Development of biomarkers for evaluating phosphate stress in *Thellungiella salsuginea*

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ABSTRACT

Phosphorus is a macronutrient required for plant growth and reproduction. Insufficient supplies of phosphate will adversely impact plant growth. In an effort to supply adequate phosphate to crops, large quantities of phosphate-rich fertilizer are applied to fields but much of the phosphate can leach from the soil as run-off, impacting water systems. Therefore, proper management of phosphate and the development of phosphate efficient genotypes of plants are strategies needed for a sustainable agriculture industry.

This thesis project focused on the development of biomarkers of phosphate stress in *Thellungiella salsuginea,* a plant highly tolerant to salt, cold and water deficit. Biomass determinations and real-time quantitative PCR were used to determine the gene expression of several genes selected as known phosphate-responsive genes from studies of phosphate starvation of the related genetic model plant *Arabidopsis thaliana.*

Thellungiella seedlings were grown on 5 and 500 μM phosphate media. The expression of several genes (*RNS1, At4, Pht1;1, Pht1;4, Pht1;5, Siz1, PHR1, WRKY75,* and *Pht2;1*) were assayed for their response to media phosphate content. *RNS1* and *At4* expression was estimated from cDNA prepared from shoot tissues while *At4, Pht1;1* and *Pht1;5* expression was determined from root tissues. In all tissue sources, significantly increased expression of *RNS1*, *At4*, *Pht1;1* and *Pht1;5* was observed under 5 μM phosphate exposure.

Two natural accessions of *Thellungiella* were used in this study with one originating from the Yukon Territory, Canada and the second from Shandong Province, China. Seedlings of both ecotypes were grown on defined media plates containing various concentrations of phosphate (0, 25, 125, 250, 500, and 2000 μM). For both accessions, the addition of as little as 25 μM phosphate led to significant increases in root and shoot biomass. Gene expression levels corresponding to *RNS1, At4* and *Pht1;1* were the highest in Yukon and Shandong *Thellungiella* grown on 0 μM phosphate media. The addition of 25 μM phosphate to the media was enough to significantly decrease transcript abundance of *RNS1, At4* and *Pht1;1.* In a test using the transfer of Yukon *Thellungiella* seedlings from high (500 μM) to low (5 μM) phosphate the expression of *At4* in roots and shoots increased 30-fold over a five-day period and only *Pht1;1* expression increased in the roots over the same time period.

RNS1 and *At4* share attributes that make them suitable biomarkers for phosphate stress in plants. Both genes are expressed in the shoots making it easier to remove tissue for monitoring gene expression, and both genes show readily discernible increases in transcript levels for determination by qPCR. At present, however, the role for their products in phosphate assimilation by plants is uncertain. This lack of knowledge is a deterrent to adopting these genes for widespread use as biomarkers. In particular, more work needs to be done to characterize factors that elicit their expression to test the specificity of their response to phosphate stress in *Thellungiella*.

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ABBREVIATIONS

µg	Microgram(s)
µL	Microlitre(s)
µM	Micromolar
µmol	Micromole
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
ANOVA	Analysis of Variance
C	Celsius
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic acid
cm	Centimetre(s)
Cq	Quantification cycle
d	Day(s)
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
g	Gram(s)
GTP	Guanosine Triphosphate
h	Hour(s)
kDa	Kilodalton(s)

kg	Kilogram(s)
L	Litre(s)
M	Molar
m	Metre(s)
mM	Millimolar
min	Minute(s)
miRNA	Micro-Ribonucleic acid
mL	Millilitre
mRNA	Messenger Ribonucleic acid
nm	Nanometre(s)
Pi	Inorganic Phosphate
pmol	Picomole
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
s	Second(s)
T-DNA	Transfer Deoxyribonucleic acid
V	Volt(s)
wk	Week(s)

LITERATURE REVIEW

I. Role and acquisition of phosphate

a. Plant metabolism and growth

Phosphate is a plant macronutrient, along with nitrogen, sulfur, potassium, calcium and magnesium. Phosphate can comprise up to 0.2% of the dry weight of a plant (Schachtman, et. al., 1998). Phosphate is a major constituent of nucleic acids in that the backbone of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) are linked by phosphodiester bonds. Adenosine diphosphate (ADP) and adenosine triphosphate (ATP) are required for photosynthesis and respiration. Free energy released from the cleavage of ATP is also required for thermodynamically non-spontaneous reactions such as nucleic acid synthesis and membrane transport. Furthermore, plants require nicotinamide adenine dinucleotide phosphate (NADPH) for certain biosynthetic reactions; guanosine triphosphate (GTP) and cyclic adenosine monophosphate (cAMP) are important signaling molecules in plants (Sugiura & Takeda, 2000). Aside from nucleic acids, phosphate is also required for phosphorylation in metabolic pathways, such as the Calvin cycle of photosynthesis, glycolysis, as well as nitrogen and sulphur assimilation (Crawford, et. al., 2000). Structurally, phosphate plays a role in plant membranes as phospholipids (Somerville, et. al., 2000).

b. Uptake and mobilization of phosphate by plants

Phosphate is moved to the rhizosphere by diffusion, which is a slow process compared to bulk flow by which nutrients can be moved from further away to the roots (Smith, 2002). At the root-soil interface, phosphate is taken up very quickly by plants, much faster than it can be replenished from surrounding soil (Smith, 2002). Therefore, plant roots are continually growing and searching for phosphate in the soil.

Root hairs reach the deepest into the soil, therefore they are thought to be the primary site of phosphate uptake (Gahoonia & Nielsen, 1998). In *Arabidopsis*, phosphate is taken into the roots at the root-soil interface via high-affinity phosphate transporters of the *Pht1* family (Shin, et. al., 2004). The phosphate is transported via the symplasm to the stele where it is loaded into xylem to be transported to the sink tissues (Sanchez-Calderon, et. al., 2010). Phosphate 1 (Pho1) is the protein thought be involved in the loading of phosphate into the xylem. Evidence for the role of Pho1 comes from a *pho1* mutant *Arabidopsis* plant. In the *pho1* mutant, phosphate is over-accumulated in the roots as it cannot be loaded into the xylem (Hamburger, et. al., 2002).

During senescence and low availability of phosphate, phosphate stores are loaded into the phloem via Phosphate 2 (Pho2) (Delhaize & Randall, 1995). Phosphate loaded into the phloem travels from the old leaves to young leaves and sink organs, such as the roots (Sanchez-Calderon, et. al., 2010).

c. Symptoms of phosphate deficiency

Phosphate is a mobile element in plants so symptoms of phosphate deficiency are seen in older leaves first. Symptoms frequently begin with the production of red and purple anthocyanin pigments in leaves, and there is an eventual development of chlorosis of the leaves (White & Hammond, 2008). Under severe phosphate deficiency chloroplast abnormalities develop (Bould, et. al., 1983). As the phosphate deficiency progresses, cell expansion and cell division rates slow and photosynthetic rates decrease (White & Hammond, 2008).

II. Introduction to soil phosphate in nature

In soil, 99% of naturally occurring phosphorus is found as phosphate in various forms, particularly free inorganic phosphate, inorganic phosphate bound to soil particles, inorganic phosphate salts, or organic phosphate esters (Sanchez-Calderon, et. al., 2010). Soils typically contain a concentration of phosphate a few magnitudes less than what is found in plant tissues (10 to 25 mM) but even fertile soils typically contain phosphate levels of only 10 μM, and soils of average fertility contain much lower concentrations of phosphate, often 2 μM (Bieleski, 1973).

The availability of phosphate in soil is influenced by many factors. Soil structure, organic matter content, temperature, soil moisture, and soil pH all influence the availability of phosphate. In alkaline conditions, inorganic phosphate readily complexes with calcium, and in acidic conditions inorganic phosphate can complex with aluminum

and iron (White & Hammond, 2008). These phosphate salts have low solubility and reduce the amount of phosphate available for plants (White & Hammond, 2008). Phosphate must be in the soluble inorganic, orthophosphate form, such as the hydrogen phosphate form $H_2PO_4^-$ in order to be available for uptake by plants (Sanchez-Calderon, et. al., 2010). Unfortunately, 20-80% of phosphate in nature is in the organic form which must be de-mineralized before it is acquired by plants (Gerke, 1993). Other factors may also influence the availability of phosphate for plants. Approximately 98% of phosphate is so firmly bound to clay, it cannot be released for use by plants (Bieleski, 1973), and organic phosphate is largely insoluble (Van Diest, 1968). All these factors contribute to the low availability of phosphate in soil.

a. Phosphate fertilizers in agriculture

It is estimated that 30 to 40% of arable lands have low phosphate concentrations that limit crop production (Franco-Zorrilla, et. al., 2004). Typically, high phosphate fertilizers are applied to land with low phosphate content to allow productive crop growth. However, when applied to soil, phosphate often forms insoluble compounds with iron, aluminum and calcium (Holford, 1997) requiring the application of significantly larger amounts of fertilizer to a field to meet plant requirements for this element (Ragothama, 1999).

Canada is the fourth largest producer and exporter of canola, a crop that contributes $13.8 billion to Canada's economy each year (Canola Council of Canada,

2008). However, canola can remove 40 kg of inorganic phosphate from one hectare of land each year (Crop Nutrient Council, 2009). Therefore, the phosphate removed must be replenished for subsequent crops and this is done by the use of phosphate fertilizers. Unfortunately, the global reserves of commercially exploitable inorganic phosphate rock are dwindling and are estimated to last for less than 150 years (White & Hammond, 2008). Fertilizers also represent a cost to farmers both from their purchase value and the energy costs associated with application (White & Hammond, 2008). Therefore, there is a need to reduce the use of inorganic phosphate fertilizers and rely on alternatives such as manure and the development of crop genotypes that are more efficient at extracting phosphate from the soil (White & Hammond, 2008)

b. Phosphate as a pollutant

The uptake of phosphate by plants is estimated to remove 10% of the phosphate applied leaving the remainder at risk of loss in runoff or immobilization by the soil (Holford, 1997). Runoff carries phosphate from soil into lakes and streams increasing the amount of phosphate in aquatic ecosystems (Bennett, et. al., 2001). These levels of phosphate lead to eutrophication of an aquatic ecosystem.

In Canada, an aquatic system is considered eutrophic at phosphorus levels of 35 to 100 parts per billion (Crop Nutrients Council, 2009). When a body of water becomes eutrophic algae over-produce leading to an "algal bloom". When the abundant algae in a bloom die their decomposition consumes oxygen in the water suffocating fish and other

aquatic organisms (Crop Nutrients Council, 2009). As a consequence, recreational and commercial fishing activities are adversely affected due to decreases in local fish stocks (Crop Nutrients Council, 2009). Eutrophication also affects human populations. Algal blooms make water unaesthetic and reduce its recreational use. Increased costs are also associated with the removal of algal toxins, foul tastes, and objectionable odours from the water in order to make it suitable for human consumption.

III. Plant response to phosphate limited conditions

a. Changes in root morphology

Plants show phenotypic plasticity in that a single genotype can yield plants with varied phenotypes under different conditions. Phosphate limitations can elicit plasticity in plant morphology. Under phosphate limiting conditions, *Arabidopsis thaliana* exhibits an increased root:shoot ratio (Williamson, et. al., 2001). This response results from proportionately more resources allocated to roots and increased root biomass and surface area for exploration of phosphate in soil (Williamson, et. al., 2001). Under low phosphate conditions, *Arabidopsis* plants reduce primary root growth in favour of lateral root growth that can see an up to 5-fold increase in density (Lopez-Bucio, et. al, 2002) This change in root growth observed under low phosphate conditions results in increased root biomass near the surface of the soil where more plant-available phosphate is found (Williamson, et. al., 2001).

In non-mycorrhizal plants such as *Arabidopsis*, root hairs make up approximately 80% of the absorptive surface (Tacconi & Abel, 2004). When grown hydroponically under conditions of extremely high phosphate (2000 mM), root hair formation can be abolished in *Arabidopsis* (Bates & Lynch, 1996). However, under low phosphate conditions root hairs may account for up to 90% of the surface area responsible for phosphate uptake (Raghothama, 1999). Thus, while experiencing low phosphate conditions *Arabidopsis* plants increase their density of root hairs and root hair length can increase 3-fold to augment the absorptive area (Bates & Lynch, 1996). Interestingly, this increase in root hair production seems to be a phosphate specific response since deficiencies of other nutrients do not elicit this response (Bates & Lynch, 1996).

b. Exudation of organic acids and phosphatases

Inorganic phosphate from rocks can be mobilized by the exudation of organic acids from the roots into the rhizosphere. These organic acids increase the availability of phosphate by mobilizing the marginally soluble phosphate salts, such as calcium phosphate, iron phosphate, and aluminum phosphate (Kirk, et. al., 1999). Anionic forms of organic acids may also mobilize phosphate through several different mechanisms including chelating metals that would otherwise immobilize phosphate, changing the soil pH to increase the solubility of phosphate, and by forming soluble, metal-chelate complexes with inorganic phosphate (Kirk, et. al., 1999). For example, *Arabidopsis* is known to exude citrate and malate, both of which are known as weak chelating agents

(Narang, et. al., 2000) and different ecotypes of *Arabidopsis* exude different amounts of organic acids. The Coimbra ecotype exudes up to three times more citrate and malate than Columbia (Narang, et. al., 2000). Hofford, et. al., (1992) proposed that the organic acids exuded by the roots into the rhizosphere are synthesized in the shoots then exported to the roots. High phosphoenolpyruvate-carboxylase activity, an enzyme involved in malate synthesis, is observed in the leaves of *Brassica napus* plants exuding organic acids from roots and an increased citrate:sugar ratio is observed in the phloem of phosphate-deficient plants relative to phosphate-sufficient plants (Hofford, et. al., 1992).

It has been suggested that *Arabidopsis* may exude acid phosphatases into the rhizosphere to breakdown the pool of organic phosphate into the "usable" inorganic form (Haran, et. al., 2000). Two acid phosphatases, PAP12 and PAP26 were purified from the secretions of phosphate starved *Arabidopsis* suspension cells and these enzymes are believed to facilitate phosphate scavenging from soil during phosphate starvation (Tran, et. al., 2010).

c. Gene expression in response to low phosphate

How plants sense phosphate status in their environment is unknown. Systems for sensing environmental phosphate status have been identified in *Escherichia coli* (Lamarche, et. al., 2008) and *Saccharomyces cerevisiae* (Mouillon & Persson, 2006). Therefore, a conserved mechanism may exist in plants for sensing environmental phosphate status (Fang, et. al, 2009). Evidence for a phosphate sensing system in the roots

comes from multiple sources. Phosphite, an analog of phosphate, is taken up by high affinity phosphate transporters in the roots but phosphite cannot be metabolized. When added to media lacking phosphate, phosphite is able to repress the low phosphate response seen in plants (Ticconi, et. al, 2001). Furthermore, Svistoonoff, et. al, (2007) have demonstrated that contact of the root tip alone with a low phosphate medium is sufficient to severely reduce root growth. Recently, forward genetics methodologies have been used to identify low-phosphate-insensitive mutants (Rouached, et. al., 2010). The mutations were mapped to *Low Phosphate Root 2 (LPR2)*, *Low Phosphate insensitive 1 (LPI1)* and *Phosphate Deficiency Response 2 (PDR2)*. These genes likely work together in monitoring the phosphate status of the growth medium and adjusting the activity of the root meristem accordingly (Rouached, et. al., 2010).

Several transcription factors have been implicated in the phosphate starvation response. These include *Phosphate starvation response 1 (PHR1), Basic Helix Loop Helix 32 (BHLH32), WRKY75,* and *Zinc Finger of A. thaliana 6 (ZAT6)* (Fig. 1).

BHLH32 is suggested to play a role in the suppression of genes involved in the low phosphate response. An *Arabidopsis bhlh32* mutant in phosphate sufficient conditions shows increased anthocyanin accumulation and increased root hair production compared to a wild type plant (Chen, et. al., 2007).

Arabidopsis WRKY75 RNAi lines show accelerated symptoms of phosphate deficiency as well as a reduction in expression of phosphate responsive genes under low

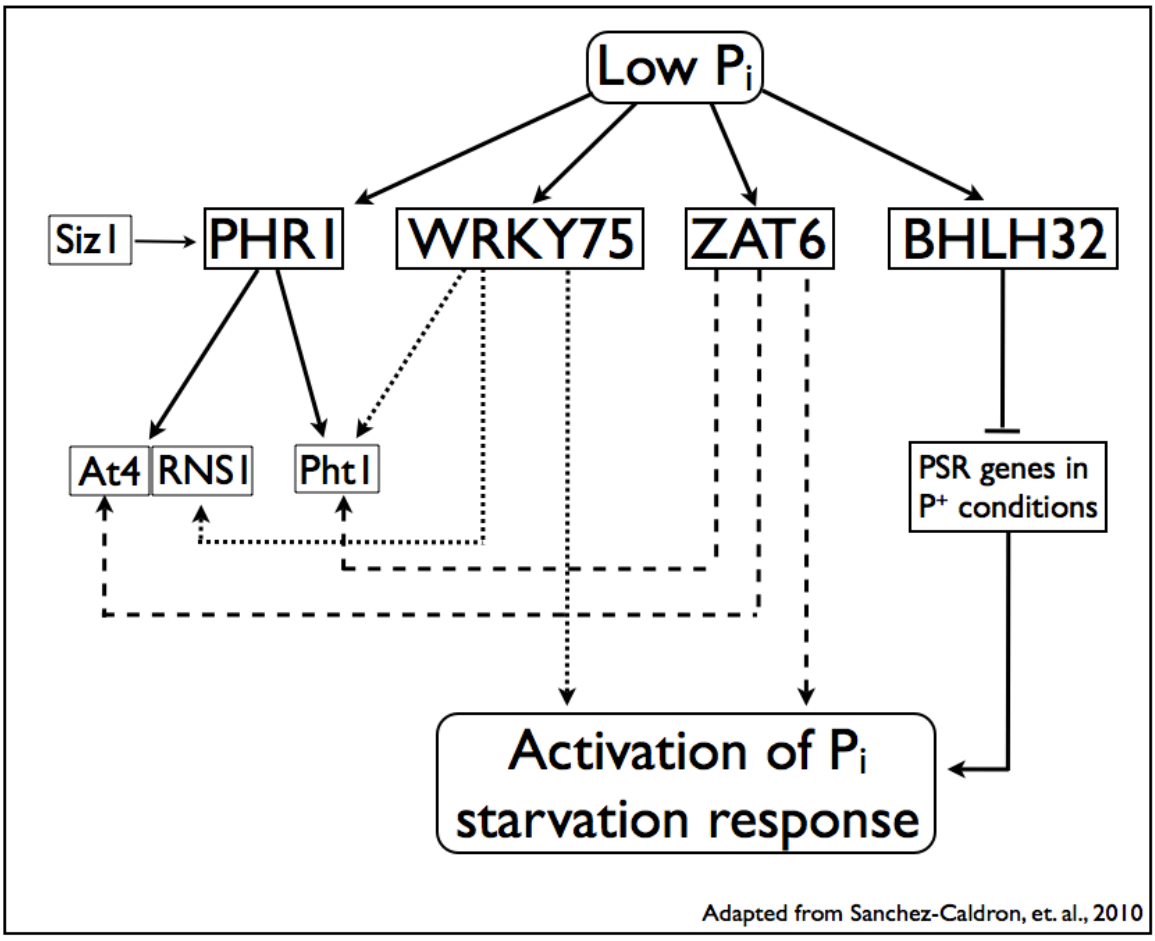
phosphate conditions. This suggests a role for WRKY75 as a positive regulator of phosphate responsive genes under low phosphate conditions (Devaiah, et. al., 2007).

Arabidopsis ZAT6 over-expression mutants show a decrease in root growth, increased anthocyanin accumulation, and an increased concentration of phosphate in the roots and shoots relative to wild type. Therefore, ZAT6 is proposed as a positive regulator of genes involved in phosphate acquisition and distribution (Devaiah, et. al., 2007).

The best characterized transcription factor of phosphate deficiency in *Arabipodsis* is *Phosphate Starvation Response 1 (PHR1).* When the ortholog of *PHR1* from laboratory green algae (*Chlamydomonas reinhardtii*) is knocked out the organism has a retarded low-phosphate-response suggesting, by analogy, an important role for PHR1 in higher plants. An *Arabidopsis phr1* mutant shows a reduction in the expression of phosphate responsive genes, further suggesting an important role for PHR1 as a positive regulator of the low phosphate responsive genes (Bari, et. al., 2006). A binding sequence for PHR1 has been observed in the promoters of several *Pht1* (high-affinity-phosphate- transporters) family members in *Arabidopsis*, suggesting PHR1 plays a role in their expression (Fang, et. al., 2009).

A simplified scheme of phosphate sensing is shown in Figure 1 (adapted from Sanchez-Caldron, et. al., 2010).

Figure 1. A proposed signaling pathway for the phosphate starvation response pathway in *Arabidopsis*. Low phosphate in the media is sensed by plants via an unknown mechanism that activates expression of *PHR1, WRKY75, ZAT6* and *BHLH32*. These transcription factors regulate phosphate stress responsive (PSR) genes to manage plant growth on low phosphate media. Signaling pathway shown is modified from Sanchez-Caldron, et. al., 2010. The use of dashed, dotted, and solid lines is intended to identify specific regulators with their proposed targets and is not intended to suggest any specific property of a given interaction.


Low Pi
Siz1
PHR1
WRKY75
ZAT6
BHLH32
At4
RNS1
Pht1
PSR genes in P+ conditions
Activation of Pi starvation response
Adapted from Sanchez-Caldron, et. al., 2010

IV. Phosphate responsive genes

a. The Pht family of phosphate transporters

Four families of *Pht* transporters have been reported in *Arabidopsis thaliana*, and follow the nomenclature *Pht1, Pht2, Pht3,* and *Pht4* phosphate transporters.

There are nine known members of the *Pht1* high-affinity phosphate transporter family in *Arabidopsis*, eight of which are known to be expressed in roots (Shin, et. al., 2004). These high affinity phosphate transporters are between 500 and 550 amino acids in length, with a mass of approximately 58 kDa. The nine members of the *Pht1* family share between 58 to 99.8% nucleotide sequence identity and 45 to 98% amino acid sequence identity (Bayle, et. al., 2011). Each member is composed of 12 hydrophobic membrane-spanning domains arranged in two, six-unit configurations separated by a large hydrophilic loop in the cytoplasm (Smith, 2002). Due to differences in the concentration gradient of phosphate from micromolar in the rhizosphere to millimolar inside the roots, phosphate uptake is an energetically unfavourable process. As such, uptake of phosphate is coupled to proton uptake using a P-type H+/Pi ATPase. The H+/Pi ATPase is capable of auto-phosphorylation of a specific aspartate residue which activates the transporter to actively transport ions across the concentration gradient. The uptake of one phosphate is coupled to the uptake of two to four protons (Sze, et. al., 1999).

Under phosphate sufficient conditions, the high-affinity phosphate transporters localize to the root tip and central cylinder (Karthikeyan et al., 2002). In low phosphate conditions, the increased affinity for phosphate is likely due increased synthesis of high-

affinity phosphate transporters and the expression of their genes is across the entire root with the highest abundance of transporters found in the plasma membranes of the root hairs, root cap, and the outer cortex (Karthikeyan et al., 2002). Mitsukawa, et. al., (1997) reported that the *Arabidopsis* transporter proteins were expressed in tobacco cell culture the maximum uptake of phosphate occurs at pH 5 and phosphate uptake was reduced by the use of an H+-ATPase inhibitor or in the presence of protonophores (lipid soluble molecules that transfer ions across membranes (McLaughlin & Dilger, 1980)). These conditions are consistent with the proton symport model of phosphate uptake in *Arabidopsis.*

Two main members of the *Pht1* family are suggested to play the largest role in phosphate uptake in *Arabidopsis, Pht1;1* and *Pht1;4* (Shin, et. al., 2006). These authors reported that a double mutant of *Pht1;1* and *Pht1;4* showed a 50% reduction in phosphate uptake under low phosphate conditions. The double mutant also shows a 75% reduction in phosphate uptake under high phosphate conditions showing that both products are necessary for phosphate uptake even when this element in not limiting. The ability of the *Arabidopsis* double mutant to take up some phosphate suggests that *Pht1;2* and *Pht1;3* also play a role in phosphate transport under high and low phosphate conditions (Shin, et. al, 2001). In the Δ*Pht1;1*Δ*Pht1;4* double mutant, expression of the gene encoding At4, a protein involved in allocation of phosphate between roots and shoots (Shin, et. al., 2006), is up-regulated (Shin, et. al., 2001). *At4* expression is a sensitive indicator of phosphate stress in *Arabidopsis* (Shin, et. al., 2006) and its increased expression is evidence that

Pht1;1 and Pht1;4 are the main transporters involved in phosphate uptake because the *Arabidopsis* mutants lacking these transporters are phosphate stressed even under phosphate sufficient conditions (Shin, et. al., 2004).

Regulation of the *Pht1* high-affinity phosphate transporters occurs at the transcriptional and post-translational level (Bayle, 2011). Transcriptional expression of *Pht1* high-affinity phosphate transporters is a response to low phosphate conditions (Karthikeyan, et. al., 2002). Subsequent control of Pht1 activity is at the post-translational level (Bayle, 2011). *Phosphate transporter traffic facilitator 1 (PHF1*) is required for the *Pht1* family members to exit the endoplasmic reticulum (Bayle, 2011). A *phf1* mutant showed an 80% decrease in phosphate uptake under normal and low phosphate conditions compared to the wild-type (Bayle, 2011). Evidence that PHF1 acts as an co-chaperon for proper protein folding of the *Pht1* high-affinity phosphate transporter family is provided by the study of a yeast analog (Bayle, 2011). Under phosphate sufficient conditions, serines 514 and 520 in the C-terminal end of *Pht1;1* are phosphorylated, preventing exit of the protein from the endoplasmic reticulum. This is an example of another level of post-translational control proposed for Pht1;1. The final level of post-translational control that has been identified thus far is through recycling of the transporter proteins from the plasma membrane. Continuous endocytosis of the plasma membrane allows for re-sorting in the complement of transporters present (Bayle, 2011). When plants are experiencing low phosphate conditions, transporters produced by the cell are directed to the plasma membrane for phosphate uptake. Under phosphate sufficient conditions, the transporters

are sorted to the vacuole and degraded. This mechanism of altering the capacity for phosphate uptake at the membrane likely prevents ion toxicity in the cell from exposure to high intracellular levels of inorganic phosphate ions (Bayle, 2011).

Other roles for the *Pht1* family of high-affinity phosphate transporters have also been suggested. Transcripts associated with the *Pht1* family of high-affinity phosphate transporters have been detected in the leaves and pollen of *Arabidopsis*. The suggested role of these transporters is for phosphate remobilization via the phloem in the leaves (Rae et. al., 2003) and for phosphate uptake by the elongating pollen tube in pollen (Mude et. al., 2002).

The Pht2 family of phosphate transporters only consists of one known member. The low-affinity phosphate transporter (Pht2;1) was discovered by sequence similarity with known bacterial and eukaryotic low-affinity phosphate transporters (Daram, et. al., 1999). Pht2;1 has a distinct structure from the *Pht1* family, Pht2;1 consists of 12 hydrophobic membrane-spanning domains but the hydrophilic linker is absent and a large extracellular hydrophilic N-terminus is present (Daram, 1999). While shown to be a H+/Pi symporter, Pht2;1 has a high Km value, and therefore has a low affinity for phosphate. Pht2;1 is not responsive to phosphate limiting conditions and is expressed in green chlorophyll containing tissue (Daram, 1999). Ferro et. al. (2002) demonstrated expression of Pht2;1 in the chloroplast membrane. The authors proposed a house keeping role for Pht2;1 where this transporter is involved in the normal phosphate distribution in the shoots of *Arabidopsis* plants.

The Pht3 family of phosphate transporters is not very well characterized. At least three members are encoded by the *Arabidopsis* genome and are characterized as putative mitochondrial phosphate transporters but their function is unknown (Rausch & Bucher, 2002; Sanzhez-Calderon, et. al., 2010).

The final family of phosphate transporters is the Pht4 family. There are six known members for the *Pht4* family, consisting of *Pht4;1*, *Pht4;2, Pht4;3, Pht4;4, Pht4;5,* and *Pht4;6* (Guo, et. al., 2007). These transporters are expressed in both the roots and the shoots of *Arabidopsis*. Pht4;1, Pht4;2, Pht4;3, Pht4;4, and Pht4;5 have been localized to the chloroplast membrane in the shoots. In contrast, Pht4;6 is localized to the golgi appartus (Guo, et. al., 2007). The authors of this study suggest the Pht4 family of phosphate transporters play a role in trafficking of phosphate between the cytosol, the chloroplast, and the golgi apparatus.

b. *Arabidopsis thaliana 4 (At4)*

Arabidopsis thaliana 4 (At4) is often used as a marker for phosphate stress in *Arabidopsis* as its expression is strongly induced in response to low phosphate (Shin et. al., 2006). In *Arabidopsis*, there are four known family members that consists of *At4, At4.1, At4.2* and *AtIPS1*. These family members are not very well conserved except for a 22 nucleotide region near the centre of the transcript. This conserved region is complimentary to a member of the miRNA 399 family which is thought to control expression of these *At4* family members (Shin et. al., 2006). Although the function of *At4*

is unknown, the *at4* mutant *Arabidopsis* accumulates a greater proportion of phosphate in the shoots rather than the roots compared to the wild-type. Constitutive expression of *At4* alters the balance of phosphate to favour the roots. Coupled with its expression in vascular tissue, the authors suggest At4 functions in phosphate allocation between the roots and shoots by phosphate re-translocation to the roots (Shin, et. al., 2006).

c. *Ribonuclease I (RNS1)*

Arabidopsis thaliana Ribonuclease 1 (RNS1) is a member of the T_2/S RNase superfamily. *RNS1* expression is associated with phosphate deficient conditions as well as mild expression in senescing leaves (Bariola, et. al., 1994). RNS1 is thought to liberate phosphate from the sugar-phosphate backbone of RNA in older leaves as part of a low phosphate responsive system and to facilitate remobilization of phosphate from senescing leaves (Bariola, et. al., 1994). The *Arabidopsis* genome predicts five family members of the RNS family designated *RNS1* through *RNS5* (Hillwig, et. al., 2008). *RNS1* and *RNS2* are responsive to phosphate whereas *RNS3* is not responsive to phosphate content in the environment but it is expressed during senescence. Hilliwig, et. al. (2008) have shown that *RNS1* is expressed in response to wounding independent of the Jasmonic Acid signaling pathway and therefore may have a defensive function in *Arabidopsis*.

V. Potential use of phosphate transporter research in agriculture

Mitsukawa et. al. (1997) demonstrated enhanced growth of cultured tobacco cells in low phosphate media when *Arabidopsis thaliana* Pht1;1 proteins were over-expressed. This has potential application in agriculture as phosphate transporters could be biochemically altered and optimized to benefit plants in low phosphate conditions. Marcel and Bucher (2006) point out that phosphate is depleted so quickly from the rhizosphere that the optimization of root traits rather than transporters would benefit plants under low phosphate conditions. However, optimization of phosphate transporter function in crop plants may benefit agriculture in the reduction of fertilizer use if crops could more efficiently or quickly take up the 90% of phosphate that is regularly lost to immobilization and runoff.

VI. Model organisms

a. *Arabidopsis thaliana* as a model organism for biological studies

Arabidopsis thaliana, commonly known as thale cress, is a small angiosperm of the mustard family that has become the organism of choice for physiological and genetic studies in plants (Meyerowitz & Somerville, 1994; Benning & McCourt, 2010). *Arabidopsis* has many desirable traits for genetic research. For example, it has a small diploid genome (120 megabases, separated into five chromosomes) that can be easily transformed by transfer DNA (T-DNA) from the organism *Agrobacterium tumefaciens* (An et al., 1986). *Agrobacterium* has been used to create thousands of T-DNA insertional

mutant lines of *Arabidopsis* that are available from public stock centers and private companies (Meinke et al, 1998).

Arabidopsis is a small plant even when mature (rosette approximately 6-10 cm in diameter, and bolt 15-20 cm in height), and its life cycle from seed to seed takes approximately six to eight weeks (Meinke et al, 1998). *Arabidopsis* is a diploid plant that is capable of self-fertilization or manual cross pollination and it produces thousands of seeds per plant making propagation of the plant very easy (McCourt & Benning, 2010). Due to its small size and comparatively fast life cycle, *Arabidopsis* can be grown in large numbers in controlled environment growth cabinets. Many trials can be completed more rapidly than with most other plants allowing a large catalog of individuals to be studied to increase sample size.

Arabidopsis research has been very productive in the past 30 years (Somerville & Koornneef, 2002; McCourt & Benning, 2010). In 1996, the *Arabidopsis* Genome Initiative was formed to establish mechanisms of coordination and cooperation between three labs responsible for the sequencing the *Arabidopsis* genome. By December 2000, most of the *Arabidopsis* genome had been sequenced (Somerville & Koornneef, 2002).

b. *Thellungiella salsuginea* as a stress tolerance model

Thellungiella salsuginea, commonly known as salt cress, is a highly stress tolerant member of the mustard family and a close relative of *Arabidopsis thaliana* (Inan, et al., 2004). *Thellungiella* has a genome size that is approximately double that of *Arabidopsis,*

and these two species share about 92% nucleotide sequence identity (Inan, et al., 2004). *Thellungiella* has other properties in common with *Arabidopsis* including a short life cycle of 8 weeks (Inan, et al., 2004) and the genome sequence has recently been released (Dassanayake, et. al., 2011). In Canada, one natural accession of *Thellungiella* is found in the subarctic region of Canada where it is exposed to cold, salt and drought (Scoggan, 1978; Warwick, et al., 2004).

Two different natural accessions of *Thellungiella* have been studied. One accession was discovered in Shandong Province of northeast China and has been referred to in the literature as the "Shandong ecotype" (Inan, et al., 2004). The other accession originated as seeds collected from the salt flats of the Yukon Territory of Canada and is referred to as the "Yukon ecotype" (Griffith, et. al., 2007).

Griffith, et al., (2007) reported that Yukon *Thellungiella* plants can complete their life cycle at 5 °C, including seed germination and production of pollen and seeds. By comparison to many crop plants, this demonstrates tolerance to cold. *Thellungiella* also demonstrates tolerance to other water deficit associated stresses.

Extreme salt tolerance is a feature of *Thellungiella*. Root and shoot growth continues even at levels up to 500 mM NaCl exposure (Inan, et al., 2004). Growth under these levels of salt can continue for several months during which time *Thellungiella* will continue to produce viable seeds (Inan, et al., 2004). In comparison, *Arabidopsis*, a glycophyte, can grow for up to 20 days in 200 mM sodium chloride but after 20 days the fresh weight decreases and the plants die (Inan, et al., 2004).

Preliminary results from our lab suggests that *Thellungiella* does not respond to low phosphate in the same way that *Arabidopsis* does. Under low phosphate conditions, *Thellungiella* has not been found to display a visible phenotype compared to high phosphate conditions when plants are propagated to maturity in soil-less media (DiBattista, 2010).The apparent hardiness of *Thellungiella* to abiotic stresses including nutrient deprivation make it a valuable and ideal candidate for the study of stress tolerance in plants.

VII. Biomarkers: properties and applications

A biomarker is "a biological response to a chemical or chemicals that gives a measure of exposure" (Peakall, 1994). There are several different types of biomarkers that are used as tools for plant research including chemicals, proteins, and genetic markers. Secondary metabolites, including pigments such as anthocyanins, have been used as chemical biomarkers of stress or for following breeding patterns in plants (Bretting & Widrlechner, 1995). Protein biomarkers have also been used as markers of stress with a well known example being the appearance of heat shock proteins upon exposure of a plant to elevated temperature (Bretting & Widrlechner, 1995).

Genetic markers were typically used for genotyping plants and plant breeders track the inheritance of different alleles from one generation to the next (Bretting & Widrlechner, 1995). Recently, Yang, et. al. (2011) demonstrated that differences in gene expression can be used as biomarkers of nutrient status in plants. The use of gene

expression as a biomarker of stress is advantageous over other methods because some easily discernable changes in gene expression occur early in the response to a stress (Brain & Cedergreen, 2008). With modern techniques, only a small sample of isolated RNA is required for analysis so sampling can be performed with minimal adverse impact to the plant. Using high-throughput techniques, all mRNA in the sample can be sequenced to provide information on transcript numbers and gene identities (Brain & Cedergreen, 2008). However, there are factors that must be considered when using gene expression as a biomarker of stress. Intrinsic sources of variability of gene expression must be considered and those factors include plant age, cell types, and time after plant exposure to a treatment condition. Therefore, the expression of certain genes may vary significantly compromising the reliability of a gene as a marker (Brain & Cedergreen, 2008). Nonetheless, through careful selection and extensive testing of candidate genes, changes in gene expression can be used as a suitable biomarker strategy (Yang, et. al., 2011).

An important property of a good biomarker for nutrient stress exposure is that it should be strongly linked to the target stress condition and not influenced by other stressors present in the environment. Ideally, the biomarker should be sensitive enough to detect modest changes in the nutrient status of the plant, it should allow for some calibration of the degree of stress, and it should be relatively invariable over the course of a day. Finally, confidence in applying a biomarker for diagnostic determinations and interpreting the results must be high so the application of any biomarker should be supported by several, independent studies reported in the literature.

VII. Quantitative real time Polymerase Chain Reaction (qPCR)

Reverse transcription (RT) in combination with Polymerase Chain Reaction (PCR) is a popular technique to analyze mRNA expression in a variety of organisms (Winer, et. al., 1999, Livak & Schmittgen, 2001; Pfaffl, 2011). The addition of a real time system to RT-PCR allows detection of rare transcripts and small changes in gene expression, making RT-PCR a very sensitive technique for gene expression studies (Morrison, et. al., 1998). Real-time RT-PCR makes use of an intercalating dye, SYBR green, that only binds the minor groove of double stranded DNA (Morrison, et. al., 1998).

Two methods for quantification of gene expression are currently used. The first method is absolute quantification (Pflaffl, 2001). This method is based on an internal or external calibration curve of known concentrations of product (DNA or RNA) to determine the absolute quantities of template in the sample. This method is considered very time consuming and requires very precise quantification of a reliable standard (Pflaffl, 2001). The standard also requires identical PCR efficiency between the source of standard material and the target cDNA. Therefore, highly validated methodologies are required for absolute quantification (Pflaffl, 2001).

The second method of quantification is a relative quantification based on expression of a target gene relative to a reference gene (Livak and Schmittgen, 2001). Pflaffl (2001) claims this method is sufficient for studying changes in gene expression. The relative method of quantification is based on an equation derived by Livak & Schmittgen in 2001. The Ct value (quantification cycle; the cycle at which fluorescence

crosses the threshold for detection) of the gene of interest is compared to the Ct value of an internal standard, as $2^{-\Delta\Delta Ct}$. Ct values correspond to the cycle number where the fluorescence level of a well on a qPCR plate crosses the threshold for detection (Morrison, et. al., 1998). Ct values are also referred to as Cq values (Bustin, et. al., 2009). Using this method, a control condition is used as a calibrator, where ΔΔCt is equal to 0 (Livak & Schmittgen, 2001). Therefore, all other gene expression is relative to the control of 1 (Livak & Schmittgen, 2001). Relative quantification is reliant on good standardized reference genes (Pflaffl, 2001). Common reference genes include *Elongation factor 1-alpha (EF1-α), polyubiquitin (UBQ), Actin (Act)* and *Tubulin (TUA or TUB)*, which were chosen in the pre-genomics era because they are suspected housekeeping genes (Czechowski, et. al., 2005). These housekeeping genes are considered to have stable, constitutive expression. However, evidence from the literature suggests there are better choices available (Czechowski, et. al., 2005). The sensitively and improvements in qPCR make it an excellent choice to study gene expression in *Thellungiella*.

METHODS AND MATERIALS

I. Plant materials and growth

a. Seeds:

Thellungiella salsuginea (Yukon ecotype) seeds were bulked from plants grown at McMaster University. The original seed stock was collected from plants obtained at Dillabaugh's grazing lease in Whitehorse, Yukon by Dr. Bruce Bennett, Wildlife Viewing Biologist with Environment Yukon. Seeds for the Shandong *Thellungiella* ecotype were a gift of Dr. Ray Bressan, Purdue University. Seeds are stored at room temperature in 1.5 mL flat-top microfuge tubes (Diamed Lab Supplies Inc., Mississauga, ON, Cat. No. SPE155-N).

b. Suppliers:

The company name and catalog number of chemicals and disposable research materials are included where the chemical or supply is first mentioned. Unless otherwise stated, the purity of the water used for all procedures (including watering plants and solution preparation) was purified by a Barnstead NANOpure II system.

c. Seed sterilization:

Approximately 50 to 70 seeds were placed in a 1.5 mL microfuge tube to which 1 mL 70% (v/v) ethanol was added. The tubes were shaken for 2 min and then the ethanol

was removed and 1 mL sterilization solution (50% (v/v) Javex Bleach™, 0.1% (v/v) Tween 20 (Sigma-Aldrich Co., Oakville, ON, Cat. No. 9005-64-5) was added. The seeds were shaken in sterilizing solution for 5 to 10 min. The solution was removed and 1 mL sterile water was added. The tubes were shaken to rinse the seeds and the water was removed. This step was repeated at least 5 times until no froth from the detergent was visible. Finally, the seeds were suspended in 0.5 mL of sterile 0.1% (w/v) Phytagel (Sigma-Aldrich Co., Oakville, ON, Cat. No. 71010-52-1) then stored overnight at 4°C.

d. Seed germination on defined media plates:

The sterilized seeds were transferred to a 100 x 20 mm cell culture dish (Corning Inc., Corning, NY, Cat. No. 430167) containing Murashige and Skoog (MS) media solidified with 5% (w/v) Phytagar. The plates were sealed with 3M micropore tape (Fisher Scientific, Ottawa, ON, Cat. No. 19-061-655) and placed in a growth chamber with 24 h light (65 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C for 7 d.

e. Seedling growth on nutrient plates:

Nutrient plates were formulated to include nutrients other than phosphate at non-limiting concentrations. The phosphate content was variable and ranged from 0 μM (no additional phosphate added) up to 2 mM. The components of the nutrient plates are listed in Table 1.

The nutrient solutions were prepared by combining all components specific to a

given phosphate formulation (Table 1) with 2% (w/v) sucrose (BioShop Canada Inc., Burlington, ON, Cat. No. SUC700), dissolving the components in a 500 mL beaker, and adjusting the final volume to 250 mL in a graduated cylinder. The pH was tested using an Orion DUAL STAR pH meter (Thermo Fisher Scientific Inc., Rockford, IL, Cat. No. 2115000) to ensure that it ranged between 5.5 and 6 (if necessary the pH was adjusted to 5.8 using 1M NaOH or 1M HCl). Finally, 0.8% (w/v) phytoblend (Caisson Labs, North Logan, UT, Cat. No. PTP01) was added and the solution was autoclaved and poured into 100 x 15 mm square plates (BD Biosciences, Mississauga, ON, Cat. No. 35112) and allowed to solidify overnight. The plates were inverted and stored at 4°C until use.

f. Seedling growth on media with 5 or 500 µM phosphate:

Approximately 5-d-old seedlings (just after the 4 leaf stage; 2 cotyledons and 2 rosette leaves) were transferred from MS plates to nutrient plates (see above) containing either 5 or 500 µM phosphate. The seedlings were grown for 10 to 14 d at 23ºC with a 12 h light/ 12 h dark diurnal cycle. Light intensity was maintained at 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ .The seedlings were harvested when the primary root of seedlings on the 500 µM phosphate plates reached the bottom of the plate. This took, on average, about 15 d. The seedlings were immediately frozen in liquid nitrogen and stored in microfuge tubes at -80°C until use. Three independent biological replicates were performed and each harvest included 93 to 674 mg of shoot tissue and 75 to 343 mg of root tissue representing 54 to 140 seedlings. The exact number of seedlings harvested in each replicate is given in Appendix A.

Table 1. Composition of defined media plates used to determine the response of *Thellungiella salsuginea* to various phosphate conditions.

			Concentration of Nutrient Media (µM Pi)						
			0	**5**	**25**	**125**	**250**	**500**	**2000**
Macronutrient Solution	[Stock] (M)	[Final] (mM)	Working Volumes (µL)						
$CaCl_2$	0.5	5.5	250	250	250	250	250	250	250
$MgSO_4$	0.5	5.5	250	250	250	250	250	250	250
Na_2HPO_4/NaH_2PO_4	0.5	Variable	0	1.25	12.5	62.5	125	250	1000
KNO_3	0.5	1.0	250	250	250	250	250	250	250
Micronutrient soultion	[Stock] (mM)	[Final] (µM)	Working Volumes (µL)						
$FeSO_4 \cdot 7H_2O$	10	100	2500	2500	2500	2500	2500	2500	2500
H_3BO_3	100	100	250	250	250	250	250	250	250
$MnCl_2$	20	20	250	250	250	250	250	250	250
$CuSO_4$	1.0	1.0	250	250	250	250	250	250	250
$ZnSO_4 \cdot 7H_2O$	3.0	3.0	250	250	250	250	250	250	250
Na_2MoO_4	0.4	0.4	250	250	250	250	250	250	250
$CoCl_2$	0.01	0.01	250	250	250	250	250	250	250

g. Testing for changes in gene expression:

After reaching the 4-leaf stage, seedlings were transferred from MS plates to nutrient plates (see above) containing either 5 µM or 500 µM phosphate. The seedlings were grown for 10 to 14 d longer at 23°C with a 12 h light/ 12 h dark cycle under a light intensity of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Seedlings were collected half-way through the light cycle and again half-way through the dark cycle. Seedlings were immediately frozen in liquid nitrogen and stored at -80°C until use. Two independent biological replicates were performed and each harvest included 129 to 236 mg of shoot tissue and 96 to 135 mg of root tissue representing 54 to 108 seedlings. The exact number of seedlings harvested in each replicate is given in Appendix A

h. Testing for gene expression at various time points during seedling growth:

Seedlings were transferred from MS plates to nutrient plates containing 500 µM phosphate just after they reached the 4-leaf stage. Plates with seedlings were placed in a growth chamber maintained at 23°C with a 12 h light/ 12 h dark cycle and light intensity of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Seedlings were grown for 7 to 10 d (until their primary root was approximately 4 cm long) then they were transferred to nutrient plates containing 5 µM phosphate and returned to the growth chamber. The seedlings were harvested at 0, 1, 2, 3, 4 and 5 d. As a control, seedlings were also transferred from 500 µM phosphate plates to 500 µM phosphate plates and harvested after 5 d to assess the effect of transfer on gene expression. Following harvest all seedlings were immediately frozen in liquid nitrogen

and stored at -80°C until use. Three independent biological replicates were performed and each harvest included 54 seedlings at each time point.

i. Monitoring gene expression in seedlings as a function of phosphate:

Seedlings (just after 4-leaf stage) were transferred from MS plates to nutrient plates containing either 0, 25, 125, 250, 500 or 2000 μM phosphate. The plates were placed in a growth chamber maintained at 23°C with a 12 h light/ 12 h dark cycle and light intensity of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The seedlings were grown for 7 to 10 d (until the primary roots of seedling on the 2000 μM phosphate plates reached the bottom of the plate) and then seedlings from all treatments were harvested. The seedlings were immediately frozen in liquid nitrogen and stored at -80°C until use. Three independent biological replicates were performed and each harvest included 296 to 530 mg of shoot tissue and 167 to 522 mg of root tissue representing 42 to 133 seedlings. The exact number of seedlings harvested in each replicate is given in Appendix A.

II. Genetic Profiling

a. RNA extraction via hot-borate method:

All solutions for RNA preparations were made up using DEPC-treated sterile water and all supplies and solutions were autoclaved for use. RNA extraction was performed using a modified hot borate method (Wan, C-Y., & Wilkins, T. A., 1994 as modified by Dr. Marc Champigny). Approximately 100 mg tissue (root or shoot) was ground into a fine

powder in liquid nitrogen using a small mortar and pestle in a fumehood. Before the powder warmed up, 400 µL of Sigma Tri reagent (Sigma-Aldrich Co., Oakville, ON, Cat. No. T9424) was added to the powder and the slurry was transferred to a 1.5 mL microfuge tube. The mortar was rinsed twice with 200 µL of Sigma Tri reagent and the volumes added to the 1.5 microfuge tube containing the slurry. The tube was vortexed hard for 30 s then centrifuged at 3000 x g for 10 min at 5°C. The supernatant was then transferred to a new 1.5 mL microfuge tube and incubated at room temperature for 10 min after which 300 µL chloroform was added to the microfuge tube. The contents of the tube were vortexed for 30 s before centrifuging at 3000 x g for 10 min at 5°C. The upper, aqueous phase was transferred to a new 1.5 mL microfuge tube, and 0.6 vol (approximately 500 µL) of isopropanol was added and the tube placed on ice for 10 min. The tube was centrifuged at 20000 x g for 20 min at 5°C, the supernatant was discarded and the pellet was allowed to air dry. The pellet was then dissolved in 100 µL water and 700 µL hot borate solution equilibrated to 60°C was added (200 mM sodium borate decahydrate, 30 mM EDTA, 1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 2% (w/v) polyvinylpyrrolidone (PVP 40000)). The tube was vortexed for 30 s then placed on ice. A volume of 65 µL 2M KCl was added to the solution, then the tube was incubated on ice for 20 min before centrifuging at 12000 x g for 20 min at 5°C. The supernatant was transferred to a new microfuge tube and 200 µL 7.5M LiCl was added. The solution was inverted end-over-end, then left overnight at -20°C. The next day the solution was centrifuged at 20000 x g for 20 min at 5°C, the supernatant was discarded and the pellet was washed once with 100 µL ice-cold 2M LiCl.

The tube was centrifuged at 20000 x g for 20 min at 5°C, the supernatant was discarded and the pellet was allowed to air dry before it was dissolved using 200 µL 10 mM Tris-HCl to which 65 µL 1M K-acetate was added. The RNA was precipitated using 2.5 volumes of 100% (v/v) ethanol and incubated at -20°C for 1 h. The solution was centrifuged at 20000 x g for 10 min at 5°C, the supernatant was discarded and the pellet was washed once using 100 µL 75% (v/v) ethanol then the pellet was allowed to air dry until transparent. The pellet was dissolved with 30 µL H_2O. RNA was quantified by measuring absorbance at 230 nm, 260 nm and 280 nm using an Ultrospec 2100 Pro. RNA was stored at -80°C. To determine concentration of RNA present in the sample, absorbance at 260 nm is measured and that value is multiplied by the dilution factor, path length (1 cm) and then that number is divided the molar extinction coefficient of 0.025 $(\mu g/ml)^{-1}$ cm^{-1} for RNA. The sample purity is assessed by the 260/280 ratio with a sample of pure RNA having a 260/280 ratio approaching 2. The absorbance is also measured at 230 nm to determine if there is any contamination from phenol, a solvent used for RNA extractions (Hutchison & Munro, 1961). RNA yields were commonly 5-50 µg with a 260/280 ratio of 1.98-2.01.

b. Removal of genomic DNA from RNA:

Approximately 1 µg of RNA was added to a 0.2 mL reaction tube (Axygen, Inc., Union City, CA, Cat. No. PCR-02-C) with 1 µL 10X reaction buffer containing $MgCl_2$ (100 mM Tris-HCl (pH 7.5 at 25°C), 25 mM $MgCl_2$, 1 mM $CaCl_2$) , 1 µL DNase 1

(Fermentas Biosciences, Burlington, ON, Cat. No. EN0521) and topped up with H_2O to a total of 9 µL. The reaction was incubated at 30°C for 30 min and then the tube was placed on ice before adding 1 µL of 50 mM EDTA. The reaction was then incubated at 65°C for 10 min to inactivate the DNaseI enzyme. Reaction products were used immediately for cDNA synthesis.

c. Use of Sigma Spectrum plant total RNA kit:

All solutions were supplied in the Sigma Spectrum Plant Total RNA kit (Sigma-Aldrich Co., Oakville, ON, Cat. No. STRN50) except DNaseI, which is provided with the appropriate buffer as part of the Sigma On Column DNase I kit (Sigma-Aldrich Co., Oakville, ON, Cat. No. DNASE10-1SET). Approximately 100 mg of tissue (root or shoot) was ground to a fine powder in liquid nitrogen in a fumehood using a small, sterile mortar and pestle. The powder was transferred to a 2.0 mL microfuge tube supplied in the kit and 500 µL of Lysis Solution was added. The tube was incubated at 56°C for 5 min then centrifuged at maximum speed for 5 min at room temperature. The lysate was transferred to a filtration column and centrifuged at maximum speed for 1 min. A volume of 750 µL of binding solution was added to the flow-through and mixed by using the repeated action of the pipette. The solution was loaded into a binding column and centrifuged at maximum speed for 1 min, the flow-through was discarded and 300 µL of Wash Solution I was added to the binding column. The column was centrifuged at maximum speed for 1 min and then 80 µL of DNase I solution (70 µL DNase I Buffer, and 10 µL DNase I) was added

to the column. After incubation at room temperature for 15 min, 500 µL of Wash Solution I was added and the then the column was centrifuged at maximum speed for 1 min. After centrifugation 500 µL of Wash Solution II were added to the column and column was centrifuged at maximum speed for 1 min. The flow-through was discarded and 500 µL of Wash Solution II was added to the column and centrifuged at maximum speed for 1 min to remove the wash solution. As a final step, a volume of 50 µL Elution Solution was added to the column, the column was incubated at room temperature for 5 min then the column was centrifuged for 1 min at maximum speed to collect the RNA. The RNA was quantified by measuring absorbance at 230 nm, 260 nm, 280 nm and 320 nm using an Ultrospec 2100 Pro. The RNA was used immediately or stored at -80°C.

d. cDNA synthesis:

DNA-free-RNA (1 µg) was added to a 0.2 mL reaction tube with 1 µL oligo-dT primer, 1 µL 10 mM dNTP mix, 1 µL M-MLV reverse transcriptase buffer (250 mM Tris-HCL pH 8.3, 375 mM KCL, and 15 mM $MgCl_2$), 40 U RNaseOUT recombinant ribonuclease inhibitor (Invitrogen Canada, Inc., Burlington, ON, Cat. No. 10777-019) and 200 U M-MLV reverse transcriptase (200 U/µL; Sigma-Aldrich Co., Oakville, ON, Cat. No. M1302) in a final reaction volume of 20 µL. The reaction tube was incubated for 50 min at 50°C and then 10 min at 94°C to deactivate the reverse transcriptase enzyme. The cDNA synthesis products were used immediately or stored at -20°C for no longer than one week. cDNA was stored at -80°C for periods exceeding one week.

e. Synthesis of primers for PCR:

Primers specific for transcripts associated with genes of interest in *Thellungiella salsuginea* were designed using DNA sequence data from Roche 454 pyrosequencing files available in the lab (unpublished). The primers were designed to be 100 to 300 bp with an optimal annealing temperature of approximately 62°C. Primers were designed using NCBI Primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were synthesized by the MOBIX lab at McMaster University and diluted to 10 pmol/µL for use following the dilution spreadsheet provided by MOBIX (http://www.science.mcmaster.ca/mobixlab/files/oligo/Primers.xls).

f. Conditions for polymerase chain reaction (PCR):

Each 50 µL PCR volume contained 34.5 µL of H_2O, 5 µL 10x PCR buffer (20 mM Tris-HCl, pH 8.8), 5 µL 25 mM $MgCl_2$, 1 µL of each 10 pmol/µL primer, 1 µL 10 mM dNTPs, 1 µL DNA and 2.5 U Taq polymerase (Sigma-Aldrich Co., Oakville, Ontario, Cat. No. D4545-1.5KU). An Eppendorf Mastercycler Thermocycler (Eppendorf, Cat. No. 5345 003558) was used for PCR reactions. The initial denaturation step was carried out at 94°C for 3 min and subsequent cycles were composed of the following steps: denaturation at 94°C for 30 s, annealing at the primer-specific temperature for 30 s and extension at 72°C for 30 s. This cycle was repeated 30 times followed by a final extension at 72°C for 3 min. The PCR products were used immediately or stored at -20°C.

g. Agarose gel electrophoresis:

Products of PCR were separated using electrophoresis on 1.2% (w/v) agarose gels. The gel was prepared using 800 µL 50X TAE (0.4 M Tris-base, pH 8.0, 0.01 M Na_2EDTA, 0.2 M NaOAc), 39.2 mL H_2O and 0.48 g agarose (BioShop Canada, Inc., Burlington ON, Cat. No. AGA001.100). The agarose gel mixture was heated in the microwave for 45 s stopping at 15 s intervals to swirl the mixture. The molten solution was allowed to cool slightly before adding 5 µL GelRed Nucleic Acid Stain (Biotium, Inc., Hayward, CA, Cat. No. 41003) and then it was poured into the mold and allowed to solidify at room temperature. Products of PCR (12 µL) were prepared for loading into the gel by the addition of 3 µL 6X loading dye (10 mM Tris-HCL pH 7.6, 0.03 % (w/v) bromophenol blue, 0.03 % (w/v) xylene cyanol FF, 60 mM EDTA, 60 % (v/v) glycerol). A 1 Kb DNA Ladder (Fermentas Biosciences, Burlington, ON, Cat. No. SM0311) was used to determine the approximate bp length of amplified products. Electrophoresis was performed at 70V for 1h.

h. Conditions for real-time quantitative polymerase chain reaction (qPCR):

A low, 96-well clear plate was used for qPCR (Bio Rad Laboratories, Hercules, CA, Cat. No. MLL9601). Each well contained a total volume of 10 µL composed of 5 µL LuminoCt SYBR Green qPCR ReadyMix (Sigma-Aldrich, Oakville, ON, Cat. No. L6544-500RXN), 0.2 µL of each 10 µM primer, 0.6 µL H_2O, and 4 µL cDNA template. A Bio Rad C1000 Thermocycler equipped with a Bio Rad CFX96 Real Time Detection

System was used for qPCR reactions. The initial denaturation step was carried out at 95°C for 3 min. The cycles were composed of a denaturation step at 95°C for 5 s followed by an annealing and extension step at 62°C for 30 s. The fluorescence from the wells was monitored at the end of each annealing and extension step. This cycle was repeated 39 times. Upon completion, a melt curve analysis was performed. For this analysis the plate was held at 62°C for 5 s then the temperature was increased by 0.5°C, 5 s increments until reaching a final temperature of 95°C. The fluorescence from the wells was monitored before increasing the temperature. Two technical replicates were conducted for each biological replicate. Internal reference genes were selected from list suggested by Czechowski et. al (2005). The real time PCR data was analyzed using Bio Rad CFX Manager (Version No. 2.1).

i. Primer validation:

Quantitative PCR reactions were set up as above, except the annealing and extension steps were run on a gradient ± 5°C of the predicted optimal melting temperature for the primers as provided by Integrated DNA Technologies. The temperature with the lowest Cq value (the point where the fluorescence crosses the threshold for detection (Heid, et. al., 1996)) is considered the optimal annealing temperature. To determine the efficiency of the reaction, an eight-point standard curve reaction plate was set up. The dilutions for the eight-point standard curve were derived from the following formula (provided in the qPCR seminar by Sean Taylor of Bio-Rad):

$$\frac{(35-Cq)}{8} \qquad \text{Equation 1.}$$

An efficiency of between 90 to 110% with a regression value of 0.98 or higher at the optimal temperature means the primer is considered valid for use in qPCR. (Bustin, et. al., 2009). Amplified products of qPCR were run on a 2% (w/v) agarose gel (1 g agarose, 50 ml 1X TAE buffer, 5 µL Gel Red nucleic acid stain; see section above for electrophoresis details) for 2.5 h at 40 V.

Primers for qPCR were designed from *Thellungiella salsuginea* (Yukon ecotype) cDNA library sequences produced from Roche 454 pyrosequencing (See Table 2 for primer sequence information). Primers were then validated by RT-qPCR using pooled cDNA derived from mRNA of *Thellungiella* seedlings.

The first step for the validation analysis involved running a temperature gradient (approximate melting temperature provided by Integrated DNA Technologies ± 5°C) with temperature changing in 1.25 °C increments to determine the optimal annealing temperature for the oligonucleotide primers. The primers were designed using NCBI primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to meet stringent temperature requirements for annealing at 62 ºC and empirical testing confirmed that the

Table 2. Primer sequence and validation information for qPCR primers sets.

Gene	Primer Sequence	Cq Value	Dilution Factor	Efficiency (%)	Points used in Standard curve	Slope
Actin 1	F: 5-'GGCGATGAAGCTCAATCCAAACG-3' R: 5'-GGTCACGACCAGCAAGATCAAGACG-3'	20	4	94	6	0.996
At5g12240	F: 5'-CTCTCTGCTTCAACCTCACCAAC-3' R: 5'-GTAGACAAAGAGAGAGAATCGGAGG-3'	22	3	108	5	0.987
UBQ5	F: 5'-CTTCGAGCTGCTTTCCGGCGAA-3' R: 5'-GGAGGTCGAGAGTTCTGACACCA-3'	14	6	109	6	0.993
EF1-α	F: 5'-TGAAGCGTGGTTACGTTGCCTCC-3' R: 5'-TGGGAGGTGAAGTTGGCTGCT-3'	18	4	106	7	0.998
RNS1	F: 5'-CCAAACCGCTCTTAACCTTAGGCA-3' R: 5'-CCGGAAGCAGACCGGTCAACA-3'	19	4	101	6	0.996
At4	F: 5'-AGGGGATGGCCCAAAACACAAGA-3' R: 5'-CGAGGGGAACCGAAGCTTGCC-3'	19	4	103	5	0.981
Pht1;1	F: 5'-CCGCCGATTTGCTCCTCATCCT-3' F: 5'-GGGATATCGGCGGCGTCTGG-3'	23	3	105	6	0.992
Pht1;4	R: 5'-CCTCGGTCGTATTTATTACCACG-3' F: 5'-CCATCACAGCTTTTGGCTCATG-3'	23	3	100	5	0.890
Pht1;5	F: 5'-GTTCACCGTCGCGTTCATCGAT-3' R: 5'-ACAGCGAATTCCTAACTCCGATCC-3'	26	2	110	6	0.986
WRKY75	F: 5'-CCGCTGTTAGCCTTTGCACTTGC-3' R: 5'-GGACTCAATGAATCATCCACGACGG-3'	24	3	110	5	0.992
Siz1	F: 5'-CGGCAACCACTGTATGCAGGTCCA-3' R: 5'-GCAGTGCTACTGGAAGTGGTAGGG-3'	22	3	112	5	0.997
PHR1	F: 5'-ATGTGGAGGCTGTCCCTTTGCCG-3' R: 5'-TCGCCAGAGAAGAAGTTGACACCG-3'	22	3	114	5	0.994
Pht2;1	F: 5'-AATTCCGCCGCGAACCCTCGA-3' R: 5'-TTCGCTCACGGAGGCAACGACG-3'	22	3	115	5	0.997
Toc1	F: 5'-ACCATACTGAGGCACCATGGCTGCT-3 R: 5'-CGGTCTCGTACGCTTCCCACATCG-3'	23	3	99	6	0.993
ACP5	F: 5'-GGGACGGTTTGATCTCGCTGCCA-3' R: 5'-AGATTCTTCCAGGTGGGTCGTCGGA-3'	17	2	109	6	0.990
SQD1	F: 5'-GCCGATGGAAGGCTTTGACAGGG-3' R: 5'-AGGCGCGGATCTCTGTTCACCA-3'	20	2	109	7	0.985

optimum temperature was indeed 62 ºC for all of the primer sets. The solutions of cDNA were then serially diluted to generate an eight-point standard curve using appropriately diluted primers (each primer set was tested for dilution using Equation 1, Method and Materials section titled Primer validation). A RT-qPCR efficiency between 90-110% with a slope regression greater than 0.98 are required to validate the use of primers for qPCR following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin, et. al., 2009). With these established parameters, all but one of the primer sets was validated for use. The exception were primers used for *Pht1;4,* which did not meet the strict requirements for validation (Efficiency of 89%). However, the amplicon generated by PCR using these primers was sequenced and the single product made matched the expected *Pht1;4* sequence providing independent verification that the primers for *Pht1;4* were amplifying the correct product (Fig. 2). The dilution factors used to make the standard curve, the slope of the line, and the PCR efficiency for the primers used are given in Table 2. The resulting amplicons generated by PCR were analyzed by agarose gel electrophoresis to ensure the length of the product matched the predicted value (Fig. 3).

Figure 2. Sequence confirmation for PCR amplicon corresponding to *Pht1;4.*

Alignment of the NCBI nucleotide sequence of *AtPht1;4* (Expected) with the sequence of the PCR product (Returned). PCR was performed using primers designed to amplify *Pht1;4* using sequence obtained from RNAseq transcriptome database (unpublished). The predicted product was a 239 bp region of *Pht1;4*. There was 92% agreement between the sequenced amplicon and the expected product with the discrepancy found at the beginning of the sequenced amplicon where read quality is poor.

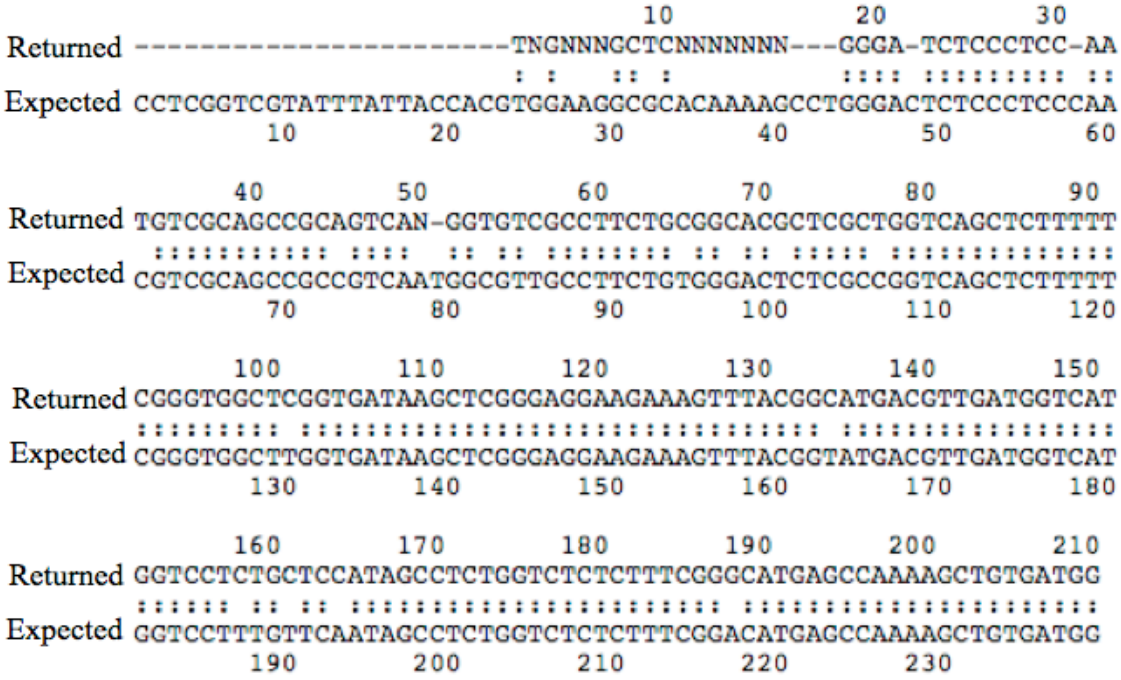
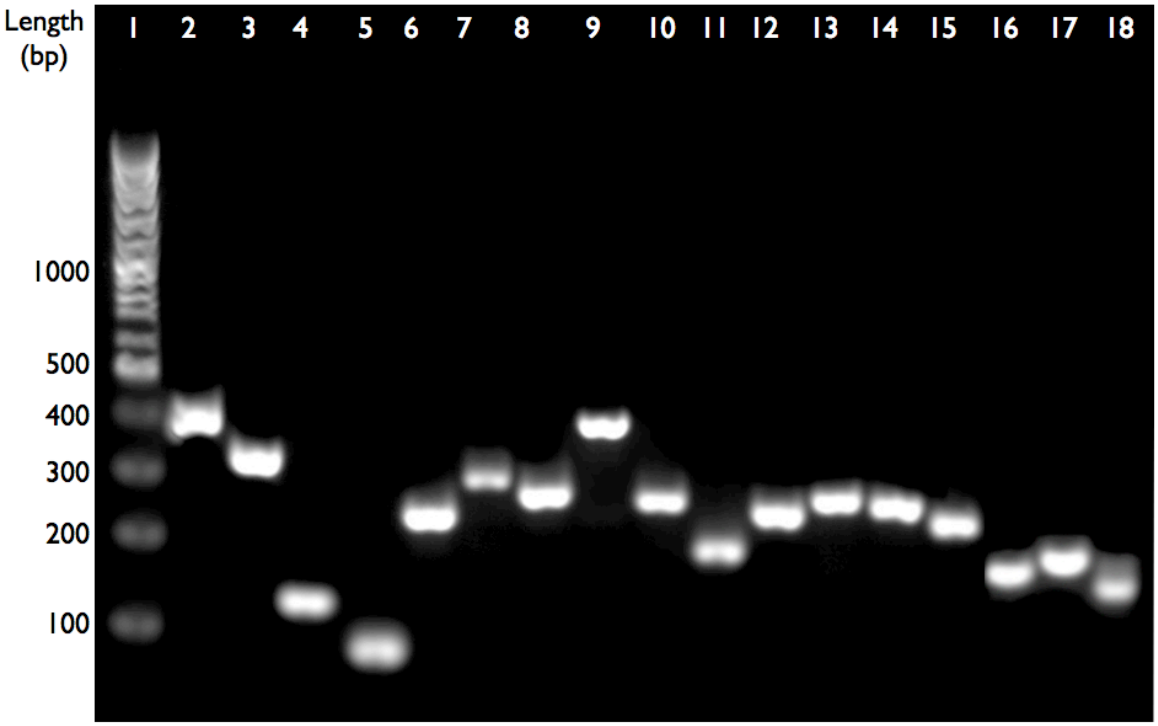

Returned ----------TNGNNNGCTCNNNNNNN---GGGA-TCTCCCTCC-AA
Expected CCTCGGTCGTATTTATTACCACGTGGAAGGCGCACAAAAGCCTGGGACTCTCCCTCCCAA
Returned TGTCGCAGCCGCAGTCAN-GGTGTCGCCTTCTGCGGCACGCTCGCTGGTCAGCTCTTTTT
Expected CGTCGCAGCCGCCGTCAATGGCGTTGCCTTCTGTGGGACTCTCGCCGGTCAGCTCTTTTT
Returned CGGGTGGCTCGGTGATAAGCTCGGGAGGAAGAAAGTTTACGGCATGACGTTGATGGTCAT
Expected CGGGTGGCTTGGTGATAAGCTCGGGAGGAAGAAAGTTTACGGTATGACGTTGATGGTCAT
Returned GGTCCTCTGCTCCATAGCCTCTGGTCTCTCTTTCGGGCATGAGCCAAAAGCTGTGATGG
Expected GGTCCTTTGTTCAATAGCCTCTGGTCTCTCTTTCGGACATGAGCCAAAAGCTGTGATGG

Figure 3. Amplicon products for qPCR primer validation.

The amplified products were run on a 2% agarose gel for 3 h at 45 volts. Lane 1, 100 bp DNA ladder; lane 2, *Actin1* (391 bp); lane 3, *At5g12240* (317 bp); lane 4, *Ubq5* (100 bp); lane 5, *EF1-α* (70 bp); lane 6, *RNS1* (216 bp); lane 7, *At4* (267 bp); lane 8, *Pht1;1* (245 bp); lane 9, *Pht1;4* (239 bp); lane 10, *Pht1;5* (377 bp); lane 11, *WKRY75* (175 bp); lane 12, *Siz1* (247 bp); lane 13, *PHR1* (253 bp); lane 14, *Pht2;1* (236 bp); lane 15, *Toc1* (250 bp); lane 16, *ACP5-1* (153 bp); lane 17, *ACP5-2* (166 bp); and lane 18, *SQD1* (137 bp).


Length
(bp)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
1000
500
400
300
200
100

III. Statistical analysis:

Nested ANOVA and post-hoc testing were performed using SAS version 9.2 (www.sas.com) to analyze all gene expression data. A Type III Sum of Squares was used to determine statistical significance. The ANOVA was considered significant if $p < 0.05$. A significant result was followed up by a Tukey post-hoc test using SAS. Full statistical results are shown in Appendix C.

RESULTS

I. *Thellungiella salsuginea* (Yukon ecotype) seedling response to low phosphate

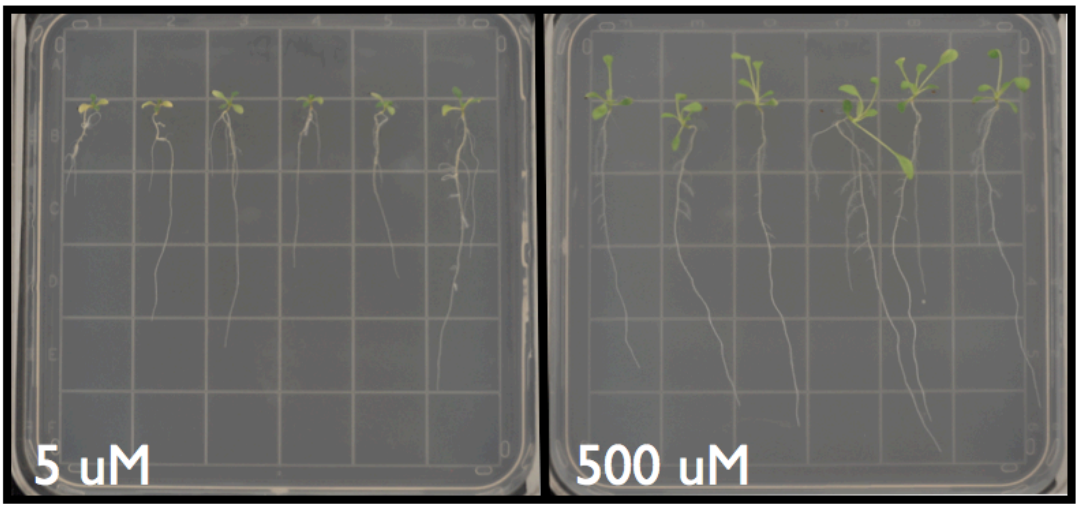
Thellungiella seedlings were grown on defined media plates under two different phosphate conditions, 5 μM and 500 μM, with all other required nutrients unaltered (See Table 1 in Methods and Materials for media formulation). These concentrations were tested to evaluate their effect on seedling growth and the expression of a number of known phosphate responsive genes from *Arabidopsis*.

a. Growth analysis

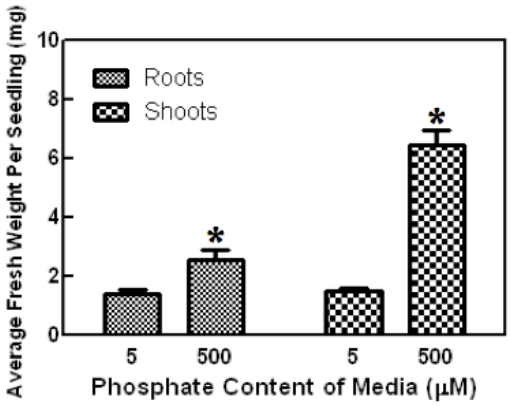
Seedlings grown on low (5 μM) phosphate were visibly smaller than plants grown on media containing a higher (500 μM) phosphate content (Fig. 4). *Thellungiella* seedlings grown under high phosphate conditions had an average shoot fresh weight of 6.44 ± 0.51 mg, about 4-fold more biomass than seedlings grown under low phosphate conditions at 1.51 ± 0.11 mg. The average root fresh biomass of the seedlings grown under high phosphate conditions was 1.8-fold greater than those grown under low phosphate conditions at 2.54 ± 0.37 mg and 1.39 ± 0.19 mg for high and low phosphate treatments, respectively (Fig. 4).

Figure 4. Response of *Thellungiella salsuginea* (Yukon ecotype) seedlings to variable phosphate. (A) Representative seedlings shown after after 15 d on defined media plates containing either 5 or 500 μM phosphate. Media contained all other required nutrients (see Materials and Methods for media preparation). (B) Average fresh weight (mg per seedling) at the time of harvest (15 d after transfer to phosphate media treatment). Mean ± standard error (SE) for three independent biological replicates (n = a minimum of 54 seedlings per treatment per replicate). Seedlings grown on the 500 μM phosphate media had significantly more root ($P = 0.05$) and shoot biomass ($P = 0.0008$).

A


5 uM
500 uM

B


Average Fresh Weight Per Seedling (mg)
10
8
6
4
2
0
Roots
Shoots
*
*
5
500
5
500
Phosphate Content of Media (µM)

b. Gene expression analysis:

i. Selection of gene subset for biomarker testing

The discernible difference in growth of seedlings exposed to the two phosphate treatments is consistent with a phosphate deficiency under 5 μM phosphate exposure. With this phenotypic response it was possible to proceed to the next step to corroborate the deficiency by independent means using an analysis of gene expression patterns. The subset of genes selected for testing was based upon a group of genes reported to be phosphate responsive in *Arabidopsis* (Bariola, et. al., 1994; Karthikeyan, et. al., 2002; Shin, et. al., 2006). For testing the following criteria were considered: 1) the gene has a history of responsiveness to phosphate in the literature or a reported role in the phosphate deficiency response pathway, 2) the gene was tightly linked to phosphate stress with no previously identified responses to other stressors, and 3) the gene could be expressed in roots or shoots but shoot localized genes are more desirable with respect to accessibility and reduced adverse impact on the plant with leaf removal as opposed to root collection . This last consideration would make estimating transcript abundance easier for future use as a biomarker of phosphate stress.

ii. Phosphate responsive gene expression in shoots

Transcripts associated with *RNS1* were detected at a significant, 25-fold higher abundance in shoots of *Thellungiella* seedlings grown for 15 days on defined media containing 5 μM phosphate compared with those grown on media containing 500 μM

phosphate ($P < 0.001$) (Fig. 5). Similarly, transcripts associated with *At4* and *WRKY75* were each detected at around 20-fold higher abundance in seedlings grown under low phosphate conditions than those grown under high phosphate conditions ($P = 0.002$ for both *At4* and *WRKY75*) (Fig. 5).

Not all genes showed statistically significant increases in expression in seedlings grown on 5 μM as opposed to 500 μM phosphate. Transcripts of *Siz1* in *Thellungiella* shoots grown under low phosphate were detected at a level double of seedlings grown under high phosphate conditions but the difference in expression is not statistically significant ($P = 0.301$). Moreover, *PHR1* and *Pht2;1* transcripts were detected in lower abundance in *Thellungiella* seedling shoots grown under low phosphate conditions compared with those on high phosphate ($P = 0.379$ and < 0.001, respectively; Fig. 7).

iii. Phosphate responsive gene expression in roots

Transcripts of *At4* were 4-fold more abundant in the roots of *Thellungiella* seedlings grown for 15 days on media containing 5 μM phosphate than those grown on media containing 500 μM phosphate ($P = 0.007$; Fig. 6). The transcripts of two high-affinity phosphate transporters were also detected in higher abundance in seedlings grown under low phosphate conditions. Transcripts of *Pht1;1* were almost 11-fold more abundant, and transcripts of *Pht1;5* were 13-fold more abundant in the roots of seedlings grown under low phosphate conditions compared to high phosphate conditions ($P = 0.002$ and 0.001, respectively; Fig. 6). Transcripts of the high-affinity phosphate transporter *Pht1;4* were

Figure 5. Transcript abundance of *RNS1*, *At4,* and *WRKY75* in the shoots of 20-day-old *Thellungiella* (Yukon ecotype) seedlings grown on defined media plates with variable phosphate. Plates contained 5 or 500 µM phosphate with all other required nutrients constant. Gene expression was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5.* Data are the mean ± standard error (SE) for three independent biological replicates (n = a minimum of 54 seedlings per treatment per replicate). $P < 0.05$ as tested by nested ANOVA.

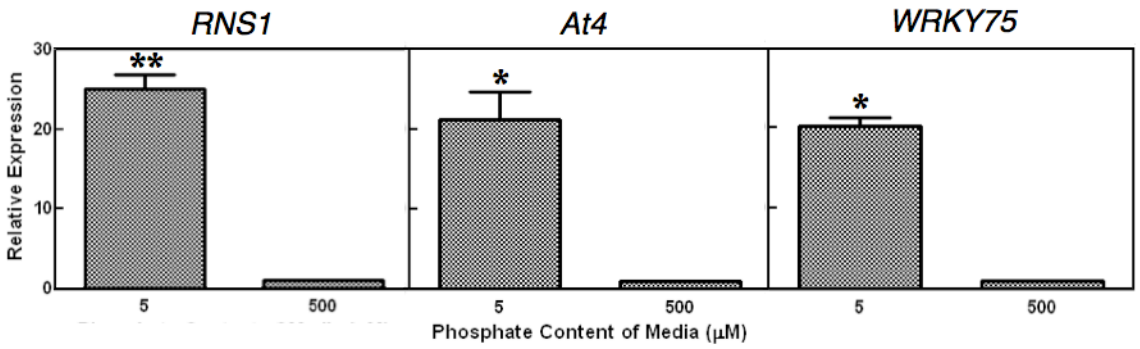

RNS1
At4
WRKY75
Relative Expression
30
20
10
0
**
*
*
5
500
5
500
5
500
Phosphate Content of Media (μM)

Figure 6. Transcript abundance of *At4*, *Pht1;1,* and *Pht1;5* in the roots of 20-day-old *Thellungiella* (Yukon ecotype) seedlings grown on modified media containing 5 or 500 µM phosphate. Gene expression was normalized to the expression of reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Data are the mean ± standard error (SE) for three independent biological replicates; n = a minimum of 54 seedlings per treatment per replicate. $P < 0.05$, as tested by nested ANOVA.

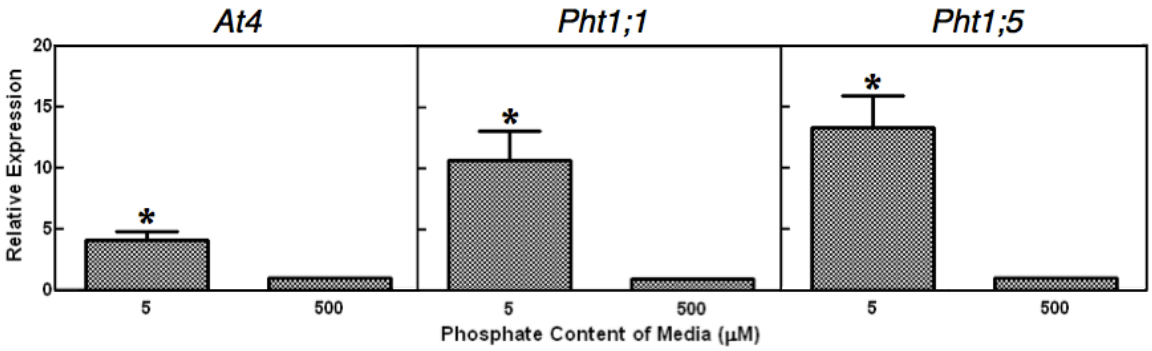
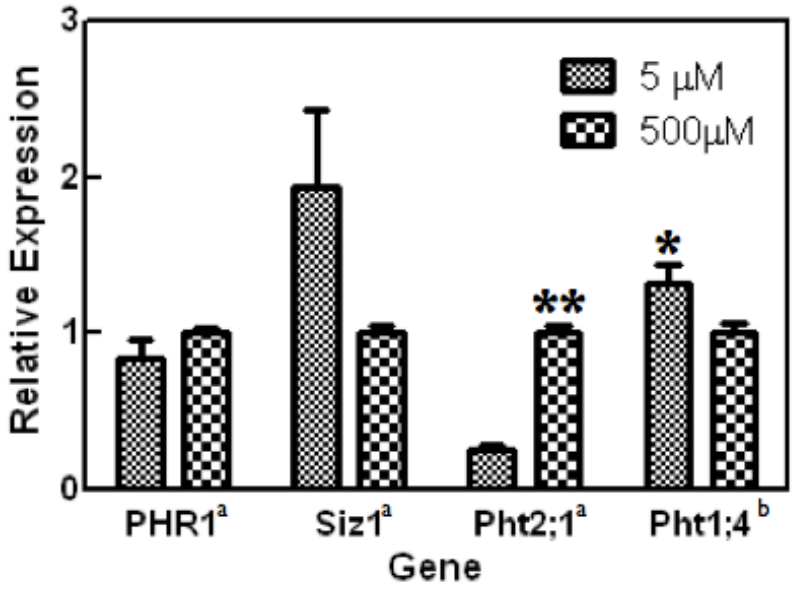

At4
Pht1;1
Pht1;5
Relative Expression
20
15
10
5
0
*
*
*
5
500
5
500
5
500
Phosphate Content of Media (μM)

Figure 7. Transcript abundance of *PHR1*, *Siz1,* and *Pht2;1* in the shoots and *Pht1;4* in the roots of 20-day-old *Thellungiella* (Yukon ecotype) seedlings grown on modified media containing 5 µM or 500 µM phosphate. Gene expression was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Data are the mean ± standard error (SE) for three independent biological replicates; n = a minimum of 54 seedlings per treatment per replicate. The difference in transcript abundance between treatments is statistically significant for *Pht2;1* and *Pht1;4* at P = 0.05 (shown by nested ANOVA). There is no significant difference in transcript abundance of *PHR* and *Siz1* in seedlings grown on high and low phosphate.



[a]Abundance in shoot tissue
[b]Abundance in root tissue

1.3 fold higher in roots of seedlings grown under low phosphate compared with those grown under high phosphate conditions (P = 0.007; Fig. 7).

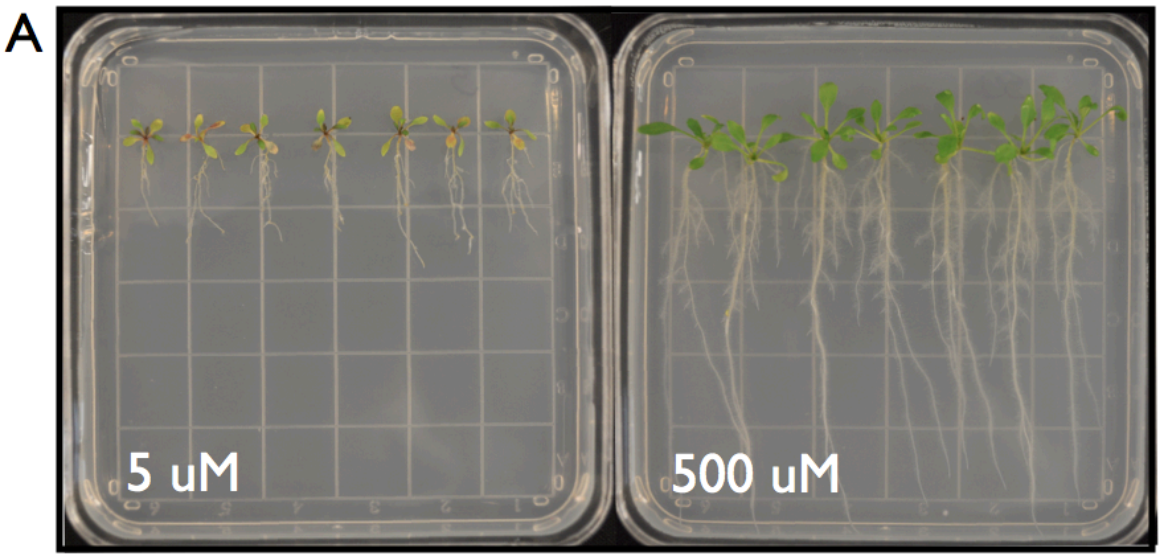
II. *Thellungiella salsuginea* (Shandong ecotype) response to low phosphate

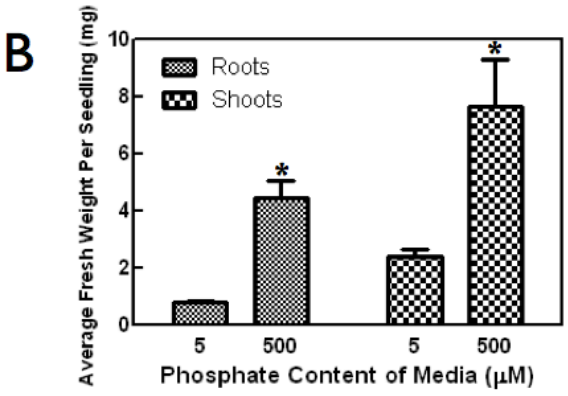
a. Growth analysis

Thellungiella salsuginea (Shandong ecotype) seedlings were grown on media plates containing a phosphate content of either 5 or 500 μM. Seedlings grown on media with 5 μM were visibly smaller than those on plates with 500 μM phosphate (Fig. 8). The average root fresh weight per seedling at 4.47 ± 0.6 mg was almost 6-fold higher for seedlings grown on plates with 500 μM phosphate as compared to the 0.8 ± 0.03 mg average root mass for seedlings grown on 5 μM phosphate. Shoot biomass was also 3.4 times greater for seedlings grown on high phosphate media (8.1 ± 1.6 mg) compared to those on low phosphate media (2.4 ± 0.25 mg) .

On average, the biomass (shoot and root) for Shandong *Thellungiella* seedlings was greater than for Yukon ecotype seedlings, particularly on the 500 uM phosphate media. The shoot biomass of Shandong *Thellungiella* was 1.6- and 1.3-fold greater than Yukon seedlings when grown on plates with 5 μM (P = 0.0275) and 500 μM phosphate (P = 0.3729), respectively. The root biomass of Shandong *Thellungiella* seedlings was, on average, 1.9 fold greater than Yukon *Thellungiella* when grown on plates with 500 μM phosphate (P = 0.523) but on plates with 5 μM phosphate the biomass of Yukon *Thellungiella* roots was 1.7-fold greater than Shandong *Thellungiella* (P = 0.0351).

Figure 8. Response of *Thellungiella salsuginea* (Shandong ecotype) seedlings to variable phosphate. (A) Representative seedlings after 15 d growth on defined media plates containing either 5 or 500 µM phosphate with all other required nutrients constant. (B) Average fresh weight (mg per seedling) at the time of harvest (15 d following transfer to treatment media). Mean ± standard error (SE) for three independent biological replicates; n = a minimum of 54 seedlings per treatment per replicate. Seedlings grown on the 500 µM phosphate media had significantly more root ($P = 0.0039$) and shoot biomass ($P = 0.0229$).


A
5 uM
500 uM


B
Average Fresh Weight Per Seedling (mg)
10
8
6
4
2
0
Roots
Shoots
*
*
5
500
5
500
Phosphate Content of Media (μM)

b. Expression of phosphate responsive genes in shoots and roots

The abundance of transcripts associated with *RNS1* was 256-fold higher ($P < 0.001$) in the shoots of seedlings grown on 5 μM phosphate compared to those grown on 500 μM phosphate. Increased abundance of transcripts associated with *At4* (73 fold higher, $P < 0.001$) was also detected in the shoots of seedlings grown on low relative to high phosphate media (Fig. 9).

In the roots, expression of *At4* was higher in seedlings grown on low (5 μM) phosphate media, though only a 10-fold increase in abundance relative to *At4* expression on high phosphate medium was detected. The expression of the high affinity phosphate transporters *Pht1;1* and *Pht1;5* was also detected at higher levels in the roots of seedlings grown on media containing 5 μM phosphate relative to 500 μM . Transcripts associated with *Pht1;1* showed a 53-fold increase in abundance in seedlings grown on low compared to high phosphate ($P = 0.0002$) while *Pht1;5* expression increased 37-fold under the same conditions ($P = 0.0008$) (Fig. 10).

Four other genes were assayed for phosphate-responsive expression but did not show a significant difference in abundance in response to low phosphate. *PHR1* and *Siz1* underwent increases in transcript abundance in *Thellungiella* shoots in response to low phosphate but their responses were variable and hence not significant ($P = 0.2423$ and $P = 0.1805$, respectively). In the roots, expression of *Pht1;4* increased 2.6-fold in seedlings grown on low phosphate (5 μM) compared with those grown on high phosphate (500 μM) media ($P = 0.1076$). In contrast, the level of *Pht2;1* transcripts was significantly higher in

Figure 9. Transcript abundance of *RNS1* and *At4* in the shoots of 20-day-old *Thellungiella* (Shandong ecotype) seedlings grown on defined media plates containing 5 or 500 μM phosphate. Media contained all other required nutrients at the same level. Gene expression was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Data are the mean ± standard error (SE) for three independent biological replicates; n = a minimum of 54 seedlings per treatment per replicate. The increased abundance of *RNS1* and *At4* in seedlings grown on low phosphate media is statistically significant at P = 0.05 as tested by nested ANOVA.

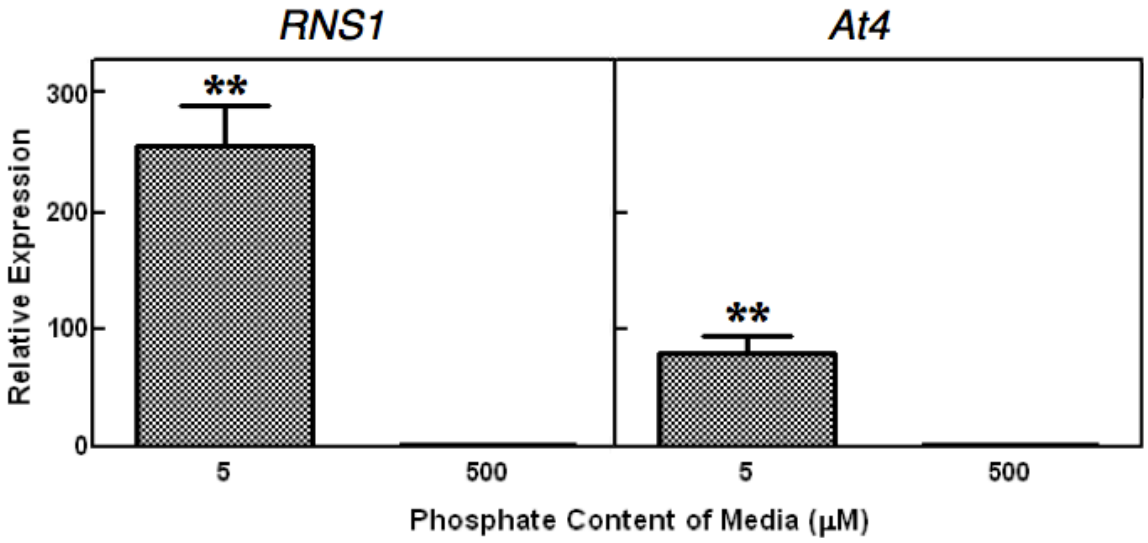

RNS1
At4
**
**
300
200
100
0
Relative Expression
5
500
5
500
Phosphate Content of Media (µM)

Figure 10. Transcript abundance of *At4*, *Pht1;1,* and *Pht1;5* in the roots of 20-day-old *Thellungiella* (Shandong ecotype) seedlings grown on defined media plates containing 5 or 500 μM phosphate. Media was prepared with all other required nutrients held constant. Gene expression was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5.* Data are the mean ± standard error (SE) for three independent biological replicates, n = a minimum of 54 seedlings per treatment per replicate. The increase in abundance of all three genes in seedlings grown on low phosphate media is statistically significant at P = 0.05 as tested by nested ANOVA.

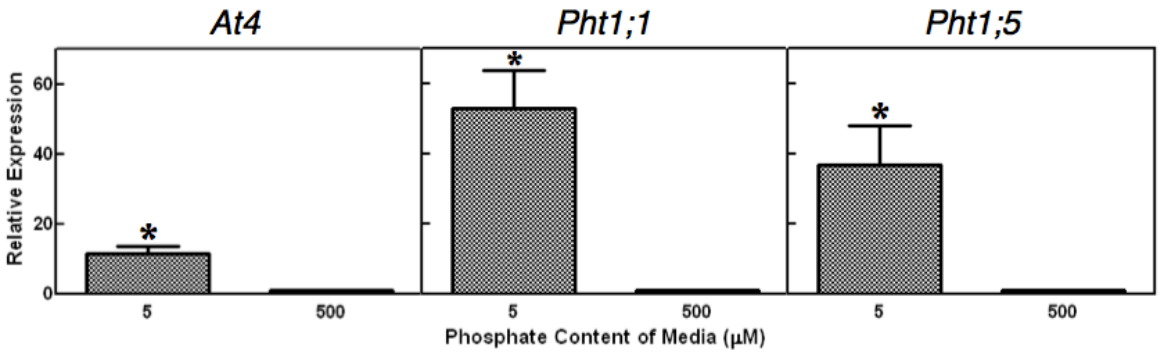
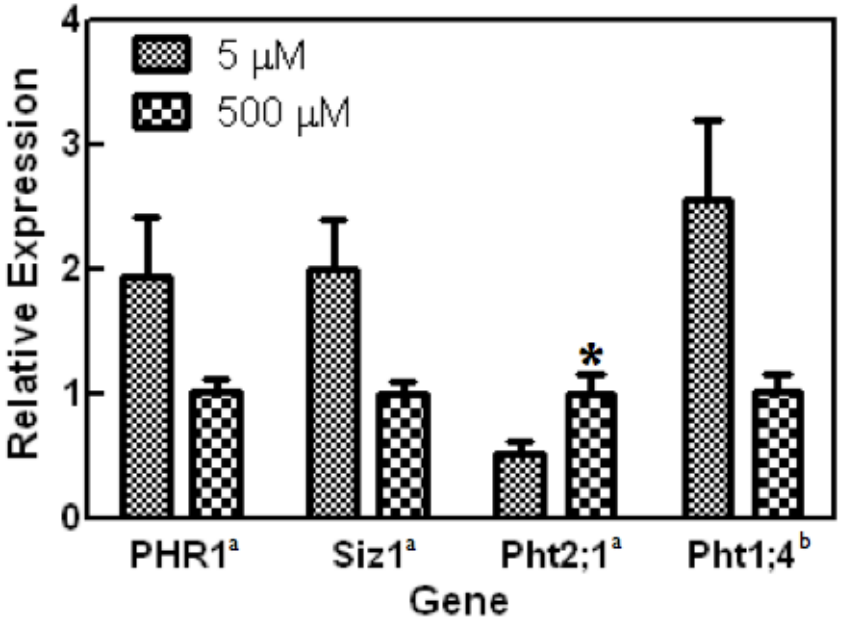

At4
Pht1;1
Pht1;5
Relative Expression
60
40
20
0
*
*
*
5
500
5
500
5
500
Phosphate Content of Media (μM)

Figure 11. Transcript abundance of *PHR1*, *Siz1,* and *Pht2;1* in the shoots and *Pht1;4* in the roots of 20-day-old *Thellungiella* (Shandong ecotype) seedlings grown on defined media plates containing 5 or 500 μM phosphate. Media contained a constant complement of other required nutrients. Gene expression was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Data are the mean ± standard error (SE) for three independent biological replicates, n = a minimum of 54 seedlings per treatment per replicate. *Pht2;1* expression is statistically significant at P = 0.05 as determined by nested ANOVA.



[a]Abundance in shoot tissue
[b]Abundance in root tissue

shoots of seedlings grown on high phosphate media compared with those grown on low phosphate media (P = 0.0339) (Fig. 11).

III. Testing for diurnal changes in gene expression in *Thellungiella* seedlings

Time of harvest can affect estimates of transcript abundance through the influence of circadian regulation (Strayer, et. al., 2000). In this study, tissue for mRNA extractions was routinely harvested midway through the light cycle. However, if this harvest time coincided with a peak or trough in the cyclical, diurnal pattern of gene expression it is possible that estimates of expression are misleading. For this study *Thellungeilla*, seedlings (Yukon ecotype) were grown on defined media plates containing either 5 μM or 500 μM phosphate until the primary root reached the bottom of the plate and seedlings were harvested either midway through the light cycle or dark cycle (referred to here as day and night, respectively). All genes previously mentioned were tested for patterns of circadian regulation except *WRKY75*, as it is reported to be influenced by other stressors (Devaiah, et. al., 2007) and was excluded from further consideration at this stage of the project as a phosphate biomarker. *PHR1, Siz1,* and *Pht2;1* were also excluded from the study because *PHR1* and *Siz1* did not show significant differential expression in response to phosphate content of the media (Fig. 5). *Pht2;1* was excluded because it showed a decrease in transcript abundance in response to phosphate stress (Fig. 7) therefore it was not included as a potential biomarker of phosphate stress.

In the shoots, *RNS1* transcripts increased in response to the low (5 μM) phosphate

treatment but the amplitude of expression of this gene was not affected by time of harvest (Fig. 12). That is, *RNS1* transcript levels estimated from tissue samples obtained at night or during the day were not significantly different for seedlings grown in media with the same phosphate content (Fig. 12). Expression of *At4* in shoots was found to be significantly different between day and night for seedlings grown in the low phosphate treatment but the lower expression level at night remains significantly higher than the transcript level in shoots of seedlings grown on high phosphate media (Fig. 12). In root tissues, expression of *At4* as well as the high affinity phosphate transporter *Pht1;5,* were the same regardless of the time of harvest (Fig. 13). The abundance of *At4* transcripts in the shoot tissue, and transcripts of *Pht1;1* in the root tissue were significantly different between mid-day harvest and night harvest. However, the significantly different expression level seen between seedlings grown on low and high phosphate media is consistent at either time point and easily seen regardless of harvest time (Fig.13). Expression of *Pht1;4* was not significantly affected by time of harvest nor phosphate content of the media ($P = 0.764$) (Fig. 13).

Toc1 is a gene with known circadian regulation in *Arabidopsis.* This gene was selected as a positive control to determine whether the scheduled harvests were suitable for identifying circadian patterns in gene expression if they strongly influenced the expression of phosphate responsive genes. Figure 11 shows that *Toc1* is expressed at different levels during the night and day and that transcript levels were consistent regardless of phosphate content of the media. This result gives evidence that a diurnal

Figure 12. Diurnal expression analysis of *RNS1, At4,* and *Toc1* in the shoots of *Thellungiella* (Yukon ecotype) seedlings grown on defined media plates containing 5 µM and 500 µM phosphate. Seedlings (20-day-old) were harvested half-way through the light cycle (day) or half-way through the dark cycle (night). Gene expression was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Data are the mean ± standard error (SE) for two independent biological replicates; n = a minimum of 54 seedlings per treatment per replicate. Significance was determined using a nested ANOVA (P = 0.05). Different letters represent significant differences between the means based upon a Tukey post-hoc test.

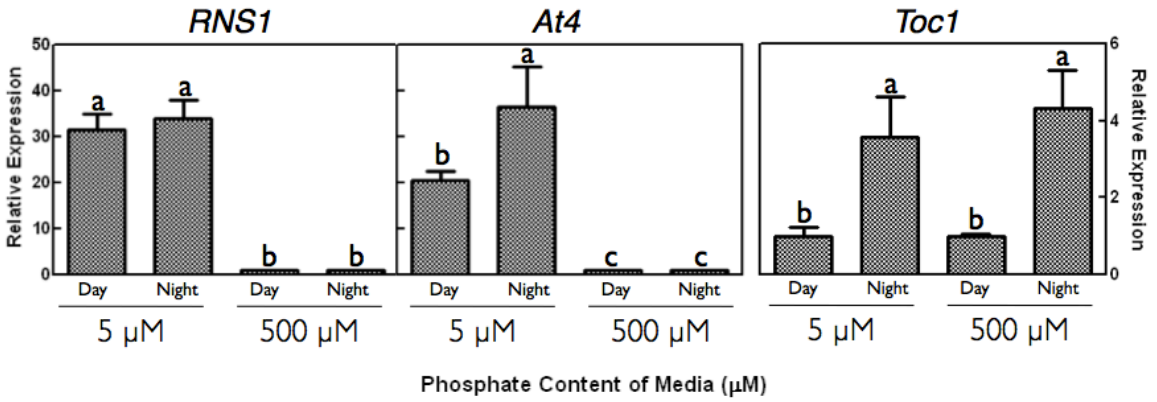
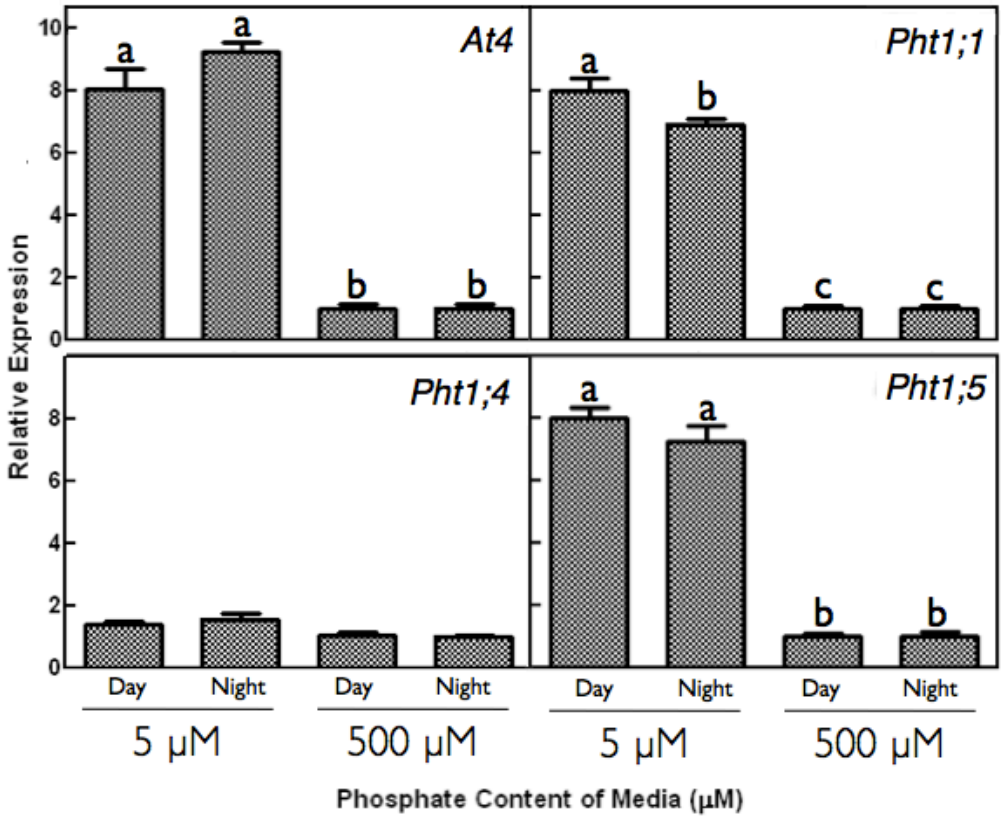

RNS1
At4
Toc1
Relative Expression
Relative Expression
50
40
30
20
10
0
6
4
2
0
a
a
b
b
b
a
c
c
b
a
b
a
Day
Night
Day
Night
Day
Night
Day
Night
Day
Night
Day
Night
5 µM
500 µM
5 µM
500 µM
5 µM
500 µM
Phosphate Content of Media (µM)

Figure 13. Diurnal expression analysis of *Pht1;1*, *Pht1;4*, *Pht1;5,* and *At4* in the roots of 20-day-old *Thellungiella* (Yukon ecotype) seedlings grown on 5 μM and 500 μM phosphate. Seedlings were harvested halfway through the light cycle (day) or halfway through the dark cycle (night). Gene expression was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5.* Data are the mean ± standard error (SE) for two independent biological replicates; n = a minimum of 54 seedlings per treatment per replicate. Data was statistically significant as determined by a nested ANOVA (P = 0.05). Different letters represent significant differences between the means based upon a Tukey post-hoc test.


At4
Pht1;1
Pht1;4
Pht1;5
Relative Expression
0
2
4
6
8
10
a
a
b
b
a
b
c
c
a
a
b
b
Day
Night
Day
Night
Day
Night
Day
Night
5 µM
500 µM
5 µM
500 µM
Phosphate Content of Media (µM)

change in gene expression would have been identified with the time points selected for this study. The differential expression of *At4* in shoots (Fig. 12) and *Pht1;1* in roots (Fig. 13) between the day and night cycles offers an indication that transcripts associated with these genes may undergo circadian changes but differences corresponding to phosphate treatments are easily distinguished nonetheless.

IV. Gene expression in response to seedling transfer from high phosphate media to low phosphate media

Thellungiella (Yukon ecotype) seedlings were assayed for changes in gene expression at various time points following transition to low phosphate media. In this study, seedlings were grown on high phosphate media (500 μM) until their primary root was approximately 4 cm long and then seedlings were carefully transferred to low phosphate media (5 μM) for a period of time (ranging from 1 to 5 days) before being harvested. A qPCR analysis was performed to quantify expression of *RNS1, At4, Pht1;1, Pht1;4,* and *Pht1;5* using shoots and roots of the seedlings. To determine whether changes in gene expression could be attributed to damage to the seedlings, a set of seedlings were transferred from high phosphate media to high phosphate media (transfer control, TC) and gene expression was assayed after 5 days (Fig. 14).

In the shoots, the abundance of *RNS1* increased two-fold after one day following transfer to the low phosphate media then its expression remained constant over the course of five days ($P = 0.9459$). In contrast, on the low phosphate media the abundance of *At4*

transcripts doubled on the first day, they increased 16-fold by the third day and then after five days the level of *At4* expression was 30-fold higher relative to day one ($P = 0.0072$). Gene expression analyses using seedlings transferred from high phosphate media to high phosphate media showed no significant change over the basal level of expression for the first day even at the end of a five day period (Fig. 15).

In roots the abundance of *Pht1;4* transcripts doubled after the first day on low phosphate media then remains unchanged for the duration of the experiment ($P = 0.746$). The high affinity transporters encoded by *Pht1;1* and *Pht1;5* showed increased transcript abundance over the five day experiment. *Pht1;1* transcripts increased 4-fold after 3 days on low phosphate media, then doubled to 8-fold relative to day 0 by day 5 ($P = 0.0348$). The abundance of *Pht1;5* doubled after one day on low phosphate media, this level increased to 5-fold by the third day then remained unchanged. However, the changes seen for *Pht1;5* expression was not statistically significant ($P = 0.2064$). The abundance of *At4* transcripts in the roots increased significantly during the five day experiment ($P < 0.001$) with a 10-fold increase between the first day and day four (Fig. 16).

Expression of *PHR1* and *Siz1*, which encode products that are important for the low phosphate response in *Arabidopsis,* were also assayed for their temporal expression following transfer from high to low phosphate. The temporal expression of *PHR1* and *Siz1* transcript abundance in *Thellungiella* shoots showed no significant difference over the five day duration of the experiment ($P = 0.2418$ and $P = 0.7951$, respectively.) (Fig. 17). This observation is consistent with the earlier experimental comparison of *PHR1* and

Figure 14. Expression analysis of phosphate-responsive genes in *Thellungiella salsuginea* (Yukon ecotype) seedlings transferred from 500 μM phosphate to 5 μM phosphate. Seedlings were grown on defined media plates containing 500 μM phosphate until their primary root length reached approximately 4 cm long (approximately 20 days post-germination). Seedlings were transferred to defined media plates containing 5 μM phosphate. Each day seedlings were harvested, RNA was extracted for cDNA synthesis and detection of gene expression by qPCR. A set of seedlings were transferred from 500 μM media plate to a fresh 500 μM media plate to serve as a Transfer Control (TC).

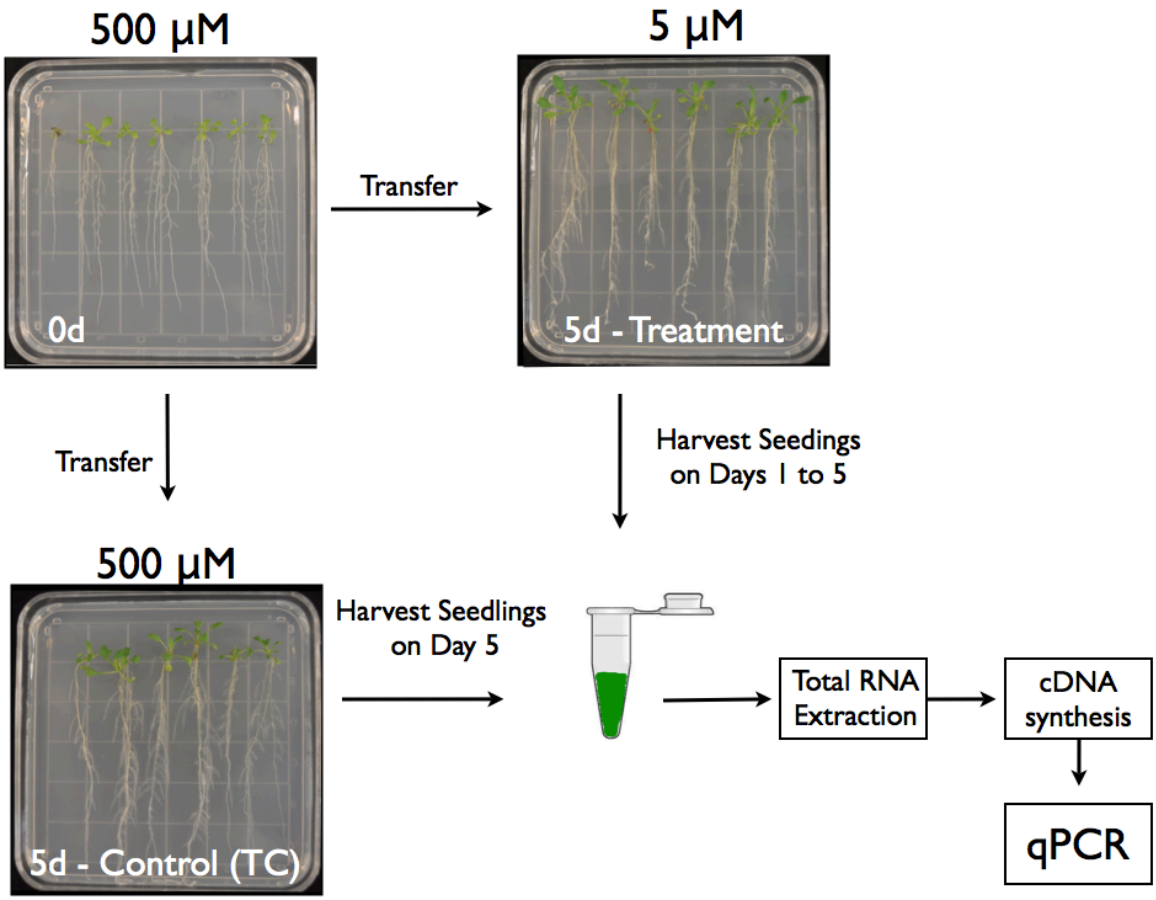

500 µM
5 µM
Transfer
0d
5d - Treatment
Transfer
Harvest Seedings
on Days 1 to 5
500 µM
Harvest Seedlings
on Day 5
Total RNA
Extraction
cDNA
synthesis
qPCR
5d - Control (TC)

Figure 15. Expression of *RNS1* and *At4* in the shoots of *Thellungiella* (Yukon ecotype) seedlings in response to low phosphate exposure. Seedlings were grown on defined media containing 500 µM phosphate until their primary root length reached approximately 4 cm. Seedlings were then transferred to media containing 5 µM phosphate for 1-5 days before harvest (TC is the transfer control, see Methods and Materials). Data are the mean ± standard error (SE) for three independent biological replicates; n = 54 seedlings per treatment per replicate. Gene expression is relative to the level of expression at day 0 and qPCR data for the target genes was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Testing by a nested ANOVA showed statistically significant difference between the means (P = 0.05). Different letters represent significant differences between the means as determined using a Tukey post-hoc test.

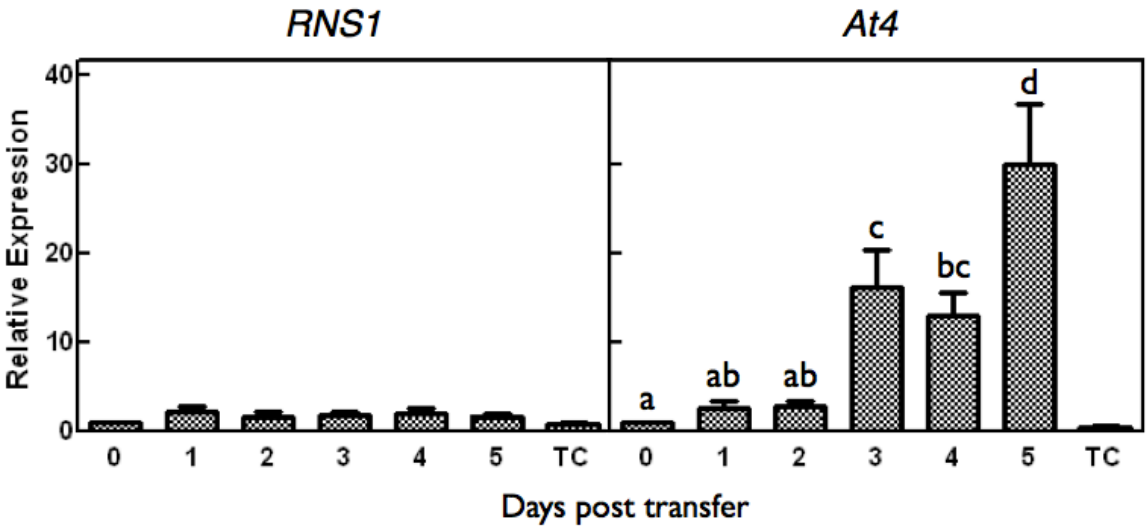

RNS1
At4
Relative Expression
40
30
20
10
0
a
ab
ab
c
bc
d
0 1 2 3 4 5 TC 0 1 2 3 4 5 TC
Days post transfer

Figure 16. Expression of *Pht1;1*, *Pht1;4*, *Pht1;5,* and *At4* in the roots *Thellungiella* (Yukon ecotype) seedlings in response to low phosphate exposure. Seedlings were grown on defined media containing 500 µM phosphate until their primary root length reached approximately 4 cm. Seedlings were then transferred to media containing 5 µM phosphate for 1-5 days before harvest (TC is the transfer control, see Methods and Materials). Data are the mean ± standard error (SE) for three independent biological replicates; n = 54 seedlings per treatment per replicate. Gene expression is relative to the level of expression at day 0 and qPCR data for the target genes was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Testing showed a statistically significant nested ANOVA (P = 0.05). Different letters represent significant differences between the means as determined using a Tukey post-hoc test.

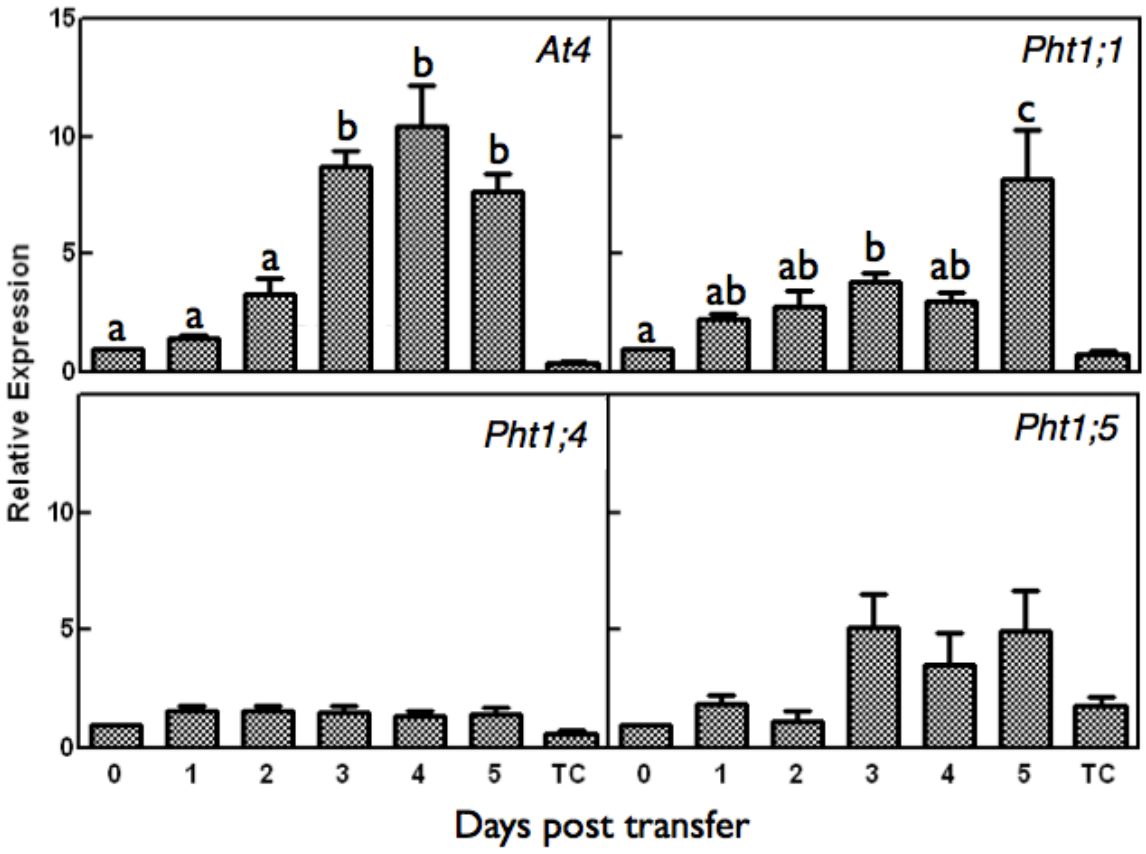
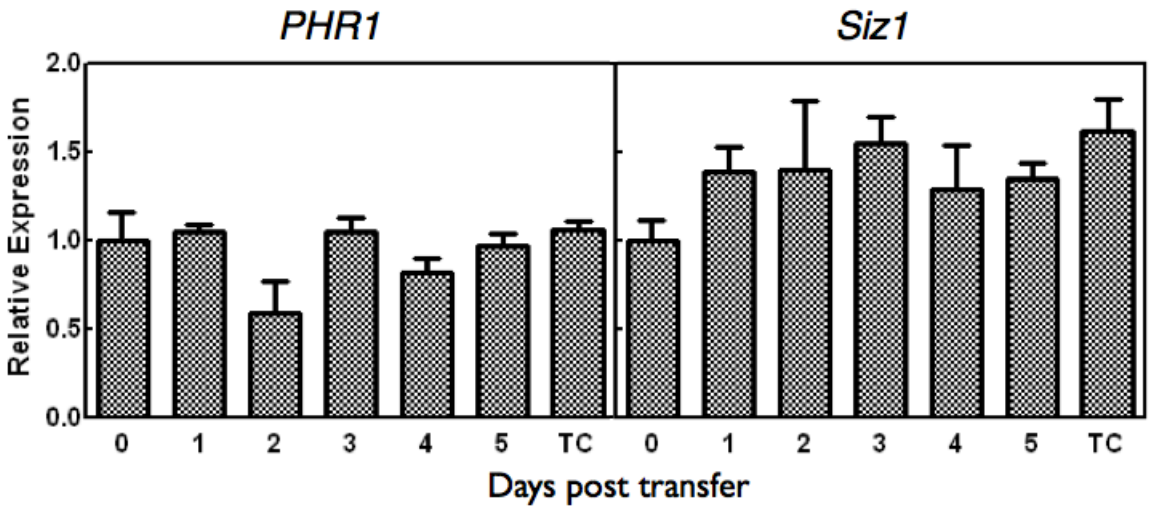

At4
Pht1;1
Pht1;4
Pht1;5
Relative Expression
15
10
5
0
a a a b b b
a ab ab b ab c
0 1 2 3 4 5 TC 0 1 2 3 4 5 TC
Days post transfer

Figure 17. Expression of *PHR1* and *Siz1* in *Thellungiella* (Yukon ecotype) seedlings in response to low phosphate exposure. Seedlings were grown on defined media containing 500 µM phosphate until their primary root length reached approximately 4 cm. Seedlings were then transferred to media containing 5 µM phosphate for 1-5 days before harvest (TC is the transfer control, see Methods and Materials). Data are the mean ± standard error (SE) for three independent biological replicates; n = 54 seedlings per treatment per replicate. Gene expression is relative to the level of expression at day 0 and qPCR data for the target genes was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. The abundance of *PHR1* and *Siz1* transcripts did not vary significantly during the experiment (P = 0.2418 and 0.7951, respectively; as shown by nested ANOVA).


PHR1
Siz1
Relative Expression
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 TC 0 1 2 3 4 5 TC
Days post transfer

Siz1 where expression results of seedling shoots on high and low phosphate showed no differences in the expression of these genes even after 15 days exposure to low phosphate media (Fig. 5). Moreover, the results of Figure 17 suggest that the lack of phosphate-responsive expression for *PHR1* and *Siz1* on low phosphate media was not because their expression underwent an increase and then returned to a basal level before sampling on day 15.

V. *Thellungiella* growth and gene expression as a function of media phosphate content

The results given in the previous sections show that seedling biomass and the expression of several genes were different between 5 and 500 μM phosphate treatments. However, phosphate responsiveness is better evaluated at variable phosphate exposures where the extent of correlation between the response (or lack thereof) and phosphate content can be determined. This study included a comparison made between the two *Thellungiella* ecotypes grown on media with variable external phosphate concentrations.

a. Yukon *Thellungiella* growth analysis at variable external phosphate

Seedlings grown on media containing no additional phosphate displayed chlorosis of the cotyledons after 10 days and they had the smallest biomass of all treatments with an average shoot weight of 3.1 ± 0.26 mg (Fig. 18). All other phosphate treatments did not produce chlorotic seedlings and the average shoot weight per seedling increased as the

concentration of phosphate in the media increased. The largest seedlings with an average biomass of 8.7 ± 0.5 mg per seedling were those grown on media containing 2000 μM phosphate. Seedlings grown with no additional phosphate in the media had the smallest roots biomass of all treatments at 2.2 ± 0.48 mg. Average root fresh weight per seedling for the seedlings grown on media containing between 25 and 500 μM phosphate showed no significant difference (average 4 ± 0.25 mg per seedling). As for shoot biomass, roots of the seedlings grown on media containing 2000 μM phosphate were the largest at 5.8 ± 0.34 mg per seedling.

b. Gene expression in response to various phosphate concentrations in Yukon *Thellungiella* shoots

Expression of *RNS1* on media with no added phosphate was variable but, on average, 120-fold higher than the expression level of seedlings grown on media containing 2000 μM phosphate, the latter yielding the lowest expression level among the phosphate treatments tested. When supplied with 25 μM phosphate, expression of *RNS1* was still significantly higher than 2000 μM at approximately 70-fold but no significant difference in transcript abundance was detected using seedlings grown on media containing 125 to 2000 μM phosphate ($P = 0.0417$) (Fig. 19).

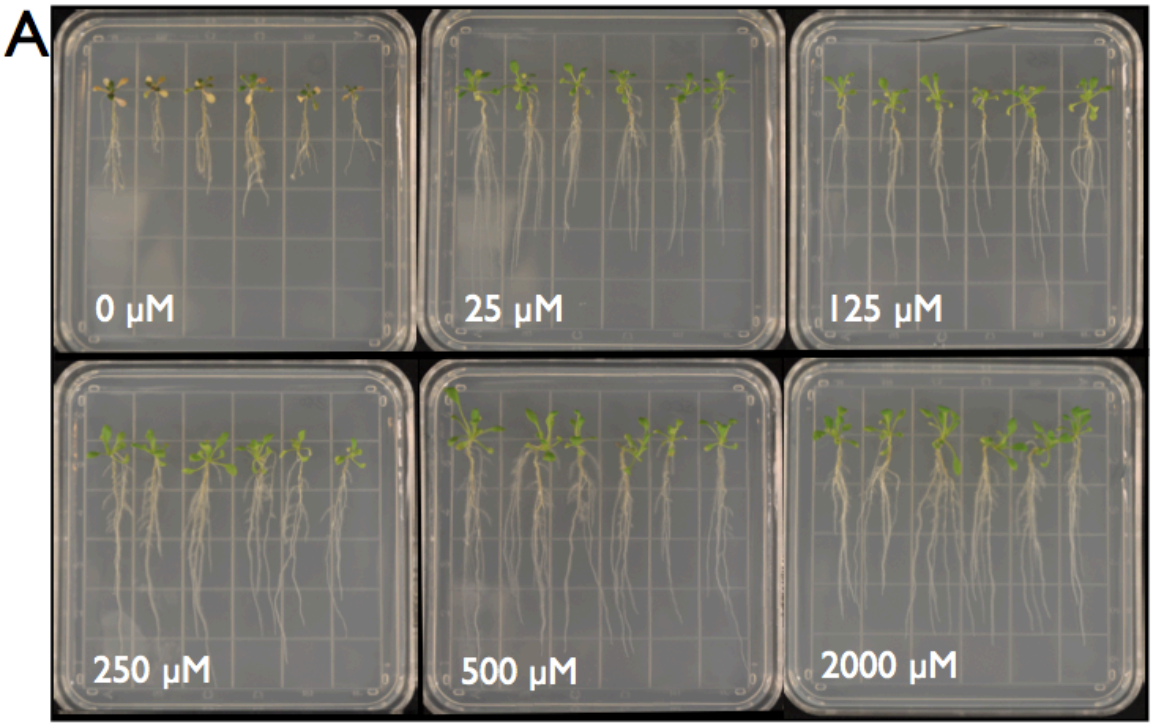
Expression of *At4* in the shoots of seedlings grown on media containing no additional phosphate was 141-fold higher than the expression of this gene in seedlings grown with 2000 μM phosphate. As was the case for *RNS1*, *At4* transcript abundance

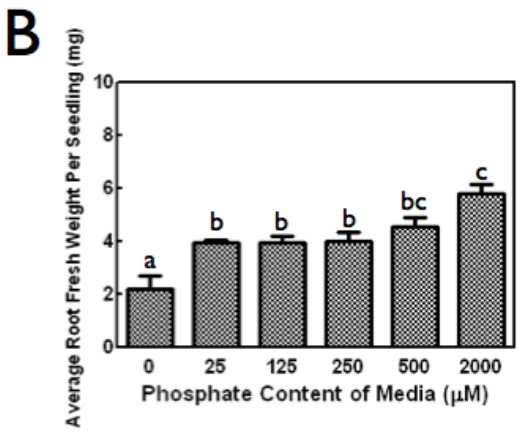
significantly decreased when additional phosphate was supplied to the media such that no significant difference was detected when phosphate content exceeded 125 μM while a media phosphate content less than 125 μM had a significant effect on the abundance of *At4* in the seedlings relative to the 2000 μM treatment ($P > 0.0001$) (Fig. 19).

c. Gene expression in response to various phosphate concentrations in Yukon *Thellungiella* roots

Phosphate content of the media had a significant effect on gene expression of *At4* and *Pht1;1* in the roots of *Thellungiella* ($P < 0.0001$ and $P = 0.0003$, respectively; Fig. 20). Transcript abundance of *At4* was increased 31-fold in seedling roots grown in media containing no additional phosphate relative to those grown on media containing 2000 μM phosphate. The presence of 25 μM phosphate was enough to significantly reduce expression of *At4* relative to the medium lacking added phosphate. Phosphate concentrations between 125 to 500 μM phosphate yielded no significant difference in *At4* transcripts but the average expression of this gene was significantly higher than seedlings grown on media containing 2000 μM phosphate. The abundance of transcripts encoding the high-affinity phosphate transporter *Pht1;1* was 30-fold higher in roots of seedlings grown on media containing no additional phosphate relative to seedlings on media supplemented with 2000 μM phosphate. *Pht1;1* abundance was significantly lower in seedlings grown on media containing 25 μM phosphate relative to no phosphate. In the case of this gene, and adding 250 μM and higher concentrations of phosphate led to no

Figure 18. Response of *Thellungiella* (Yukon ecotype) seedlings to variable phosphate content in the media. (A) Representative seedlings grown for 15 d on defined media containing no added phosphate (0 μM) up to 2000 μM. (B). Average root fresh weight (mg) and (C) average shoot fresh weight (mg) at the time of harvest. Data are the mean ± standard error (SE) for three independent biological replicates; n = a minimum of 42 seedlings per treatment per replicate. Data was statistically significant as determined by nested ANOVA (P = 0.05). Different letters represent significant differences between means from a Tukey post-hoc test.


A
0 µM
25 µM
125 µM
250 µM
500 µM
2000 µM


B
Average Root Fresh Weight Per Seedling (mg)
0
2
4
6
8
10
a
b
b
b
bc
c
0
25
125
250
500
2000
Phosphate Content of Media (µM)

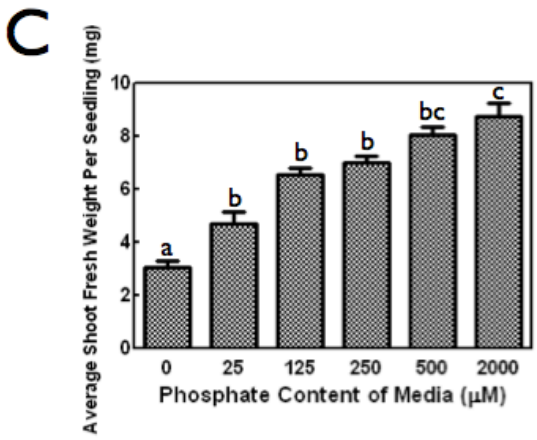

C
Average Shoot Fresh Weight Per Seedling (mg)
0
2
4
6
8
10
a
b
b
b
bc
c
0
25
125
250
500
2000
Phosphate Content of Media (µM)

Figure 19. Expression of *RNS1* and *At4* in the shoots of *Thellungiella* (Yukon ecotype) seedlings exposed to variable phosphate concentrations (0 to 2000 μM) for 15 d. Data are the mean ± standard error (SE) for three independent biological replicates; n = a minimum of 42 seedlings per treatment per replicate. Gene expression for all treatments is reported relative to the qPCR data for the 2000 μM treatment. qPCR data was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Data was shown to be significant using a nested ANOVA (P = 0.05). Different letters represent significant differences between the means following a Tukey post-hoc test.

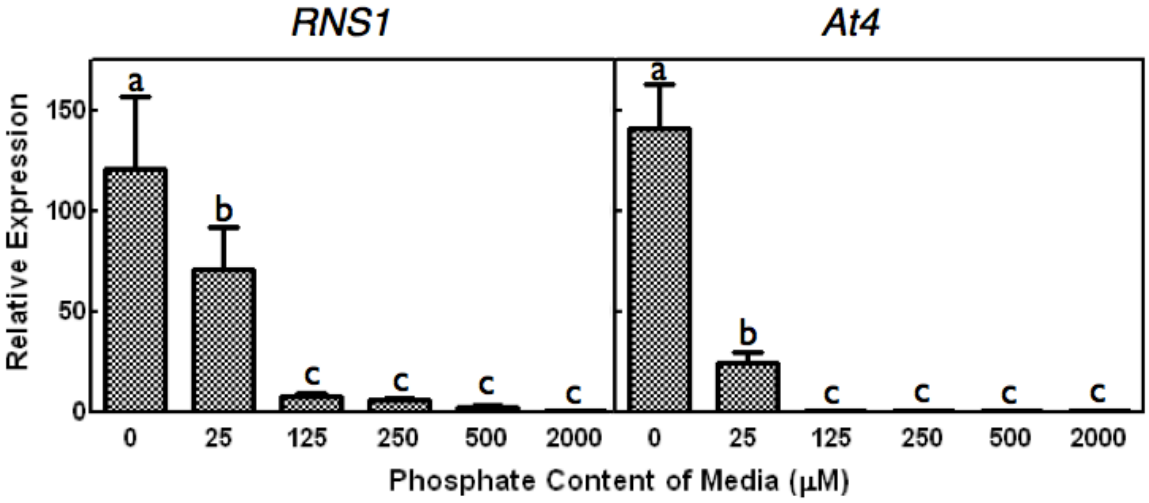
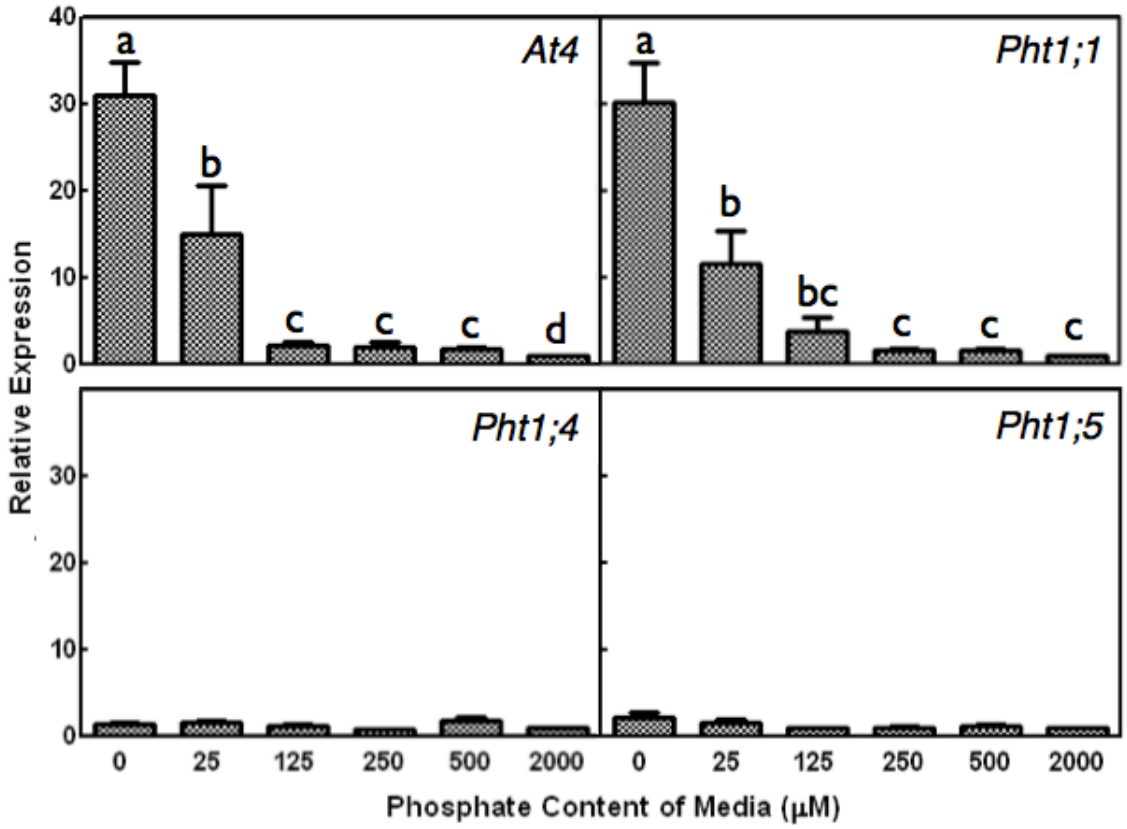

RNS1
At4
Relative Expression
150
100
50
0
a
b
c
c
c
c
a
b
c
c
c
c
0
25
125
250
500
2000
0
25
125
250
500
2000
Phosphate Content of Media (µM)

Figure 20. Expression of *Pht1;1*, *Pht1;4*, *Pht1;5,* and *At4* in the roots of *Thellungiella* (Yukon ecotype) seedlings exposed to variable phosphate concentrations (0 to 2000 μM) for 15 d. Data are the mean ± standard error (SE) for three independent biological replicates; n = a minimum of 42 seedlings per treatment per replicate. Gene expression for all treatments is reported relative to the qPCR data for the 2000 μM treatment. qPCR data was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Data was shown to be significant using a nested ANOVA (P = 0.05). Different letters represent significant differences between the means following a Tukey post-hoc test. No statistically significant difference related to treatments was found for *Pht1;4* and *Pht1;5* (P = 0.0895 and 0.5543, respectively).


At4
Pht1;1
Pht1;4
Pht1;5
a
b
c
c
c
d
a
b
bc
c
c
c
Relative Expression
40
30
20
10
0
0
25
125
250
500
2000
Phosphate Content of Media (µM)

significant difference in *Pht1;1* transcript abundance in the roots relative to the 2000 µM treatment (Fig. 20).

In contrast to the expression behaviour of the genes described above, phosphate content of the media had no significant effect on expression of *Pht1;4* and *Pht1;5* across the treatments tested (P = 0.0895 and P = 0.5543, respectively; Fig. 20). A modest two-fold increase in the expression of *Pht1;5* was detected in the roots of seedlings grown with no added phosphate as compared to 25 µM phosphate but otherwise the level of *Pht1;5* expression was unchanged at the higher levels of phosphate used. Transcript abundance associated with *Pht1;4* was the same across all treatments.

d. Shandong *Thellungiella* growth analysis at variable external phosphate

Shandong *Thellungiella* seedlings that were grown on media lacking additional phosphate were the smallest of all the treatments with an average shoot and root fresh weight of 3 ± 0.16 mg and 1.3 mg ± 0.16 per seedling, respectively. As was the case for the Yukon *Thellungiell*a, these seedlings also displayed slight chlorosis of the cotyledons (Fig. 21). However, the treatments thereafter yielded seedlings with similar fresh biomass and overall phenotype with respect to uniformly green shoots and having roots that were visually indistinguishable between phosphate treatments. The average shoot biomass measurement for seedlings grown on media exceeding 25 µM was between 8 to 10 mg and their average root biomass was 5 mg (Fig. 21).

e. Gene expression in response to various phosphate concentrations for Shandong *Thellungiella* shoots

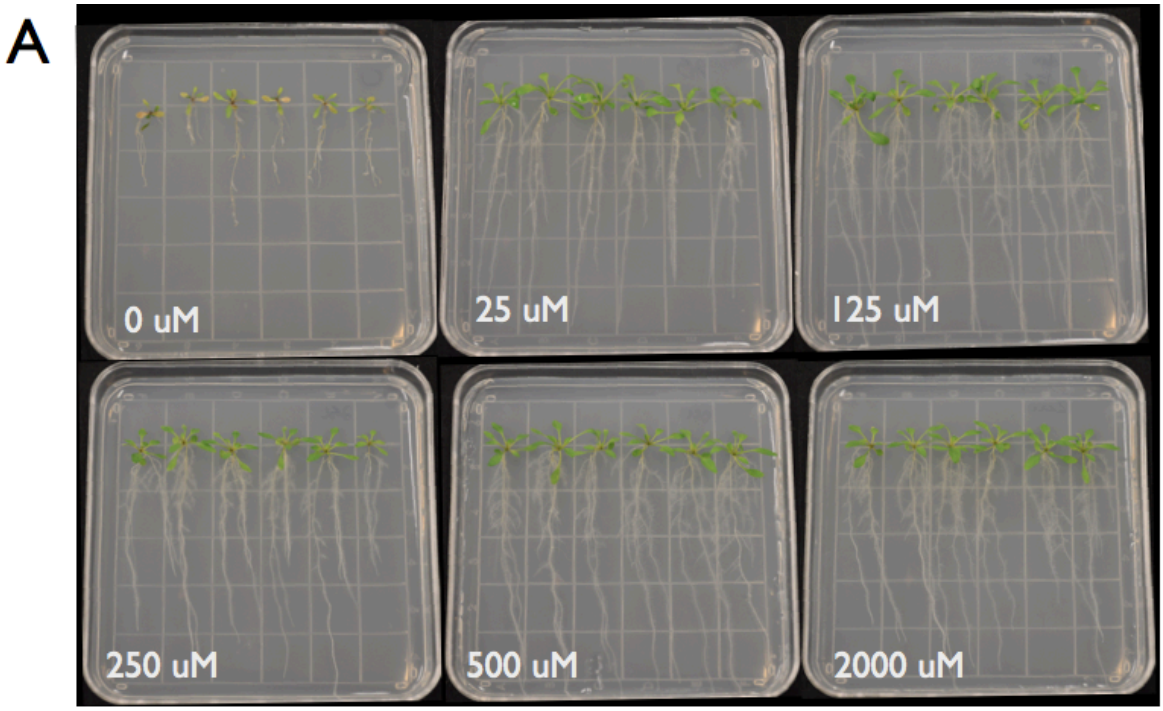
Expression of *RNS1* and *At4* in *Thellungiella* shoots was affected by phosphate content of the media ($P < 0.0001$ and $P = 0.0314$, respectively) with their transcript abundance in seedlings grown with no additional phosphate being significantly higher than levels detected in seedlings grown under the other treatments (Fig. 22). In both cases, seedlings grown on media containing 25 μM phosphate or higher showed significantly reduced levels of expression relative to the no phosphate added treatment. The use of media containing 125 μM or higher phosphate showed no significant difference in the expression of *RNS1* or *At4* in shoots and the level of expression was very low relative to the sample lacking added phosphate (Fig. 22).

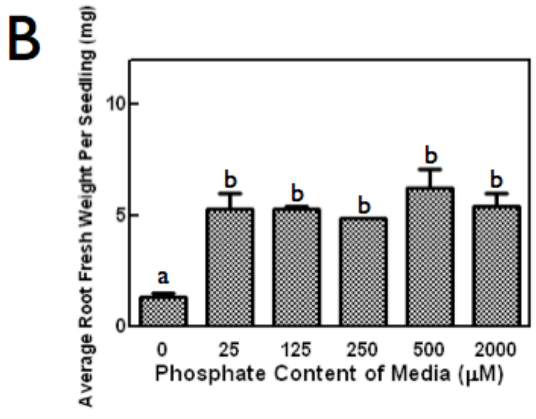
f. Gene expression in response to various phosphate concentrations in Shandong *Thellungiella* roots

The phosphate content of the media had a significant effect on the abundance of transcripts in the roots of Shandong *Thellungiella* seedlings associated with *At4* ($P = 0.0004$), *Pht1;1* ($P = 0.0005$) and *Pht1;5* ($P = 0.0013$). However, expression of *Pht1;4* was not significantly affected by phosphate content of the media ($P = 0.8332$) (Fig. 23).

At4 and *Pht1;1* expression showed a similar pattern in response to various media tested in that the lack of added phosphate led to significantly increased expression relative to media with 125 μM or more phosphate present. (Fig. 23). In Shandong *Thellungiella*

Figure 21. Response of *Thellungiella* (Shandong ecotype) seedlings to variable phosphate content in the media. (A) Representative seedlings grown for 15 d on defined media containing no added phosphate (0 μM) up to 2000 μM. (B) Average root fresh weight (mg) and (C) average shoot fresh weight (mg) at the time of harvest. Data are the mean ± standard error (SE) for two independent biological replicates; n = a minimum of 43 seedlings per treatment per replicate. Data was statistically significant as determined by nested ANOVA (P = 0.05). Different letters represent significant differences between means from a Tukey post-hoc test.


A
0 uM
25 uM
125 uM
250 uM
500 uM
2000 uM


B
Average Root Fresh Weight Per Seedling (mg)
10
5
0
a
b
b
b
b
b
0
25
125
250
500
2000
Phosphate Content of Media (µM)

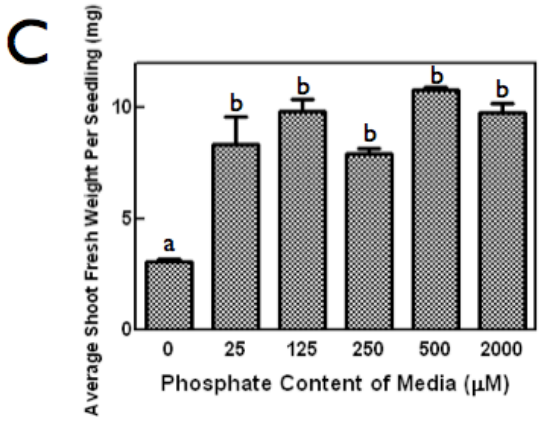

C
Average Shoot Fresh Weight Per Seedling (mg)
10
5
0
a
b
b
b
b
b
0
25
125
250
500
2000
Phosphate Content of Media (µM)

Figure 22. Expression of *RNS1* and *At4* in the shoots of *Thellungiella* (Shandong ecotype) seedlings exposed to variable phosphate concentrations (0 to 2000 μM) for 15 d. Data are the mean ± standard error (SE) for two independent biological replicates; n = a minimum of 43 seedlings per treatment per replicate. Gene expression for all treatments is reported relative to the qPCR data for the 2000 μM treatment. qPCR data was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Data was shown to be significant using a nested ANOVA (P = 0.05). Different letters represent significant differences between the means following a Tukey post-hoc test

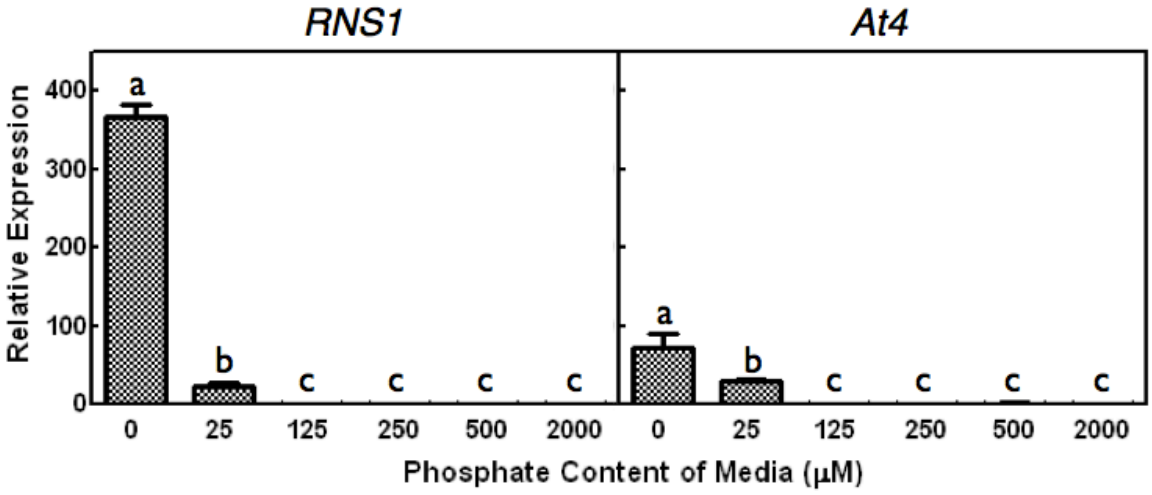
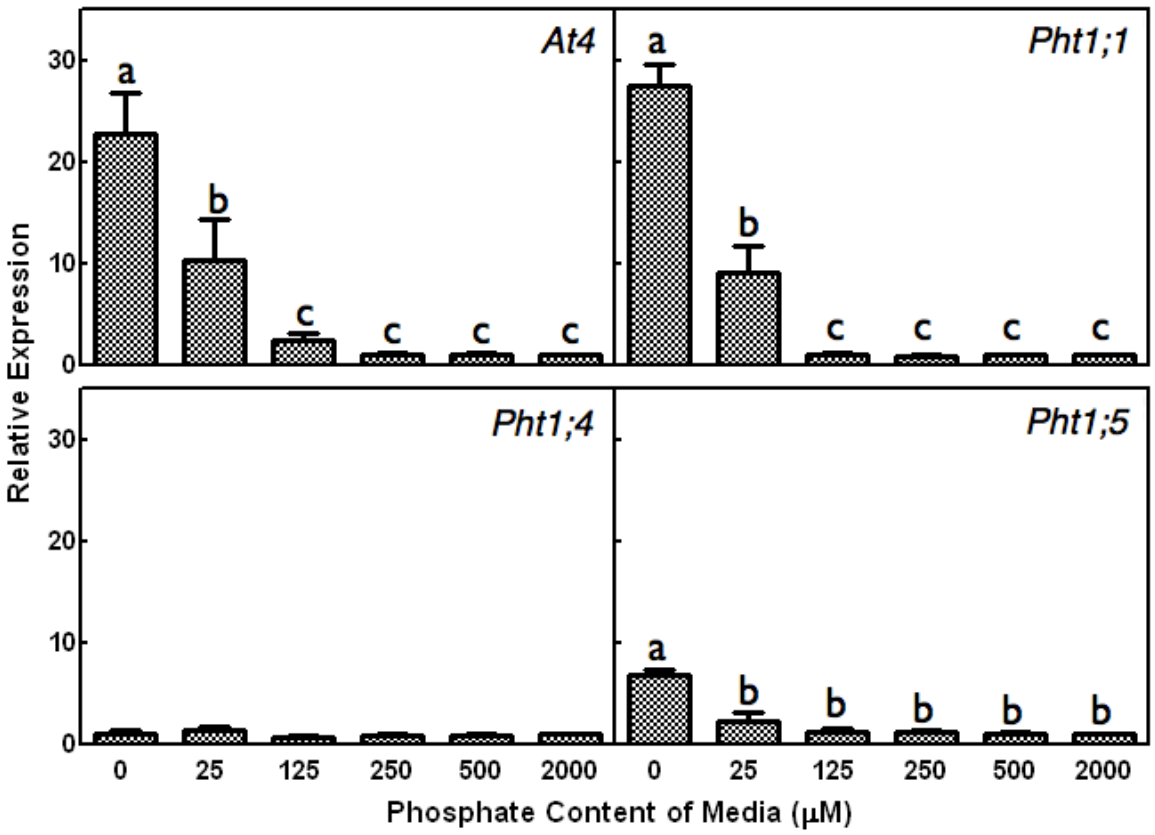

RNS1
At4
Relative Expression
400
300
200
100
0
a
b
c
c
c
c
a
b
c
c
c
c
0
25
125
250
500
2000
0
25
125
250
500
2000
Phosphate Content of Media (µM)

Figure 23. Expression of *Pht1;1*, *Pht1;4*, *Pht1;5,* and *At4* in the roots of *Thellungiella* (Shandong ecotype) seedlings exposed to variable phosphate concentrations (0 to 2000 μM) for 15 d. Data are the mean ± standard error (SE) for two independent biological replicates; n = a minimum of 43 seedlings per treatment per replicate. Gene expression for all treatments is reported relative to the qPCR data for the 2000 μM treatment. qPCR data was normalized to the reference genes *Actin1*, *At5g12240, EF1-α,* and *UBQ5*. Data was shown to be significant using a nested ANOVA (P = 0.05). Different letters represent significant differences between the means following a Tukey post-hoc test. No statistically significant difference related to treatments was found for *Pht1;4* (P = 0.8332).


At4
Pht1;1
Pht1;4
Pht1;5
Relative Expression
0
10
20
30
a
b
c
c
c
c
a
b
c
c
c
c
a
b
b
b
b
b
0
25
125
250
500
2000
Phosphate Content of Media (µM)

seedlings the expression of *Pht1;5* was only significantly higher on media containing no additional phosphate relative to all of the other treatments tested where levels were similar and low. Finally, there was no significant difference in *Pht1;4* transcript levels in roots of seedlings subjected to any of the phosphate treatments (Fig. 23).

VI. Comparison of transcript abundance by the RNAseq transcriptome database to qPCR data

The RNAseq transcriptomic database is comprised of data compiled from cDNA libraries produced from Yukon and Shandong four-week old vegetative plants grown under controlled environment conditions at McMaster University that were fertilized weekly and mature, flowering *Thellungiella* plants harvested from unmanaged fields in the Yukon. The transcriptome RNAseq data was compared to data analyzed by qPCR. It is important to note that the qPCR data was generated from seedlings grown in cabinets on media containing either 5 μM or 500 μM phosphate.

We predict that the expression patterns associated with reference genes should have similar expression levels across all samples and treatments irrespective of where the plants are growing. In this comparison, we selected several candidate reference genes based upon published reports of their suitability in this role (Czechowski, et. al., 2005). Expression of *At5g12240, UBQ5,* and *EF1-α* do not differ significantly regardless of the condition or location from which the plant was harvested and the same conclusion is reached whether using qPCR or RNAseq data (P = 0.2031, 0.9231 and 0.1614, respectively; Table 3). In

contrast, *Actin1* differs in the number of transcripts between the field and the cabinet plants (P = 0.0177) as sources while no significant difference in abundance were detected between seedlings grown on 5 μM or 500 μM phosphate (P = 0.5185). This suggests that using *Actin1* as a reference gene should be approached with caution as it shows differential expression in some tissue sources (See columns difference in abundance (qPCR) and difference in abundance (Transcriptome) in Table 3).

In contrast to the reference genes, the expression patterns associated with the phosphate-responsive genes mentioned in this thesis are predicted to be significantly different between fertilized (cabinet) and non-fertilized (field phosphate content 26 ppm) plants due to low phosphate availability in the Yukon (Guevara, et. al. 2012, submitted) (Table 3). The abundance of *RNS1, At4,* and *Pht2;1* transcripts in the RNAseq transcriptome differ significantly between the cabinet and field plants (P = 0.0043, 0.0074 and 0.0164, respectively). These differences in abundance seen in the RNAseq transcriptome agree with the qPCR data which detected significant differences in the abundance of transcripts associated with *RNS1, At4* and *Pht2;1* in seedlings grown on 5 μM phosphate compared to 500 μM phosphate (See columns difference in abundance (qPCR) and difference in abundance (Transcriptome) in Table 3). Several genes, *Pht1;1, Pht1;4, Pht1;5,* and *WRKY75*, were detected in significantly higher abundance in seedlings grown on 5 μM phosphate compared to 500 μM phosphate. However, no significant difference in the abundance of transcripts of *Pht1;1, Pht1;4, Pht1;5,* and *WRKY75* were detected between field and cabinet grown plants (P = 0.0522, 0.1551,

0.8593, 1.000 respectively). *PHR1* and *Siz1* show no significant difference in transcript abundance in the RNAseq transcriptome or qPCR data collected for seedlings.

To determine if potential phosphate responsive genes could be selected from the transcriptome based upon differences in abundance, two candidate genes were selected for testing. *SQD1* and *ACP5* were assayed by qPCR for differential expression in seedlings grown on 5 µM or 500 µM phosphate after confirming their abundance in the transcriptome. The abundance of *SQD1* transcripts differs significantly between field and cabinet plants, whereas the abundance of *ACP5* transcripts do not. qPCR results compliment the findings of the transcriptome as transcripts of *SQD1* were significantly more abundance in seedlings grown on 5 µM phosphate compared to seedlings grown on 500 µM phosphate. No significant difference in the abundance of *ACP5* transcripts were detected in seedlings grown on 5 µM phosphate relative to seedlings grown on 500 µM phosphate (Table 3).

Table 3. Comparison of transcriptome data to qPCR gene expression data.

Gene	Locus ID	qPCR Analysis			Transcriptome Analysis			Reference
		Cq value		Difference[1]	Abundance (# transcripts/ million)		Difference[4]	
		High Pi	Low Pi	P value	Cabinet[2]	Field[3]	P value	
Reference Genes								
Actin 1	AT2G37620	31.8 ± 0.4	32.2 ± 0.4	0.5185	1360 ± 74	1000 ± 66	**0.0177**	Czechowski et. al., 2005
At5g12240	AT2G37620	32.7 ± 0.5	32.1 ± 0.5	0.4439	8 ± 1	11 ± 2	0.2031	Czechowski et. al., 2005
UBQ5	AT2G37620	16.8 ± 0.4	16.4 ± 0.5	0.5560	634 ± 77	624 ± 45	0.9231	Czechowski et. al., 2005
EFl-α	AT2G37620	14.8 ± 0.3	13.4 ± 0.7	0.2221	2867 ± 170	2242 ± 386	0.1614	Czechowski et. al., 2005
At5g15710	AT2G37620	n/a	n/a	n/a	17 ± 4	17 ± 7	1.000	Czechowski et. al., 2005
UBQ10	AT2G37620	n/a	n/a	n/a	7915 ± 1311	7892 ± 148	0.9888	Czechowski et. al., 2005
GAPDH	AT2G37620	n/a	n/a	n/a	1915 ± 78	1847 ± 105	0.6164	Czechowski et. al., 2005
Phosphate Responsive Genes								
RNS1	AT2G02990	27.6 ± 0.6	20 ± 0.6	**<0.0001**	N.D[a]	38 ± 13	**0.0043**	Bariola, et. al., 1994
At4	AT5G03545	26.6 ± 0.3	20.9 ± 0.5	**0.0002**	N.D[a]	18 ± 7	**0.0074**	Shin, et. al., 2006
Pht2;1	AT3G26570	24.3 ± 0.6	25 ± 1.1	**0.0339**	114 ± 37	296 ± 32	**0.0164**	Daram, et. al., 1999
SQD1	AT4G33030	21.7 ± 0.4	16.6 ± 1.2	**0.0157**	54 ± 7	164 ± 50	**0.0495**	Hammond, et. al., 2003
Pht1;1	AT5G43350	25.4 ± 0.2	22.3 ± 0.5	**0.0021**	N.D[a]	3 ± 1	**0.0522**	Karthikeyan, et. al., 2002
Pht1;4	AT2G38940	29.8 ± 0.3	28.9 ± 0.4	**0.0007**	7 ± 1	17 ± 7	0.1551	Karthikeyan, et. al., 2002
Pht1;5	AT2G32830	26.8 ± 0.4	23.5 ± 0.4	**0.001**	9 ± 4	10 ± 3	0.8593	Nagarajan, et. al., 2011
WRKY75	AT5G13080	29.6 ± 0.1	24 ± 0.4	**0.0002**	1 ± 1[b]	1 ± 0.6	1.000	Devaiah, et. al., 20007
PHR1	AT4G28610	25.7 ± 0.4	24.9 ± 0.1	0.2423	52 ± 12	57 ± 12	0.7852	Nilsson, et. al., 2007
Siz1	AT5G60410	26.9 ± 1.3	24.6 ± 0.9	0.1805	148 ± 17	136 ± 9	0.6005	Miura, et. al., 2005
ACP5	AT3G17790	18.3 ± 0.3	17.7 ± 0.4	0.2964	4 ± 1	87 ± 49	0.0989	Hammond, et. al., 2003

[a]No transcripts were detected
[b]Transcript only detected in 1 of 4 libraries
[1]Significance by nested ANOVA of gene expression data (3 independent biological replicates)
[2]Average ± standard error of the mean of 3 different libraries from the transcirptome
[3]Average ± standard error of the mean of 4 different libraries from the transcirptome
[4]Significance determined by unpaired t-test using transcript number

DISCUSSION

I. Growth analysis of *Thellungiella salsuginea*

Thellungiella seedlings grown without supplemental phosphate in their growth medium show the lowest root and shoot biomass relative to the plants grown with phosphate added (Figs. 18 and 21). Root fresh weight does not differ significantly between Yukon *Thellungiella* seedlings grown on nutrients plates containing 25 to 500 μM phosphate while shoot biomass rose incrementally with increasing phosphate content of the media. Seedlings grown on nutrient plates containing 2000 μM phosphate had the largest biomass (Fig. 18). Thus phosphate content of the media has a direct and positive effect on the growth of *Thellungiella*. This conclusion is not surprising as it has been well documented that *Arabidopsis* shows a reduction in shoot biomass as the phosphate content of the media decreases and primary root elongation is inhibited by as much as 50% at 1 μM phosphate relative to 500 μM phosphate (Bates & Lynch, 1999; Lopez-Bucio, et. al., 2002). What is unusual about the *Thellungiella* response to low phosphate is the roots show comparatively good primary root elongation and no significant reduction in average root biomass when some supplemental phosphate is available in the medium even at a concentration as low as 25 μM (Fig. 18). Thus the classic phosphate starvation response of shorter primary roots and increased production of lateral roots observed in *Arabidopsis* grown on 50 μM phosphate media compared to 500 μM media is not replicated by

Thellungiella (Bremner, 2009). The second noteworthy observation was a differential response to low phosphate between the two natural accessions of *Thellungiella*.

Shandong ecotype *Thellungiella* seedlings resembled Yukon plants in that the seedlings were smallest when grown on media containing no additional phosphate. However, when additional phosphate was added to the media (25 μM or more) there was no statistically significant difference in seedling biomass; Shandong root and shoot biomass measurements are larger than Yukon *Thellungiella* seedlings regardless of the phosphate level present (Fig. 21). Yukon *Thellungiella* seedlings were larger than the Shandong *Thellungiella* seedlings when no additional phosphate was present in the medium (0 μM phosphate; Figs. 18 and 21). Specifically, at 0 μM phosphate, the biomass of Yukon ecotype seedling roots was 1.7-fold greater than Shandong seedlings although the average biomass of the shoots is approximately 3 mg in both Yukon and Shandong seedlings. This difference in the seedling root growth behavior of the two accessions is interesting and may relate to different efficiencies in phosphate uptake between the two ecotypes. This observation should be carried through in an analysis of mature plants to see if this trait contributes to increased fitness of Shandong plants when they are growing with a media phosphate content greater than 0 μM. At the level of seedlings this differential response is amenable to screening. For example, the genetic basis responsible for superior growth of Shandong relative to Yukon plants on media containing more than 0 μM phosphate could be identified by association based mapping between Yukon X Shandong recombinant inbred lines (RILs) that are available (unpublished).

II. Biomarkers for phosphate use efficiency: the application of qPCR for gene expression analysis.

The classic phosphate starvation response shown by *Arabidopsis* roots has made it possible to readily discern a phosphate deficiency phenotype for this species (Williamson, et. al., 2001). However, the lack of a strong root starvation phenotype between *Thellungiella* seedlings grown on media containing 25 μM or more phosphate makes it difficult to conclude, based upon visual indicators, that the seedlings are experiencing any phosphate deficiency over this range of treatment conditions. This lack of visible, morphological phenotype is very interesting as it strongly suggests that *Thellungiella* has a more efficient coping mechanism for dealing with variable phosphate in its environment than does *Arabidopsis*. Moreover, crop plants are recognized for showing low overall phosphate nutrient efficiency making this an important target for crop improvement (Baligar, et. al., 2001). Engineering phosphate deficiencies in plants are most easily done using defined nutrient conditions. While nutrient plates may work well for seedlings, particularly for species that do not tolerate hydroponics, culture plates are seldom suitable for growing a large number of plants to maturity. However, knowledge gained by using seedlings may not readily translate into a greater understanding of how phosphate deficiencies reduce yield of mature crop plants.

We have observed that Yukon Thellungiella plants do not grow well on many types of media that we have tested (unpublished). This has made it difficult to generate phosphate deficient mature plants. As a consequence, this study has used seedlings

growing on defined culture media as a source of tissues for characterization. Despite these precautions, only the absence of added phosphate seemed to elicit a barely discernible phenotype which limits the use of visual indicators in diagnosis of a phosphate deficiency. With the main indication of phosphate deficiency in seedlings only being decreased biomass at 0 μM phosphate, an alternative trait that could be used to assay a phosphate deficiency was needed, such as a genetic biomarker. Thus the next stage of this project was to identify molecular indicators that could be used to report on the phosphate nutrition status of Yukon and Shandong seedlings exposed to varying external phosphate levels.

Detection of transcript abundance by conventional PCR uses the product from the plateau stage of a PCR amplification cycle and these products are detected on an agarose gel. This process results in poor precision of gene detection since amplicon abundance is determined visually and qualitatively rather than through quantitative means (Valasek & Repa, 2005). Despite measures to optimize amplification and render this procedure more quantitative, it is highly subject to experimental error and relatively insensitive to true, albeit minor, biological variation (Ferre, 1992). In contrast, quantitative (or real-time) PCR allows for detection of differences in gene expression to be collected during the exponential phase of amplification thereby allowing for a more accurate determination of gene expression (Valasek & Repa, 2005). qPCR allows for exact transcript numbers to be reported and fluorescence detection during the course of amplification allows for greater sensitivity and reproducibility in quantifying relative or absolute transcript abundance over a very large dynamic range (Valasek & Repa, 2005). qPCR is not without limitations

in that it still relies upon the use of primers and it yields only a single target per reaction well. The use of transcriptomics by next generation sequencing technology overcomes these limitations. Transcriptomics creates a simultaneous "snapshot" of all genes expressed in a sample with great sensitivity and dynamic range. Unfortunately, the process is very expensive compared with qPCR and requires extensive bioinformatics for post-processing and mapping of the sequences produced (Jain, 2011).

III. Gene expression of seedlings in response to media with varying phosphate content

Thellungiella (Yukon ecotype) seedlings exhibit differential gene expression of several classical phosphate-responsive genes identified from studies of *Arabidopsis*. In the shoots of seedlings grown on low phosphate (5 μM) media, expression of *RNS1* is seen at 25-fold higher levels than seedlings grown on high phosphate (Fig. 5). Differential expression of *RNS1* in phosphate deficient 10 day-old *Arabidopsis* seedlings was reported by Bariola, et. al. (1999) when this group compared plants on 0 uM (deficient) and 1.25 mM (sufficient) phosphate-containing media. In a later study, Hammond, et. al, (2003) identified a 60-fold higher abundance of *RNS1* transcripts in 28-day-old *Arabidopsis* plants after removal of phosphate from the hydroponic media compared to 250 μM phosphate in which the plants were initially grown. *At4* transcript abundance is also higher in response to phosphate deprivation with approximately 20 times more *At4* associated transcripts on low (5 μM) relative to high (500 μM) phosphate media (Fig. 5). Shin, et. al. (2006) reported that the gene product encoded by *At4* plays a role in phosphate allocation

between the shoots and the roots during phosphate starvation and hence up-regulation of its expression in this study is not unexpected. Nagarajan, et. al. (2011) reported strong up-regulation of *At4* (50-fold) expression in phosphate deficient 12-day-old *Arabidopsis* when seedlings where grown on 0 mM phosphate compared to 1.25 mM phosphate. *WRKY75,* encodes a transcription factor that has been shown to influence root development and the expression of phosphate-responsive genes. *WRKY75* shows increased abundance in 25-day-old *Arabidopsis* plants grown on media with no phosphate compared with plants grown on 1 mM phosphate (Devaiah, et. al., 2007). My works shows *WRKY75* transcripts were 20-fold more abundant in Yukon *Thellungiella* seedlings grown on media containing 5 μM phosphate compared to seedlings grown on 500 μM phosphate (Fig. 5). However, increased expression of *WRKY75* is not specific to phosphate stress as it is responsive to other stressors including biotic stress (Devaiah, et. al., 2007). Given the lack of strong support for phosphate association in the literature and absence of transcripts in our field transcriptome database (see Table 3), work on this transcription factor was discontinued after the initial experiments on high and low phosphate (see Figs. 4 and 5).

Expression of the high affinity phosphate transporters was expected to be higher in roots relative to shoots given the reports on the expression of these genes in *Arabidopsis* (Shin, et. al., 2004; Nagarajan, et. al., 2011). Significant increased abundance of *Pht1;1* and *Pht1;5* transcripts was detected in roots; their increased expression on low relative to high phosphate implicates them in the phosphate uptake and management of *Thellungiella* seedlings (Fig. 6). Interestingly, there was comparatively little up-regulation of *Pht1;4*

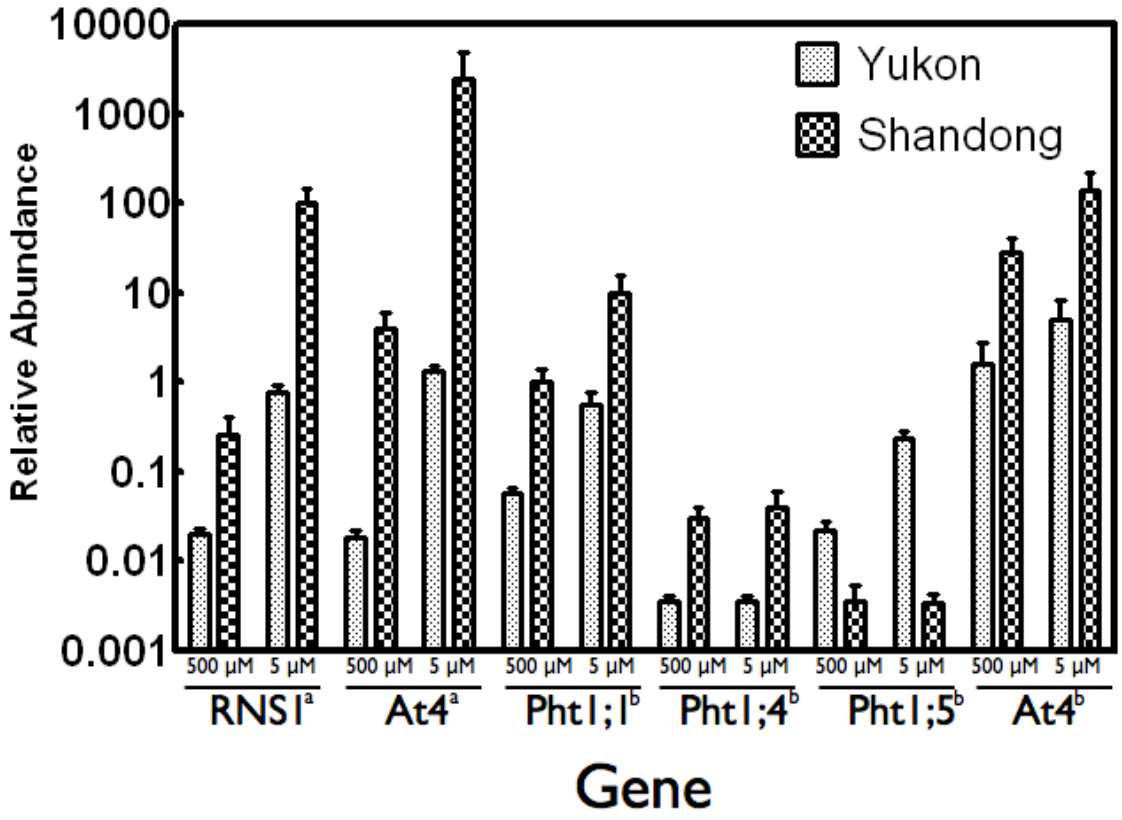
associated expression (Fig. 7) and yet the product of this gene shows increased abundance under phosphate deficient conditions and is considered to be an important phosphate transporter in *Arabidopsis* (Shin et. al., 2004). *At4* transcripts were also detected in higher abundance in the roots (Fig. 6). This increase in *At4* expression under low phosphate conditions is also observed in *Arabidopsis* (Nagarajan, et. al 2011).

Three other genes were selected as potential candidates for markers of phosphate stress not necessarily based upon expression behavior in *Arabidopsis* but because they have documented roles in the low phosphate response pathway of *Arabidopsis*: *Siz1, PHR1,* and *Pht2;1* (Sanchez-Caldron, et. al., 2010). Transcripts of *Siz1*, a gene encoding an E3 SUMO ligase, were two-fold more abundant in seedlings grown on low as opposed to high phosphate and transcript levels of *PHR1*, a transcription factor, showed no difference (Fig. 7). These genes have not been reported as showing differential expression in *Arabidopsis* in response to low phosphate (Miora, et al, 2005; Nilsson, et. al, 2007). *Pht2;1* encodes a low-affinity phosphate transporter reported to be localized to the chloroplast membrane in *Arabidopsis* where it is suggested to play a role in phosphate allocation in phosphate-deficient plants (Versaw & Harrison, 2002). However, in the shoots of *Thellungiella* seedlings the transcript abundance of *Pht2;1* decreased when seedlings were grown on media containing less phosphate (Fig. 7). Based upon these initial differential gene expression results, *RNS1, At4, Pht1;1,* and *Pht1;5* were selected as the main targets for further study to identify candidate phosphate stress markers in *Thellungiella*.

Shandong *Thellungiella* seedlings displayed a very similar expression pattern to that of the phosphate responsive genes discussed above for Yukon *Thellungiella* but there were some noteworthy differences between the two ecotypes with respect to transcript abundance. For example, the expression of *RNS1, At4, Pht1;1,* and *Pht1;4* are nearly 10-fold higher in Shandong seedlings compared to Yukon seedlings grown under the same low phosphate conditions (see Figs. 5 and 9). Moreover, Figure 24 shows a comparative profile of genes expressed in Yukon and Shandong *Thellungiella* normalized to the same reference genes. The reference genes all showed the same level of expression in cDNA samples prepared from the two genotypes (Table 4) but the target genes *RNS1, At4, Pht1;1,* and *Pht1;5* did not. Indeed, Figure 24 shows that for any given phosphate concentration the abundance of *RNS1, At4, Pht1;1,* and *Pht1;4* transcripts were all higher in Shandong compared with Yukon *Thellungiella* seedlings (Fig. 24). However, *Pht1;5* transcripts are more abundant in the Yukon seedlings compared to the Shandong seedlings (Fig. 24). These differences in abundance are likely not due to differences in total mRNA extracted from Shandong and Yukon *Thellungiella* seedlings. Support for this assertion is found in the similar Cq values of the various reference genes given in Table 4 that shows the abundance of transcripts associated with the reference genes in the two ecotypes to be similar. As a result, one can conclude that differences in the transcript abundance of the phosphate-responsive genes are likely due to genotype and not a variable abundance of extracted RNA or mRNA.

The differences in the amplitude of expression of phosphate responsive genes between the Yukon and Shandong ecotypes of *Thellungiella* could reflect natural biological differences between these accessions. The Shandong *Thellungiella* plants have adapted to a location with a temperate climate, a longer growing season, and more precipitation (Dedrick & Weretilnyk, 2007) than the Yukon ecotype. Phosphate is also more abundant in soils of Shandong Province (71 ppm) than in the Yukon (26

Figure 24. Summary of relative transcript abundance of phosphate responsive transcripts in *Thellungiella* seedlings (Yukon and Shandong ecotypes). Data represents the relative abundance of the tested phosphate responsive genes compared to each other in the roots and shoots of 20-day-old *Thellungiella* seedlings grown on defined media plates containing all required nutrients and either 5 μM or 500 μM phosphate. The abundance of each gene was normalized to the reference gene *UBQ5*. Data are the mean ± standard error (SE) for three independent biological replicates (n = a minimum of 54 seedlings per treatment per replicate).



[a]Abundance in shoot tissue
[b]Abundance in root tissue

Table 4. Comparative Cq values of the internal reference genes used for qPCR in both Yukon and Shandong *Thellungiella* seedlings.

Gene	Locus ID	Tissue	Mean Cq ± SE	
			Yukon[a]	Shandong[b]
Actin 1	At2g37620	Shoot	32.5 ± 1.9	33.8 ± 1.76
Actin 1	At2g37620	Root	33.4 ± 3.0	34.3 ± 1.88
At5g12240	At5g12240	Shoot	32.3 ± 2.4	32.9 ± 0.93
At5g12240	At5g12240	Root	29.8 ± 2.7	29.1 ± 0.64
UBQ5	At3g62250	Shoot	13.0 ± 0.66	12.8 ± 1.78
UBQ5	At3g62250	Root	12.0 ± 1.3	13.1 ± 0.67
EF1-α	At5g60390	Shoot	14.0 ± 0.91	13.9 ± 1.25
EF1-α	At5g60390	Root	12.9 ± 1.4	12.8 ± 1.33

[a]Average ± standard error of the mean of 3 independent biological replicates
[b]Average ± standard error of the mean of 2 independent biological replicates

ppm) (Zhang, et. al., 2005; Guevara, et. al., 2012 submitted). Phosphate tends to be limiting in natural habitats (Bieleski, 1973) and may be an important factor for both accessions. The differences in climate and soil conditions between the two geographic locations may have led to variations in their adaptive capacity to take up and manage phosphate. A reflection of this difference in phosphate physiology, uptake and/or metabolism could explain the differential transcript abundance associated with the phosphate responsive genes between the two ecotypes under comparable phosphate conditions. Evaluating the response of this class of genes in other natural accessions of *Thellungiella* may be one way of determining the extent of plasticity associated with this nutrient response.

Ideally, genes used as biomarkers should faithfully report on the treatment being tested and show as little influence as possible to other controlled and/or uncontrolled factors in the environment. The expression of many genes in plants is regulated by circadian rhythms (Strayer, et. al., 2000; Covington, et. al., 2008). This regulation could account for anomalous levels of expression simply because the harvest time was routinely mid-way through the light cycle. However, when the expression of the phosphate-responsive genes *RNS1, At4, Pht 1;1,* and *Pht1;5* was tested for circadian changes, the only significant day-night differences were found for transcripts associated with *At4* in the shoots and *Pht1;1* in the roots (Figs. 12 and 13). The differential expression of *At4* in shoots (Fig. 12) and *Pht1;1* in roots (Fig. 13) between the day and night cycles offers an indication that transcripts associated with these genes may undergo circadian changes but

differences corresponding to phosphate treatments are easily distinguished nonetheless. This group of genes all show increased transcript abundance when seedlings were grown on low phosphate media and this response was not obscured by the influence of harvest time (Figs. 12 and 13). This expression pattern contrasts to that of *Toc1*, a gene known to have circadian regulation in *Arabidopsis* (Strayer, et. al., 2000). Serving as a positive control in this study, the expression level of *Toc1* is highest in *Thellungiella* samples collected during the night (Fig. 12; Strayer, et. al., 2000) and there is no difference in the pattern seen when seedlings are grown on media with different phosphate content (Fig. 12). The expression patterns of the phosphate responsive genes and *Toc1* are consistent with the results obtained for *Arabidopsis* through the bio-array resource for plant biology (Toufihi, et. al., 2005).

The timing of transcript accumulation relative to the onset of phosphate starvation is an important consideration in selecting appropriate biomarkers. Changes in gene expression that are transient may lead to misleading determinations of stress if the molecular biomarker is not stably expressed (Brain & Cedergreen, 2008). To address this concern, seedlings were grown on high phosphate media and transferred to low phosphate media for a period of five days. The expression of *RNS1* in shoots of *Thellungiella* seedlings doubled after the first day following transfer to low phosphate media and then its expression remained constant for the duration of the 5-day experiment. The study by Muller et. al. (2004) reported that *RNS1* expression in *Arabidopsis* went from a stable, high level under limiting phosphate conditions to a low and unchanging level within a 24

hour period after replenishing the phosphate supply. This suggests that the expression pattern of this gene leads to a rapid threshold transcript abundance that is stably maintained until the phosphate content of the environment is changed. In contrast, the expression of *At4* increased over the course of the experimental period (Fig. 15). Muller et. al. (2004) also identified different behavior associated with *At4* in *Arabidopsis* expression under their experimental conditions. When *At4* expression was monitored after resupply of phosphate it showed a steady decline in expression over the experimental time course suggesting that unlike *RNS1* expression, *At4* transcript abundance diminishes with time. A longer time course to determine if the *At4* transcripts reach a plateau and/or eventually decrease would be useful if *At4* is to be used as biomarker to ensure that time of sampling has no effect on the estimates of abundance. This type of variability should not exclude *At4* as a marker if protocols for standardized sampling can be followed to ensure that seedlings or plants are sampled in a suitable window of time following exposure to low phosphate media.

In the roots of Yukon *Thellungiella* seedlings the expression of *Pht1;1* and *Pht1;5* increased during the time-course of the experiment (Fig. 16). This result was not unexpected as phosphate deficient *Arabidopsis* seedlings show increased abundance of transcripts associated with high-affinity phosphate transporters (Karthikeyan, et. al., 2002). However, *Pht1;4* encodes a central high affinity phosphate transporter in *Arabidopsis* and it did not show up-regulation in *Thellungiella* during the course of my experiment (Fig. 16). A modest (ca 2-fold) but insignificant increase in *Pht1;4* expression

was detected after 24 h on low phosphate media but thereafter transcript abundance was constant. In contrast, Karthikeyan, et. al., (2002) provided evidence of strong (ca 25-fold) up-regulation of *Pht1;4* transcripts in phosphate-deficient *Arabidopsis* seedlings. Aside from different use of plant species, one noteworthy difference between experimental conditions is that Karthikeyan and co-workers used 4-week-old plants rather than seedlings. Future studies should look at more mature plants to see if the differences in gene expression patterns associated with *Pht1;4* in *Thellungiella* can be attributed to developmental factors.

As discussed in preceding sections, *PHR1* and *Siz1* showed no significant differential response in expression to variable phosphate in the media (Fig. 7). It is possible that early up-regulation of their expression was missed given the two-week duration of these experiments. This would be consistent with their roles as encoding products regarded as early response factors in the phosphate deficiency response pathway (Fig. 1). In the seedlings transferred from high to low phosphate, *PHR1* and *Siz1* expression was followed daily for five days. The expression levels for both genes were similar throughout the duration of the experiment (Fig. 17). This observation is consistent with *Siz1* and *PHR1* expression in *Arabidopsis* as they do not show differences in abundance in response to phosphate media content. Miura et. al., (2005) showed that *Siz1* is not up-regulated in 7-day-old *Arabidopsis* seedlings over a period of 2 days following exposure to 0 mM phosphate after transfer from 1.25 mM phosphate. Moreover, Nilsson et. al., (2007) reported that *PHR1* expression in 21-day-old *Arabidopsis* plants was the

same when grown on 2 μM and 40 μM phosphate. In the absence of evidence for differential expression, it is possible that these genes are under post-transcriptional regulation.

Expression of *RNS1*, *At4*, *Pht1;1* and *Pht1;5* show very similar patterns in *Thellungiella* (Yukon ecotype) seedlings grown on media containing various concentrations of phosphate (Fig. 19 and 20). When 25 μM of phosphate was present in the medium transcript abundance associated with these genes decreased to approximately one-half the level found when no phosphate was added and at 125 μM (or higher phosphate) their expression was significantly lower. Karthikeyan et. al., (2002) also reported decreased expression of the high-affinity phosphate transporters in *Arabidopsis* as the phosphate content of their media increased. However, in their study the fold-changes in expression levels relative to 0 μM phosphate content were much higher than those reported in this thesis (125-fold compared to 30-fold, respectively for *Pht1;1*). This difference suggests that *Thellungiella* may have a naturally greater ability to take up phosphate than *Arabidopsis*. This is not unexpected given the stress tolerant nature of *Thellungiella* but better evidence in support of this proposal would be provided by a comparative phosphate-uptake study using these two species.

In general, Shandong *Thellungiella* seedlings display an almost identical pattern of phosphate-responsive gene expression and transcript abundance numbers as Yukon *Thellungiella*. One exception is the expression of *RNS1* in seedlings grown with no additional phosphate (Figs. 19 and 22). The relative expression of *RNS1* in Shandong

seedlings grown on media with no additional phosphate is 3.5-fold greater than *RNS1* transcript levels detected in comparable Yukon seedlings. The higher baseline for *RNS1* expression in Shandong *Thellungiella* and the proposed role for RNS1 suggests that Shandong *Thellungiella* seedling shoots are more efficient at recycling phosphate than Yukon *Thellungiella.* Recycling nutrients is an important strategy in plants for managing limiting resources so biochemical and physiological comparisons between the two ecotypes with respect to this capacity could help identify molecular mechanisms underlying plant nutrient use efficiency for phosphate.

IV. Transcriptomics as a method to identify phosphate responsive genes, molecular markers and internal reference genes

The main impediment to finding *Thellungiella*-specific phosphate responsive genes is that there are too few studies in the literature that allow for easy assembly of a list of potential biomarkers of stress. Transcriptomics (aka transcriptome profiling or RNAseq) is a feasible approach to find potential phosphate-responsive genes in *Thellungiella*. These comprehensive lists of expressed genes should provide a larger and more appropriate pool of molecular markers to evaluate phosphate stress in this species than using an extrapolation of phosphate-responsive genes identified from research on *Arabidopsis*. There are several strategies that could be used to identify this class of genes. Using plants grown in a growth cabinet exposed to phosphate treatments as a basis for comparison, one could identify correlations between genes showing differential

expression in response to external phosphate. It is also possible to make comparisons of gene expression between cabinet grown plants to plants growing under natural conditions where phosphate limitations are common. In addition to treatment/location-specific correlations with differences in gene expression it should be possible to identify genes whose expression patterns overlap between cabinet treatments and the field. Those genes showing large differences in expression levels that correlate to environmental phosphate content can be tested by other methods (eg. qPCR) to determine if they are suitable markers for phosphate stress in *Thellungiella*.

Table 3 offers a small-scale test of the strategy involving field and cabinet comparisons of transcript abundance. The Table also assesses the reliability of transcript abundance comparisons across qPCR and RNAseq platforms using several genes, many of which were used in this study as reference genes as well as those targeted as phosphate-responsive candidate genes. Ideally, a good method of gene identification and quantitative abundance should show agreement regardless of the method of quantification.

In Table 3 the transcript abundance for several genes is estimated by reporting the qPCR-based Cq numbers and, where available, the number of transcripts found in the transcriptome database for *Thellungiella* plants grown under control conditions in cabinets and from mature, flowering plants collected in 2005/2006 at Yukon field sites (2 libraries from 2005 field plants, 2 libraries from 2006 field plants and 3 libraries from cabinet grown plants were assembled in this study; unpublished). The Cq number is a reflection of abundance with a lower number indicative of a highly expressed gene (conversely, a high

Cq indicates low transcript abundance) and where identical Cq numbers for any given gene from different tissue sources means no quantitative difference in expression.

Determination of reference genes may be suitable using data obtained from the RNAseq transcriptome. Genes that do not show statistically significant differences in abundance across different treatments are considered good reference genes for genetic studies (Czechowski, et. al., 2005). From the data available in Table 3, *UBQ5* and *EF1-α* appear to be the best validated references genes thus far since no statically significant difference in abundance is seen when transcript abundance is assayed by qPCR and the RNAseq transcriptome numbers are also invariable. In contrast, *Actin1* is not as consistent as a reference gene as its transcript abundance in the cabinet grown plants is significantly higher than in the field plants. Since *At5g15710* and *UBQ10* show no significant difference in abundance in the transcriptome database these genes should be tested as candidate reference genes for future studies ($p = 1.000$ and 0.9888, respectively).

A phosphate-responsive gene is predicted to show statistically significant differences in transcript abundance when a plant is grown in environments varying with respect to phosphate availability. Table 3 lists several phosphate responsive genes assayed via qPCR and provides the number of transcripts detected in the RNAseq transcriptome. *RNS1* and *At4* show significantly increased abundance of transcripts when seedlings were grown on 5 μM phosphate compared to 500 μM phosphate, and similarly, significantly increased transcript levels in unfertilized plants in the field compared to well fertilized plants grown in the cabinet. This suggests they are good candidates for biomarkers of

phosphate stress in *Thellungiella*. Transcripts associated with *Pht2;1* are higher in the field plants compared to cabinet plants but lower in seedlings grown on low phosphate in cabinets. *Pht1;1, Pht1;4, Pht1;5* and *WRKY75* transcripts all increase in seedlings grown on 5 μM phosphate compared to 500 μM phosphate but their abundance was not significantly different in unfertilized field plants compared to well fertilized cabinet-grown plants. Discrepancies between markers tested by qPCR compared to the transcriptome database may be due to the use of seedlings for the experiments conducted by qPCR as opposed to the use of mature plants in compiling the transcriptome database.

In theory the transcriptome database should allow for selecting phosphate responsive genes without *a priori* knowledge of a role in phosphate starvation. However, at present there is a limited capacity for queries of this nature as there are no transcript profiles in the database for plants that have been deliberately deprived of phosphate to serve as a reference. Given this limitation, only two genes that were not part of the initial trials using seedlings grown on high and low phosphate were selected to determine how well the data would correspond to qPCR information. The genes *SQD1* and *ACP5* are known to be phosphate responsive in *Arabidopsis* (Essigmann, et. al., 1998; del Pozo, et. al., 1999). The transcriptome database predicts that *SQD1* is phosphate responsive and *ACP5* shows no significant difference in transcript abundance between field and cabinet-grown plants (Table 3). When qPCR was performed on 20-day-old seedlings grown on 5 and 500 μM phosphate *SQD1* expression showed a differential response but *ACP5* did not. This comparison albeit limited, shows the database and qPCR information to be in

agreement. In the future more extensive comparisons made on a transcriptome-wide scale should allow for the discovery of *Thellungiella*-specific phosphate responsive genes that could be validated by independent comparison using qPCR.

V. Genetic markers of phosphate stress in *Thellungiella*

There are several good candidate genes for markers of phosphate stress in *Thellungiella.* The significant phosphate-responsive increase in transcript abundance, rapid response to low phosphate exposure, and history of phosphate responsiveness in *Arabidopsis* (Karthikeyan et. al., 2002; Shin, et. al., 2004; Shin, et. al., 2006) all make the high-affinity phosphate transporters, *Pht1;1* and *Pht1;5*, excellent candidates for phosphate responsive markers in *Thellungiella.* With seedlings grown on nutrient plates it was relatively easy to obtain and process shoots and roots for RT-qPCR. However, collecting the roots for RNA extraction from mature plants grown on soil is a tedious process where tissue adhering to soil is lost and harvesting is usually destructive to the remainder of the plant. Therefore, using molecular markers in the roots is not the best choice to monitor phosphate responsive changes in gene expression.

RNS1 and *At4* also have a history of phosphate responsiveness (Bariola et. al., 1999; Shin, et. al., 2006) and they have the distinct advantage of showing differential regulation in shoots. In this study both genes show strong responses to low phosphate in the form of readily detectable and statistically significant changes in transcript abundance. The transcript level associated with both genes is amenable to quantification by RT-qPCR

using minimal leaf tissue so it may be feasible to "assay" plants repeatedly over time to study changes in gene expression with prolonged exposure to phosphate starvation.

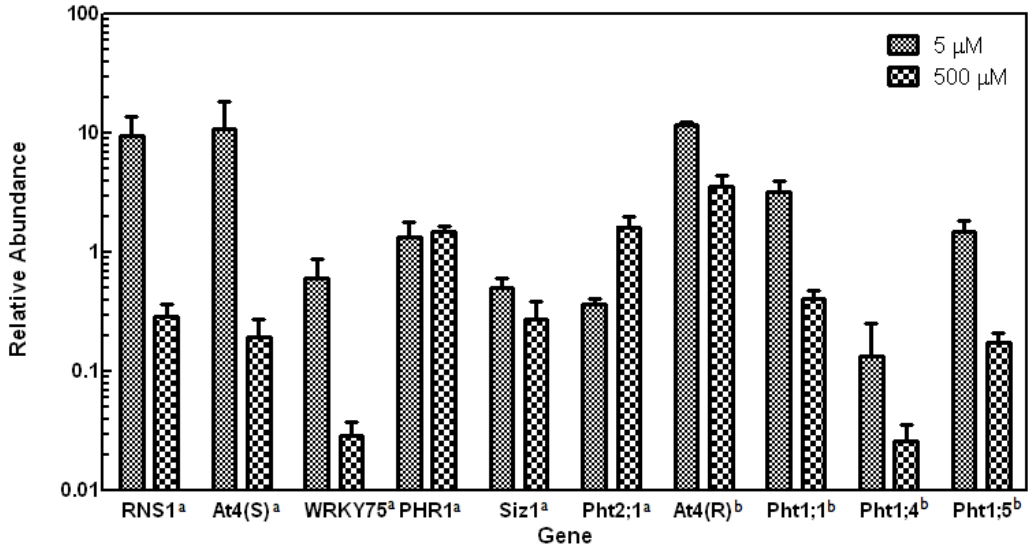
WRKY75 is a known phosphate-responsive gene for *Arabidopsis* and it also showed increased transcript abundance in the shoots of Yukon *Thellungiella* exposed to low phosphate (Fig. 5). One deterrent to its use is that it has been shown to respond to other stresses, including biotic stress (Devaiah, et. al., 2007). Use of the gene to monitor phosphate deficiency stress requires a more careful assessment of factors affecting *WRKY75* expression in *Thellungiella*. *Siz1* and *PHR1* are two other documented early phosphate-responsive genes (Miura, et. al, 2005; Nilsson, et. al., 2007). However, neither gene shows an increase in transcript abundance associated with low phosphate conditions in *Thellungiella* seedlings (Fig. 7 and 11), suggesting they are either not involved in the phosphate starvation responses of this species or they may be post-transcriptionally regulated. The results of this study do not support their use as expression markers of phosphate stress for *Thellungiella*.

Extensive research in *Arabidopsis* suggests that Pht1;4 is an important phosphate transporter and that *Pht1;4* expression is increased 25-fold when plants are grown under low phosphate conditions (Muchhal, et. al., 1996; Mitsukawa, et. al., 1997; Karthikeyan et. al., 2002; Shin, et. al., 2004; Shin, et. al., 2006). In view of these studies, it was surprising that *Pht1;4* expression was only modestly increased in response to low phosphate in *Thellungiella* seedlings (Figs. 7 and 11). *Pht1;4* transcript abundance in both Yukon and Shandong *Thellungiella* seedlings is also much lower than that associated with

the other transporters genes namely *Pht1;1* and *Pht1;5* (Fig. 25). One explanation is that Pht1;4 may not play as significant of a role in phosphate uptake in *Thellungeilla* but this difference may also be a consequence of using seedlings as opposed to mature plants. That is, Pht1;4 may play a role in the uptake and transport of phosphate by a more mature plant. An additional factor to consider is that the primers for *Pht1;4* amplification were not validated to the appropriate efficiency standards (see Methods and Materials). However, the amplicon that was generated was sequenced and only one product that matched the *Arabidopsis Pht1;4* sequence was identified (Fig. 2). With this sequence confirmation it is reasonable to conclude that the lack of increased transcripts in *Thellungiella* seedlings is supported by the qPCR data despite the lack of primer validation. Nonetheless, more research is necessary to determine the role of Pht1;4 in *Thellungiella*. In the short term it should be possible to address whether *Pht1;4* expression behavior is anomalous in mature plants by looking at its transcript abundance across the various transcriptomes now being assembled in our laboratory.

As previously stated, a good biomarker must be sensitive to changes in the phosphate content of the media and relatively insensitive to other environmental conditions. Figure 25 shows why *RNS1* and *At4* are the most suitable candidates for biomarkers of phosphate stress in *Thellungiella* based upon the data of this research. Expression of *RNS1* and *At4* undergoes a larger fold-increase than the other genes tested under low phosphate conditions. *RNS1* and *At4* have a larger dynamic range of differential expression allowing for more sensitivity in

Figure 25. Summary of relative transcript abundance for phosphate responsive genes in *Thellungiella* (Yukon ecotype) seedlings. The expression data shown in Figures 5, 6 and 7 were all normalized to the qPCR expression data associated with the reference gene *UBQ5*. Data are the mean ± standard error (SE) for three independent biological replicates (n = a minimum of 54 seedlings per treatment per replicate).



[a]Abundance in shoot tissue
[b]Abundance in root tissue

identifying differential transcript levels using a broad range of media phosphate content. In this regard, *Pht1;1* expression also appears to be a good biomarker for phosphate stress based upon differential expression in response to low phosphate. However, the difference in abundance of *Pht1;1* transcripts in seedlings grown on high and low phosphate is only a factor of four. In contrast, *RNS1* and *At4* transcripts show 25-fold changes in abundance under the same treatment conditions. As a consequence, changes in the expression of *RNS1* and *At4* should be easier to detect than those associated with *Pht1;1.* Figure 25 also shows why using shoot *At4* expression as a biomarker is preferable to monitoring its expression in roots. In roots, *At4* transcripts show a modest 3-fold difference between seedlings grown on high and low phosphate media whereas this gene shows a 25-fold change in relative abundance in shoots of the same plants. When grown on low phosphate, the abundance of *At4* transcripts is the same in the roots and the shoots. However, when grown on high phosphate, the abundance of *At4* is much lower in the shoots than the roots. These fold differences in gene expression should make changes in *At4* transcripts easier to detect particularly by the highly sensitive quantification procedures available with qPCR.

CONCLUSION

This research was conducted to explore the expression response of known *Arabidopsis* phosphate-responsive gene markers in *Thellungiella* in order to identify suitable genetic markers for phosphate stress in this highly stress tolerant plant. Quantitative-PCR is a reliable and sensitive approach for quantifying gene expression using cDNA produced from *Thellungiella* grown on media containing a variety of phosphate concentrations. *RNS1* and *At4* are two genes with a history of phosphate responsiveness in *Arabidopsis* and both showed reproducible, phosphate-responsive behavior for *Thellungiella* seedlings on low phosphate media. As such *RNS1* and *At4* were deemed to be the best candidates for genetic markers of phosphate stress in *Thellungiella* seedlings. The next steps would be to evaluate their suitability as markers of phosphate stress in mature *Thellungiella* plants and their specificity for phosphate as the source of stress. In the future, other candidate genes such as those encoding PAP12 and PAP26 should be considered due to their roles in phosphate homeostasis (Hurley, et. al., 2010; Tran, et. al., 2010). However, it may be that genetic profiling via transcriptomics will offer a better, more comprehensive species-specific list of phosphate-responsive genes. Analysis of these genes and the role of their products under phosphate limiting conditions would enable us to better understand how *Thellungiella* thrives in environmentally stressful, nutrient poor conditions. An increased understanding of the mechanisms underlying these

traits can be used to improve the nutrient use efficiencies of Canadian crops such as the related oilseed, canola.

Appendix A - Fresh weight and number of seedlings

Yukon high and low phosphate

Tissue	Concentration of Phosphate in Media	Trial (Biological Replicate)	Total Pooled Weight (mg)	Number of Seedlings per Trial
Shoot	5 µM	1	93	70
		2	104	70
		3	240	140
	500 µM	1	400	54
		2	306	54
		3	674	108
Root	5 µM	1	75	70
		2	98	70
		3	240	140
	500 µM	1	138	54
		2	102	54
		3	343	108

Shandong high and low phosphate

Tissue	Concentration of Phosphate in Media	Trial (Biological Replicate)	Total Pooled Weight (mg)	Number of Seedlings per Trial
Shoot	5 µM	1	408	140
		2	291	133
		3	288	133
	500 µM	1	660	60
		2	458	60
		3	306	54
Root	5 µM	1	112	140
		2	115	133
		3	102.5	133
	500 µM	1	323	60
		2	282	60
		3	180	54

Testing for diurnal regulation

Tissue	Concentration of Phosphate in Media	Trial (Biological Replicate)	Time of Harvest	Total Pooled Weight (mg)	Number of Seedlings per Trial
Shoot	5 µM	1	Day	137	108
		2	Day	144	108
		1	Night	138	108
		2	Night	129	108
	500 µM	1	Day	236	54
		2	Day	221	54
		1	Night	188	54
		2	Night	201	54
Root	5 µM	1	Day	128	108
		2	Day	135	108
		1	Night	100	108
		2	Night	96	108
	500 µM	1	Day	112	54
		2	Day	122	54
		1	Night	111	54
		2	Night	114	54

Yukon growth as a function of media phosphate content

Trial	Pooled Root Fresh Weight (mg)	Pooled Shoot Fresh Weight (mg)	Number of Seedlings
BioRep1			
0 uM	400	450	126
25 uM	522	530	133
125 uM	210	380	54
250 uM	200	383	54
500 uM	280	513	60
2000uM	260	411	42
BioRep2			
0 uM	244	391	133
25 uM	223	243	54
125 uM	241	352	54
250 uM	253	398	54
500 uM	275	433	54
2000uM	293	402	48
BioRep3			
0 uM	223	358	133
25 uM	208	301	54
125 uM	167	296	48
250 uM	176	318	48
500 uM	221	436	57
2000uM	246	387	48

Shandong growth as a function of media phosphate content

Trial	Pooled Root Fresh Weight (mg)	Pooled Shoot Fresh Weight (mg)	Number of Seedlings
BioRep1			
0 uM	160	349	108
25 uM	325	516	54
125 uM	292	563	54
250 uM	267	414	54
500 uM	383	579	54
2000uM	290	491	48
BioRep2			
0 uM	125	311	107
25 uM	208	328	46
125 uM	250	446	48
250 uM	231	393	48
500 uM	289	590	54
2000uM	206	405	43

Appendix B - Raw data from qPCR

Yukon high and low phosphate

Trial	RNS1	At4 (Shoots)	Pht1;1	Pht1;4	Pht1;5	At4 (Roots)	PHR1	Siz1	Pht2;1	WRKY75
BioRep1										
Tech 1 High	1	1	1	1	1	1	1	1	1	1
Tech 1 Low	27.8	15.63	5.67	1.6	8.9	5.29	0.9	3.6	0.2	20.2
Tech 2 High	1	1	1	1	1	1	1	1	1	1
Tech 2 Low	19.1	17.46	5.35	1.1	6.8	6.92	1.1	3.4	0.2	24.9
BioRep2										
Tech 1 High	1	1	1	1	1	1	1	1	1	1
Tech 1 Low	26.88	34.1	10	1.6	10.9	3.11	0.4	1	0.3	17
Tech 2 High	1	1	1	1	1	1	1	1	1	1
Tech 2 Low	29.3	30	9.24	0.9	11.2	4.42	0.66	1.1	0.28	18
BioRep3										
Tech 1 High	1	1	1	1	1	1	1	1	1	1
Tech 1 Low	19.36	16.55	13.6	1.5	22.8	2.3	1.2	1.1	0.25	19.2
Tech 2 High	1	1	1	1	1	1	1	1	1	1
Tech 2 Low	29.7	13.13	20.7	1.2	19.4	2.54	0.8	1.4	0.33	21.3

Shandong high and low phosphate

Trial	RNS1	At4 (Shoots)	Pht1;1	Pht1;4	Pht1;5	At4 (Roots)	PHR1	Siz1	Pht2;1
BioRep1									
Tech 1 High	1	1	1	1	1	1	1	1	1
Tech 1 Low	161	37.6	37.4	1	11.6	14	0.6	0.9	0.3
Tech 2 High	1	1	1	1	1	1	1	1	1
Tech 2 Low	180.9	41.2	20.7	1.2	19.4	2.54	0.8	1.4	0.16
BioRep2									
Tech 1 High	1	1	1	1	1	1	1	1	1
Tech 1 Low	307	110.4	100	5.2	85.8	19.1	2.2	3.4	0.48
Tech 2 High	1	1	1	1	1	1	1	1	1
Tech 2 Low	396.1	117	53.8	3.24	34.5	12.3	1.9	3	0.66
BioRep3									
Tech 1 High	1	1	1	1	1	1	1	1	1
Tech 1 Low	237.1	96.2	50.6	2.5	49.9	10	2.1	1.8	0.8
Tech 2 High	1	1	1	1	1	1	1	1	1
Tech 2 Low	256.3	77.9	55.4	2.3	19.7	11.46	4	1.5	0.7

Yukon diurnal regulation

Trial	RNS1	At4 (Shoots)	Pht1;1	Pht1;4	Pht1;5	At4 (Roots)	Toc1
BioRep1							
Tech 1 High Day	1	1	1	1	1	1	1
Tech 1 Low Day	29.2	26	6.96	1.66	7.2	7.87	1
Tech 2 High Day	1	1	1	1	1	1	1
Tech 2 Low Day	26.6	18.35	8.37	1.13	8.56	7.78	1
Tech 1 High Night	1	1	1	1	1	1	3.6
Tech 1 Low Night	37.8	47.8	7.28	2.1	6.3	8.79	2.4
Tech 2 High Night	1	1	1	1	1	1	3.1
Tech 2 Low Night	43	54.69	7.01	1.3	6.4	9.33	1.89
BioRep2							
Tech 1 High Day	1	1	1	1	1	1	1
Tech 1 Low Day	41.5	19.96	8.65	1.54	7.5	9.8	1
Tech 2 High Day	1	1	1	1	1	1	1
Tech 2 Low Day	29.1	18.35	8.12	1.23	8.7	6.8	1
Tech 1 High Night	1	1	1	1	1	1	7.25
Tech 1 Low Night	30.1	21.8	6.51	1.67	7.9	10.12	6.6
Tech 2 High Night	1	1	1	1	1	1	3.32
Tech 2 Low Night	25.2	22.2	6.97	1.25	8.4	8.71	3.41

Yukon timing of response to low phosphate

Trial	RNS1	At4 (Shoots)	Pht1;1	Pht1;4	Pht1;5	At4 (Roots)	PHR1	Siz1	Pht2;1
BioRep1									
Tech 1 Time 0	1	1	1	1	1	1	1	1	1
Tech 1 Time 1	1.07	1.29	2.11	2.19	1.45	1.17	0.96	1.12	1.49
Tech 1 Time 2	0.6	1.33	1.61	0.93	0.32	1.68	0.27	0.66	0.76
Tech 1 Time 3	1.07	10.2	3.2	0.66	1.16	6.49	1.15	1.13	5.3
Tech 1 Time 4	0.5	4.76	2.6	1.61	0.41	6.48	0.63	0.75	1.8
Tech 1 Time 5	1.1	24.1	5.73	1.2	1.35	5.8	0.81	1.11	3.6
Tech 1 TC	1.05	0.51	0.55	0.01	0.92	0.18	0.91	1.2	1.5
Tech 2 Time 0	1	1	1	1	1	1	1	1	1
Tech 2 Time 1	1.1	1.52	2.31	2.31	2.2	1.48	1.1	1.8	1.8
Tech 2 Time 2	0.7	1.63	1.81	1.53	0.46	2.4	0.32	0.81	0.89
Tech 2 Time 3	1.15	4.6	3.4	0.91	2.43	7.57	0.86	1.7	3.32
Tech 2 Time 4	0.6	5.3	4.3	2.21	0.51	8.49	0.79	0.98	2.01
Tech 2 Time 5	1.2	22.1	7	3.01	1.57	7	0.9	1.31	3.46
Tech 2 TC	0.93	0.503	0.63	0.01	1.32	0.28	1.13	1.5	1.7
BioRep2									
Tech 1 Time 0	1	1	1	1	1	1	1	1	1
Tech 1 Time 1	1.005	1.28	1.5	1.14	1.31	1.64	1	1.24	1.16
Tech 1 Time 2	0.98	3.62	1.65	1.15	0.51	3	0.84	1.92	0.96
Tech 1 Time 3	0.85	12.3	3.3	2.02	4.3	10.77	0.98	1.55	1.13
Tech 1 Time 4	1.88	17.19	1.8	0.76	2.2	11.2	0.9	1.64	0.67
Tech 1 Time 5	1.34	20.73	4.04	1.23	3.05	10.08	1.05	1.43	0.85
Tech 1 TC	0.9	0.65	0.41	0.9	1.6	0.46	1.05	2	0.81

Yukon timing of response to low phosphate con't

Trial	RNS1	At4 (Shoots)	Pht1;1	Pht1;4	Pht1;5	At4 (Roots)	PHR1	Siz1	Pht2;1
BioRep2 (con't)									
Tech 2 Time 0	1	1	1	1	1	1	1	1	1
Tech 2 Time 1	1.1	1.1	1.8	1.32	1.61	1.2	1.14	1.39	1.29
Tech 2 Time 2	1.09	3.16	1.93	1.35	0.83	2.2	0.95	2.22	1.05
Tech 2 Time 3	0.95	11.01	3.65	1.4	3.9	8.73	1.22	1.83	1.4
Tech 2 Time 4	1.68	15.6	2.3	1.01	2.5	10.32	0.98	1.78	0.79
Tech 2 Time 5	1.24	18.95	4.32	1.4	3.55	9.4	1.13	1.54	0.9
Tech 2 TC	0.8	0.59	0.63	1.1	1.3	0.34	1.15	1.79	0.88
BioRep3									
Tech 1 Time 0	1	1	1	1	1	1			
Tech 1 Time 1	4.17	5.54	2.52	1.28	3.44	1.84			
Tech 1 Time 2	2.95	2.3	4.28	2.01	3.32	5.36			
Tech 1 Time 3	3.7	31.6	4.44	1.82	9.93	9.71			
Tech 1 Time 4	3.85	13.69	2.98	1.007	8.56	18.17			
Tech 1 Time 5	2.59	63	10.42	0.77	12.42	6.25			
Tech 1 TC	0.42	0.1	1.07	0.88	8.6	0.45			
Tech 2 Time 0	1	1	1	1	1	1			
Tech 2 Time 1	4.38	4.8	3.22	1.4	1.44	1.34			
Tech 2 Time 2	3.62	4.9	5.39	2.51	1.32	5.4			
Tech 2 Time 3	3.11	27.1	5.2	2.43	8.73	9.2			
Tech 2 Time 4	3.92	21.2	3.99	1.45	6.9	8.17			
Tech 2 Time 5	2.91	31	17.57	0.89	7.7	7.65			
Tech 2 TC	0.98	0.51	1.4	0.52	3.3	0.65			

Yukon various phosphate concentration

Trial	RNS1	At4 (Shoots)	Pht1;1	Pht1;4	Pht1;5	At4 (Roots)	WRKY75
BioRep1							
Tech 1 0 uM	187.4	186.5	38.9	1.62	3.36	21.02	10.4
Tech 1 25 uM	110.7	27.9	15.7	1.93	2.64	34.2	9.78
Tech 1 125 uM	11.93	0.8	2.27	1.3	0.82	2.64	3.54
Tech 1 250 uM	8.56	1.3	1.522	0.66	0.15	1.56	1.8
Tech 1 500 uM	4.85	0.83	1.98	2.18	0.76	2.2	3.18
Tech 1 2000 uM	1	1	1	1	1	1	1
Tech 2 0 uM	135.2	135.34	24.3	1.53	5.1	34.05	8.4
Tech 2 25 uM	86.9	17.9	13.7	1.75	3.2	29.8	6.23
Tech 2 125 uM	9.83	0.9	3.04	1.2	1.42	3.4	2.83
Tech 2 250 uM	6.34	1.2	1.82	0.78	0.21	2.1	2.31
Tech 2 500 uM	3.01	0.85	1.22	1.98	0.97	1.25	1.89
Tech 2 2000 uM	1	1	1	1	1	1	1
BioRep2							
Tech 1 0 uM	208	207	17.79	0.67	1.22	39.94	6.76
Tech 1 25 uM	122	30.88	0.33	0.9	1.04	5.34	12.5
Tech 1 125 uM	12.04	0.53	0.5	0.89	0.53	1.3	7.17
Tech 1 250 uM	9.13	1.47	0.88	0.48	1.17	1.8	3.45
Tech 1 500 uM	5.08	0.67	1.23	1.2	1.18	2.39	3.48
Tech 1 2000 uM	1	1	1	1	1	1	1

Yukon various phosphate concentration con't

Trial	RNS1	At4 (Shoots)	Pht1;1	Pht1;4	Pht1;5	At4 (Roots)	WRKY75
BioRep2 (con't)							
Tech 2 0 uM	171	157	24.21	0.89	1.74	19.54	12.36
Tech 2 25 uM	92.8	46.31	0.43	1.1	1.61	11.6	16.56
Tech 2 125 uM	9.81	0.66	0.55	1.09	1.01	1.24	12.1
Tech 2 250 uM	7.13	0.98	1.06	1.31	2.01	1.2	5.45
Tech 2 500 uM	3.54	0.89	0.89	1.09	1.98	1.6	4.6
Tech 2 2000 uM	1	1	1	1	1	1	1
BioRep3							
Tech 1 0 uM	10.5	77.15	48.5	2.6	0.87	28.51	27.75
Tech 1 25 uM	6.17	11.18	16	2.74	0.62	3.2	24.52
Tech 1 125 uM	1.9	1.24	7.4	0.96	0.87	2.09	18.28
Tech 1 250 uM	2	0.59	2.07	0.55	0.84	1.28	18.35
Tech 1 500 uM	0.894	0.55	1.83	2.57	0.8	1.57	8.08
Tech 1 2000 uM	1	1	1	1	1	1	1
Tech 2 0 uM	12.53	81.97	27.9	1.31	1.12	42.76	10.4
Tech 2 25 uM	7.46	13.57	23.41	0.98	1.08	6.23	7.91
Tech 2 125 uM	2.33	1.56	9.3	2.27	1.17	2.55	7.84
Tech 2 250 uM	2.76	0.74	2.18	0.76	1.3	1.82	12.35
Tech 2 500 uM	1.02	0.81	2.51	2.32	1.42	1.96	6.61
Tech 2 2000 uM	1	1	1	1	1	1	1

Shandong various phosphate concentration

Trial	RNS1	At4(Shoot)	Pht1;1	Pht1;4	Pht1;5	At4(Root)
BioRep1						
Tech 1 0 uM	335.4	94.6	26.1	1.06	7.8	12.2
Tech 1 25 uM	13.4	26.94	3.85	0.96	0.82	14.3
Tech 1 125 uM	1.37	1.36	1.21	0.36	1.97	3.36
Tech 1 250 uM	1.18	1.38	0.74	0.44	1.15	1.01
Tech 1 500 uM	1.34	1.86	0.87	0.53	0.89	1.05
Tech 1 2000 uM	1	1	1	1	1	1
Tech 2 0 uM	402.7	112.25	33.71	0.4	5.91	21.1
Tech 2 25 uM	16.6	33.45	5.5	0.51	1.2	19.5
Tech 2 125 uM	1.48	1.44	1.4	1.02	1.22	3.97
Tech 2 250 uM	1.26	1.55	0.98	0.58	1.67	1.429
Tech 2 500 uM	1.42	2.04	1.13	0.73	1.15	1.4
Tech 2 2000 uM	1	1	1	1	1	1
BioRep2						
Tech 1 0 uM	382.7	36.55	24.7	1.86	6.26	30.32
Tech 1 25 uM	25.5	26.59	14.76	2.7	2.14	1.99
Tech 1 125 uM	0.4	0.86	0.704	0.64	0.94	0.95
Tech 1 250 uM	0.22	0.26	0.58	0.98	0.74	0.64
Tech 1 500 uM	0.62	0.66	0.92	1.58	0.57	0.81
Tech 1 2000 uM	1	1	1	1	1	1
Tech 2 0 uM	350.55	47.3	25.8	1.18	7.5	27.4
Tech 2 25 uM	33.1	34.7	12.35	1.4	4.9	5.68
Tech 2 125 uM	1.4	1.29	1.3	1.09	1.14	1.84
Tech 2 250 uM	1.23	1.26	1.18	1.58	1.35	1.33
Tech 2 500 uM	0.71	0.74	1.25	0.46	1.52	1.05
Tech 2 2000 uM	1	1	1	1	1	1

Appendix C - Statistical analysis of seedling fresh weight

Tissue	**p-value**
Yukon High-Low	
Roots	0.05
Shoots	0.0008
Shandong High-Low	
Roots	0.0039
Shoots	0.0229
Yukon Magnitude	
Roots	0.0003
Shoots	<0.0001
Shandong Magnitude	
Roots	0.0067
Shoots	0.008

Appendix D - Statistical analysis of gene expression results from qPCR

Gene Yukon High-Low	p-value	F-ratio	Degrees of Freedom
RNS1	<0.0001	301.91	1
At4(Shoot)	0.0002	153	1
WRKY75	0.0002	171.21	1
Pht1;1	0.0021	49.53	1
Pht1;4	0.0007	90.25	1
Pht1;5	0.001	73.69	1
At4(Root)	0.0074	25.21	1
PHR1	0.3739	1	1
Siz1	0.3005	1.41	1
Pht2;1	<0.0001	608	1

Gene Shandong High-Low	**p-value**	**F-ratio**	**Degrees of Freedom**
RNS1	<0.0001	710.8	1
At4(Shoot)	<0.0001	574.7	1
Pht1;1	0.0002	184	1
Pht1;4	0.1076	4.26	1
Pht1;5	0.0008	82.18	1
At4(Root)	0.0084	23.38	1
PHR1	0.2423	1.88	1
Siz1	0.1805	2.63	1
Pht2;1	0.0339	10.05	1

Gene	p-value	F-ratio	Degrees of Freedom
Yukon Day-Night			
RNS1	0.0048	24.91	3
At4(Shoot)	0.005	78.93	3
Pht1;1	<0.0001	333.13	3
Pht1;4	0.1677	22.05	3
Pht1;5	0.0006	71.52	3
At4(Root)	<0.0001	898.03	3
Toc1	0.1067	4.01	3
Timing of Expression			
RNS1	0.9459	0.27	5
At4(Shoot)	0.0072	5.53	5
Pht1;1	0.0348	3.51	5
Pht1;4	0.764	0.51	5
Pht1;5	0.2064	1.71	5
At4(Root)	<0.0001	17.45	5
PHR1	0.2418	1.89	5
Siz1	0.7951	0.46	5

Gene	p-value	F-ratio	Degrees of Freedom
Yukon Magnitude			
RNS1	0.0417	3.3	5
At4(Shoot)	<0.0001	54.05	5
Pht1;1	0.0003	11.81	5
Pht1;4	0.0895	2.5	5
Pht1;5	0.5543	0.83	5
At4(Root)	<0.0001	14.7	5
Shandong Magnitude			
RNS1	<0.0001	71.38	5
At4(Shoot)	0.0314	5.42	5
Pht1;1	0.0005	27.23	5
Pht1;4	0.8332	0.4	5
Pht1;5	0.0013	18.7	5
At4(Root)	0.0004	5.67	5

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