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Original Article

Acclimation of the crucifer Eutrema salsugineum to phosphate limitation is associated with constitutively high expression of phosphate-starvation genes

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ABSTRACT

***Eutrema salsugineum*, a halophytic relative of *Arabidopsis thaliana*, was subjected to varying phosphate (Pi) treatments. *Arabidopsis* seedlings grown on 0.05 mM Pi displayed shortened primary roots, higher lateral root density and reduced shoot biomass allocation relative to those on 0.5 mM Pi, whereas *Eutrema* seedlings showed no difference in lateral root density and shoot biomass allocation. While a low Fe concentration mitigated the Pi deficiency response for *Arabidopsis*, *Eutrema* root architecture was unaltered, but adding NaCl increased *Eutrema* lateral root density almost 2-fold. *Eutrema* and *Arabidopsis* plants grown on soil without added Pi for 4 weeks had low shoot and root Pi content. Pi-deprived, soil-grown *Arabidopsis* plants were stunted with senescing older leaves, whereas *Eutrema* plants were visually indistinguishable from 2.5 mM Pi-supplemented plants. Genes associated with Pi starvation were analysed by RT-qPCR. *EsIPS2*, *EsPHT1;4* and *EsPAP17* showed up-regulated expression in Pi-deprived *Eutrema* plants, while *EsPHR1*, *EsWRKY75* and *EsRNS1* showed no induction. Absolute quantification of transcripts indicated that *PHR1*, *WRKY75* and *RNS1* were expressed at higher levels in *Eutrema* plants relative to those in *Arabidopsis* regardless of external Pi. The low phenotypic plasticity *Eutrema* displays to Pi supply is consistent with adaptation to chronic Pi deprivation in its extreme natural habitat.**

Key-words: Arabidopsis thaliana; Thellungiella salsuginea; absolute RT-qPCR; extremophile; halophyte; phosphate transporter; phosphorus; stress tolerance.

INTRODUCTION

Inorganic phosphate (Pi) is a form of phosphorus (P) taken up by plants to meet their requirements for this macronutrient (Sánchez-Calderón *et al.* 2010). However, Pi is naturally limiting for plant growth in many soils, rendering the availability of this nutrient low for crops and native plants. Insufficient Pi can lead to a 30 to 40% reduction in crop yield, requiring the routine use of Pi-containing fertilizer to improve crop productivity (Ramaekers *et al.* 2010; Miura 2013). However, rock Pi stocks are a non-renewable and rapidly depleting natural resource (Cordell *et al.* 2009; Fixen & Johnston 2012). An alternative strategy to enhance the Pi nutrient status of crops involves improving the capacity of crops to acquire and use Pi.

With Pi as a frequently limiting resource, plants have evolved mechanisms to cope with Pi-deficient conditions by maximizing Pi uptake and regulating Pi use (Vance *et al.* 2003). Under Pi-deficient conditions, many plants show attenuated primary root elongation accompanied by lateral root proliferation and increased root hair density (Bates & Lynch 1996; Williamson *et al.* 2001; Abel 2011; Péret *et al.* 2014). The reduced expression of genes encoding the high-affinity Pi transporters, *PHT1*;*1* and *PHT1*;*4*, was associated with reduced Pi uptake in Pi-deprived *Arabidopsis* (Misson *et al.* 2004; Shin *et al.* 2004). Conversely, the overexpression of *PHT1*;*1* leads to increased Pi content in *Arabidopsis* seedlings (Wang *et al.* 2014). Thus, changes in root architecture and increased contributions of Pi transporters have been proposed as responses that can enhance the capacity of plants to extract Pi from the environment (Mudge *et al.* 2002; Versaw & Harrison 2002). These adjustments, leading to enhanced Pi uptake, benefit the plant through an increased capacity for roots to explore soil and scavenge Pi more effectively when soil Pi content is low (Linkohr *et al.* 2002; Abel 2011), although the down-regulated expression of transporters is necessary to prevent potential toxicity when external Pi is high (reviewed in Lambers *et al.* 2011). Once Pi is taken up by roots, transporters mobilize and distribute Pi between cells and cellular compartments (Versaw & Harrison 2002; Nagarajan *et al.* 2011; Nussaume *et al.* 2011). The metabolism of plants starved for Pi undergoes modifications that can mitigate the perturbing effects of low Pi. For example, Pi-starved plants show increased activities of purple acid phosphatases (Tran *et al.* 2010), ribonucleases (Bariola *et al.* 1994; Tran & Plaxton 2008) and enzymes that catalyse glycolytic bypasses such as pyrophosphate-dependent

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phosphofructokinase and phosphoenolpyruvate carboxylase (Duff *et al*. 1989; Plaxton & Tran 2011).

The adjustments made by plants require higher-order regulation and Pi sensing systems that serve to co-ordinate the appropriate Pi management responses with a given plant Pi status (Abel 2011). For *Arabidopsis*, the primary root tip has been implicated as a sensor of external Pi with low Pi conditions leading to a decrease in the size of the quiescent centre and reduced cell proliferation at the root tip (Sánchez-Calderón *et al*. 2005; Sánchez-Calderón *et al*. 2006; Svistoonoff *et al*. 2007). The changes associated with meristematic activities at the primary root tip leads to altered root architecture under low relative to high Pi conditions with seedlings featuring shortened primary roots, increased lateral roots and proliferation of root hairs.

Gene expression profiling in *Arabidopsis* deficient for Pi and the study of specific *Arabidopsis* mutants defective in Pi sensing, uptake or assimilation have contributed to our current understanding of the molecular responses of plants to low Pi (Abel 2011; Chiou & Lin 2011). For example, genes involved in Pi uptake, internal Pi mobilization and lipid metabolism as influenced by plant Pi status are directly regulated by the R2R3 MYB transcription factor AtPHR1 (Rubio *et al*. 2001; Bustos *et al*. 2010; Pant *et al*. 2015). Genes regulated by AtPHR1 include *AtIPS2* (*At4*), a gene that produces a non-coding RNA, *AtRNS1* encoding a ribonuclease and *AtPAP17* that encodes a purple acid phosphatase (Nilsson *et al*. 2007; Bustos *et al*. 2010). The expression of *AtIPS2* as well as *AtPHT1*;*4*, the latter encoding a high-affinity Pi transporter, is also positively regulated by another transcription factor, AtWRKY75 (Devaiah *et al*. 2007). While transcriptional regulation of the Pi-starvation response may be complex, for *Arabidopsis*, the positive correlation between Pi deficiency and an increased expression of several genes, including *AtIPS2* (Burleigh & Harrison 1999), *AtRNS1* (Bariola *et al*. 1994), *AtPAP17* (del Pozo *et al*. 1999) and *AtPHT1*;*4* (Muchhal *et al*. 1996), is sufficiently consistent that their expression can be used as biomarkers in Pi-starvation response studies (Daram *et al*. 1999; Versaw & Harrison 2002; Shin *et al*. 2004; Ticconi *et al*. 2004; Hurley *et al*. 2010).

Broadening the scope of plants used for comparisons with taxonomically diverse species holds promise for developing a greater appreciation for the variety of means by which plants manage their uptake and use of Pi (Lambers *et al*. 2011; Gibon 2014). For example, several traits that contribute to efficient Pi mining and proficient Pi use have been determined using the Proteaceae *Banksia* spp. and *Hakea prostrata*, plants that are native to Pi-deprived soils of Australia (Denton *et al*. 2007; Lambers *et al*. 2011). These traits include specialized cluster roots that secrete large amounts of organic acids to increase the bioavailability of mineral-bound P under low Pi conditions, a high photosynthetic rate per unit leaf area at low internal Pi concentration, membrane remodelling to reduce phospholipids and increase galactolipids and a high capacity to remobilize and recover Pi from senescent organs (Lambers *et al*. 2011; Kuppusamy *et al*. 2014; Sulpice *et al*. 2014).

We report on the growth and expression of Pi-associated genes in the extremophile crucifer *Eutrema salsugineum* (synonymous with *Thellungiella salsuginea*) grown under varying Pi conditions. *Eutrema* is a close relative of *Arabidopsis* and *Brassica* species, and the accession we used is native to the Yukon Territory of Canada. By comparison with soils that are cropped, the alkaline and saline soil of this region is low in most nutrients including Pi (Day 1962) but high in Ca and Mg (Guevara *et al*. 2012), conditions expected to exacerbate the availability of Pi to plants. Our work shows that *Eutrema* displays important differences compared with *Arabidopsis* when both species are exposed to low Pi conditions including increased vigour under conditions that provoke a classic Pi-starvation response in *Arabidopsis*. We hypothesize that *Eutrema* is adapted to environments where Pi availability is a chronic source of stress and constitutive Pi management, like salt tolerance, is required to confer greater fitness.

MATERIALS AND METHODS

Plant material

Inbred *E. salsugineum* (Pall.) Al-Shehbaz & Warwick (Yukon accession) seeds were used (Champigny *et al*. 2013) and *Arabidopsis thaliana* (L.) Heynh. (Col-0 accession) seeds were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University.

Seedling growth conditions and measurements

Seeds were surface sterilized, re-suspended in 0.1% (w/v) Phytagel (Sigma-Aldrich, Oakville, ON, Canada), stratified in the dark for 48 h at 4 °C and then plated on MS basal salt and vitamins medium (Sigma-Aldrich) solidified with 0.65% (w/v) Phytagel. Seeds germinated under a 24 h photoperiod at 23 °C and 60 μmol photons $m^{-2} s^{-1}$ then at 3 to 4 d post germination (dpg) seedlings were transplanted to nutrient media in 100 × 100 × 15 mm polystyrene BD Falcon™ Integrid plates (BD Biosciences, Mississauga, ON, Canada). The plates were positioned vertically in a growth chamber under a 12 h photoperiod (150 μmol photons $m^{-2} s^{-1}$) at 23 °C.

Defined nutrient medium was composed of variable Pi (no added Pi, 0 up to 2.0 mM Na-PO_4) with the following: 0.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM KNO_3, 0.1 mM $FeSO_4$, 0.1 mM H_3BO_3, 20 μM $MnCl_2$, 1 μM $CuSO_4$, 3 μM $ZnSO_4$, 1 μM Na_2MoO_4, 0.1 μM $CoCl_2$ and 2% (w/v) sucrose. Phytagel (Lot No. 028K0079) or Phytoblend (Lot No. 20801110; Caisson, North Logan, UT, USA) were used at 0.8% (w/v). The Pi content of Phytagel was 1.1 μmol g^{-1}, and for Phytoblend, it was 0.8 μmol g^{-1}. The low iron medium contained 0.01 mM $FeSO_4$, and the salt-supplemented medium had 100 mM NaCl.

The time course from high to low Pi conditions was performed by growing *Eutrema* seedlings on 0.5 mM Pi nutrient media until their primary roots were approximately 40 mm long (7 to 10 d), and then the seedlings were transferred to 0.05 or 0.5 mM Pi nutrient plates. For diurnal experiments, seedlings at 15 dpg were harvested at the midpoint of the light cycle or the midpoint of the dark cycle. Harvested root and

shoot tissue was weighed, flash-frozen in liquid nitrogen and then stored at −80 °C.

Root growth metrics were scored from photographic images using ImageJ 1.42q (Wayne Rasband, National Institutes of Health, USA).

Plant growth conditions and measurements

Stratified seeds were sown on a mix of 71% (v/v) sphagnum peat moss (Premier Tech, Rivière-du-Loup, PQ, Canada), 10% (v/v) coarse perlite (Therm-O-Rock, New Eagle, PA, USA), 5% (v/v) medium vermiculite (Therm-O-Rock), 14% (v/v) Turface® (PROFILE, Buffalo Grove, IL, USA) and 1.85% (w/v) dolomitic limestone (National Lime & Stone, Findlay, OH, USA) producing a pH 6.5 to 7 soil mix. The soil was autoclaved, and then AquaGro 2000L (Aquatrols, Paulsboro, NJ, USA) was added to a final concentration of 0.1% (v/v). The nutrient content of the soil mix is detailed in Table S1. The soil was saturated with a solution containing 5.5 mM $Ca(NO_3)_2$, 5.5 mM KNO_3, 2.3 mM $MgSO_4$, 1.1 mM Fe-EDTA, 51 μM H_3BO_3, 10 μM $MnCl_2$, 0.9 μM $ZnCl_2$, 0.32 μM $CuCl_2$ and 0.11 μM Na_2MoO_4, where 2.5 mM KH_2PO_4 was replaced by variable quantities of KCl to adjust the soil from high to low Pi and maintain K^+ content. Seeds (treated as mentioned earlier) were sown in 4.4 × 7.7 cm pots. *Arabidopsis* plants were grown at 22 °C under a 12 h photoperiod (200 μmol photons $m^{-2} s^{-1}$), and *Eutrema* plants were grown under a 21 h (250 μmol photons $m^{-2} s^{-1}$), 22 °C day and 3 h, 10 °C night (Wong *et al.* 2005). Plants were watered with de-ionized H_2O as needed. Tissue was harvested from vegetative plants at weekly intervals, shoot and root fresh weights (FWs) were measured and samples were flash-frozen in liquid nitrogen for storage at −80 °C. Samples were dried for 48 h at 80 °C for dry weight (DW) measurements. Relative growth rates were calculated from dry weight measurements according to Hoffmann & Poorter (2002).

Anthocyanin content in leaf tissue was measured as described by Kim *et al.* (2003). To account for differences in water content of different tissues, the Versaw & Harrison (2002) Pi determination method was modified in the use of dry and not fresh tissue. Dry root or leaf tissue (50 mg) was ground with liquid nitrogen, and the powder extracted with 1% (v/v) acetic acid. After centrifugation at 10 000 *g* for 10 min, Pi in the supernatant was quantified by the phosphomolybdate colorimetric method (Martin & Tolbert 1983). Pi determinations using dried and fresh tissue were compared, and Pi content estimates from dried tissue are approximately 20% higher ($r^2 = 0.96$), likely due to Pi released from phosphoesters (Leloir & Cardini 1957). Seed P content was determined using the Phytic Acid/Total Phosphorus Kit (Megazyme, Wicklow, Ireland) following the manufacturer's protocol.

Total RNA extraction

RNA was isolated from 100 mg of tissue using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) followed by an on-column DNase digestion step for seedlings. For 4-week-old plants, a scaled-down version of the TRIzol®/hot borate method was used (Champigny *et al.* 2013), and genomic DNA was removed using the Ambion® TURBO DNA-*free*™ kit (Life Technologies, Burlington, ON, Canada) as per manufacturer's instructions. RNA quantity and integrity were determined using an RNA Nano 6000 chip on a Bioanalyzer 2100 instrument (Agilent, Santa Clara, CA, USA).

Gene expression analysis

First-strand cDNA synthesis was performed using the M-MLV reverse transcriptase kit (Sigma-Aldrich) with 2 μg total RNA and oligo dT_{20}. Species and gene-specific primers for RT-qPCR were designed with Primer-BLAST® (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the TAIR10 *Arabidopsis* (www.arabidopsis.org) or *Eutrema* (www.phytozome.net/thellungiella.php) reference genomes and evaluated using mfold® DNA Folding Form (http://mfold.rna.albany.edu/). Primers are listed in Table S2. RT-qPCR reactions (10 μL) included 2 μL cDNA synthesis product, 200 nM primer and 5 μL LuminoCt® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich) and were performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). The optimum annealing temperature was established using a temperature gradient, and primer efficiency was determined using an eight-point standard curve. All primer combinations met the MIQE guidelines of 90 to 110% efficiency (Bustin *et al.* 2009; Taylor *et al.* 2010). Reactions lacking cDNA template or reverse transcriptase were also performed at the optimized conditions. A minimum of three biological replicates consisting of three technical replicates each were analysed. Amplicon sequences were verified by Sanger sequencing.

For relative quantification, RT-qPCR data were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001) on the Bio-Rad CFX Manager 2.0 platform. Unless otherwise stated, expression data are reported relative to the results for the highest Pi treatment. Four reference genes served as internal controls (Table S2).

For absolute RT-qPCR, standard curves relating the number of template copies to C_T values were generated using known quantities of cDNA templates (Lu *et al.* 2012). Templates were generated by conventional PCR (primer sequences in Table S3); the amplicons were separated by agarose gel electrophoresis, purified and then quantified fluorometrically using the QuantiFluor™ dsDNA kit (Promega, Madison, WI, USA) following the manufacturer's directions and 100 ng μL^{-1} λ-DNA for the standard curve. A 10-fold serial dilution series of each cDNA template, quantified in absolute terms, was then used to generate templates ranging from 1 to 1×10^8 copies μL^{-1}. The template dilution series was subjected to RT-qPCR using primers listed in Table S3. RT-qPCR was performed under optimized conditions to obtain the C_T values for the standard curves.

Statistics

Data were analysed using mixed effects analysis of variance (ANOVA) where interactions at $P < 0.05$ (*) were deemed

significant. *P* values lower than 0.01 were denoted by multiple asterisks: **<0.01 or ***<0.001. Least significant difference (LSD) was used for multiple comparisons, and the same letters indicate no statistically significant difference. Smooth regression analysis at 95% confidence interval (grey shade) and line of best fit (black line) were performed for Figs 2a,b,c, 4c, and 5c,d. Shoot allocation (Fig. 3b) was obtained from the least square mean from an analysis of covariance (ANCOVA) with seedling root FW as the dependent variable and the shoot FW as the covariate (McConnaughay & Coleman 1999; Poorter *et al.* 2012). For leaf and root Pi content (Figs 3c & 8e), a type III multivariate ANOVA (MANOVA) Pillai test statistic was employed. R version 2.13.1 was used for all statistical tests (R Core Team 2014).

RESULTS

Eutrema seedling growth in low Pi media

Eutrema seedlings grown on sterile gel media supplemented with 0.05 mM Pi had a root architecture and shoot size that was similar to those grown on 0.5 mM Pi (Fig. 1a). *Arabidopsis* seedlings grown in parallel displayed a classical Pi deficiency phenotype of shortened primary roots, proliferation of lateral roots and noticeably reduced shoot biomass on the 0.05 mM Pi versus 0.5 mM Pi medium (Fig. 1a). Given the lack of a clear Pi deficiency phenotype for *Eutrema* seedlings, we analysed seedling growth patterns including primary root elongation, the length and density of lateral roots and the shoot biomass allocation in response to varying Pi conditions. *Eutrema* seedlings grown on low Pi (0.05 mM) treatment plates showed a modest, 1.3-fold slower rate of root growth compared with the roots of seedlings on 0.5 mM Pi medium (Fig. 1b) and reached the bottom of the plate within 10 d. In contrast, *Arabidopsis* seedlings grew 2.8-fold slower on low Pi relative to high Pi medium, and seedling roots did not reach the bottom of the plate by 10 d.

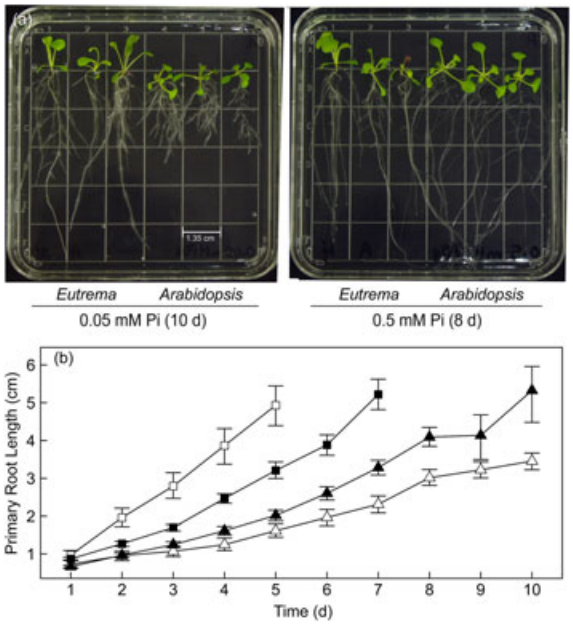

Figure 1. Pi deficiency does not inhibit *Eutrema* primary root growth. (a) Representative seedlings on nutrient media supplemented with 0.05 or 0.5 mM Pi. (b) Primary root length of *Eutrema* (closed) and *Arabidopsis* (open) grown on 0.05 (△, ▲) or 0.5 (□, ■) mM Pi media for up to 10 d (mean ± SE, $n = 10$). The figure shows one of two independent experiments, all with similar results.

Significant differences in seedling root growth were apparent by day 4 (Fig. 1b). Total root length for *Eutrema* seedlings grown 4 d with and without added Pi ranged from 20 to 80 mm with the contribution of lateral roots providing the major source of variability (Fig. 2a,b). *Eutrema* seedlings showed no difference in lateral root density associated with Pi treatment, although seedlings on high Pi with longer primary roots tended to have the lowest lateral root density (Fig. 2c). For *Arabidopsis*, total root lengths ranged from 20 to 100 mm, but no overlap was seen between measurements for seedlings on 0.5 mM Pi and those grown on 0 or 0.05 mM Pi media (Fig. 2a, b). Moreover, *Arabidopsis* seedlings on 0.5 mM Pi had significantly longer total roots, including longer primary and lateral roots, and a lower lateral root density relative to seedlings grown on 0.05 mM Pi media (Fig. 2a,b,c).

The difference in the Pi-responsive root growth behaviour between *Eutrema* and *Arabidopsis* is summarized by the norms of reaction plots of Fig. 2d. *Eutrema* root length increased through equivalent contributions of primary and lateral roots, whereas this was only true for *Arabidopsis* seedling roots grown on 0.5 mM Pi. Furthermore, *Eutrema* primary and lateral root growth was comparatively unresponsive to Pi treatment. In contrast, when comparing seedling growth on 0, 0.05 and 0.5 mM Pi, *Arabidopsis* primary and lateral roots were significantly shorter on 0 and 0.05 mM Pi where the primary root length contributed about 20% more to total root length than did the lateral roots. Together, the data shown in Fig. 2 are consistent with Pi deprivation reducing both primary and lateral root length and increasing lateral root density in *Arabidopsis* as reported by Linkohr *et al.* (2002). However, by these criteria, the inhibitory impact of low Pi on total root growth, specifically on the length of *Eutrema* lateral and primary roots, was comparatively modest relative to the striking differences observed for *Arabidopsis* (Figs 1 & 2).

Eutrema and *Arabidopsis* seedlings accumulated the least total biomass in the absence of added Pi and the most biomass in 0.5 mM Pi conditions (Fig. 3a). However, *Eutrema* seedlings showed significantly greater biomass at all the Pi levels tested with a significant, 2-fold higher biomass relative to *Arabidopsis* when no Pi was added to the medium. Moreover, the distribution of biomass between roots and shoots differed between the two species (Fig. 3b). *Arabidopsis* seedlings allocated proportionately more biomass to roots than shoots under low Pi relative to high Pi conditions, while *Eutrema* seedlings showed no Pi-responsive change in biomass allocation. The lack of differential biomass between the roots and shoots of *Eutrema* seedlings is evidence that the above-ground and below-ground organs of *Eutrema* are served equally in terms of allocated resources under Pi starvation. When expressed on a dry weight basis, *Eutrema* seedling roots consistently had a lower Pi content compared with *Arabidopsis* seedlings irrespective of Pi treatment level (Fig. 3c). However, relative to *Arabidopsis*,

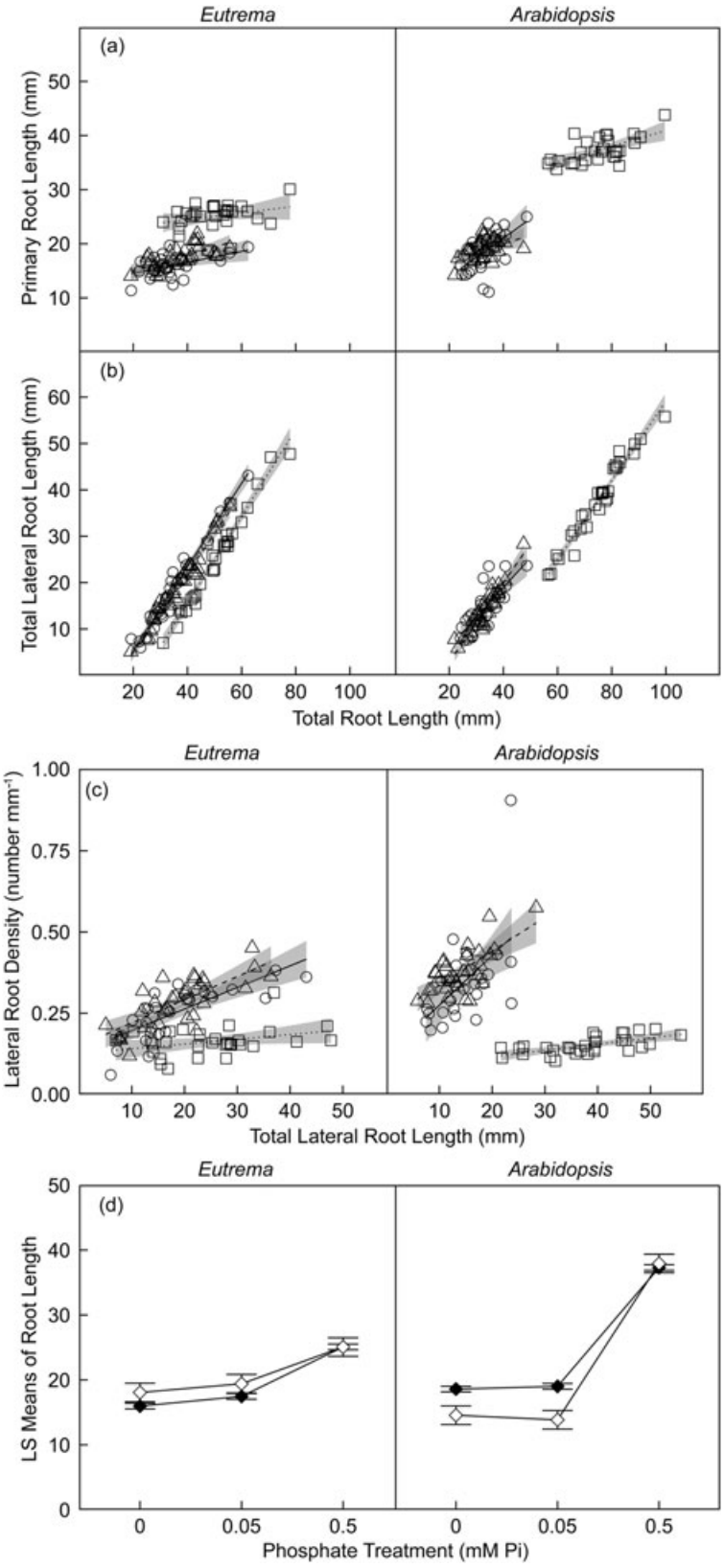

Figure 2. Pi deficiency does not alter *Eutrema* root architecture. (a) Primary root length versus total root length, (b) lateral root length versus total root length and (c) lateral root density versus lateral root length of seedlings measured 4 d after transfer to treatment plates containing 0 (○), 0.05 (△) or 0.5 (□) mM Pi ($n = 30$). (d) Least square (LS) mean ± SE from MANOVA of primary root length (◊, $n = 30$) and lateral root length (♦, $n = 30$) with Pi treatment as the dependent variable. This figure shows one of three independent experiments, all with similar results.

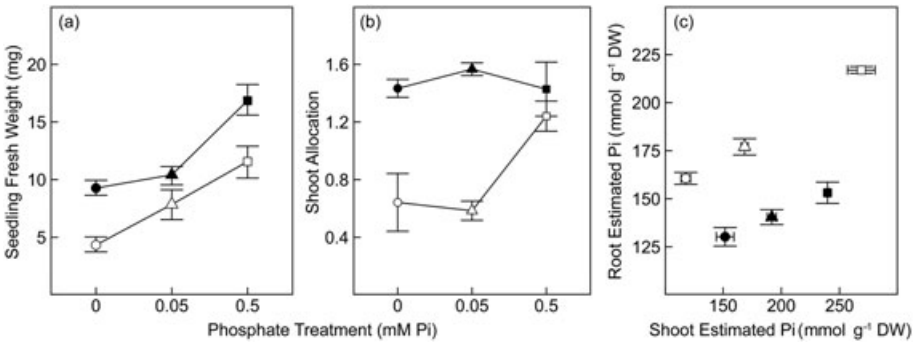

Figure 3. *Eutrema* and *Arabidopsis* seedlings show different biomass and Pi allocation in response to low Pi. (a) Seedling FW, (b) shoot allocation and (c) Pi content of *Eutrema* (closed) and *Arabidopsis* (open) seedlings. Data mean ± SE with $n \geq 15$, where n is a pool of 6 seedlings grown for 6 d on medium supplemented with 0 (○, ●), 0.05 (△, ▲) or 0.5 (□, ■) mM Pi. Shoot allocation is the proportion of shoot to root biomass corrected for ontogenetic drift (Materials and Methods). Symbols for genotypes are connected with a line for ease of comparison and not to indicate intermediate values. This figure shows one of three independent experiments, all with similar results.

Eutrema seedlings had a greater biomass (Fig. 3a) and, as such, had a higher Pi content per seedling (Table S4). This outcome indicates that *Eutrema* seedlings removed more Pi from the same gel-based medium than *Arabidopsis* seedlings.

Effect of external iron concentration on Pi-starved Eutrema seedlings

Ward *et al.* (2008) reported that *Arabidopsis* primary root growth inhibition under Pi starvation is due to Fe toxicity because seedlings developed long primary roots on low Pi medium when the Fe concentration in the plates was reduced from 0.1 to 0.01 mM. We also found that reducing the Fe concentration in the medium to 0.01 mM for 0.05 and 0.5 mM Pi treatments led to *Arabidopsis* roots that were similar in appearance (Fig. 4a,b). Decreasing the Fe concentration in the media improved primary root elongation for *Eutrema* seedlings at both Pi concentrations, but the outcome differed in one important respect, primary roots of *Eutrema* seedlings on high Pi with high Fe grew as well as those roots on low Pi with low Fe (Fig. 4b). Another key difference between the species was

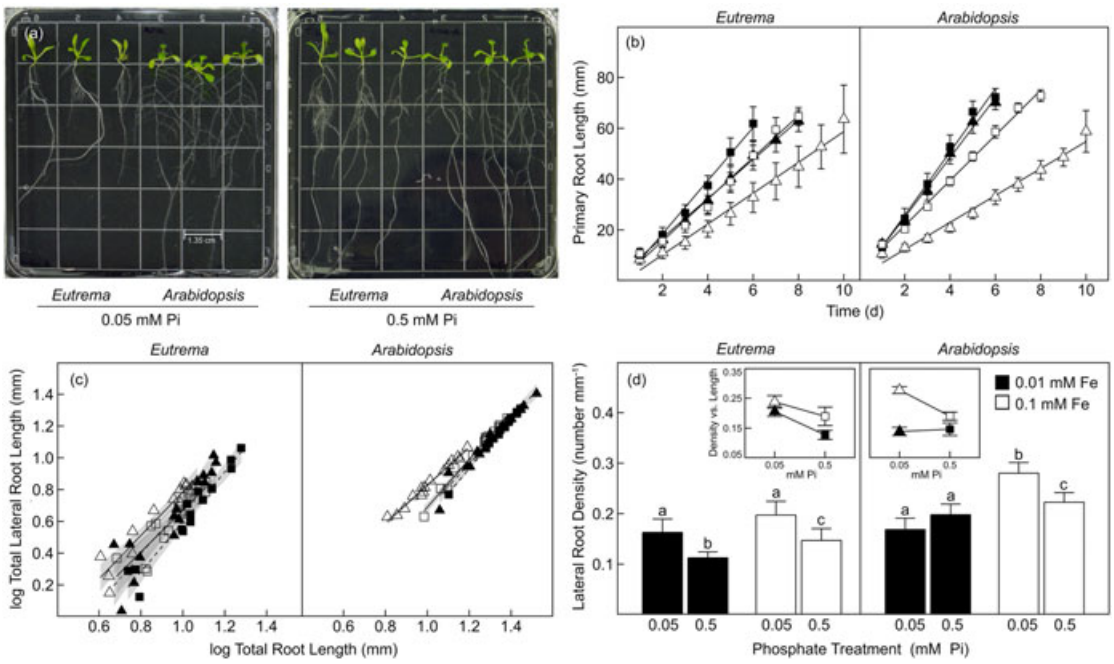

Figure 4. *Eutrema* and *Arabidopsis* seedlings respond differently to Fe in Pi-deficient media. (a) Seedlings on media with 0.01 mM Fe. (b) Primary root length over time and (c) day 6 lateral root length versus total root length for seedlings on 0.05 mM Pi + 0.1 mM Fe (△), 0.5 mM Pi + 0.1 mM Fe (□), 0.05 mM Pi + 0.01 mM Fe (▲) or 0.5 mM Pi + 0.01 mM Fe (■). (d) Day 6 lateral root density for plants grown on media containing 0.01 mM Fe (filled bars) and 0.1 mM Fe (open bars). Figure 4d inset is the plot of estimated marginal means for lateral root density and length as independent and dependent variables, respectively (symbols as per b,c). For panels b and d, the mean ± SE for $n \geq 15$ is shown. The figure shows one of three independent experiments, all with similar results.

that higher concentrations of Pi supported faster growth rates for *Eutrema* primary roots whether the Fe concentration of the medium was high or low, whereas for *Arabidopsis*, the impact of decreasing Fe on root elongation was independent of Pi concentration.

Using seedling root measurements for day 6, we examined the effects of high Fe on the growth and density of lateral roots (Fig. 4c,d). Total root and total lateral root length of *Eutrema* seedlings were unaffected by the Fe concentration in the medium, and a reduction in Pi was associated with increased lateral root density that was independent of Fe concentration. In contrast, *Arabidopsis* seedlings on low Pi with high Fe had significantly decreased total root and total lateral root length and reduced lateral root density with either low Pi or a combination of low Fe with high or low Pi. The response curves (insets, Fig. 4d) relate changes in both lateral root density and lateral root length with Pi and Fe and underscore that low Pi is a critical factor for eliciting a high root density phenotype for *Eutrema* seedlings, whereas the concentrations of both Pi and Fe must be manipulated to alter this trait in *Arabidopsis* seedlings.

Response of Eutrema to Pi deprivation and high salt

Eutrema is a halophyte, so seedling growth was tested on plates containing 100 mM NaCl with 0.05 or 0.5 mM Pi (Fig. 5). *Eutrema* seedlings grew more slowly on plates containing 100 mM NaCl relative to plates without added salt but grew faster on 0.5 mM Pi compared with 0.05 mM Pi when salt was present (Fig. 5a,b). As expected, *Arabidopsis* seedlings showed poor growth on these plates even with 0.5 mM Pi present (Fig. 5a,b). Measurements on day 8 showed that salt decreased lateral root length for seedlings of both species under low or high Pi treatments (Fig. 5c). However, Fig. 5d shows that the relative contribution of lateral roots to total root length for *Eutrema* did not change when plants were grown on 0.05 or 0.5 mM Pi, while for *Arabidopsis*, the reduction in total root length with salt represented, in large part, the reduced contribution of lateral roots. For *Eutrema*, the addition of 100 mM NaCl to the high Pi medium led to the same lateral root density as seedlings on low or high Pi that were not exposed to salt. Salt added to the low Pi medium led to a significant increase in lateral root density resulting in a root architecture resembling the Pi-starvation phenotype of *Arabidopsis* (Fig. 5e). This outcome is consistent with the hypothesis that *Eutrema* seedlings exposed to 100 mM NaCl and low Pi were deprived for Pi. In contrast, for *Arabidopsis*, the addition of salt to either Pi concentration led to an increase in lateral root density.

Expression of Pi-associated genes in Eutrema seedlings on a low Pi regime

The low Pi phenotype for *Eutrema* seedlings was comparatively modest relative to the response of *Arabidopsis* seedlings grown under the same conditions (Fig. 1a). This raised a question of whether our growth conditions actually deprived

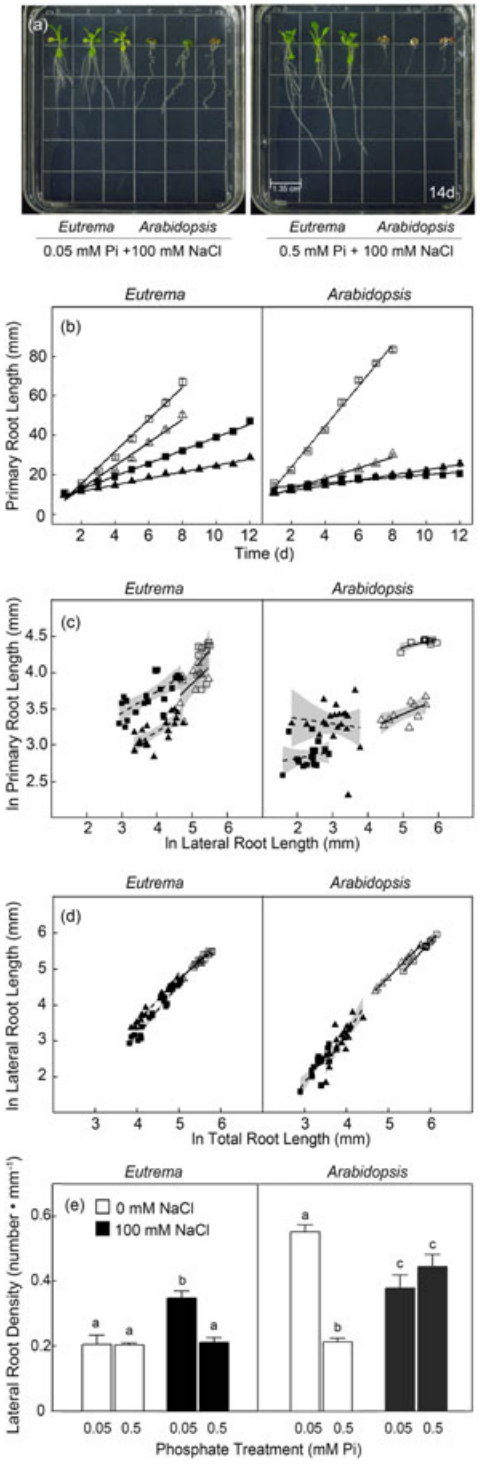

Figure 5. *Eutrema* seedlings under Pi-deficient conditions show increased lateral root density with salinity. (a) Seedlings grown with 100 mM NaCl. (b) Primary root length on media with 0.05 mM Pi (△), 0.05 mM Pi + 100 mM NaCl (▲), 0.5 mM Pi (□) or 0.5 mM Pi + 100 mM NaCl (■); each point is the mean ± SE, $n \geq 33$. Day 8 measurements ($n \geq 10$) for (c) primary root length versus lateral root length, (d) total root length versus lateral root length (symbols are means and as per panel b) and (e) lateral root density means ± SE for seedlings grown on media without NaCl (open bars) or with 100 mM NaCl (filled bars). The figure shows one of three independent experiments, all with similar results.

Eutrema seedlings of Pi. As such, we monitored the expression of several genes orthologous to *Arabidopsis* Pi-starvation inducible (PSI) genes including *EsIPS2*, *EsRNS1* and *EsWRKY75* by RT-qPCR (Fig. 6).

EsIPS2 and *EsRNS1* transcript abundance was over 20-fold greater in *Eutrema* shoots (Fig. 6a) and 4-fold greater in roots (Fig. 6b) from seedlings on 0.005 mM Pi compared with 0.5 mM Pi plates. With respect to transcription factors, *EsWRKY75* transcript abundance underwent a 20-fold and 4-fold increase in *Eutrema* seedling shoots and roots, respectively, when grown on low Pi, but we found no difference in the expression of *EsPHR1* using the same samples (Fig. 6). In *Arabidopsis*, *AtPHR1* either is only modestly responsive to external Pi (Rubio *et al.* 2001; Morcuende *et al.* 2007) or shows no increase in expression under Pi-deficient conditions (Nilsson *et al.* 2007); the latter situation is consistent with our observations for *Eutrema*.

AtPHT1;4 and AtPHT1;1 are considered to be the two main high-affinity Pi transporters in *Arabidopsis*, and their genes both show strong Pi-responsive up-regulation in *Arabidopsis* roots under Pi-limiting conditions (Shin *et al.* 2004). For *Eutrema*, however, *EsPHT1;4* transcripts were only 1.3-fold more abundant in roots of seedlings grown under 0.005 mM Pi compared with those on 0.5 mM Pi nutrient media (Fig. 6b), and there is no *PHT1;1* ortholog present in the *Eutrema* genome (www.phytozome.net/thellungiella.php). The apparent absence of a gene encoding PHT1;1 bore further investigation and Fig. S1 is a phylogeny of the *PHT1* gene family in several Brassicaceae. This phylogeny shows that there is a single copy of *PHT1;4* in *Eutrema* along with several copies of other genes encoding putative high-affinity Pi transporters including several genes encoding orthologs of *PHT1;3*. With negligible change in *EsPHT1;4* expression and no ortholog for *PHT1;1*, it is possible that *EsPHT1;4* is chiefly post-translationally regulated in *Eutrema* or other high-affinity Pi transporters may preferentially operate in this species. The *Eutrema* genome encodes a single ortholog annotated as the low-affinity Pi transporter, *EsPHT2;1* (www.phytozome.net/thellungiella.php). PHT2;1 is involved in translocation of Pi from older to younger leaves (Versaw & Harrison 2002). Wheat *TaPHT2;1* shows increased expression in Pi-starved plants (Guo *et al.* 2013). We determined *EsPHT2;1* expression and found it to be 4-fold lower in shoots of seedlings grown on 0.005 mM Pi relative to 0.5 mM Pi media (Fig. 6a).

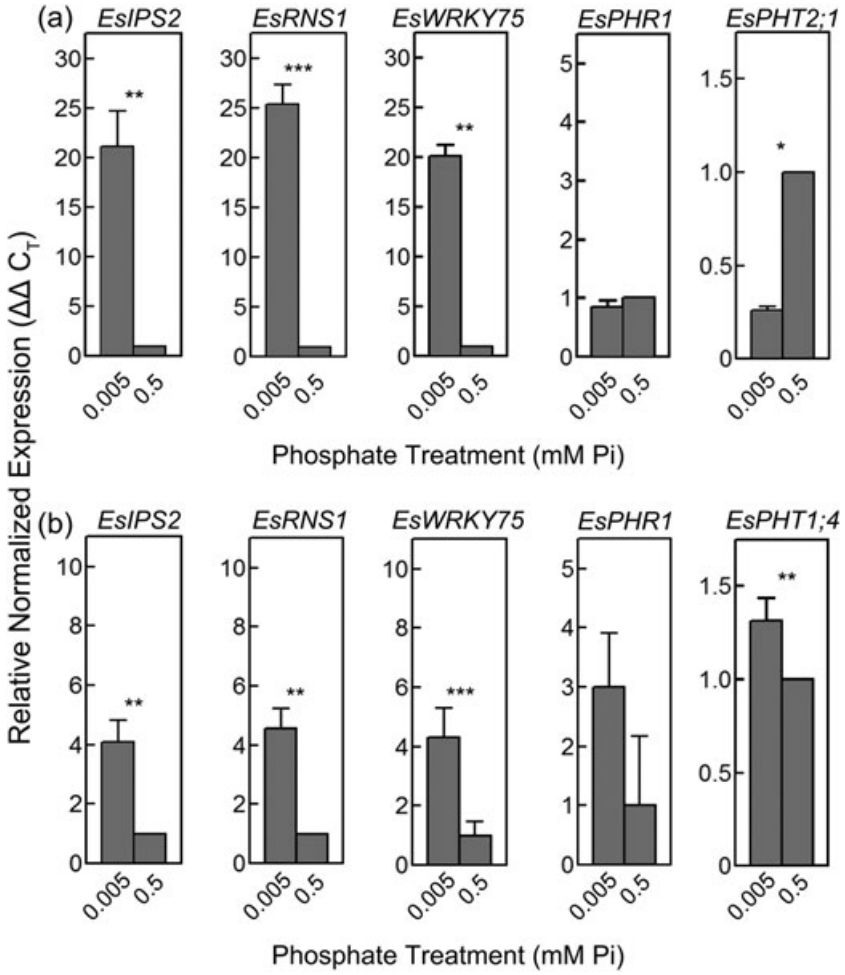

Figure 6. Increased expression of PSI genes in *Eutrema* seedlings grown on low Pi. (a) Shoot and (b) root tissues were used for cDNA preparation and RT-qPCR measurements. Transcript abundance reported as mean fold-change ± SE relative to 0.5 mM Pi-treated seedlings with $n = 3$ where n is a pool of twelve 8-day-old seedlings with each pool from a replicate experiment.

The induction of several PSI genes, most notably *EsIPS2*, is evidence that the seedlings on low Pi media were responding to a deficiency in Pi. To titre this response, we exposed seedlings to a range of Pi concentrations in the media (0 to 2.0 mM Pi) and quantified *EsIPS2* transcripts by RT-qPCR. The most significant induction of *EsIPS2* transcripts that we observed was 141-fold in shoots of *Eutrema* seedlings grown on media containing no added Pi compared with 2.0 mM Pi and levels of Pi in excess of 0.125 mM were sufficient to significantly reduce its expression (Fig. 7a). In comparison, *EsIPS2* expression

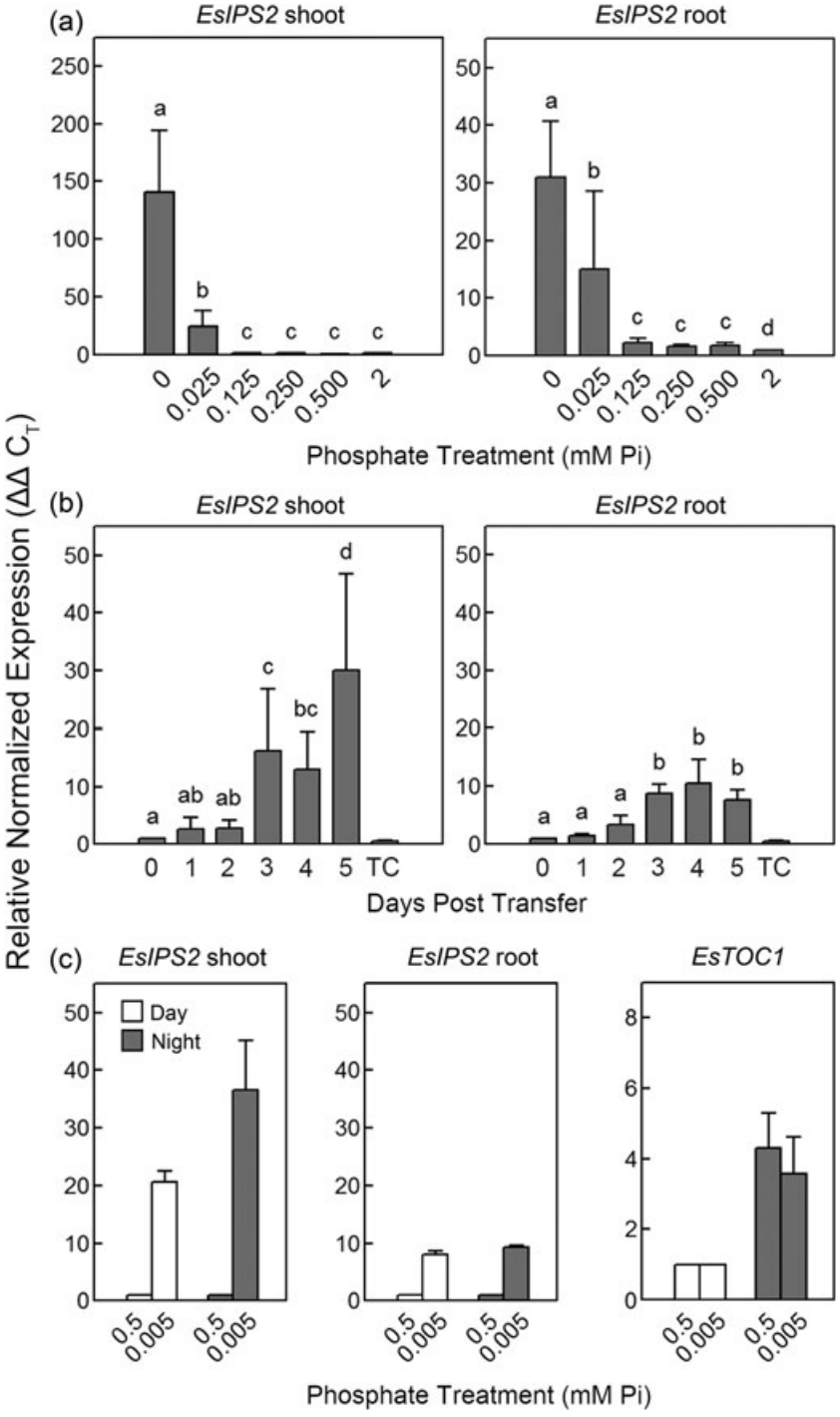

Figure 7. *EsIPS2* expression of *Eutrema* seedlings is responsive to changes in external Pi. (a) *EsIPS2* transcript abundance as a function of Pi content of medium. Data are normalized to expression of *EsIPS2* on 2 mM Pi. (b) *EsIPS2* expression following transfer of seedlings from 0.5 to 0.05 mM Pi-containing plates with data reported relative to expression on day 0. TC, transfer control, seedlings moved from 0.5 onto 0.5 mM Pi plates. (c) Diurnal expression analysis of *EsIPS2* in seedlings. Mean fold-expression ± SE reported relative to day or night, 0.5 mM Pi. For *EsTOC1*, normalized expression for night was relative to day and used as positive control for circadian expression. Transcript abundance reported as mean ± SE and $n = 3$ (a, b) or $n = 2$ (c), where n is a pool of 12 seedlings with each pool from an independent experiment.

was increased 31-fold in *Eutrema* roots growing in medium with no added Pi, and low but detectable transcript expression was observed even for seedlings on 2 mM Pi (Fig. 7a). On a timescale, *EsIPS2* expression doubled in seedling shoots within 1 d of transfer from 0.5 to 0.05 mM Pi media, and expression further increased about 30-fold by 5 d (Fig. 7b). In roots, the relative increase in the expression of *EsIPS2* was generally lower than that observed in shoots but also increased significantly within 5 d following transfer of seedlings from high to low Pi. We observed no discernible increase in *EsIPS2* expression with a mock transfer of seedlings and sampling during the day or night did not alter the results (Fig. 7c).

Impact of Pi deficiency on phenotype of soil-cultivated Eutrema plants

Eutrema and *Arabidopsis* plants were grown on a low Pi soil mix for 4 weeks (Table S1). *Arabidopsis* plants under the 0 mM Pi treatment (no Pi added) showed senescent older leaves and reduced rosette biomass relative to plants fertilized with a nutrient solution containing 2.5 mM Pi (Fig. 8a,b). In contrast, *Eutrema* plants fertilized with either 0 or 2.5 mM Pi were indistinguishable, and there was no difference in *Eutrema* rosette biomass (Fig. 8a,b) or the relative growth rate of plants associated with Pi during the 4 week period (Fig. 8c). Anthocyanin content was determined, and no correlation between leaf anthocyanin content and treatment was found for *Eutrema* plants, whereas *Arabidopsis* plants lacking added Pi showed a 4-fold increase in anthocyanins relative to plants treated with 2.5 mM Pi (Fig. 8d).

A reduction in Pi content is an early response to low Pi availability (Raghothama 1999; Al-Ghazi *et al*. 2003). Given the lack of phenotypic changes for *Eutrema* plants experiencing low Pi conditions, we estimated the Pi content of leaves and roots. *Eutrema* grown with 2.5 mM Pi had a higher shoot and root Pi content relative to the same tissues in *Arabidopsis* plants (Fig. 8e). This outcome suggests that *Eutrema* roots extract more Pi from the growth medium when higher Pi is available. However, there was no difference with respect to Pi content for shoot and root tissue between *Eutrema* and *Arabidopsis* plants grown with the 0 mM Pi solution. Of interest, while the low Pi conditions did not lead to differences in leaf or root Pi content, the *Arabidopsis* plants showed reduced shoot biomass, whereas *Eutrema* plants showed no difference (Fig. 8b). The lack of adverse impact for *Eutrema* with low internal Pi indicates this species must manage lower Pi levels more efficiently to mitigate deficiency symptoms, whereas *Arabidopsis* plants at a comparably low internal Pi content are showing signs of Pi shortage (Fig. 8a,e). These results indicate that mechanisms underlying Pi management for the two species are different.

Pi-associated transcriptional response of soil-cultivated Eutrema plants

In the absence of added Pi, there was little phenotypic evidence that *Eutrema* plants were experiencing a Pi limitation except for a lower level of Pi in leaves and roots (Fig. 8). We then examined the expression of known *Arabidopsis* PSI gene orthologs in *Eutrema* plants. *EsIPS2* transcript levels increased 40-fold and 23-fold in the leaves and roots, respectively, for plants grown without added Pi (0 mM Pi) relative to the 2.5 mM Pi treatment (Fig. 9), an indication that the plants were responding to low Pi. In contrast, the transcript abundance of *EsWRKY75* was unchanged while *EsRNS1* and *EsPHR1* expression was higher in the roots but not in the leaves of *Eutrema* plants grown in 0 mM Pi relative to those given the 2.5 mM Pi treatment (Fig. 9). However, the orthologous genes in *Arabidopsis* were all more highly expressed in shoots but not roots of Pi-deprived plants. We next compared the expression of *EsPHT1;4*, *EsPHT1;5* and *EsPHT1;8*. All three of these *PHT1* genes showed increased transcript abundance in *Eutrema* roots of plants grown without added Pi relative to those grown with 2.5 mM Pi, with *EsPHT1;5* showing the greatest expression difference at almost 230-fold and *EsPHT1;4* the least at 4-fold (Fig. 10). We also used RT-qPCR to estimate transcript abundance associated with the gene having the highest degree of sequence similarity with *AtPHT1;3* (Thhalv10003183; *EsPHT1;3*). The transcript abundance of this gene was 70-fold higher in *Eutrema* roots for plants grown without added Pi compared with 2.5 mM Pi (Fig. 10). The expression of *EsPHT2;1* was determined in leaves, and we found no Pi-associated differential expression of *EsPHT2;1* (Fig. 10). The orthologous purple acid phosphatase (*PAP*) genes *EsPAP7*, *EsPAP8*, *EsPAP13*, *EsPAP15* and *EsPAP17* were all significantly up-regulated in *Eutrema* roots when plants were grown under 0 mM Pi rather than 2.5 mM Pi conditions (Fig. 11). Interestingly, of this group, only *PAP17* is strongly induced by Pi limitation in *Arabidopsis* seedlings (Misson *et al*. 2005; Morcuende *et al*. 2007).

With the exception of *EsIPS2*, we observed modest changes in the expression of PSI genes in leaves (Fig. 9). However, more significant increases in the expression of these genes, including Pi transporter genes, were found in roots of *Eutrema* grown with no Pi added (Figs 9 & 10). For *Arabidopsis* (Karthikeyan *et al*. 2002) and tomato (Muchhal & Raghothama 1999), the expression of genes encoding Pi transporters is positively correlated with Pi transporter activity. If this holds true for *Eutrema*, then the expression data of Fig. 10 indicate that plants deprived of Pi have an increased capacity for Pi uptake, allocation and/or recycling. It is possible that these changes could lead to a sufficient supply of Pi in leaves to reduce the induction of PSI genes. However, the leaf Pi content of 0 mM Pi *Arabidopsis* and *Eutrema* plants is comparably low, so we would expect to see leaf PSI gene induction in both species and not just *Arabidopsis* (Figs 8d & 9). Alternatively, this outcome could also be explained if PSI gene transcript abundance was already high in leaves of 2.5 mM Pi-treated *Eutrema*, thereby reducing the need for more transcripts under stress.

To address this possibility, we quantified the absolute number of transcripts associated with *EsIPS2*, *EsRNS1*, *EsWRKY75* and *EsPHR1* in leaves of *Eutrema* plants grown under 0 or 2.5 mM Pi conditions. Table 1 shows that plants treated with 2.5 mM Pi expressed *EsRNS1*, *EsWRKY75* and *EsPHR1* at levels similar to or higher than the increased transcript levels of their orthologs in Pi-starved *Arabidopsis* plants.

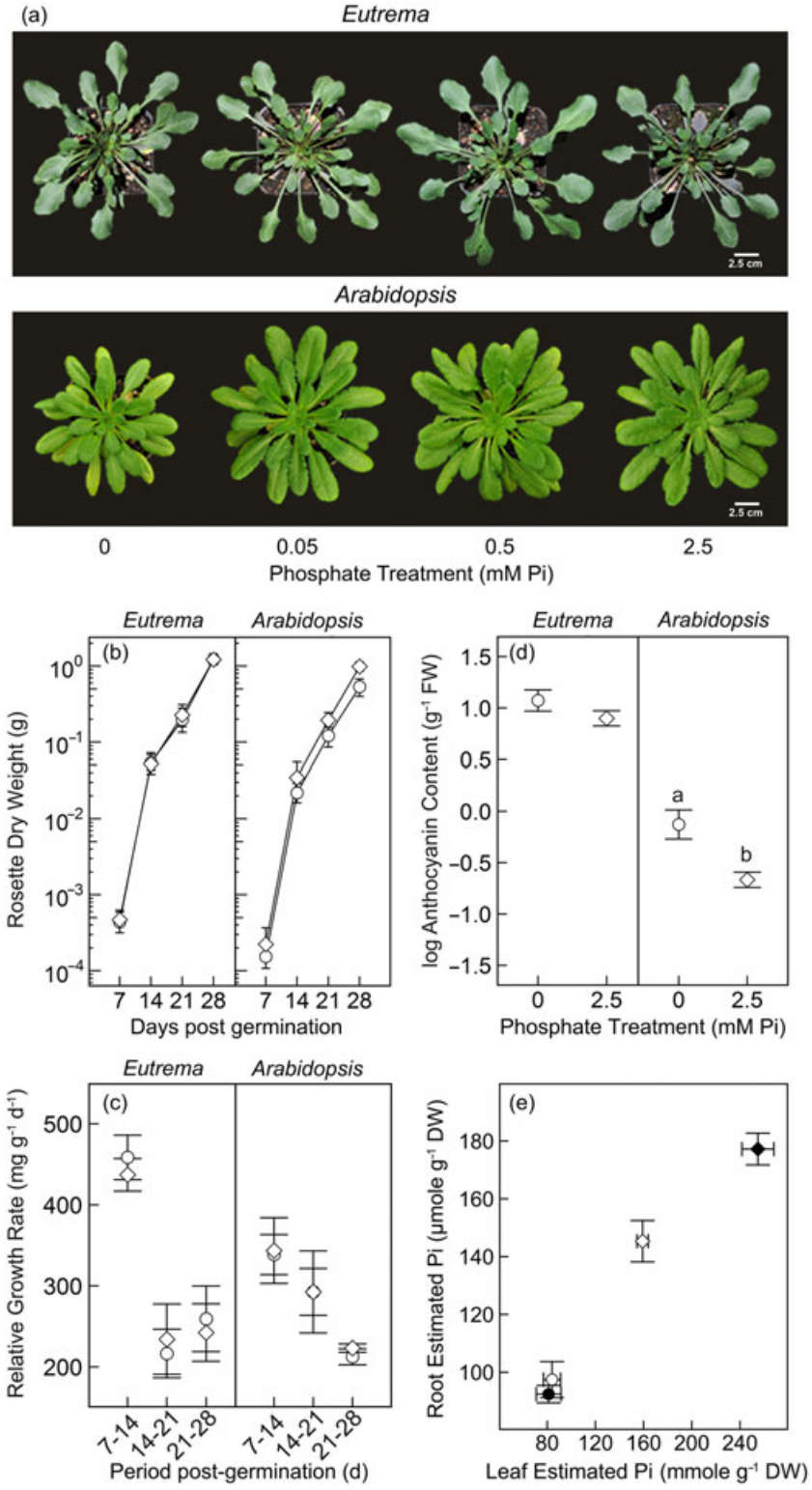

Figure 8. Phenotypic responses of soil-grown *Eutrema* and *Arabidopsis* plants to Pi deprivation. (a) Representative 4-week-old plants treated using nutrient solution without Pi (0 mM Pi) and containing 0.05, 0.5 or 2.5 mM Pi. Triplicate determinations performed for each of three, independent, biological replicates (data are mean ± SE) describing (b) rosette biomass and (c) relative growth rate of plants grown without Pi (0 mM Pi, ○) or with 2.5 (◊) mM Pi. (d) Leaf anthocyanin content of 4-week-old plants (data are mean ± SE, $n \geq 8$ from a minimum of two independent experiments for each treatment). (e) Pi content of leaves and roots of 4-week-old *Eutrema* (closed) and *Arabidopsis* (open) plants (data are mean ± SE, $n \geq 6$) exposed to 0 (○,●) or 2.5 (◊,◆) mM Pi. One of two experiments is shown; both had similar results.

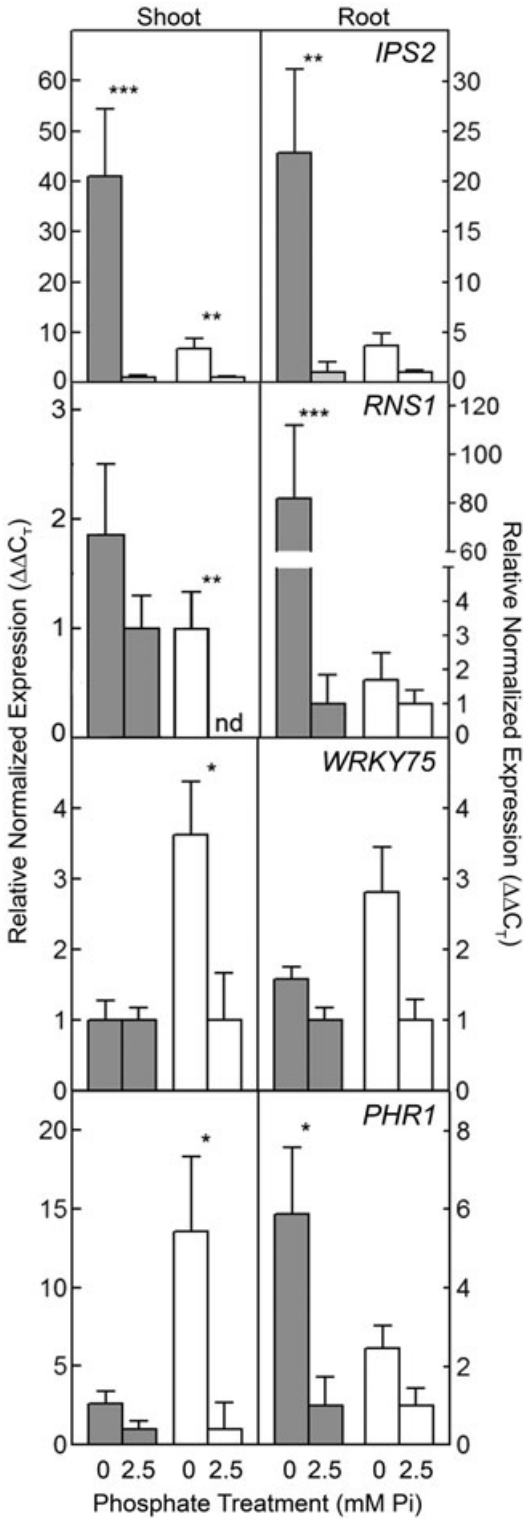

Figure 9. Genes involved in the Pi-starvation response are differentially expressed in the *Eutrema* and *Arabidopsis* plants. Relative transcript abundance was estimated by RT-qPCR for shoots and roots of 4-week-old *Eutrema* (closed, $n \geq 11$) and *Arabidopsis* (open, $n \geq 5$) plants. Gene expression values are presented as mean fold-differences ± SE relative to 2.5 mM Pi-treated plants. As *RNS1* transcript was not detected (nd) in the shoots of 2.5 mM Pi-treated *Arabidopsis* plants, transcript abundance at 0 mM Pi was set to 1. RT-qPCR measurements included three technical repeats.

This outcome is noteworthy because 2.5 mM Pi is a condition under which *Arabidopsis* showed little to no detectable transcripts associated with these genes (Fig. 9). For plants grown with 0 and 2.5 mM Pi, *EsRNS1*, *EsWRKY75* and *EsPHR1* transcript abundance in *Eutrema* leaves was variable but not statistically different, whereas *EsIPS2* transcripts were significantly different (Table 1). These findings agree with the relative abundance results shown in Fig. 9.

DISCUSSION

Low Pi elicits a determinate developmental programme for primary and lateral roots in *Arabidopsis* that ultimately alters root system architecture (Sánchez-Calderón *et al.* 2005). In soil, available Pi generally decreases with depth, so a shortened primary root with many lateral roots is considered to be an adaptive trait to Pi limitation (Williamson *et al.* 2001). However, that adaptive mechanism does not satisfactorily explain the response of *Eutrema* roots to low Pi. Low Pi did not alter *Eutrema* seedling root system architecture, manipulating Fe did not alter this response and only low Pi in combination with 100 mM NaCl increased lateral root density to where *Eutrema* roots resembled the Pi-starvation phenotype shown by *Arabidopsis* (Fig. 5). The lack of comparable phenotypic changes for *Eutrema* under low Pi suggests that Pi nutrient stress is not a major factor instigating alterations to root development.

The *Eutrema* accession used in this study is found in the semi-arid Yukon, a region that is frequently exposed to water deficits (Guevara *et al.* 2012), a stress where having longer roots would be advantageous for water acquisition (Ho *et al.* 2005). Water availability may be the more determining stressor for *Eutrema* in its native habitat, and hence, the development of long roots may be a specialization where limited plasticity for this trait would be advantageous. Lengthening lateral roots (Fig. 2b) may provide a suitable solution to improving the nutrient scavenging capacity for *Eutrema* given that Pi levels tend to be higher near the soil surface. Alternatively, an elevated Pi content of seeds is another mechanism that would be advantageous in that it would allow *Eutrema* seedlings to establish under low Pi conditions and facilitate the growth of mature plants. This is an adaptive trait reported for plants that have evolved on soils deficient for P (Lambers *et al.* 2011). *Eutrema* seeds, at about 22 μg per seed, resemble the average mass of *Arabidopsis* seeds, and therefore, both species have minimal seed storage capacity for P as Pi or phytate (Table S4; Stevenson-Paulik *et al.* 2005). We compiled Pi estimates for *Eutrema* and *Arabidopsis* seeds, total seedlings and entire rosettes (Table S4). Seed total P content for *Eutrema* was approximately 2-fold higher compared with that for *Arabidopsis* seed, but the 110 ng per seed only contributes about 1% of seedling (shoot plus root) Pi and only 0.01% of the estimated Pi in the total rosette of a 4-week-old plant. Thus, *Eutrema* seeds are a negligible source of P, and growth of the plant is reliant upon the acquisition of Pi from the environment to meet requirements for this nutrient.

Pi starvation also alters sucrose movement, leading to increased photosynthate allocation to roots favouring root

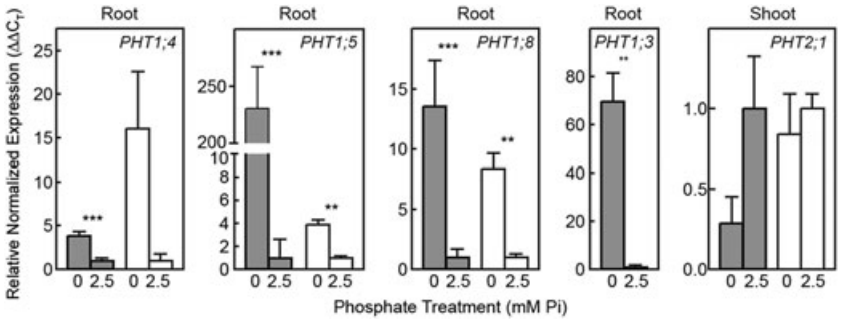

Figure 10. Comparative expression of genes encoding Pi transporters (PHTs) in *Eutrema* and *Arabidopsis* plants. RT-qPCR was used to analyse gene expression in roots (*PHT1;4*, *PHT1;5*, *PHT1;8* and *PHT1;3*) and shoots (*PHT2;1*) of 4-week-old *Eutrema* (closed, $n \geq 11$) and *Arabidopsis* (open, $n \geq 5$) plants. Gene expression values are presented as mean fold-differences ± SE relative to 2.5 mM Pi-treated plants. RT-qPCR measurements included three technical repeats.

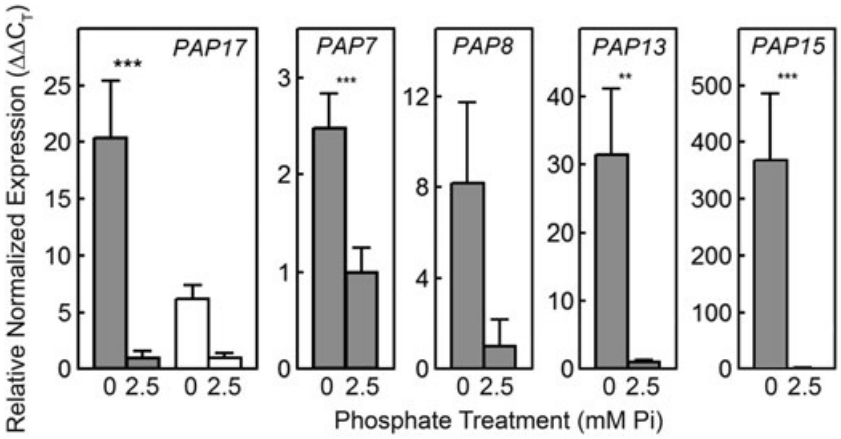

Figure 11. Purple acid phosphatase (*PAP*) gene expression in *Eutrema* and *Arabidopsis* plants in response to Pi treatment. RT-qPCR was used to analyse *PAP* gene expression in *Eutrema* (closed, $n \geq 11$) and *Arabidopsis* (open, $n \geq 5$) roots of 4-week-old plants during Pi limitation. Gene expression values are presented as mean fold-differences ± SE relative to 2.5 mM Pi-treated plants. RT-qPCR measurements included three technical repeats.

Table 1. Absolute transcript abundance of genes expressed in leaves of *Eutrema* and *Arabidopsis* plants

Gene	Treatment (mM Pi)	Transcript abundance (10^3 copies μg^{-1} RNA)		
		Eutrema	*Arabidopsis*	*P* value
IPS2	2.5	0.1 ± 0.0	nd	3.28 ± 10^{-4}
	0	12.9 ± 2.6	0.4 ± 0.1	
RNS1	2.5	9.6 ± 4.1	nd	6.53 ± 10^{-6}
	0	30.2 ± 12.9	10.3 ± 0.4	
WRKY75	2.5	11.7 ± 4.5	0.2 ± 0.2	9.47 ± 10^{-3}
	0	13.7 ± 1.8	7.7 ± 2.1	
PHR1	2.5	49.7 ± 18.9	nd	1.07 ± 10^{-2}
	0	39.6 ± 13.8	6.7 ± 4.7	
EF1a	2.5	2157.4 ± 315.4	1079.6 ± 116.1	0.16
	0	3275.6 ± 1237.1	1073.2 ± 292.5	

The transcript numbers ± SE were determined from plants watered with a nutrient solution without Pi (0 mM) or containing 2.5 mM Pi. *P* values were determined using ANOVA with $\log_{10}$ transcript abundance as dependent variable and the interaction of species and treatment as independent variable (*n* was a minimum of three plants for each species). One of two experiments with similar results is shown.
nd, transcripts not detected using RT-qPCR after 35 cycles.

biomass over shoot biomass (Hammond & White 2008; Wang *et al.* 2010). This change is believed to improve the foraging capacity of the plant for a deficient soil resource. We observed reduced shoot allocation in Pi-deprived *Arabidopsis* seedlings (Fig. 3b), a finding in agreement with reports by other groups (Hermans *et al.* 2006). However, shoot allocation remained constant between low and high Pi conditions for *Eutrema* seedlings (Fig. 3b). Remarkably, seedlings and soil-grown *Eutrema* plants with low internal Pi maintained their above-ground biomass under conditions where *Arabidopsis* was showing signs of Pi deficiency (Figs 3 & 8). Phosphate efficiency (PE) describes the capacity of a plant to maximize its biomass under low Pi conditions (Föhse *et al.* 1988; Wang *et al.* 2010), and our data show that over the 4 week experimental period, *Eutrema* undergoes no significant change in biomass between Pi sufficient and deficient conditions (Fig. 8b). Unchanged biomass and relative growth rates for plants grown on low and high Pi are findings consistent with the conclusion that *Eutrema* has a high PE.

With respect to gene expression, *EsIPS2*, *EsRNS1* and *EsPHR1* all showed Pi-responsive expression in roots of plants grown on low Pi medium but not in the leaves of the same plants (Fig. 9). Presumably, the lack of Pi-responsive expression in leaves is a consequence of their high basal level of expression in leaves, a level that is not affected by external Pi (Table 1). Moreover, this outcome predicts that a higher number of differentially expressed genes with Pi starvation would be associated with the root transcriptome, an expectation not supported by work with other species. Transcriptome profiling of *Arabidopsis* and *Lupinus albus* grown on variable Pi has identified more differentially expressed genes in leaves relative to roots (Wu *et al.* 2003; Bustos *et al.* 2010; O'Rourke *et al.* 2013). Interestingly, in rice, more genes are differentially regulated in the leaves than the roots, at least during early stages of Pi deficiency, but the opposite is observed when rice is exposed to long-term Pi starvation (Oono *et al.* 2011).

Conceivably, an important mechanism enabling *Eutrema* plants to thrive in the extreme soil conditions of its native habitat is its constitutively heightened capacity to utilize Pi in leaves. This would explain the lack of discernible decrease in biomass of *Eutrema* plants grown on low Pi soil despite having leaves with an internal Pi content comparable with that of *Arabidopsis* plants that showed visible signs of Pi deficiency (Fig. 8). It is difficult, however, to extrapolate findings for plants grown in controlled environments to explain how plants cope in their native habitat (Guevara *et al.* 2012). The alkaline soil with high Ca and Mg content is expected to reduce Pi solubility likely requiring plants to improve Pi accessibility through organic anion (malate/citrate) excretion (Sánchez-Calderón *et al.* 2010). Champigny *et al.* (2013) reported on comparisons made between the transcriptomes of *Eutrema* leaves of plants grown in growth cabinets and those from plants collected at a Yukon field site. We used their data in a meta-analysis to compare the absolute transcript numbers associated with *EsIPS2*, *EsRNS1*, *EsWRKY75*, *EsPHR1* and *EsPHL1* (*PHR1*-like) in field and cabinet-grown plants (Fig. S2). The chamber-grown plants in that study were fertilized weekly, while the field plants were in soil of undetermined Pi status in that soil at the base of the plants was not analysed. In this study, we showed that *EsIPS2* is reproducibly responsive to low Pi exposure in shoots of *Eutrema* seedlings (Fig. 6) and leaves of 4-week-old plants (Fig. 9). The elevated expression of *EsIPS2* in one of three field plants tested is consistent with only one of the three field plants profiled as experiencing sufficiently low Pi to elevate transcripts associated with this gene. The field plant with the highest number of *EsIPS2* transcripts also had the highest number of *EsRNS1* transcripts. However, *AtRNS1* is responsive to osmotic stress (Raghothama 1999; Morcuende *et al.* 2007) and the field plants grow in a highly saline environment (Guevara *et al.* 2012), and thus, elevated *EsRNS1* expression is not likely correlated solely to soil Pi. Nonetheless, several studies have found that the expression of *AtWRKY75* is differentially regulated by Pi (Devaiah *et al.* 2007) and *AtPHR1* is not highly Pi responsive (Rubio *et al.* 2001; Morcuende *et al.* 2007). The field plants corroborate our controlled environment chamber studies in that there was no differential expression for *EsWRKY75* or *EsPHR1* between the three field plants and three well-fertilized cabinet plants. That is, neither *EsWRKY75* nor *EsPHR1* showed higher expression in the field plants, including the field plant with the most abundant *EsIPS2* expression. *EsWRKY75* transcript abundance was of equally low abundance in cabinet and field plants, while transcript numbers associated with *EsPHR1* are higher and also equal between plants fertilized in cabinets and plants collected in the field. The activity of the transcription factor AtPHR1 can be replaced by a functionally redundant protein called AtPHL1 (Bustos *et al.* 2010). However, Fig. S2 shows the same outcome with respect to transcript abundance associated with *EsPHL1* as was found for *EsPHR1*. In *Eutrema*, *EsPHR1* and *EsPHL1* are encoded by single nuclear genes (www.phytozome.net/thellungiella.php), and to date, no other genes that complement the function of these genes have been identified.

In summary, under Pi-deficient conditions, *Eutrema* plants show remarkably few of the classical symptoms of Pi starvation. The only consistent trait for plants exposed to variable Pi, whether as seedlings in culture or mature plants on soil, was a positively correlated increase in Pi content with increased Pi in the medium and an inverse correlation in *EsIPS2* transcript abundance. Another commonality was an apparently constitutive expression of several *Eutrema* orthologs of *Arabidopsis* PSI genes. Our work suggests that response to nutrient deprivation, like the constitutive expression of genes associated with salinity, is not a plastic trait but may be 'hard-wired' into the regulatory network of *Eutrema* (Kawecki & Ebert 2004; Taji *et al.* 2004; Wong *et al.* 2006). The mechanism(s) by which extremophile plants cope with Pi limitation, seemingly without major structural modifications, offers insights that can be used to develop crops that are more Pi use efficient.

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Supporting Information

Supplementary Fig. **S1**. Molecular phylogenetic analysis of plant high-affinity phosphate transporters (PHT1 family). *Eutrema salsugineum*, *Schrenkiella parvula*, *Brassicca rapa* Chiifu-401, *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Capsella rubella*, and *Carica papaya* are the species included in the analysis. Amino acid sequences were obtained from phytozome.net, thellungiella.org, and arabidopsis.org, aligned with MUSCLE, and trimmed with trimAl. Unrooted phylogenetic inference was performed with the PROTGAMMAWAG model with 4 rate categories, 200 inferences, and 1000 standard bootstrap replicates using RAxML version 8.0.25.

Supplementary Fig. **S2**. Meta-analysis of transcriptome data shows that several phosphate starvation genes are up-

regulated in field-grown *Eutrema* plants compared with cabinet-grown plants. Data presented are derived from three *Eutrema* plants collected from a field site in the Yukon in 2005 (+,*,⊠) and three control plants grown in the cabinet (Δ, □,○). Each symbol represents RNA-Seq quantification of expression of a particular gene in an individual plant as reported in Champigny *et al.* (2013).

Supplementary Table S1. Macro- and micro-nutrient composition of custom soil mix and soil sampled from Yukon field sites (60° 55.928′ N, 135° 10.249′ W).

Supplementary Table S2. Primers used for real-time absolute and relative RT-qPCR amplification from cDNA prepared from *Eutrema* and *Arabidopsis*, including optimum primer annealing temperatures, primer efficiencies, and regression coefficients.

Supplementary Table S3. Primers used to amplify cDNA fragments by conventional PCR. Amplicons were quantified by fluorometry and then used as standards for absolute RT-qPCR.

Supplementary Table S4. Estimates of P content for *Eutrema* and *Arabidopsis* seeds and tissues. Values are mean ± SE.