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Phylogenetic analysis of NADP-malic enzyme and its expression in *Arabidopsis thaliana* guard cells

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Abstract

Decreased crop yield as a result of insufficient freshwater supply is a distressing problem in world agriculture today. Plants lose water via transpiration through pores located in the epidermis on the underside of the leaf referred to as stomata. The aperture of such a pore is regulated by two adjacent guard cells that swell and subside as water follows ions that are pumped into and from the cell in response to changing environmental conditions. Our hypothesis that one of the six NADP-malic enzyme isoforms in *Arabidopsis thaliana* is guard-cell specific and plays a role in the mechanism of closing the stomata was tested by isolating guard cell mRNA from *A. thaliana* and determining the relative expression of the six NADP-ME isoforms in whole leaves and guard cell protoplasts via RT-PCR. The optimization of a guard cell protoplast isolation protocol allowed for the use of very pure mRNA in these experiments. Our results suggested that all six of the NADP-ME isoforms in *A. thaliana* are expressed in whole leaves, and that but at2g19900 are expressed in the guard cell protoplasts. There was no evidence of a guard cell-specific isoform. Additionally, we conducted a phylogenetic analysis of malic enzyme sequences among plant species in order to test the hypothesis that the C4 form of malic enzyme evolved independently from the forms found in C3 plants. This hypothesis was supported by our preliminary phylogenetic analysis. 3

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PHYLOGENETIC ANALYSIS OF NADP-MALIC ENZYME AND ITS
EXPRESSION IN *ARABIDOPSIS THALIANA* GUARD CELLS

by

Erin Horning

A Senior Thesis Submitted to the

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ABSTRACT

Decreased crop yield as a result of insufficient freshwater supply is a distressing problem in world agriculture today. Plants lose water via transpiration through pores located in the epidermis on the underside of the leaf referred to as stomata. The aperture of such a pore is regulated by two adjacent guard cells that swell and subside as water follows ions that are pumped into and from the cell in response to changing environmental conditions. Our hypothesis that one of the six NADP-malic enzyme isoforms in *Arabidopsis thaliana* is guard-cell specific and plays a role in the mechanism of closing the stomata was tested by isolating guard cell mRNA from *A. thaliana* and determining the relative expression of the six NADP-ME isoforms in whole leaves and guard cell protoplasts via RT-PCR. The optimization of a guard cell protoplast isolation protocol allowed for the use of very pure mRNA in these experiments. Our results suggested that all six of the NADP-ME isoforms in *A. thaliana* are expressed in whole leaves, and that but at2g19900 are expressed in the guard cell protoplasts. There was no evidence of a guard cell-specific isoform. Additionally, we conducted a phylogenetic analysis of malic enzyme sequences among plant species in order to test the hypothesis that the C4 form of malic enzyme evolved independently from the forms found in C3 plants. This hypothesis was supported by our preliminary phylogenetic analysis.

INTRODUCTION

Decreased crop yield as a result of insufficient freshwater supply is a vastly distressing problem in world agriculture today. Approximately 70% of the fresh water available globally is used for agricultural purposes, providing about 40% of the total food that is produced world-wide. Much present-day irrigation is done using finite aquifer resources, a practice that will soon lead to increased dependence on other freshwater sources (Somerville and Briscoe, 2001). Insufficient water supply may impose large-scale reductions in crop yield and limit crop expansion outside of areas currently used for agricultural purposes in the near future (Chaves and Oliveira, 2004). This poses a major problem, as the world food requirement is expected to double with an increase in population of 2.5 billion people by the year 2020 (Somerville and Briscoe, 2001).

Stomata are pores located in the epidermis on the underside of plant leaves. These pores form the interface between the atmosphere and the leaf, and serve as the passageway through which the plant exchanges gases, including oxygen, carbon dioxide, and water, with the environment. Each pore is encircled by a pair of guard cells that swell and contract in response to changing light, water availability, temperature, the CO_2 availability, and hormonal stimuli (Shope *et. al.*, 2003). Plants lose water in excess via transpiration through their stomata. Guard cell activity effectively regulates stomatal aperture in response to environmental conditions, allowing the plant to maximize the intake of carbon dioxide for use in photosynthesis while minimizing water loss.

Irrigation systems have been established for crops in order to prevent desiccation-induced growth inhibition and plant death (Somerville and Briscoe, 2001). Under usual irrigation conditions, crops perceive the condition of constant water surplus, consequently taking no measures to restrict water loss through the stomata. Although somewhat more efficient crops have been obtained through selective breeding, research is conducted with haste in order to toward discover other mechanisms with which to alter guard cell response as a result of rapidly depleting freshwater sources.

In the past, agricultural advances were designed around favorable environmental conditions, focusing on drought-tolerance: the capacity to adjust to dry conditions (Laporte *et. al.*, 2002). Today, sub-optimal conditions must be considered as the global climate trends toward more arid conditions, with higher temperatures and irradiance, that may accompany global warming (Chaves and Oliveira, 2004). A drought-avoidant phenotype that allows for the conservation of water throughout plant development is desired (Laporte *et. al.*, 2002).

The mechanism by which the guard cells contract relies on osmotically generated turgor pressure. It has been known for over half a century that the concentration of potassium ions in guard cells increases as the stomata open and that specialized pumps transport K^+ ions into the cell. More recently, however, the amount of K+ being shuttled into the guard cell has been quantified. 5-73% of the net positive electrical influx has been found to be balanced by chloride ions that diffuse into the cell in multiple plant species (Outlaw and Lowry, 1977). The rest of the cation uptake is balanced by organic anions. A predominant organic

anion in guard cells is malate, a dicarboxylic acid that is synthesized in the cytosol of guard cells from starch that is stored in the chloroplasts as catalyzed by phospho*enol*pyruvate carboxylase. Malate is stored in the guard cell vacuole during stomatal opening (Outlaw *et. al*., 1981). The increased osmotic pressure in the guard cell that forms as a consequence of this ion relocation results in the influx of water, instigating an increased cell volume and turgor pressure (Outlaw and Lowry, 1977). The cell wall that faces the stomatal pore is thicker and less elastic than that found away from the pore. This difference in rigidity causes the guard cells to bow away from one another as they swell, thereby increasing the aperture of the stomatal pore (Jones *et. al.*, 2003).

To close the stomatal pore, potassium ions must first be transported out of the cell from the cytoplasm. Chloride ions then follow via facilitated diffusion. The fate of organic anions such as malate, however, is currently unclear (Outlaw *et. al.*, 1981, Laporte *et. al., 2001*). Evidence exists that supports the hypothesis that malic enzyme located in guard cells and neighboring subsidiary cells facilitates the digestion of malate during stomatal closure. As ions are removed from the guard cell, water exits via osmosis and the cell becomes more flaccid. This change in conformation causes the guard cells to further cover the stomatal pore (Shope *et. al.*, 2003).

NADP-malic enzymes (NADP-MEs) comprise a group of proteins that catalyze the oxidative decarboxylation of L-malate to pyruvate, CO_2, and NADPH in the presence of a divalent cation. NADP-malic enzymes are frequently encountered in nature as a result of pyruvate, CO_2, and NADH/NADPH being

required participants in a large number of metabolic pathways. NADP-MEs are known to participate in C_4 and CAM photosynthesis. Other biological functions, however, remain largely unknown at this point, although many speculations- from involvement in lipid biosynthesis to plant defense to daytime guard cell closure- have been proposed and supported (Wheeler *et. al.*, 2005).

In a recent study (Laporte *et. al.*, 2002), the expression maize NADP-ME in tobacco resulted in different stomatal activity and water use. Maize NADP-ME-transformed tobacco plants exhibited decreased stomatal conductance while gaining more fresh mass per unit water consumed than the wild type did. The transformed tobacco plants also experienced growth and a rate of development that was comparable to the wild type plants. The fact that the expression of maize NADP-ME likely changed the metabolism of malate in tobacco guard cells, and that water was conserved in the production of equal plant mass, suggests that malic enzyme may be capable of playing a role in breaking down malate. The behavior of the transgenic tobacco plants suggests that malic enzyme may play a role in the mechanism of stomatal closure. It has been hypothesized that this role involves either 1) decreasing the intracellular concentration of malate in guard cells, consequently reducing the ion concentration and therefore the osmotic potential, or 2) increasing the partial pressure of CO_2 active in the stomatal cavity, which is known to trigger stomatal closure (Laporte *et. al.,* 2002).

The hypothesis that it is possible to increase water-use efficiency by coaxing guard cells to decrease their aperture despite optimal conditions through the over-expression of NADP-ME is currently being addressed using *Arabidopsis*

thaliana, a C3 dicotyledonous plant. Six NADP-ME genes have been identified in *Arabidopsis thaliana* based on sequence similarity to the maize Me1 gene, as well as the occurrence of NADP-ME motifs in the sequences as determined by Pfam and Prosite (M. Laporte, personal communication). The six isoforms are at1g79750, at2g13560, at2g19900, at4g00570, at5g25880 and at5g11670. Four of these isoforms have been independently identified as such by Wheeler *et. al.* (2005) (at1g79750, at2g19900, at5g25880, and at5g11670).

Although the genetic resources available for *Arabidopsis thaliana* are well-developed and make it desirable as a model organism for the study of guard cell processes, it is very difficult to isolate sizable, pure preparations of guard cells from this species (Pandey *et. al,* 2002). A reason for this is that as a result of the inherent characteristic of stomatal development and spacing (Zhao and Sack, 1999), guard cells make up only a very small fraction of the total cells found in the leaf. *Arabidopsis thaliana* has a considerably different tissue texture than similar plant species, and epidermal peels consistently have considerable amounts of mesophyll cells adhering to them (Pandey *et. al.,* 2002). We have modified a same-day, small-scale guard cell isolation protocol devised by Pandey *et. al*. (2002) and Leonhardt *et. al*. (2004) for the purpose of isolating pure preparations of guard cells for this study of NADP-ME expression.

The function of the six NADP-ME isoforms in *Arabidopsis thaliana* is largely unknown. We are completing multiple experiments in order to determine the role of those genes in normally functioning *Arabidopsis thaliana* plants for the

purpose of gaining an understanding of how genetically altering NADP-ME in the plants would decrease water loss. Our goals are:

1) to determine where and to what degree each NADP-ME isoform is expressed in the plant. This first experiment is completed by isolating tissues from various parts of the plant, specifically the whole leaf and the guard cells, and performing RT-PCR and qualitatively analyzing reaction products. We have hypothesized that there is one form of NADP-ME that is expressed only in guard cells.

2) to investigate how the six *Arabidopsis thaliana* NADP-ME isoforms are related to malic enzyme sequences in other species of the plant kingdom through phylogenetic analysis.

METHODS

Isolation of *Arabidobsis thaliana* Guard Cell Protoplasts (GCPs): same-day, small-scale isolation protocol.

This protocol was modified from a protocol originally developed by Pandey *et. al*. (2002) with transcription inhibitors included as suggested in a protocol developed by Leonhardt *et. al* (2004). The inclusion of the transcription inhibitors actinomycin D and cordycepin in order to minimize the induction of stress-induced gene transcription during the extended isolation protocol is optional.

The protoplast isolation solutions were as follows:

Basic solution: One small-scale protocol requires 300ml, which consists of 5 mM MES hydrate, 0.5mM $CaCl_2$, 0.5mM $MgCl_2$, 10μM KH_2PO_4, 0.5 mM L-ascorbic acid, 0.01% cordycepin (Sigma), 0.0033% actinomycin D (Sigma), and the osmolarity is adjusted to 550 mmol/kg by addition of D-sorbitol. pH to 5.5 using Tris base.

Enzyme solution 1: One small-scale protocol required 15ml, which consisted of 0.7% cellulysin cellulase *Trichoderma viride* (Calbiochem) , 0.1% (w/v) PVP-40, 0.25% (w/v) bovine serum albumin, 0.5mM L-ascorbic acid, 0.1% cordycepin, and 0.0033% actinomycin D, all dissolved in 55% (v/v) basic solution and 45% (v/v) distilled water.

Enzyme solution 2: One small-scale protocol required 15ml, which consisted of 1.3% (w/v) Onozuka RS cellulase (Research Products International Corp.), 0.0075% (w/v) Pectolyase Y-23 (Sigma), 0.25% (w/v) bovine serum

albumin, 0.5mM L-ascorbic acid, 0.1% cordycepin, and 0.0033% actinomycin D in basic solution. The pH of this solution was reduced to pH 2.5 for 5 minutes with HCl just prior to use. The pH is then raised to pH 5.5 using KOH.

Twenty fully expanded leaves were harvested from the base of the plant. The leaves were placed in approximately 100ml of cold tap water after excision of the midvein with a razorblade, and blended twice for 30 s in a Waring blender. The mixture was poured through a 200μm Spectrum® nylon filter (Spectrum Laboratories, Inc.) in an apparatus attached to an aspirator to remove broken mesophyll and epidermal cells. Any large green specks of mesophyll tissue were removed from the filter with forceps. All remaining peels were thoroughly rinsed with deionized water until the foam produced during the blending step was gone. Using a spatula, all peels were scraped from the mesh into a flask containing enzyme solution 1 to be shaken at 27°C, 140rpm, for 40 minutes in darkness. After 40 minutes, 37.5ml of basic solution was added to the mixture, and it was shaken under the same conditions for five minutes to provide an intermediate osmolarity between enzyme solutions 1 and 2 and prevent sudden plasmolysis of fragile guard cells.

The digested peels were once again poured through a fresh 200μm Spectrum® nylon filter in the same apparatus as before, and rinsed with 10-15ml of basic solution. The peels were scraped from the mesh with a spatula into a flask containing enzyme solution 2. The flask was shaken at 20°C, 60rpm, for 40 minutes in darkness, then again at 20°C, 40rpm, for 55 minutes in darkness. The

flask was swirled by hand for several seconds upon completion of the final digestion step in order to better the liberation of GCPs from the peels.

The contents of the flask were poured through a 20μm Spectrum® nylon filter in the same apparatus as before. Filters of this size were used in order to allow only the guard cell protoplasts to flow through into the filtrate. The peels that remain on the filter were rinsed with 150ml of basic solution as a final attempt to release GCPs from them. The filtrate contained the GCPs. It was at this time that the GCPs were most readily viewed under an Olympus BH-2 microscope at 1000X magnification. When this microscopic view revealed excessive debris or mesophyll presence, further filtration may be desired using a 10μm Spectrum® nylon filter.

The filtrate was collected in four 50ml centrifuge tubes, and centrifuged for five minutes at 1000 **g**. All but 1-1.5ml of supernatant was removed using a pipette, as the pellet tends to be quite fragile. The pellet in each tube was resuspended, 20-25ml of basic solution was added to each tube, and an identical centrifugation step is performed. All but 1-1.5ml of supernatant is removed with a pipette. The 4-8ml that remains was combined and evenly distributed between 1.5ml Eppendorf microcentrifuge tubes and centrifuged again for five minutes at 1000 **g**. All but about 0.5ml of supernatant was removed using a pipette, and the pellet was once again resuspended and combined until all resuspended pellet fits into one 1.5ml Eppendorf microcentrifuge tube. This tube was centrifuged for five minutes at 1000 **g**, all supernatant was removed, and the pellet was flash-

frozen by submerging the closed tube in liquid nitrogen. For long-term storage, the protoplasts may be placed in a -80°C freezer.

The frozen protoplast pellet was ground using a mortar and pestle on liquid nitrogen. The Qiagen® RNeasy Plant Mini Kit was used following the manufacturer's directions in the third edition RNeasy Mini Handbook for isolation of RNA from plant and fungi beginning at step 3 with the introduction of ground protoplast material to a solution containing buffer RLT and β-Mercaptoethanol. DNase digestion with the RNase-Free DNase set is completed following the directions on page 99 of the Handbook.

50-100mg of whole *A. thaliana* leaves were placed in an Eppendorf tube and submerged in liquid nitrogen. RNA was extracted from the leaves following the same protocol as above. All extracted RNA was quantified using the Beckman Coulter DU-530 spectrophotometer to measure the absorbance at 260nm an 280nm.

Isolation of GCPs: overnight, small-scale isolation protocol.

This protocol was modified from a protocol originally developed by Pandey *et. al.* (2002). The basic solution was prepared as it was in the same-day, small-scale isolation, above. 25mL of enzyme solution was prepared containing the following: 0.65% Onozuka RS cellulase (Research Products International Corp.), 0.35% Macerozyme R10 (Research Products International Corp.), 0.25% BSA and 0.001% kanamycin.

The leaves were harvested and blended as described for the same-day, small-scale isolation. The blended mixture was poured through a 200μm Spectrum® nylon filter and rinsed first with deionized water, then basic solution. The peels were transferred to a flask containing the enzyme solution, and were shaken at $22^{o}C$ at 44rpm for 12-13 hours. The shaken solution was then poured through a 20μm Spectrum® nylon mesh and processed as described for the same-day, small-scale isolation from this point onward.

Growth of sterile plants in nutrient-rich media.

MS media (Murashige and Skoog, 1962) including 0.8% agar is prepared and poured into magenta boxes to a depth of approximately 1 inch under a laminar flow hood. Wild type *Arabidopsis thaliana* seeds are sterilized in a 20% bleach solution for twenty minutes. Seeds are rinsed with autoclaved water under the laminar flow hood just prior to planting. 3-4 seeds are equally spaced on the surface of the MS media using sterile forceps under the laminar flow hood. Magenta boxes are closed before removal from the hood. Boxes are placed under constant light conditions until harvesting leaves for the same-day, small-scale isolation, above.

Growth of plants in Conviron E-15 chambers.

Plants were grown in soil in Conviron E-15 chambers. The light level in the chambers was about 500 μE m^{-2} s^{-1} and the relative humidity was over 60%.

The day and night temperatures were 22°C and 16°C, respectively, and the day length was 12 hours light and 12 hours dark.

Optimization of reverse transcription-polymerase chain reactions.

Primers that flanked one or two introns were designed for each of the six NADP-ME isoform loci (table 1). All RT-PCR was assembled using Promega AccessQuick RT-PCR System. Reagent concentrations and reaction conditions were adjusted until each RT-PCR resulted in only one expected band. The amount of RNA used in each reaction was kept constant (5ng RNA), as was the amount of AMV Reverse Transcriptase (5 units) and AccessQuick Master Mix (1X). Additionally, six steps of the RT-PCR program remain constant between loci, and are as follows:

1) 48°C for 45 minutes

2) 94°C for 2 minutes

3) 94°C for 15 seconds

4) Varies

5) 72°C for 45 seconds

6) Varies

7) 68°C for 7 minutes

8) 4°C forever

LOCUS	Left Primer	Right Primer	Reaction Conditions
At5g11670	TCCGAGAT ACAACAAG GGACT T 0.2μm	CTTCCTGA AAATGCTC CCATAC 0.2 μm	4) 61°C for 1 minute 6) 33 times to 3
At5g25880	GAGGCCAC AAGGTCTTT ACATC 0.8 μm	TGTTTGTA CCCACATC AATGGT 0.8 μm	4) 64.5°C for 1 min 6) 33 times to 3
At4g00570	CCGCAGGT AGATATGA TTGTCA 0.4 μm	GGTACTCC TCTCCTTCC AACCT 0.4 μm	4) 63.5°C for 1 min 6) 28 times to 3
At2g19900	GGGGACAG CATCAGTTG TTT 0.2 μm	CAGATTTT CTTGCGGC TTTC 0.2 μm	4) 59.9°C for 1 minute 5) 44 times to 3
At2g13560	TCTTTTAT CTGGTGCT CCCATT 0.2 μm	CAAAGTCG GGTACTCT GGATTC 0.2 μm	4) 60.5°C for 1 minute 6) 33 times to 3
At1g79750	CATCCAGG TCATTGTT GTCACT 0.2 μm	CCATAGTT CTGCTTGA CTGCTC 0.2 μm	4) 61.5°C for 1 minute 6) 35 times to 3

TABLE 1: Unique reaction concentrations and reaction conditions for each of the

six *Arabidopsis thaliana* NADP-ME loci.

A 1% agarose gel was assembled using 0.5X TBE buffer. 5µl of RT-PCR product with 1 µl of 6X load dye was loaded into each well. The positive and negative control RT-PCR products were run on separate gels. Each gel was run at 100V.

Preliminary phylogenetic analysis of NADP-malic enzyme.

An unrooted strict consensus of 31 most parsimonious trees was found by performing a parsimony analysis of malic enzyme sequences. A BLAST search against maize malic enzyme was conducted in order to search for homologous sequences within the kingdom Viridiplantae. Homologous sequences were analyzed with BLAST 2 Sequences (www.ncbi.nlm.gov/blast/bl2seq/wblast2.cgi) in order to remove duplicate or nearly duplicate sequences. Sequences were also included based on their bit score. Homologous sequences were aligned in ClustalW (www.ebi.ac.uk/clustalw/). Analysis of the aligned sequences by eye using ClustalW allowed for the detection of signal sequences in some of the alignments. TargetP (www.cbs.dtu.dk/services/TargetP/) was used in order to identify chloroplast and mitochondrial signal sequences. These sequences were removed and resulting sequences were once again aligned in ClustalW. Aligned sequences were exported to PAUP* (Swofford, 2001) for parsimony analysis. Bootstrap analysis was performed using 500 bootstrap replicates and 100 random addition replicates.

RESULTS

Guard cell protoplast isolations were attempted under multiple conditions, the resultant 260/280 absorbance and RNA yield of which have been tabulated (Table 2). Isolation 1 was conducted following an overnight protocol that required the shaking step with a hybrid of enzyme solution 1 and 2 be extended to 8-10 hours. This isolation resulted in a relatively large amount of impure RNA. Under 1000X inspection, only approximately 80% of the guard cell protoplasts were intact. Of that 80%, many of them were flaccid. Debris included broken protoplast fragments and bacteria, as well as other plant tissues, including mesophyll cells that fit through the 20μm filter perhaps due to their flaccid state. Isolation 2, 3, and 4 followed the same-day, small-scale isolation protocol discussed above, however transcriptional inhibitors Actinomycin D and Cordycepin were included only in Isolation 4. Isolation 2 was conducted three times following identical procedures. Each time, leaves from sterile plants that were grown in MS media under constant light were used. Average values are presented in table 2. Sterile growth in media consistently resulted in protoplast isolations with very few guard cell protoplasts (microscopic views reveal a number of protoplasts approximately 20% of what was seen in Isolation 1) and, subsequently, very little RNA. Isolation 3 was conducted using leaves from plants grown in soil under long-day conditions. These plants yielded the more total RNA than Isolations 1 and 2. When viewed under the microscope, approximately 95% of the protoplasts present were unlysed and the only debris present was protoplast fragments and bacteria.

Isolation 4 was conducted using leaves grown form plants grown in soil under short-day conditions. These plants appeared to be the healthiest of any that had been used in the previous isolations. The same-day, small-scale guard cell protoplast isolation protocol conducted during Isolation 4 resulted in individual spherical protoplasts as was visible by inspection under 1000X magnification (figure 1). Approximately 95% of the guard cell protoplasts present at the end of the protocol were unlysed. Debris in the final preparation included fragments from lysed protoplasts and bacteria. Debris appeared to be minimal during inspection. This isolation resulted in the most guard cell RNA as well as the best 260/280 ratio. The results of this protocol were more comparable to those of the overnight protocol used in Isolation 1 than any of the other over-day protocols.

Variation existed in the location of expression between the six NADP-ME isoforms in *Arabidopsis* thaliana, as was visible in a 1% agarose gel of the RT-PCR products that illustrated the relative expression of the six isoforms in the *Arabidopsis thaliana* whole leave and guard cell protoplast (figure 3). The expected band for the At5g11670 locus, at 297bp, was very bright. Through inspection by eye, it appeared that the band in lane 2 (whole leaf) was brighter than that in lane 3 (GCP). All other bands for this locus appeared in the negative control reactions (figure 4).

The expected band size for at5g25880 was 241bp. On the gel, these bands appeared to be equally bright in both whole leaf and GCP reactions. All other bands for this locus appeared in the negative control reactions. The expected band size for at4g00570 was 212bp. The band in the whole leaf RT-PCR reaction

appeared to be slightly brighter than that in the GCP reaction. There was no negative control included for this locus in this report. No additional bands appeared in the lanes containing the products for these reactions.

The expected band size for at2g19900 was 185bp. There did not appear to be a band of this size in the reaction assembled using GCP RNA (lane 9). There was some banding in the negative control reactions for this locus that did not appear in the reactions containing RNA. The expected band size for at2g13560 was 291bp. The band at this length was much brighter in the reaction containing whole leaf RNA than in the reaction containing GCP RNA. There was a second, lighter band at approximately 260bp in lane 10 that did not appear in the negative control reaction. Another band at approximately 125bp was present in lane 11 that does not appear in the negative control.

The expected band size for at1g79750 was 278bp. The band at this size was much brighter in the lane 12, with the reaction containing whole leaf RNA, than in lane 13, with the reaction containing GCP RNA. Another band was present at approximately 240bp that was equally bright in both lanes. These bands did not appear in the negative control reactions.

A rectangular unrooted phylogenetic tree of NADP-ME in plants that is a strict consensus of 31 most parsimonious trees was constructed (figure 5). The six *Arabidopsis thaliana* isoforms were scattered throughout the tree. The form of malic enzyme found in C_4 plants, including *Zea mays* and *Sorghum bicolor*, were found in a clade together. This clade was relatively well supported with bootstrap values of 91 and 96 over a pair of the branches. The clade containing

multiple Flavaria species includes C_3, C_4, and C_3-C_4 intermediate plants. An

unrooted circular phylogenetic tree (figure 6) showing data from the same

analysis allows for better visualization of individual clades.

FIGURE 1: Isolated guard cell protoplasts as viewed at 1000X magnification. Protoplasts were isolated following a same-day, small-scale isolation protocol with transcriptional inhibitors adapted from methods developed by Pandey et. al. (2002) and Leonhardt et. al. (2004). Approximately 95% of the protoplasts were intact at the end of the protocol. Debris included broken protoplast fragments and bacteria. No mesophyll cells were observed in the final preparation.

	260nm/280nm ratio	Concentration RNA (ng/µl)
Isolation 1	2.091	144.4
Isolation 2	1.206	23.54
Isolation 3	2.033	192.0
Isolation 4	2.111	272.8

TABLE 2: 260nm/280nm absorbance ratios and concentrations of RNA extracted from guard cell protoplasts using the Qiagen® RNeasy Plant Mini Kit as measured using the Beckman Coulter DU-530 spectrophotometer. Isolation 1 was conducted following a modified overnight protocol that required 8-10 hours of total shaking time. Isolations 2-4 were conducted following the same-day, small-scale protocol outlined above. The tabulated values for Isolation 2 are averages from three isolations conducted using plants grown under sterile conditions in MS media that yielded similar results. Isolation 3 was conducted using plants grown in soil under long-day growth conditions. Isolation 4 was conducted using plants grown in soil under short-day growth conditions.
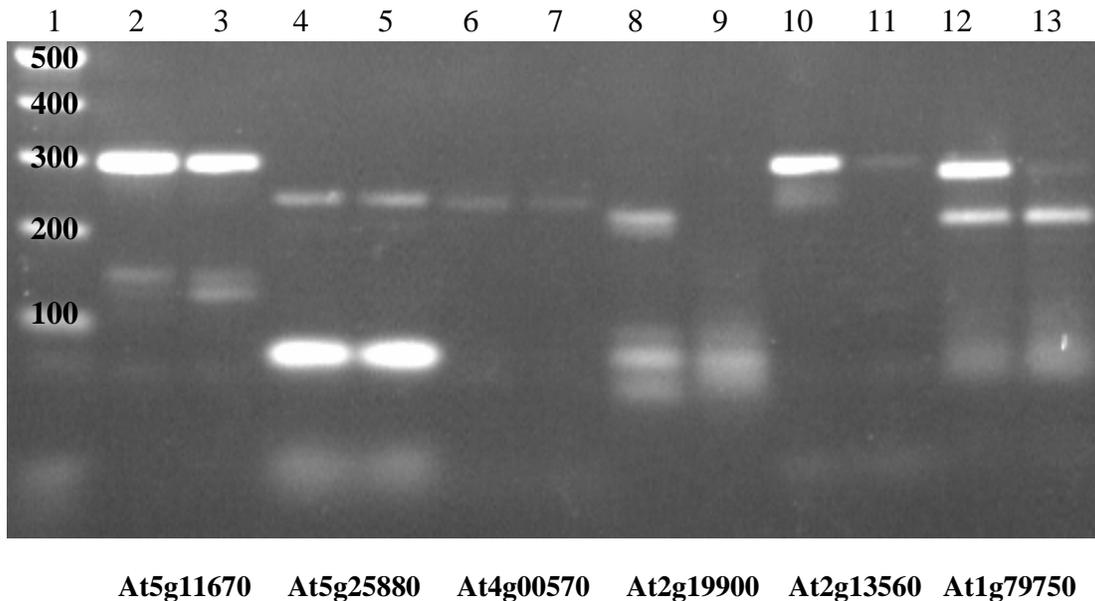
FIGURE 2: Results of an RT-PCR experiment in 1% agarose gel to determine the

relative expression of the six putative NADP-ME genes in *Arabidopsis thaliana*

guard cells and whole leaves. For each locus, the whole leaf reaction is pictured

on the left and the guard cell protoplast reaction is pictured on the right. Five ng

of total RNA was used for each RT-PCR reaction. The expected product sizes

for amplification from mRNA (listed first) and genomic DNA (listed second) for

each locus are as follows: At5g11670 294bp, 437bp; At5g25880 241bp, 384bp;

At4g00570 212bp, 628bp; At2g19900 185bp, 419bp; At2g13560 291bp, 399bp;
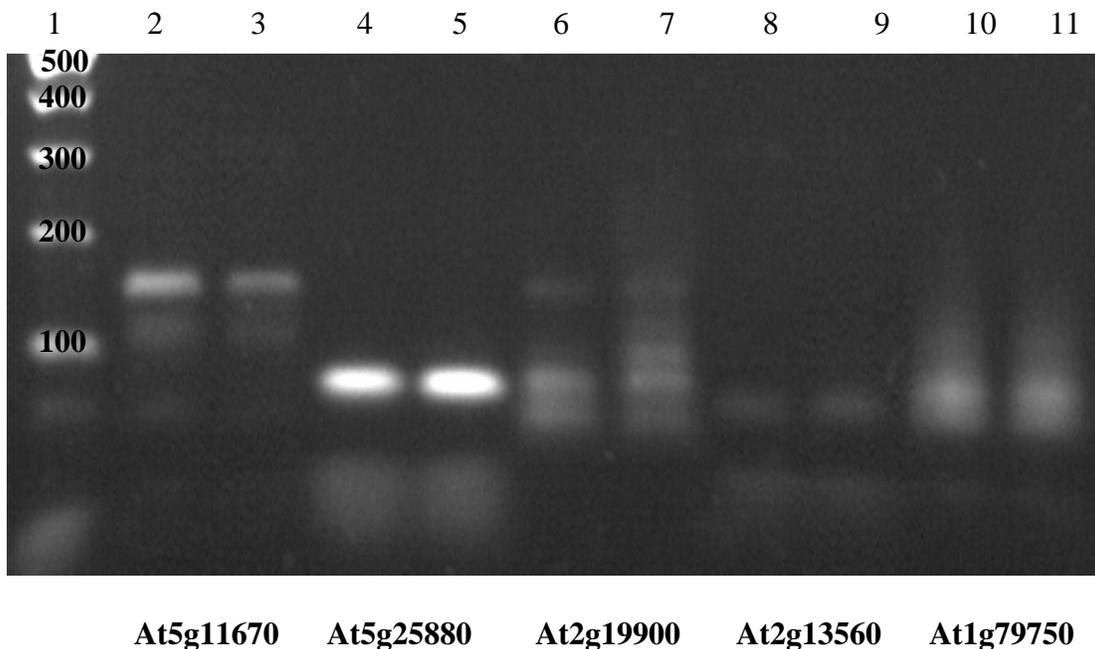
At1g79750 278bp, 512bp.

FIGURE 3: Negative control reactions (RNA template excluded) in 1% agarose gel for the RT-PCR conducted to determine the relative expression of the six NADP-ME isoforms in *Arabidopsis thaliana* whole leaves and guard cells. For each locus, the whole leaf negative control reaction is pictured on the left, and the guard cell protoplast negative control reaction is pictured on the right.
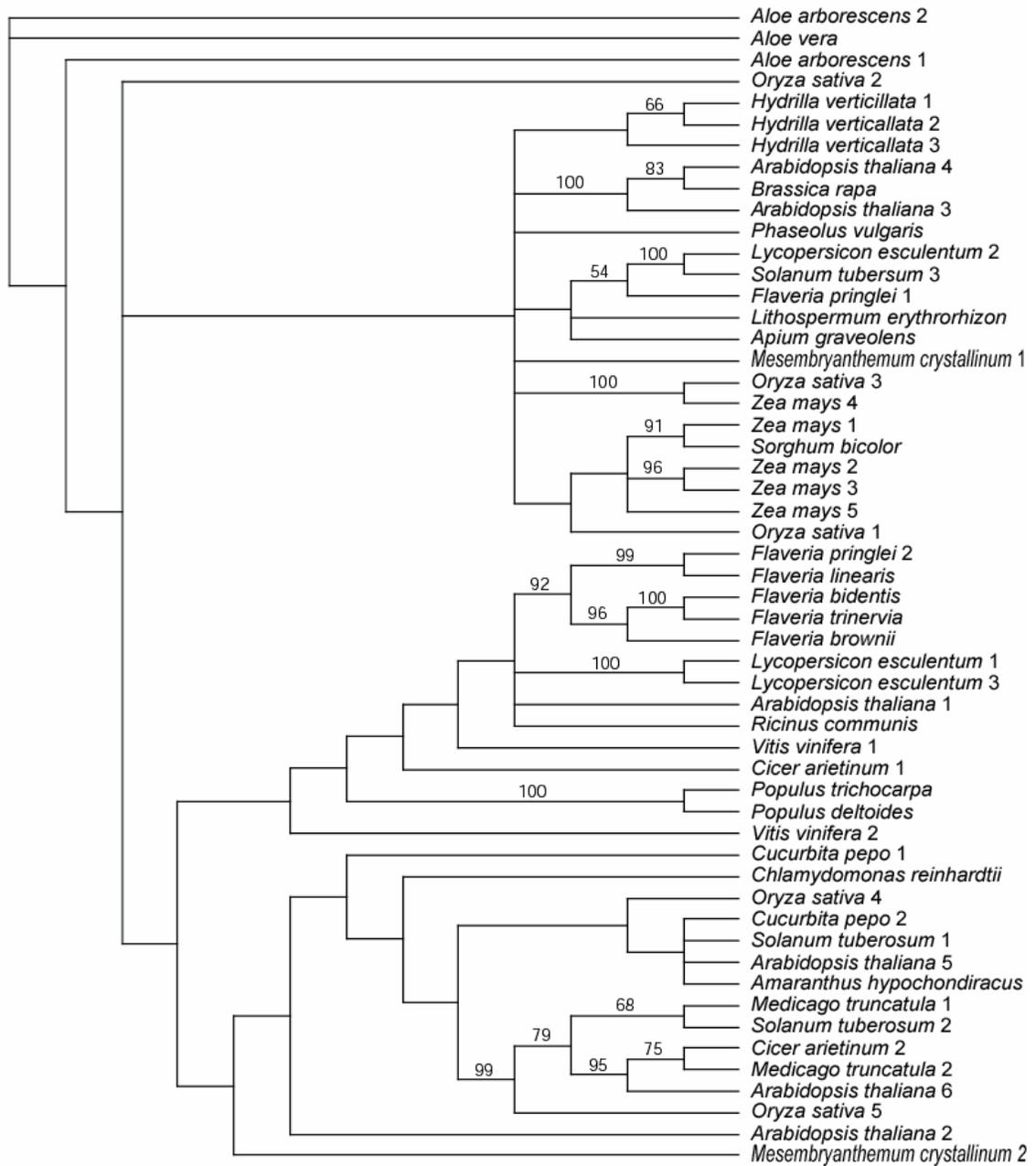
FIGURE 4: Rectangular unrooted phylogenetic tree of plant NADP-ME. A strict consensus of 31 most parsimonious trees was found using parsimony analysis of malic enzyme sequences. Bootstrap analysis was performed using 500 bootstrap replicates and 100 random addition replicates. Bootstrap values greater than 50% are found above the branches. C.I.= 0.598.
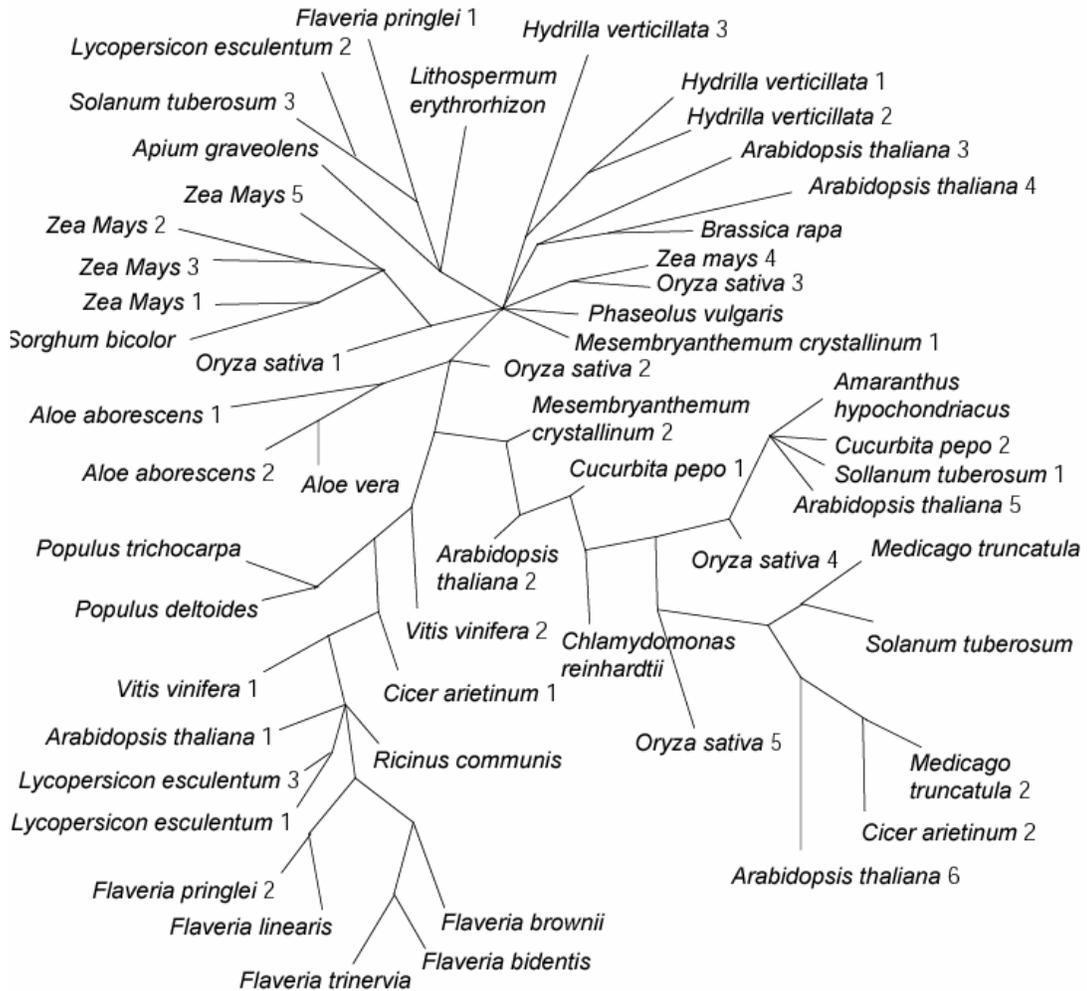
FIGURE 5: A circular unrooted phylogenetic tree of plant NADP-ME. This tree was constructed during the same analysis that was conducted to construct the rectangular tree above (figure 5). Bootstrap values in the rectangular tree apply here.

DISCUSSION

The guard cell protoplast isolation was conducted under multiple conditions that differed in terms of the yield and purity of RNA obtained. Although Isolation 1 yielded a relatively large amount of RNA with little contamination, as is apparent by the high 260/280 ratio, it is undesirable for a couple reasons. First, the overnight protocol results in a protoplast preparation that is less pure than that which results from the same-day, small-scale protocol. The presence of unwanted tissues may alter the results of gene expression analysis by RT-PCR, as RNA from all tissues experiences nondiscriminatory amplification.

Second, the extended shaking time alters the gene expression in the protoplasts in response to the stress that the cells must endure during physical isolation and enzymatic digestion of the cell wall. The transcription inhibitors Cordycepin and Actinomycin D may be included to minimize the induction of stress-inducible genes (Leonhardt *et. al.,* 2004), but when conducting a sensitive analysis of gene expression, all precaution must be taken in order to preserve the "transcript environment" in the guard cell that is present during normal growth conditions. Leonhardt *et. al.* (2004) tested the effectiveness of Cordycepin and Actinomycin D as transcription inhibitors of stress-induced genes by performing RT-PCR using cDNA from guard cell RNA from protoplasts prepared in the presence or absence of these inhibitors. Monitoring the expression of the stress-induced gene phenylalanine ammonia-lyase (*PAL*), it was seen that *PAL* was highly induced in guard cells isolated in the absence of the inhibitors, while it was very low, but still present, in guard cells isolated in the presence of the inhibitors.

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Similar results were obtained for other stress-induced genes, including *COR47*, several heat shock proteins, and glutathione S-transferase (Leonhardt *et. al.,* 2004). No evidence has been found at this time that suggests that Cordycepin or Actinomycin D completely inhibits the expression of any stress-induced gene.

Very little, highly protein-contaminated, RNA was isolated from these leaves used in Isolation 2. One can possibly explain the lack of RNA from this protocol by considering a possible relative lack of guard cells in the leaves of a plant grown under these sterile conditions. When grown in MS media, the plant does not depend as heavily on photosynthesis to produce the sugars necessary for growth. A decreased dependence on photosynthesis may translate into a decreased dependence on stomata and guard cells, the vehicles used for gas exchange with the environment.

The same-day nature of Isolation 3 allows for a reduced incubated shaking time, subsequently decreasing the amount of stress-induced transcription that can take place. The condensed shaking time also limits the amount of mRNA degeneration that can occur. The stability of mRNA in plants varies depending on the gene from which the mRNA was transcribed. Although the half-life of plant mRNA covers a wide range of times, from a minimum of less than one hour to a maximum on the order of days, it is suspected that the average mRNA transcript has a half-life of several hours (Gutierrez *et. al.,* 2002). The decreased amount of degenerated mRNA that results from a shortened incubation time helps the normal "transcript environment" to be maintained.

Isolation 4 was conducted using the same-day, small-scale isolation protocol. The only difference between this and Isolation 3 was the growth conditions of the plants from which the leaves were isolated. These plants were grown under short-day conditions (12 hours under light), in which they flourished, producing hearty plants nearly twice the size as those grown under long-day conditions. There was no mesophyll contamination. This isolation provided the most RNA, as well as the most pure RNA. Using the same-day, small-scale guard cell protoplast isolation protocol with plants grown under short-day conditions in soil is optimal for increasing the yield and purity of RNA.

The RT-PCR analysis of the six isoforms of NADP-ME in normal *Arabidopsis thaliana* plants was performed in an attempt to determine the relative expression of each isoform in the whole leaf and guard cell tissues. Difficulties in optimizing all six of the reactions to produce only the expected band using the same RT-PCR program and the same reagent concentrations lead to reactions that allow only for the comparison of leaf to guard cell protoplast NADP-ME expression at each individual locus. No two loci share the same RT-PCR program and reagent concentrations.

Through visualization of the RT-PCR products by gel electrophoresis (figure 3), it is possible to qualitatively assess the relative expression of each NADP-ME isoform in the whole leaf and the guard cell protoplast. At5g11670, At2g13560, and At1g79750 are all expressed more in the whole leaf than in the guard cell protoplasts. At5g25880 and At4g00570 are expressed to about the same level in the whole leaf and guard cell protoplasts. It appears that At2g19900

is not expressed in the guard cell protoplast, while it is expressed in the whole leaf. These data do not support the hypothesis that there is a guard cell-specific isoform of NADP-ME. Although the reaction conditions of our RT-PCR did not allow for the side-by-side comparison of the expression of each NADP-ME isoform in *Arabidopsis thaliana*, a study by Wheeler *et. al.* (2005) suggested that At5g11670 was the most highly expressed isoform in whole leaves.

This gene expression project will continue into the future in order to provide more reliable data. Currently, not all of the primer pairs are designed to provide only the expected band when the RT-PCR is optimized. The presence of multiple bands in the negative control show that some primer is being tied up in processes other than amplifying RNA, potentially altering the final intensity of the expected bands. New primers will be designed and work will continue toward optimizing reactions in order to provide optimized reactions for each of the six loci under the same conditions so that they can be compared with each other. Gel analysis software will be used to quantitatively measure the intensity of each band on the gel. The products of the currently optimized RT-PCR reactions will be TopoTA cloned (Invitrogen Corp.) in order to verify the identity of the expected bands and determine the identity of the nonspecific bands. Because the presence of guard cells, although few relative to other cell types, in the whole leaf preparation may somewhat alter the intensity of the whole leaf bands on the gel, effort may be put toward adapting a mesophyll isolation protocol for the isolation of pure mesophyll preparations.

The phylogenetic tree (figure 5, figure 6) forms the basis of a preliminary analysis of the evolutionary history of malic enzyme in Viridiplantae. At this time, it is possible to conclude that because a two C_4 plants (*Zea mays, Sorghum bicolor*) are found in a clade together, the share a common evolutionary history. The six *Arabidopsis thaliana* isoforms of NADP-ME are scattered throughout the phylogenetic tree, suggesting that these isoforms are more closely related to the malic enzyme genes of other species than they are to each other. The evolutionary events that occurred resulting in the six *A. thaliana* NADP-ME isoforms are more likely to have occurred in a distant ancestor than in a recent ancestor. This evidence supports the hypothesis that each isoform has a different function within *A. thaliana*, rather than having redundant functions.

This preliminary phylogenetic analysis will be continued by first conducting a more thorough homology search prior to tree construction with the intent of including broad gene sampling, including gymnosperm and animal malic enzyme in the analysis. This inclusion may increase the statistical support behind the tree. A maximum likelihood analysis will be conducted.

Based upon thises studies, we conclude that:

1. Using the same-day, small-scale guard cell protoplast isolation protocol with plants grown under short-day conditions in soil is optimal for increasing the yield and purity of RNA.

2. There does not appear to be a guard cell-specific isoform of NADP-ME in *Arabidopsis thaliana.*

3. The malic enzyme sequences in *A. thaliana* likely evolved in a distant

   ancestor and do not share function.

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