CSPD (Roche, Basel, Switzerland), according to the manufacturer's protocol.

Determination of *SlPAP1* gene copy number and gene family members using Southern blot analysis

As primer PAP01 was located at the splice site of intron 1, another primer PAP05 (5′-ATAACACAAGGAGATCATGTGGG-3′), located slightly downstream of primer PAP01, was used to synthesize a DIG-labeled probe for Southern blot analysis. Restriction enzymes *Eco*RI, *Xba*I and *Hin*dIII (New England Biolabs, Ipswich, MA, USA) were chosen as their restriction sites are not present in the probe (TPAP-1000) or the corresponding sequence in the tomato genome. Ten micrograms of DNA were digested overnight, separated by electrophoresis on 0.8 % agarose gel, blotted onto a nylon membrane and hybridized to the DIG-labeled antisense *SlPAP1* probe (TPAP-1000; Supplementary Figure 1). For high stringency conditions, the membrane was hybridized at 42 °C for 16 h, washed twice in low stringency buffer at room temperature for 15 min and twice in high stringency buffer at 68 °C for 15 min. For low stringency conditions, the membrane was hybridized at 39 °C for 16 h and then washed four times in low stringency buffer, twice at room temperature for 15 min and twice at 65 °C for 15 min. Signals were detected using the chemiluminescence substrate CSPD (Roche, Basel, Switzerland), according to the manufacturer's protocol. For gene copy number reconstructions (Croy et al. 1993), the 1, 2, 5 and 10 haploid genome equivalents of plasmid pGEM-T/TPAP-500 DNA were used. The plasmid pGEM-T/TPAP-500 was digested with EcoRI to release the TPAP-500 fragments. The size of tomato genome is approximately 1,000 Mbp per haploid genome (Arumuganathan and Earle 1991), and the size of the pGEM-T/TPAP-500 DNA is approximately 3.5 kbp. Thus, 35 pg of pGEM-T/TPAP-500 DNA is approximately equal to a single copy of SlPAP1 in 10 μg of tomato genomic DNA.

Sequence analysis of *SlPAP1*

A homologous sequence search was performed against the NCBI non-redundant nucleotide database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments were made using the BioEdit program. Protein sequence analysis was performed using ExPASy (http://www.expasy.org/tools/). Multiple sequence alignments and determination of phylogenetic relationships

were performed using MEGA program version 6 and a neighbor-joining tree with 1,000 bootstrap replicates.

GenBank accessions

The *SlPAP1* cDNA and predicted amino acid sequence have been submitted to GenBank under the accession number (KP406520). The following PAP cDNA sequences were retrieved from the NCBI GenBank nucleotide database: tobacco (*Nicotiana tabacum*): *NtPAP4* (AB017966), *NtPAP12* (AB017967), *NtPAP19* (AB017968) and *NtPAP21* (AB084124); *A. officinalis*: AoPAP32 (AF126255); soybean (*Glycine max*): *GmPAP* (AF200824); sweet potato (*I. batatas*): *IbPAP1* (AF200825), *IbPAP2* (AF200826) and *IbPAP3* (AJ006870); Arabidopsis (*A. thaliana*): *AtPAP10* (AF492662), *AtPAP12* (AF492664) and *AtPAP26* (AY842026); kidney bean (*Phaseolus vulgaris*): *kbPAP* (AJ001270); and tomato (*Solanum lycopersicum*): *SlPAP2* (XR_182419), *SlPAP3* (XM_004243777), *SlPAP4* (XM_004251686).

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Conflict of interest All the authors declare that they have no conflict of interest.

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