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Lily pollen alkaline phytase is a histidine phosphatase similar to mammalian multiple inositol polyphosphate phosphatase (MINPP)

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Abstract

Phytic acid is the most abundant inositol phosphate in cells; it constitutes 1–5% of the dry weight of cereal grains and legumes. Phytases are the primary enzymes responsible for the hydrolysis of phytic acid and thus play important roles in inositol phosphate metabolism. A novel alkaline phytase in lily pollen (LlALP) was recently purified in our laboratory. In this paper, we describe the cloning and characterization of LlALP cDNA from lily pollen. Two isoforms of alkaline phytase cDNAs, *LlAlp1* and *LlAlp2*, which are 1467 and 1533 bp long and encode proteins of 487 and 511 amino acids, respectively, were identified. The deduced amino acid sequences contains the signature heptapeptide of histidine phosphatases, -RHGXRXP-, but shares <25% identity to fungal histidine acid phytases. Phylogenetic analysis reveals that LlALP is most closely related to multiple inositol polyphosphate phosphatase (MINPP) from humans (25%) and rats (23%). mRNA corresponding to *LlAlp1* and *LlAlp2* were expressed in leaves, stem, petals and pollen grains. The expression profiles of *LlAlp* isoforms in anthers indicated that mRNA corresponding to both isoforms were present at all stages of flower development. The expression of *LlAlp2* cDNA in *Escherichia coli* revealed the accumulation of the active enzyme in inclusion bodies and confirmed that the cDNA encodes an alkaline phytase. In summary, plant alkaline phytase is a member of the histidine phosphatase family that includes MINPP and exhibits properties distinct from bacterial and fungal phytases.

Keywords: *Lilium longiflorum*; Liliaceae; Easter lily; Pollen grains; Cloning; Alkaline phytase; Recombinant expression; Multiple inositol polyphosphate phosphatase (MINPP)

1. Introduction

Phytases are a class of phosphatases that catalyze the sequential hydrolysis of phytic acid (**1**) to less phosphorylated inositol phosphates and, in some cases, to inositol (Wodzinski and Ullah, 1996; Mullaney and Ullah, 2003; Oh et al., 2004). Phytic acid (**1**) is the most abundant inositol phosphate in cells (Reddy et al., 1982). Thus, phytases, the primary enzymes responsible for the hydrolysis of phytic acid (**1**), play an important role in the metabolism of inositol phosphates, a class of compounds involved in signal transduction and calcium regulation (Shears, 1998, 2001; Raboy, 2003).

A number of phytases have been isolated in plants and microorganisms (Mullaney and Ullah, 2003; Konietzny and Greiner, 2002). Phytases have been classified on the basis of pH optima (acid and alkaline), catalytic mechanisms (histidine acid phosphatase-like phytase, purple acid phosphatase-like phytase, β-propeller phytase, and cysteine phosphatase-like phytase (Chu et al., 2004)), and specificity of hydrolysis of the first phosphate group (3-phytase, 6-phytase and, more recently, 5-phytase) (Wodzinski and Ullah, 1996; Konietzny and Greiner, 2002; Mullaney and Ullah, 2003; Oh et al., 2004; Barrientos et al., 1994). Although acid phytases, especially from fungal and bacterial sources, have been extensively studied, investigations on alkaline phytases are few (Wodzinski and Ullah, 1996; Konietzny and Greiner, 2002; Mullaney and Ullah, 2003; Oh et al., 2004). Alkaline phytase was first reported in

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the pollen grains of cattail, *Typha latifolia*, Easter lily, *Lilium longiflorum* and legume seeds (Hara et al., 1985; Scott and Loewus, 1986). Alkaline phytase from *L. longiflorum* (LlALP) exhibits unique catalytic properties compared to acid phytases including narrow substrate specificity, specificity of hydrolysis (first phosphate hydrolyzed at D-5 rather than D-3 or D-6) (Fig. 1), calcium ion requirement, inhibitor effect of EDTA and final product produced ($InsP_3$ rather than Ins) (Scott and Loewus, 1986; Baldi et al., 1988; Barrientos et al., 1994; Jog et al., 2005). More recently, alkaline phytases from bacterial sources, including *B. subtilis*, *B. amyloliquefaciens*, and *B. licheniformis*, were discovered and shown to exhibit similar biochemical characteristics (Kerovuo et al., 1998, 2000; Kim et al., 1998; Shin et al., 2001). In addition, X-ray crystallography structure of the bacterial alkaline phytase from *B. amyloliquifaciens* revealed that the molecular structure was unique; the enzyme has a six-bladed propeller structure rather than the α/β domain structure found in acid phytases (Ha et al., 2000; Shin et al., 2001). In animal tissues, multiple inositol polyphosphate phosphatase (MINPP; also abbreviated MIPP), a histidine phosphatase, catalyzes the hydrolysis of $InsP_6$, $InsP_5$, and $InsP_4$ (Ali et al., 1995; Craxton et al., 1997; Chi et al., 1999).

Although the presence of phytic acid (**1**) was discovered in plants in 1885 (reviewed in Posternak, 1965) and the presence of phytase activity in plants has been known since 1905 (reviewed in Konietzny and Greiner, 2002), details at the molecular level have been slow in coming. To date, two plant phytases have been isolated, characterized, purified and sequenced, namely the purple acid phosphatase-phytase from soybean (Hegeman and Grabau, 2001) and the acid phytase from maize (Maugenest et al., 1997). The purification of alkaline phytase from plant sources has been a challenge because of its instability and contamination with non-specific phosphatases. Little is known about the catalytic details, structure/activity relationships or the role of the enzyme in inositol phosphate metabolism in plants. Recently, we completed the purification of alkaline phytase from lily pollen (Garchow et al., 2006). In this paper, we present the amino acid sequence of internal peptides and describe the preparation and cloning of the cDNA encoding LlALP using amino acid sequence information and PCR strategy. The expression levels of alkaline phytase in different tissues of Easter lily, as well as changes in expression during pollen grain formation, were investigated to gain an understanding of the physiological significance of LlALP.

2. Results and discussion

2.1. Amino acid sequences of internal peptides

LlALP from *L. longiflorum* pollen was purified in our laboratory using a multi-step procedure involving selective precipitation by heat and ammonium sulfate followed by anion exchange and chromatofocusing chromatography and, finally, gel-electrophoresis (Garchow et al., 2006). Separation by 2-D gel electrophoresis followed by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometric analysis indicated the presence of several isoforms (Garchow et al., 2006). The MALDI-TOF data did not indicate if the isoforms differed in primary amino acid sequences, or posttranslational modifications, or both. In addition, the MALDI-TOF peptide mass maps did not match any known phytases or phosphatases.

Amino acid sequence information of internal peptides was obtained by Edman degradation (four peptides) and electrospray ionization-tandem mass spectrometry (ESI-MS/MS) (Table 1). Three peptides were sequenced de novo from their fragment ion spectrum (Table 1). Database searches with the BLASTP algorithm (Altschul et al., 1997) revealed that the amino acid sequences did not match any known plant protein. However, six of the seven peptides shared identity with deduced amino acid sequences in *Arabidopsis thaliana* (annotated as a putative histidine acid phosphatase, HAP, GenBank Accession No. NP_563856) and/or *Oryza sativa* (annotated as putative multiple inositol polyphosphate phosphatase, MINPP, GenBank Accession No. XP_470115) (Table 1) with percentage identity greater than 77%.

2.2. Cloning and sequence analysis of cDNA encoding alkaline phytase from lily pollen

Amino acid sequence information of internal peptides described above was used to design forward and reverse degenerate primers. The primers were oriented based on

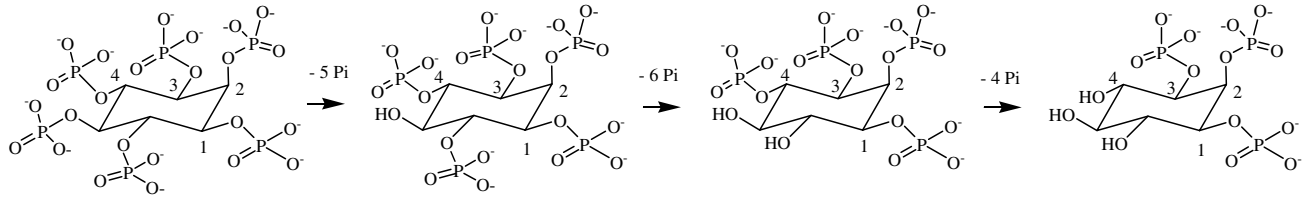


Fig. 1. Specificity of hydrolysis of phytic acid by lily pollen alkaline phytase.

Table 1
Internal peptide sequences of purified alkaline phytase

Peptide name	Peptide sequence	Homology to	Identity
Edman degradation			
Peptide 1	NDEPFDVR	No known protein	None detected
Peptide 2	GEDELY	HAP-family protein, *Arabidopsis*	100%
Peptide 3	AFSVISE	Putative MIPP, rice	88%
Peptide 4	GYGNSVNYR	Putative MIPP, rice	100%
		HAP-family protein, *Arabidopsis*	87%
ESI-MS/MS			
Peptide 5	AFSVISESR	Putative MIPP, rice	88%
Peptide 6	FFDSCETYK	Putative MIPP, rice	77%
Peptide 7	EQIVEPHLK	Putative MIPP, rice	90%

Amino acid sequences of internal peptides were obtained by Edman degradation (Peptides 1–4) or ESI-MS/MS (Peptides 5–7). Three pairs of residues I/L, K/Q and F/Msx (oxidized methionine) are isobaric (identical mass) and therefore cannot be unambiguously assigned. Database searches were conducted with the BLASTP algorithm using the peptide sequences as query. Note that Peptides 3 and 5 have 7 identical amino acids.

the location of the peptides in the rice sequence that showed highest homology (GenBank Accession No. XP_470115). The full length cDNA encoding LlALP was obtained by a two-stage process using RNA extracted from pollen grains of *L. longiflorum*. In the first stage, a truncated cDNA was synthesized by RT-PCR using multiple permutations (total of 30) of degenerate forward and reverse primers in the amplification step (details in Section 3). One set of degenerate gene-specific primers (Primers 1 and 2) yielded a single product of 836 bp; the predicted amino acid sequence of the putative LlALP-encoding cDNA shared a high degree (89%) of sequence identity with putative rice MINPP. In the second stage, the complete full-length sequence of the cDNA, including the 3′ and 5′ flanking regions, was obtained using mRNA and 3′ and 5′ RACE and nested PCR techniques. The 3′-RACE experiments yielded two products (420 bp and 520 bp), the complete nucleotide sequences of the full length isoforms are shown in Fig. 2. Nucleotide sequences of the amplified full-length cDNAs were determined with multiple primers with overlapping regions covering the full length. Both strands of amplified cDNA were sequenced to ensure accuracy.

Nucleotide sequences of cDNA isoforms *LlAlp1* and *LlAlp2* possess open reading frames of 1467 and 1533 bp (Fig. 2) in length, respectively. Compared to typical plant genes the 5′ untranslated regions (UTR) of *LlAlp1* and *LlAlp2* (9 nucleotides long) were quite short (the average length in dicots is 98 nucleotides and in monocots is 113 (Kochetov et al., 2002), but the AUG codon at position 10 appears to be an authentic translation start codon since it is in an optimum context with purines at −3 and +4 positions (Joshi et al., 1997)). In the 3′ untranslated region, there were 192 nucleotides in *LlAlp1* and 294 in *LlAlp2*. The mRNA also has a distinct 21 bp long poly(A) tail indicating that it is complete at the 3′ end (Fig. 2). A putative polyadenylation signal, -CACTG-, found in many plant species is observed 67 bp upstream of the poly(A) tail (Joshi, 1987). The 5′ UTRs and the coding regions of *LlAlp1* and *LlAlp2* cDNAs show a very high degree of conservation (89%), but at the 3′ end both the coding regions and UTRs differ (Fig. 2).

The deduced amino acid sequence indicates that *LlAlp1* and *LlAlp2* code for polypeptides LlALP1 and LlALP2 containing 489 and 511 amino acids, respectively (Fig. 2). The predicted peptide sequence of the two isoforms shows 97% identity. All seven peptide sequences from Edman degradation and ESI-MS/MS of the purified enzyme were present in the deduced polypeptide sequence of *LlAlp1* and *LlAlp2* cDNAs. The conserved sequence, -RHGXRXP-, present at the active site of histidine phosphatases (usually near the N-terminal) and the dipeptide, HD, near the C-terminal are both present in the translated region indicating that LlALP belongs to the family of histidine phosphatases (Van Etten et al., 1991). The predicted amino acid sequences of the cDNAs possess 10 cysteine residues. Although LlALP shares many biochemical and catalytic similarities with alkaline phytase from bacteria, the amino acid sequence data presented here (Fig. 2) reveal that at the amino acid level they share few similarities.

The calculated molar mass of LlALP1 and LlALP2 was found to be 53.8 kDa and 56.2 kDa, respectively, and these values are consistent with the band observed in SDS–PAGE during purification (Garchow et al., 2006). The isoelectric points were predicted (the pI determination program available at http://seqtool.sdsc.edu/) to be 6.50 for LlALP1 and 7.04 for LlALP2. At least 2 putative *N*-glycosylation sites were predicted between amino acids 250–253 and 410–413 in LlALP1 and LlALP2 by the program Prosite (http://us.expasy.org) (Falquet et al., 2002). Many potential sites for phosphorylation and myristoylation were also found. LlALP has previously been shown to be a membrane-bound protein (Baldi et al., 1988). The program PSORT (http://us.expasy.org) predicted the presence of three transmembrane segments in alkaline phytase. The program SignalP (http://us.expasy.org) (Jannick et al., 2004; Bendtsen et al., 2004; Henrik et al., 1997) predicted a cleavage site between amino acids 21 and 22 (AAA-ND). Analysis with the program Prosite (Falquet et al., 2002) suggested that LlALP2 possesses a C-terminal signal peptide (amino acids 509–511) targeted to microbodies.

The presence of two isoforms of alkaline phytase transcripts could be due to the occurrence of two distinct genes or alternative splicing of the primary transcript; efforts to resolve this issue are ongoing.

2.3. Phylogenetic analysis

Database searches with *LlAlp1* and *LlAlp2* cDNA nucleotide sequences revealed little (<20%) homology to any known plant phytase or protein sequenced to date. The highest homology was to a nucleotide sequence from

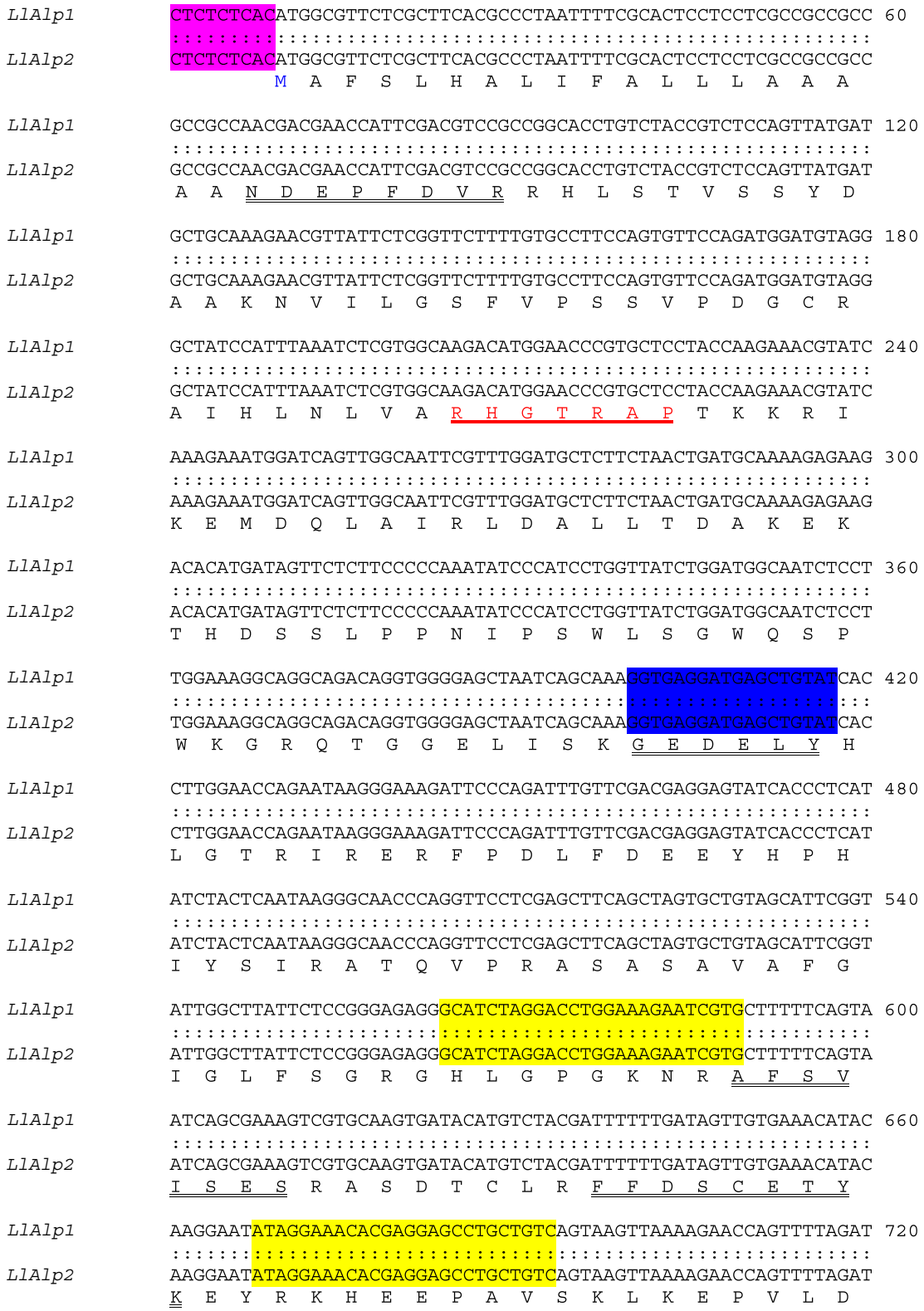


Fig. 2. Nucleotide sequences of cDNAs *LlAlp1* and *LlAlp2* and deduced polypeptide sequences. Partial cDNA encoding alkaline phytase was amplified by employing **Primer 1** and **Primer 2** (blue). The 5′ ends were amplified by a GeneRacer primer and the reverse gene-specific primer, **Primer 3** (yellow) followed by nested amplification employing the GeneRacer nested primer and the reverse gene-specific **Primer 4** (yellow). Nested amplification of the 3′ ends employed the forward gene-specific **Primer 5** (dark red) and the GeneRacer primer followed by the gene-specific **Primer 6** (dark red) and GeneRacer nested primer. The 5′ UTR is shown in magenta and the 3′UTR is shown in turquoise. PolyA tails are shown in teal. Deduced polypeptide sequence of LlALP2 where it differs from LlALP1 is shown in green. Seven peptide sequences of the purified enzyme are underlined (double). The septapeptide histidine phosphatase motif and the dipeptide HD are shown in red and underlined. The putative polyadenylation signal, CACTG, is underlined.

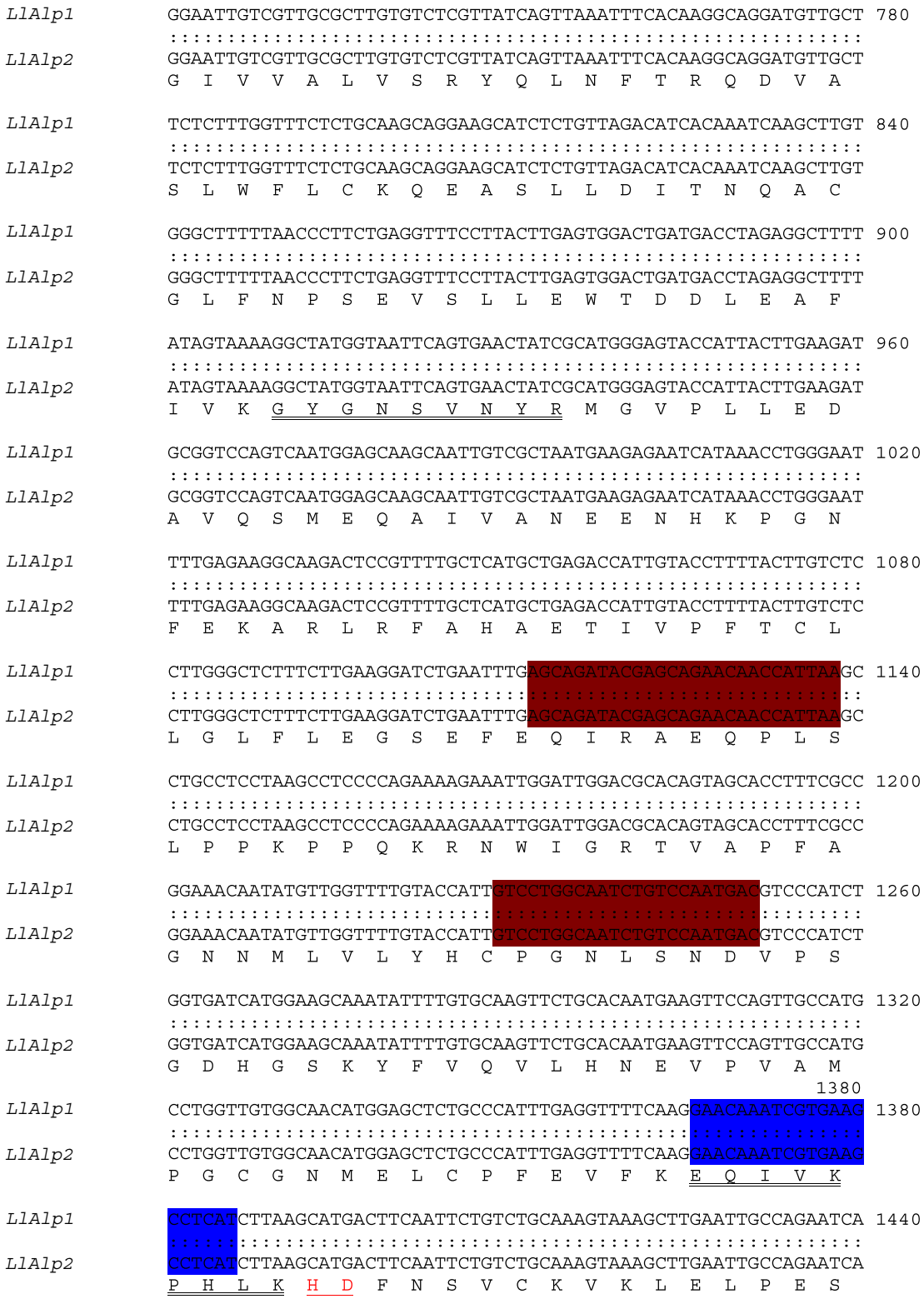
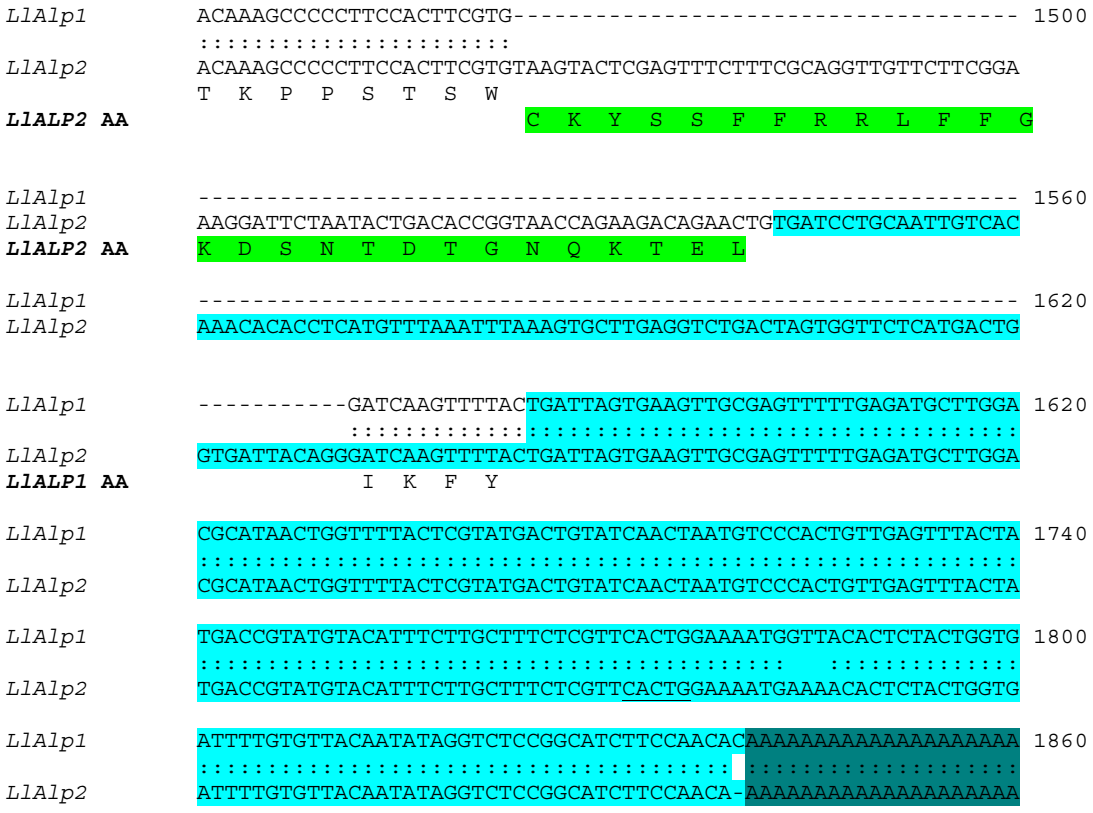


Fig. 2 (*continued*)

the *O. sativa* genome (cDNA clone) (81% identity) designated as putative MINPP. The other top scoring match was a sequence from the *A. thaliana* genome (58% identity) designated as MINPP-like histidine acid phosphatase. Comparison of deduced amino acid sequences revealed that the most closely related enzymes were putative MINPP from rice (65% identity) and MINPP-like HAP from *Arabidopsis* (60% identity). MINPP from rat, mouse, and humans showed identities of 24–26%. A high degree of homology was found in sequences surrounding the active



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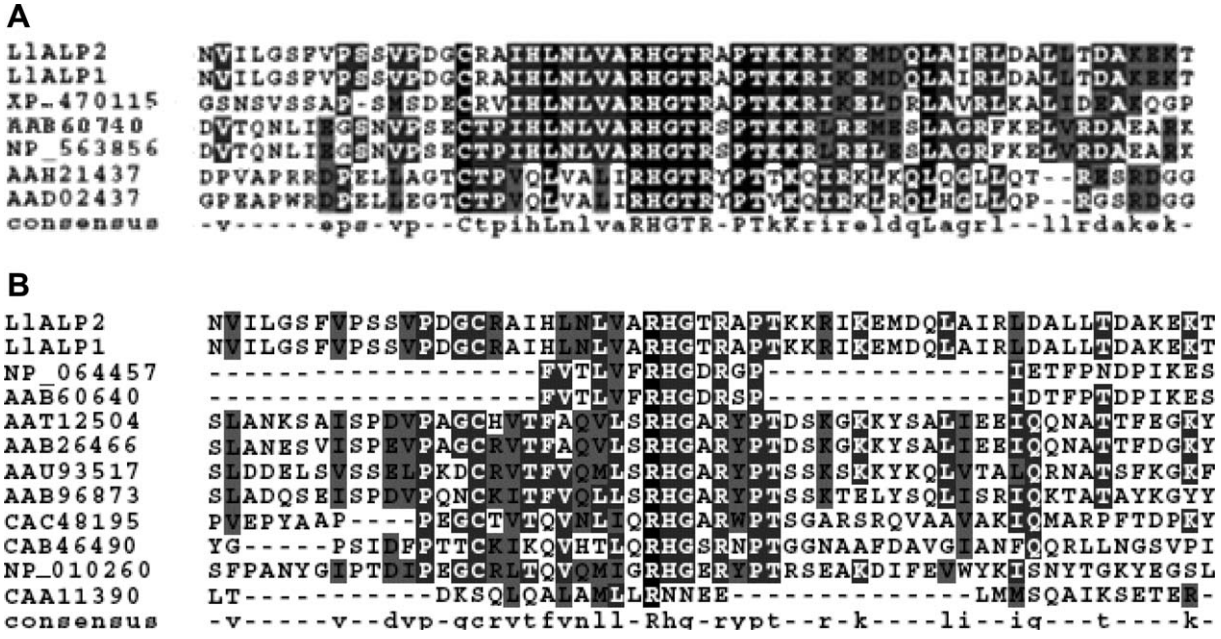

Fig. 3. Alignment of deduced amino acid sequences surrounding the histidine phosphatase active site peptide -RHGTXAX- of *LlAlp1* and *LlAlp2* and (A) selected MINPPs and (B) selected acid phytases and phosphatases. Completely conserved residues are in black, identical residues in dark grey and similar residues in light grey. The homologs are designated by their accession numbers and abbreviations. (A) XP_470115, *O. sativa* putative MIPP; AAB60740, *A. thaliana* HAP; NP_563856, *A. thaliana* HAP family; AAH21437, *M. musculus* MIPP; AAD02437, *H. sapiens* MIPP. (B) NP_064457, *R. norvegicus* prostatic acid phosphatase; AAB60640, *H. sapiens* prostatic acid phosphatase; AAT12504, *A. oryzae* phytase; AAB26466, *A. ficuum* phytase; AAU93517, *A. fumigatus* phytase; AAB96873, *T. thermophilus* phytase; CAC48195, *P. lycii* phytase; CAB46490, *S. pombe* acid phosphatase; NP_010260, *S. cerevisiae* Dia3p; CAA11390, *Z. mays* root phytase.

site peptide, -RHGXRXP- (Fig. 3). Although many fungal and bacterial phytases are histidine phosphatases, LlALP shares less homology with these phytases (Fig. 3B) than with MINPP from animal sources (Fig. 3A). Sequences with scores greater than 100 were imported into Molecular Biology Workbench (www.workbench.sdsc.edu) and a phylogenetic tree was generated employing ClustalW (Fig. 4) (Felsenstein, 1993). The phylogenetic tree indicated that there are three main branches of histidine phosphatases, the LlALPs, putative plant MINPPs from *Arabidopsis*, maize and rice and the animal MINPPs form one branch, the maize acid phytases and the prostatic phosphatases form a second branch, and the yeast acid phosphatases and acid phytases form a third (Chi et al., 1999).

2.4. *LlAlp1 and LlAlp2 mRNA are expressed in tissues of lily pollen*

The expression of *LlAlp1* and *LlAlp2* mRNA in different tissues and at different stages of anther development of *L. longiflorum* was examined by semi-quantitative RT-PCR. In an attempt to find a suitable constitutive housekeeping

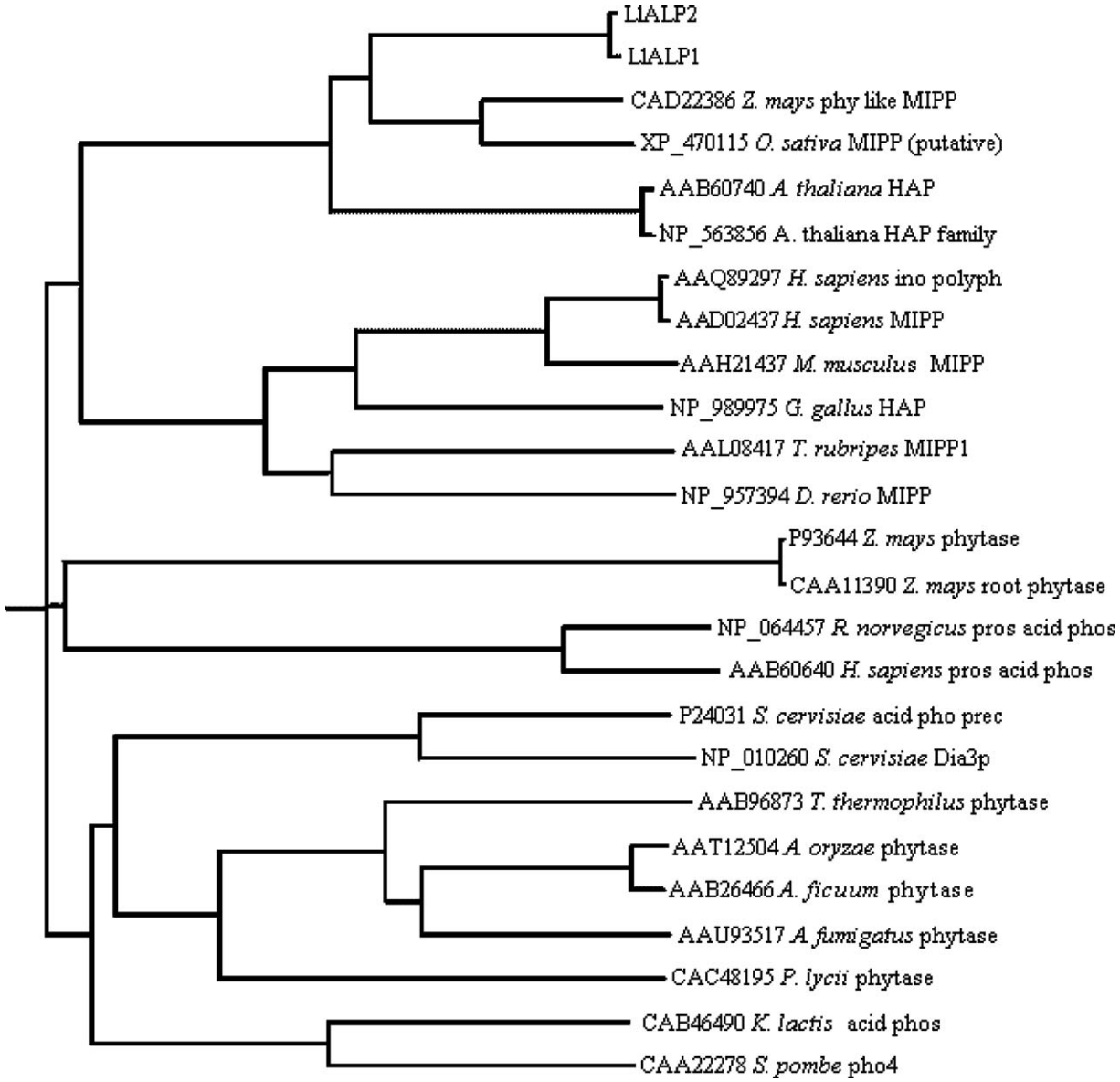

Fig. 4. Phylogenetic tree of selected histidine phytase and phosphatase sequences. The tree was generated by ClustalW program at http://seqtool.sdsc.edu. The homologs are designated by their accession numbers and abbreviations. CAD22386, *Z. mays* root MIPP with phytase activity; XP_470115, *O. sativa putative* MIPP; AAB60740, *A. thaliana* HAP; NP_563856, *A. thaliana* HAP family protein; AAQ89297, *H. sapiens* multiple inositol polyphosphate phosphatase; AAD02437, *H. sapiens* MIPP; AAH21437, *M. musculus* MIPP; NP_989975, *G. gallus* HAP; AAL08417, *T. rupripes* MIPP1; NP_957394, *D. rerio* MIPP; P93644, *Z. mays* phytase; CAA11390, *Z. mays* root phytase; NP_064457, *R. norvegicus* prostatic acid phosphatase; AAB60640, *H. sapiens* prostatic acid phosphatase; P24031, *S. cerevisiae* acid phosphatase precursor; NP_010260, *S. cerevisiae* Dia3p; AAB96873, *T. thermophilus* phytase; AAT12504, *A. oryzae* phytase; AAB26466, *A. ficuum* phytase; AAU93517, *A. fumigatus* phytase; CAC48195, *P. lycii* phytase; CAB46490, *K. lactis* acid phosphatase; CAA22278, *S. pombe* pho4.

gene that is expressed at a constant level to normalize variations during experimental work, the transcript levels of actin 2/actin 8 (An et al., 1996), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitin were examined. Preliminary experiments were conducted with primers for actin (from Arabidopsis, GenBank Accession No. AAB37098), GAPDH (from *L. longiflorum*, GenBank Accession No. ABC47829), and ubiquitin (from *L. longiflorum*, GenBank Accession No. AAF21992). Under the RT-PCR conditions employed transcript levels of actin and GAPDH varied significantly in different tissues; ubiquitin transcript levels varies less and therefore was used for normalization.

Oligo(dT)$_{15}$-primed cDNA was synthesized from equal amounts of total RNA from different tissues using reverse transcriptase and amplified with isoform-specific primers or ubiquitin-specific primers (Primers 15 and 16). Since the 5′ ends of the two isoforms are conserved, Primer 10 was used as forward primer. The isoforms differ at the 3′ end (Fig. 2) therefore isoform-specific reverse primers, Primer 13 for *LlAlp1* and reverse Primer 12 for *LlAlp2* were employed and the amplified cDNA products were separated by agarose gel electrophoresis.

The expected 1100-bp product from *LlAlp1 and LlAlp2* transcripts were detected in leaves, stem, petals and pollen grains of *L. longiflorum* indicating that mRNA corresponding to both isoforms, *LlAlp1* and *LlAlp2* cDNA, were expressed in all tissues examined (Fig. 5A). Pollen grains are viable for extended periods of time so the stability of mRNA transcripts in pollen grains of different ages were examined. When the level of *LlAlp1* and *LlAlp2* transcripts in pollen collected in 2004 was compared with that collected in 1988, there was no noticeable difference suggesting that, in lily pollen, mRNA corresponding to *LlAlp1* and *LlAlp2* are stable for extended periods of time. The expression profiles of *LlAlp* isoforms in anthers as a function of flower development (Fig. 5B) indicated that *LlAlp1* and *LlAlp2* transcripts were present at relatively constant levels at all stages of development, from early stage (2.5 cm) to fully developed bud (10 cm) (Fig. 5B).

2.5. Expression of *LlAlp2* cDNA in *Escherichia coli*

The *LlAlp2* cDNA was expressed in *E. coli* to confirm the enzymatic activity of the cloned cDNA and as a potential source of substantial quantities of the active enzyme for future structural and biochemical studies. The coding region of *LlAlp2* (1533 bp) was inserted into pET30a(+), a prokaryotic expression vector in which expression of the target-gene is under the control of a strong T7/lac promoter. *E. coli* strain BL21(DE3) cells were transformed with the pET30a(+) plasmid DNA construct. Cells were induced with isopropyl-β-D-thiogalactoside (IPTG) and samples of induced and un-induced cells were collected at different time points. Cell lysis followed by fractionation and alkaline phytase assay indicated that the active enzyme was located in the pellet containing inclusion bodies (IB); no activity was detected in the soluble fraction. Fig. 6 compares the time-dependent changes in cell growth and

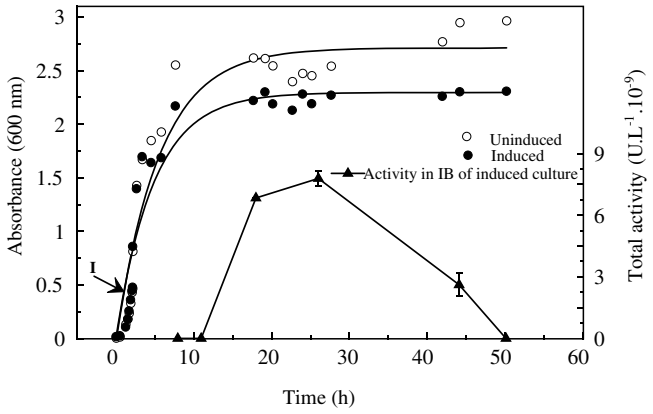

Fig. 6. Time dependent growth of *E. coli* strain BL21(DE3) and accumulation of alkaline phytase activity. Cultures were grown to A_{600} of 0.45 and protein expression was induced (I) with IPTG (1 mM). Growth of the induced and uninduced cultures was monitored by measuring A_{600}. Samples were collected at different time points from 6 h to 48 h after induction. Cells were lysed and the phytase activity in the soluble and inclusion body fractions of induced and uninduced cells was determined. Error bars represent ±SEM, $n = 2$.

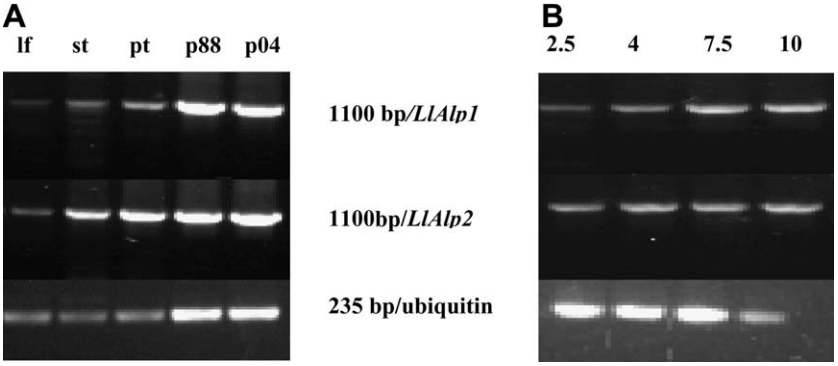

Fig. 5. Semi-quantitative RT-PCR analysis of gene expression of *LlAlp1* and *LlAlp2*. (A) Different tissues of lily plant; leaf (lf), stem (st), petal (pt), pollen 1988 (p88), pollen 2004 (p04). (B) Different stages of bud development 2.5 cm, 4.0 cm, 7.5 cm, 10.0 cm. Ubiquitin was amplified in parallel experiments to normalize variations during experiments. Data are representative of three different experiments.

phytase activity in un-induced and induced cells. No detectable accumulation of phytase activity was observed in the soluble fraction or in inclusion bodies for up to 16 h after induction with IPTG. Maximum accumulation was observed 24 h after induction (22 U L^{-1}) after which time alkaline phytase activity decreased. No activity was detected in the un-induced sample at any time point. The expression of the alkaline phytase cDNA in *E. coli* confirmed that the cloned sequence encodes an enzyme that catalyzes phytic acid (**1**) hydrolysis at pH 8.0. Although recombinant proteins in IBs are frequently inactive (Georgiou and Valax, 1990), enzyme activity has been observed in IB-based endoglucanase D (Tokatlidis et al., 1991) and β-galactosidase (Worrall and Goss, 1989) suggesting that in some cases the protein deposited in IBs are in the native conformation. The alkaline phytase isoform *LlAlp2* cloned into pET30a(+) yielded active enzyme in IBs of *E. coli* strain BL21(DE3), in contrast to the majority of inclusion body-localized enzymes (Ellis and Hart, 1999; Tsumoto et al., 2003).

In summary, LlALP contains the histidine phosphatase conserved sequence, -RHGXRXP-, and therefore belongs to the histidine phosphatase family of enzymes unlike alkaline phytases from bacteria (Oh et al., 2004). Although phosphatases containing -RHGXRXP- are often referred to as histidine acid phosphatases, it may be more accurate to call them histidine phosphatases as the MINPP subgroup in this family exhibit pH optima above 7.0 (Craxton et al., 1995; Chi et al., 1999). Comparison of alkaline phytase from plants with MINPP from animals reveals some interesting catalytic characteristics. Both groups exhibit alkaline pH optima (8.0 for LlALP and 7.6 for animal MINPP) and hydrolyze inositol phosphates with four or more phosphate groups; when phytic acid (**1**) is the substrate, $InsP_3$ (**4**) is the final product produced (Craxton et al., 1995). Whereas the first phosphate hydrolyzed by LlALP is at the D-5 position (Barrientos et al., 1994), MINPP from rat shows less specificity and hydrolyzes phosphates from D-3, D-5 and D-6 positions to yield a mixture of $InsP_5$s (Craxton et al., 1995). In addition, MINPP from animal sources do not need Ca^{2+} for activity whereas LlALP shows minimal activity in the absence of calcium (Nogimori et al., 1991; Craxton et al., 1995; Chi et al., 1999). Furthermore, the addition of chelating agent EDTA strongly inhibits hydrolytic activity of LlALP whereas enzyme assays of animal MINPP are often conducted in the presence of 1 mM EDTA, 0.5 mM EGTA or both (Craxton et al., 1997). In addition to phytic acid (**1**), MINPP from animals are able to hydrolyze Ins(1,3,4,5)P_4 to Ins(1,4,5)P_3, Ins(1,3,4,5,6)P_5 to Ins(1,4,5,6)P_4 and finally to Ins(1,4,5)P_3, and $InsP_6$ to a mixture of $InsP_5$s. MINPP from animals also hydrolyzes $PPInsP_5$ and $(PP)_2InsP_4$ (Craxton et al., 1997). The substrate specificity as well as the specificity of hydrolysis of LlALP towards inositol phosphates such as $InsP_5$ and $InsP_4$ remain to be determined. The differences in catalytic properties between LlALP and MINPP from animals could be due to the differences in amino acid sequences.

The mechanism of catalysis of a number of histidine phosphatases is well understood and therefore can be used as a model to propose a mechanism for alkaline phytase (Van Etten, 1982; Ostanin et al., 1992, 1994; Ostanin and Van Etten, 1993). The presence of the consensus motif of histidine phosphatases, -RHGXRXP-, suggests that, in the first step, catalysis may occur by the attack of a nucleophilic histidine and the formation of a phosphohistidine intermediate accompanied by the release of the dephosphorylated inositol phosphate. In the second step, the cleavage of the phosphoramide bond of phosphohistidine by attack of a water molecule and release of the phosphate completes the hydrolytic reaction. The decomposition of the phosphohistidine intermediate is generally the rate determining step although this may vary with substrate and reaction conditions (Ostanin et al., 1992, 1994; Ostanin and Van Etten, 1993). The concentration of protonated histidine decreases with increase in pH, therefore, in the first step, His^{67} in alkaline phytase would be a better nucleophile at pH 8 than at acidic pH. Research work with acid phosphatase mutants suggests that the two positively charged arginine residues separated by three amino acids around histidine in the consensus motif (Arg^{66} and Arg^{70} in alkaline phytase) may be involved in binding the negatively charged phosphates (Ostanin et al., 1992, 1994). In prostatic acid phosphatase and *E. coli* phosphatase, the aspartic acid in the distant -HD- sequence is believed to donate a proton to the leaving alcohol thus making it a better leaving group; although the adjacent histidine does play a role in catalysis, the precise nature of the involvement of histidine was not clear (Ostanin et al., 1992, 1994; Ostanin and Van Etten, 1993). Proton donation by aspartic acid in the -HD- sequence (His^{442} and Asp^{443} in alkaline phytase) can occur efficiently at high pH if the carboxylic acid has an altered high pK_a or if the attack is assisted by general acid catalysis. The pK_a values of amino acid side chains in proteins vary considerably depending on the microenvironment in the protein (Bugg, 1977; Forsyth et al., 2002). The pK_a value of aspartic acid, generally around 3.4 ± 1.0, can be elevated to greater than 9 (around 9.9 in reduced thioredoxin) by hydrophobic microenvironment, the presence of negatively charged residues in close proximity, and/or the presence of hydrogen bond acceptors (reviewed in Forsyth et al., 2002). Alternatively, the adjacent histidine, His^{442}, could function as a proton donor to aspartate or as a general acid catalyst. Additional experiments are needed to resolve this issue.

In the context of pH-dependent phosphatase activity, it is worthwhile to note two related points. First, human prostatic acid phosphatase is highly active against phosphomonoester over a wide pH range, 3–8 (Ostanin et al., 1994; Van Etten, 1982, and references therein). When the pH dependence of K_m and V_{max} was measured, V_{max} was nearly constant from pH 3 to nearly 8 (Van Etten, 1982). The author suggested that the "typical" bell shaped curve generally observed in pH-rate profiles was due to the effect of substrate ionization on K_m values (Van Etten, 1982).

Supporting this argument was the observation that when differing extent of ionization was taken into account, K_m and V_{max} was constant between pH 3 and 8 (Van Etten, 1982; Ostanin et al., 1994). These experimental data led to the suggestion, and subsequent confirmation, that for side chains to function as nucleophiles or proton donors over a range of pH the pK_a values of side chains in the active site of acid phosphatases may be altered (Ostanin et al., 1994). Second, historically, enzymes known as acid phosphatase were so defined on the basis of pH optimum with the substrate *para*-nitrophenyl phosphate which exhibits an unusually low pH optimum because of the low pK_{a2} (Van Etten, 1982, 1993). When *para*-nitrophenyl phosphate was the substrate, alkaline phytase exhibited a pH optimum of 7.0, one pH unit less than with phytate (Jog et al., 2005).

2.6. Concluding remarks

The structural characteristics presented here suggest that alkaline phytase from *L. longiflorum* is a unique enzyme that differs from bacterial alkaline phytase, MINPP from animals and fungal histidine phosphatase. The final product produced by alkaline phytase, Ins(1,2,3)P_3, (**4**) contains a *cis* trisphosphate arrangement and exhibits antioxidant properties; it has been shown to inhibit iron-catalyzed free radical formation by chelating iron in an unique manner (Hawkins et al., 1993; Phillipy and Graf, 1997). The unusual catalytic properties suggest that the enzyme has the potential to be useful as a feed and food supplement (Jog et al., 2005). The physiological role of alkaline phytase in plant cells, as well as the role of the enzyme in inositol metabolism remains to be established.

3. Experimental

3.1. Plant material

Pollen grains from 1991 and 1988 harvests were a kind donation from Dr. Frank Loewus, Washington State University, Pullman, WA. Pollen grains from the 2004 harvest were collected from un-opened lily buds (14–15 cm) just before anthesis as described by Loewus and Loewus (1990). Briefly, *L. longiflorum* plants were maintained in a greenhouse at 20 °C/24 °C (night/day). The buds were cut open with clean razor blades and the anthers were allowed to dehisce and air dry on a tray lined with waxed paper in a single layer. After the thecae had hardened, pollen grains were separated from dried thecae with a sieve and stored in a loosely capped bottle at −20 °C. Anthers from buds of different lengths were collected as follows: Buds of different length (2–10 cm) were removed from the plants and the petals carefully separated. The anthers were cut from the buds with a clean razor blade, immediately flash frozen in liquid N_2 and stored at −80 °C until use.

3.2. Peptide sequencing

Alkaline phytase was purified as described previously (Garchow et al., 2006). Amino acid sequences of seven peptides (Peptides 1–7) were determined by Edman degradation or ESI-MS/MS. Peptide sequencing by Edman degradation was conducted as follows: protein bands from a 2-D gel (Garchow et al., 2006) were excised and subjected to in-gel reduction, alkylation and tryptic digestion as described previously (Garchow et al., 2006). Prior to Edman sequencing, the tryptic digest was separated by HPLC on a Vydac C18 column, 1.0 mm × 150 mm, using a linear aqueous-acetonitrile gradient. Selected peptides were sequenced on an Applied Biosystems model 494 protein sequencer. This analysis was performed at the Protein Chemistry Core Facility, Howard Hughes Medical Institute, Columbia University, New York, NY.

De novo sequencing by liquid chromatography–tandem mass spectrometry (LC/MS/MS): sequence analysis was performed by microcapillary reversed-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer (Chittum et al., 1998). Briefly, the procedure is as follows: protein bands from 2-D gel (Garchow et al., 2006) were excised and subjected to in-gel reduction, *S*-carboxyamidomethylation, and tryptic digestion. The resulting mixture was separated by a reversed-phase microcapillary column and introduced directly into the electrospray ionization (ESI) source of a quadrupole ion trap mass spectrometer. The ion trap was set for one survey full scan and MS/MS on the most abundant four precursor ions in that scan: collision energy of 30, isolation width of 2.5 amu, dynamic exclusion for 120 s. The MS–MS spectra were correlated with known peptide sequences in databases using the algorithm Sequest (Eng et al., 1994), followed by manual interpretation with the aid of the program, FuzzyIons (Chittum et al., 1998). This sequence analysis was performed at the Harvard Microchemistry Facility, Harvard University, Cambridge, MA.

3.3. Isolation of RNA and mRNA

Total RNA was isolated from 70 mg of tissue (frozen lily pollen, petals, fresh leaves, stem and anthers from buds of different lengths) with RNAeasy Plant Mini Kit (Qiagen, CA). For RACE procedures, mRNA was used as the template. mRNA was isolated from 250 μg of total RNA using the Oligotex mRNA Mini Kit (Qiagen, CA) per the manufacturer's instruction.

3.4. Preparation of alkaline phytase cDNA

The amplification of an internal section of the mRNA sequence was conducted by RT-PCR. Degenerate primers with low degeneracy were designed based on the amino acid sequences of the seven peptides (Table 1). The first strand of cDNA was synthesized from 200 ng of total

RNA (from lily pollen) and gene-specific degenerate primer (Primer 2) at 42 °C for 55 min using Superscript III RT (Invitrogen, CA) per manufacturer's instructions. The resulting cDNA was used as a template for PCR amplifications using multiple permutations (total of 30) of degenerate forward and reverse primers. Amplification employing the degenerate forward primer (Primer 1, GG\ideoxyI\GARGAYGARCT\ideoxyI\TA) and degenerate reverse primer (Primer 2, RTG\ideoxyI\GGYTC\ideoxyI\AC\ideoxyI\ATYTTYTG) (35 cycles at 94 °C for 30 s, 45 °C for 1 min and 72 °C for 1 min) yielded a 856 bp alkaline phytase cDNA fragment.

RACE procedures were used to amplify the 3′ and 5′ ends of lily pollen alkaline phytase cDNA. Gene-specific primers were designed based on the sequence of the 856 bp alkaline phytase partial cDNA obtained above. GeneRacer kit (Invitrogen, CA) was used to perform 5′ RACE and 3′ RACE. The GeneRacer Oligo(dT) primed cDNA was synthesized by using Superscript III Reverse transcriptase per manufacturer's instructions. The 5′ end was amplified using GeneRacer 5′ primer and a gene-specific reverse primer (Primer 3, ACAGCAGGCTCCTCGTGTTTCCTAT) for the first round of amplification per manufacturer's instructions. For nested amplification, GeneRacer 5′ nested primer and gene-specific reverse primer (Primer 4, GCACGATTCTTTCCAGGTCCTAGATG) were employed. The 658 bp 5′ RACE product contained the methionine start codon and a 9 bp long 5′ untranslated region.

The amplification of the 3′ end of the cDNA was performed employing the forward gene-specific primer (Primer 5, GCAGATACGAGCAGAACAACCCATT) and reverse GeneRacer 3′ primer under the same conditions as described for the 5′ end. Nested amplification was carried out employing the forward nested gene-specific primer (Primer 6, GTCCTGGCAATCTGTCCAATGAC) and the reverse nested GeneRacer 3′ primer.

3.5. Construction of an alkaline phytase expression plasmid

cDNA corresponding to the full-length coding region was amplified by high fidelity, touch-down, PCR. The forward primer containing the *Bgl*II site, in bold (Primer 7, AGCC**AGATCT**AATGGCGTTCTCGCTTCACGC) was synthesized based on the sequence near the N-terminal. The reverse primers containing *Eco*RI sites, in bold (Primer 9 for *LlAlp1*, CGGT**GAATTC**TCAGTAAAACTTGATCCACG, and Primer 8 for *LlAlp2*, GGT**GAATT**CTCACACTTTCGTCTTCGGGTTACCGGT) were designed based on the sequence surrounding the termination codon. Oligo(dT)$_{15}$-primed cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen, CA) per manufacturer's instruction. The PCR was carried out using *Pfu* DNA polymerase (Stratagene, CA) per manufacturer's instruction. The purified products were ligated into two pET 30a (+) expression vectors (Novagen, CA) between the restriction sites *Bgl*II and *Eco*RI and the recombinants containing the inserts were sequenced to confirm correct insertion.

3.6. Semi-quantitative RT-PCR for expression analysis

To specifically amplify transcripts from each gene, we took advantage of the presence of the variable region in the 3′ coding sequence of *LlAlp1* and *LlAlp2*. Gene-specific reverse primer (Primer 13, AGAACAACCTGCGAAAGAAACTCG) specific for *LlAlp1* and Primer 12 (ACACTTTCGTCTTCGGGTTAC) specific for *LlAlp2* were synthesized based on the variable region of the two isoforms. Oligo(dT)$_{15}$-primed first strand cDNA was synthesized with SuperScript reverse transcriptase III (Invitrogen, CA) and the cDNA was amplified employing a forward primer (Primer 10, CAAAGGTGAGGATGAGCTGTAT) and reverse primer (Primer 13) specific for *LlAlp1* or a forward primer (Primer 10) and reverse primer (Primer 12) specific for *LlAlp2*. To normalize for experimental errors parallel experiments were performed with ubiquitin-specific forward primer (Primer 15, GAAACTCACCACCACGAACCAC) and reverse primer (Primer 16, AAGCAGCTGGAAGATGGACGTACT).

3.7. Expression of alkaline phytase in E. coli

Escherichia coli strain BL21(DE3) was transformed with 2–10 ng pET30a(+)/*LlAlp2* plasmid DNA construct. Transformed cells were grown until the A_{600} reached 0.4–0.6, induced with IPTG (1 mM) and harvested at different times as indicated in Fig. 6. The cells were cooled on ice for 15 min followed by centrifugation at 5000*g* for 30 min at 4 °C. Cell pellet was resuspended in lysis Buffer A (10 mM Tris–HCl, pH 7.0, containing reduced glutathione, 0.5 mM, 5 ml per gram of wet cell pellet). Lysozyme (1 mg/ml of lysis buffer) was added to the suspension followed by incubation on ice for 30 min. Cells were sonicated (Bransonic 12, 117 V, 50/60 Hz, 80 W) in an ice bath with 6 bursts of 10 s each with a delay of 10 s between each burst. Lysed cells were centrifuged at 20,000*g* for 30 min at 4 °C to separate the soluble fraction from cell debris and inclusion bodies. The insoluble pellet was resuspended in Buffer A in a volume equal to the lysis buffer used. The soluble fraction was fractionated with $(NH_4)_2SO_4$ and the protein pellet (41–56% $(NH_4)_2SO_4$) was resuspended in Buffer A (Jog et al., 2005). The soluble fraction and the inclusion body suspensions were assayed for alkaline phytase activity.

3.8. Alkaline phytase activity assay

Alkaline phytase activity was determined by measuring the amount of inorganic phosphate released by the enzyme (Jog et al., 2005). The standard assay mixture contained Tris–HCl buffer (100 mM, pH 8.0), KCl (0.5 M), $CaCl_2$ (1 mM), substrate sodium phytate (1 mM), NaF (10 mM), and an aliquot of enzyme preparation in a total

volume of 250 μl. The assay mixture was incubated at 37 °C for 1 h and the reaction was stopped by the addition of 50 μl of 50% trichloroacetic acid. Inorganic phosphate released was quantified by the addition of ammonium molybdate–ascorbic acid solution (700 μl of 1:6 solution of 10% (w/v) ascorbic acid and 0.42% ammonium molybdate (w/v) in 0.5 M H_2SO_4) followed by incubation at 37 °C for 1 h. The absorbance at 820 nm was measured and the inorganic phosphate concentration was determined from a calibration curve using KH_2PO_4 as the standard. Enzyme units are in katals (the amount of enzyme that releases 1 mol of inorganic phosphate per s under these conditions). The nucleotide sequences reported in this paper have been submitted to GenBank Data Base with the following Accession Nos. DQ437678 and DQ437677.

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