Growth and Phospholipid Analysis of *Thellungiella salsuginea* Exposed to Varying Environmental Phosphate

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ABSTRACT

In global soils phosphorus is both the most abundant yet the least available essential macronutrient for plants. Phosphorus is a component of nucleic acids, some signalling molecules, membrane lipids and, as ATP, for metabolic control and energy transfer. Consequently, a phosphorus deficiency impacts many aspects of growth and development leading to plants that are smaller in stature, develop slowly and yield less than plants with sufficient phosphate.

Diversity among native plants with respect to phosphorus use can allow researchers to identify traits meriting consideration for crop improvement. Thus the objective of this thesis research was to study the response of a native crucifer found in the Yukon Territory, *Thellungiella salsuginea,* to various concentrations of external phosphate. Seedlings were grown on nutrient medium containing 0.05, 0.1, 0.2 or 0.5 mM phosphate. No significant differences were found with respect to root elongation rates for concentrations below 0.2 mM phosphate but root elongation was 1.4-fold faster for seedlings grown on 0.5 mM phosphate. *Thellungiella* seedlings grown on low (0.05 mM) phosphate medium showed a 2.5-fold increased biomass allocation to roots when compared to plants on high (0.5 mM) phosphate medium consistent with an increased allocation to root mass under low phosphate seen with plants optimizing foraging for limiting nutrients. Even at the lowest external Pi content no seedlings showed the classic phosphate starvation response typified by short primary roots with many lateral branches.

The lack of a starvation phenotype raised questions of how *Thellungiella* allocates phosphate to major sinks, one being phospholipids. Therefore, a ^{31}P-NMR profiling strategy was developed for leaf phospholipids. This approach required more tissue than was available from seedlings so rosette leaves were harvested from five-week-old plants grown in PromixBX and watered weekly with a low (0.05 mM) or high (1.0 mM) phosphate solution. Shoot fresh weight was not significantly different between treatments and visual inspection revealed no overt phenotypic differences. Shoot tissue was solvent-extracted to yield polar and non-polar phases. The polar phase of plants grown under low phosphate conditions contained significantly less (1.3-fold) inorganic phosphate (Pi) than plants watered with 1.0 mM phosphate ($P < 0.05$). Non-polar phases dissolved in 5% (w/v) sodium cholate produced high resolution spectra by ^{31}P-NMR. Spectra from low and high phosphate-treated plant samples usually showed five peaks tentatively identified as free Pi, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine. Statistical analysis indicated that only the putative phosphatidylinositol peak showed treatment-related differences, with low phosphate plants showing about 50% of the lipid species relative to high phosphate plants.

Use of the Electronic Reference To Access In Vivo Concentrations (ERETIC) method was tested on a limited number of extracts and results show this to be a promising approach for quantifying phospholipid species profiled by ^{31}P-NMR. Moreover, this profiling protocol was successfully extended to *Arabidopsis* tissue producing spectra with a signal-to-noise comparable to those from *Thellungiella* samples despite using 2-fold less tissue. In the case of *Arabidopsis*, only four distinct peaks were identified with the

one attributed to phosphatidylinositol apparently absent or below the detection limits. The difference in spectra found between *Thellungiella* and *Arabidopsis* plants warrants further investigation.

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ABBREVIATIONS

µL	Microlitre(s)
µM	Micromolar
µmol	Micromole
^{1}H-NMR	Proton NMR
^{31}P-NMR	Phosphorus-31 NMR
ADP	Adenosine Diphosphate
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
APase(s)	Acid phosphatase(s)
AtNMT3	Arabidopsis N-methyltransferase enzyme
ATP	Adenosine Triphosphate
C	Celsius
cm	Centimetre
d	Day(s)
DGDG	Digalactosyldiacyglycerol
DW	Dry Weight
ERETIC	Electronic Reference To Access In Vivo Concentrations
ESI-MS/MS	Electrospray Ionization-Tandem Mass Spectrometry
FID	Free Induction Decay
FW	Fresh Weight
g	Gram(s)
GTP	Guanosine Triphosphate
h	Hour(s)
L	Litre(s)

MANOVA	Multivariate Analysis of Variance
MGDG	Monogalactosyldiacyglycerol
mL	millilitre(s)
mM	Millimolar
mmol	Millimole
mol	Mole
NMR	Nuclear Magnetic Resonance
O_2	Oxygen
PA	Phosphatidic Acid
PC	Principal Component
PC	Phosphatidylcholine
PCA	Principal Component Analysis
PE	Phosphatidylethanolamine
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
Pi	Inorganic Orthophosphate
PK_c	Cytosolic Pyruvate Kinase
PPi	Pyrophosphate
PPi-PFK	Pyrophosphate-dependent phosphofructokinase
ppm	Parts per million
PSI	Phosphate-starvation Induced
RNA	Ribonucleic Acid
rpm	Rotations per minute
s	Second(s)

SL	Sulfoquinovosyldiacylglycerol
TFs	Transcription factors
UDP	Uracil-diphosphate
wk	Week(s)
WT	Wild-type

LITERATURE REVIEW

The Role of Phosphorus *in planta*

Plants require mineral nutrients to support normal growth and development. Phosphorus, along with nutrients like nitrogen and potassium is considered to be an essential macronutrient for plants (Wu et al., 2003). Phosphorus is a major component of phospholipids, nucleic acids and coenzymes (Wu et al., 2003). Phosphate, as polyphosphoinositide (derived from the membrane lipid) and the second messenger phosphatidic acid, is also used in signal transduction pathways of plants (van Leeuwen, Ökrész, Bögre, & Munnik, 2004). Phosphorous is also a central component of energy storage and is required for almost all metabolic processes, notably photosynthesis and respiration (Duff, et al., 1989; Theodorou & Plaxton, 1993). In this role, -phosphorus is essential for energy transfer and is required for every metabolic reaction that is thermodynamically non-spontaneous (Knowles, 1980). Free energy released from the cleavage of the phosphoric anhydride of nucleoside triphosphate (particularly ATP) is coupled to the displacement of the phosphorus from monoesters, diesters and anhydrides (Knowles, 1980). Examples of enzymes that catalyze these displacements are phosphokinases, phosphatases, phospholipases and nucleases. These classical metabolic enzymes operate in plants along with the metabolic bypass enzyme pyrophosphatase that cleaves the high-energy phosphoanhydride bond to yield two phosphate molecules. These reactions illustrate the ubiquitous and essential nature of phosphorus in metabolic regulation (Plaxton, 2004).

Phosphate Availability and Implications

Phosphorus is implicated in almost all vital biological processes and it is likely the most readily abundant nutrient in the lithosphere (Ticconi & Abel, 2004). This element is primarily taken up by plants as inorganic orthophosphate (Pi) (Duff et al., 1994). However, it is the inorganic orthophosphate form that is the least bioavailable nutrient in natural soils and the least abundant form with approximately 20 to 80% of soil phosphates present as non-mineralized organic phosphates (Franco-Zorrilla et al., 2004; Raghothama, 1999). In acidic soils (ca. 30% of global soils), Pi readily complexes with ions such as aluminum, while in alkaline soils it assumes an uneven and patchy distribution (Raghothama, 1999). It has been estimated that between 30 to 40% of arable land has sufficiently low levels of Pi as to limit crop production (Franco-Zorrilla et al., 2004). To combat low Pi, often in otherwise fertile soils, phosphate-rich fertilizers are applied to croplands to prevent Pi-starvation and promote high crop yields. However, a sizable portion of the phosphate added forms insoluble compounds in the soils requiring the addition of almost four times the necessary levels of fertilizers to the field (Raghothama, 1999). An excess amount of fertilizer ensures that soluble Pi levels greatly surpass necessary levels but this surplus of soluble Pi increases the incidence of phosphate leaching in run-off from fields into surrounding waters (Bennett et al., 2001). Phosphate-laden run-off leads to eutrophication and unchecked algal growth or algal "blooms." High levels of algae result in oxygen depletion, extirpation of native aquatic species, and potential human toxicity due to algal toxin production (Bennett et al., 2001).

There are also severe economic implications to phosphate deficiency in arable lands. For example, one hectare of canola can remove up to 40 kg of phosphate from the soil which can easily and quickly deplete the soil in the absence of a regular annual application of fertilizer (Crop Nutrients Council, 2009) Given that Canada is the world's 4th largest producer of canola oil and the leading exporter of canola oil (Agriculture and Agri-food Canada, 2007), canola contributes $13.8 billion dollars to the Canadian economy (Canola Council of Canada, 2008). As such, a reduction in available phosphate has the potential to severely limit Canada's canola production and export and damage the economy. For canola, the current yield increase in response to phosphate fertilizers is a function of many factors including soil type, but the average increase is approximately 10%; crop returns were maximized with the addition of 10 to 20 kg per hectare following results comparing yield at 154 field sites distributed across Alberta (McKenzie et al., 2003). These authors note that the practice before the 1960s was to fertilize heavily but that since 1975, fertilizer use has dropped to where the amount of P imported as fertilizer for the three Prairie Provinces is equivalent to the P exported in grain. However, this study also showed comparable (ca 10%) yield benefits for two other major crops (wheat and barley) indicating that phosphate applications at some level remains a necessary input to improve crop productivity in Western Canada.

Phosphate Deficiency-Induced Root Alterations

The low availability of phosphate in soil has many consequences on plant growth and development. Plants must be able to explore the soils to exploit the spatial variability

in phosphate deposits and the consequence is a change in root growth patterns (Lynch & Brown, 2001). In *Arabidopsis*, a commonly observed phenomenon under low phosphate growing conditions is altered root morphology; the root-shoot ratio is increased and lateral root formation is enhanced (Ticconi & Abel, 2004). An increase in lateral root growth coupled with inhibition of primary root growth enables greater exploration of the nutrient-rich upper soil in a process called topsoil foraging (Jiang et al., 2002). Concurrent with the larger-scale architectural changes is an increase in root hair density that is unique to phosphate deficiency (Ma et al., 2001; Ma et al., 2003). Increased root hair density increases both total root surface area and the absorptive surface area of the roots in *Arabidopsis* (Lopez-Bucio et al., 2002). Approximately 70 to 80% of root area is supplied by root hairs, which are the dominant sites of phosphate uptake in non-mycorrhizal plants (Ticconi & Abel, 2004). Architectural changes in root growth and morphology allow plants to explore the soil to find and uptake phosphate during times of deficiency.

Phosphate Deficiency-Induced Metabolic Alterations

When plants are under phosphate stress conditions, many molecular alterations occur to help preserve the viability of the organism. To maintain adequate levels of free phosphate within the cell, Pi is liberated from a variety of compounds. Phosphate-starved cells undergo a decrease in RNA content and free nucleotides such as ATP and GTP (Shimano & Ashihara, 2006). These changes are attributed to increases in DNase, RNase and nucleotidase activity, decreases in RNA synthesis, and increases in the activity of

nucleotide salvage pathways during phosphate stress (Shimano & Ashihara, 2006). Recycling purine nucleotides is believed to provide the cell with small amounts of energy that it can use to survive until phosphate is no longer limiting.

Plant acid phosphatases (APases) are a class of enzyme involved in Pi recycling that are induced in plants in response to phosphate deprivation (Plaxton, 2004). These enzymes catalyze a hydrolysis reaction that release Pi from phosphate monoesters (Duff et al., 1994). Acid phosphatases can be intracellular or extracellular. Extracellular, or secreted acid phosphatases, can liberate Pi from soil organic phosphates for uptake by plant roots. In contrast, intracellular acid phosphatases are vacuolar and function to scavenge internal Pi for use in critical metabolic processes (Yun & Kaeppler, 2001). Some APases such as phosphoenolpyruvate (PEP) phosphatase or 3-phosphoglycerate phosphatase are part of a substrate-specific class of APases and perform more specialized metabolic functions (Duff et al., 1994). However, many APases are non-specific and can catalyze the release of Pi from a variety of substrates.

Phosphate starvation can also result in the production and excretion of organic acids to the rhizosphere (Raghothama, 1999). PEP carboxylase is induced in response to Pi deprivation and the activity of this enzyme leads to the production of organic acids that may be secreted to the external environment (Plaxton, 2004). The organic acids, typically malic and citric acid, acidify the soil which helps release Pi from insoluble rock phosphates (Raghothama, 1999).

When plants are Pi-starved a discernible decrease in cellular Pi, ATP and ADP occurs (Shimano & Ashihara, 2006; Theodorou & Plaxton, 1993). Cellular ATP and

ADP content decrease 4- and 10-fold, respectively, whereas Pi shows a dramatic 44-fold decrease in response to Pi-deprivation (Duff et al., 1989; Plaxton, 2004). Many of the enzymes in classical glycolysis require phosphate or adenylates as co-substrates (Plaxton, 2004). Consequently, phosphate deprivation can severely restrict carbon flux through glycolysis.

Phosphate has a role in carbohydrate metabolism. In the initial steps of glycolysis in plants sucrose is hydrolysed to produce glucose and fructose with both serving as substrates for the production of fructose-6-phosphate (fructose directly and glucose indirectly) (Plaxton, 2004). Sucrose synthase is an adenylate-independent enzyme that produces UDP-glucose from sucrose and then UDP-glucose pyrophosphorylase phosphorylates UDP-glucose to glucose-1-phosphate. The latter reaction requires pyrophosphate (PPi) as a co-substrate (Plaxton, 2004). Cellular levels of PPi remain elevated and independent of externally applied phosphate and, as such, are associated with reactions (and enzymes) that are inherently insensitive to a phosphate deficiency (Duff et al., 1989).

Plants are now known to contain phosphate-starvation inducible by-pass enzymes that allow critical metabolic reactions to proceed during a phosphate deficiency (Theodorou & Plaxton, 1993). A PPi-dependent enzyme, PPi-dependent phosphofructokinase (PPi-PFK), is induced in response to phosphate starvation: PPi-PFK catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate concurrent with the release of Pi. This is a reaction traditionally catalyzed by ATP-dependent PFK when Pi is plentiful (Duff et al., 1989) and PPi-PFK serves as a by-pass

to the ATP-dependent PFK when Pi is limiting. Moreover, PEP phosphatase was first discovered in 1989 as an ATP-independent bypass to cytosolic pyruvate kinase (PK_c) (Duff et al, 1989). When phosphate is non-limiting and ADP is plentiful, PK_c catalyzes the formation of pyruvate from PEP which is coupled to the production of ATP. Under conditions of low Pi, PEP phosphatase undergoes a 10-fold increase in activity and offers a vacuolar bypass to PK_c (Duff et al., 1989; Theodorou & Plaxton, 1993; Ticconi & Abel, 2004). While this bypass can be viewed as energetically wasteful because it is not coupled to the production of ATP it does allow for the conversion of PEP to pyruvate that is necessary for other critical metabolic reactions. PEP can also be catabolised via phosphoenolpyruvate carboxylase (PEPC). This is another Pi-starvation inducible enzyme that catalyzes the production of oxaloacetate from PEP while bypassing PK_c (Theodorou & Plaxton, 1993). Oxaloacetate is rapidly converted to malate that can be excreted as an organic acid or transported to the vacuole where it is converted to pyruvate. Another phosphate-dependent reaction of glycolysis is the conversion of glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphoglycerate (1,3-P_2-glycerate) via the phosphorylating NAD-G3P dehydrogenase (Duff et al., 1989). The 1,3-P_2-glycerate is then converted to 3-PGA via 3-PGA kinase which is dependent on ADP as a co-substrate. A non-phosphorylating NADPH-G3P dehydrogenase is induced when phosphate is scarce and bypasses these two reactions to directly produce 3-PGA from G3P (Duff et al., 1989).

Mitochondrial respiration also has the potential to be hindered by the low levels of Pi, ADP, and ATP that occur during periods of phosphate deprivation. Bypasses exist,

however, to maintain electron transport via non-phosphorylating alternative pathways (Theodorou & Plaxton, 1993). The rotenone-insensitive pathway is the bypass to Complex I in mitochondrial electron transport and the cyanide-resistant bypass offers an alternative to cytochrome oxidase (Theodorou & Plaxton, 1993). These bypasses maintain Krebs cycle function and O_2 consumption while producing adequate ATP for short-term stress survival (Rychter & Mikulska, 1990).

Lipids are a large and complex class of molecules implicated in cell structure, storage, signalling and metabolism (Meyerowitz & Somerville, 1994). Phospholipids are a class of glycerolipids that are composed of a glycerol backbone to which two fatty acids are esterified at positions *sn*-1 and *sn*-2 (Peterson & Cummings, 2006). A phosphate group that is present at the *sn*-3 position is attached to a polar head group (Peterson & Cummings, 2006). In mammalian cells, phospholipids comprise approximately 60% of the total lipids while glycolipids (such as galactolipids) and/or sphingolipids are approximately 10% of the cellular total (Han & Gross, 2005). Non-polar lipids and lipid metabolites together can contribute up to 40% of total cellular lipids but this value depends heavily on sub-cellular location, cell type and cellular condition (Han & Gross, 2005). *Arabidopsis* roots and seeds have a similar lipid class distribution: phospholipids comprise the majority of cellular lipids while glycolipids constitute the remaining 10%. Leaf lipids, however, are dominated by the galactolipids monogalactosyldiacyglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). These lipids are localized to the plastid membranes (Bonaventure et al., 2003). Other membrane leaf lipids include phosphatidylglycerol (PG, 12%), phosphatidylcholine (PC, 11.2%),

phosphatidylethanolamine (PE, 10%), phosphatidylinositol (PI, 5.5%), the primarily plastidic non-phosphate sulfolipid is sulfoquinovosyldiacylglycerol (SL, 4.3%) and the minor contributor phosphatidylserine (PS, 0.9%) (Bonaventure et al., 2003).

Membrane phospholipids sequester approximately one-third of the organic phosphate within plants (Andersson et al., 2003). *Arabidopsis* and *Avena sativa* growing under phosphate deficient conditions both accumulate galactolipids in extraplastidic membranes (Andersson et al., 2003; Hartel et al., 2000). For *Avena sativa*, DGDG content in shoot and root plasma membranes increases from 12% and 29% to 46% and 70%, respectively. This change in DGDG content is concomitant with decreases in all classes of phospholipids except phosphatidic acid (Andersson et al., 2003). This response is consistent with plants replacing phospholipids with galactolipids under phosphate stress in order to recycle much-needed phosphate and preserve the structure and integrity of the plasma membrane.

Profiling Lipid Composition in Plants: Current Approaches

To gain an understanding of plants as a dynamic and complex network of elements, interactions across various levels and components must be elucidated. To avoid the limitations of any single experimental approach, data from the transcriptome, the proteome and the metabolome must be integrated to gain a true understanding of how changes at one level can affect the entire organism (Sauer et al., 2007). At present, even these approaches have been further refined as targets of enquiry. For example, for lipid composition and content, together describing the lipidome, has been demonstrated to

change in response to environmental stresses and external conditions (Kuiper, 1985). Lipidomics provides a "snapshot" of an organism's lipid content at a given point in time allowing for the identification, quantification and functional determination of lipid species (Han & Gross, 2003). Lipidomics employs many different techniques to study the fluxes in lipid composition and metabolism under defined circumstances, such as under control conditions and in response to environmental stress.

Electrospray ionization coupled to tandem mass spectrometry (ESI-MS/MS) is a commonly used platform to assess lipid composition. It is a soft ionization (less energy is imparted to the molecules and thus, fewer fragmentations occur) followed by mass spectrometry (Peterson & Cummings, 2006). A crude lipid mixture is expelled through a very small nozzle that is held at a high potential (Gaskell, 1997). This imparts a positive charge to the emerging droplets (Gaskell, 1997). The ionized lipids are then fragmented and separated based on their mass-to-charge ratios and, in this way, lipid classes and acyl chains can be resolved and identified. This technology has furthered understanding of the roles of lipids in plant stress responses (Li, et al., 2006; Welti et al., 2002). For example, fatty acid saturation is reduced and overall glycerolipid quantity is increased in response to cold stress. This change in lipid composition is believed to reduce freezing-induced hexagonal formations that invert phospholipids and can lead to membrane rupture and ion leakage (Welti et al., 2002). Furthermore, it was determined that *Arabidopsis* deficient in the major phospholipase, PLDα, show reduced PC hydrolysis and reduced PA accumulation in response to cold stress, which may protect mutant plants from freezing-

induced nonbilayer and hexagonal phase formations that can result from high proportions of PA and PE or PG in membranes (Welti et al., 2002).

Nuclear magnetic resonance (NMR) is another analytical technique that can be used to determine the lipid content and composition of tissues. NMR works on the principle that nuclei spin about an axis, thus generating a small magnetic field (Jacobsen, 2007). In the presence of a larger, externally applied field, such as that inside a spectrometer, a nucleus will attempt to align itself with the external field (B_0). This results in precession or circular motion. This precession occurs at the Larmor frequency (ν) and is unique to a nucleus of a given element (Jacobsen, 2007). In this way, the spectrometer can be "tuned" to detect only the nucleus of interest. The spinning nuclei within a sample are hit with a radio frequency pulse that makes them all precess in unison, pointing in the same direction (x-y plane) at the same time. This creates a very short-lived and measurable magnetic field that induces a current in a coil surrounding the NMR tube containing the sample. The nuclei desynchronize quickly and return to alignment with the external field (z-axis) and the signal will die down as the nuclei become undetectable (Jacobsen, 2007). This echo after the pulse is called a free induction decay (FID) and contains all of the frequencies of the nuclei within the sample. A Fourier transform will sort out the individual frequencies and display them as individual peaks. The chemical environment of a given nucleus at a particular position within the molecule is the slight perturbation in the external magnetic field produced by the smaller magnetic fields of neighbouring nuclei. This can shift the resonant frequency on the order of parts per million (ppm) of the fundamental frequency (Jacobsen, 2007). Therefore, NMR is a

structural tool where each peak within the spectrum represents a nucleus in a specific and unique chemical environment.

Phosphorus-31 is 100% abundant in nature and only one phosphorus nucleus is present in each phospholipid. Each phospholipid species will therefore produce a single peak in the resultant spectrum. Furthermore, each phospholipid headgroup possesses a different chemical shift, facilitating identification and quantification (London & Feigenson, 1979). ^{31}P-NMR is a powerful tool that can be used to determine changes in the phospholipid fraction of the lipidome in response to various external stresses. Jouhet *et al.* (2003) used ^{31}P-NMR to determine phospholipid fluxes in response to Pi deprivation and made the surprising discovery that PC content of membranes actually increases transiently in early Pi starvation. Furthermore, the reduction in PE content in response to Pi stress suggests that PE is converted to PC; PC is then hydrolyzed to DAG which is the precursor to the galactolipids DGDG and MGDG, the predominant membrane lipids during Pi-starvation (Jouhet, Maréchal, Bligny, Joyard, & Block, 2003).

Phosphate Starvation-Induced Transcriptional Alterations

Phosphate starvation induces alterations in gene expression. These phosphate starvation inducible (PSI) genes are differentially regulated both spatially and temporally (Misson et al., 2005; Wu et al., 2003). An *Arabidopsis* microarray of 6172 genes shows that about 29% of genes are up- or down-regulated in response to phosphate stress (Wu et al., 2003). Seedlings were grown for 30 d in liquid culture before being transplanted to media containing low (0 mM) or high (2.5 mM) levels of phosphate. Most PSI genes are

induced in either roots or leaves; however some genes undergoing induction show spatial overlap. A majority of the genes followed the same general pattern with induction of gene expression detected 6 h after phosphate removal followed by maximal expression by 48 h and a decline in expression by 72 h (Wu et al., 2003). As expected, not all genes follow this expression pattern with some responding earlier or later to phosphate starvation and others switching from up- to down-regulated or vice versa (Wu et al., 2003).

PSI genes span a broad range of functional classes (Wu et al., 2003). Genes encoding Calvin cycle components such as the Rubisco small subunit and phosphorylating G3PDH are repressed as are enzymes associated with the photosystems (Wu et al., 2003). When phosphate is limiting, genes encoding nitrate reductase, nitrite reductase and other key enzymes of nitrogen metabolism are down-regulated as are genes involved in starch, fatty acid and lipid synthesis (Wu et al., 2003). This implies that energetically demanding growth processes are slowed when phosphate is limiting. Genes encoding enzymes implicated in bypasses to glycolysis and electron transport are up-regulated under phosphate stress to maintain flux through glycolysis and to produce trace amounts of ATP for critical metabolic processes (Theodorou & Plaxton, 1993; Wu et al., 2003). As is the case for many synthetic reactions, protein synthesis is depressed in phosphate deficient plants and in the study by Wu et al, (2003) 42 ribosomal genes were highly down-regulated along with a key transcription factor eIF2. Protease-encoding genes, however, were strongly up-regulated in leaves and roots indicating that proteins are likely degraded from senescing tissues to recycle amino acids for re-use in other tissues or when Pi is no longer limiting. Approximately 30% of the represented

transcription factors (TFs) also showed altered expression patterns during phosphate starvation. The *PHR1* eukaryotic Pi metabolism regulator was up-regulated along with seven other MYB-related proteins (Wu et al., 2003). Three members of the SCARECROW family, which are TFs for root development, also showed altered expression. Signal transduction gene expression was also affected by Pi starvation, emphasizing the importance of stress-signalling pathways in the plant response to phosphate availability. Genes that were reported to be up- or-down regulated relative to unstressed controls were members of all common groups of signal-transduction related genes including calmodulin-dependent protein kinase (CDPK) or CDPK-related protein kinases (Wu et al., 2003). CDPKs are implicated in plant response to general environmental stresses. Ethylene, auxin and gibberellin response genes showed differential regulation under phosphate stress conditions with the expression of some genes showing up-regulation and others down-regulation. The different expression patterns indicate that Pi stress affects multiple, hormone-mediated responses. For example, ethylene and auxin are known to increase root hair number and length and gibberellins are implicated in primary root growth inhibition. As such, the altered expression of genes whose products contribute to hormone related metabolism and signalling is not surprising given the complex physiological and developmental changes accompanying the plant's response to Pi stress (Jiang et al., 2007).

Transcriptional analysis of phosphate starvation induced alterations in gene expression was expanded from the 8,100 genes profiled by Wu et al. (2003) to the full 22,810 genes corresponding to the entire *Arabidopsis* genome in 2005. As seen in

previous transcriptional analyses, expression patterns varied both temporally and spatially (Misson et al., 2005). While there was some overlap in induced and repressed genes in leaves and roots after long-term Pi starvation, most genes showed tissue-specific expression patterns (Misson et al., 2005). Approximately 84% of the induced genes and 75% of the repressed genes had functional annotations. The classification of these genes with respect to the roles of their products revealed some interesting patterns of spatio-temporal regulation. The Pht1 family of Pi transporters is induced with *(Pht1;4)* being up-regulated in the short (3, 6, and 12 h), medium (1 and 2 d) and long (5, 10 and 15 d) term. This response is expected given that *(Pht1;4)* is a high affinity phosphate transporter that allows for efficient uptake of Pi from the growth medium and has been suggested to play a role in remobilization of Pi within the plant (Misson et al., 2004). Other genes encoding a mitochondrial ADP/ATP antiporter (*Pht3;2)*, a glucose 6P/Pi translocator, a Pi xylem loader (*PHO1)* and sulphate transporters are induced after longer-term stress and these late-induced genes appear to help the plant use Pi more efficiently under Pi-starvation (Bari et al., 2006; Hammond et al., 2003; Misson et al., 2005). Genes encoding products involved in lipid biosynthesis were also induced in response to phosphate starvation. For example, genes encoding enzymes responsible for phospholipid degradation and the non-phosphate galactolipid MGDG are induced after short-term Pi stress, implying that plants can rapidly sense and respond, in this case, to an absence of Pi in the growth medium (Misson et al., 2005). The DGDG synthase gene *DGD1* is primarily responsible for the synthesis of chloroplastic DGDG (Kelly & Dörmann, 2002) but a second, DGD1-independent DGDG synthase gene, *DGD2*, is

induced under phosphate stress. The product of *DGD2* is responsible for the synthesis of non-chloroplastic DGDG under Pi starvation (Hartel et al., 2000; Kelly & Dörmann, 2002). Both of the genes encoding DGDG synthase are up-regulated after medium- and long-term stress, possibly to maintain Pi recycling via galactolipid synthesis. Additionally, the sulfolipid synthase genes UDP-sulfoquinovose synthase and UDP-sulfoquinovosyl:DAG sulfoquinovosyltransferase are induced after long-term Pi deprivation (Misson et al., 2005). In terms of prolonged Pi depletion sulfolipids can replace phosphatidylglycerol in membranes (Misson et al., 2005).

The Suitability of *Arabidopsis* as a Model Organism in Stress Tolerance Studies

Arabidopsis was first proposed as a model organism by Frederich Laibach in 1907 (Somerville & Koornneef, 2002). Interest in this unassuming Brassica species waxed and waned until the late 1970s; it was at this time that *Arabidopsis* research enjoyed a revival (Koornneef & Meinke, 2010) and in 1998 it was declared a model organism for genome studies (Meinke et al., 1998).

Arabidopsis possesses many characteristics that make it an ideal model for research. These attributes include a wide geographic distribution that has led to a variety of ecotypes showing different phenotypic characteristics of interest (Bressan et al., 2001; Meinke et al., 1998), it is easily propagated as it can complete its entire lifecycle in six weeks when under continuous light conditions and yields approximately 5000 seeds per plant, and it can be grown on defined-nutrient agar plates, hydroponically or in a soil medium (Meinke et al., 1998). Unlike many model organisms, both plant and animal,

Arabidopsis is easily transformed making it extremely valuable for genetic screening, gene identification and functional annotation (Koornneef & Meinke, 2010). In 2000 full genome sequencing was completed and this provided the identification of over 25,000 unigenes on five chromosomes (Arabidopsis Genome Initiative, 2000). A complete genome has created opportunities for numerous genetic studies and comparative genomics studies. Gene sequences from 178 different plant species have led to the identification of evolutionarily conserved genes within the Plant Kingdom and a set of Brassica-specific genes (Lin et al., 2010). Furthermore, lineage-specific genes have been identified within *Arabidopsis* that may be integral to the biological functions and pathways that distinguish *Arabidopsis* from other species (Lin et al., 2010).

As a glycophyte, *Arabidopsis* is unable to withstand extreme conditions (Bressan et al., 2001). While this may limit the use of this plant for the studies of abiotic stress tolerance, molecular and genetic studies have led researchers to hypothesize that stress tolerant halophytes may use many of the same mechanisms found in glycophytes and that slight variations in the regulation of these mechanisms leads to the significant increases in stress tolerance in halophytes (Taji et al., 2004). This hypothesis is difficult to test in the absence of a highly tolerant model to use for comparison. In light of this consideration, a stress tolerant, halophytic model organism is required that possesses similar attributes of a model plant exemplified by Arabidopsis.

Thellungiella salsuginea **as a Stress Tolerant Model Organism**

The salt cress, *Thellungiella salsuginea,* is a crucifer that is rapidly becoming the new standard for stress tolerance studies. *Thellungiella* is a member of the *Brassicaceae* family and it possesses many of the favourable traits that made its close relative, *Arabidopsis,* ideal for genetic, physiological and biochemical studies (Bressan et al., 2001). *Thellungiella* is similar in size to *Arabidopsis* and it completes its lifecycle in six to eight weeks (Inan et al., 2004), it is prolific producing 4000-8000 seeds per plant (Bressan et al., 2001), the *Thellungiella* genome is quite small at less than twice the size of *Arabidopsis* and preliminary EST analyses shows 90 to 95% identity between *Arabidopsis* and *Thellungiella* sequences making it ideal for comparative genomic analyses (Wong et al., 2005). Furthermore, *Thellungiella* is transformable via floral dip and this has led to the creation of an extensive mutant library (Amtmann, 2009).

It is the extremophilic nature of this crucifier, however, that makes it particularly suitable for use as a model for stress tolerance. *Thellungiella* can withstand temperatures as low as -19°C (Griffith et al., 2007) and it is drought tolerant, remaining viable after losing 40% of its fresh weight in water (Wong et al., 2006). True to its name, salt cress can tolerate salinity up to 500 mM NaCl (Wong et al., 2006). In contrast, *Arabidopsis* cannot survive at salt concentrations of 300 mM or higher (Bressan et al., 2001).

The similarities between *Thellungiella* and *Arabidopsis* allow researchers of this extremophile to investigate salt cress by drawing on decades of established knowledge and tools. These two crucifers are genetically, metabolically and morphologically similar and it is believed that most stress tolerance pathways used by halophytes are present in *Arabidopsis* (Taji et al., 2004). Consequently, any insights into abiotic stress tolerance

gained from *Thellungiella* can be applied to other *Brassica* species, particularly crop plants (Wong et al., 2005).

Thesis Objectives and Hypothesis

The objective of my work was to evaluate the effect of external phosphate on the growth of *Thellungiella salsuginea* with particular emphasis on the leaf membrane lipid composition. This study entailed developing a protocol suited to profiling phospholipids. Establishing a consistent method of plant growth, lipid extraction, sample preparation and analysis is an extremely important step in determining whether *Thellungiella* would be a suitable model species to study Pi nutrient use efficiency. Model species that perform well under Pi levels considered limiting for most plants would be valuable towards identifying traits needed to improve phosphate assimilation for Canada's crop plants. For example, a protocol that provides comprehensive lipid profiles can be used to determine whether a lipid fingerprint exists that can associate specific lipid profiles with phosphate stress.

H_0: *Thellungiella salsuginea* does not respond to phosphate deficiency by altering its lipid composition; there will be no change in phospholipid species in response to the low phosphate treatment.

MATERIALS AND METHODS

Plant Material and Growth

Seeds

Thellungiella salsuginea (Yukon ecotype) seed stock was collected originally from plants grown in the Takhini Salt-Flats in Whitehorse, Yukon by Dr. Bruce Bennett, Wildlife Viewing Biologist with Environment Yukon. Seeds used in this study are descendents from the Yukon seed collections bulked from plants that have been propagated under controlled environment conditions at McMaster University. As such, the seeds represent progeny of a mixed population of plants and no assumptions of genetic homogeneity were made. Collected seeds are routinely stored in 1.5-mL flat-top graduated microfuge tubes (Diamed Lab Supplies Inc., Mississauga, ON, cat. no. SPE155-N). For long-term storage, microfuge tubes containing seeds were stored in 50mL BD Falcon™ polypropylene screw-cap, conical tubes (BD Biosciences, Franklin Lakes, NJ, cat. no. 352098) with Drierite dessicant (W.A. Hammond Drierite Company, Ltd., Xenia, OH).

Seeds for the *Arabidopsis thaliana* wild-type CS 60000 and a T-DNA SALK line SALK_062703 were purchased from the Arabidopsis Biological Resource Center (ABRC) at the University of Ohio (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). The mutant line has been shown to be deficient in the expression of a gene encoding phosphoethanolamine *N*-methyltransferase

(M. Macleod, 2010) also annotated as AtNMT3 and hence the mutant line is referred to as *atnmt3* in this thesis.

Materials

Sources of chemicals and disposable materials used for this research are disclosed in this section when their application is first described. Unless otherwise stated, the purity of the water used for all procedures including the preparation of solutions and watering plants was the equivalent of double-distilled as prepared from a Barnstead Nanopure II System.

Seed Surface Sterilization

Approximately 0.1 mL of seeds were transferred to a 1.5-mL microfuge tube (Diamed). Seeds were rinsed first with approximately 1 mL of 70% (v/v) ethanol then the ethanol was discarded and replaced with a solution made of 50% (v/v) sodium hypochlorite (Javex Bleach™) and 0.1% (v/v) Triton X-100 (Sigma-Aldrich Co., Oakville, ON, cat. no. 9002-93-1). The hypochlorite/Triton X-100 solution was removed and seeds were rinsed at least five times with 1-mL volumes of H_2O and then the seeds were stored overnight at 4°C in a sterile 0.1% (w/v) solution of Phytagel (Sigma-Aldrich Co., Oakville, ON, cat. no. 71010-52-1).

Plant Growth

Nutrient Agar Plate Preparation

A complete nutrient solution was modified to contain different concentrations of phosphate. Four concentrations tested ranged from 0.05 mM phosphate ($LP_{0.05}$ for "low") to 0.5 mM phosphate ($HP_{0.50}$ for "high"). Table 1 outlines the formulations used for the modified nutrient agar plates. Solutions were made by combining all components in a 500-mL beaker. Each nutrient solution was brought to a final volume of 250 mL using H_2O then sucrose (BioShop) was added to a final concentration of 2% (w/v) and Phytagel (Sigma-Aldrich) to a final concentration of 0.8% (w/v). The pH of the medium was 5.5 to 6.0 and was not adjusted. Once autoclaved, sterile media was poured into 100 x 15 mm polystyrene BD Falcon™ Integrid plates (BD Biosciences, cat. no. 35112). Once solidified, plates were inverted and stored at 4°C until used. Typically, each of the four phosphate treatments yielded five replicate plates for a total of twenty plates across all treatments for each experiment.

Planting Medium Preparation

A 6:1 (v/v) mixture of Promix BX soil (Premier Horticulture Inc., Quakertown, PA) and Turface (Profile Product LLC, Buffalo Grove, IL) was prepared to a total volume of seven litres and combined with 3.5 L H_2O and sterilized. Upon cooling, this mixture was thoroughly mixed and lightly packed into black, rectangular 7.7 x 4.4 cm plastic pots (The Lerio Corp, Mobile, AL).

Table 1: Composition of nutrient agar solutions used to determine the responses of *Thellungiella* seedlings to various concentrations of phosphate.

Solutions were made to a final volume of 250 mL and, with the exception of phosphate, the provision of all other macro- and micro-nutrients was held constant across all treatments.

			Concentration of Nutrient Media *(mM Pi)*			
			0.5	**0.1**	**0.2**	**0.05**
Macro-nutrient solution	*[Stock] (M)*	*[Final] (mM)*	*Working Volumes (mL)*			
$CaCl_2$	0.5	0.5	0.25	0.25	0.25	0.25
$MgSO_4$	0.5	0.5	0.25	0.25	0.25	0.25
[a]Na-PO_4	0.5	Variable	0.25	0.10	0.05	0.025
KNO_3	0.5	1.0	0.5	0.5	0.5	0.5
Micro-nutrient solution	*[Stock] (mM)*	*[Final] (μM)*	*Working Volumes (mL)*			
$FeSO_4 \cdot 7H_2O$	10	100	2.5	2.5	2.5	2.5
H_3BO_3	10	100	2.5	2.5	2.5	2.5
$MnCl_2$	2	20	2.5	2.5	2.5	2.5
$CuSO_4$	0.1	1	2.5	2.5	2.5	2.5
$ZnSO_4 \cdot 7H_2O$	0.1	3	7.5	7.5	7.5	7.5
Na_2MoO_4	0.02	0.4	5.0	5.0	5.0	5.0
$CoCl_2$	0.001	0.01	2.5	2.5	2.5	2.5

[a]4.97 g Na_2HPO_4 and 2.07 g NaH_2PO_4 dissolved in 100 ml H_2O

Plant Fertilizer Preparation

Pot-grown plants were fertilized once weekly with a modified Hoagland's solution (Hoagland, 1920). Two solutions were made that contained either a 0.05 mM or 1.0 mM phosphate concentration (1 M KH_2PO_4; Table 2). With the exception of varying phosphate composition, both solutions contained the same concentrations of all other critical macro- and micro-nutrients. The pH of the final solution was pH 5.5 (without adjustment). New fertilizer was prepared weekly from refrigerated stocks by adding each component to a 2-L beaker and then the solution was brought up to a final volume of 1 L with H_2O. It is important to note that micronutrients are prepared first as a single, stock solution.

Seed Germination

Sterilized Yukon seeds were sown on 100 x 20 mm cell culture dishes (Corning Incorporated, Corning, NY, cat. no. 430167) containing Murashige and Skoog (MS) agar media which is 0.43% (w/v) Murashige and Skoog salts, 0.6% (w/v) Phytagel and 0.1% (v/v) Murashige and Skoog Vitamins, supplemented with 2% (w/v) sucrose (Murashige & Skoog, 1962). Plates were sealed with 3M Micropore paper tape (1/2"x10yds, Fisher Scientific, Ottawa, ON, cat. no. 19-061-655) and seeds were stratified for 48 h at 4°C in the dark. Following stratification, plates were incubated at 22°C with a 24 h photoperiod and a photon flux density of 60 to 65 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Table 2: Composition of fertilizer solutions used to determine the response of *Thellungiella* plants to varying concentrations of phosphate.

Fertilizer solutions were made to a final volume of 1 L from stock solutions.

Micronutrients were prepared first as a single stock solution.

			1.0mM Pi	**0.05mM Pi**
Nutrient Solutions	*[Stock] (M)*	*[Final] (mM)*	*Working Volume (mL)*	
$Ca(NO_3)_2$	1.0	5.5	5.5	5.5
KNO_3	1.0	5.5	5.5	5.5
$MgSO_4$	1.0	2.3	2.3	2.3
KH_2PO_4	1.0		1.1	0.05
KCl	1.0	1.05	-	1.05
Fe-EDTA	See below		1.1	1.1
Micro-nutrient solution	See below		1.1	1.1

Nutrient Solution	*Mass Fe/working volume (mg)*
Fe-EDTA	5.5 mg Fe
Micronutrient Solution	*Mass/L of stock (g)*
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnCl_2$	0.11
$CuCl_2 \cdot 2H_2O$	0.05
$NaMoO_4 \cdot 2H_2O$	0.025

Seedling Growth on Nutrient Plates

Once seeds germinated and the seedlings were of an appropriate size (approximately 4 to 6 d of incubation), six were selected at random and transferred to treatment plates. Transfers were performed aseptically in the sterile flow bench and seedlings were positioned along the second gridline from the top (Figure 1). Plates were sealed with 3M Micropore tape and placed at a 45° incline in a controlled environment chamber set at 22°C with a 12 h photoperiod and a photon flux density of 150 to 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Plates were photographed daily at the same time and roots were measured using ImageJ 1.42q (Wayne Rasband, National Institutes of Health, USA).

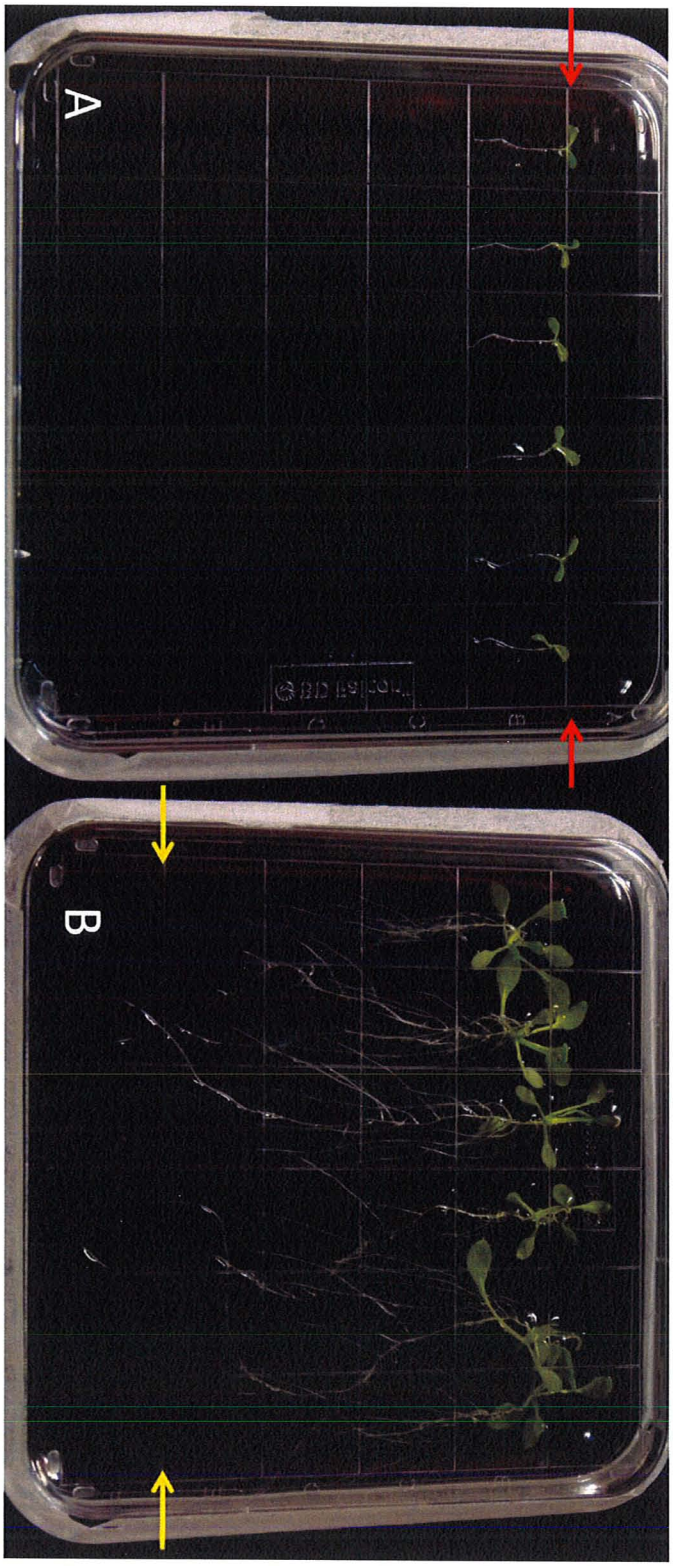
Plants were harvested when a majority of roots for the seedlings within a treatment extended down the plate to reach the second-to-last gridline (Figure 1). Seedlings from each plate within a treatment were harvested individually and as quickly as possible to minimize the time the plants were off the agar. Shoots and roots were separated, pooled by treatment plate and tissue type into pre-weighed 1.5-mL microfuge tubes and fresh weight was measured. Tubes were immediately flash-frozen in liquid nitrogen and stored at -80°C until use. Four biological replicates were harvested over the course of this project.

Seedling Growth on Soil Media

Seeds were germinated on MS plates (prepared as described above) and plates were incubated for 7 d before seedlings were transferred to pots.

Figure 1. Representative *Thellungiella* seedlings immediately following transfer to nutrient plates (A) and at time of harvest (B).

A. Seedlings are placed along the second gridline indicated by the red arrows to allow for maximum root extension. B. Seedlings are harvested when a majority of the roots have extended past the gridline indicated by the yellow arrows.


A
B

Seedlings of the same approximate size were selected for transplant. Seedlings were grown in a Conviron CMP 3244 controlled environment chamber (Conviron, Winnipeg, MB) with a 21 h light/3 h dark photoperiod at 22°C/10°C.Photon flux density was 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Flats were randomly assigned either a high (1.0 mM) or a low (0.05 mM) phosphate fertilizer treatment and were labelled accordingly. Plants were watered as needed with a 10-mL syringe and fertilized weekly with the appropriate treatment fertilizer, beginning one week after transplant to allow the young plants time to acclimate to the new conditions before starting the treatment. Plants were grown for a total of 5 wk from the date of transplant.

Plants were harvested by removing all leaves (including petioles) and pooling tissue to produce 5-g portions representing the pooled material from 5 to 7 plants. Tissue was weighed, wrapped in foil and flash frozen in liquid nitrogen for storage at -80°C until use.

Lipid Extraction

Small-scale (50 mg) Extraction

The general metabolite extraction protocol established by Fiehn et al. (2000) was modified slightly to accommodate 50 mg of tissue. Frozen, stored seedling tissue representing a specific nutrient plate treatment was pooled to yield 50 mg in a 1.5-mL microfuge tube. Liquid nitrogen was added to the tube and the tissue was ground using a sterile Eppendorf micropestle (Sigma-Aldrich Co., Oakville, ON, cat. no. Z317314). Just prior to the evaporation of the liquid nitrogen, 350 µL of 100% HPLC-grade methanol

(Caledon Laboratories Ltd, cat. no. 6701-7) was added. Grinding continued until a uniform slurry formed; 14.5 µL of 1M NaCl was added to the slurry and the microfuge tube was placed in a 70°C shaking water bath (100 rpm) for 15 min. The tube was then centrifuged at 4°C in an Eppendorf 5415D Microfuge (Eppendorf AG, Hamburg, Germany, cat. no. 0061966) at 14,000*g* for 3 min. Following centrifugation, the supernatant was removed and transferred to a new 1.5-mL microfuge tube (Diamed). The pellet was resuspended with 187.5 µL of chloroform and heated to 37°C for 5 min in a shaking water bath. The resuspended material was vortexed and centrifuged as described previously, The supernatant was removed and pooled with the initial supernatant and 350 µL H_2O was added. The tubes were vortexed to mix the contents and centrifuged at 8000 rpm for 5 min at room temperature. The upper phase containing the polar metabolites was transferred to a Wheaton V-vial (Wheaton, Millville, NJ, cat. no. 986254) while the lower phase containing non-polar metabolites was transferred to a 2-mL screw-cap glass vial (Gerresheimer, Cat. No. 60910, Rochester, NY). Both phases were evaporated to dryness under a stream of N_2 in an N-Evap Analytical Evaporator (Organomation, Westbury, NY, model no. 111). Dried samples were sealed with Parafilm and stored at -20°C for future use.

Large-scale (5 g) Extraction

The metabolite extraction protocol used by Bligh and Dyer (1959) was modified slightly in accordance with Fiehn et al. (2000). A previously frozen 5 g aliquot of tissue from a given treatment was transferred to a pre-chilled mortar filled with liquid nitrogen.

Prior to the complete dissipation of the liquid nitrogen, the tissue was ground into a powder with a pre-chilled pestle. A 5-mL volume of chilled 100% HPLC grade methanol was added to the mortar and the resulting slurry transferred to a 15-mL Corex™ tube. A second 5-mL volume of methanol was then used to rinse the mortar and the slurry was added to the contents in the Corex™ tube. The tubes were capped and centrifuged in an International clinical centrifuge in the fume hood at setting 6 for 10 min (International Clinical Equipment, Needham, MA, model CL). The supernatant was removed to a 50-mL BD Falcon™ polypropylene conical centrifuge tube (BD Biosciences). The pellet was resuspended with 10 mL of chloroform, the tube was vortexed to mix the contents and then the tube was centrifuged as described above. The supernatant was removed and pooled with the first supernatant. A volume of 5 mL H_2O was added to the Falcon™ tube containing the pooled supernatants, the tube was vortexed to mix the contents and then the tube was centrifuged as above. The upper, polar phase and lower, non-polar phases were transferred to separate, 16 x 150 mm borosilicate glass disposable culture tubes (Fisherbrand®, cat. no. 14-961-31) and evaporated to dryness under a stream of N_2 gas in an N-Evap Analytical Evaporator. The dried samples were stored at -20°C for future use. Table 3 outlines the details of samples that were used for extraction.

Arabidopsis Tissue

Arabidopsis plant material was grown and harvested as indicated by Mitchell Macleod as outlined in his MSc Thesis (2010). Briefly, *Arabidopsis* seeds were surfaced sterilized, germinated and transferred to pots as described above. Plants were grown in a

Conviron CMP 3244 controlled environment chamber with a 12 h light/12 h dark photoperiod at a constant temperature of 21°C. Photon flux density was 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Plants were watered as needed and fertilized biweekly with a 1 g/L of 20-20-20 all purpose fertilizer. Leaf tissue from wild-type and the *atnmt3* mutant *Arabidopsis* line was harvested and frozen into two aliquots (2.4 g and 2.1 g, respectively). This tissue was subjected to the same metabolite extraction procedure as described above for *Thellungiella* tissue. However, one important alteration was made in that the solvent volumes were reduced by a factor of two to account for the reduced sample mass.

Phospholipid Profiling by NMR

Pot-Grown Plants

For the initial experiment, dried non-polar samples were dissolved in 700 µL of the NMR solvent deuterated chloroform (chloroform-d) and transferred to a standard 5-mm diameter NMR tube. A Bruker DRX 500 500MHz spectrometer was used, operating at 202.45 MHz for ^{31}P nuclei. The following parameters were used: 30° excitation pulse, width 12.5 µs; spectral/sweep width 122.2645 ppm (25 KHz); acquisition time 0.5 s; relaxation delay 4.5 s; repetition rate (acquisition time + relaxation delay) 5 s. The number of scans ranged from approximately 1100 to almost 5000 depending on both time available and the signal-to-noise ratio (SNR) as judged by Dr. Bob Berno, manager of the NMR facility and spectrometer operator.

Table 3. Summary of tissue samples prepared for analysis by ^{31}P-NMR. An asterisk indicates that this tissue extract was spiked with an internal standard that obscured a region of interest and, as such, was unsuitable for data analysis and hence not considered a replicate of Trial 3.

Trial	Replicate	Date Extracted	Date of NMR Analysis
1	1	November, 2009	November 30, 2009
1	2	December 3, 2009	December 8-11, 2009
3	N/A*	March 16, 2010	March 26, 2010
2	1	April 22, 2010 #2	May 5, 2010
3	1	April 22, 2010 #3	April 27, 2010
1	3	May 28, 2010	June 1, 2010
4	1	June 16, 2010	June 22, 2010
4	2	July 16, 2010	July 20, 2010
3	2	July 29, 2010	August 5, 2010

To potentially narrow resonances and increase the SNR in subsequent experiments, samples dried by N_2 evaporation were dissolved in a 5% (w/v) sodium cholate solution with D_2O as the solvent (London & Feigenson, 1979). Samples also contained 10 mM EDTA disodium salt to chelate multivalent cations such as Ca^{2+} and further narrow resonances. Scanning was carried out using the same hardware and parameters as previously described.

Desiccated extracts prepared from *Arabidopsis* tissue was dissolved in the presence of sodium cholate and Na_2EDTA and scanned using the same parameters as described above for *Thellungiella* tissue. However, because of the reduced concentration of lipids relative to solvent, 12000 scans were acquired to ensure that SNR of the spectra would be comparable to that of *Thellungiella* scans.

Once spectra were obtained, deconvolution was performed. Deconvolution is a data processing technique that eliminates spurious noise peaks by fitting a Lorentzian distribution to automatically and/or manually picked peaks. The areas under the resultant Lorentzian curves were used to determine the contribution of the area of each peak to the total area of all peaks detected in a given sample.

Quantification of lipid species is achieved via **E**lectronic **Re**ference **T**o Access **I**n Vivo **C**oncentrations (ERETIC). Briefly, an external standard is scanned separately to generate a reference peak. This synthesized peak is then inserted into the spectrum acquired from the sample of interest (Akoka et al., 1999). A variety of potential ERETIC external standards were created, including dimethyl PE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N,N-dimethyl). Dimethyl PE was not soluble in D_2O and therefore

could not be used. A new standard solution was made by dissolving 0.054 g of phosphocholine chloride calcium salt tetrahydrate (Sigma-Aldrich) in 700 µL of D_2O. This standard solution therefore contained 163 µmol of phosphocholine and provided a strong, clean signal (see Results and Discussion for further detail). The standard was scanned with the same parameters described for the samples and the resultant phosphocholine peak was subjected to a Gaussian line fit (i.e. deconvoluted). The area of the fitted peak was so great that it rendered the rest of the spectrum invisible so the area corresponding to phosphocholine was scaled down on the instrument by a factor of 300 (from 163 µmol to 0.543 µmol) and the peak associated with the lower content was inserted into the spectra. Only singlet peaks were present in previous *Thellungiella* spectra, indicating that only one phosphorus atom is present in each molecule of an unknown, so this provided a way to convert peak areas to Pi contents. Content was calculated using the following equation:

$$Sample\ Peak\ Content = \left(\frac{[(peak\ area\ of\ sample)(Standard\ content)]}{(Peak\ area\ of\ Standard)} \div mg\ DW\right) \times 1000$$

Dry weight was considered to be ca 8% of fresh weight (Meyerowitz & Somerville, 1994; Somerville & Browse, 1991)

Internal Phosphate Determination Assay

The phosphate content of leaf samples was assayed in accordance with the colourimetric method of Martin and Tolbert (1983). Briefly, a set of phosphate standards were created to span 0 to 25 nmol of phosphate/reaction tube to which 500 µL of

ammonium molybdate solution (0.5% $(NH_4)_6Mo_4O_{24}$, 1.2 N H_2SO_4 and 2% ascorbic acid) was added. These tubes were incubated for 90 min at 37°C to produce a colourimetric reaction (Martin & Tolbert, 1983).

Polar phase fractions that had been stored desiccated were dissolved with 1 mL H_2O and diluted a further 100-fold with H_2O. To a 50 µL volume of the 100-fold diluted fraction, 450µL of H_2O was added and 500 µL of ammonium molybdate solution. These tubes were incubated along with the tubes containing the series of phosphate standards (Martin & Tolbert, 1983). Following the incubation, the absorbance of each tube was read using a spectrophotometer set at A_{820}. The phosphate content of polar phase extracts was quantified by comparison to the standard curve generated by the standards and the content of phosphate in $\mu mol \cdot g^{-1}$ FW was determined.

Statistical Analyses

Statistical analyses were performed using multiple statistical packages. A linear regression on daily root measurements was performed using GraphPad Prism 4 (www.graphpad.com). The slopes of the regression lines were tested for equality using ANCOVA, where significance was assessed at $\alpha = 0.05$. Multiple comparisons were accounted for by using a Bonferroni correction. An allometric plot of natural log transformed root and shoot biomass was made using GraphPad Prism 4. An ANCOVA was performed on these transformed root and shoot data using Type III Sum of Squares in PASW Statistics 18 (formerly SPSS; www.spss.com) to determine if the slopes of the regression lines were significantly different at $\alpha = 0.05$. The significance for

measurements of internal Pi content was assessed using a one-way ANOVA performed in PASW Statistics 18 (Type III Sum of Squares) and $\alpha = 0.05$. A post hoc Scheffé test was used to determine the source of the significant difference, since the Scheffé test is a conservative test that allows for unequal sample sizes (Ruxton & Beauchamp, 2008). A comparison was considered significant if $p < 0.05$. All Student's unpaired, two-tailed t-tests were performed in GraphPad Prism 4, with $\alpha = 0.05$.

Mulivariate analysis of variance (MANOVA) on peak area as an estimate of component abundance was performed in PASW Statistics 18. A Type III Sum of Squares design with $\alpha = 0.05$ was used. The Wilks' Lamba test statistic was used to determine significant differences in mean values. A significant multivariate result was followed up by examination of the univariate tests for each dependent variable (peak). A univariate result was considered significant if $p < 0.05$. Full univariate results are shown in Appendix A.

RESULTS

Plant Growth in Response to Nutrient Treatments

Seedlings Grown on Defined Nutrient Plates

Thellungiella salsuginea (Yukon genotype) plants were grown under four defined nutrient conditions that only differed with respect to phosphate concentrations: 0.5mM, 0.2mM, 0.1mM and 0.05mM (see Table 1, Materials and Methods section for media formulations). Testing the different phosphate contents was carried out to characterize the response of the plants to these concentrations. Ideally, the outcome of this comparison would identify whether the same or different responses are shown by the seedlings, whether the responses between replicates were reproducible and whether the number of treatments under evaluation could be reduced to only two concentrations of phosphate.

Seedling root growth was monitored with measurements carried out until the roots reached the lower grid line positions of the plates (ca 5 cm). The length of time for roots to reach this length varied (Figure 2A; 2BI) from approximately 7 d on 0.5 mM phosphate to 10 d on 0.05 mM phosphate. The only statistically significant difference between treatment responses was for seedlings on 0.5 mM phosphate whose roots and shoots consistently grew faster than seedlings plated on 0.05, 0.1 or 0.2 mM phosphate. Figure 2(Panels II, III) also show an interesting aspect of the response of *Thellungiella* to phosphate in that a dilution of 0.5 mM phosphate to 0.2 mM reduces growth significantly

but any further dilutions of the same magnitude (two successive 2-fold dilutions) elicit no further changes with respect to shoot biomass or rates of root elongation.

Given the consistent difference seen between the highest concentration of phosphate tested and the other levels and the lack of significant different in responses among the lower three concentrations, we selected the high phosphate ($HP_{0.5}$; 0.5mM) and low phosphate ($LP_{0.05}$; 0.05mM) treatments for the remainder of the work reported in this thesis. Figure 3 shows representative *Thellungiella* seedlings at 4 and 7 d of growth on their respective $HP_{0.5}$ and $LP_{0.05}$ treatment plates. Seedlings grown for the same length of time on $LP_{0.05}$ plates had shorter roots than those on $HP_{0.5}$ media (Fig. 2BIII), but the difference in final length was only significant at 7 d ($P = 0.0265$). Moreover, no discernible differences were seen in shoot coloration (Figure 3) of seedlings on $LP_{0.05}$ or $HP_{0.5}$ media. Figure 3 together with the data shown in Figure 2, shows seedling development to be delayed on $LP_{0.05}$ relative to $HP_{1.0}$ medium. Specifically, using daily primary root measurements the rate of root elongation for $LP_{0.05}$ and $HP_{0.5}$ plants are 0.58 $\pm$ 0.03 $cm \cdot d^{-1}$ and 0.84 $\pm$ 0.03 $cm \cdot d^{-1}$, respectively (Figure 4). These rates are highly significantly different ($P < 0.0001$) and indicate the growth rate of $LP_{0.05}$ plants was reduced by about 30% when compared to $HP_{0.5}$ plants.

Shoot and root fresh weights were taken immediately upon harvest. Dry weights are not available as the tissue was flash-frozen for future metabolite and lipid extraction. Shoot and root fresh weights for $LP_{0.05}$ plants are significantly greater than those plants grown under $HP_{0.5}$ conditions ($P = 0.0009$ and $P = 0.0023$, respectively; Figure 5).

Figure 2. Growth responses of *Thellungiella* seedlings grown on defined nutrient media with varying concentrations of phosphate. Panel A shows the mean time to harvest, primary root elongation rate and shoot fresh weight of seedlings. Panel B is the graphical representation of (I) mean days to harvest, (II) shoot fresh weight and (III) primary root elongation rate.

Data represent the mean ± standard error (SE) of a minimum of 40 seedlings representing four independent experimental trials (biological replicates). Where ANOVA indicated that treatment had a significant effect on the measurements a Scheffé post hoc comparison was applied to determine significance among the means. Treatments followed by the same bolded letters are not significantly different.

A

Phosphate Content of Growth Media *mM*	Time to Harvest *d*	Primary Root Elongation Rate $cm \cdot d^{-1}$	Shoot Fresh Weight *g*
0.05	10.33 ± 1.15 **a**	0.58 ± 0.03 **a**	0.012 ± 0.0015 **a**
0.1	9.75 ± 0.5 **a**	0.57 ± 0.01 **a**	0.012 ± 0.0006 **a**
0.2	9.5 ± 0.58 **a**	0.61 ± 0.02 **a**	0.012 ± 0.0005 **a**
0.5	7.25 ± 0.5 **b**	0.84 ± 0.03 **b**	0.007 ± 0.0005 **b**

B

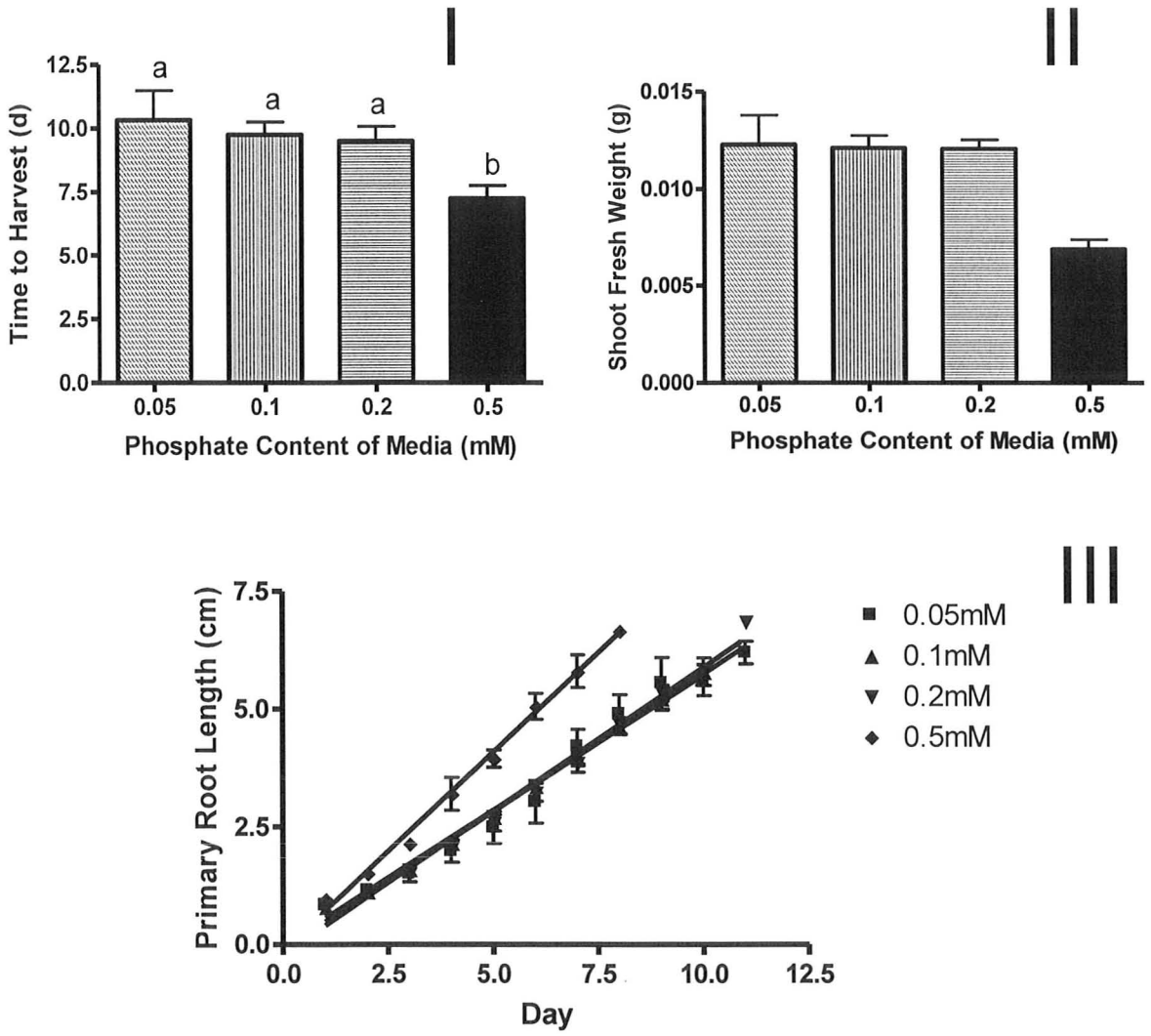


Figure 3. Representative *Thellungiella* (Yukon genotype) seedlings after growing 4 d (A) and 7 d (B) on $LP_{0.05}$ (0.05mM Pi) and $HP_{0.5}$ (0.5mM) defined nutrient media plates.

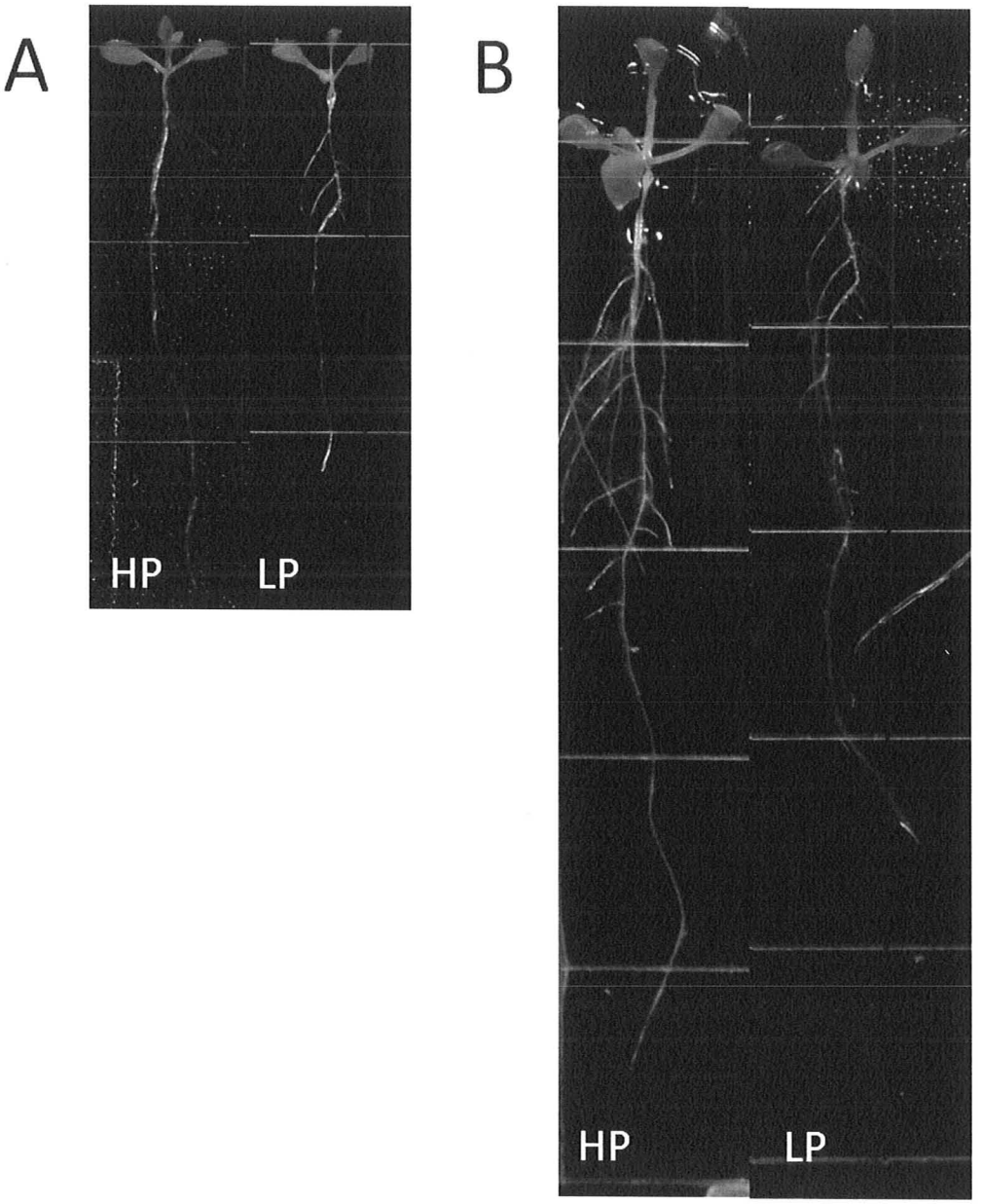
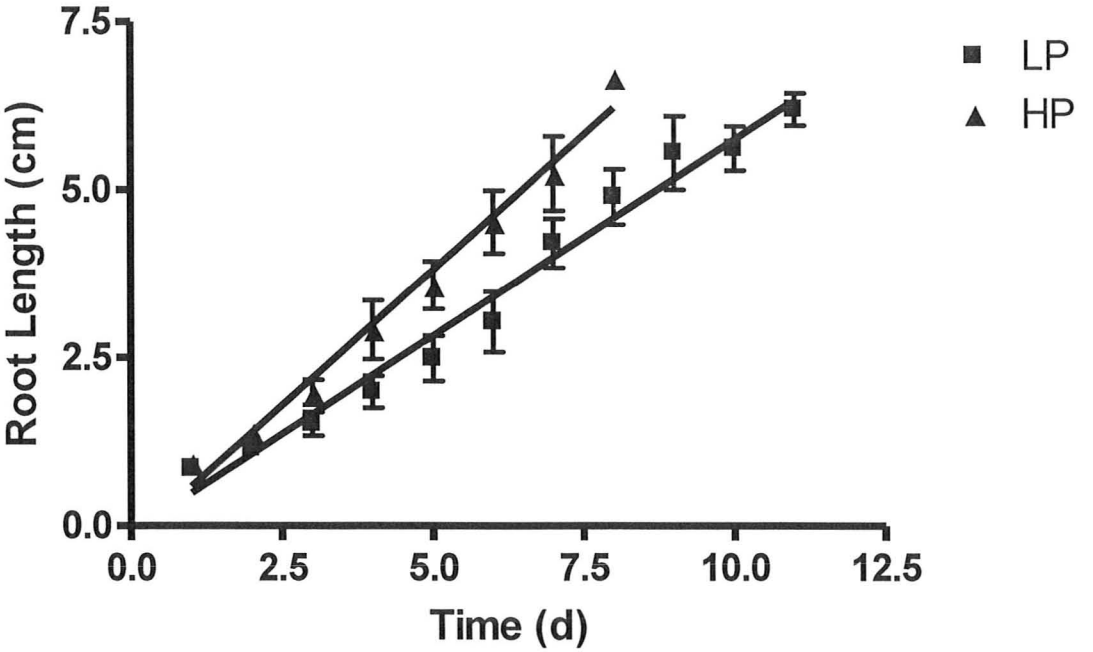

A
HP
LP
B
HP
LP

Figure 4. Primary root elongation of *Thellungiella* seedlings growing on $LP_{0.05}$ (0.05 mM) and $HP_{0.5}$ (0.5 mM) defined nutrient media plates. A linear regression performed on daily primary root length measurements for plants grown on $HP_{0.5}$ ($r^2 = 0.98$) or $LP_{0.5}$ ($r^2 = 0.98$) media shows a highly significant difference ($P < 0.00079$) in primary root growth rate (0.84 $cm \cdot d^{-1}$ and 0.58 $cm \cdot d^{-1}$ for $HP_{0.5}$ and $LP_{0.05}$ seedlings, respectively).

Data represent the mean ± standard error (SE) of a minimum of 40 seedlings across four independent experimental trials (biological replicates).

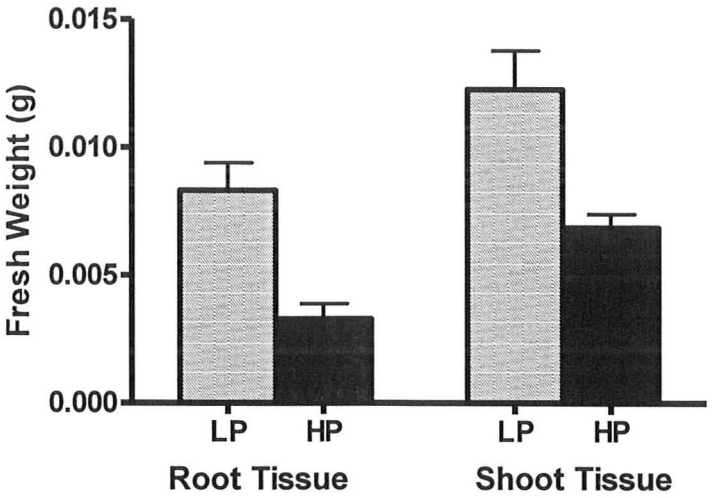

7.5
5.0
2.5
0.0
Root Length (cm)
0.0
2.5
5.0
7.5
10.0
12.5
Time (d)
LP
HP

To provide sufficient material for extraction the $LP_{0.05}$ plants remained on plates for three additional days until the length of the seedling roots approximated the length of the $HP_{0.5}$ seedling roots at harvest (ca 5 cm). This longer time probably accounts for the increased shoot biomass. Indeed, when plants are visually compared on the same day (Figure 3) the shoots are virtually indistinguishable from one another, despite obvious differences in root length. However, the mean specific root length (the ratio of root length to root mass) of harvest seedlings across the four trials is significantly higher for $HP_{0.5}$ plants (3529 $cm \cdot g^{-1}$) than for $LP_{0.05}$ plants (836.4 $cm \cdot g^{-1}$) ($P = 0.0383$; Appendix B) and this could be a consequence of differences in root growth pattern.

An allometric plot of natural log transformed shoot versus root fresh weight can indicate differences in biomass allocation. Allometry refers to the changes in proportions of traits or characteristics in relation to changes in the overall size of the organism (Gedroc et al., 1996). Plants grown in different environments can grow at different rates and the developmental age of the plant will not be consistent with the chronological age (Gedroc et al., 1996). Therefore, a simple, static root-to-shoot ratio may represent differences in allocation which are actually the result of normal developmental (ontogenetic) changes (McConnaughay and Coleman, 1999). An allometric plot accounts for time indirectly by directly comparing root and shoot fresh weight. In this way, progression along the curve, to higher values of each trait, is the developmental trajectory (Klingenberg, 1998). Figure 6 shows data for plants grown for seven days on media with different phosphate concentrations (same experiment and data set shown in Fig. 2B).

Figure 5. Root and shoot fresh weights of *Thellungiella* seedlings at harvest after growing on $LP_{0.05}$ and $HP_{0.5}$ defined nutrient media.

To reach the same approximate root length at harvest, $LP_{0.05}$ seedlings remained on media for an additional three days, on average, and to a maximum of four additional days. Despite the shorter roots, the $LP_{0.05}$-grown seedlings have greater shoot and root biomass at harvest ($P = 0.0009$ and $P = 0.0023$, respectively). Data represent the mean ± standard error (SE) of a minimum of 40 seedlings across four independent experimental trials (biological replicates).


0.015
0.010
0.005
0.000
Fresh Weight (g)
LP
HP
LP
HP
Root Tissue
Shoot Tissue

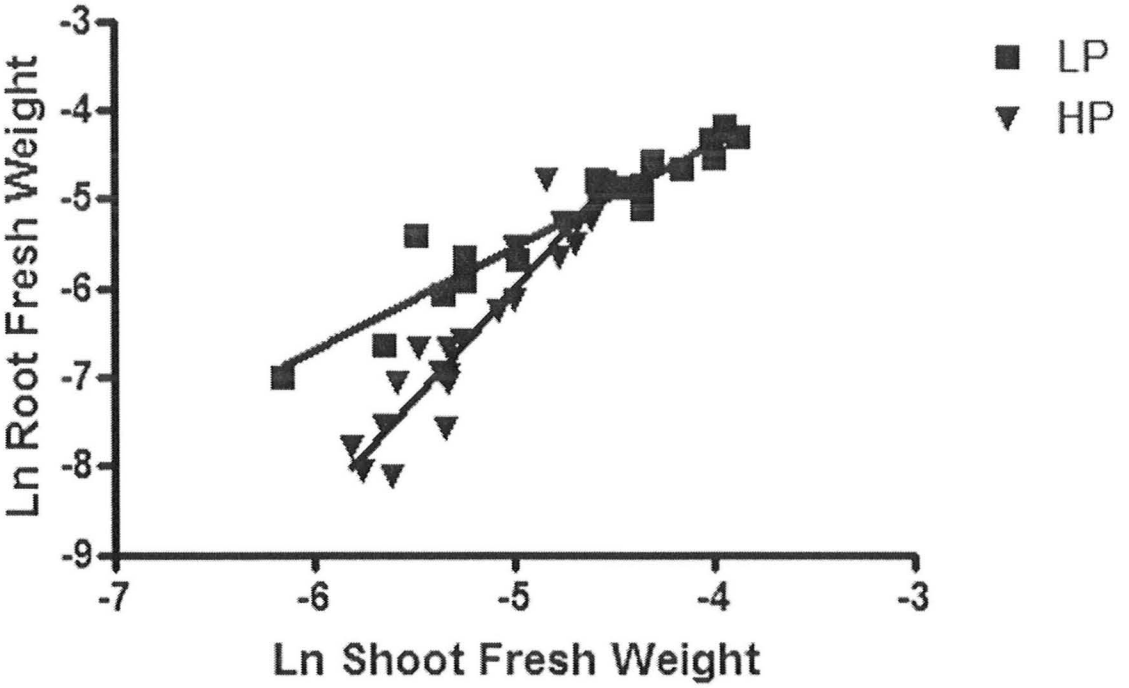
The plot of this data produces different slopes, an outcome consistent with the plants showing differences in biomass allocation in response to the $LP_{0.05}$ and $HP_{0.5}$ treatments. An analysis of covariance confirmed that treatment does have a significant effect on the relationship between root and shoot fresh weights ($P = 0.0039$). At an earlier developmental stage (i.e., smaller seedlings) at equivalent shoot fresh weights, $LP_{0.05}$ seedlings allocate more towards root biomass than $HP_{0.5}$ seedlings. The different slopes and intersecting regression lines would suggest that allocation between roots and shoots as a function of available P may change as the plants age.

Soil-Grown Plants

Thellungiella plants grown on PromixBX were harvested at 5 wk post germination (one wk on MS plates and four wk on PromixBX). Watering was done once-weekly with a modified Hoagland's solution formulated with 0.05 mM or 1.0 mM phosphate to maintain consistency with the treatments reported in the *Arabidopsis* literature (Hartel et al., 2000; Muller et al., 2007) (see Materials and Methods) and plants from both treatments were harvested at the same time. During the entire treatment period no obvious treatment-associated differences in the appearance of the plants were observed. Figure 7 shows representative 5-week old plants from each treatment at the time of harvest. Total above-ground biomass was harvested and weighed but it was not possible to separate root tissue from the potting medium without significantly damaging the root system. However, Figure 8 shows that there was no significant, treatment-related

Figure 6. An allometric plot of shoot and root fresh weight measurements shows evidence of differences in biomass allocation for *Thellungiella* seedlings grown on $LP_{0.05}$ and $HP_{0.5}$ defined nutrient media. Each data point represents pooled shoot and root tissue from one treatment plate in one trial representing a maximum of five individuals. Four independent experimental trials (biological replicates) were included in this analysis. Measurements were taken at the day of harvest and age ranges from 9 to 11 d for $LP_{0.05}$ (■) seedlings or 7 to 8 d for $HP_{0.5}$ (▼) seedlings.

The linear regressions for data of $LP_{0.05}$ and $HP_{0.5}$-grown seedlings are $r^2 = 0.92$; $n = 19$ and $r^2 = 0.8982$; $n = 23$, respectively. The slopes for $LP_{0.05}$ and $HP_{0.5}$ measurements are 1.15 ± 0.08 and 2.50 ± 0.18, respectively. The two slopes are significantly different (ANCOVA, $P = 0.004$).


Ln Root Fresh Weight
-3
-4
-5
-6
-7
-8
-9
-7
-6
-5
-4
-3
Ln Shoot Fresh Weight
LP
HP

difference in shoot fresh weight measurements between $LP_{0.05}$ and $HP_{1.0}$ plants at the date of harvest.. However, Figure 8 shows that there was no significant, treatment-related difference in shoot fresh weight measurements between $LP_{0.05}$ and $HP_{1.0}$ plants at the date of harvest.

Phosphate Content in Polar Extracts of Leaves

The free Pi content of polar phase fractions prepared from leaves of plant watered with modified Hoagland's nutrient solution is shown in Figure 9 and results of specific trials is given in Table 4. Figure 9 shows that leaf tissue from plants grown under $HP_{1.0}$ conditions have a significantly greater internal concentration of free Pi than that of plants grown under $LP_{0.05}$ conditions ($P = 0.05$ by Student's t-test). The absolute Pi content varied between trials but in each case the average content of Pi was higher in leaves of plants grown under $HP_{1.0}$ as opposed to $LP_{0.05}$ conditions (Table 4). In general, greater variability in Pi content was found for determinations made using leaves of $LP_{0.05}$ grown plants irrespective of the trial.

Lipid Profiling of *Thellungiella* Shoot Biomass

The lipid phase from the previously extracted 500mg of plate-grown tissue was evaporated to dryness then re-dissolved in a volume of chloroform-d that represented the minimal amount necessary for analysis by the instrument. No signal above the noise

Figure 7. Representative five-week old *Thellungiella* plants fertilized once weekly with a modified Hoagland's solution containing a 0.05 mM (A) or 1.0 mM (B) phosphate concentrations.


A
B
1 cm

Figure 8: Mean shoot fresh weights for five-week old *Thellungiella* plants at harvest following once-weekly fertilizer treatment with a modified Hoagland's solution containing 0.05 mM ($LP_{0.05}$) or 1.0 mM ($HP_{1.0}$) phosphate. Mean shoot weights were 1.27 ± 0.09 g with n = 26 and 1.17 ± 0.08 g with n = 27 for $LP_{0.05}$ and $HP_{1.0}$ grown plants, respectively. Four independent experimental trials (biological replicates) were included in the analysis. No significance difference related to treatment was found by Student's t-test (P = 0.40).

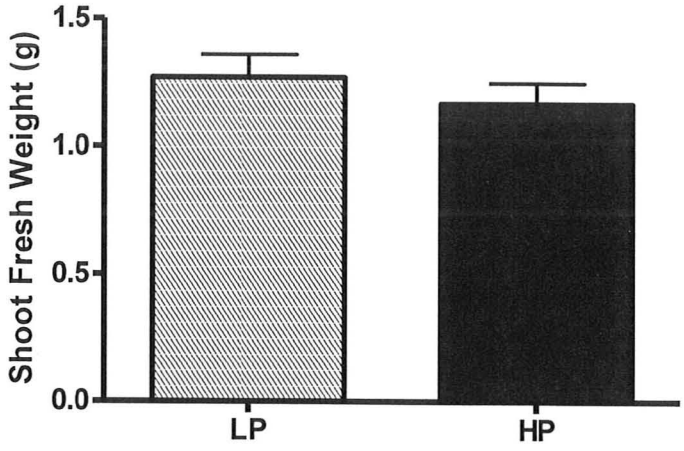

Shoot Fresh Weight (g)
1.5
1.0
0.5
0.0
LP
HP

Figure 9: Tissue Pi content of five-week old Thellungiella plants irrigated once weekly with a modified Hoagland's solution containing 0.05 mM ($LP_{0.05}$) or 1.0 mM ($HP_{1.0}$) phosphate. Mean +/- SE for n = 7 representing four independent experimental trials (biological replicates). The data used in this analysis is a summary of the trial data reported in Table 4. The difference between the means is statistically significant at P = 0.05 as shown by a Student's unpaired t-test.

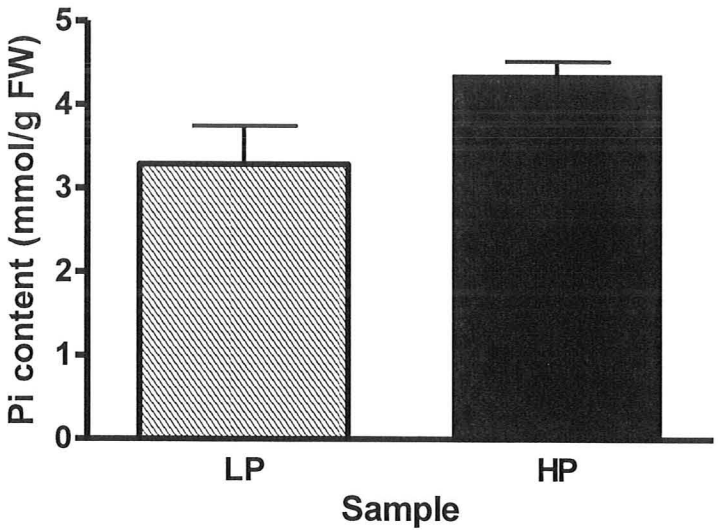

Pi content (mmol/g FW)
5
4
3
2
1
0
LP
HP
Sample

Table 4. Internal Pi content of *Thellungiella* leaf tissue reported for individual experimental trials.

Date of extraction and date of ^{31}P-NMR analysis are also listed to relate internal Pi content to specific lipid profiles. All values reported are mean ± standard error of duplicate assays.

Experimental Trial #	**Date of NMR Analysis**	$HP_{1.0}$	$LP_{0.05}$
		Internal Pi (µmol/g FW)	
1	Nov 30, 2009	3.89 ± 0.00	1.82 ± 0.01
	Dec 8-11, 2009	4.27 ± 0.02	3.05 ± 0.01
	June 1, 2010	4.00 ± 0.01	1.70 ± 0.01
2	May 5, 2010	4.74 ± 0.02	4.33 ± 0.01
3	Apr 27, 2010	5.04 ± 0.02	4.93 ± 0.00
	Mar 26, 2010	4.55 ± 0.04	3.41 ± 0.01
4	June 22, 2010	4.05 ± 0.01	3.79 ± 0.00
Overall Mean		4.36 ± 0.16	3.29 ± 0.46

threshold was detected (data not shown). This outcome indicates that there was not enough lipid material present in the sample for a successful analysis by NMR.

The sample mass used for non-polar phase preparation was increased 10-fold. This volume was not easily accommodated by small seedlings grown on defined culture medium so mature plants were produced in a soil-less medium to maximize biomass available for lipid profiling. Research done by Andersson et al. (2006) in *Avena sativa* demonstrate lipid composition changes after 2 wk on low phosphate, with the most extreme changes occurring after 4 wk of growth. Longer term exposure to 0.05 or 1.0 mM phosphate was therefore expected to produce a lipid profile that more faithfully represented a steady-state response to the treatment conditions. Desiccated lipid fractions of $LP_{0.05}$ and $HP_{1.0}$ samples were re-dissolved in chloroform-d and analyzed via ^{31}P-NMR. Representative scans are shown in Figure 10. These initial scans were disappointing in that the SNR is quite low, as evidenced by the broad peaks and pervasive background, and individual peaks are not clearly resolved.

The addition of bile salts (detergents) such as cholate act has been reported to improve the solubilisation of lipid membranes in analytical studies (London & Feigenson, 1979). Non-polar phases of extracts from leaves of *Thellungiella* plants fertilized with a high phosphate treatment were divided into two identical aliquots with one aliquot dissolved with chloroform-d and the second dissolved in an aqueous 5% (w/v) solution of sodium cholate. The addition of a detergent led to narrowed line widths and clearly resolved, sharp, distinct peaks relative to the profile produced by the same sample without sodium cholate (Fig. 11).

Figure 10. Representative spectra from a ^{31}P-NMR analysis of non-polar fractions prepared from leaf tissue from five-week old *Thellungiella* plants watered once weekly with modified Hoagland's containing 0.05 mM (A) or 1.0 mM (B) phosphate. Sample tissue biomass used for extraction was 5 g and the dried non-polar phase residue was dissolved in chloroform-d for analysis. The profile shows a poor signal-to-noise ratio and broad, poorly resolved peaks.

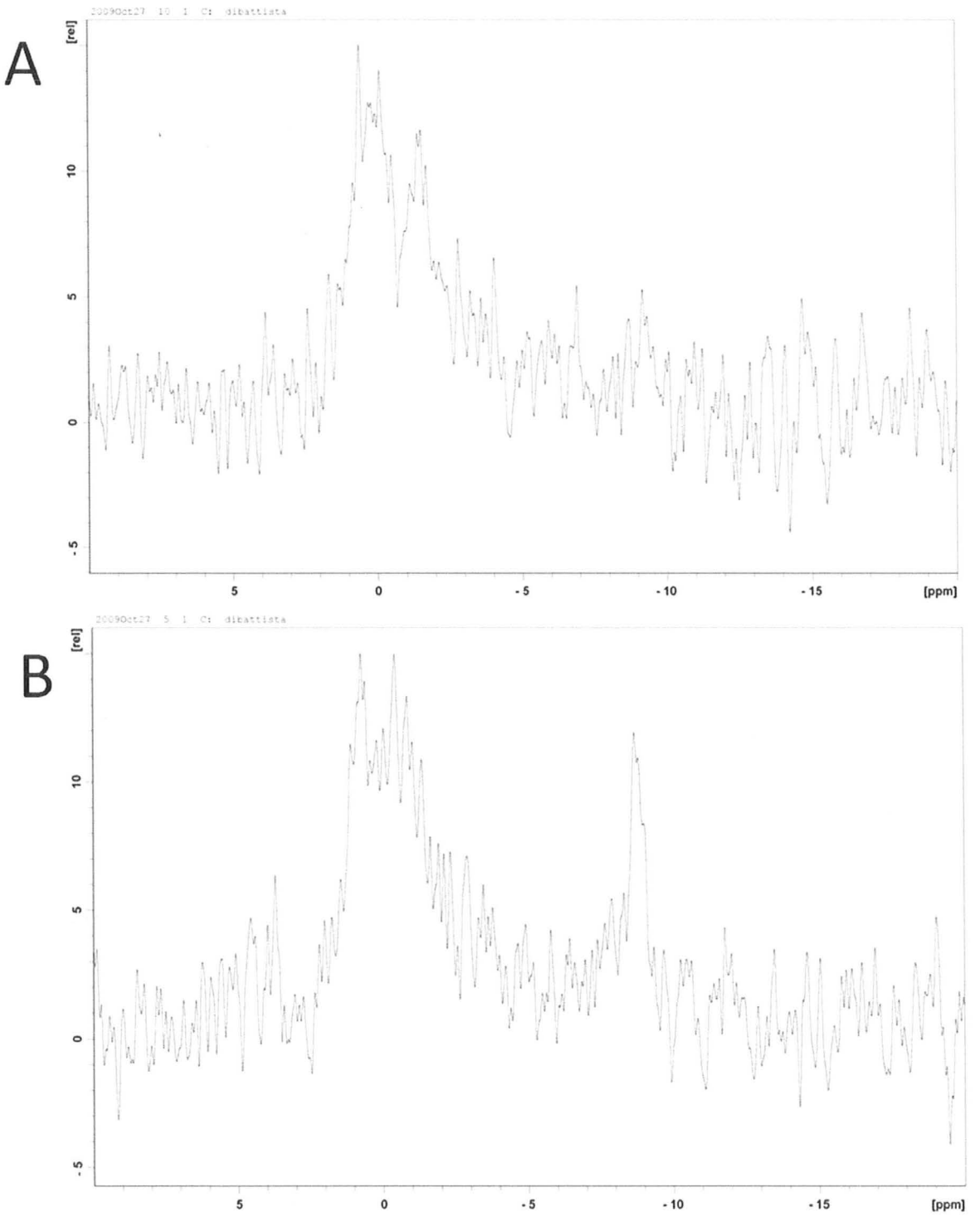
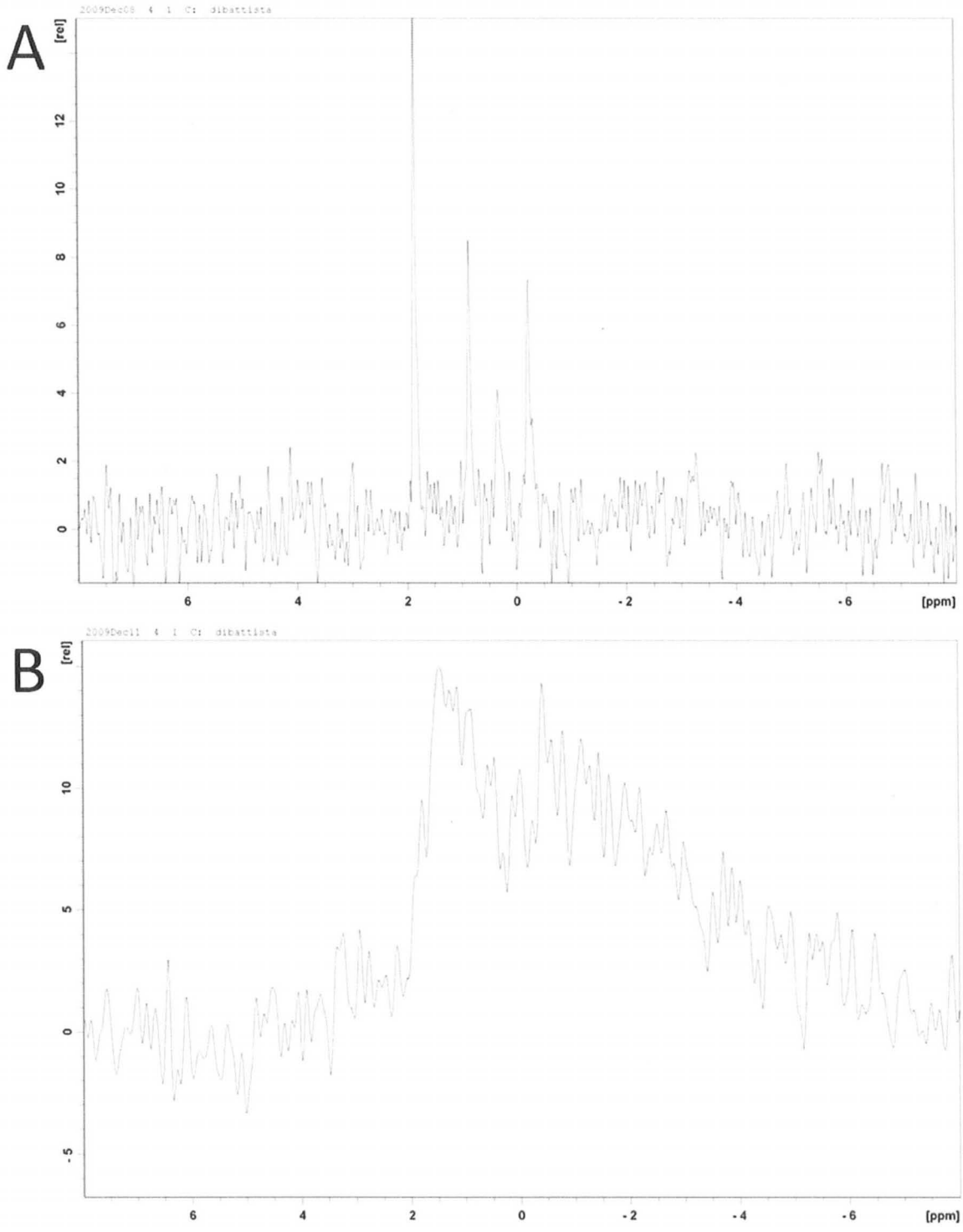

A
2009Oct27 10 1 C: dibattista
[rel]
10
5
0
-5
5
0
-5
-10
-15
[ppm]
B
2009Oct27 5 1 C: dibattista
[rel]
10
5
0
-5
5
0
-5
-10
-15
[ppm]

Figure 11. ^{31}P-NMR spectra of a non-polar phase extracted from leaves of 5-week old *Thellungiella* fertilized once weekly with modified Hoagland's solution containing 1.0 mM phosphate (HP). Ten g leaf tissue was used, the extract was divided, dried and each half (equivalent to 5g fresh weight) was dissolved in aqueous sodium cholate (A) or chloroform-d (B) to determine the effect of these solvents on spectral resolution.


A
2009Dec08 4 1 C: dibattista
[rel]
12
10
8
6
4
2
0
6
4
2
0
-2
-4
-6
[ppm]
B
2009Dec11 4 1 C: dibattista
[rel]
10
5
0
-5
6
4
2
0
-2
-4
-6
[ppm]

Profiling of Non-Polar Fractions Using ^{31}P-NMR

Total leaf tissue was harvested from five-week-old plants grown on ProMix B media and fertilized once weekly with a modified Hoagland's solution containing either 0.05 mM ($LP_{0.05}$) or 1.0 mM ($HP_{1.0}$). The tissue (5 g) was solvent-extracted and the non-polar phases were dried and dissolved in 5% (w/v) aqueous sodium cholate for ^{31}P-NMR analysis. Representative NMR spectra before and following deconvolution are shown in Figure 12. These spectra each show five well-defined peaks visible above background noise. However, the relatively high baseline indicates the SNR is not as high as can be achieved by ^{1}H-NMR (Dr. Bob Berno, personal communication).

A total of nine ^{31}P-NMR experiments comprised of samples from four independent trials were performed. Figure 12, panels A and C are the respective deconvoluted versions of actual spectra shown in panels B and D. The process of deconvolution fits a Lorentzian line to the peaks thereby reducing noise. Samples of deconvoluted and actual spectra from non-polar extracts prepared from leaves of $LP_{0.05}$ (Figure 12A, B) and $HP_{1.0}$ (Figure 12C, D) treated plants show five peaks at approximately the same chemical shift positions. However, the intensity (peak area) associated with these peaks is different and this difference can be related to the variation in the abundance of specific chemical components in the sample that contribute towards specific peaks. For ease of discussion, the peaks occupying similar positions in different spectra are designated by numbers 1 through 8. The peaks may or may not represent the same chemical component in different samples.

Figure 12. Representative ^{31}P-NMR spectra of non-polar fractions prepared from leaves of five-week old *Thellungiella* plants fertilized once weekly with a modified Hoagland's solution containing either 0.05 mM ($LP_{0.05}$; panels A, B) or $1.0_{1.0}$ mM ($HP_{1.0}$; panels C, D) phosphate. Deconvolutions are shown in panels A ($LP_{0.05}$) and C ($HP_{1.0}$) and actual spectra are shown in panels B ($LP_{0.05}$) and D ($HP_{1.0}$). Deconvoluted spectra show five peaks at approximately the same chemical shift positions (numbered 1 through 5).

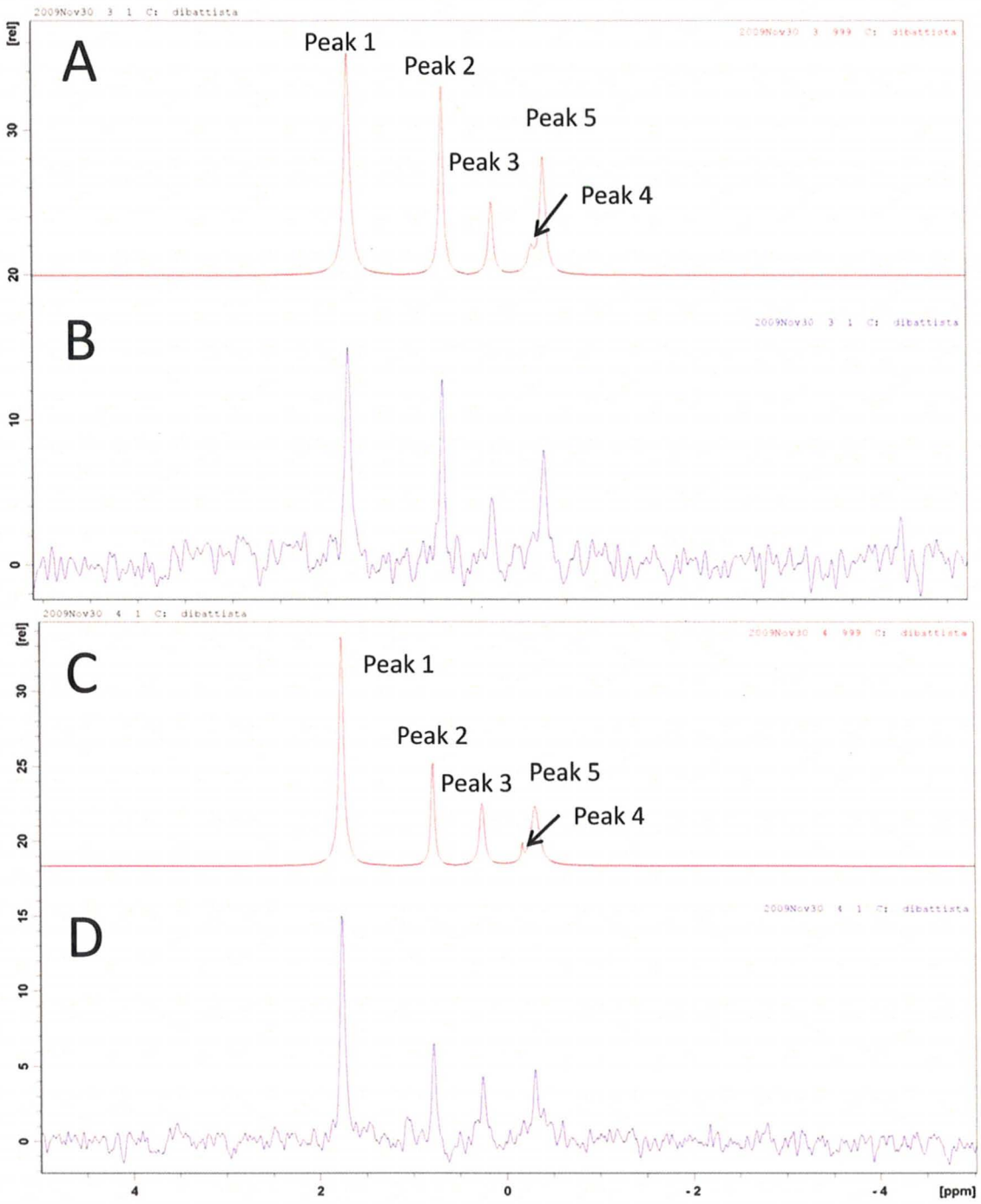

A
Peak 1
Peak 2
Peak 3
Peak 5
Peak 4
B
C
Peak 1
Peak 2
Peak 3
Peak 5
Peak 4
D
[rel]
[ppm]

Table 5 summarizes the chemical shift range for specific peaks detected in spectra and gives the percent area of individual peaks relative to the total peak area for all peaks of a given spectrum. As this table shows, while 15 of 18 spectra contain the same five peaks, there is variation with respect to the number of peaks detected in various samples. For example, in spectra for replicates in Trials 2 and 3 for $LP_{0.05}$ samples a unique sixth peak was observed albeit not at the same ppm position. Also, variation in the percent contribution of a specific peak to the total peak area for a spectrum was found for comparably positioned peaks across replicates and trials, and within and across treatments. However, several qualitative generalizations can be made based upon the data reported in Table 5. Firstly, Peak 1 occupies the largest proportion of the total area across all spectra for both phosphate fertilizer treatments. Secondly, the area occupied by Peak 2 is the next highest in $LP_{0.05}$ plants, followed by the area occupied by Peak 5. In spectra obtained from $HP_{1.0}$ plants this trend in relative abundance is reversed with Peak 5 occupying a greater area than Peak 2. Peak 3 is, on average, the fourth-largest contributor to spectral area in both $LP_{0.05}$ and $HP_{1.0}$ plants. Finally, the areas associated with peak 4 suggest that this component is least abundant in extracts of $HP_{1.0}$ plants as opposed to $LP_{0.05}$ plants. This latter peak shows variability in intensity in that spectra from $LP_{0.05}$ and $HP_{1.0}$ plant samples show an almost 5 to 6-fold range in area and it was even below the detection limits in two $HP_{1.0}$ spectra. Statistical analyses will increase the rigor of the qualitative observations.

Table 5. Summary of chemical shift ranges for peak identification and the relative area of peaks as estimates of abundance of non-polar P-metabolites detected in extracts of leaves of five-week old *Thellungiella* plants and analyzed by ^{31}P-NMR. Peak Area of a given peak is expressed as a percentage of total area of all peaks in the spectrum. Blank cells indicate that a given peak was not detected in the spectrum. Mean +/- SE where n is a minimum of 2 peak area measurements.

0.05 mM phosphate fertilizer

		Peak ID No.						
		1	2	3	4	5	6	7
Trial	Replicate	Peak Area (%)						
1	1	39.6	24.7	10.5	4.5	20.6		
	2	43.9	22.4	8.7	12.2	12.8		
	3	61.9	14.1	6.6	15.7	1.6		
Mean ± SE		48.5 ± 6.9	20.4 ± 3.2	8.6 ± 1.1	10.8 ± 3.3	11.7 ± 5.5		
2	1	55.6	15.8	9.5	6.2	11.8	1.0	
	2	49.7	17.8	11.3	4.7	16.4		
Mean ± SE		52.7 ± 2.9	16.8 ± 1.0	10.4 ± 0.9	5.5 ± 0.8	14.1 ± 2.3		
3	1	56.0	19.1	10.6	6.1	8.2		
	2	49.4	17.4	7.5	4.1	20.1		1.6
Mean ± SE		52.7 ± 1.7	18.2 ± 0.4	9.1 ± 0.8	5.1 ± 0.5	14.1 ± 3.0		
4	1	53.9	15.6	7.4	3.5	19.6		
	2	50.4	17.4	7.7	4.5	20.0		
Mean ± SE		52.2 ± 1.7	16.5 ± 0.9	7.5 ± 0.2	4.0 ± 0.5	19.8 ± 0.2		

1.0 mM phosphate fertilizer

		Peak ID No.							
		1	2	3	4	5	6	7	8
Trial	Replicate	Peak Area (%)							
1	1	46.8	15.09	13.6	1.3	22.5			
	2	35.0	21.8	18.5		24.8			
	3	64.2	13.7	5.8	1.6	14.7			
Mean ± SE		48.7 ± 8.5	17.1 ± 2.4	12.6 ± 3.7	1.4 ± 0.1	20.7 ± 3.1			
2	1	43.5	20.8	12.6	3.3	19.9			
	2	36.0	21.3	11.2		31.6			
Mean ± SE		39.74 ± 3.7	21.0 ± 0.2	11.9 ± 0.7	3.3	25.7 ± 5.8			
3	1	57.1	13.5	6.9	8.1	12.2			2.2
	2	56.9	15.4	7.9	5.4	14.5			
Mean ± SE		57.0 ± 0.1	14.5 ± 1.0	7.4 ± 0.5	6.7 ± 1.4	13.4 ± 1.2			
4	1	59.5	15.3	9.1	1.8	14.2			
	2	41.8	17.1	11.7		29.4			
Mean ± SE		50.7 ± 8.8	16.2 ± 0.9	10.4 ± 1.3	1.8	21.8 ± 7.6			

Prior to the statistical analysis of the NMR data, the validity of the peaks must be established. As mentioned above, there are three peaks that are unique to the spectra in which they appear. These peaks are present at 0.431 ppm, 0.214 ppm and 1.052 ppm found in samples of $LP_{0.05}$ (Trial 2, replicate 1), $LP_{0.05}$ (Trial 3, replicate 2) and $HP_{1.0}$ (Trial 3, replicate 1) spectra and are labelled peaks 6 to 8, respectively. The area contribution of these peaks relative to the total spectral area ranges from negligible (0.982% of total) as in the case of the peak at 0.431 ppm to substantial as in the case of the peak at 1.052 ppm (compare to peak 4 percent contribution). However, because of their appearance in only one spectrum (i.e. not in replicate scans) these peaks were considered spurious and were not included in statistical analyses.

A multivariate analysis of variance (MANOVA) was performed as outlined in Materials and Methods. Table 6 shows the results of the MANOVA. This analysis shows the effect of treatments to be significant. Given this significant difference the univariate main effects were examined. A significant univariate effect was obtained for peak 4 ($P = 0.02$) indicating that peak area is greater in $LP_{0.05}$ plants as opposed to $HP_{1.0}$ plants. Full univariate results can be found in Appendix 1.

A principal component analysis was performed to elucidate any patterns in the data that are not evident in univariate space (Dr. Susan Dudley, personal communication). Three principal components were extracted that, cumulatively, accounted for over 98% of the variance in the data. Figure 13, panels A and B, show the projections of the data for peak areas and the data for treatments irrespective of component peaks on the factor

Table 6. MANOVA results for treatment and trial effects on peak area. Significance for multivariate effect for treatment is denoted by an asterisk.

Effect	Wilks' λ	F-value	df	P-value
Treatment	0.178	5.528	5	0.030*
Trial	0.164	1.048	15	0.459

planes in panels C and D. An ANOVA on the first three principal components (synonymous with "factors" in Statistica) reveals a significant treatment-related change in factor 2 ($P = 0.04$). Comparisons of Figure 13A and 13B suggests that the source of significant changes could be the positioning of peaks 1 and 5 at high positive values of factor 2 and of peaks 2 and 4 at lower, more negative values of factor 2. Examination of the projection of the treatment data on the factor plane given in Figure 13, panels C and D, show peak contributions from plants grown in high levels of phosphate ($HP_{1.0}$) grouping together at positive values of factor 2 whereas those of data from $LP_{0.05}$ plants tend to group together at negative values of factor 2. These data indicate that the results of the $HP_{1.0}$ treatment contribute to the positions of peaks 5 and 1 at positive values of factor 2 and those of the $LP_{0.05}$ treatment that contribute to the positions of peaks 4 and 2 at negative values of factor 2.

The addition of the external ERETIC standard peak allows quantification of chemical components beyond reporting simply the percent contribution of an individual peak area as a function of total area occupied by all peaks of a spectrum. Table 7 summarizes the chemical shift range as a peak identifier and tissue content (reported in $nmol \cdot mg^{-1}DW$) of non-polar chemicals contributing to peaks detected in spectra. Content of the various components range from a low of 0.2 $nmol \cdot mg^{-1}DW$ (Table 7, 1.0 mM phosphate treatment) to a high of 2.8 $nmol \cdot mg^{-1}DW$ (Table 7 10, 0.05 mM and 1.0 mM phosphate treatments). A comparison of the mean content for non-polar P-containing metabolites of both treatments is consistent with the conclusion that $HP_{1.0}$ plants consistently have a higher content of components associated with four of the five peaks

Figure 13: Principal component (PC) analysis of data relating to peak areas and phosphate fertilizer treatment for five-week-old *Thellungiella* plants. Panels A and B depict the projection of peak area data contributions on the factor plane with PC 2 separating and grouping peaks 2 and 4 at negative values and 1 and 5 at positive values. Peak reference numbers (1 through 5) correspond to those given in Figure 12. Panels C and D show the projection of treatment data on the factor plane with fertilizer treatment contributing to the clustering of data for relative peak contributions towards total area with points associated with $HP_{1.0}$ plants (High) at positive values of PC 2 and those for $LP_{0.05}$ plants (Low) at negative values of PC 2.

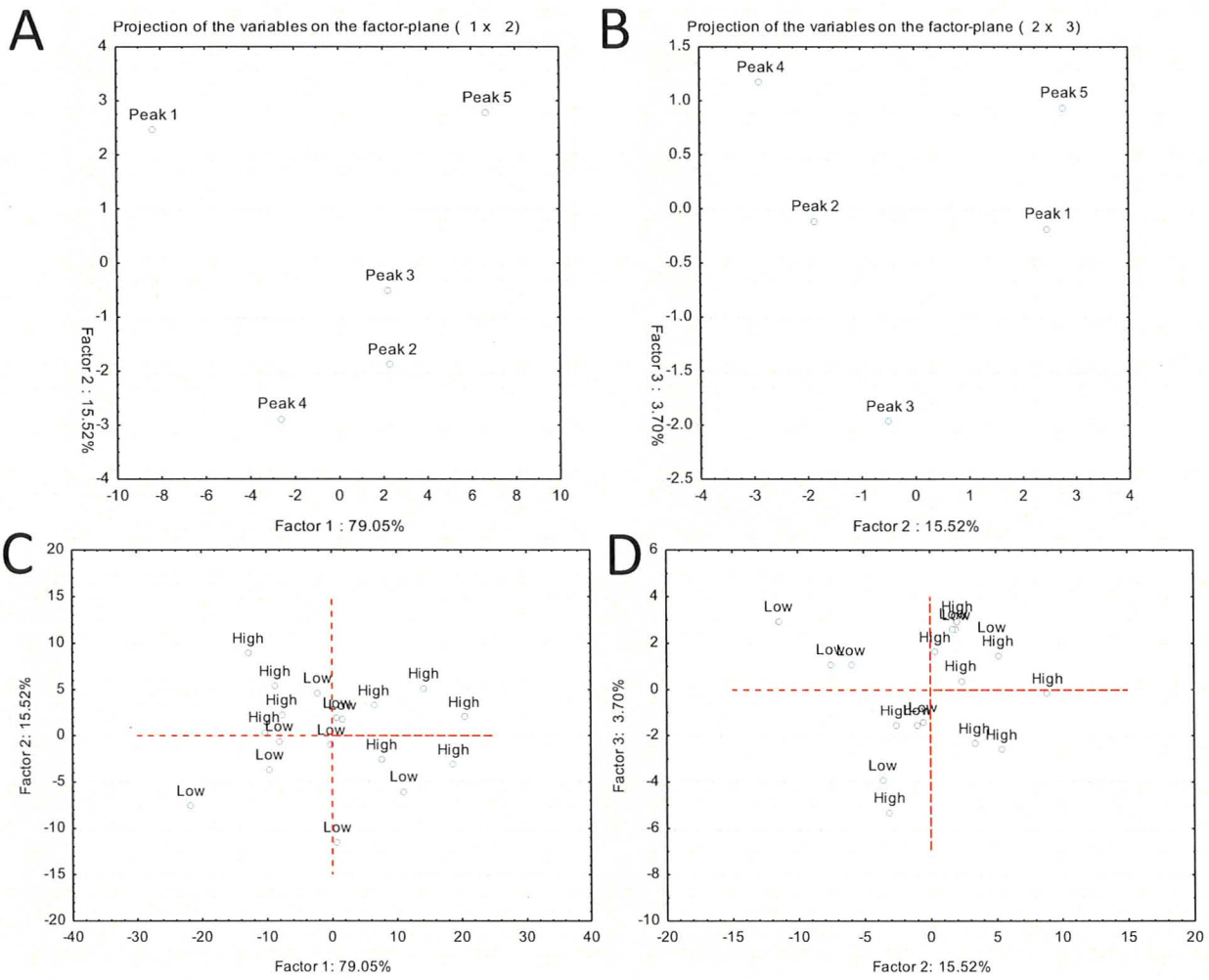

A
Projection of the variables on the factor-plane (1 x 2)
Peak 1
Peak 5
Peak 3
Peak 2
Peak 4
Factor 2 : 15.52%
Factor 1 : 79.05%
B
Projection of the variables on the factor-plane (2 x 3)
Peak 4
Peak 5
Peak 2
Peak 1
Peak 3
Factor 3 : 3.70%
Factor 2 : 15.52%
C
Factor 2: 15.52%
Factor 1: 79.05%
D
Factor 3: 3.70%
Factor 2: 15.52%

Table 7. Chemical shift values and tissue content of non-polar phospho-metabolites extracted from leaves of 5-week-old *Thellungiella* plants as analyzed by ^{31}P-NMR. Plants were watered once weekly with a modified Hoagland's solution containing either 0.05 mM or 1.0 mM phosphate. Content is reported as nmol·mg^{-1}DW and was estimated using the ERETIC external standard method (see Materials and Methods for details). ERETIC was only applied to a subset of the samples. Blank cells represent the failure to detect a given peak in a given spectrum.

0.05 mM phosphate fertilizer

		Peak ID No.						
		1	2	3	4	5	6	7
Trial	Replicate	Content (nmol·mg^{-1}DW)						
1	3	3.09	0.70	0.33	0.78	0.08		
2	1	2.63	0.75	0.45	0.30	0.56	0.05	
3	2	4.17	1.49	0.95	0.40	1.37		
4	1	2.14	0.76	0.33	0.18	0.87		0.07
4	2	2.29	0.66	0.31	0.15	0.83		
Mean ± SE		2.8 ± 0.3	0.9 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.8 ± 0.2		

1.0 mM phosphate fertilizer

		Peak ID No.							
		1	2	3	4	5	6	7	8
Trial	Replicate	Content (nmol·mg^{-1}DW)							
1	3	2.44	0.52	0.22	0.06	0.56			
2	1	2.65	1.27	0.77	0.20	1.21			
3	2	1.97	1.16	0.61		1.73			
4	1	3.77	1.02	0.52	0.35	0.96			
4	2	4.03	1.04	0.62	0.12	0.97			
Mean ± SE		2.8 ± 0.4	1.0 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	1.1 ± 0.2			

(with the exception being peak 4). A MANOVA does not reveal any significant differences in tissue content of non-polar P-metabolites ($P = 0.66$), however, this result must be interpreted with caution since only a subset of the data was subjected to ERETIC quantification hence n is low.

Profiling of Non-Polar Fractions From Arabidopsis Using ^{31}P-NMR

Two samples of *Arabidopsis* leaf tissue (a 2.4 g sample from wild-type and a 2.1 g sample from SALK_062703 mutant plants) were extracted following the same procedure as that used for *Thellungiella* leaf samples including the use of 5% (w/v) aqueous sodium cholate. SALK_062703 mutants contain a T-DNA insertion in *atnmt3* a gene encoding a putative phosphobase methyltransferase enzyme implicated in phosphocholine biosynthesis. Lipid profiles and phosphatidylcholine content could aid in elucidating the role of this gene in the phosphocholine synthesis pathway. Figure 14 shows the spectra and the spectral deconvolutions obtained from these extracts of wild-type (A) and SALK_062703 mutants (B). Table 8 reports the chemical shifts, percent area and the estimated content of contributing chemical components for each peak. The spectra from both *Arabidopsis* samples show similar general features: four peaks are resolved with the peak around 1.94 ppm highest followed by the peak at 0.94 ppm, then the peak between -0.119 and -0.133 ppm and, finally, the peak around 0.4 ppm.

While it may not be possible to draw conclusions about differences between the two *Arabidopsis* genotypes, the two *Arabidopsis* spectra show four distinct peaks at comparable ppm positions corresponding to peaks 1, 2, 3, and 5 for *Thellungiella* (Fig. 12). In this regard, the apparent lack of peak 4 in both *Arabidopsis* spectra is striking.

Figure 14. Spectra and spectral deconvolutions of ^{31}P-NMR analysis of non-polar extracts from leaves of *Arabidopsis* (A) wild-type and (B) SALK_062703 mutant plants. Lower spectra in individual panels are the actual spectra and their spectral deconvolutions are immediately above.

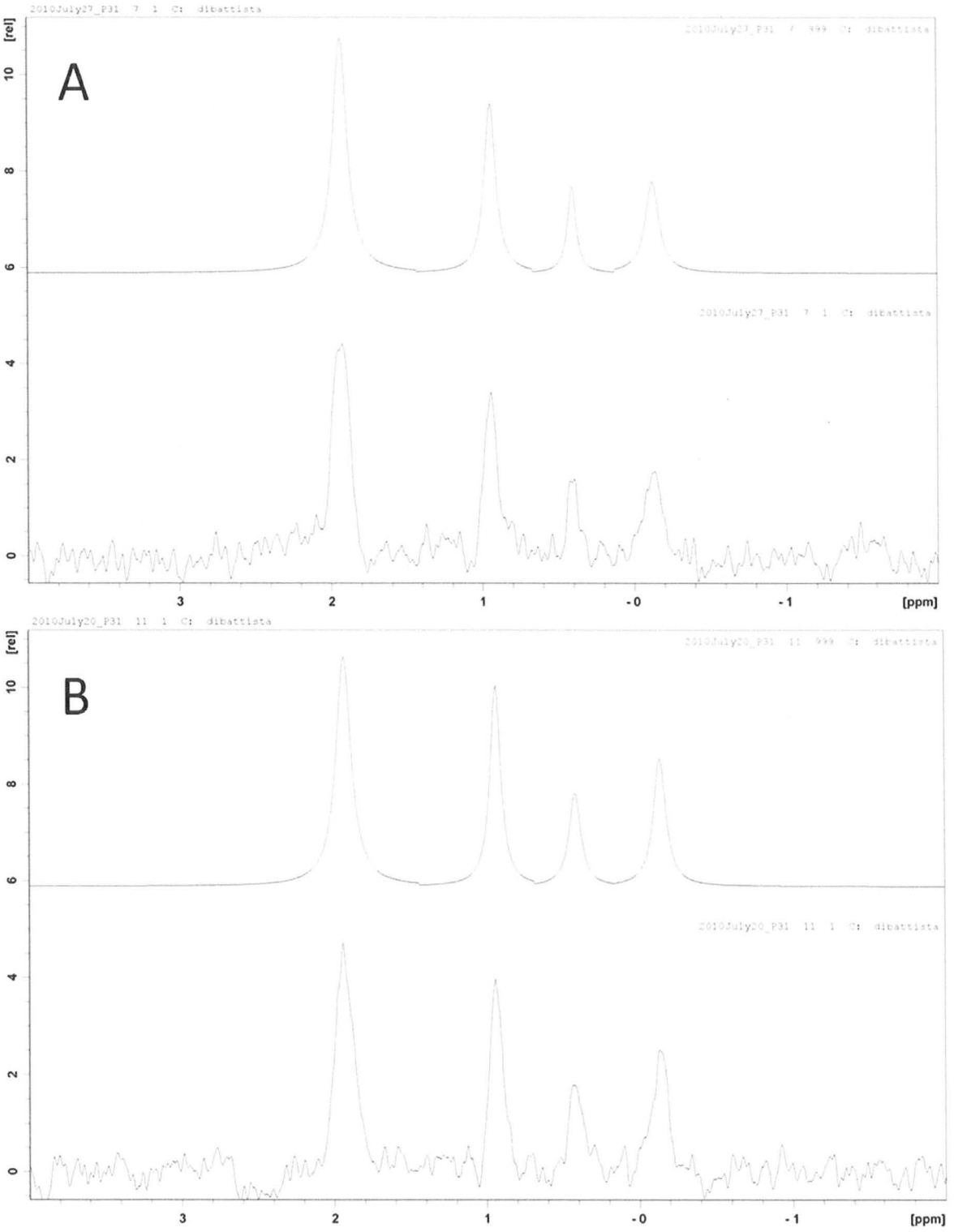

A
B
[rel]
10
8
6
4
2
0
3
2
1
-0
-1
[ppm]

Table 8. Chemical shift values, percent composition of each peak as a function of total peak area and estimated tissue content of non-polar P-metabolites as determined from leaf extracts of *Arabidopsis* wild-type and SALK_062703 mutant plants. Data represents a single determination for each sample and for both samples four peaks were resolved following deconvolution of spectra.

	Wild-type				SALK_062703			
Chemical Shift *(ppm)*	1.94	0.94	0.42	-0.13	1.94	0.95	0.41	-0.12
Peak Area (%)	42.10	25.35	13.31	19.24	46.71	26.15	10.07	17.06
Content ($nmol \cdot mg^{-1}DW$)	6.01	3.62	1.90	2.75	6.67	3.74	1.44	2.44

Pilot Studies

Profiling of Polar and Non-Polar Fractions Using ^{1}H-NMR

A 500 mg sample of shoot tissue from seedlings grown on defined, sterile media was extracted and the polar phases were dried then dissolved in methanol-d for analysis by ^{1}H-NMR. The results acquired by the instrument are shown in Figure 15. The spectra produced are very complex and in this trial the signals comprising the spectra associated with the $LP_{0.05}$ samples were, for some reason, uniformly lower than the sample prepared from the $HP_{1.0}$. This discrepancy could have arisen from a poorer extraction efficiency of the $LP_{0.05}$ leaf tissue or because of genuine differences in polar metabolite content. Increasing the amplitude of the signals arbitrarily by scaling the spectrum by a factor of three makes some components in the $LP_{0.05}$ and $HP_{1.0}$ spectra more similar (Fig. 15). Nonetheless, there are distinct differences in the relative abundances of some peaks including those present between 2.5 and 1.0ppm and at 5.3ppm. However, the structural nature of NMR means that multiple peaks can belong to a single compound and many different compounds can have protons in similar environments. As such, it is difficult to deconvolute the individual compounds contributing to the same and different peaks within a complex mixture using ^{1}H-NMR. Despite a promisingly high SNR demonstrated by very narrow peaks the complex array of plant metabolites containing many protons per molecule prevents further use of ^{1}H-NMR of polar metabolites.

The non-polar fractions prepared from the same tissue source were dissolved in chloroform-d and the lipid composition was analysed by ^{1}H-NMR. Figure 16 shows that qualitative differences in peaks are found between the acquired spectra of the $LP_{0.05}$ and

$HP_{1.0}$ samples. For example, the region around 1.6-1.7 ppm (as indicated by the black arrow) contains a singlet peak in the $HP_{1.0}$ spectrum and a visibly different structure, possibly a doublet or two overlapping peaks, in the $LP_{0.05}$ spectrum. The large doublet structure to the right (green arrows) shows an interesting quantitative difference; the smaller peak is more abundant in the $LP_{0.05}$ sample than in the $HP_{1.0}$ sample while the larger peak in the doublet is less abundant. Because of the 100% natural abundance of ^{1}H and sensitivity of the spectrometer to this nucleus, peaks are narrow, noise is minimal and SNR is high. However, as is the case for ^{1}H-NMR of polar compounds, ^{1}H-NMR of lipid compounds was not pursued in this study due to the complexity of the spectra arising from an abundance of proton-carrying chemicals present and the difficulty of deconvoluting peaks to identify the chemical source(s) responsible.

Figure 15: Spectra from ^{1}H-NMR analysis of polar metabolites prepared from 500 mg of shoot tissue of seedlings grown on defined nutrient plates containing either 0.05 mM (A) or 0.5 mM (B) phosphate regime. Figure C shows the results of arbitrarily scaling the $LP_{0.05}$ spectrum (red) three-fold to make overall signal more comparable to $HP_{1.0}$ (blue). Arbitrarily increasing the scale three-fold for $LP_{0.05}$ samples made qualitative and quantitative differences more obvious (as indicated by arrows).

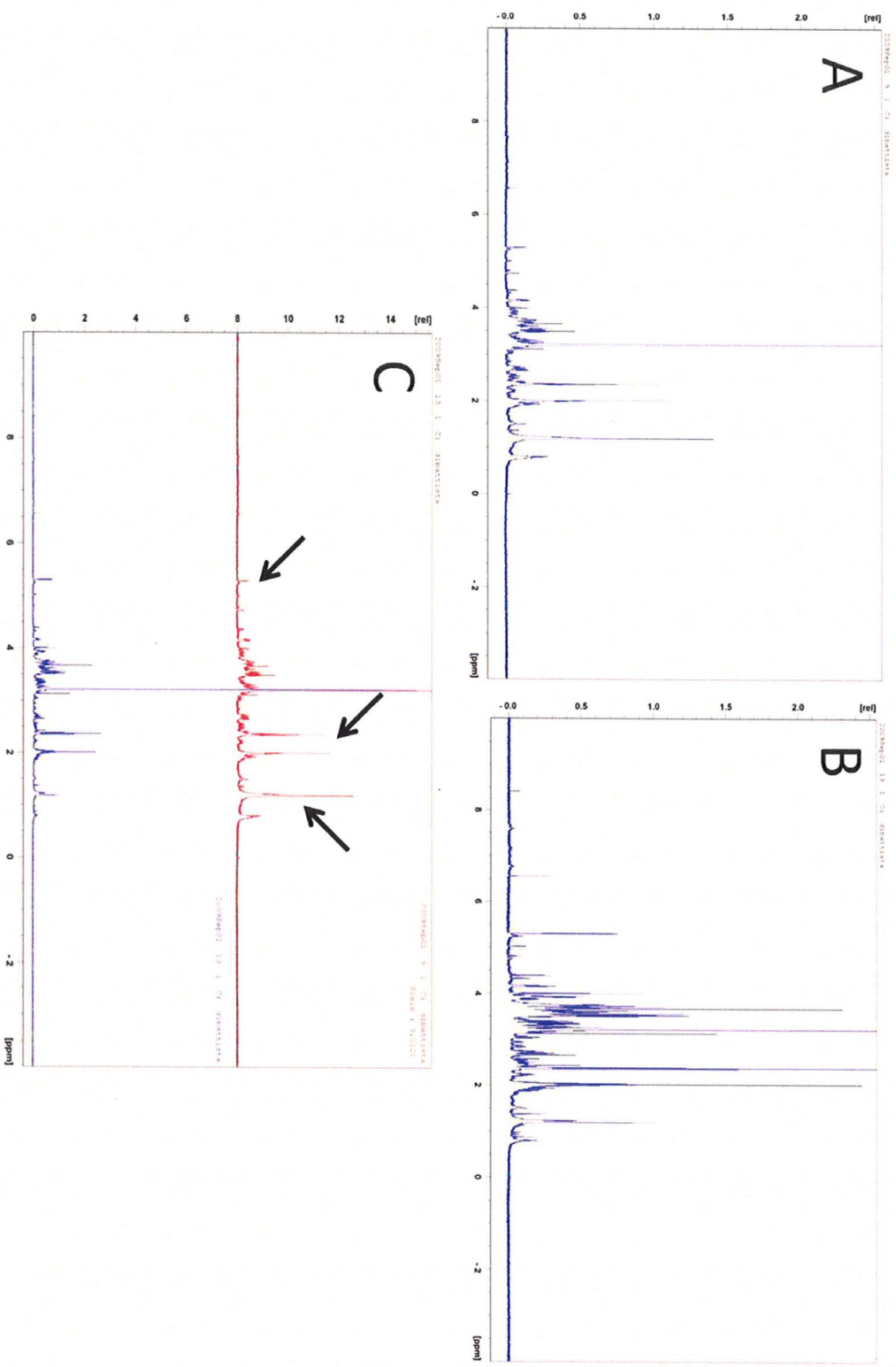
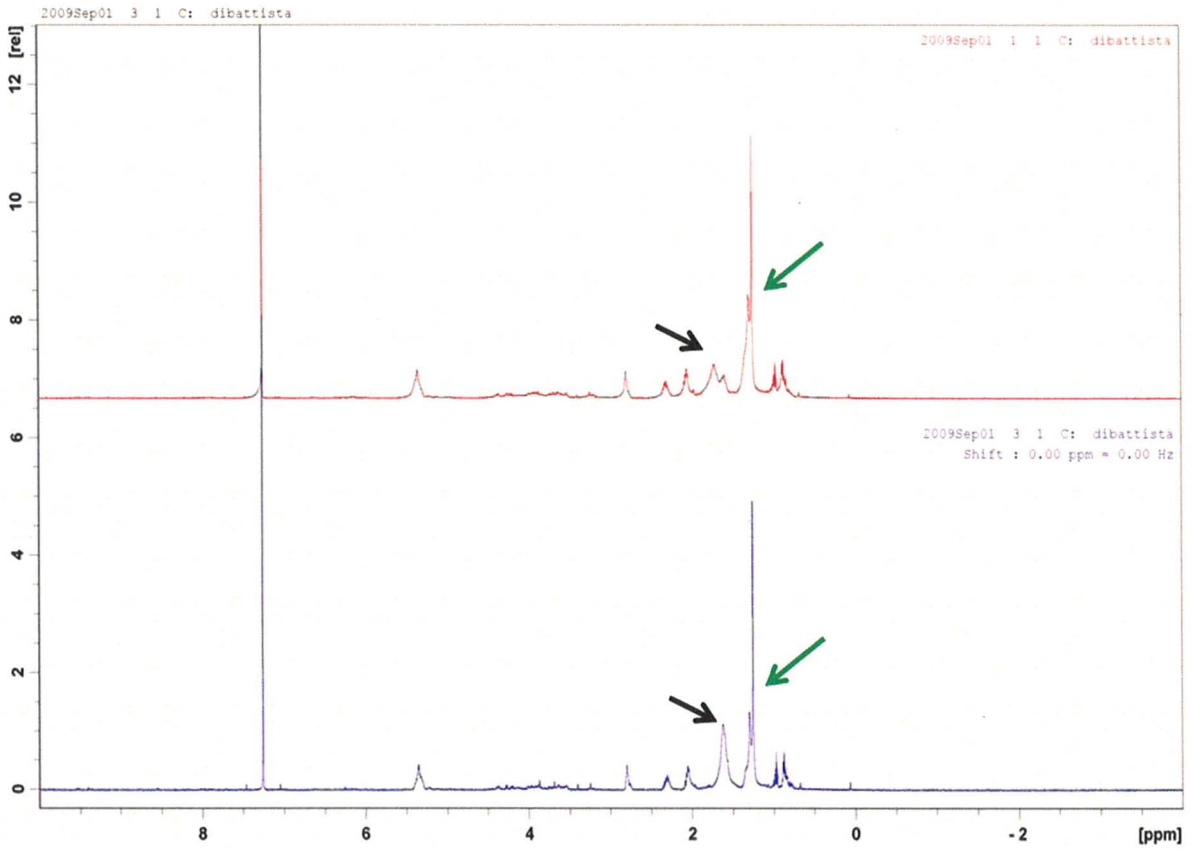

A
B
C
[rel]
[ppm]

Figure 16: Representative ^{1}H-NMR spectra of non-polar extracts from shoot tissue of *Thellungiella* seedlings grown on $LP_{0.05}$ (red) or $HP_{1.0}$ (blue) phosphate nutrient agar plates. Spectra show qualitative and quantitative differences; with respective examples indicated by black and green arrows. Sample size of tissue used for extraction was 500 mg. ^{1}H-NMR was performed twice on non-polar extracts.


2009Sep01 3 1 C: dibattista
2009Sep01 1 1 C: dibattista
2009Sep01 3 1 C: dibattista
Shift : 0.00 ppm = 0.00 Hz
12 [rel]
10
8
6
4
2
0
8
6
4
2
0
-2
[ppm]

DISCUSSION

Plant Growth in Response to Nutrient Treatments

Thellungiella seedlings were grown on four different concentrations of phosphate (Table 1, Materials and Methods). To determine an appropriate "high" or "low" concentration for testing, the effects of the various phosphate levels on seedling growth were examined. Several indicators show that the 0.5 mM phosphate treatment led to a significantly different growth of seedlings on defined nutrient media compared to any of the remaining three treatment levels. Mean number of days to harvest is significantly lower, primary root elongation rate is significantly higher and shoot fresh weight at harvest is significantly lower for seedlings grown on 0.5 mM Pi relative to those on 0.2, 0.1 or 0.05 mM phosphate (Figure 2). What was unexpected was that the reduction of external phosphate from 0.2 mM to 0.05 mM did not lead to significant differences among these plant responses with respect to these measurements. Given the apparent lack of difference in the response of Thellungiella to 0.2 mM and 0.05 mM concentrations of Pi, the "low" phosphate exposure was selected as 0.05 mM (the $LP_{0.05}$ treatment) to serve as a comparison to the response at 0.5 mM (hence $HP_{0.5}$ treatment).

Representative *Thellungiella* (Yukon genotype) seedlings grown on $LP_{0.05}$ and $HP_{0.5}$ nutrient media (Figure 3) show differences in root length with $LP_{0.05}$ plants being, on average, 30 % shorter than their $HP_{0.5}$ counterparts after 7 d. Indeed, the days required for plants to reach harvest length (ca 5 cm) is significantly greater for $LP_{0.05}$ plants. However, these data are simply a snapshot of these plants at a particular point in time. In

order to understand changes in root length in response to different concentrations of phosphate, these plants must be examined as dynamic systems. To address this consideration, Figure 4 is a plot of primary root measurement over time and shows that $LP_{0.05}$ roots elongate at a rate that is ca 70% of the rate observed for $HP_{0.5}$ seedlings. This reduction in growth rate is well supported in the literature. Ma et al. (2001) reported a 30% reduction in primary root growth rate of *Arabidopsis* seedlings grown in a low (1 μM) phosphate medium relative to high phosphate (1 mM) (Ma et al., 2001). However, the inhibition of primary root elongation has been reported in other literature as more severe. For example, *Arabidopsis* seedlings grown on 5 μM Pi media show only 15% the root elongation rate of seedling grown on 500 μM Pi media (Misson et al., 2005). Furthermore, primary roots of seedlings grown on 1 μM Pi plates and can reach just over 50% of the root length of seedlings grown on media containing higher (500 μM) phosphate (Lopez-Bucio et al., 2002). For *Thellungiella*, the mean primary root length of $LP_{0.05}$ plants can range from ca 60 % of the $HP_{0.5}$ primary root length on day 4 to over 70% on day 7 (Fig. 2BIII).

Inhibition of primary root elongation is a well-documented phosphate-starvation response that is accompanied by an increase in lateral root growth (Lynch & Brown, 2001; Z. Ma et al., 2003; Misson et al., 2005; Raghothama, 1999). Increased lateral root growth is a hallmark of topsoil foraging where increasing the root surface area can increase the soil exploration capacity of the plant and provide a greater absorptive surface when Pi is found (Lynch & Brown, 2001). Lateral root number and density were not measured in this thesis, however, specific root length (root length per root mass) was

lower for $LP_{0.50}$ plants (Appendix B), which suggests that root mass is being allocated to lateral root structures (Lynch & Brown, 2001).

Another classic phosphate-starvation response is the increased allocation of plant biomass to the roots (Lynch & Brown, 2001; Raghothama, 1999; Ticconi & Abel, 2004). Root-shoot ratios are frequently reported as measurements to describe this response but they can be misleading when plants of the same age versus the same size are compared (McConnaughay & Coleman, 1999). Specifically, apparent increases in root-shoot ratio can result from normal changes during plant growth (ontogenetic drift) or because different environments can affect the growth of the plant resulting in a different developmental age than the chronological age would predict (Gedroc, et al., 1996; McConnaughay & Coleman, 1999). An allometric plot of $LP_{0.05}$ and $HP_{0.5}$ seedling shoot versus root mass accounts for ontogenetic drift by removing the confounding effects of time. This allows for comparisons to be made across treatments for seedlings at the same developmental stage which can reveal true plasticity in resource allocation. The plot of these measurements for $LP_{0.05}$ and $HP_{0.5}$ seedlings have significantly different slopes (Fig. 6) and this indicates that *Thellungiella* seedlings show greater resource allocation to roots in response to $LP_{0.05}$ as opposed to $HP_{0.5}$ conditions.

Thellungiella shows developmental adjustments associated with a plant responding to detecting locally reduced levels of phosphate in the growth medium as evidenced by an increase in resource allocation to root biomass, primary root inhibition and increased lateral root allocation. This response may be modest in comparison to the classic phosphate starvation stress response of *Arabidopsis* but suggests, nonetheless, that

Thellungiella senses the reduced phosphate concentration of the growth medium and alters root architecture in a manner consistent with an improved capacity for topsoil foraging and soil exploration. Given the stress-tolerant nature of *Thellungiella*, it is perhaps not surprising that this low phosphate concentration does not elicit the stronger phosphate starvation response seen in *Arabidopsis*.

In terms of root and shoot growth, *Thellungiella* does not appear to differentiate between 0.2 mM and 0.05 mM external Pi (Figure 2). While the exact mechanism by which plants sense Pi is still unknown, it is thought to be bipartite. Plants are thought to sense Pi both in terms of whole plant internal status and local external Pi concentration at the roots (Abel et al., 2002). Root tip cells are very responsive and split plate design experiments have shown that primary root elongation slows noticeably when roots encounter a patch of low Pi medium (Ticconi & Abel, 2004). In other studies, the internal root Pi content is significantly lower in starved plants following 3 d of complete Pi-starvation than in control *Arabidopsis* plants provided with sufficient (2.5 mM) phosphate (Wu et al., 2003). Moreover, *Arabidopsis* shows changes in the expression of PSI genes in roots after 3 h on low Pi medium and as rapidly as 1 h post-removal of Pi from the growth medium (Misson et al., 2004; Ticconi & Abel, 2004). Together, these results demonstrate the localized sensing of external Pi and the rapid root-specific responses. No statistically significant difference was found for *Thellungiella* seedlings on 0.2 mM 0.1 mM or 0.05 mM Pi with respect to root elongation rates, days to harvest and shoot fresh weight (Fig. 2). For *Thellungiella* seedlings the similarity in growth response on media containing 0.05 and 0.2 mM Pi suggests that a low Pi threshold was never

reached by the lowest Pi concentration used. In future studies, this experiment should be repeated at even lower Pi levels to see when or even if *Thellungiella* shows a classic Pi starvation response. An accompanying measurement of the internal Pi content of leaves would also help determine if the plants compensate by storing available Pi in tissues when Pi content in the environment is low.

Many different levels can be examined for indications that phosphate exposure has elicited changes in a plant species. That is, changes in transcript expression, metabolism and/or development could conceivably all offer unique and potentially complementary views of plant responses to phosphate. Such a detailed, integrative body of work is beyond the scope of this thesis necessitating a more focussed target for examination. Two considerations made profiling phospholipids a potentially important focus for our work. Firstly, phospholipids comprise a large metabolic sink for phosphate and secondly, a substantive body of literature amassed for *Arabidopsis* has shown many changes to take place in this species in response to various external phosphate concentrations (Hartel et al., 2000; Li et al., 2006; Plaxton et al., 2004). These considerations led us to expect phosphate stress-responsive changes in the lipid composition of *Thellungiella*. This experimental approach necessitated the development of a protocol that delivered comprehensive, reproducible lipid profiles in order to determine if a consistent treatment-specific lipid fingerprint could be identified. The extraction and ^{31}P-NMR analysis protocol developed in this thesis can be used to determine lipid abundance as both a relative composition (percent of spectral area) and as an absolute determination of content (nmol·mg^{-1}DW). While data obtained from plants

suggested some heterogeneity in lipid composition, treatment-related changes in phospholipid composition showed statistically significant differences (Tables 5 and 6). Moreover, this approach was tested with Arabidopsis tissue and it has proven suitable for use in this species. For Arabidopsis the mass of tissue available was smaller and so the successful resolution of well-resolved peaks in spectra for these samples show that smaller quantities of leaf tissue can be used in NMR analyses. Scaling down sample size opens the possibility that the application of lipid profiling by NMR could be used for seedlings and other tissues where available biomass may be typically lower than leaf tissue of mature plants.

Five-week old plants grown on Promix BX medium fertilized weekly with a modified Hoagland's solution containing either 0.05 mM ($LP_{0.05}$) or 1.0 mM ($HP_{1.0}$) phosphate were used to scale up the tissue available for NMR analysis. No differences were detected in the development or biomass of the rosettes for plants grown on either treatment (Figs. 7 and 8). This is unexpected given previous research indicating that plants grown under low phosphate conditions are smaller (Wu et al., 2003; Li et al., 2006). It is possible that any Pi that is not taken up by plants following the weekly addition of fertilizer accumulates in the soil medium only to be removed by the roots at a later date. This would suggest that the plants experiencing low phosphate are never truly deficient for this nutrient so that the $LP_{0.05}$ treatment is simply not low enough to be classified as stressful by *Thellungiella* and does not adversely impact plant growth. However, leaf number may be a useful indicator of plant responses to external Pi and is something to be investigated in future studies.

Internal Pi content of shoot tissue was measured to determine any treatment-related differences in the Pi content of the leaves. Internal Pi content is known to decrease in shoot tissue when plants are grown in a medium with a reduced phosphate concentration (Hammond et al., 2003; Lopez-Bucio et al., 2002; Muller et al., 2007; Shimano & Ashihara, 2006). Typically, this decrease in internal Pi content is significant. For example, for Arabidopsis after 48 h of growth on low (5 μM) phosphate media the whole plant internal Pi levels were 68% less than those in control plants grown at 500 μM Pi (Misson et al., 2005). Not all organs of a plant respond similarly to Pi starvation as determined by tissue Pi content. For example, the internal Pi content of roots of Pi-starved *Arabidopsis* plants decreased by over 20% relative to control, untreated plants after only 24 h exposure to Pi starvation conditions but leaf Pi levels remained unchanged even after 3 d of Pi starvation, (Wu et al., 2003). However, *Arabidopsis* seedlings cultured on a defined medium under various concentrations of Pi for 10 d show an almost 5-fold decrease in internal Pi concentration in leaf tissue from seedlings grown on 0.1 mM media relative to 1.0 mM media (Hartel et al., 2000). Given the differences in tissue content of Pi as a function of treatment reported in different sources for even a single species, it is, a priori, difficult to predict whether Pi content for *Thellungiella* should or will change in response to Pi treatments. As such, we determined the Pi content using the polar phases of leaf extracts from *Thellungiella* and this source shows a significant ($P < 0.05$), albeit modest 1.3-fold difference in free internal Pi content with the lower level consistently found in leaves of plants watered with 0.05 mM phosphate fertilizer (Fig. 9, Table 4).

Müller *et al.* (2007) report that plants grown for four weeks on a 0.05 mM Pi inert medium (Rockwool) contained less than 1 $\mu mol \cdot g^{-1}FW$ free Pi. In contrast, Table 4 shows that the mean $LP_{0.05}$ leaf Pi content is 3.29 $\mu mol \cdot g^{-1}FW$ and that only two of the values approach 1 $\mu mol \cdot g^{-1}FW$. Interestingly, Müller *et al.*, (2007) also reported that internal Pi content for *Arabidopsis* plants grown for four weeks on 1.0 mM Pi-containing medium lies between 5 and 10 $\mu mol \cdot g^{-1}FW$, while Table 4 shows that the leaf Pi content following fertilizer treatments of 1.0 mM phosphate ranges from 3.89 to 5.04 $\mu mol \cdot g^{-1}FW$. Whether *Thellungiella* accumulates more Pi in leaves than *Arabidopsis* or not when Pi is limiting in the environment would be important to establish in future experiments done with both species in parallel. Given that *Thellungiella* is such a stress-tolerant organism and one that has adapted to saline and alkaline soils, this capacity may confer an adaptive advantage. Part of the mechanism underlying this ability for *Thellungiella* may involve maintaining a high tissue Pi content when Pi availability is low enough to lead to phosphate deficiency stress and a lower tissue Pi content in *Arabidopsis*. Uptake is a critical component for increasing the Pi content of plants, particularly when soil Pi availability is low. Canola, a member of the Brassica family to which *Thellungiella* belongs, is known to acidify the rhizosphere via the excretion of organic acids (Bolland, 1997; McKenzie et al., 2003). Acidification of the rhizosphere solubilises Pi from otherwise-insoluble sources such as rock phosphate, making this source of Pi available for uptake (Raghothama, 1999). It is possible that *Thellungiella* uses a similar strategy to maintain elevated internal Pi when external Pi is low and this possibility should be tested in future studies. Moreover, plants can improve their capacity for Pi uptake by high

affinity Pi transporters that are membrane-associated. These transporters function, primarily, in translocating Pi from the growth medium to the roots when Pi content in the external environment is low (Karthikeyan et al., 2002). During Pi starvation, *Arabidopsis* and tomato have been shown to increase the number of these transporters to improve the influx of Pi into the plant suggesting that the number of high affinity transporters limits the capacity for Pi uptake (Raghothama, 1999). It is possible that *Thellungiella* has a superior capacity for Pi uptake in reduced Pi environments by virtue of having a constitutively higher number of high-affinity Pi transporters associated with the root membranes.

While five-week-old plants are more mature than seedlings they show vegetative and not reproductive development. In future studies *Thellungiella* plants should be grown to maturity to determine if the external phosphate concentrations adversely impact reproduction. Monitoring time to flowering, seed production and seedling vigor may reveal alterations that do not show up in vegetative plants dominated by a rosette and could include changes at other levels including Pi content, Pi uptake capacity and composition of the lipidome. Ideally, *Thellungiella* plants grown under $LP_{0.05}$ and $HP_{1.0}$ conditions would be subjected to transcriptional analysis given that PSI genes are well-documented in *Arabidopsis*. These changes in gene expression could be used to infer whether *Thellungiella* uses the same biochemical pathways as *Arabidopsis* under conditions of low Pi. Moreover, a comparison including quantitative and qualitative differences in documented patterns of expression for other species as a consequence of low phosphate could reveal novel mechanisms of stress tolerance for *Thellungiella*.

Lipid Profiling Using ^{31}P-NMR Spectroscopy

Increasing Spectral Resolution

Figure 10 shows that using chloroform-d as a solvent results in spectra that have poor SNR, the peaks are broad and not resolved. While increasing the number of scans will increase SNR this is not a practical solution given that the spectrometer is heavily used by others. Increasing sample mass is possible but this not a suitable option given limitations to growing space for the plants and the labour involved in treating the plants. At present, approximately five plants are required for 5 g of leaf tissue and this requires pooling young, emerging leaves with older, fully expanded (and potentially senescing) leaves. Biochemical differences resulting from different developmental stages, physiological or metabolic roles (i.e. source versus sink leaves) within a plant have the potential to introduce variation in lipid composition. For example, phospholipid content is known to decrease in older tissues because of reduced synthesis and increased phospholipase activity (Borochov et al., 1982). Additionally, because multiple plants from a genetically heterogeneous source must be pooled to achieve the required sample mass, there is great potential for variation within treatments and across replicates. Further increasing the sample mass will simply exacerbate these concerns by introducing more variation. It should be kept in mind, however, that both control and treatment batches of leaves are, at least in theory, subject to the same sources of variability and we surmise that this is true given that MANOVA did not reveal statistical differences between replicates of a given treatment.

The solution that we tested to improve the quality of the specta involved the use of solvents. Detergents and bile salts such as cholate and oxycholate solubilise lipid membranes by destroying the bilayer structure and promoting the formation of small, mixed micelles (London & Feigenson, 1979; Schubert & Schmidt, 1988). Phospholipid headgroups within a lipid mixture can be spectroscopically differentiated because the inclusion of a detergent retards lipid aggregation and produces narrow resonances (London & Feigenson, 1979). Sodium cholate easily dissolves desiccated lipids and does not produce stable foams with the same frequency as SDS or Triton X-100 making cholate more suitable for some applications including NMR (London & Feigenson, 1979). It is known that the chemical shift of a given molecule is affected by the NMR solvent (Meneses & Glonek, 1988). Choosing sodium cholate means that chemical shift values can be compared to those well-tested and cited values reported in papers (particularly the paper by London and Feigenson, 1979) arising from studies that helped to pioneer the use of detergent to increase spectral quality. The addition of a detergent such as sodium cholate means that high resolution spectra can be obtained while requiring a lower magnetic field strength, lower sample mass and/or fewer scans. In this way, the protocol can be optimized for both scan time and sample mass and can potentially be applied to other plant species and tissues where sample mass may be at a premium. Figure 11 show how the inclusion of sodium cholate produced high quality spectra with clearly resolved peaks in isolation or as shoulders.

Peak Contribution to Spectral Area

Figure 10 shows that there is considerable variation in the contribution of various peaks to total spectral area. Seeds used in this thesis represent the progeny of a mixed population, and as such, it is expected that there is a certain degree of genetic heterogeneity. However, as indicated by the MANOVA results (Table 6), treatment does have a significant effect on peak contribution to spectral area while variation between replicates does not. The univariate main effects for treatment effects indicate a significant result for peak 4 which contributes less to the total spectral area in $HP_{1.0}$ plants relative to spectra from $LP_{0.05}$ plants (Appendix A). It should be noted that some spectra contained extra, unique peaks in addition to the expected four or five. These peaks contributed minimally to the spectral area and were considered to be spurious and were probably the result of the heterogeneous nature of the seed stocks used or a transient change in growth chamber conditions.

A principal component analysis was performed to help elucidate any patterns in the data that were not seen in univariate space. A scree plot of the eigenvalues indicated that the first three PCs should be retained. Figure 13 offers evidence that PC 2 is most meaningful in describing the variance in the data and an ANOVA performed on the factors also indicated that factor 2 shows a significant, treatment-related difference. Figure 13 shows that peaks 2 and 4 group together at low, negative values of PC 2 while peaks 1 and 5 group together at high, positive values of PC 2. Projecting the treatments onto the factor plane shows that peak contributions from plants grown under a high phosphate regime group primarily at positive values for PC 2; peak contributions for $LP_{0.05}$ plants group primarily at negative values. As such, PC 2 can be considered a

"treatment" component and it is along this principal component that peak contributions segregate by fertilizer phosphate concentration. The grouping of peaks 1 and 5 along PC 2 is driven by the peak area contributions of $HP_{1.0}$ plants, while the grouping of peaks 2 and 4 is driven by areas of these peaks for $LP_{0.05}$ plants. A biplot of peaks on opposing ends of PC2 can reveal patterns of peak area difference not evident in the univariate results of the MANOVA.

Biplots of peak area contributions shows the same trends discussed above (Figure 17). Figure 17A is a biplot of areas contributed by peaks 1 and 4 and shows, with a few exceptions, that $LP_{0.05}$ peak 4 contributions to spectral area are greater than peak 4 $HP_{1.0}$ contributions, including two very large $LP_{0.05}$ peak area values. Figure 17B shows a biplot of peaks 5 and 2. Peak 5 areas for spectra of plants grown under the $LP_{0.05}$ treatment are generally lower than peak 5 areas under the $HP_{1.0}$ treatment. In other words, percent areas are found in a narrow region and do not separate by treatment for peaks 1 and 2 (30 to 65% and 13 to 25%, respectively). In contrast, percent areas for peaks 4 and 5 span a wider range (0 to 16% and 1 to 36%, respectively) and loosely group by treatment.

In summary, the data is variable but statistical analyses showed some patterns that are significant. Peaks 4 and 5 of the NMR spectra appear to contribute the most to the variation in the data with only peak 4 found to be significant in the univariate MANOVA results (Appendix A). The biplots also showed treatment-specific differences in peak area contribution towards total spectal area for peak 5 (but univariate MANOVA results reveal a peak 5 P-value of 0.139). While this is not significant under the criteria used in

this experiment, increasing sample size could improve the confidence supporting this judgement regarding the significance of peak 5.

Peak Identification and Rationale

^{31}P-NMR is ideally suited to phospholipid studies owing to the single phosphorus nucleus that resides in the headgroup. The electronic environment of each phosphorus nucleus is sufficiently different that each nucleus resonates at a unique frequency giving each phospholipid a unique chemical shift for identification (London & Feigenson, 1979). Peaks resolved in this study have been tentatively identified using published chemical shift tables of lipid extracts in aqueous sodium cholate in conjunction with published data on the lipid composition of *Arabidopsis* (Bonaventure et al., 2003; Devaiah et al., 2006; Meyerowitz & Somerville, 1994).

The chemical shift of a given compound can vary depending on solvent, temperature, detergent concentration, acyl chain composition and pH (London & Feigenson, 1979; Schiller, et al., 2007; Vyssotski, et al., 2009). London and Feigenson (1979) report a chemical shift variation of ± 0.08 ppm using mixtures of known lipid standards so it is conceivable for the chemical shifts of species in a natural lipid mixture to vary to a greater extent.

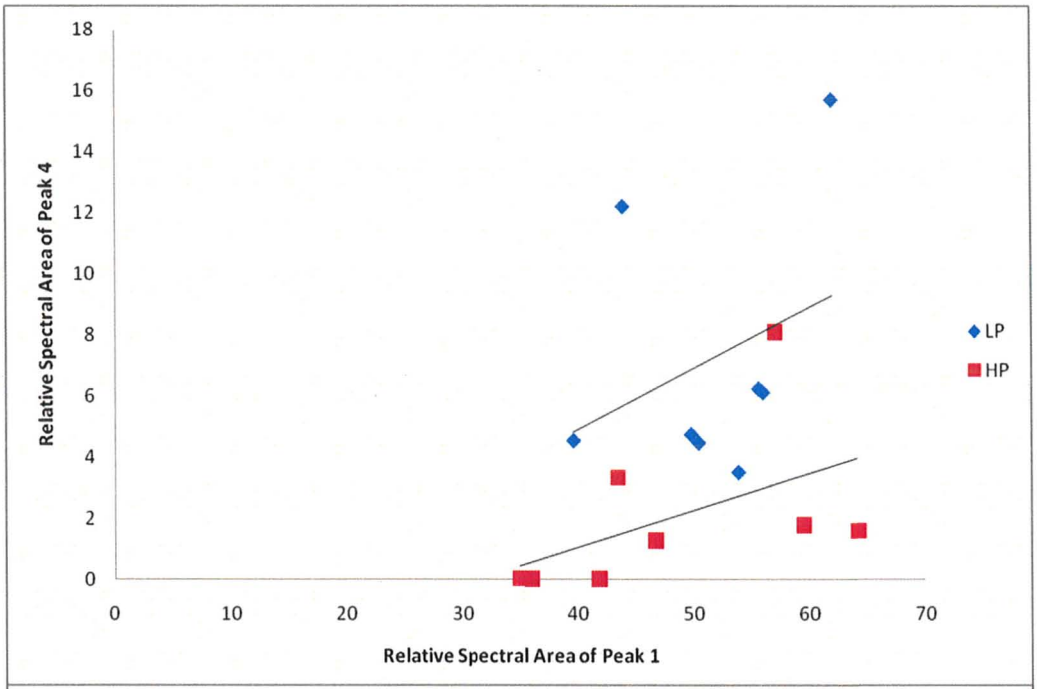
Comparing peak positions relative to others in a spectrum can therefore be useful in identifying components contributing to peaks. However, verification using certified, authentic standards is necessary to identify components with a high degree of confidence. We did not use internal standards so tentative identifications were made based upon published data for phospholipids.

Figure 17. Biplots of the peak areas for those peaks that contribute to the variance along principal component 2.

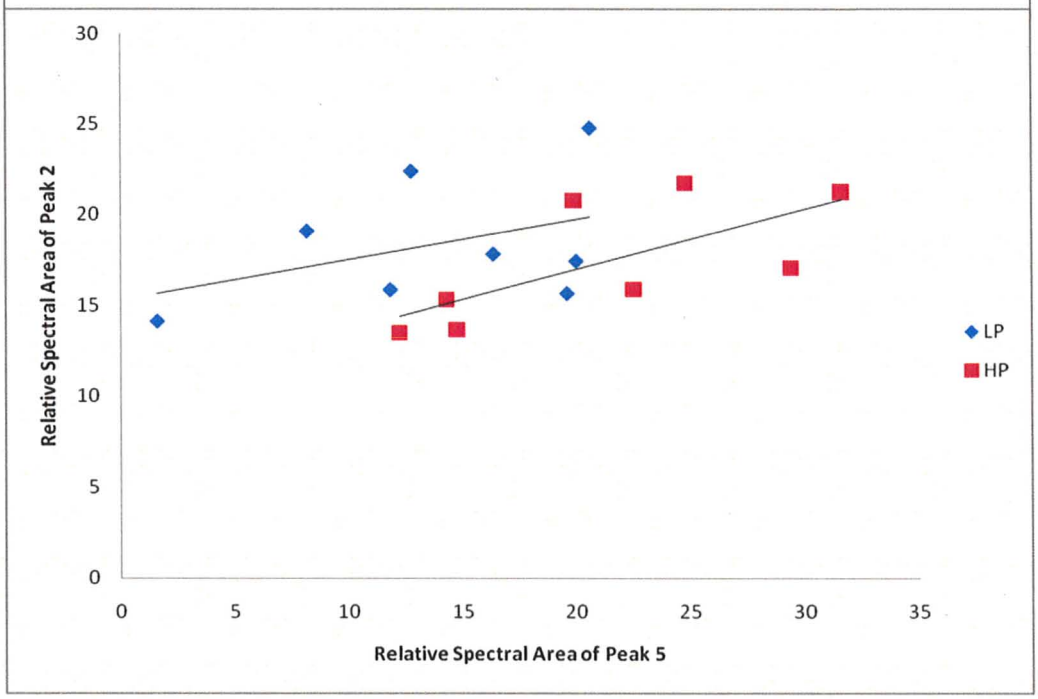
Biplots showing peak areas of $LP_{0.05}$ (♦) and $HP_{1.0}$ (■) fertilized plants for peaks 1 and 4 (A) and peaks 5 and 2 (B).

Slope and r^2 for $LP_{0.05}$ fertilized plants are 0.2016; 0.1082 and 0.2223; 0.1684 for panels A and B, respectively. Slope and r^2 for $HP_{1.0}$ fertilized plants are 0.1208; 0.2415 and 0.3323; 0.4907 for panels A and B, respectively.

A


Relative Spectral Area of Peak 4
Relative Spectral Area of Peak 1
LP
HP
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
50
60
70

B


Relative Spectral Area of Peak 2
Relative Spectral Area of Peak 5
LP
HP
0
5
10
15
20
25
30
0
5
10
15
20
25
30
35

Peak 1 (1.77-1.949 ppm) is potentially free Pi. A Pi peak occurs at δ = 2.0 to 2.2 ppm in an artificial lipid mixture (London & Feigenson, 1979), δ = 1.9 to 2.3 in artificial mixtures (Vyssotski et al., 2009), and δ = 2.2 ppm in an artificial mixture (Schiller et al., 2007). While free Pi was detected in the polar extract by colourimetric assay, it is possible that phosphatase activity was not quenched during the extraction. Internal phosphatases are used to recycle and scavenge Pi (Plaxton, 2004). It has been demonstrated that phosphatase activity can persist, even after "killing" tissues by boiling in 80% methanol prior to extraction; post-mortem phosphatase activity in potato, tobacco and *Chlorella* can persist for 3 min and, in a model disk system using partially-purified potato phosphatases and substrates, can be equivalent to full enzyme activity for 1 min at 25°C (Bieleski, 1964).

Peak 2 is likely phosphatidylglycerol (PG). Published chemical shift data report that PG is present at δ = 0.43 ppm (London & Feigenson, 1979) and 0.52 ppm (Schiller et al., 2007). Peak 2 is present in $LP_{0.05}$ and $HP_{1.0}$ samples between 0.77 and 0.956 ppm; the chemical shift deviation from published spectra is noticeable but it is the next major lipid class reported to be present at a lower frequency than Pi. PG is one of the most abundant phosphate-containing glycerolipids in leaf non-polar extracts comprising between 28 and 36% of total leaf membrane phospholipids (Bonaventure et al., 2003; Devaiah et al., 2006). On average, excluding Pi (peak 1), peak 2 contributes 37% ($LP_{0.05}$) and 33% ($HP_{1.0}$) to spectral area; this agreement with *Arabidopsis* literature (Bonaventure et al., 2003; Devaiah et al., 2006) giving considerable credence to the identification. Furthermore, the distance between peak 2 and peak 3 in a given $LP_{0.05}$ and $HP_{1.0}$

spectrum is around 0.53 ppm. This is close to the chemical shift difference reported between PG and phosphatidylethanolamine (PE) of 0.47 ppm (Schiller et al., 2007) and 0.43 ppm (London & Feigenson, 1979). Given that the peaks in the *Thellungiella* spectra are much broader than those of an artificial lipid spectrum, the positional variation between peaks 2 and 3 is within an acceptable range (Dr. Bob Berno, personal communication).

Peak 3 is potentially PE. In addition to its positional relationship to PG, PE is the next major class of phospholipid to be found at a lower frequency among leaf phospholipids than PG (Diehl, 2001; London & Feigenson, 1979; Schiller et al., 2007; Vyssotski et al., 2009). In *Arabidopsis* leaf membranes, PE is the third-largest contributor to lipid composition generally representing between 20 to 25% of phospholipids (Bonaventure et al., 2003; Devaiah et al., 2006). Peak 3 is the third-largest contributor to spectral area ranging from 18% ($LP_{0.05}$) to 20% ($HP_{1.0}$) which suggests that the identification is correct.

The spectral results suggest that peak 5 is phosphatidylcholine (PC). In all published spectra, PC is found at the lowest frequency (i.e. the most negative ppm value between -0.55 and -0.65 ppm (London & Feigenson, 1979; Schiller et al., 2007; Vyssotski et al., 2009). In *Thellungiella* spectra, PC is probably present between -0.123 and -0.31 ppm. However, the chemical shift difference between PE and PC in these spectra (~ 0.55 ppm) is very close to that reported in the above references (~ 0.60 ppm). PC is one of the most abundant phosphate-containing glycerolipids. Of the phosphoplipids, it comprises between 31 to 34% of the total leaf phospholipids

(Bonaventure et al., 2003; Devaiah et al., 2006). After peak 1 (Pi) and peak 2 (PG), peak 5 (PC) makes the greatest contribution to total spectral area. On average, peak 5 contributes 30 % ($LP_{0.05}$) and 39% ($HP_{1.0}$) to spectral area (excluding the spectral area of Pi).

Finally, data indicate that peak 4 could be phosphatidylinositol (PI) the major lipid class immediately adjacent (at a higher frequency) to PC. PI resonates between -0.35 and -0.40 ppm (London & Feigenson, 1979; Schiller et al., 2007), which is at a lower frequency than the values reported here (-0.023 to -0.21 ppm). However, in the literature, PE and PI are separated by approximately 0.40 ppm (London & Feigenson, 1979; Schiller et al., 2007), which is in agreement with the value of 0.44 ppm obtained from the *Thellungiella* spectra. Peak 4 is the lowest peak with respect to contribution by area which corresponds to PI in *Arabidopsis* literature where it comprises between 7 to 14% of total phospholipids (Bonaventure et al., 2003; Somerville & Koornneef, 2002). *Thellungiella* $LP_{0.05}$ and $HP_{1.0}$ spectra show, on average, 14% and 7% contributed by peak 4, respectively. PI and PS are found in a similar spectral region but PS is present at much lower quantities than PI in *Arabidopsis* (13.9% vs 2.3% of total phospholipids) so it is unlikely that PS would be detected exclusive of PI (Bonaventure et al., 2003).

PI is a minor component of the leaf lipid membrane. PI can be phosphorylated by a PI-kinase to produce a phosphatidylinositolmonophosphate (PIP) which is, in turn, phosphorylated to produce phosphatidylinositolbisphosphates (PIP_2) (Meijer & Munnik, 2003). The PIP_2 lipid $PI(4,5)P_2$ is the precursor for many signalling molecules, including 1,4,5-triphosphate (IP_3) and diacylglycerol. PC has been observed to increase in times of

phosphate deprivation (Jouhet et al., 2003) and it can be hydrolyzed to produce DAG, a precursor to the galactolipids MGDG and DGDG that increase in abundance during phosphate stress. The observed accumulation of PI by $LP_{0.05}$ plants may represent an analogous transitory state in *Thellungiella*. Perhaps one of the more striking outcomes of growing *Thellungiella* plants at 0.05 and 1.0 mM phosphate is that the plants did not show a difference in shoot mass or morphology after five weeks. Thus any differences seen in lipid PC and PI in low versus high Pi appear to have been tolerated well by this plant.

In summary, ^{31}P-NMR is an analytical tool that can be used to more fully profile phospholipids in this species in order to provide a more comprehensive and dynamic picture of lipid alterations. These determinations, coupled with internal Pi assays, can link changes in the lipid profile with the internal Pi status of the plant providing a more complete picture of *Thellungiella*'s capacity to grow under different phosphate conditions.

Peak Quantification

NMR offers the benefit over some analytical tools in that it is an inherently quantitative technique (Jacobsen, 2007). The intensity of the signal is in direct proportion to the sample concentration/content; therefore, one mole of phosphorus will always be "seen" the same way. This stands in direct contrast to mass spectrometric methods where quantification must be targeted requiring identification of a chemical component and the extensive use of internal standards (Devaiah et al., 2006). For example, quantifying

phospholipids via mass spectrometric techniques requires the addition of multiple internal standards representing each major class of lipid to be quantified because the ionization efficiency varies by both by lipid class and experimental conditions. Ideally these standards should be non-naturally occurring phospholipids to avoid obscuring sample peaks and to ensure that an accurate concentration is being reported (Brugger et al., 1997).

As outlined in the Materials and Methods section, ERETIC is a convenient way to quantify the phospholipid species in a sample. Table 7 shows the lipid content of each *Thellungiella* sample in nmol·mg^{-1}DW. Similar to peak areas, it is clear that there is variation in phospholipid content both within treatments and across treatments. However, preliminary MANOVA of this subset of data showed no significant, treatment- or trial-related differences. However, differences are found when this data is compared to those reported in the *Arabidopsis* literature (Li et al., 2006). Mean phospholipid content expressed on a DW basis are consistently higher in *Arabidopsis* leaf tissue from 7 d old seedlings grown under 0.5 mM or 1.0 mM phosphate than in *Thellungiella* as summarized in Table 9. The phospholipid quantification results given for *Arabidopsis* in Table 9 are comparable to those reported in a cold-acclimation and freezing study that used well-fertilized 5-wk-old control (i.e. non-stressed) plants (Welti et al., 2002). For *Arabidopsis* PG is present at ca 23 nmol·mg^{-1}DW, PE at ca 12 nmol·mg^{-1}DW, PI at ca 5 nmol·mg^{-1}DW and PC at ca 23 nmol·mg^{-1}DW. These values are about an order of magnitude different between the reported *Arabidopsis* estimates and the values we obtained for *Thellungiella*. If this outcome is valid, *Arabidopsis* would contain

approximately 15 to 40 times more of any given lipid (depending on its class). Losses during extraction procedures may account for some of the discrepancies between *Thellungiella* phospholipid content and reported *Arabidopsis* content. The addition of an internal recovery standard can quantify this loss, allowing for adjustments during calculation of the lipid content during the various stages of preparation. However, a consistent recovery of ca 3%, as reported for here for *Thellungiella*, is highly unlikely given other extractions for lipids carried out in the lab (Table 7; Dr. Elizabeth Weretilnyk, personal communication). Moreover, our estimates for *Arabidopsis* phospholipid content are also anomalously low (see discussion below). Thus, a more likely explanation is that the ERETIC method for quantification underestimated the lipid content. The ERETIC peak is generated from a pure sample and is very narrow. When it is inserted into the sample spectrum, it is not subjected to the same noise-related line-broadening that the biological peaks are. Therefore, the ERETIC peak may not accurately quantify phospho-metabolite peaks. A solution could be to artificially broaden the standard peak in accordance with the width (more specifically, the full-width at half-max) of the largest peak (in this case, peak 1). In this way the standard peak can more accurately represent and quantify a true biological peak.

Application of the Protocol to Arabidopsis Leaf Extracts

Arabidopsis leaf tissue was subjected to the protocol of extraction, sample preparation and ^{31}P-NMR analysis developed to profile *Thellungiella* phospholipids. The *Arabidopsis* spectra (Figure 14) show narrow, clean resonances, comparable to those

achieved with twice the sample mass used in the preparation of spectra from *Thellungiella* tissues. Indeed, peak width is equal to that of *Thellungiella* spectra (data not shown). *Arabidopsis* spectra show four well-resolved peaks, corresponding to peaks 1, 2, 3 and 5 found in *Thellungiella* spectra (Table 8). These peaks have been tentatively identified earlier in this discussion as Pi, PG, PE and PC, respectively. Peak areas of the components of the spectra follow the same general pattern of contribution to total spectral area found in both *Thellungiella* (Table 8) and the aforementioned *Arabidopsis* literature (Bonaventure et al., 2003; Devaiah et al., 2006; Li et al., 2006). Lipid content in $nmol \cdot mg^{-1}DW$, however, is between 2- and 4-fold greater for phospholipid classes in *Arabidopsis* than in *Thellungiella* (Tables 7 and 8). Interestingly, phospholipid content is between 3- and 10-fold lower than is reported in the literature (Tables 8 and 9). This observation gives weight to the conclusion that ERETIC is not accurately quantifying phospholipids present in our samples. Peak widths, an indicator of SNR, are comparable in *Arabidopsis* and *Thellungiella* spectra. This implies that ERETIC is being applied to spectra of both species equally, without differentially impacting or being impacted by the SNR. Apparent qualitative differences in phospholipid content between the *Thellungiella* and *Arabidopsis* samples analyzed in this experiment therefore raise a question about what lipid class comprises the bulk of the membrane lipids of *Thellungiella*. Glycerolipids such as MGDG and DGDG are the predominant lipid in chloroplast membranes; however, phosphate starvation increases the proportion of galactolipids in the plasma membrane and induces expression of sulfolipid synthesis genes in *Arabidopsis* (Hartel et al., 2000; Li et al., 2006; Misson et al., 2005). Given that

Thellungiella is a naturally stress-tolerant organism that grows in extreme conditions, a greater basal contribution by non-phosphate glycerolipids to the plasma membrane may be an adaptation to low phosphate in the field. Investigating the galactolipid and sulfolipid content of *Thellungiella* membranes and introducing an internal standard to ^{31}P-NMR analyses of phospholipids would help address any outstanding questions regarding interspecies differences.

Immediately noticeable upon inspection of *Arabidopsis* spectra is the absence of the peak found in position 4. This peak was tentatively identified as PI in *Thellungiella*. While PI is reported to be a minor component of the plasma membrane, it constitutes 7 to 14% of the plasma membrane; our inability to detect this peak in *Arabidopsis* spectra is therefore unexpected (Devaiah et al., 2006; Li et al., 2006; Welti et al., 2002). The non-destructive nature of NMR means that samples can be taken to a mass spectrometry facility and re-analyzed. In addition to quantitative information, NMR can generate the *a priori* information required to perform targeted mass spectrometry that, in turn, helps validate peak predictions regarding identity and even indicates acyl chain composition. For this particular investigation, a precursor ion scan of *m/z* 241 could detect the presence of PI at levels that are undetected by NMR (Taguchi et al., 2005).

Table 9. Phospholipid content of *Thellungiella* leaves estimated in this study compared with published results for *Arabidopsis.* Values reported for *Thellungiella* are mean ± SE for six replicates over four trials. *Thellungiella* plants were fertilized weekly with 1.0 mM (High) or 0.05 mM (Low) phosphate and harvested at five wk. Values reported for *Arabidopsis* are mean ± SE of 5 replicates. *Arabidopsis* plants were grown on media containing 0.5 mM (High) or 0 mM (Low) and harvested at one wk. Dry weight was assumed to be 8% of fresh weight, based on Browse and Somerville (1993).

	External Phosphate Treatment			
	High		Low	
	Phospholipid Content *(nmol·mg^{-1}DW)*			
Phospholipid Class	*Thellungiella*	*Arabidopsis*[a]	*Thellungiella*	*Arabidopsis*[a]
PG	0.87 ± 0.1	29 ± 1.1	0.95 ± 0.4	21 ± 1.6
PE	0.46 ± 0.1	21 ± 0.6	0.54 ± 0.1	15 ± 1.1
PI	0.34 ± 0.1	6 ± 0.3	0.18 ± 0.1	5 ± 0.5
PC	0.79 ± 0.2	28 ± 0.8	1.11 ± 0.2	23 ± 0.5

[a]*Arabidopsis* data is taken from Li et al., (2006)

CONCLUSIONS

In conclusion, an important outcome of this research was the development and testing of a protocol for phospholipid profiling by ^{31}P-NMR. This protocol of lipid extraction and analysis is amenable to profiling lipids in *Thellungiella* and *Arabidopsis* and can be adapted, with some modification in terms of scans, to using samples of reduced mass. While NMR is not as sensitive as mass spectrometric methods, in this work it served as an exploratory tool to specifically profile phospholipids and its potential for quantification of lipids was tested. In conjunction with mass spectrometry, the next steps would involve confirming peak identifications and acyl chain compositions by mass spectrometry to fully exploit the power of NMR approaches. Together, these technologies could create a comprehensive lipid profile of *Thellungiella* under various environmental conditions. Through the use of phospholipid profiling, transcriptomics, and morphological observations, a more complete picture of *Thellungiella* as a stress-tolerant model organism can be assembled. Understanding the traits that confer tolerance to stresses such as phosphate deprivation will prove critical in improving the nutrient use efficiency of Canada's crop plants.

Appendix A – Full Univariate MANOVA Results

Complete univariate results for MANOVA on peak area as a percentage of total spectral area.

Between-Subjects Factors

		N
Treatment	1	9
	2	9
Trial	1	6
	2	4
	3	4
	4	4

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	1.000	65207.111[a]	5.000	6.000	.000
	Wilks' Lambda	.000	65207.111[a]	5.000	6.000	.000
	Hotelling's Trace	54339.260	65207.111[a]	5.000	6.000	.000
	Roy's Largest Root	54339.260	65207.111[a]	5.000	6.000	.000
Treatment	Pillai's Trace	.822	5.528[a]	5.000	6.000	.030
	Wilks' Lambda	.178	5.528[a]	5.000	6.000	.030
	Hotelling's Trace	4.607	5.528[a]	5.000	6.000	.030
	Roy's Largest Root	4.607	5.528[a]	5.000	6.000	.030
Trial	Pillai's Trace	1.212	1.084	15.000	24.000	.418
	Wilks' Lambda	.164	1.048	15.000	16.965	.459
	Hotelling's Trace	2.988	.930	15.000	14.000	.557
	Roy's Largest Root	2.097	3.355[b]	5.000	8.000	.063
Treatment * Trial	Pillai's Trace	1.262	1.162	15.000	24.000	.361
	Wilks' Lambda	.074	1.779	15.000	16.965	.127
	Hotelling's Trace	8.377	2.606	15.000	14.000	.041
	Roy's Largest Root	7.893	12.629[b]	5.000	8.000	.001

a. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level.

c. Design: Intercept + Treatment + Trial + Treatment * Trial

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	1.000	65207.111[a]	5.000	6.000	.000
	Wilks' Lambda	.000	65207.111[a]	5.000	6.000	.000
	Hotelling's Trace	54339.260	65207.111[a]	5.000	6.000	.000
	Roy's Largest Root	54339.260	65207.111[a]	5.000	6.000	.000
Treatment	Pillai's Trace	.822	5.528[a]	5.000	6.000	.030
	Wilks' Lambda	.178	5.528[a]	5.000	6.000	.030
	Hotelling's Trace	4.607	5.528[a]	5.000	6.000	.030
	Roy's Largest Root	4.607	5.528[a]	5.000	6.000	.030
Trial	Pillai's Trace	1.212	1.084	15.000	24.000	.418
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	Roy's Largest Root	2.097	3.355[b]	5.000	8.000	.063
Treatment * Trial	Pillai's Trace	1.262	1.162	15.000	24.000	.361
	Wilks' Lambda	.074	1.779	15.000	16.965	.127
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	Roy's Largest Root	7.893	12.629[b]	5.000	8.000	.001

a. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level.

c. Design: Intercept + Treatment + Trial + Treatment * Trial

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Peak1	359.482[a]	7	51.355	.545	.783
	Peak2	76.027[b]	7	10.861	1.025	.470
	Peak3	61.318[c]	7	8.760	.878	.555
	Peak4	211.782[d]	7	30.255	3.733	.030
	Peak5	395.213[e]	7	56.459	1.120	.421
Intercept	Peak1	44049.596	1	44049.596	467.850	.000
	Peak2	5400.668	1	5400.668	509.584	.000
	Peak3	1652.214	1	1652.214	165.694	.000
	Peak4	347.021	1	347.021	42.821	.000
	Peak5	5440.133	1	5440.133	107.938	.000
Treatment	Peak1	27.428	1	27.428	.291	.601
	Peak2	2.968	1	2.968	.280	.608
	Peak3	12.506	1	12.506	1.254	.289
	Peak4	62.448	1	62.448	7.706	.020
	Peak5	130.570	1	130.570	2.591	.139
Trial	Peak1	170.865	3	56.955	.605	.627
	Peak2	27.337	3	9.112	.860	.493
	Peak3	23.606	3	7.869	.789	.527
	Peak4	39.661	3	13.220	1.631	.244
	Peak5	134.140	3	44.713	.887	.481
Treatment * Trial	Peak1	166.635	3	55.545	.590	.635
	Peak2	43.207	3	14.402	1.359	.311
	Peak3	20.572	3	6.857	.688	.580
	Peak4	82.753	3	27.584	3.404	.061
	Peak5	106.433	3	35.478	.704	.571
Error	Peak1	941.532	10	94.153		
	Peak2	105.982	10	10.598		
	Peak3	99.715	10	9.971		
	Peak4	81.040	10	8.104		
	Peak5	504.003	10	50.400		

Total	Peak1	46385.322	18			
	Peak2	5833.915	18			
	Peak3	1898.886	18			
	Peak4	676.738	18			
	Peak5	6403.916	18			
Corrected Total	Peak1	1301.014	17			
	Peak2	182.009	17			
	Peak3	161.033	17			
	Peak4	292.822	17			
	Peak5	899.216	17			

a. R Squared = .276 (Adjusted R Squared = -.230)

b. R Squared = .418 (Adjusted R Squared = .010)

c. R Squared = .381 (Adjusted R Squared = -.053)

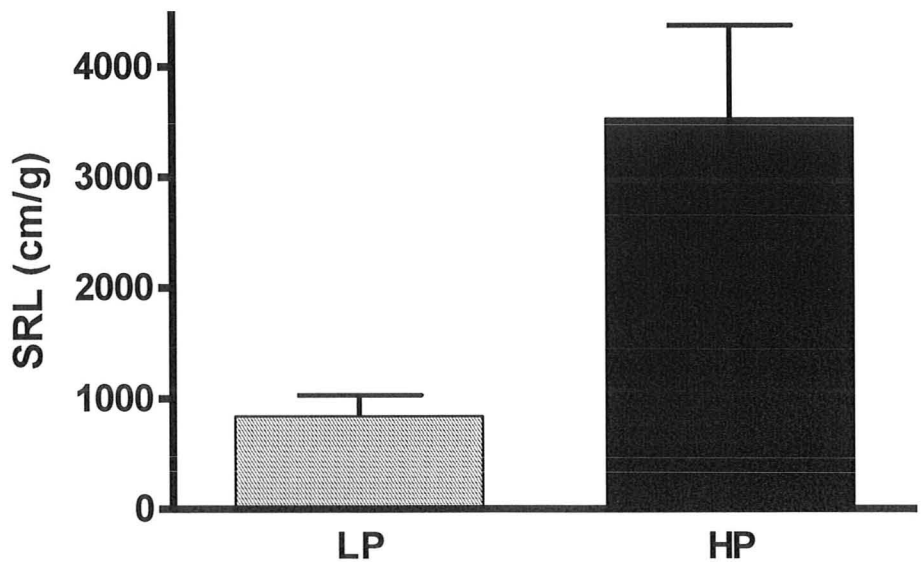
d. R Squared = .723 (Adjusted R Squared = .530)

e. R Squared = .440 (Adjusted R Squared = .047)

Appendix B – Specific Root Length

Specific root length, reported as $cm \cdot g^{-1}$, was calculated using the total primary root length and total root fresh weight at harvest for each trial, and reported as the average across a minimum of three trials ± SE. Specific root length is significantly higher for $HP_{0.5}$ plants ($P = 0.0383$).

	Specific Root Length (cm/g)	
Trial	$LP_{0.05}$	$HP_{0.5}$
1	N/A	3498.45
2	1215.66	5070.92
3	707.62	4347.91
4	585.89	1202.34
Overall Mean	836.39 ± 192	3529 ± 839



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