Proteomic analysis of alterations in the secretome of Arabidopsis thaliana suspension cells subjected to nutritional phosphate deficiency

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A proteomic approach was applied to compare the secretome (culture filtrate proteome) of phosphate-sufficient (+Pi) and Pi-deficient (−Pi) Arabidopsis thaliana suspension cell cultures. Secretomes harvested from the +Pi and −Pi cells yielded dissimilar 2-DE maps. PMF via MALDI-TOF MS resulted in the identification of 50 protein spots representing 37 discrete proteins having unique gene identities. A total of 24 Pi-starvation responsive proteins were identified, with 18 of these being up-regulated and six down-regulated. Secreted proteins up-regulated by the −Pi cells included a ribonuclease involved in Pi scavenging from extracellular nucleic acids, as well as enzymes of cell wall modification, proteolysis, pathogen responses, and ROS metabolism. Enzyme activity assays and immunoblotting demonstrated that a pair of purple acid phosphatase isoforms having subunit Mr’s of 65 and 55 kDa was also secreted by the −Pi cells. Semiquantitative RT-PCR was used to assess the relationship between mRNA levels and relative amounts of selected secretome proteins. The results indicate that transcriptional control is but one of many factors contributing to Arabidopsis Pi starvation responses, and highlight the importance of parallel biochemical/proteomic studies of −Pi plants.

Keywords:
Arabidopsis / Peptide mass fingerprinting / Phosphate starvation / Secreted protein

1 Introduction

Phosphate (Pi) is a crucial but limiting macronutrient for plant growth and metabolism. Agricultural Pi deficiency is alleviated by the massive application of Pi fertilizers. However, Pi assimilation by fertilized crops is inefficient and unsustainable, as the majority of applied Pi becomes insoluble in the soil or runoffs into and pollutes nearby surface waters. The projected depletion of nonrenewable global rock-Pi reserves by the year 2100 [1] has prompted plant scientists to develop strategies and molecular tools for engineering Pi-efficient crops. This goal necessitates our thorough understanding of the intricate molecular and biochemical adaptations of Pi-deprived (−Pi) plants.

Plants respond to Pi starvation by increasing their root growth at the expense of shoot growth, and by forming lateral roots or root clusters, thereby increasing the surface area for absorption of limiting soil Pi [1]. −Pi plants also up-regulate alternative bypass enzymes to Pi- and adenylate-dependent glycolytic and respiratory electron transport reactions, and scavenge and conserve Pi by replacing membrane phospholipids with amphipathic galacto- and sulfonyl lipids [2–5]. In addition, secreted ribonuclease (RNS), phosphodiesterase, and acid phosphatase (APase) cooperate in the catabolism of soil-localized nucleic acids to mobilize Pi [3, 4,
6, 7], which is made available for root uptake by Pi-starvation inducible (PSI) high-affinity Pi transporters [8]. – Pi plants also up-regulate phosphoenolpyruvate (PEP) carboxylase which can result in the root excretion of large quantities of malic and citric acids [1, 2]. This acidifies the rhizosphere and contributes to the solubilization and assimilation of mineral Pi from the environment.

Important insights into plant Pi starvation responses have been acquired through extensive transcriptomic analyses that have revealed hundreds of PSI genes encoding proteins that are believed to help – Pi plants reprioritize internal Pi use and maximize Pi acquisition from the soil [8–18]. However, these results must be balanced by the fact that transcript abundance does not necessarily reflect cognate protein levels [2, 19–21], and that transcript profiling provides no information about either the subcellular location of gene products, or PTMs that may be essential for their function, transport, and activation. A proteomic study of – Pi rice seedlings documented numerous alterations in root protein expression that had not been revealed in earlier transcriptomic studies [20]. Similarly, AtPAP26 encodes a vacuolar purple APase that is markedly up-regulated by – Pi Arabidopsis thaliana [21]. However, AtPAP26 transcripts are constitutively expressed in Pi-sufficient (+Pi) and – Pi Arabidopsis indicating that transcription exerts little influence on AtPAP26 polyptide levels relative to translational and/or proteolytic controls [21]. Therefore, it is crucial that transcriptomic studies are integrated with proteomic, enzymological, and metabolomic analyses so that the full suite of the metabolic/biochemical adaptations of – Pi plants can be fully understood.

Owing to the availability of genomic resources we recently initiated biochemical and molecular studies of – Pi Arabidopsis suspension cells and seedlings [21]. Although suspension cell cultures provide a robust model for assessing the biochemical adaptations of – Pi plants [5, 7, 21–25], few reports describe the use of cell cultures for investigating Arabidopsis Pi starvation responses. In this study, we employ 2-DE, MS, and immunoblotting to identify secretome alterations due to Pi-deprivation of Arabidopsis suspension cells.

2 Materials and methods

2.1 Plant material and cell viability assay

Heterotrophic A. thaliana (cv. Landsberg erecta) suspension cells were cultured at 25 °C in the dark as previously described [21]. For secretome analysis, 50 mL of a 7-day +Pi culture was used to inoculate 450 mL of fresh media containing 0 or 5 mM K2HPO4. Cells were harvested after 7 days by filtration, frozen in liquid N2, and stored at −80 °C. Cell culture filtrates (CCFs) were processed as described below. Double staining with fluorescein diacetate and propidium iodide was used to respectively discriminate between living (stained green) and dead cells (stained red) via fluorescence microscopy using an Axio Imager Z1 Fluorescence Microscope (Carl Zeiss) as previously described [25].

2.2 Protein extraction and secretome preparation for 2-DE

Quick-frozen cells (1 g) were ground to a powder under liquid N2 and homogenized (1:2 w/v) in ice-cold buffer (50 mM Na-acetate, pH 5.6, 1 mM DTT, 1 mM PMSF, 1% insoluble PVP), and clarified by centrifugation at 14 000 × g and 4 °C for 10 min. The corresponding CCF (∼1 L obtained from replicate 500 mL cultures) was rapidly concentrated to about 100 mL with a Pellicon and further concentrated using an Amicon Ultra-15 (10 kDa cut-off; Millipore Canada) until the final protein concentration was ≥1 mg/mL.

2.3 Enzyme, protein, and Pi assays

All enzymes were assayed at 25 °C by continuously monitoring NADH oxidation at 340 nm using a Gilford 260 recording spectrophotometer. APase assay conditions were 50 mM Na-acetate, pH 5.6, 5 mM PEP, 10 mM MgCl2, 0.2 mM NADH, and 3 U of rabbit muscle lactate dehydrogenase in a final volume of 1 mL. Aldolase and PEP carboxylase activities were determined using coupled assays [26, 27]. One unit of activity is defined as the amount of enzyme resulting in the use of 1 μmol of substrate/min. Protein concentrations were determined with a CBB G-250 dye-binding method using bovine γ-globulin as the standard, whereas CCF Pi levels were determined at 660 nm as previously described [7].

2.4 Immunoblotting

SDS-PAGE mini-gels were prepared and electroblotted onto PVDF membranes as previously described [7] and probed using IgGs described in the relevant figure legends. Immunoreactive polypeptides were visualized using a HRP-conjugated secondary antibody and ECL detection (ECL Plus, GE Healthcare). Immunoblots probed with anti-(AtPAPI2 or AtPAP26)-immune serum were pretreated with sodium-m-periodate [7] to oxidize antigenic glycosylated side chains of glycoproteins. All immunoblots were performed in triplicate with representative results shown in the various figures.

2.5 2-DE and image analysis

All 2-DE equipment and reagents were from GE Healthcare. Concentrated CCF proteins were precipitated with a 2D Clean-Up Kit. Following centrifugation, pellets were solubilized in 50 μL of rehydration buffer (6 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer (pH 4–7), and 50 mM DTT), and proteins quantified using the 2D Quant Kit. IEF was conducted at 20 °C using IPG strips (pH 4–7 L, 7 cm) and an Etan IPGphor II system. Protein (50 μg in 125 μL) was loaded into the IEF tray and passive rehydration carried out overnight under a layer of mineral oil. IEF was performed as follows: 250 V for 15 min, and 1000 V for 6 h, and 8000 V for a total of 40 000 V·h. Focused strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, containing 6 M urea,
30% glycerol, 2% SDS, and 1% DTT, followed by incubation for 15 min in the same buffer without DTT, but containing 2.5% iodoacetamide. Equilibrated strips were placed on 12% SDS-gels (10 cm x 10.5 cm x 1 mm) and sealed with a 3% agarose solution. SDS-PAGE was performed with a MiniVE system at 5 mA for 30 min and then 30 mA until the tracking dye reached the bottom of the gel. Gels were stained with CBB R-250. For each condition analyzed, three replicate gels were prepared from three independent samples. Gels were scanned using a desktop scanner (Epson perfection 600) and image analysis performed with PDQuest software (version 7.0; BioRad). After background subtraction and spot detection, spots were matched and normalized using the method of total density in gel image. The statistical significance of quantitative data was determined using the Student's t-test ($n = 3$, $p < 0.05$), and the obviously different protein spots defined as $p < 0.05$ and change in amount at least two-fold.

### 2.6 In-gel digestion, MS, and database searching

Protein-staining spots were excised from 2-DE gels, diced into -1 mm pieces, and destained in 50 mM ammonium bicarbonate in 50% ACN. Proteins were reduced with 10 mM DTT in 100 mM ammonium bicarbonate and alkylated in 100 mM ammonium bicarbonate containing 55 mM iodoacetamide. Samples were dehydrated using 100% ACN and digested with 10 μL of 20 ng/μL of trypsin (sequencing-grade, Promega) in 50 mM ammonium bicarbonate at 37°C for 5 h. Peptides were gel extracted with a 1% formic acid/2% ACN solution, followed by two extractions with 50% ACN. All three extractions were pooled and evaporated to ~10 μL using a SpeedVac.

PMF by MALDI-TOF MS was performed using a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems). Each pooled tryptic digest (0.5 μL) was mixed with an equal volume of CHCA matrix (Sigma; 5 mg/mL in 70% ACN, 0.1% TFA, and 10 mM diammonium citrate) and spotted onto the MALDI target. Spectra were acquired in positive ion reflector mode under 17 000 kV accelerating voltage and a mass range of 800–3500 Da. Internal calibration was performed using trypsin autolysis fragments at $m/z$ 842.5100, 2211.1046, and 2807.3000. LC-MS/MS was performed as previously described [28]. Briefly, digestions were injected onto an LC Packings C18 PepMap 100 column (75 μm x 150 mm, particle size 3 μm, pore size 100 Å), equilibrated with 0.1% formic acid using a Waters CapLC XE chromatography system connected to a Waters QTOF Global mass spectrometer. The column was developed at 0.2 μL/min with a 5–80% ACN gradient over 35 min. Data acquisition and analyses were performed using Waters MassLynx 4.0 software.

Protein identification was performed by searching in the National Center for Biotechnology Information non-redundant database (NCBIInr) using both MASCOT (http://www.matrixscience.com) and MS-FIT (http://www.prospector.ucsf.edu/). The following parameters were used for the database searches: 50 ppm mass tolerance, one allowed trypsin mis-cleavage, carbamidomethylation of Cys (fixed modification), and Met oxidation (variable modification). For a positive identification, the identified protein must rank as the top hit in both search programs, match at least four peptides, cover ≥10% of the total sequence, and generate a MOWSE score greater than the significant threshold, at the $p = 0.05$ level (MOWSE score >60 using MASCOT and ≥103 with MS-FIT).

### 2.7 RNA extraction and RT-PCR

Total RNA was extracted from +Pi and –Pi cells using an RNaseasy kit (Qiagen) and semiquantitative RT-PCR performed as described [27] using gene specific primers listed in Table S1 (Supporting Information). On column DNase treatment was incorporated to eliminate genomic DNA. RNA samples were assessed for purity by their $A_{260}/A_{280}$ ratio and integrity by resolving 1 μg of total RNA on a 1.2% w/v denaturing agarose gel. Normalization of RNA for the purpose of equal starting material during RT was done for each sample by density measurement of 28S RNA bands from the above gel (scanned using Image) software from the National Institute of Health, USA.

### 3 Results and discussion

#### 3.1 Optimization of +Pi and –Pi Arabidopsis cell cultures, and influence of Pi starvation on cell growth and viability

When subcultured in the presence of 5 mM Pi, the CCF of 7-day Arabidopsis cells contained 1.7 ± 0.3 mM Pi ($n = 3$, ±SEM), indicating that the cells were still completely +Pi. By contrast, the 1.25 mM Pi of conventional Murashige–Skoog media [29] was inadequate for maintaining +Pi batch-cultured Arabidopsis suspension cells; 7 days following their subculture into this media: (i) Pi was undetectable in the CCF, and (ii) biomass accumulation was reduced by at least 25% relative to cells subcultured for 7 days in 5 mM Pi. The inability of conventional Murashige–Skoog media to maintain plant (including Arabidopsis) suspension cells fully +Pi for at least 1 wk in batch culture has been well documented [5, 7, 21, 23, 24]. All subsequent studies were conducted using optimized 7-day –Pi and +Pi Arabidopsis suspension cells subcultured into media containing 0 and 5 mM Pi, respectively.

The –Pi cells attained about 50% of the fresh weight of the corresponding +Pi cells (about 25 and 50 g of –Pi and +Pi cells were respectively obtained/500 mL culture). Growth inhibition of the –Pi cells was correlated with depletion of CCF Pi to undetectable levels within 1-day following subculture of 7-day +Pi cells into –Pi media. Cellular viability quantification using fluorescein diacetate/propidium iodide double staining and fluorescence microscopy
revealed that >95% of the 7-day-old –Pi cells were still alive (Fig. S1 in Supporting Information). Similarly, the vast majority (>90%) of Brassica napus suspension cells remained viable for at least 21 days following their subculture into –Pi media (although negligible growth occurred beyond 9-day), at which point they began to enter into programmed cell death [23].

To assess possible secretome contamination by soluble intracellular proteins, immunoblots of a clarified cell extract, and concentrated +Pi and –Pi secretomes were probed with antibodies raised against the cytoplasmic marker enzymes PEP carboxylase and cytosolic aldolase. Immunoreactive polypeptides comigrating with the respective purified antigens were only observed on immunoblots of cell lysates (Fig. 1). Similarly, PEP carboxylase and aldolase activities were readily assayed in cell lysates, but were not detected in the concentrated CCFs (Fig. 1). The combined results (Figs. 1 and S1) indicate that proteins localized in the +Pi or –Pi secre- tome were actively secreted, and not artifacts due to cell lysis.

3.2 Influence of Pi-starvation on secreted APase activity and immunoreactive polypeptides

A ubiquitous biochemical marker of plant Pi stress is the up-regulation of intracellular and secreted APases [1, 3, 4, 7, 21, 23, 24]. Extractable (intracellular) and secreted (concentrated CCF) APase activities of the –Pi cells used in the current study were respectively about four- and six-fold greater than that of the corresponding +Pi cells (Fig. 2A and results not shown). Similarly, –Pi tomato suspension cells exhibited eight-fold greater APase activity in their CCF relative to +Pi cultures [7, 23]. Time-course studies revealed that the activity of both intracellular and secreted APases of the –Pi Arabidopsis cells maximized 6–7-days following their subculture into –Pi media [21] (results not shown). This was paralleled by the appearance of 65 and 55 kDa immunoreactive polypeptides on immunoblots of –Pi but not +Pi secretomes, respectively, probed with rabbit antibodies raised against the Arabidopsis purple APases, AtPAP12, and AtPAP26 (Fig. 2B). Our current efforts include the purification, molecular/biochemical characterization, and functional analysis of secreted AtPAP12 and AtPAP26 isoforms (subunit Mₚ = 65 and 55 kDa, respectively) from the CCF of the –Pi Arabidopsis cells (H. Tran, W. Qian, D. Wang, W. Plaxton, unpublished data). Secreted APases are believed to play a pivotal role in root Pi-scavenging from soil-localized Pi-esters [1, 3, 4, 7, 23, 24].

![Figure 1](image1.png)

**Figure 1.** Immunological detection of PEP carboxylase and cytosolic aldolase in clarified Arabidopsis suspension cell extracts. Concentrated CCF proteins from +Pi and –Pi cells (CCF, 30 µg each), a clarified cell extract from +Pi cells (CE, 5 µg), and homogenous castor seed PEP carboxylase (PEPC) and cytosolic aldolase (ALD; 10 ng each) [26, 27] were resolved by SDS-PAGE and electroblotted onto PVDF membranes. Immunoblots were probed with a 500-fold dilution of affinity-purified rabbit anti-(castor seed PEP carboxylase or cytosolic aldolase)-IgG [26, 27]. Immunoreactive polypeptides were detected using a HRP-conjugated secondary antibody and ECL detection. Corresponding PEP carboxylase and aldolase activities appear below the respective lanes.

![Figure 2](image2.png)

**Figure 2.** APase activities (A) and immunological detection of secreted purple APase isoforms (B) in CCFs of +Pi versus –Pi Arabidopsis suspension cells. CCFs were harvested and concentrated as described in Section 2, and (A) assayed for APase activity and (B) subjected to immunoblot analysis using anti-(AtPAP12 or AtPAP26)-IgGs. (A) All APase activities represent the means ± SEM of n = 3 separate flasks. (B) Concentrated CCF proteins from the +Pi and –Pi cells (5 µg/lane), as well as homogenous AtPAP26 (20 ng) [21] were resolved by SDS-PAGE and electroblotted onto PVDF membranes as previously described [7]. Immunoblots were probed with a 5000-fold dilution of rabbit anti-(AtPAP12 or AtPAP26) immune serum as indicated. Immunoreactive polypeptides were detected using a HRP-conjugated secondary antibody and ECL detection. Lanes labeled “+Pi” and “–Pi” denote CCF proteins from +Pi and –Pi cultures, respectively. The migration of various Mₛ standards, in kDa, is indicated.

3.3 Protein separation by 2-DE and identification by MS

Initially, pH 3–10 IPG strips were employed for IEF of concentrated CCF proteins prior to second dimension SDS-PAGE. As all proteins focused within the 4–7 pH range
subsequent 2-DE was conducted using pH 4–7 IPG strips (Fig. 3). 2-DE of the +Pi and −Pi secretomes yielded reproducible protein patterns between the three sets of independent culture repetitions. Although the respective patterns were relatively distinct, an obvious overlap of some spots was observed (Fig. 3). About 110 CBB-staining spots were detected in total following 2-DE of the +Pi and −Pi secretomes. PMF via MALDI-TOF MS was performed on 64 spots, including all 46 spots whose abundance changed by ≥2-fold following Pi-stress, and resulted in 50 protein assignments representing 37 different proteins having unique gene identities (Table 1). LC MS/MS was performed on four spots (#10, 13, 15, and 44; Fig. 3) and confirmed identifications obtained using PMF (Table S2 in Supporting Information). Thirteen different proteins identified following 2-DE of the −Pi secretome appeared to be absent in the +Pi secretome, whereas levels of five different proteins of the +Pi secretome were up-regulated by ≥2-fold in response to Pi stress (Fig. 3, Table 1). By contrast, 13 of the identified proteins were secreted at similar levels irrespective of Pi nutritional status, whereas six proteins present in the +Pi secretome appeared to be down-regulated by ≥2-fold in response to Pi stress (Fig. 3, Table 1). The discrepancy between the experimental and theoretical values observed for some proteins (Table 1) might be explained by proteolytic degradation of polypeptides, PTM events, or variability arising from alternate splicing of mRNAs. Similar levels of discrepancy between the predicted and experimental Mr and pI of proteins identified by MS have been noted in previous studies [30, 31].

Although 2-DE coupled with MS is a powerful tool for investigating stress-induced proteome modifications, a disadvantage of this technique is that key protein players of low abundance may be masked by more plentiful proteins. For example, although APases were secreted in response to Pi stress (Fig. 2), no APase was identified following MS analysis of all CBB-250 staining spots that were up-regulated in the −Pi secretome. This is likely due to the low concentration of APase polypeptides in the −Pi secretome, and corroborates studies demonstrating that secreted APases of −Pi plants exhibit relatively high specific activities (>300 U/mg) [7, 24].

3.4 Some of the proteins identified in the secretome of Arabidopsis suspension cells lack predicted signal peptides

About 40% of proteins identified from the +Pi and −Pi secretomes lack putative N-terminal secretory signal peptides (Table 1). Several of the same proteins including dehydroascorbate reductase 1, phosphoglycerate kinase, and enolase have been localized to the Arabidopsis cell wall [30, 32–35]. As discussed by Slabas et al. [33], the results imply that certain cytoplasmic proteins without obvious targeting signals are secreted to perform specific extracellular functions.

3.5 Proteins secreted by −Pi Arabidopsis perform diverse functions

Accumulation of the glycolytic enzymes phosphoglycerate mutase and enolase in the CCF of the −Pi cells implies that they are multifunctional proteins. This is not unreasonable since various extracellular animal proteins with functions unrelated to glycolysis were eventually discovered to be encoded by glycolytic enzyme genes [36]. For example, enolase is a structural protein of the mammalian eye lens and in yeast is a heat shock protein that may confer thermo-tolerance [36]. Enolase was also up-regulated in response to cold stress in Arabidopsis, anoxia, cold, and heat shock in Echinochloa phyllopogon, and Pi-deprivation in maize [31, 36]. Apart from its glycolytic role, enolase likely functions as a general stress protein that protects cellular components at the structural level [36].

![image](image_url)
Table 1. Functional characterization of proteins identified following 2-DE of the CCF secretome from 7-day old +Pi and −Pi Arabidopsis suspension cells

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<th>Protein assignment and gene ID</th>
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<td>Observed</td>
<td>Predicted (^{b)})</td>
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**Cell wall modifying**

Expansin-like protein, At3g45970

| 73 (34) | 7 | 24 | ≥7.0/35 | 8.3/28.7 | − |

\(\text{β}-\text{Fructofuranosidase 5, At1g55120}^{\text{b)}}\)

| 67 (8)  | 8 | 14 | 5.8/73  | 5.5/67.3 | ++ |

Glycosyl hydrolase family 17 protein, At5g58090

| 99 (13\(^{c)}\)) | 12 | 23 | 5.4/56  | 5.6/52.6 | 0 |

Glycosyl hydrolase family 17 protein, At1g64760

| 76 (12) | 7 | 17 | 5.0/57  | 5.0/52.6 | 0 |

Glycosyl hydrolase family 17 protein fragment, At4g34480

| 72 (23) | 6 | 19 | 5.5/46  | 5.4/37.8 | 0 |

Glycosyl hydrolase family 3 protein, At5g10560

| 63 (16) | 4 | 8  | 5.2/54  | 6.0/87.1 | 0 |

Galactosyltransferase family protein, At3g14960

| 138 (17) | 14 | 22 | 5.4/58  | 0         | |

**Glycosyl hydrolase family 17 protein fragment, At4g34480**

| 117 (35) | 13 | 39 | 5.1/33  | 5.1/32.0 | 0 |

Xyloglucan endo-1,4-\(\beta\)-D-glucanase, At4g30270

| 75 (42)  | 10 | 31 | ≥7.0/32 | 8.4/30.9 | − |

Polygalacturonase, At3g16850

| 61 (10\(^{d)}\)) | 6 | 18 | 5.9/66  | 5.5/49.1 | − − |

RGP1\(^{d)}\), At3g02230

| 156 (28) | 14 | 28 | 5.7/41  | 5.6/40.6 | ++ |

RGP2\(^{d)}\), At5g15650

| 172 (28) | 14 | 37 | 5.7/41  | 5.8/40.9 | ++ |

RGP3\(^{d)}\), At3g08900

| 170 (28) | 14 | 37 | 5.7/41  | 5.4/41.3 | ++ |

Xyloglucan endotransglycosylase 6, At4g25810

| 117 (35) | 13 | 39 | 5.1/33  | 5.1/32.0 | 0 |

Xyloglucan endotransglycosylase 6, At4g25810

| 87 (36)  | 6 | 20 | 5.2/33  | 5.1/32.0 | ++ |

LysM domain GPI-anchored protein 2, At2g17120

| 61 (22)  | 5 | 15 | 6.3/50  | 5.9/37.7 | 0 |

Monocopper oxidase-like protein, At5g51480

| 146 (1)  | 15 | 27 | 6.3/89  | 5.9/66.5 | ++ |

| 135 (2)  | 15 | 21 | 6.3/93  | 0         | |

| 99 (3)   | 14 | 20 | 6.2/81  | 0         | ++ |

**Defense/detoxifying**

Glutathione transferase 8, At1g78380

| 81 (47)  | 7 | 28 | 5.8/27  | 5.8/25.7 | ++ |

Dehydroascorbate reductase 1, At1g19570

| 87 (46)  | 7 | 35 | 5.6/27  | 5.6/23.7 | ++ |

Fe superoxide dismutase 1, At4g25100

| 70 (49)  | 4 | 19 | 6.5/26  | 6.1/23.8 | 0 |

Mn superoxide dismutase 1, At3g10920

| 81 (50)  | 5 | 29 | 6.3/26  | 8.5/25.4 | + |

Glutathione reductase, At3g54660

| 65 (21)  | 7 | 17 | 6.4/55  | 8.0/60.8 | 0 |

NADPH-dependent thioredoxin reductase 2, At2g17420

| 96 (33)  | 7 | 27 | 6.3/36  | 6.3/40.0 | + |

Peroxidase, At5g64120

| 125 (6)  | 10 | 37 | ≥7.0/70 | 8.6/34.9 | − |

Peroxidase 17, At2g22420

| 97 (27)  | 10 | 31 | 5.1/41  | 5.1/36.7 | + |

Peroxidase 53, At5g06720

| 78 (11)  | 5 | 26 | 4.7/50  | 4.7/35.0 | + |

Peroxidase 58, At5g19880

| 77 (26)  | 4 | 18 | 5.0/41  | 5.1/35.4 | 0 |

**Glycolysis**

Phosphoglycerate mutase, At3g08590

| 179 (9)  | 16 | 26 | 5.7/64  | 5.5/60.7 | ++ |

Enolase, At2g36530

| 100 (15\(^{c)}\)) | 9 | 17 | 5.7/55  | 5.5/47.7 | ++ |

| 100 (14) | 12 | 27 | 5.6/55  | 0         | |

Phosphoglycerate kinase, At1g79550

| 126 (30) | 7 | 21 | 5.7/41  | 5.5/42.1 | − − |

**N-Metabolism**

Amidase family protein, At5g07360

| 66 (7)   | 7 | 9  | 5.7/78  | 8.1/72.2 | ++ |

Glutamine synthetase, At3g17820

| 70 (29)  | 4 | 11 | 5.7/42  | 5.7/38.6 | ++ |

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Table 1. Continued

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<td>87 (4)</td>
<td>10</td>
<td>17</td>
<td>6.7/81</td>
<td>5.9/79.4</td>
</tr>
<tr>
<td></td>
<td>107 (5)</td>
<td>11</td>
<td>18</td>
<td>≥7.0/71</td>
<td></td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
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<tr>
<td>Cyclase-family protein, At4g34180 ◊</td>
<td>94 (37)</td>
<td>7</td>
<td>30</td>
<td>6.6/34</td>
<td>6.0/28.4</td>
</tr>
<tr>
<td></td>
<td>94 (38)</td>
<td>9</td>
<td>33</td>
<td>6.3/34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80 (39)</td>
<td>5</td>
<td>22</td>
<td>6.3/31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>98 (41)</td>
<td>9</td>
<td>33</td>
<td>6.1/34</td>
<td></td>
</tr>
</tbody>
</table>

A unique number was assigned to spots identified as the same protein and migrating to the same coordinate upon 2-DE, while different spot numbers were assigned to spots identified as the same protein and migrating to different coordinates (Fig. 3). Protein spots reproducibly detected only under −Pi or +Pi conditions are indicated by (1) and (2), respectively, whereas proteins up-regulated or down-regulated by at least two-fold in the CCF of −Pi cells are indicated by (+) and (−), respectively; (0) corresponds to proteins exhibiting similar expression under +Pi and −Pi conditions. Genes containing a coding sequence for a putative transit peptide (as predicted by SignalP, version 3.0) are indicated with a diamond ◊.

a) MOWSE score: statistical probability of a positive identification of predicted proteins calculated by MASCOT (http://www.matrixscience.com) with 50 ppm mass tolerance and one permissible missed cleavage (MOWSE<sup>b</sup>). All identifications were corroborated using MS-FIT (http://www.prospector.ucsf.edu/) (MOWSE<sup>c</sup>) as outlined in Section 2.

b) Calculated using the EXPASY pI/<M<sub>r</sub> tool (http://www.expasy.org/tools/pi_tool.html).

c) Spots identified by both PMF and LC MS/MS (Table S2 in Supporting Information).

d) PMF via MALDI-TOF MS matched corresponding peptides from RGP1–3, and did not allow for discrimination between these three very closely related isoforms.

The role of the PSI secreted RNS1 in Pi scavenging from extracellular nucleic acids has been well documented in −Pi Arabidopsis and tomato [4, 6]. RNS1 hydrolyzes extracellular RNA into mononucleotides, thereby allowing secreted APases to cleave Pi from resultant Pi-monoesters for its uptake by PSI high-affinity Pi transporters of the plasmalemma. RNS1 was the most abundant protein in the CCF of −Pi Arabidopsis cells (Fig. 3B, spots 43–45). As is the case with several of the other identified proteins, RNS1 may be subject to PTM since multiple spots of similar <M<sub>r</sub> were detected following 2-DE (Fig. 3).

Cell wall modifying enzymes located in the CCF included xyloglucan endotransglycosylase 6 (XTR6), several glycosyl hydrolases, as well as an expansin, and polygalacturonase (Table 1). Many of these proteins contain predicted N-terminal targeting sequences and several have been localized to the Arabidopsis cell wall [30, 32, 33, 35, 37]. Those specific to the Pi starvation response included β-fructofuranoside 5, a monocopper oxidase-like protein, a reversibly glycosylated polypeptide (RGP), and an XTR6 isoform (spot 36; Table 1). Three spots corresponding to a monocopper oxidase-like protein were also up-regulated in the −Pi secretome (Fig. 3B, Table 1). Although little is known about its physiological role(s), the monocopper oxidase-like protein encoded by At5g51480 is believed to exist as a GPI-anchored protein of the Arabidopsis plasma membrane [38]. GPI-anchored proteins targeted to the plant cell surface may function in extracellular matrix remodeling and/or signaling. Since the phosphatidylinositol moiety of their GPI anchor is susceptible to cleavage by specific phospholipases, GPI-anchored proteins exist in both soluble and membrane-associated forms [38].

RGPs are plant-specific proteins that catalyze self-glycosylation reactions [39, 40]. These proteins are highly conserved, with five RGP genes identified in Arabidopsis. Because RGP1–3 are so closely related (95% amino acid
sequence identity) our PMF data was unable to discriminate as to which RGP isozyme occurred in the −Pi secretome (Fig. 3B, Table 1). RGP self-glycosylation promotes RGP complex formation and association with the Golgi [40]. In Arabidopsis, the maximal concentration of RGP1 transcripts and protein occurred in roots and suspension cell cultures where it was suggested to function in cell wall modification [39].

XTR6 forms part of a large gene family of Arabidopsis xyloglucan endotransglycosylases/hydrolases [41]. XTRs possess xyloglucan endohydrolase and endotransglycosylase activities which respectively catalyze the hydrolysis of xyloglucan polymers and transfer of the newly generated reducing ends to adjacent xyloglucan chains. A specific XTR was up-regulated in the CCF of Arabidopsis suspension cells treated with fungal elicitors [42], indicating a role in pathogen defense. Similarly, an XTR6 isoform was up-regulated in the −Pi secretome (spot 36, Fig. 3B; Table 1).

The majority of proteins accumulating in the −Pi secretome function in cellular defense and/or ROS detoxification (Table 1). Proteomic studies of −Pi maize and rice roots also demonstrated the up-regulation of many antioxidant enzymes [20, 31], and corroborate transcriptomic studies documenting numerous PSI Arabidopsis genes involved in cell defense and oxidative stress amelioration [10–13]. Secreted antioxidant enzymes identified in the current study included glutathione transferase, dehydroascorbate reductase, and Fe and Mn superoxide dismutase (Table 1).

Numerous extracellular peroxidase isozymes exist in plants and likely function in pathogen responses and oxidative stress resistance. Peroxidases promote the oxidative cross-linking of cell wall polymers thereby forming a physical barrier that may hinder pathogen penetration. Several peroxidases accumulated in the CCF of Arabidopsis suspension cells treated with fungal elicitors [42], and peroxidases also occur in the cell wall [32, 35, 37]. Although two peroxidases (At5g64120 and At5g19880) were transcriptionally induced in −Pi Arabidopsis seedlings [11, 12], the peroxidase encoded by At5g64120 was down-regulated in the −Pi secretome (Table 1). By contrast, peroxidase 17 and 53 (respectively encoded by At2g22420 and At5g06720) accumulated in the CCF of the −Pi cells, suggesting that the −Pi cells experienced oxidative stress.

Secreted proteases are involved in maturation of enzymes, signaling, protein turnover, and pathogen defense [35, 42], and may also contribute to secretome remodeling according to nutritional Pi status [22]. Of the five proteases we identified in the Arabidopsis secretome, three were up-regulated in response to Pi starvation, including two leucine aminopeptidases and a serine carboxypeptidase (Table 1). A serine carboxypeptidase was also secreted by Arabidopsis cultures treated with fungal elicitors [42]. Interestingly, transcript profiling has identified a PSI subtilisin-like serine protease in Arabidopsis, despite the Arabidopsis genome encoding 56 subtilisin-like proteases [11]. Our understanding of these proteins is limited, although they have been implicated in protein turnover, response to pathogen infection, and control of plant development. It is notable that within 48 h of resupply of 2.5 mM Pi to −Pi tomato suspension cells, CCF APase activity and immunoreactive polypeptides corresponding to a pair of secreted PSI purple APase isozymes disappeared [22]. Their disappearance was correlated with the de novo synthesis and secretion of a pair of CCF-localized serine proteases. Biotechnological strategies for engineering Pi-efficient crops should consider the possibility that PSI protein overexpression in transgenic plants may be enhanced by modified protease expression and/or the design of protease-resistant PSI proteins.

3.6 Semiquantitative RT-PCR analysis of selected transcripts

Recent studies of plant Pi-starvation responses have focused on identifying genes displaying enhanced transcription during Pi stress [8–18]. However, alterations in transcript abundance do not necessarily translate into a correlated change in protein amount or enzymatic activity, or vice versa [2, 19–21]. Indeed, only two (XTR6 and RNS1) of the 18 identified proteins that accumulated in the −Pi secretome (Table 1) have been documented to be transcriptionally induced in −Pi Arabidopsis [6, 11]. This was further investigated by semiquantitative RT-PCR analysis of selected transcripts from the +Pi and −Pi Arabidopsis cells (Fig. 4). RNS1, AtPAP17, and AtPAP26 were employed as positive controls since their mRNAs have been documented to either markedly increase (AtPAP17, RNS1) or remain invariant (AtPAP26) when Arabidopsis is subjected to Pi deprivation [6, 21]. The RNS1, XTR6, and cyclase transcripts correlated well with relative levels of the corresponding polypeptides in the +Pi and −Pi secretomes (Figs. 3 and 4, Table 1). In contrast, monocopper oxidase-like protein, dehydroascorbate reductase-1, enolase, phosphoglycerate mutase, polygalacturonase, serine carboxypeptidase-50, and leucine aminopeptidase-1 transcripts were expressed at similar levels irrespective of nutritional Pi status, whereas the corresponding polypeptides were only detected in the +Pi (polygalacturonase) or −Pi (remaining enzymes) secretomes. Similarly, the purple APase AtPAP26 was markedly up-regulated by −Pi Arabidopsis, but AtPAP26 transcripts were constitutively expressed (Fig. 4) [21]. In silico analysis of Arabidopsis microarray data using Genevestigator (www.genevestigator.ethz.ch/) confirmed that: (i) RNS1 (At2g02990) and AtPAP17 (At3g17790) are significantly induced in −Pi tissues, whereas (ii) all other genes that we analyzed via RT-PCR (Fig. 4) have generally high basal transcript levels and are constitutively expressed in all tissues examined to date. Thus, transcriptional control appears to exert less impact on levels of certain intracellular and secreted proteins up-regulated in −Pi Arabidopsis, relative to translational and post-translational controls that influence protein synthesis and degradation.
stress response. Future studies using knockout lines should prove invaluable in this regard. Our study also highlights the importance of complementing transcriptomics with proteomics, as the combined datasets provides a more robust depiction of how alterations in gene expression may be linked to adaptive changes in the metabolism of −Pi plants.

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The authors have declared no conflict of interest.

5 References


