Feeding hungry plants: The role of purple acid phosphatases in phosphate nutrition

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Review

Feeding hungry plants: The role of purple acid phosphatases in phosphate nutrition

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ABSTRACT

Phosphate (Pi) is an essential, but limiting macronutrient that plays critical roles in plant metabolism and development. Plants have evolved an intricate array of adaptations to enhance Pi acquisition and utilization from their environment. The availability of the complete genome sequence of the model plant *Arabidopsis thaliana*, together with a wide assortment of related genomic resources, has significantly advanced our understanding of the adaptations of Pi-starved plants. Information on the genetic identity, subcellular location, biochemical properties, and probable functions of acid phosphatases involved in the Pi metabolism of Pi-starved *Arabidopsis* is beginning to emerge. Acid phosphatases catalyze the hydrolysis of Pi from a broad range of phosphomonoesters with an acidic pH optimum. The *Arabidopsis* genome encodes 29 different purple acid phosphatases whose expression is influenced by various developmental and environmental factors. Pi starvation induces de novo synthesis of several extra- and intracellular *Arabidopsis* purple acid phosphatase isozymes; AtPAP12 and AtPAP26 appear to be the principal root-secreted acid phosphatases that scavenge Pi from extracellular Pi-esters, whereas the dual-targeted AtPAP26 is the predominant intracellular acid phosphatase that functions in vacuolar Pi recycling by Pi-starved *Arabidopsis*. The identification and functional characterization of intracellular and secreted purple acid phosphatase isozymes upregulated by Pi-deprived plants may help develop strategies for engineering Pi-efficient crops, thereby minimizing the use of unsustainable Pi fertilizers in agriculture.

Contents

Abbreviations: AtPAP, *Arabidopsis* purple acid phosphatase; HMW, high molecular weight; PEP, phosphoenolpyruvate; miRNA, microRNA; Pi, orthophosphate.

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1. Introduction

Orthophosphate (Pi) plays a central role in virtually all major metabolic processes in plants, including photosynthesis and respiration. It is also a key structural constituent of important biomolecules such as ATP, NADPH, nucleic acids, phospholipids, and sugar-phosphates. However, the soluble Pi concentration of many soils ranges from about 1 to 10 μM, far lower than the intracellular Pi concentrations (5–20 mM) required for optimal plant growth [1–3]. Maximizing the yield of most modern crop varieties therefore relies on the extensive application of Pi fertilizers. Of the estimated 40 million metric tons of Pi fertilizer currently applied worldwide each year, less than 20% is absorbed by crops, whereas Pi-runoff from fertilized fields into nearby surface waters results in environmentally destructive processes such as aquatic eutrophication and blooms of toxic cyanobacteria [1]. Unlike the global N cycle, Pi fertilizers mostly rely on extraction from non-renewable rock-Pi reserves, derived from fossilized bone deposits. The application of Pi fertilizers is quite expensive, particularly in developing countries, and the problem is exacerbated in tropical and subtropical regions containing acidic soils, as metal cations (e.g., Al^{3+}) in these soils form insoluble complexes with Pi, thereby greatly reducing the availability of Pi to the plants. The projected depletion of global rock-Pi reserves within the next 80 years raises an interesting dilemma in the face of the world population explosion [1,4]. In order to ensure agricultural sustainability and a reduction in Pi fertilizer overuse, plant and soil scientists must address the need to bioengineer Pi-efficient transgenic crops. The design of effective biotechnological strategies to enhance crop Pi acquisition necessitates our detailed understanding of Pi-starvation inducible gene expression and the complex biochemical adaptations of Pi-deficient plants. The aim of this article is to provide a brief overview of the remarkable adaptations of Pi-starved plants, with an emphasis on our current understanding of the properties and roles of purple acid phosphatase isozymes that facilitate the acclimation of the model plant *Arabidopsis thaliana* (mouse-eared cress) to nutritional Pi deprivation.

1.1. Plant phosphate nutrition is determined by root–soil interactions

Phosphorus is assimilated by plants in its fully oxidized state, $H_2PO_4^-$ or HPO_4^{2-}. Pi is the least accessible macronutrient in many natural soils because it readily forms insoluble calcium salts in alkaline soils, or complexes with iron and aluminium oxides in acidic soils, rendering it inaccessible for root uptake [4,5]. Extensive soil Pi reserves also exist in the form P-esters derived from decomposing biomaterial, referred to as organic-Pi [4,5]. Plants therefore face two primary challenges in acquiring Pi from their environment. The first is the solubility of free Pi, which plants can improve by secretion of large amounts of organic acids into the rhizosphere that saturate soil anion exchange capacity [1,4,5]. Secondly, roots possess the ability to mobilize Pi from soil organic-Pi pools via the secretion of hydrolytic enzymes that free esterified-P [1,4,5]. However, which soil organic-Pi pools are accessible to roots remains unclear, and most plants appear to be unable to utilize phytate (*myo*-inositol hexaphosphate), an abundant organic-Pi component of certain soils, as a source of nutritional Pi [6–8].

2. The plant phosphate starvation response

Plants have evolved the ability within species-dependent limits to tolerate extended periods of Pi deprivation by eliciting a complex array of morphological, physiological and biochemical adaptations, collectively known as the Pi-starvation response. The Pi-starvation response arises in part from the coordinated induction of hundreds of Pi-starvation inducible genes that reprioritize internal Pi use and maximize external Pi acquisition [1,4,5,9]. The evolution of elaborate strategies to enhance the acquisition and use of Pi from the environment allows many plants to effectively acclimate to periods of nutritional Pi deficiency.

2.1. Morphological adaptations of Pi-deprived plants

Extending the root's surface area for phosphate absorption underlies the morphological adaptations of phosphate starved plants that include: (i) increasing the root:shoot growth ratio, (ii) alterations in root architecture and diameter, (iii) a shift from primary to lateral root growth, which promotes the exploration of topsoil for available Pi, and (iv) increased root hair growth and density [1,3,10]. For example, Pi-starved *Arabidopsis* seedlings have elongated root hairs and a 5-fold increase in root hair density accounting for up to 70% of the total root absorptive area [10,11]. An analogous adaptive strategy of most Pi-starved vascular plants is the formation of symbiotic associations between their roots and beneficial mycorrhizal fungi.

2.2. Mycotrophic versus non-mycotrophic plants

Phosphate-acquisition by mycotrophic plants is significantly enhanced by the presence of arbuscular mycorrhizae formed between soil-inhabiting fungi of the order *Glomales* and roots of about 90% of vascular plants (excluding *Cheonpodiaceae*, *Cruciferae*, *Cyperaceae*, *Junaceae*, and *Proteaceae* families) [12]. This symbiosis develops in roots when the fungus colonizes the apoplast and cells of the cortex, accessing photosynthate (sucrose) supplied by the host plant. However, disruption of beneficial mycorrhizal associations due to Pi-fertilization and soil tilling has been an undesirable consequence of modern agriculture. It is notable that many non-mycotrophic plants such as *Fagopyrum esculentum* (buckwheat), *Lupinus albus* (white lupin), and *Hakea prostrata* (harsh hakea) are notorious for their ability to thrive on Pi-deficient soils. This reflects the view that relative to mycotrophic species, the non-mycotrophs have evolved mechanisms that allow more efficient acclimation to low Pi conditions [13]. Non-mycotrophic members of the *Proteaceae* such as white lupin and harsh hakea form proteoid roots when cultivated under Pi-starved conditions. Proteoid roots are clusters of short lateral roots that can absorb Pi at a faster rate than non-proteoid roots, thus enhancing Pi uptake [1]. As a member of the *Cruciferae*, *Arabidopsis* does not form mycotrophic associations and is thus an ideal model species for studying the molecular and biochemical adaptations of Pi starvation in non-mycotrophic

plants. Future studies concerning morphological adaptations to Pi starvation and downstream signaling need to address the specificity of the transcriptional networks induced by Pi starvation. For example, it is unclear if modifications in root architecture and morphology are mediated by the same transcription factor networks that control plant molecular and biochemical adaptations to Pi starvation. Answers to such questions would aid in developing plants that can tailor induction of specific aspects of the Pi-starvation response, for example induction of hydrolases and altered root architecture without concomitant decreases in growth.

2.3. Transcriptional and post-transcriptional responses to Pi starvation

Many elements of the Pi-starvation response are controlled at the transcriptional level, and Pi-deprived plants extensively remodel their transcriptome and proteome in ways that coordinate the requisite metabolic and morphological adaptations. A large collection of microarray data regarding plant, and particularly *Arabidopsis*, transcriptional responses to Pi starvation has shed light on the molecular identity and regulation underlying many classical biochemical and physiological adaptations to Pi deprivation [10,14–18]. Pi-starvation inducible gene expression is highly coordinated in a temporal and tissue-specific manner [10]. The marked reduction in cytoplasmic Pi pools that accompanies prolonged Pi starvation is met by a highly specific response that differs in roots and shoots of *Arabidopsis* [14,17]. Despite the induction of 600–1800 genes across all tissues, there appears to be only an approximate 25% overlap between those specifically induced in the root and shoot, implying strong tissue-specific adaptations to Pi starvation. Transcriptional repression also plays a critical role in determining the Pi-starvation response, with 250–700 genes having decreased transcript accumulation in response to Pi starvation. Again tissue-specificity plays a key role in this response with only a 5–10% overlap in repressed gene expression between shoots and roots [14,15,17].

Post-transcriptional mechanisms also make an important contribution to the control of Pi-starvation inducible gene expression and activity. This is reflected by proteomic profiling of Pi-starved *Oryza sativa* (rice), *Zea mays* (corn), and *Arabidopsis* plants demonstrating that transcript abundance of various genes is not always indicative of protein accumulation during Pi deprivation [19–22]. One of the best characterized examples of post-transcriptional mechanisms in the *Arabidopsis* Pi-starvation response is the regulatory module comprising the transcription factor PHR1, the E2 ubiquitin conjugase UBC24 (also known as PHO2), the *microRNA399* (*miR399*), and the non-coding RNA *At4* [10,23–25]. During Pi sufficiency, UBC24 is expressed and functions in regulating systemic Pi homeostasis by decreasing the expression of Pi-starvation inducible genes, including high affinity Pi transporters of the plasmalemma [24]. Upon Pi starvation PHR1 is induced by an unknown mechanism and activates expression of the phloem-mobile *miR399* by up to 1000-fold. Binding of *miR399* to complementary bases of *UBC24* transcripts leads to the destruction of *UBC24* mRNA, resulting in low levels of UBC24 E2 ubiquitin conjugase activity, and the consequent accumulation of its downstream protein targets [23]. Expression of the ribo-regulator *At4* is strongly induced during prolonged periods of Pi starvation [24,26]. *At4* binds to complementary bases of *miR399*, thereby inhibiting its silencing of *UBC24* mRNA. This allows UBC24 levels to rapidly adjust to the dynamic balance of Pi supply and demand [24,26]. The ability of *At4* to antagonize *miR399* activity suggested a novel regulatory paradigm known as 'target mimicry'; *i.e.*, an RNA analogous to a competitive enzyme inhibitor effectively sequestrates *miR399*-charged silencing complexes so that they cannot act on *UBC24* transcripts. A key challenge will be to identify the initial substrate(s) of UBC24 which remain unknown. Likewise, the activity of the small-ubiquitin like modifier (SUMO) E3 ligase *AtSIZ1* appears to be critical to Pi deficiency responses [27], but none of its substrates have been identified. Future studies must focus on these downstream post-transcriptional mechanisms as they may define novel Pi-starvation responsive genes and new targets for biotechnological engineering of Pi-efficient crops.

Reversible phosphorylation and differential glycosylation are also emerging as important post-translational modifications for controlling the activity of and/or subcellular targeting of diverse enzymes upregulated during Pi-starvation. As discussed below, upregulation of phosphoenolpyruvate (PEP) carboxylase is an archetypical metabolic adaptation of Pi-starved plants (Fig. 1) [1,3,28–32]. The simultaneous induction and *in vivo* phosphorylation-activation of the PEP carboxylase isozyme AtPPC1 contributes to the metabolic adaptations of Pi-starved *Arabidopsis* [30]. Interestingly, the most responsive Pi-starvation inducible *Arabidopsis* transcripts include those encoding both PEP carboxylase protein kinase isozymes (*AtPPCK1* and *AtPPCK2*) [14,15,30]. Similarly, corn cultivars resistant to Pi starvation upregulate phosphoprotein phosphatase type-2A catalytic subunits [21], whereas alterations to *in vivo* protein phosphorylation patterns were documented in *Brassica napus* (oilseed rape) suspension cells responding to Pi deficiency [33]. A challenging goal for future research will be to document the functional consequences of reversible protein phosphorylation in the signaling and metabolic pathways involved in plant acclimation to nutritional Pi deprivation.

2.4. Biochemical adaptations of Pi-starved plants

Plants employ a host of biochemical adaptations as part of their Pi-starvation response. These include anthocyanin accumulation in leaves, and the induction of high affinity Pi transporters and alternative bypass enzymes to the Pi- or adenylate-dependent reactions of glycolysis and mitochondrial respiration (Fig. 1). Anthocyanin accumulation has frequently been observed in shoots of Pi-deficient plants and is believed to protect chloroplasts from photoinhibition [2,3,10]. The upregulation of high affinity Pi transporters of the plasma membrane is another important component of the plant Pi-starvation response (Fig. 1) [3,9]. These transporters are induced when the external Pi concentration is low, and actively assimilate Pi against a steep concentration gradient, as the soil Pi concentration can be 10,000-fold lower than that of root cells [9]. High affinity Pi transporters of *Arabidopsis* belong to the nine member PHT1 family and consist of Pi/H^+ symporters with 12 transmembrane domains [34]. While all nine members are responsive to Pi starvation, each appears to have a certain degree of tissue-specific expression, with some expressed in epidermal and root hair cells while others are expressed in stelar cells of the root [35]. Consistent with this, knockout of *Pht1;4* or *Pht1;1* results in decreased Pi acquisition during Pi deficiency [36], while knockout of *Pht1;1* (*PHO1*) also results in failure to accumulate Pi in shoots during Pi sufficiency due to its role in xylem Pi loading [37].

The reorganization of cellular metabolism in a manner that conserves limited pools of adenylates and Pi is another important biochemical adaptation of Pi-deprived plants. This is accomplished by altering the organization of glycolysis, mitochondrial respiration, and tonoplast H^+-pumps allowing adenylate and Pi-dependent reactions to be bypassed during Pi starvation [2,29]. Several of these bypasses facilitate respiration and vacuolar pH maintenance during extended periods of Pi starvation by using pyrophosphate in performing cellular work, while simultaneously conserving ATP and recycling Pi (Fig. 1). Glycolytic bypass enzymes such as pyrophosphate-dependent phosphofructokinase and PEP

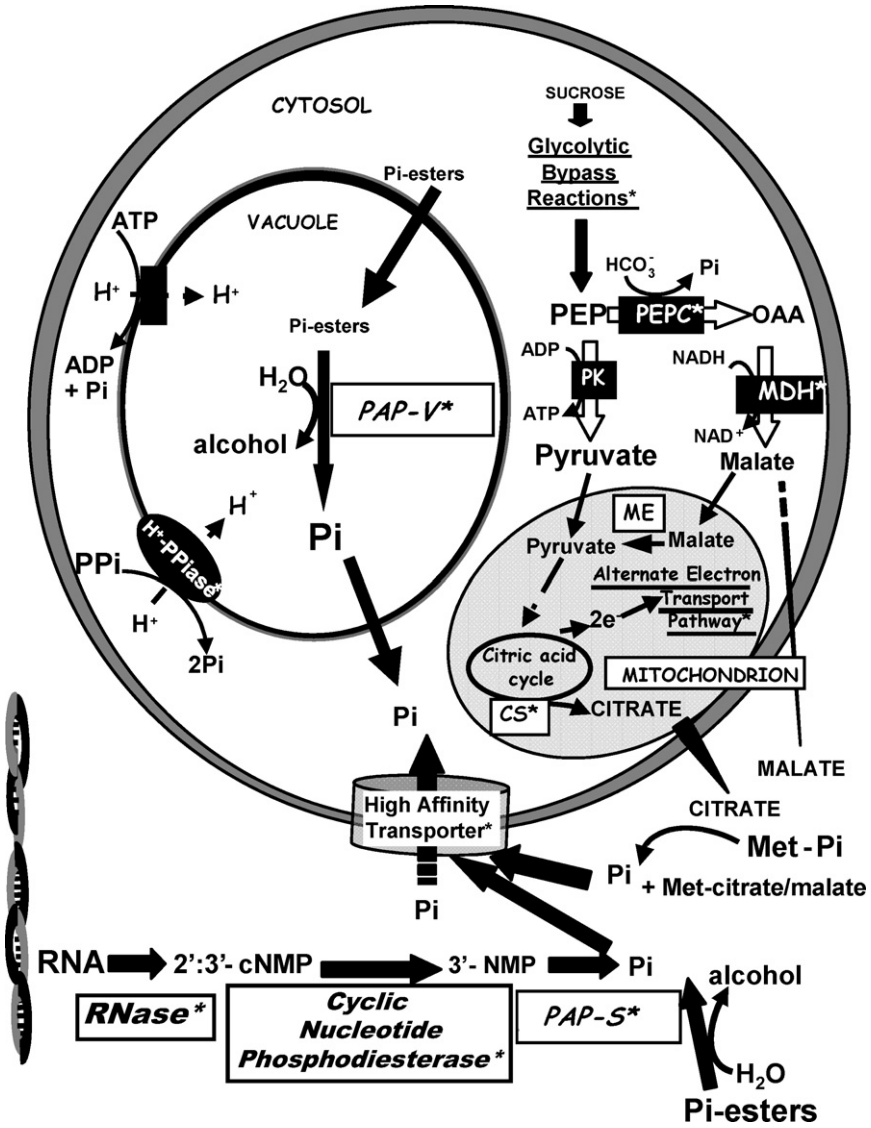


Fig. 1. A model suggesting various adaptive metabolic processes (indicated by asterisks) that are believed to help plants acclimate to nutritional Pi deficiency. Alternative pathways of cytosolic glycolysis, mitochondrial electron transport, and tonoplast H^+-pumping facilitate respiration and vacuolar pH maintenance by Pi-starved plant cells because they negate the dependence on adenylates and Pi, the levels of which become markedly depressed during severe Pi starvation. Large quantities of organic acids produced by PEP carboxylase (PEPC), malate dehydrogenase (MDH), and citrate synthase (CS) may also be secreted by roots to: (i) increase the availability of mineral bound Pi (by solubilizing Ca-, Fe- and Al-phosphates = 'Met–Pi'), and (ii) increase the availability of organic-Pi and its amenability to hydrolysis by secreted acid phosphatases [51]. A key component of this model is the critical secondary role played by metabolic Pi recycling systems such as PEPC during Pi deprivation. During Pi deprivation vacuolar purple acid phosphatases (PAP-V) are believed to recycle Pi from non-essential intracellular Pi-esters. Similarly, secreted purple acid phosphatases (PAP-S) likely function to scavenge Pi from extracellular Pi-monoester and nucleic acid fragment pools for its eventual uptake by Pi-starvation inducible high affinity Pi transporters of the plasma membrane.

carboxylase also promote metabolic Pi recycling, as Pi is a byproduct of their reactions [29]. The PEP carboxylase catalyzed bypass of cytosolic pyruvate kinase also results in the synthesis of organic acids from glycolytic metabolites (Fig. 1). This is critical for the anaplerotic replenishment of tricarboxylic cycle intermediates, as well as the root secretion of organic acids, a common response to Pi starvation [1,3,29,31,32]. Organic acid secretion is believed to aid in chelating metal cations that immobilize Pi (e.g., Al^{3+}, Ca^{2+}, $Fe^{2/3+}$), thus increasing soil solution Pi concentrations by as much as 10- to 1000-fold [4,5]. Organic acid secretion also increases the ability of secreted acid phosphatases to scavenge Pi from soil localized organic-Pi-esters [5,8]. However, the effectiveness of different organic acids in soil Pi mobilization depends upon the form and amount of the particular anion being released. Citrate appears to be the most effective relative to other organic anions, such as malate [5,8].

Plants also increase the efficiency of Pi use during Pi starvation via upregulation of Pi-starvation inducible hydrolases that scavenge Pi from non-essential P-esters. Classical Pi-starvation inducible hydrolases include non-specific phospholipases, ribonucleases, and acid phosphatases [2,3,9,38–40]. Phospholipase induction is accompanied by the replacement of membrane phospholipids with amphipathic sulfonyl- and galactolipids [3,41,42]. Phospholipids are a dynamic and indispensable P-reserve during Pi starvation [43]. Knockout of phospholipase activity or the downstream synthases required for membrane lipid remodeling results in the impaired development of Pi-starved *Arabidopsis* seedlings [41,44].

Roots and suspension cell cultures of Pi-depleted plants also induce the secretion of ribonucleases, phosphodiesterases, and acid phosphatases which participate in systematic Pi mobilization from soil localized organic-P, including nucleic acids (Fig. 1) [1–5,9,45].

Arabidopsis ribonuclease-1 transcript and secreted protein levels are highly induced in response to Pi deficiency [22,40] and *Arabidopsis* plants cultivated on nucleic acids as their sole source of exogenous Pi grow just as well as Pi-fertilized control plants [38,46].

3. Plant acid phosphatases

Acid phosphatases catalyze the hydrolysis of Pi from a broad and overlapping range of Pi-monoesters with an acidic pH optimum, and function in the production, transport, and recycling of Pi [39]. Eukaryotic acid phosphatases exist as a wide variety of tissue- and/or cellular compartment-specific isozymes that vary in their physical and kinetic properties. The *Arabidopsis* genome encodes over 50 putative acid phosphatases including 10 vegetative storage protein type acid phosphatases, four phosphatidic acid phosphatases, and one histidine acid phosphatase [47]. The unexpected diversity of vascular plant acid phosphatases poses a fascinating biological question in its own right. This is complicated by the fact that not all acid phosphatases appear to function as metabolic enzymes. For example, a vegetative storage protein accumulates to almost 40% of total soluble protein in leaves of depodded *Glycine max* (soybean) plants, but contributes less than 1% to extractable acid phosphatase activity (while a single point mutation can increase its acid phosphatase activity by up to 20-fold) [48].

The upregulation of intracellular and secreted acid phosphatase activity has long been recognized as a biochemical hallmark of plant Pi deprivation [39]. Vacuolar acid phosphatases of Pi-starved plant cells likely remobilize and recycle Pi from expendable intracellular Pi-monoesters and anhydrides (Fig. 1). This is accompanied by marked reductions in cytoplasmic P-metabolites during extended Pi deprivation [39]. Extracellular acid phosphatases belong to a group of Pi-starvation inducible hydrolases secreted by Pi-deprived plants. They hydrolyze Pi from external organophosphates, that can comprise up to 80% of total soil P [3,46]. Induction of intracellular and secreted acid phosphatase activity has been correlated with *de novo* acid phosphatase synthesis in several Pi-depleted plants, including *Brassica nigra* (black mustard), *Lycopersicon esculentum* (tomato), and *Arabidopsis* suspension cells and seedlings [49–54]. The genetic and functional redundancy of plant acid phosphatases necessitates greater study, with particular focus on the largest group, the purple acid phosphatases.

3.1. Purple acid phosphatases

Purple acid phosphatases received their colorful nomenclature owing to their distinctive purple or pink color in solution. This results from a charge transfer transition at about 560 nm from the metal-coordinating tyrosine to the metal ligand Fe(III) [55]. Purple acid phosphatases belong to a metallophosphoesterase superfamily that includes phosphoprotein phosphatases and exonucleases. All contain five blocks of conserved metal ligating residues, although the location, number and identity of the residues differ between the family groups [47,55]. Members of the purple acid phosphatase family contain seven metal ligating residues (**D**xG–G**D**XX**Y**–GN**H**(D/E)–VXX**H**–G**H**X**H**; bold letters indicate metal ligating residues, dashes indicate separation between blocks) that form dimetallic active sites and are highly conserved amongst bacterial, mammalian, and plant purple acid phosphatases [47,55]. Despite conservation of metal ligating residues, mammalian purple acid phosphatases contain a Fe(III)–Fe(II) active site whereas plant purple acid phosphatases typically contain an Fe(III)–X(II) active site where X is either Zn^{2+} or Mn^{2+} [55,56]. The availability of these metals in mammalian or plant cells implies that divalent metal cation specificity may provide a form of functional purple acid phosphatase specialization [56].

The structure of purple acid phosphatase catalytic sites and domains are also highly conserved [55,56]. Bacterial, mammalian and plant purple acid phosphatases all contain catalytic domains that consist two sandwiched β–α–β–α–β motifs, with almost perfect alignment and order of the conserved metal ligating residues [47,56]. Despite the conservation of catalytic domains, mammalian and plant purple acid phosphatases tend to differ in their oligomeric structure [55,56]. All mammalian purple acid phosphatases that have been biochemically characterized to date exist as 35 kDa monomers consisting solely of a catalytic domains [57]. However, a recent bioinformatics study identified a putative high molecular weight (HMW) purple acid phosphatase gene in mammals, insects and nematodes [57]. The predicted amino acid sequence of these putative HMW animal purple acid phosphatase isozymes are closely related to the approximate 55-kDa HMW purple acid phosphatase characterized from plants. Although mammalian-like low molecular weight purple acid phosphatases exist in plants [58,59], the *Arabidopsis* genome encodes a relatively large family of putative HMW oligomeric purple acid phosphatases composed of 45–74 kDa subunits (Fig. 2) consisting of an N-terminal non-catalytic domain fused to a C-terminal catalytic domain which is structurally related to monomeric low molecular weight purple acid phosphatases [55,56]. Although most HMW plant purple acid phosphatases exist as homodimers [53,55,60–63] several HMW purple acid phosphatases secreted by Pi-starved plant cells are monomeric (Table 1) [50,54]. Dimeric HMW acid phosphatases form either through disulfide bridges or via non-covalent interactions [54,55,60,62]. It is not yet understood how oligomeric structure impacts purple acid phosphatase function, or even why plant purple acid phosphatases appear to exist in two disparate oligomeric states.

Most purple acid phosphatases that have been biochemically characterized have been classified as non-specific acid phosphatases that catalyze Pi hydrolysis from a broad spectrum of Pi-esters [47,55]. However, mammalian purple acid phosphatases expressed in macrophages and spleen cells after phagocytosis probably play a role in the generation of reactive oxygen species via a Fenton reaction involving the Fe(II) of the active site [56]. Similarly, (i) several plant purple acid phosphatases that exhibit significant acid phosphatase activity also have alkaline peroxidase activity that is unaffected by acid phosphatase inhibitors [50,51,53,58], and (ii) overexpression of a soybean purple acid phosphatase, *GmPAP3*, increased tolerance to oxidative damage imposed during salinity stress [64]. Mammalian and bacterial purple acid phosphatases also function as phosphotyrosyl phosphatases, implying a role in signal transduction [65–67]. Likewise, a purple acid phosphatase (NtPAP12) of *Nicotiana tabacum* (tobacco) cell walls is highly active against phosphotyrosylated peptides [65], whereas a variety of acid phosphatases from other plant sources have significant activity with phosphotyrosine or other phosphoamino acids as substrates [50,51,53,63,68]. Interestingly, transgenic expression of NtPAP12 resulted in altered cell wall composition and enhanced β-glucan synthase activity, implying that this tobacco purple acid phosphatase isozyme might function as a protein phosphatase involved in the control of cell wall biosynthesis [69].

3.2. Phosphate starvation inducible purple acid phosphatases

Many studies have focused on the role that plant purple acid phosphatases might play in Pi scavenging and recycling from Pi-esters and anhydrides during Pi starvation. Pi starvation induces temporal and tissue-specific expression of purple acid phosphatases [14,17,47,52,70,71]. The transcription factors PHR1, WRKY75, and ZAT6 have been implicated in the control of Pi-starvation inducible purple acid phosphatase expression [72–74], while other studies have revealed Pi-starvation inducible pur-

Table 1
Properties and proposed roles of functionally and/or biochemically characterized *Arabidopsis* purple acid phosphatases.

AtPAP designation	AGI code	Upregulated by Pi starvation?		Subcellular localization	Best substrate	Physical properties			Proposed function(s)	Refs.
		Transcript	Protein			Native M_r (kDa)	Subunit M_r (kDa)	λ_{max} (nm)		
AtPAP12	At2g27190	Yes	Yes	Secretome	PEP	130	60	520	Extracellular Pi scavenging	[22,54,70]
AtPAP15	At3g07130	No	No	n.d.*	Phytic acid	58	60	n.d.	(i) Ascorbate biosynthesis, (ii) Pi mobilization from phytic acid	[7,84,85]
AtPAP17	At3g17790	Yes	n.d.	n.d.	pNPP**	n.d	34	n.d.	(i) Pi mobilization, (ii) ROS metabolism	[22,53,54,58]
AtPAP23	At4g13700	No	No	n.d.	ATP	n.d.	52	n.d.	Flower: (i) Pi metabolism and (ii) Fe/Mn homeostasis	[80]
AtPAP26-V	At5g34850	No	Yes	Cell vacuole	PEP	100	55	520	Intracellular Pi recycling	[53,86]
AtPAP26-S1	At5g34850	No	Yes	Secretome	PEP	65	55	500	Soil Pi scavenging	[54]
AtPAP26-S2	At5g34850	No	Yes	secretome	PEP	65	55	n.d.	Soil Pi scavenging	[54]

* Not determined.
** Para-nitrophenyl-P.

ple acid phosphatases that are controlled by post-transcriptional mechanisms (Table 1) [22,53,54]. In contrast, fertilizing Pi-deficient plants with Pi quickly represses Pi-starvation inducible purple acid phosphatase genes while inducing specific proteases that appear to target intracellular and secreted Pi-starvation inducible purple acid phosphatases [53,68,75]. Identification and characterization of Pi-starvation inducible purple acid phosphatases is required to define the molecular mechanisms underlying this archetypical plant response to Pi starvation, as well as to identify suitable targets for improving crop Pi acquisition.

A variety of intracellular and secreted acid phosphatase isozymes that are upregulated following plant Pi deprivation were demonstrated to be purple acid phosphatases. These have been biochemically characterized from several species including tomato [50,51], lupin [62,76], *Phaseolus vulgaris* (bean) [59], tobacco [77], and *Arabidopsis* [53,54,58]. White lupin secretes copious amounts of acid phosphatase activity from its proteoid roots when subjected to Pi starvation [76]. The transcript and corresponding protein levels of the secreted acid phosphatase responsible for this activity are highly induced upon Pi starvation [76]. The protein exists as a glycosylated homodimer composed of 70 kDa subunits, and although not directly determined to exhibit a purple or pink color in solution (or a dimetallic active site), it shares high amino acid sequence identity (about 65%) with AtPAP12 [76]. This similarity extends to its promoter region, which can direct enhanced gene expression under Pi-starved conditions. The secreted acid phosphatase isozyme of *Lupinus luteus* (yellow lupin) roots is orthologous to AtPAP26 (Table 1) [62]. As with AtPAP26 (see below), this lupin AtPAP26 ortholog is constitutively transcribed regardless of Pi nutritional status [62].

Three acid phosphatases purified to homogeneity from Pi-starved tomato suspension cell cultures were markedly upregulated in response to Pi deficiency of tomato seedlings [52]. They are *bona fide* purple acid phosphatases as they have: (i) a pink color in solution, (ii) amino acid sequence similarity to putative or previously characterized plant purple acid phosphatases, (iii) insensitivity to tartrate[1], and (iv) substantial acid phosphatase activity [50,51]. All three are glycoproteins exhibiting broad acid phosphatase substrate specificity and an acidic pH-activity optimum. Two of these Pi-starvation inducible purple acid phosphatases are secreted into the rhizosphere as 84 and 57 kDa monomers [50,52]; the third was a novel heterodimer composed of an equivalent ratio of 63 and 57 kDa subunits, and is most likely localized to the cell vacuole [51]. The biochemical characterization of the intra- and extracellular Pi-starvation inducible tomato purple acid phosphatase isozymes indicates their probable physiological role in Pi scavenging and Pi recycling by Pi-deprived tomato. Decreased cytoplasmic P, a consequence of prolonged Pi starvation, is met by a highly specific response that involves temporal- and tissue-specific synthesis of Pi-starvation inducible tomato purple acid phosphatase isozymes [52]. These results corroborated aforementioned *Arabidopsis* transcriptomic studies suggesting that some Pi-starvation inducible genes have temporal as well as tissue-specific expression [14,16,17,78]. Most recently, a purple acid phosphatase upregulated by Pi-depleted bean plants was characterized and identified as PvPAP3 by mass spectrometry [59]. This 34 kDa monomeric purple acid phosphatase had a broad pH-activity profile and was insensitive to tartrate. Its deduced amino acid sequence was highly similar to other purple acid phosphatases, with AtPAP8 being closest relative in *Arabidopsis*. *PvPAP3* was highly induced in both leaves and roots of Pi-deficient bean plants. The transient expression of *35S:PvPAP3-GFP* constructs in *Allium*

[1] Lack of inhibition of acid phosphatase activity by L-tartrate is a characteristic kinetic feature of plant and non-plant purple acid phosphatases.

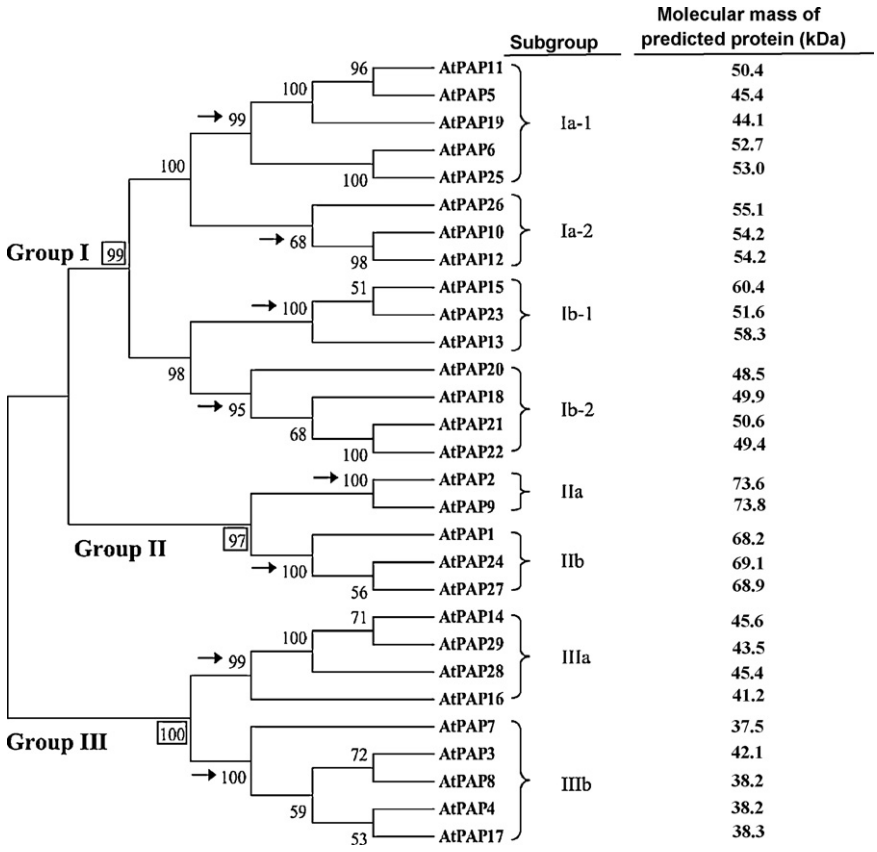


Fig. 2. A classification scheme for *Arabidopsis* purple acid phosphatases based on clustering analysis of amino acid sequences. The clustering analysis used amino acid sequences of 19 predicted purple acid phosphatases and those of 10 purple acid phosphatases (AtPAP3, AtPAP7–AtPAP13, AtPAP17, AtPAP18) derived from cDNA analysis. The main groups (groups I, II, and III) are further divided to yield the eight subgroups (second column). The bootstrap values for the three main groups are boxed, whereas those for the eight subgroups are indicated by arrows. The predicted molecular masses of the deduced polypeptides are listed in the third column. Figure modified from [47].

cepa (onion) epidermal cells indicated that it is secreted into the apoplast. PvPAP3 was most active with ATP as a substrate, suggesting that it may function in the adaptation of common bean to Pi starvation through the use of extracellular ATP as a Pi source from the environment [59]. Both animal and plant cells secrete ATP into the extracellular matrix, and extracellular ATP is essential for maintaining plant cell viability [79].

4. The complex world of *Arabidopsis* purple acid phosphatases

The *Arabidopsis* purple acid phosphatase (AtPAP) family is encoded by 29 genes, although only 28 appear to be actively transcribed [47,80]. The AtPAPs have been classified into three distinct phylogenetic groups according to their deduced amino acid sequences (Fig. 2) [47]. Groups I and II are comprised of oligomeric HMW AtPAPs, with group I consisting of purple acid phosphatases of slightly smaller monomer size than group II. Group III consists of the monomeric low molecular weight mammalian-like AtPAPs. Transcript profiling of the *AtPAP* family revealed that while most are expressed in all tissues, seven members are predominantly expressed in flower tissue [80]. Although some *AtPAP* transcripts accumulate in response to stress [22,47,54,58,70], cell-specific expression patterns of *AtPAPs* remain elusive. There is also a paucity of information regarding the subcellular location of most AtPAPs. Plant purple acid phosphatases have been localized in mitochondria (GmPAP3) [64], the cell vacuole (AtPAP26) [81], the cell wall (AtPAP10, NtPAP12) [65,82], and the secretome (AtPAP10, AtPAP12, and AtPAP26) [22,54,70,83]. This is curious given that all purple acid phosphatases characterized to date contain transit peptides and are glycosylated, implying that they are all initially targeted to the golgi where glycosylation occurs.

Although the molecular and biochemical properties of a variety of plant purple acid phosphatases have been well documented, their precise physiological functions have not been resolved [3,55]. To date, only *AtPAP15*, *AtPAP23*, and *AtPAP26* have been functionally characterized in transgenic *Arabidopsis* [80,81,84]. AtPAP15 is the only member of the AtPAP family that has thus far been shown to possess significant phytase activity (Table 1) [84,85]. AtPAP15 is thought to be involved in ascorbate synthesis via production of *myo*-inositol [85]. Lower phytase activity was exhibited in extracts of *AtPAP15* T-DNA knockout mutants, but unaltered activity was observed with the generic acid phosphatase substrate *para*-nitrophenyl-P relative to wild-type controls [85]. AtPAP15 also appears to play an important role in mobilizing Pi from phytate reserves during seed or pollen germination [84]. Constitutive overexpression of AtPAP15 containing a carrot extracellular targeting peptide in soybean plants significantly improved the growth and P efficiency of resulting transformants when cultivated on sand containing phytate as the sole source of external P [7], therefore confirming the phytase activity of AtPAP15. In contrast to AtPAP15, AtPAP23 is a non-specific acid phosphatase that is predominantly expressed in flower apical meristems, but becomes restricted to petals and anther filaments in fully developed flowers [80]. Despite its highly specific expression pattern and demonstrated acid phosphatase activity of its protein product, *atpap23* knockout and overexpression lines had unaltered extractable acid phosphatase activity and were indistinguishable from wild-type plants in the development of flower or other organs [80]. Although alterations in AtPAP23 expression levels did not result in any obvi-

ous phenotype, the Fe and Mn content of the overexpressed lines was significantly elevated relative to wild-type plants. Thus, apart from its putative *in vivo* role as a non-specific acid phosphatase, AtPAP23 possibly functions in Fe and Mn homeostasis in flower development and metabolism (Table 1) [80].

4.1. Phosphate starvation inducible Arabidopsis purple acid phosphatases

In contrast to the wealth of *AtPAP* genomic and transcript expression data comparatively little information is available on the specific AtPAP isozyme(s) that contribute to intra- versus extracellular Pi scavenging by Pi-deprived *Arabidopsis*. AtPAP17 is one of the few Pi-starvation inducible purple acid phosphatases that has been purified and characterized from Pi-starved *Arabidopsis* seedlings [58]. AtPAP17 exists as a low molecular weight (34 kDa) monomeric purple acid phosphatase and is transcriptionally induced in roots and leaves of Pi-depleted *Arabidopsis* (Table 1) [58]. The *AtPAP17* promoter contains a binding site for AtPHR1, a transcription factor involved in the *Arabidopsis* Pi-starvation response; thus, the upregulation of AtPAP17 during Pi deprivation appears to be mainly controlled at the transcriptional level [58]. *AtPAP17* transcripts also accumulate in response to oxidative or salt stress, and AtPAP17 is a bifunctional enzyme that has both acid phosphatase and alkaline peroxidase activity. This suggests that AtPAP17 could be involved in the metabolism of reactive oxygen species during general stress, rather than playing significant Pi recycling or scavenging roles in Pi-starved *Arabidopsis* [58]. The determination of the subcellular localization of AtPAP17, as well as the phenotypic impact that modifying its expression has on transgenic *Arabidopsis* will help to fully establish its function(s) during Pi deprivation.

An initial biochemical approach to identify and characterize specific acid phosphatase isozymes involved in Pi recycling or scavenging by Pi-starved *Arabidopsis* involved: (i) purification and characterization of intracellular and secreted acid phosphatases upregulated by Pi-starved *Arabidopsis*, coupled with (ii) bioinformatic analysis of peptide mass fingerprint and/or N-terminal amino acid sequence data obtained with the purified native acid phosphatases [53,54]. Suspension cell cultures have been invaluable in this regard since a relatively large biomass of *Arabidopsis* cells cultured under a well defined nutritional regime (and their surrounding liquid media containing secreted proteins), can be obtained over a relatively short period of time.

4.2. AtPAP26 is the predominant intracellular acid phosphatase upregulated by Pi-deprived Arabidopsis suspension cell cultures and seedlings

Recent biochemical, proteomic, and T-DNA knockout mutant studies provide convincing evidence that AtPAP26 is the predominant intracellular (vacuolar), as well as a major secreted acid phosphatase isozyme upregulated by Pi-starved *Arabidopsis*. There was a 5-fold increase in extractable acid phosphatase activity of Pi-deprived *Arabidopsis* suspension cells that was paralleled by a similar increase in the amount of a 55 kDa anti-(AtPAP26)-IgG immunoreactive polypeptide [53]. This was concomitant with a 35-fold reduction in the intracellular free Pi concentration relative to Pi-replete cells (Fig. 3(A)). The protein responsible for this activity was purified to homogeneity from the Pi-starved cells and identified as AtPAP26 by N-terminal sequencing and BLAST analysis [53]. Analysis of the cell vacuole proteome of *Arabidopsis* leaves [86], as well as transient expression of an *AtPAP26-GFP* fusion construct coupled with imaging via epifuorescence microscopy [81] have confirmed AtPAP26's vacuolar localization (designated as AtPAP26-V). Purified AtPAP26-V is a *bona fide* purple acid phosphatase, existing as a 100 kDa homodimer composed of 55 kDa glycosylated subunits (Table 1) [53]. AtPAP26-V has optimal acid phosphatase activity at pH 5.6 and broad substrate selectivity, and was thus hypothesized to recycle Pi from intracellular P-metabolites in Pi-starved *Arabidopsis*. As AtPAP26-V exhibits alkaline peroxidase activity, it might also participate in the metabolism of reactive oxygen species, particularly in senescing tissue where vacuolar integrity is compromised. Recent analyses of a homozygous *atpap26* loss-of-function T-DNA mutant (discussed below) [81] corroborated the earlier biochemical study [53] indicating that AtPAP26-V is the principal contributor to intracellular acid phosphatase activity in shoots and roots of Pi-starved *Arabidopsis* seedlings.

4.3. AtPAP12 and a pair of AtPAP26 glycoforms are secreted by Pi-deprived Arabidopsis

The identification and characterization of secreted acid phosphatases of Pi-starved *Arabidopsis* was recently accomplished [54]. A significant increase in secreted acid phosphatase activity of Pi-deprived *Arabidopsis* roots and suspension cell cultures has been correlated with the absence of detectable extracellular Pi, and the appearance of 60- and 55 kDa immunoreactive polypeptides on immunoblots of secretome proteins probed with rabbit antibodies raised against AtPAP26 and AtPAP12, respectively (Fig. 3(B)) [22,54]. The secreted acid phosphatases of Pi-starved *Arabidopsis* suspension cells were fully purified, identified, and characterized as an AtPAP12 homodimer and AtPAP26 monomer composed of glycosylated 60- and 55 kDa subunits, respectively [22]. The former result corroborates earlier work [70] with *Arabidopsis* transformed with a construct encoding the *AtPAP12* promoter and its signal peptide fused in-frame to a *GFP* gene. The transformed plants secreted small amounts of the fusion protein into the rhizosphere when subjected to nutritional Pi deficiency [70]. Secreted AtPAP12 and AtPAP26 are true purple acid phosphatases, as the purified enzymes were pink in solution and insensitive to tartrate inhibition [54]. Their overlapping but non-identical substrate selectivities and pH-activity profiles, and high specific acid phosphatase activities are consistent with the hypothesis that their combined activities allow Pi-starved *Arabidopsis* to efficiently scavenge Pi from a wide range of extracellular P-esters over a broad pH range. Indeed the growth of wild-type *Arabidopsis* seedlings on media containing 5 mM glycerol-3-P (an effective *in vitro* substrate of AtPAP12 and AtPAP26 purified from Pi-starved *Arabidopsis* [53,54]) as their sole source of exogenous P was indistinguishable from that of Pi-fertilized seedlings (B. Provost, H. Tran, & W. Plaxton, unpublished data). An unexpected outcome of this research was the discovery that AtPAP26 is dual-targeted to both the cell vacuole and secretome during Pi deprivation. This has been corroborated by the absence of 55 kDa immunoreactive AtPAP26 polypeptides on immunoblots of shoot/root extracts or secretomes of homozygous T-DNA tagged *atpap26* mutant seedlings cultivated under Pi-sufficient or Pi-deficient conditions [81]. The secreted AtPAP26 of Pi-starved *Arabidopsis* exists as a pair of distinct glycoforms (AtPAP26-S1 and AtPAP26-S2) that differentially bind to lectins such as Concanavalin-A and *Galanthus nivalis* agglutinin [54]. Heterogeneity at a conserved C-terminal glycosylation site could potentially account for the differential targeting of AtPAP26 during Pi deficiency [55,62]. Glycosylation is an important post-translational modification that influences enzyme localization, stability, and/or kinetic properties. Differential glycosylation also appears to influence the substrate specificities of AtPAP26-S1 and -S2 [54]. This may be due to steric hindrance of substrate binding to AtPAP26. The varying sizes and dynamics of different glycans can result in substantial shielding of functionally important protein domains, modulate the interactions of glycoconjugates with other molecules, and influence the rate of protein conformational

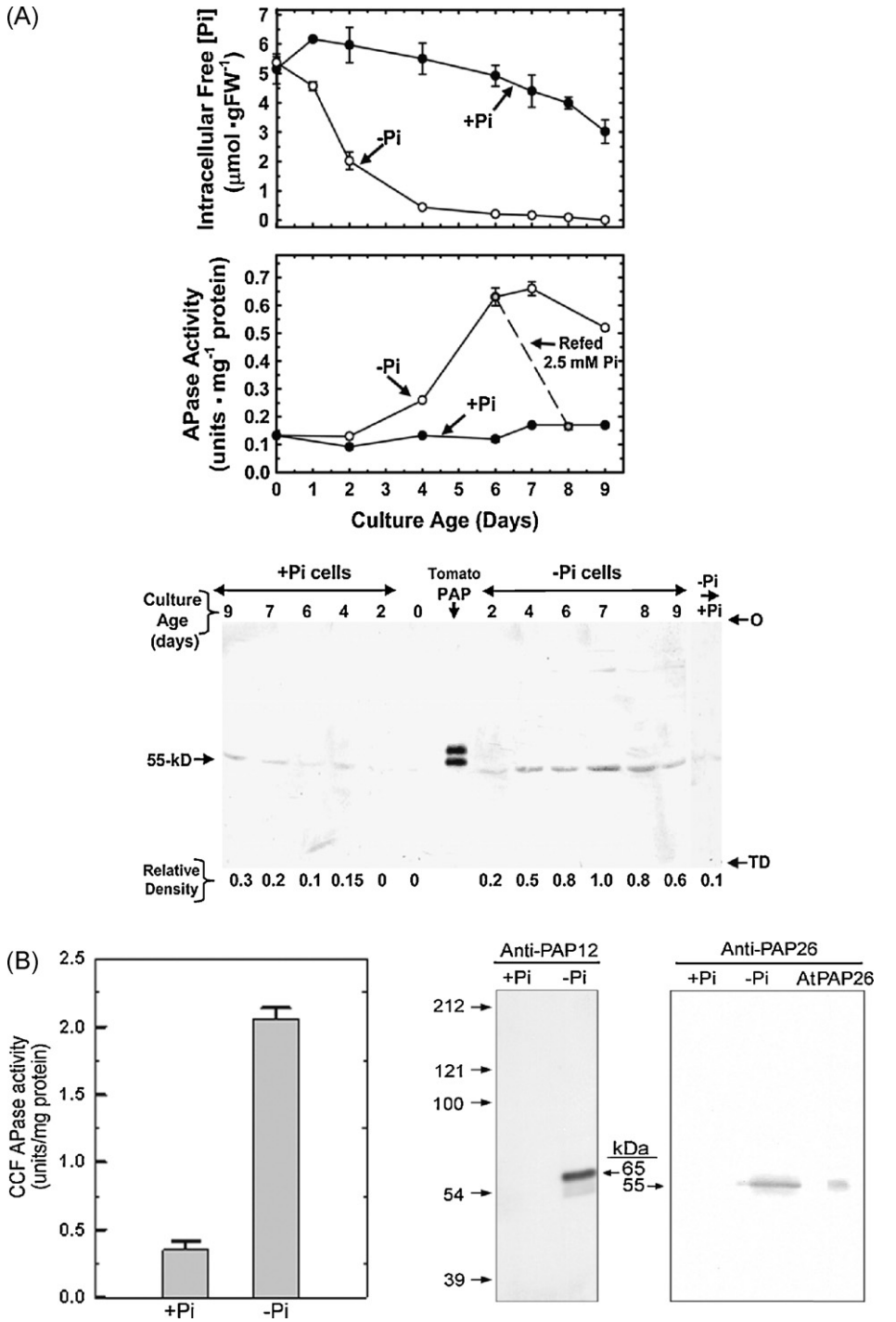


Fig. 3. The upregulation of intracellular and secreted AtPAPs in Pi-deficient *Arabidopsis* suspension cells. Cells cultured for 7 days in Pi-replete nutrient media were subcultured into media containing 5 mM Pi (+Pi) or 0 mM (−Pi). (A) Time courses for intracellular Pi depletion (top panel), increased acid phosphatase activity (middle panel), and purple acid phosphatase immunological detection (bottom panel) from clarified extracts of the Pi-sufficient and Pi-starved *Arabidopsis* cells. Purified intracellular PAP (50 ng) from Pi-starved tomato cells [51] and clarified cell-extract proteins (15 μg) from the *Arabidopsis* cells were resolved by SDS-PAGE and blot transferred to a poly(vinylidene) difluoride (PVDF) membrane. The immunoblot was probed with an anti-(tomato intracellular PAP)-IgG [51], and immunoreactive peptides were detected using an alkaline-phosphatase linked secondary antibody and chromogenic staining. "−Pi → +Pi" denotes extracts from 6-day-old Pi-starved cells that were resupplied with 2.5 mM Pi and cultured for an additional 2 days. Figure reproduced from [53] (Copyright American Society of Plant Biologists; www.plantphysiol.org). (B) Acid phosphatase activities and immunological detection of secreted purple acid phosphatase isozymes in concentrated cell culture filtrates (CCFs) of 7-day-old Pi-sufficient versus Pi-starved *Arabidopsis* suspension cells. All acid phosphatase activities represent the means ± SEM of $n = 3$ separate flasks. Concentrated CCF proteins (5 μg/lane), as well as homogeneous AtPAP26 (20 ng) [53] were resolved by SDS-PAGE and electroblotted onto PVDF membranes. Immunoblots were probed with a 5000-fold dilution of rabbit anti-(AtPAP12 or AtPAP26) immune serum as indicated. Immunoreactive polypeptides were detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescent detection. Lanes labeled '+Pi' and '−Pi' denote CCF proteins from Pi-sufficient and Pi-starved cultures, respectively. The migration of various M_r standards, in kDa, is indicated. Figure from [22]; Copyright Wiley-VCH Verlag GmbH & Co. KGaA; reproduced with permission.

changes. The determination of glycan structures and linkages at each N-linked glycosylation site is required to test the hypothesis that the dual targeting and kinetic differences of the AtPAP26-V, -S1, and -S2 glycoforms of Pi-starved *Arabidopsis* arise from differential glycosylation. The use of glycosylation to control enzyme turnover, localization, association with binding partners, and activity may transcend purple acid phosphatases and Pi starvation. Differential glycosylation, as determined by primary amino acid sequence and environmental conditions, may allow cells to generate an assortment of glycoforms resulting in a single gene product exhibiting diverse dynamic and functional activities.

4.4. Post-transcriptional control of AtPAP26 versus transcriptional control of AtPAP12 expression

Although AtPAP26-V, -S1, and -S2 polypeptides are markedly upregulated by Pi-starved *Arabidopsis*, semi-quantitative RT-PCR revealed that *AtPAP26* transcripts are relatively abundant and invariant irrespective of nutritional Pi status (Table 1) [22,53,54]. Transcript profiling of the *AtPAP* family confirmed that *AtPAP26* transcripts are constitutively expressed in all tissues [80]. This has been corroborated by several recent proteomic studies that have documented a variety of intracellular and secreted proteins that are also controlled post-transcriptionally mainly at the level of protein accumulation in plants responding to changes in environmental Pi availability [19–22]. This highlights the need to integrate transcript profiling with parallel biochemical and proteomic analyses of the plant Pi-starvation response, as the combined datasets provide a more robust depiction of how alterations in gene expression may be linked to adaptive changes in the metabolism of Pi-depleted plants. This is especially pertinent for our understanding of the plant Pi-starvation response in which many recent studies have focused on identifying genes whose transcripts differentially accumulate during Pi deprivation [14–18,71–75]. Furthermore, transcript profiling provides no information about either the subcellular location of gene products, or post-translational modifications that may be essential for their function, transport, or activation.

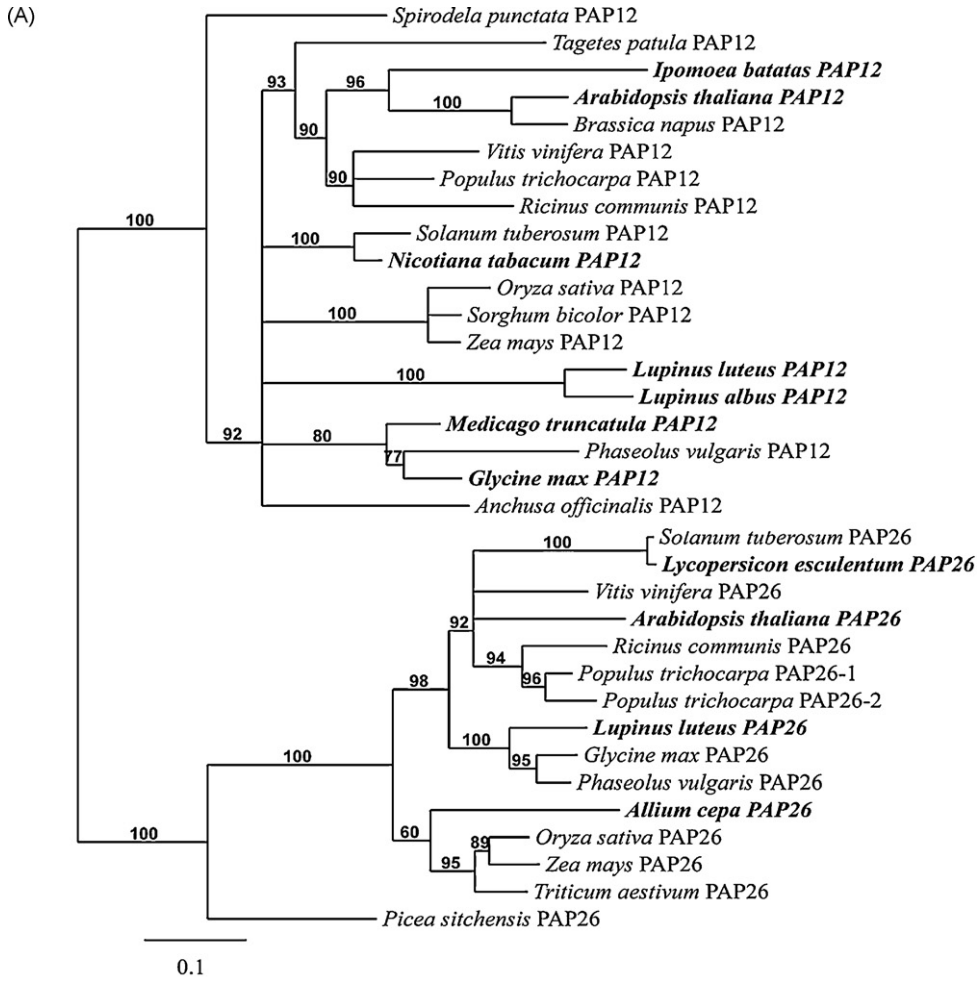
As discussed above (Section 2.3), post-transcriptional processes play a crucial role in the control of Pi-starvation inducible gene expression, particularly those played by the microRNA *miR399* and ubiquitin E2 conjugase UBC24 [23–25]. Plant microRNAs can disrupt gene expression by a combination of mRNA 'silencing' (transcript cleavage) and translational repression [87]. Splice variants of *AtPAP10* preferentially associate with polysomes during Pi starvation [47], AtPAP26 exists as vacuolar and secreted glycoforms [53,54], whereas turnover of extracellular Pi-starvation inducible tomato purple acid phosphatases appears to be mediated by serine proteases that are induced and secreted upon Pi-resupply to Pi-depleted cells [68]. It stands to reason that the regulation of AtPAP26 abundance by nutritional Pi status could be controlled by any of these processes without concomitant changes in *AtPAP26* transcripts. Post-transcriptional control of gene expression has been well established as a key control point in yeast responding to oxidative stress, as well as glucose or amino acid starvation [88–90]. Hypoxic roots of corn, heat stressed *Triticum aestivum* (wheat), and dehydration stressed *Arabidopsis* react similarly [91–93]. In all cases, the pool of actively translating ribosomes greatly decreases under stress and is accompanied by differential phosphorylation of translation initiation factors and ribosomal proteins indicative of a translationally incompetent state [94]. A general survey of the expression and phosphorylation status of the translational machinery during Pi starvation of *Arabidopsis* would be extremely informative. In any event, constitutive transcriptional expression of key Pi-metabolizing enzymes such as AtPAP26 is hypothesized to help 'prime the system', thereby accelerating their rate of biosynthesis upon any subsequent exposure to suboptimal environmental Pi levels. It is also important to note that vacuolar and secreted AtPAP26 isoforms display significant product inhibition by Pi [53,54]. The intracellular Pi concentration of Pi-sufficient *Arabidopsis* (up to 10 mM, assuming 1 g FW ≈ 1 ml) would exert significant feedback inhibition of AtPAP26's acid phosphatase activity *in vivo*. Conversely, the >10-fold reduction in intracellular Pi levels in Pi-depleted tissues should effectively relieve AtPAP26 from Pi inhibition and thus contribute to its enhanced acid phosphatase activity. In contrast to AtPAP26, the synthesis of AtPAP12 and AtPAP17 during Pi deprivation appears to be mainly controlled at the transcriptional level (Table 1) [47,54,58,70]. Although the collective results provide insights into the adaptive biochemical mechanisms that Pi-depleted *Arabidopsis* employs to scavenge Pi from intra- and extracellular organic-Pi, a major challenge will be to fully dissect the signaling pathways involved in the upregulation of purple acid phosphatases such as AtPAP12 and AtPAP26 during Pi starvation.

4.5. Functional analysis of AtPAP26 in transgenic Arabidopsis

The *in planta* function of AtPAP26 was assessed by molecular, biochemical, and phenotypic characterization of a homozygous T-DNA insertional *atpap26* mutant [81]. Loss of *AtPAP26* expression resulted in the elimination of *AtPAP26* transcripts and 55 kDa immunoreactive AtPAP26 polypeptides. This was correlated with: (i) a 9- and 5-fold decrease in extractable shoot and root acid phosphatase activity, respectively, (ii) no change in shoot or root acid phosphatase activity following Pi deprivation, and (iii) a 40% reduction in secreted acid phosphatase activity from Pi-starved *Arabidopsis* seedlings. The *atpap26* mutant seedlings had impaired shoot and root development during Pi deprivation (correlated with 35% and 50% lower levels of free and esterified Pi, respectively, in leaves of 3-week-old plants). They also accumulated more anthocyanin in leaves relative to Col-0 wild-type plants when cultivated on Pi-deficient soil. By contrast, no differences with respect to wild-type plants were noted during growth of *atpap26* mutant seedlings under Pi-replete conditions, or during nitrogen or potassium deprivation [81]. Loss of AtPAP26 function also failed to influence the development of *Arabidopsis* seedlings subjected to paraquat-induced oxidative stress, suggesting that *in vitro* alkaline peroxidase activity of purified AtPAP26-V [53] has little *in vivo* relevance during Pi starvation. This is consistent with AtPAP26-V's vacuolar localization [86], in which an acidic pH of about 5.5 would promote optimal acid phosphatase activity of AtPAP26-V (acid phosphatase activity optimum = pH 5.6), but prevent its peroxidase activity (peroxidase activity optimum = pH 9.0) [53]. It therefore appears that while AtPAP26 plays an indispensable Pi recycling and scavenging role in *Arabidopsis* acclimation to Pi deprivation, it is expendable in Pi-fertilized *Arabidopsis*, or during other macronutrient deficiencies or oxidative stress.

5. AtPAP12 and AtPAP26 orthologs in other plant species

Phylogenetic analysis of AtPAP12 and AtPAP26 orthologs revealed their grouping into distinct monophyletic groups corresponding to two known plant purple acid phosphatase subfamilies (Fig. 4(A)) [55]. AtPAP12 and AtPAP26 share 57% amino acid sequence identity, as compared to much higher identities (64–88%) with their respective orthologs from other plant species [54]. The high degree of sequence identity across dicots and monocots implies an important and conserved function for AtPAP12 and AtPAP26 orthologs in vascular plants. *In silico* analysis of the deduced AtPAP12 and AtPAP26 polypeptides predicted that the mature proteins have molecular masses of 50- and 51 kDa, respectively. The 10 and 4 kDa discrepancies with the respec-



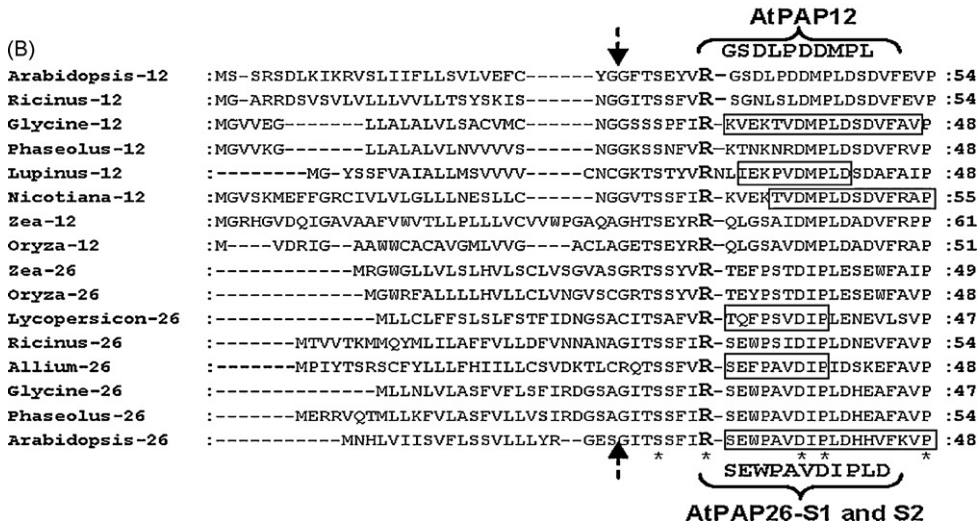


Fig. 4. Bioinformatic analysis of AtPAP12 and AtPAP26 with orthologs from other plants. Phylogenetic analysis of AtPAP12 and AtPAP26 orthologs (A) and alignment of their deduced N-terminal sequences (B) were performed using ClustalX. Orthologs that have been biochemically and/or functionally characterized to date are indicated with a bold font in panel A and originate from sweet potato (*I. batatas*) [97], tobacco (*N. tabacum*) [65,69], yellow lupin (*L. luteus*) [62], white lupin (*L. albus*) [76], barrel medic (*M. trunculata*) [98], soybean (*G. max*) [60], tomato (*L. esculentum*) [51], and onion (*A. cepa*) [95]. Only bootstrap probability values of ≥60% (over 100 replicates) are indicated at the branching points. The scale bar indicates 0.1 substitutions per site. Figure modified from [54]. (B) N-terminal sequences obtained by automated Edman degradation of secreted AtPAP26-S1, AtPAP26-S2, and AtPAP12 purified from the cell culture filtrates of Pi-starved *Arabidopsis* suspension cells [54] are shown in bold font. The N-terminal sequences obtained for the 55-kDa subunit of AtPAP26-V [53], the 57-kDa subunit of purple acid phosphatases from tomato (*Lycopersicon*) [51], tobacco (*Nicotiana*) [65], and soybean (*Glycine*) [60], the 52-kDa subunit of an onion (*Allium*) acid phosphatase [95], as well as the 70-kDa subunit of an acid phosphatase secreted by proteoid roots of Pi-deprived white lupin (*Lupinus*) [76] are enclosed with rectangles. Identical amino acids are denoted by an asterisk. Arrows indicate the predicted transit peptide cleavage site (using Signal P). Figure modified from [51].

tive subunit molecular masses of the purified native AtPAP12 and AtPAP26-V, -S1, and -S2 as estimated by SDS-PAGE [53,54] can be explained by glycosylation. Using the prediction program Signal P, the N-terminus of the deduced AtPAP12 and AtPAP26 polypeptides contain a 28- and 22-amino acid transit peptide, respectively (Fig. 4(B)). By contrast, the N-terminal sequence of the mature polypeptides for AtPAP12 and AtPAP26 begins at position 38 and 31, respectively, implying actual transit peptides of 37 and 30 amino acids [53,54]. Interestingly, the transit peptides of secreted AtPAP12 and its soybean (*Glycine max*) ortholog, the vacuolar and secreted AtPAP26 isoforms, and AtPAP26 orthologs isolated from onion (*Allium cepa*) bulbs, and Pi-starved tomato (*Lycopersicon esculentum*) cell cultures are all processed at the same site, beginning after an invariant Arg residue (Fig. 4(B)) [50,51,53,54,60,95]. Alignment of the N-terminal amino acid sequences of the various orthologs reveals that there are highly conserved amino acid residues at the −6, −5, and −3 positions (T/S, S, and Y/F) with respect to the R cleavage site (Fig. 4(B)). It will be interesting to determine whether these residues are required for efficient transit peptide cleavage by a transit peptidase ortholog that processes the various precursors into mature PAP polypeptides.

6. Concluding remarks

A host of remarkably adaptive mechanisms contribute to the survival of Pi-deprived plants. The availability of the complete genome sequence of the model plant *Arabidopsis*, together with a wide assortment of genomic resources (including high throughput transcript profiling, and a large collection of T-DNA insertional mutants) has significantly advanced our understanding of the plant Pi-starvation response. However, effective integration of these powerful genomic tools with proteomic, enzymological/biochemical, and metabolomic approaches will be needed to fully elucidate the plant Pi-starvation response, including novel Pi signaling components and signal transduction pathways.

Purple acid phosphatases are obvious targets for engineering Pi-efficient crops, since they play a crucial role in Pi recycling and scavenging by Pi-starved plants. Additional studies on the function(s) of AtPAP10 and AtPAP17 in the *Arabidopsis* Pi-starvation response are warranted. However, AtPAP12 and AtPAP26 account for the bulk of acid phosphatase activity secreted by Pi-deficient *Arabidopsis* suspension cells and seedlings and are highly active against a wide range of P-ester substrates over a broad pH range (Fig. 3(B)) [54,81], making them ideally suited for scavenging Pi from the organic-Pi pool prevalent in many soils. Owing to microbial activity, the application of Pi fertilizers increases both the inorganic and organic P content of agricultural soils, thereby affecting the amount of organic-Pi that is available to hydrolysis by purple acid phosphatases [96]. It will therefore be of considerable interest to determine whether the efficiency of Pi fertilizer application can be improved by the overexpression of secreted PAPs such as AtPAP12 and AtPAP26 in genetically engineered crop plants.

The investigation of an AtPAP12 and AtPAP26 double knockout mutant is currently underway in our laboratory and will help to verify their roles in extracellular Pi scavenging by Pi-starved *Arabidopsis*. Overexpression of secreted acid phosphatases can improve plant biomass and Pi accumulation [4]. However, these attempts have either focused upon acid phosphatases with significant phytase activity, or on those with extracellular roles, but unknown intracellular functions. Conversely, AtPAP26 not only has an extracellular role, but is also a dual-targeted enzyme that accumulates in the cell vacuole of Pi-depleted *Arabidopsis* [22,53,54,81]. It will thus be of some interest to examine the Pi metabolism and growth characteristics of *PAP26* overexpressors cultivated on soils containing various P-esters as their sole source of exogenous P.

The biochemical and molecular properties, expression patterns, and subcellular location of plant purple acid phosphatases indicate that this group of enzymes have been significantly modified during the evolution of vascular plants, generating novel isozymes suited for a wide variety of metabolic, developmental, and/or environmental situations. Nevertheless, our understanding of this enzyme family is fragmentary and many crucial questions remain unanswered. For example: (i) why are there so many more different purple acid phosphatase genes in plants than animals? (ii) What is the functional significance of the non-catalytic N-terminal domain of the HMW plant purple acid phosphatases such as AtPAP12 and AtPAP26, or the alkaline peroxidase activity of purple acid phosphatases such as AtPAP17 and AtPAP26? and (iii) what role(s) do post-translational modifications including differential glycosylation play in influencing the turnover, subcellular targeting, and/or kinetic properties of purple acid phosphatases such as AtPAP26? These studies should yield additional insights into the physiological roles of this important enzyme class, and are relevant to ongoing efforts to engineer Pi-efficient transgenic crops that can utilize the P already in the soil, without adding more P than is needed, thereby lessening costs and pollution.

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