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Increased expression of NADP malic enzyme in the guard cells of *Arabidopsis* plants

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INCREASED EXPRESSION OF NADP MALIC ENZYME IN THE GUARD

CELLS OF *ARABIDOPSIS* PLANTS

by

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Thesis

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Molecular and Cellular Biology

Thesis Committee:

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Ypsilanti, Michigan
DEDICATION

To Shivani and Dhiraj
ACKNOWLEDGEMENTS

This acknowledgement is much more than a vote of thanks to all those towards whom I wish to express my sincere gratitude. First and foremost I wish to thank my advisor Dr. Marianne Laporte, whose continuous guidance, support, patience, and encouragement at every step throughout the study has made this research a success. I want to extend my sincere thanks to my committee members, Dr. James Vandenbosch and Dr. Daniel Clemans, for their valuable advice.

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ABSTRACT

Plants lose their water as transpiration by stomatal opening, which is governed by guard cells surrounding the stomata. Influx of ions such as K⁺, Cl⁻, from neighboring cells into guard cells, and malate synthesis within guard cells increases turgor pressure opening the stomata. Stomata close when K⁺ and Cl⁻ efflux out, and the cytosolic isoform of NADP ME convert malate to pyruvate. We hypothesize that stomatal closure can be controlled by increased NADP ME activity in guard cells. More than one of the homozygous transgenic Arabidopsis plants that are transformed with a guard cell promoter driving expression of maize NADP ME have been obtained by herbicide screening. Molecular studies have confirmed presence of the transgene in these transformants. Enzyme assays shows higher ME activity in these transformants, indicating an active form of maize ME. Such transgenic plants would help to determine effects of increased ME activity on plant water loss.
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INTRODUCTION

Water is the most precious resource for sustained agricultural production. Natural rainfall is still the most important source of water for farms. Of the water that is available for use, about 70% is already used for agriculture (Somerville and Briscoe, 2001). Approximately 40% of the world’s food crops are grown under irrigation (Johnson et al., 2001). Many rivers no longer flow all the way to the sea, 50% of the world's wetlands have disappeared, and many major groundwater aquifers are being mined unsustainably (Somerville and Briscoe, 2001). When plants are grown under irrigation conditions, they use the supplied water rapidly because no water deficit exists and therefore the plants perceive no water deficit. Therefore it is important to practice dry farming that depends on moisture conservation strategies and judicious use of available water for irrigation. Water is a critical component of photosynthesis, the process by which plants manufacture their own food from carbon dioxide and water in the presence of light. Water is one of the many factors that can limit plant growth. Plants take in carbon dioxide through their stomata--microscopic openings on the undersides of leaves. Photosynthetic carbon dioxide fixation by plants is associated with a large amount of water loss through transpiration (Somerville and Briscoe, 2001). Altering stomatal aperture in plants has the potential to improve plant water use characteristics under irrigation. The manipulation of guard cell organic anion metabolism is a novel mechanism to modulate stomatal aperture while maintaining the underlying environmental responsiveness of stomatal movement. A vast quantity of irrigation water could be saved by the development of plants that use water conservatively under well-watered conditions.
**Stomata and Transpiration**

Gas exchange mainly takes place through microscopic pores, the stomata. Two highly differentiated epidermal cells surrounding the pore, named guard cells, control stomatal aperture, allowing the plant to cope under diverse environmental conditions. They try to maintain the conflicting needs of sufficient internal CO$_2$ concentration for photosynthesis and of preventing excessive transpirational water loss (Hosy et al., 2003). Opening of the central pore is accomplished via increase in guard cell solute content, which generates a turgor pressure that causes the cells to swell and the stomata to open (Schroeder, 2003). The larger the pore, the greater the stomatal conductance-to-water-vapor, the more water is lost from the plant. This process is reversed during stomatal closure (Schroeder, 2003). The volume of the guard cell determines the size of stomatal aperture, from which plants lose the majority of their water due to transpiration. Blue-light wavelengths of daylight, detected by zeaxanthin (a carotenoid), activates proton pumps in the guard cell membranes. The proton pumps then extrude protons from the cytoplasm of the cell, creating a "proton motive force" (an electrochemical gradient across the membrane). The proton motive force is responsible for opening voltage-operated channels in the membrane, allowing positive K$^+$ ions to flow passively into the cell from the surrounding tissues (Salisbury and Ross, 1992). The dominant cation accumulated in guard cells during stomatal opening is potassium (Schroeder et al., 1994). To balance this influx of the K$^+$ ions, a counter ion pool, including Cl$^-$ and malate$^{2-}$, accumulates in the guard cells (Raschke, 1975; Zeiger, 1983). Water passively follows these ions into the guard cells and their turgidity increases; all this leads to the opening of the stomata (Salisbury and Ross, 1992). During stomatal opening there is an influx of malate in guard cells from the apoplast (Ritte and Raschke, 2003). Malate is also synthesized in the cytosol from
starch stored in the guard-cell chloroplast by a unique, regulated form of phosphoenolpyruvate carboxylase (Du et al., 1997) (Figures 1 and 2).

**Figure 1: Guard cell malate**
Shows the exchange of ions in the guard cells during the stomatal opening and closing and the production of malate within the guard cells.

**Figure 2: Stomatal pore**
The figure is an electron microscopy of the wild-type *Arabidopsis* stomata pattern. Many of the stomata are seen in various stages of pore formation (Geisler et al., 2000).
Changes in the activity of phosphoenolpyruvate carboxylase in transgenic plants result in changes in the kinetics of stomatal movement (Gehlen et al., 1996; Asai et al., 2000). When stomata are open, malate is stored in the vacuole of guard cells (Gotow et al., 1985; Pie et al., 1996). Interestingly, malate appears to be continually synthesized and degraded in guard cells of open stomata (Asai et al., 2000; Outlaw et al., 2002). Stomatal closure begins with the release of K\(^+\) out of guard cells through outward rectifying potassium channels on the guard cells (MacRobbie, 1997). These channels are encoded by genes of the *Shaker* family, which is composed of nine members in *Arabidopsis*. Three of them, KAT1 and KAT2, which encode inwardly rectifying channels, and GORK, which encodes an outwardly rectifying channel, display high expression levels in guard cells (Hosy et al., 2003). The fate of malate is not very clear during stomatal closure. Malate may be extruded from guard cells during closure (Van Kirk and Raschke, 1978) and/or may be converted to pyruvate in guard and/or epidermal cells by an isoform of NADP-malic enzyme (Outlaw et al., 1981). This could be further supported by the fact that all the different forms of NADP-ME in *Arabidopsis* were seen to have more specific activity in leaves than in any other parts of the plant relating it to the stomatal activities (Wheeler et al., 2005) (Figure 3).
The highest amount of specific activity, in most of the different types of NADP malic enzyme, was seen in the crude protein extract of the leaf compared to the stem, root or flower of the *Arabidopsis* plant (Wheeler et al., 2005).

The NADP-ME levels in various cells of *Vicia* leaflets were found to be much higher in guard cells and epidermal cells than in the photosynthetic parenchyma (Outlaw et al., 1981). This evidence supports the hypothesis that a form of ME located specifically in guard cells and the cells around them may facilitate malate degradation during stomatal closure (Outlaw et al., 1981).

**NADP Malic Enzyme**

NADP Malic enzyme (E.C. 1.1.1.40) is a widely distributed enzyme involved in different metabolic pathways in prokaryotes and eukaryotes (Drincovich et al., 2001). Its major function is oxidative decarboxylation of malate, generating NADPH, CO₂ and pyruvate. In animals, the cytosolic NADP-ME generates reducing equivalents for the biosynthesis of long chain fatty acids.

\[
\text{Malate} + \text{NADP}^+ \rightarrow \text{pyruvate} + \text{CO}_2 + \text{NADPH} + \text{H}^+
\]

There are multiple isoforms, photosynthetic and non-photosynthetic, of NADP malic enzyme in CAM, C₃, and C₄ plants located in the chloroplast or cytoplasm (Edwards...
and Andreo, 1992). In C₄ plants, two forms of NADP-ME have important metabolic roles. A cytosolic isoform is thought to function in conjunction with phosphoenolpyruvate carboxylase to regulate intracellular pH. A second, chloroplastic form of NADP-ME is found specifically in the bundle sheath chloroplasts in C₄ photosynthetic plants such as maize. The nuclear encoded NADP-ME is synthesized in the cytoplasm as a precursor with a transit peptide that is removed upon transport into the chloroplast stroma (Rothermel and Nelson, 1989). The C₄ protein that is positioned in the chloroplast of bundle sheath cell plays a role in photosynthesis by decarboxylating malate shuttled from the neighboring mesophyll cells. The CO₂ released is then refixed by Ribulose bisphosphate carboxylase-oxygenase, which eliminates the photorespiratory loss of CO₂ in plants (Rothermel and Nelson, 1989). The biological role of NADP-ME, apart from being involved in C₄ and CAM photosynthesis, remains elusive. Plastidic nonphotosynthetic isoforms in C₃ plants were suggested to be involved in plant defense responses and in lipid biosynthesis. Cytosolic isoforms have also been linked to plant defense responses, to lignin biosynthesis by providing NADPH and also to control the cytosolic pH by balancing the synthesis and degradation of malate (Wheeler et al., 2005).

Etiolated leaves of the C₄ plant maize contain a major isozyme, type1, of NADP-dependent malic enzyme with maximum activity at pH 7.45 with a molecular weight of 280,000, and an NAD-dependent activity about 1/50 the NADP-dependent activity. The dominant isozyme of young green leaves, type2, however, has a pH optimum of 8, and a molecular weight of 280,000. It is also more stable and exhibits an appreciable NAD-dependent activity (1/5–1/7 the NADP activity). Both isozymes show linear kinetics, dependence on Mn or Mg ions, similar Kₘ (NADP⁺), and the typical increase of Kₘ for L-malate with increasing pH values. Type 1 isozyme of
maize is assumed to be cytosolic (Pupillo and Bossi, 1979). Generally, other forms of the enzyme, which are thought to be cytosolic, have lower pH optima than the chloroplast enzyme (Edwards and Andreo, 1992). Type 2 corresponds in each property to the chloroplast enzyme of bundle-sheath cells. It is present at a low level in etiolated leaves and develops to a high specific activity (up to 100 nmol min\(^{-1}\) mg protein\(^{-1}\) by 150 h illumination) during photosynthetic differentiation, replacing the type 1 form (Pupillo and Bossi, 1979).

In addition to its metabolic role in C\(_3\) and C\(_4\) plants, NADP-ME has been proposed to have a role in the biochemistry of stomatal movement (Outlaw et al., 1981; Laporte et al., 2002). A cytosolic isoform of NADP ME in the guard cells in C\(_3\) plant wheat has been confirmed by immunolocalization (Maurino et al., 1997), suggesting that malate is converted to pyruvate by this cytosolic ME in guard cells during stomatal closure (Outlaw et al., 1981). Over expression of C\(_4\) ME in tobacco using a mannopine synthase promoter, present in guard cells as well as in other cells, resulted in a decrease in the stomatal aperture. However, it was not possible from these studies to conclude that it was the ME activity in guard cells specifically that was responsible for the decrease in aperture (Laporte et al., 2002).
Present Study

The present study focuses on the important role of malic enzyme in a crucial issue of water balance within plants. The study is on the expression and analysis of NADP ME within guard cells in Arabidopsis plants. Arabidopsis (C₃ type) is a small plant, easy to transform and quick-growing. It has been completely sequenced, and a wide variety of mutants of these plants are available to the academic community, making it apt for research. A key component of the study was to develop transgenic Arabidopsis plants that over-express maize NADP ME using a guard cell specific promoter. C₄ NADP-ME from maize leaves, which has been very well studied, was used. Its corresponding cDNA was the first to be determined among plant NADP-ME (Drincovich et al., 2001). Its kinetic parameters, molecular properties and amino acid residues essential for catalysis have been determined. The chloroplast enzyme of bundle-sheath cell, type2, which is highly expressed in the young leaves of the maize plant, has a high pH optimum (pH 8) under high malate, which favors the more active tetramer form, whereas lower pH (pH 7) favors the dimer form (Edwards and Andreo, 1992). However, to ensure its settlement in the guard cell cytoplasm, the cDNA obtained from the maize ME RNA was amplified without the transit peptide region. In the study of guard cell cytoplasmic pH changes in Arabidopsis in response to the plant hormones Abscisic Acid (ABA) and Methyl Jasmonate (MJ), it was seen that alkalization of the guard cell cytoplasmic pH was important for hormone-related stomatal closure. The estimated cytoplasmic pH (Cyt pH) for untreated guard cell protoplasts was 7.33± 0.04 (Cyt pH± Standard error), and 7.47± 0.02 or 7.68± 0.02 after a 30 min treatment with 20 mM ABA or MJ, respectively, leading to stomatal closure (Dontamala et al., 2004). The functional matured maize protein, without the chloroplast transit peptide, has been expressed and purified from Escherichia coli
Enzyme kinetics of this purified chloroplast maize malic enzyme has been fully determined at different pH levels. The activity of the type 2 chloroplast form of the maize malic enzyme in the *Arabidopsis* guard cell at pH 7.35 (Dontamala et al., 2004) was seen to be comparable to its optimum activity at pH 8 (Figure 4), which provides a strong basis to choose this enzyme for expressing it in *Arabidopsis* guard cell (Alaina Tabaczynski and Marianne Laporte, personal communication).

**Figure 4: Maize NADP-ME activity in *Arabidopsis* guard cell**

The Figure represents the activity of Maize Malic enzyme with its Vmax and Km values. The enzyme activity was measured at its optimum pH 8 and the *Arabidopsis* guard cell pH 7.35 (Alaina Tabaczynski and Marianne Laporte)

The primary aim was to produce an expression cassette with an *Arabidopsis* KAT2 promoter (NCBI Accession Number AJ288900), driving expression of the maize NADP-ME cDNA (ZmCh1ME1, J05130) using the pMP535 vector. The KAT2 promoter has been well studied and is seen to be expressed in guard cells of all the aerial organs of a plant. Earlier KAT1 was the only inward K⁺ channel shown to be expressed in *Arabidopsis* guard cells, where it was proposed to mediate a K⁺ influx that enables stomatal opening. But later another *Arabidopsis* K⁺ channel, KAT2, was found to be expressed in guard cells. More than KAT1, KAT2 displays functional
features resembling those of native inward K\(^+\) channels in guard cells (Pilot et al., 2001). Another important reason to choose KAT2 over KAT1 was that KAT2 is expressed only in aerial organs and never in the roots. In mature leaves, the activity of KAT2 was mainly detected in guard cells and minor veins (Pilot et al., 2001) (Figure 5).

Figure 5: KAT2 expression pattern
A. The Northern Blot shows the increased amount of KAT2 compared to KAT1 in the aerial organs, the rosette leaves (RL), caulinary leaves (CL) and the stem (S) than in the roots (R) of Arabidopsis. B. Shows the KAT2 promoter activity in the leaves using the GUS reporter gene in Arabidopsis (Pilot et al., 2001).

A KAT2 promoter region sourced from the genomic DNA of Arabidopsis thus serves the purpose of guard cell expression. The vector, pMP535, is derived from the minibinary vector pCB302 that has been used to accomplish transformation of Arabidopsis (Xiang et al., 1999). This is a novel vector that is comparable to the size of other minibinary vectors (5.4kb). It has unique and extended restriction sites in the multiple cloning sites, which increases its utility. It contains a multiple cloning site abutting an E9 gene terminator, and also contains an herbicide selectable marker cassette (Michael Prigge, University of Michigan personal communication). Once the expression cassette was complete, it was used to transform competent Agrobacterium
through freeze thaw technique (Holsters et al., 1987). The advantages of
Agrobacterium-mediated transformation have been documented many times. Among
the most important is the integration of a single or low number of copies of a defined
T-DNA fragment without rearrangement or with minimal rearrangements. These
factors are expected to result in better and more reliable transgene expression.
Another very important advantage of Agrobacterium-mediated transformation is the
possibility of marker gene elimination by its segregation from the transgene of
interest. This relies on the independent integration of two separate T-DNA fragments
(Przetakiewicz et al., 2004). Evidence shows that among various lines including
AGL1 of Agrobacterium tested for plant in vitro transformation/regeneration, the
highest transformation efficiency was obtained with the disarmed hypervirulent strain
AGL1 (Chabaud et al., 2003). Therefore the hypervirulent strain of Agrobacterium,
AGL1, was selected for transformation in the present study. The resultant
Agrobacterium was used to transform Arabidopsis plants by the Floral dip method
(Clough and Bent, 1998). Seeds collected from these transgenic plants were screened
with herbicide Finale (Glufosinate) to test the herbicide resistance on the transformed
plants, which is conferred by the BAR gene present on the pMP535 vector. The
positive transgenic plants were further analyzed using PCR to confirm presence of the
transgene. Homozygous lines of the transgenic plants were obtained from further
generations. A spectrophotometric assay, designed to detect the production of
NADPH by showing an increase in absorbance at 340 nm (Laporte et al., 2002), was
performed to confirm that the NADP ME protein is active in the transgenic plants.
Western blotting was further performed, using anti-his antibodies, on the crude
enzyme extract of the transgenic plants to confirm the presence of the histidine-tagged
maize ME.
Hypothesis

The hypothesis of this study is that the guard cell specific expression of C₄ maize malic enzyme in C₃ Arabidopsis plant using guard cell biased promoter KAT2 will lead to a decrease in malate ion pool within the guard cells in a dose-dependent manner. This may result in reduced stomatal aperture in Arabidopsis. The implication of this decrease in the malate pool in guard cells will be on the reduced water loss by transpiration. The experiments were built on previous studies that engineered tobacco plants with a maize NADP ME cDNA using a constitutive promoter, which resulted in altered stomatal function (Laporte et al., 2002). The main objective of the study is to produce transgenic Arabidopsis plants over-expressing maize NADP ME in their guard cells. A second important aspect of the study is to determine the effects of the increased enzyme on the guard cell malate content and the stomatal aperture. This could be understood by comparing the presence of the protein, by determining its enzyme activity, and by comparing the phenotype of the transgenic plants with the wild-type plants. These experiments will allow exploring the effects of varying guard cell malate content on the stomatal function.
METHODS AND MATERIAL

Amplification of the promoter, KAT2

Wild-type *Arabidopsis* genomic DNA was extracted from healthy *Arabidopsis* leaf tissue. Tissues from 1-2 leaves were placed with 200µl of extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, and 0.5% SDS) in the Eppendorf tube and grounded using a pestle attached to a drill at its highest speed. The sample was vortexed and centrifuged at highest speed (14000rpm) for 2 mins at room temperature. 150µl of this supernatant was added to 150µl of isopropanol in a new Eppendorf tube and vortexed. The sample was incubated at room temperature for 2 mins to help precipitate DNA in isopropanol and centrifuged again for 2 mins at room temperature at maximum speed (14000rpm). The pellet obtained was washed with 200µl of 70% ethanol and air-dried. The pellet was dissolved in 100µl of autoclaved distilled water and then centrifuged again for 2 mins at highest speed at room temperature, and the supernatant was quantified for the DNA concentration and purity. This DNA was used as a template to amplify the KAT2 promoter. A complete promoter region of 2258bp was amplified using left primer

5’CGATCCCGGGGAAAAACGCAAAGTGAATCCTCT 3’ having the *XmaI* restriction site and the right primer

5’CGATCATATGAGGTAGTATAAATATAGTGA 3’ with the *NdeI* restriction site just upstream of the ATG start codon at 2259bp. The PCR reaction mix was made using Invitrogen reagents, 2.5µl of 10X buffer, 0.5µl of 10 mM dNTPs, 1µl of 50 mM MgSO4, 0.5µl of 10 pm/µl right primer, 0.5µl of 10 pm/µl left primer, 0.5µl of template 118 ng/µl with 0.1µl of Platinum taq polymerase, and the volume made
up to 25µl using autoclaved distilled water. Platinum taq was used because it adds on the polyA tails at each end of the amplified product, which is required to TOPOclone the PCR product into the pCRII-TOPO vector. It is also a high fidelity enzyme and reduces any mistakes added on into the PCR product. The PCR cycle was programmed at 95 °C for 2 mins, and 25 cycles of 95 °C for 40 sec, 50 °C for 30 sec and 72 °C for 2 mins with a final extension of 72 °C for 7 mins.

**Maize malic enzyme DNA**

RNA was extracted from healthy maize plant leaf tissue using the Qiagen RNase easy plant minikit following the manufacturer’s protocol. The RNA was then reverse transcribed to obtain the cDNA. RTPCR was set up using 1µl of 10mM dNTPs, 0.2µl of 10 pm/µl of left primer 5’ CGCCACCATGCTGTCCAC 3’, which starts at 108bp, 0.2µl of 10 pm/µl of right primer 5’ CCATCCCGTTGATAGATTT 3’, which starts at 2075bp, and 6.9µl of double distilled water, and the reaction mix was incubated at 65 °C for 5 mins and then left on ice for a minute. To this mix was further added 4µl of 5X first standard buffer, 1µl of 0.1 M of DTT, 1µl of RNase out, 5000u (40 u/µl), which acts as a Ribonuclease inhibitor, and 1µl of superscript III RNaseH RT, 2000u (200 u/µl) from Invitrogen. The RT reaction was carried out at 50 °C for 60 mins and 70 °C for 15 min. The mix was treated with 1µl of 200units RNaseH (Invitrogen) and incubated at 37 °C for 20 mins. RNaseH (RibonucleaseH) is an endoribonuclease that specifically hydrolyzes the phosphodiester bonds of RNA that is hybridized to DNA. The malic enzyme gene was amplified using the cDNA obtained from the ME RNA from maize. The transit peptide of the gene was not included in the cDNA. In maize, nuclear encoded NADP-ME is synthesized in the cytoplasm as a precursor with a transit peptide. The amino-terminal extension encodes the transit peptide, which is
removed upon import into the chloroplast. The maize NADP-ME sequence contains two possible processing sites. The stretch of acidic residues from positions 68 to 77 that lies between the two possible processing sites is neither characteristic of chloroplast transit peptides nor found at the amino termini of known malic enzymes. It could have functional significance for either region (Rothermel and Nelson, 1989).

**Figure 6: Nucleotide sequence and amino acid residue of Maize chloroplastic NADP-ME**
The above figure shows the Nucleotide sequence and the amino acid residue of the transit peptide and part of the mature protein of maize malic enzyme. The cDNA of the ME protein was amplified starting at 298bp (Rothermel and Nelson 1989).

Based on the above study the cDNA of ME protein was amplified starting at 298bp coding for the mature protein, starting at the amino acid position 62, which is followed by a start codon of the mature protein (Detarsio et al., 2002) (Figure 6). Cleaving the first 298bp of the gene, which coded for the transit peptide, ensured that the matured protein of predicted size of 62-kilo daltons would remain in the cytoplasm, failing to get targeted into the chloroplast. The primers used were 5’CGATCATATGGCGATGGTCTCCAACGCGGAGA 3’, which had the *NdeI* site on the 5’ end, while the C- terminal has the *NcoI* his-tagged primer 5’ CGATCCATGGCTACACCACCACCACCACCACCACCAGGTAGTTGCGGCGTAGGACGGG G 3’. The primer was tagged with histidine on the C- terminal end for ease in seeking the presence of this protein in western blot in later studies. The reaction mix was made using Invitrogen reagents, 2µl of 10X buffer, 0.5µl of 10 mM dNTPs, 1µl of 50 mM MgSO₄, 0.5µl of 10 pm/µl right primer containing the histidine tag, 0.5µl of 10
pm/µl left ME NdeI primer, 0.2µl of 66 ng/µl cDNA template and 0.1µl of platinum taq polymerase with the total volume brought to 20µl using autoclaved distilled water. The PCR conditions were 94 °C for 4 mins, 29 cycles of 94 °C for 30 sec, 65 °C for 30 sec, 68 °C for 4 mins, and 68 °C for 7 mins, final extension. Both the PCR products were then separated on a 1% agarose gel using 0.5X TBE buffer at 120volts to check for the appropriate bands.

**TOPO Cloning and Sequencing**

2µl of the fresh PCR product of KAT2 and ME were then TOPOcloned into pCRII-TOPO vector following the protocol by Invitrogen life technologies. The use of platinum taq polymerase added on the polyA tails/overhangs onto the product on both ends, and that was done to help the product insertion in the pCRII-TOPO vector that has polyT tails or overhangs on it, hence the name TOPO TA cloning. Invitrogen TOPO TA cloning kit was used for the purpose (Figure 7).
The map above shows the features of the pCRII-TOPO vector and the sequence surrounding the TOPO Cloning site (Invitrogen).

The TOPO Cloned product was then used to transform chemically competent one shot TOP10F' *E.coli* bacteria, provided with the TOPO TA kit, following the kit protocol. Once in the bacteria i.e. TOP10F' cells, the bacteria were then screened for positive transformants with the help of blue white screening on LB and kanamycin plates. Isolated white colonies were then picked, and overnight liquid cultures using one
colony each in 15ml of regular LB media (10 gms/L Tryptone, 5 gms/L yeast, 10 gms NaCl) with 15ul of kanamycin 50 mg/ml were grown in shaking water bath at 150rpm at 37 °C overnight. Kanamycin helped screen the bacteria that were transformed with pCRII-TOPO vector, as this vector has the kanamycin resistant gene present on it. The bacterial cultures were then used to extract plasmid DNA using Promega SV minprep DNA extraction kit1 using the kit protocol. The extracted DNA was sent in for sequencing at the University of Michigan DNA Sequencing core with the M13 reverse and M13 forward primers that can obtain a sequence from the pCRII-TOPO vector since it has the N- terminal coding sequence of the LacZα gene. Custom sequencing primers were designed at every 500bp of the sequence that would span the entire DNA. The obtained sequence from the core was then analyzed for any PCR induced errors and the presence of the restriction sites at both the ends using NCBI Nucleotide Blast and software Sequencher 4.6 (Gene codes).

**Insert DNA formation**

An error-free sequence of both KAT2 and ME DNA once obtained was then cleaved out of the pCRII-TOPO vector by restriction enzyme digestion. Restriction Enzyme digest was performed on KAT2 pCRII-TOPO construct by using 11.62µl (301 ng/µl) of the KAT2 pCRII-TOPO DNA, 5µl of 10X bufferB, 0.63µl of 5 u/µl XmaI (Promega) and 32.75µl of autoclaved distilled water. The reaction was incubated at 25 °C for 2 hrs. The mixture was cleaned up using Wizard SV GEL and PCR CLEAN UP SYSTEM by Promega. Because the incubation temperatures of the two enzymes to be used were different after clean up and quantification, the DNA was again digested using 32.60µl of cleaned and XmaI digested DNA (3.0 µg/ml), 5ul of 10X bufferD, 1.25ul of 10 u/ul Ndel (Promega) and 11.15µl of autoclaved distilled water.
The mix was incubated for 2 hrs at 37 °C. Similarly the ME gene was also cleaved out of pCRII-TOPO vector by performing a double restriction enzyme digest on 10µl (301 ng/µl) of ME pCRII-TOPO DNA, 5µl of 10X buffered and 1.5µl of 10 u/µl NdeI and NcoI (Promega), with volume brought up to 50µl using autoclaved distilled water. The mix was incubated at 37 °C for 2 hrs. After both the DNAs were digested, the product was separated on a 0.8% Agarose gel using 0.5X TBE buffer running the gel at 109volts. 25µl of product was loaded in each well. Once the bands were separated out of pCRII-TOPO they were gel purified using Wizard SV gel and PCR clean up system by Promega to obtain 20.55 ng of ME digested and purified DNA and 29 ng of digested and purified KAT2 DNA.

These two gel purified products, having the required site at the ends, were then ligated at the NdeI site of both the DNAs. The ligation mix was made at the ratio of 1:1 ((50 ng of ME * 2.26kb of KAT2) / (1.7kb of ME)) * (1:1) = 67.6 ng of KAT2. The Ligation reaction contained 2.4µl of ME (20.55 ng/µl), 2.3µl of KAT2 DNA (29 ng/µl), 2µl of 10X T4 DNA ligase buffer with 10 mM ATP, and 1µl of T4 DNA ligase 400,000u/ml from New England Biolabs, with the volume brought up to 20µl with autoclaved distilled water. The reaction was carried out at 16 °C overnight and was heat inactivated at 65 °C for 10 mins.

**Amplification and sequencing of the insert**

The ligated product was amplified by PCR with the PCR mix containing 1µl of 50 mM MgSO₄, 2.5µl of 10X buffer, 0.6µl 10 mM dNTPs, 0.5µl of 10 pm/µl ME NcoI his-tag right primer, 0.5µl of left 10 pm/µl KAT2 XmaI primer, 6µl of the ligated product, and 0.1µl of Platinum taq polymerase, with the total volume brought to 25µl using autoclaved distilled water. The PCR conditions were 94 °C for 2 mins, 35
cycles of 94 °C for 30 sec, 64 °C for 30 sec, 68 °C for 4 mins, and 68 °C for 7 mins final extension. The product was checked on 1% Agarose gel to confirm its correct size. The product was TOPOcloned again into pCRII-TOPO vector and transformed into TOP10F’ bacterial cells. The transformed cells were then screened for positive transformants, and overnight cultures were set up in a similar way to what was stated above. DNA was extracted from the bacterial cultures using Promega SV minprep DNA extraction kit1. The DNA samples were sent in for sequencing at the University of Michigan DNA Sequencing core with the M13 reverse, forward, and sequencing primers designed at every 500bp. The sequences obtained from the core were then checked by using Sequencher 4.6 (Gene codes) and NCBI blast for any PCR errors, for the presence of the start and stop codons, and for the presence of the sites at the 5’ and 3’ end required for the insertion into pMP535, of the ligated product. The whole of the above process was done with replicates to ensure that at least one correct sequence of the ligated product was obtained.

**Formation of the final construct**

After getting a correct sequence, the ligated product was digested out of pCRII-TOPO using XmaI and NcoI restriction enzymes. The RE reaction was made using 12.1µl of DNA of the ligated product (KAT2+ME) (248 ng/µl), 5µl of 10X BufferB, 0.75µl of 5 u/µl XmaI (Promega), 1.5µl of 10 u/µl NcoI (Promega), and 30.65µl of autoclaved distilled water. The reaction was incubated at 37 °C for 2 hrs. The minibinary vector pMP535, provided by Michael Prigge at the University of Michigan (Figure 8), was also digested with the same set of enzymes.
The reaction mix contained 37.97µl of vector DNA (79 ng/µl), 5µl of 10X BufferB, 0.75µl of 5 u/µl XmaI (Promega), 1.5µl of 10 u/µl NcoI (Promega) and 4.78µl of autoclaved distilled water. The reaction was incubated at 37 °C for 2 hrs. After digesting both the products, they were gel purified and then ligated at the ratio of 5:1. 

\[
((50 \text{ ng of pMP535} \times 3.75kb \text{ of KAT2+ME}) / (5.4kb \text{ of pMP535})) \times (5:1) = 173.6 \text{ ng of insert (KAT2+ME)}.
\]

The ligation reaction was made with 4.16µl of pMP535 vector (12 ng/µl), 6.42µl of insert DNA (27 ng/µl), 2µl of 10X T4 DNA ligase buffer, and 1.5µl of T4 DNA ligase, with the volume brought up to 20µl with autoclaved distilled water and carried out at 16 °C overnight and then heat inactivated at 65 °C for 10 mins. The ligated product, which now had pMP535, KAT2, and ME, was used to transform JM109 bacterial cells using the freeze-thaw transformation technique.
Transformation of *Agrobacterium tumefaciens* and *Arabidopsis thaliana*

The miniprep DNA obtained from the JM109 cells was used to transform *Agrobacterium tumefaciens*. The AGLI lines of *Agrobacterium tumefacies* were transformed. The chemically competent *Agrobacterium* cells (100µl) were transformed using the freeze-thaw technique with 1µg of the whole construct DNA (Höfgen and Willmitzer, 1988). The transformed cells were then screened on LB (10 g/L tryptone, 5 gms/L yeast extract, 5 gms/L NaCl, 15 gms/L agar), with kanamycin 50 mg/ml and ampicillin 100mg/ml plates at 28 °C. The transformed bacteria were confirmed of the presence of the transgene using colony PCR. The PCR mix was made using Promega PCR core systemII PCR kit, 1.2µl of 25 mM MgCl2, 2µl of 10X buffer, 0.4µl 10 mM dNTPs, 0.5µl of nos terminator 5’GTCGACGATCGTTCAACATTTGG 3’ left (10 pm/µl), nos terminator 5’CCCGGGCGATCTAGT AACATAGATGA 3’ right primer (10 pm/µl) and 0.1µl of taq polymerase, with the total volume brought to 20µl using autoclaved distilled water. A part of the bacterial colony was then touched by a tip and then dipped in the PCR mix; this acted as a template for the reaction. The PCR conditions were set to 94 °C for 2 mins, 30 cycles of 94 °C for 30 sec, 57 °C for 30 sec, 72 °C for 4 mins, and 72 °C for 7 mins final extension. Colony PCR was also carried out using the KAT2 Xmal and ME his-tagged Ncol primers to check for the presence of complete insert. The product was separated on 1% Agarose gel. These tested colonies were used to make overnight cultures with 15µl of LB media (10 g/L tryptone, 5 gms/L yeast extract, 5 gms/L NaCl) with 15µl kanamycin 50 mg/ml and 15µl of ampicillin 100 mg/ml in a shaking incubator, 200rpm at 28 °C. Both the antibiotics were used for the screening of the transformed *Agrobacterium* since the AGL1 strain of *Agrobacterium*
is ampicillin resistant, while the minibinary vector pMP535 is kanamycin resistant. Larger overnights of 350ml of LB (10 g/L tryptone, 5 gms/L yeast extract, 5 gms/L NaCl) were started using 3.5ml of these smaller overnights and 3.5ml of the selective agents. The cultures were grown at 28 °C with good aeration in a shaking incubator at 200 rpm to a density of 0.8-1.0 (OD$_{600}$). The cells from these larger overnights were used to transform *Arabidopsis* plants. Healthy *Arabidopsis* plants, variety Columbia, with unopened flowers buds were used for transformation. The plants were transformed using the Floral dip method (Clough and Bent, 1998). The plants were dipped again in the fresh transformed *Agrobacterium* culture after an interval of 6 days to increase the efficiency of transformation. The plants were then grown regularly in the growth chambers.

**Screening for transformants**

Seeds were collected from the transformed plants every other day. They were screened for positive transformants in two ways.

A) Seeds collected were dried, and about 5-6 seeds were sown on the surface of moist soil, Metro mix 360, in square pots (5.5 × 5.5 cm) and vernalized for 48 hrs at 4 °C to synchronize germination (Mengiste et al, 1997). The plants were grown in the controlled growth conditions of 8 hours light temperature 20 °C with light level averaging 417 PAR. (PAR = photosynthetic active radiation, in mmol per m$^2$ per second) and 15 °C of 16 hours dark with a relative humidity of 60%. The plants were sprayed with herbicide Finale diluted 1:1000 with water, containing 5.78% glufosinate, every other day for a week.

B) The seeds were also screened by planting them into the Murashige and Skoog media (Murashige and Skoog, 1962) containing Glufosinate ammonia. 40 seeds per plate were surface sterilized with 20% bleach for 20 mins and then washed with
autoclaved distilled water. They were then plated on to petri dishes containing 50ml of 1/2X regular MS basal salt media from the Phytotechnology labs, 2.17 gms/L of media with 15 gms/L sucrose and 7.5 gms/L Agar, containing 10 mg of Glufosinate ammonia, original concentration of 5.78% Glufosinate in Finale. 5-6 plates containing 40 seeds in each plate of each line were made. The plates were kept inverted for maximum exposure of seeds to the herbicide at the growth conditions of 22 °C for 8 hrs light period and 16 °C for 16 hrs in dark. After the seeds germinated, the plates were made upright and the plants were allowed to grow; later, each surviving plant was transplanted into individual regular MS media boxes.

**Detection of the inserted gene**

The positive transformants were then detected for the presence of the inserted gene by PCR. Genomic DNA extracted from these plants, using the same procedure of DNA extraction as stated above, was amplified using Promega PCR core systemII PCR kit. The reaction contained 1.2µl of 25 mM MgCl₂, 2µl of 10X buffer, 0.4µl of 10 mM dNTPs, 0.5µl of 10 pm/µl right primer containing the his-tag 5'CGATCCATGGCTACACCACCACCACCACCACCACCACCACGGGTAAGTTGCAGGTAGACG 3', 0.5µl of 10 pm/µl left KAT2 sequencing primer 5' TTTGATCTACTTAACGATAGTGAA 3' starting at 1576bp, 2µl of genomic DNA, and 0.1µl of taq polymerase, with the total volume brought to 20µl with autoclaved distilled water. The PCR conditions were 94 °C for 4 mins, 25 cycles of 94 °C for 30 sec, 54 °C for 30 sec, 68 °C for 4 mins, and 68 °C for 7 mins final extension. The PCR product was then separated on a 1% Agarose gel to check for presence of the band.
Screening for homozygous line/Genetic analysis of transformants

The seeds obtained from the T1 generation of transgenic plants were then screened for homozygous lines. This was done to obtain the optimum amount of enzyme activity in the plant. The seeds were screened using the MS finale media technique of screening as stated above.

Detection of the transcript

RNA was extracted from healthy homozygous transformant leaf tissue using Qiagen RNase easy plant minikit following the manufacturer’s protocol. RT-PCR reactions were set up using 12.5 µl of 2X Acess Quick RT-PCR master mix, 0.5 µl of 10 pm/µl maize ME left primer starting at 909bp 5' TGACCCATCAGTTTGCTTGC 3', 0.5 µl of 10 pm/µl maize ME right primer starting at 1603bp 5’AATGCGAGGTTGGGTTTGAC3’, 20 ng of extracted RNA and 0.2 µl AMV reverse transcriptase, with total volume brought to 25 µl with autoclaved distilled water. The reaction was set at 48 °C for 45 mins, 95 °C for 2 mins, 30 cycles of 95 °C for 30 secs, 55 °C for 30 secs, 72 °C for 30 secs and 72 °C for 5 mins final extension. The samples were then separated on 1% agarose gel to check the presence of the cDNA band.

Enzyme assay

Enzyme assay was carried out to measure the activity of ME by providing substrates malate and NADP and measuring the formation of NADPH in the spectrophotometer. Crude protein was extracted from 500 mg of fresh green fully grown leaf tissue of a 6 week old plant, both wild-type and transgenic *Arabidopsis* plants. Tissue was crushed using a glass homogenizer with 1492.5 µl of cold extraction buffer containing 50 mM
Hepes, pH 8.0, 2.5 mM EDTA, 5 mM MgCl2, 1%PVPP (Polyvinyl propylene), 7.5µl of 1 mM DTT, and 15µl of protease inhibitor Cocktail set III from Cal Biochem (1:100 dilution). The buffer used was at pH 8.0, which is the approximate pH of the stroma of an illuminated chloroplast where malic enzyme is located. After collecting the supernatant from this extract, enzyme assays were further carried out. An NADP assay cocktail was prepared comprising 895µl of assay buffer, the assay buffer composed of 50 mM Hepes, pH 8.0, 2.5 mM EDTA, and 4 mM Malate, with 40µl of 10 mM NADP and 5µl of 1mM DTT. The same assay cocktail was used as blank where the volume was brought up to 1ml using the assay buffer instead of the protein extracts. 20µl of the protein extract was then added to the assay mix, and the reaction was measured for 15 mins. This was done to ensure that there was no more background activity once the reaction was initiated with MgCl2. This reaction was measured at 340nm, because NADPH absorbs light at 340nm and the increase in the absorbance was being measured to ensure the formation of NADPH in the reaction. After reading the background activity, the reaction was initiated with 40µl of 500 mM MgCl2, final concentration of 20 mM in each assay, and measured at 340nm for 15 mins. Rise in the Mg dependent conversion of NADP to NADPH was recorded.

**Protein quantification and specific activity**

After measuring the activity of the protein it was important to find out the actual quantity of the protein in the crude extract and to measure the specific activity of the transgenic enzyme. Bio-Rad protein assay dye reagent, Coomasie Brilliant Blue G-250, 1:5 dilutions, was used to determine the color change. The protein samples were then measured at 430nm and the readings were plotted against a BSA (0.1 mg/ml)
standard curve to determine the specific activity. Statistical analysis was further carried out on the specific activity data using Microsoft Excel.

Detection of the protein by Immunoblotting

SDS-PAGE

The crude protein extract was further analyzed for the presence of the his-tagged ME protein. The crude enzyme extract was first separated depending on the size of the proteins in an electric field by performing Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Protein samples were diluted in the Tris Glycine SDS sample buffer (2X) containing 63mM Tris HCl, 10% Glycerol, 2% SDS, and 0.0025% Bromophenol Blue pH 6.8 with 5% β-mercaptoethanol and heated at 99 °C for 5 mins. The SDS denatures and unfolds the proteins by wrapping around the hydrophobic portions of the protein. SDS binds at a ratio of ~1.4 g SDS per gram of protein. The resultant SDS protein complexes are highly negatively charged and migrate through the gel based on their size rather than charge. β-mercaptoethanol (β-ME) that is used as the reducing agent completely unfolds the denatured proteins into their subunits by cleaving the disulfide bonds between cysteine residues. The protein samples were then loaded on the precast Novax 10%, 10 well Tris Glycine gel. 20µl of the diluted and heat-treated protein sample was loaded in each well, with 10µg of protein in a well, and then separated using BioRad SDS triglycine 10X buffer, with a working concentration of 1X running buffer at 95Volts for one hour using the XCell Surelock gel box from Invitrogen. The purified malic enzyme with the C-terminal his-tag and the N- terminal his-trx tag was used as a positive control for the anti his antibodies. The control (0.6 µg/µl) was diluted with assay buffer (1:16) before being diluted with the Tris Glycine SDS sample buffer and
heat-treated. The gel was then stained in Molecular Probes Coomassie Fluor Orange protein gel stain, by Invitrogen, for 45 mins on a rotary shaker.

**Immunoblotting/ Western Blot**

Proteins were separated on SDS-PAGE as described above and transferred on to the nitrocellulose membrane using semi-transfer buffer (25 mM Tris, 150 mM Glycine, 10% Methanol) at 125mAmps and 11volts for 90 mins. The membrane was then washed in 1X TBS buffer (10 mM Tris-Cl pH 7.5 and 150 mM NaCl) (2× 10 mins). To prevent non-specific antibody binding, the blot was incubated in 50ml Blocking buffer (3% (W/V) BSA in TBS) for 1hour. This procedure allows saturation of all non-specific protein binding sites on the blots. The blot was then washed in TBS Tween/Triton (20 mM TrisCl pH 7.5, 500 mM NaCl, 0.05% Tween 20, 0.2% TritonX 100) (2× 10 mins) and again washed with 1X TBS for 10 mins to remove the excess blocking buffer. The blot was then incubated in α-His antibody (12.5µl of Penta His Antibody in 25ml Blocking buffer), shaking on a rotary shaker for two hours. The membrane was again washed (2× 10 mins) in TBS Tween/Triton and once in 1X TBS for 10 mins. The membrane was incubated in secondary Antibody (Goat α Mouse IgG Alkaline Phosphatase conjugate) for 1 hour at room temperature. Excess secondary Ab was then washed out using TBS Tween/Triton (4× 10 mins). 1-step NBT/BCIP substrate was added on the membrane for detecting the protein by chemiluminescence reaction. The membrane was left in the fluorescence solution for 5 mins without shaking, and the blot was observed for the desired bands.
Protoplast isolation and western blot

(Pandey et al., 2002; Leonhardt et al., 2004)

Protoplast isolation was carried out using twenty fully expanded leaves from soil grown *Arabidopsis* transformants and wild-type plants. The leaves were placed in approximately 100ml of cold tap water after excision of the mid-vein, and blended twice for 30 sec in a Waring blender. The mixture was poured through a 200 µm Spectrum® nylon filter 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 to remove the foam produced by the blending. All peels were scraped from the mesh into a flask containing 15ml of enzyme solution 1, which consists of 0.7% cellulysin cellulase, *Trichoderma viride*, 0.1% (w/v) PVP-40, 0.25% (w/v) bovine serum albumin, 0.5 mM 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. This mixture of the peel in enzyme solution 1 was left in a shaking incubator at 27 °C with 140rpm, for 40 minutes in the dark. After 40 minutes, 37.5ml of basic solution (5 mM MES hydrate, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 10 µM KH₂PO₄, 0.5 mM L-ascorbic acid, 0.01% cordycepin, 0.0033% actinomycin D, osmolarity adjusted to 550 mmol/kg using D-sorbitol; pH 5.5 (using Tris base) to a total volume of 300ml) was added to the mixture. The mixture was then shaken under the same conditions for five minutes to provide an intermediate osmolarity between enzyme solutions 1 and 2 and also to prevent sudden plasmolysis of fragile guard cells. The digested peels were again poured through a fresh 200 µm 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 15ml of enzyme solution
comprising 1.3% (w/v) Onozuka RS cellulase, 0.0075% (w/v) Pectolyase Y-23, 0.25% (w/v) bovine serum albumin, 0.5 mM L-ascorbic acid, 0.1% cordycepin, and 0.0033% actinomycin D, pH 5.5. The flask was shaken at 20 °C, 60rpm, for 40 minutes in the dark and again at 20 °C, 40rpm, for 55 minutes in the dark. The flask was swirled by hand for several seconds upon completion of the final digestion step in order to increase the liberation of GCPs (Guard cell protoplast) from the peels. The contents of the flask were poured through a 20 µm Spectrum® nylon filter in the same apparatus as before. The peels that remained on the filter were rinsed with 150ml of basic solution as a final attempt to release GCPs. It is at this time that the GCPs in the filtrate are most readily viewed under a microscope. When a microscopic view reveals excessive debris or mesophyll presence, further filtration may be desired using a 10 µm Spectrum® nylon filter. The filtrate was collected in four 50ml centrifuge tubes, and centrifuged for five minutes at 1000g. 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 in 20-25ml of basic solution followed by an identical centrifugation step. Again all but 1-1.5ml of supernatant was removed. The 4-8ml of the supernatant and pellet that remained in the five tubes was then combined and evenly distributed between 1.5ml Eppendorf microcentrifuge tubes and centrifuged again for five minutes at 1000g. All but about 0.5ml of supernatant was removed, and the pellet was once again resuspended in basic solution so that it fits into one 1.5ml Eppendorf microcentrifuge tube. This tube was centrifuged for five minutes at 1000g, all supernatant was removed, and the pellet was collected. Protein was extracted from the isolated protoplast and quantified. Around 6 µg of the extracted protein of each line and wild-type was separated by SDS-PAGE. Western blots were performed on these separated protoplast protein using the above procedure.
RESULTS

Vector Construct

The primary step in the study was to develop a clone or a vector construct that could be used to target the gene of interest into *Arabidopsis* plant. The resulting vector (Figure 9) consisted of the KAT2 promoter and the maize ME gene (4kb) ligated into the minibinary vector pMP535 (5.4kb). pMP535 is a novel vector consisting of the multiple cloning site and the E9 terminator. It also has the herbicide resistance gene (BAR) that was used for screening the transformed plants in later studies. The KAT2 promoter, which is a guard cell biased promoter, ensured that the gene of interest, maize ME, was targeted to the stomatal guard cell. Maize ME gene was selected on the basis of its effect on stomatal phenotype of tobacco plant when expressed with a constitutive promoter. It was also found to be active at the guard cell pH 7.35. It was his-tagged at the C- terminal so that it could be detected in the western blot analysis.

![Figure 9: Vector construct: pMP535, 5405 base pairs](image)
The vector construct had the guard cell specific promoter KAT2 gene that will lead expression of the C- terminal histidine tagged maize ME gene in the guard cells of transformed *Arabidopsis* plants.
Sequencing

The insert DNA was analyzed for any PCR-induced mistakes using Sequencher 4.6 (Gene Codes). A complete contig was obtained (Figure 10) using the sequencing primers and the sequences obtained from the University of Michigan DNA Sequencing Core. These sequences were compared with the KAT2 promoter AJ288900 and ZmCh1ME1, J05130 as reference sequences from the NCBI database. This analysis was done to ensure that the insert would translate into an unmutated matured protein.

Figure 10: Complete contig of the insert sequence

The figure shows the complete alignment of the sequences obtained from the sequencing core and the sequences from NCBI with their accession numbers. It also shows the alignment of the sequencing primers used with respect to the reference and core sequences. The primers are represented by the base number at which they start and the direction in which they run.
Restriction enzyme digestion

To confirm the presence of the complete construct or the ligated product, the isolated DNA from the transformed JM109 *E.coli* cells was digested using the restriction enzymes *XmaI* (5’) and *NcoI* (3’). The presence of the vector band at 5.4kb and the complete insert at 4kb (2258bp of KAT2 and 1745bp of ME DNA) confirmed the ligation of the insert and the vector (Figure 11). This complete construct was then used to transform chemically competent AGLI strain of *Agrobacterium tumefaciens*.

![Figure 11: Restriction enzyme digest of the complete construct](image)

The gel showed the presence of the two expected bands in lane 2 and 3 of sizes 5.4kb, which are pMP535 vector and the 4kb insert (KAT2 and ME) after digesting with *XmaI* and *NcoI* restriction enzymes. The largest band seen was the undigested DNA. Lane 1 shows the 1kb+ DNA ladder (Promega).

Agrobacterium transformation

The transformed *Agrobacterium*, which were selected on the LB (10 g/L tryptone, 5 gms/L yeast extract, 5 gms/L NaCl, 15 gms/L), Kan, Amp plates, were further confirmed as positive transformants with the help of colony PCR. The presence of the nopaline synthase (nos) terminator band at 259bp (Figure 12B) that is present on the
vector and also the complete insert band of 4kb (Figure 12A) assured the transformation of the *Agrobacterium* with the desired vector, insert construct.

![Image of gel electrophoresis](https://example.com/gel.png)

**Figure 12: Transformed *Agrobacterium***

A. Colony PCR showed the presence of the complete insert at 4kb in *Agrobacterium* cells in lane 2, 3, 4, 7, 8, and 9. B. Colony PCR showed the nos terminator at 259bp in lanes 2-5, which was present on pMP535 vector in transformed *Agrobacterium*

**Selection of positive *Arabidopsis* transformants**

Positive transformants were selected both by growing plants in soil and in media and screening them with the herbicide Finale. The plants that survived the glufosinate-screening test and looked green and healthy were selected as positive transformants. The plants that were untransformed could not sustain the herbicide and bleached and died (Figure 13A). Wild-type plants were used as negative control for the screening. The resistant plants were then transplanted into new pots containing the same type of soil. They were grown individually and fertilized to obtain seeds that were used further for analysis of the transformants (Figure 13B).
A. Herbicide resistant *Arabidopsis* plants looked green even after spraying Finale while others looked bleached and dead. B. Each surviving plant or transformant was then transplanted into a new individual pot with the same type of soil and fertilized for better growth.

Screening of plants in media was done by germinating seeds in MS media containing Finale for obtaining positive transformants. All the seeds germinated in about 2-3 weeks’ time, but not all of them survived. Only the resistant ones showed growth while the nontransformed ones bleached and died (Figure 14A). The seeds were initially tested in MS finale media plates to screen a large number of seeds at once. After transformed plants were obtained, they were transplanted into individual boxes of MS media without Finale (Figure 14B). Seeds were collected from these plants for further analysis. Wild-type seeds were also used as negative control. Although the floral dip transformation technique has a 1% transformation rate, more than one line of transgenic plants were obtained successfully using both the screening methods.
Figure 14: Screening in media
A. Positive transformants showed germination and growth into green plants as compared to the untransformed ones that bleached and died. B. Each survived plant was then transplanted into individual boxes containing regular MS media.

Detection of the inserted gene

Once the positive transformants were obtained, they were further screened for the presence of maize ME gene. The plants were screened for the presence of the insert, i.e., a part of KAT2 gene, 682bp and the ME gene, 1745bp, which together make a 2.4kb band. Whole construct DNA, i.e. insert and pMP535, was used as control (Figure 15). All of the plants that survived the Finale test showed the presence of the gene of interest, confirming them to be positive transformants.
Figure 15: Detection of inserted gene

Lane 2, 3, 4, and 5 in the gel showed the presence of a part of the KAT2 and ME insert, which is 2412bp in size from 5 different transgenic plants. Lane 6 contains wild-type DNA used as negative control and showed no band. While lane 7 is the positive control and showed the same size band as the insert DNA.

Screening for homozygous line/Genetic analysis of transformants

Once positive transformants (T0 generation) were obtained, the progeny were then analyzed to obtain T1 generation transgenic plants. Around 20 positive transformants of the T1 generation of each line were then selected for further genetic analysis. The homozygous heterozygous ratio, or the genetic ratio, was 1:2:1 in these T1 generation plants. This type of screening was done to eliminate any heterozygotes and obtain homozygous transformants that exhibit the highest amount of protein expression in them and show its effect on the stomatal biology. The other advantage of homozygous screening is that it eliminates the need to screen plants for positive transformants in further generations. Around 40-50 seeds (T2 generation) were collected from each T1 line plant and then screened again with glufosinate (Finale) using the technique of screening on media. When all the seeds of the T2 generation of a plant survived the screening, that plant was determined to be a homozygote and was selected for further analysis (Figure 16A). While those plants of which some but not all seeds germinated, that plant was determined to be heterozygous plant and was eliminated (Figure 16B).
Figure 16: Homozygous screening
A. All the seeds of T2 generation showed germination and growth on the MS finale media dishes confirming a homozygous T1 plant. B. Some of the seeds of T2 generation showed germination; some showed bleaching and no growth, confirming the T1 generation plant as a heterozygote.

Detection of the transcript

RT-PCR studies were performed on the RNA extracted from the different lines of homozygous transgenic plants. All the transgenic lines showed the presence of the ME gene transcript (Figure 17) at ~ 700bp while the wild-type plant, which was used as a negative control, failed to show the presence of the transcript. This confirms the transcription of the gene into its respective RNA.
Enzyme assay

Once the homozygous lines were obtained, whole leaves from individual plants of these lines were used for extraction of crude protein for malic enzyme assays. Wild-type plants grown in same conditions and of same age and growth stage were selected for the comparative analysis. Fully expanded leaves of 6-7 weeks old, soil grown plants that were about to bolt, were selected for assays. Substrates malate and NADP were provided in the reaction, and formation of NADPH was measured at 340nm in the spectrophotometer after initiation with Mg. NADPH-ME is very specific to the divalent cation Mg and needs it in order to perform its reaction. The reaction was measured for 15 mins without MgCl₂ to eliminate any background activity. After the background was stabilized, the reaction was initiated with 20 mM MgCl₂, and the rise in the Mg dependent conversion of NADP to NADPH was recorded. Multiple plants of each line were assayed and they were compared to more than one wild-type plant. This was done by performing enzyme assays both on transgenic line plants and a
wild-type plant, grown under the same conditions, to make a true comparison in the expression of the enzyme based on the age and the growth stage of that plant (Figure 18). Enzyme assays were performed in duplicates on more than one plant of each transgenic line and same number of wild-type plant.

Figure 18: Enzyme assay
The above shows the rise in the formation of NADPH measured at 340nm. It is a graphical representation of an assay performed on one plant from each line and a wild-type plant. (A) Shows enzyme assays on line1 plants with comparison to the wild-type plant. (B) Shows assay on line2 plants and wild-type plants. (C) Shows assay on line5 and wild-type plants. (D) Shows assay on line6 and wild-type plant.
Protein concentration and specific activity

Specific activity of ME in the crude protein extract was measured against the BSA standard curve (Figure 19) after determining the total protein concentration of both the wild-type and the transgenic protein in the extract.

The equation obtained from the standard curve was used to find the concentration of the total protein in the extract. Specific activity of ME was calculated using the equation

\[ \left( (\Delta A_{340\text{ME}/\text{min}}) - (\Delta A_{340\text{bg}/\text{min}}) \right) \times (1\text{ mol NADPH}/6270\text{OD.L.cm}) \times (1\text{ cm}/1) \times (1\times10^{-3}\text{L}/1) \times (1\text{ ml/x mg protein}) \times (1/0.02\text{ml}) \times (10^6\mu\text{mol}/1\text{mol}) \]

where the $1\times10^{-3}\text{L}/1$ is the assay volume 1ml and the $1/0.02\text{ml}$ is the $20\mu\text{l}$ sample used in the assay mix. Statistical analysis on specific activity of each line and its corresponding wild-type plant was performed to analyze significance in the difference of enzyme activity (Table 1, Figure 20). Statistical data on all the transgenic lines do not show a significant rise in activity ($P>0.05$) in any of the transgenic lines. The
reason is due to the variation in individual assay data of wild-type and transgenic plants of each line. This could be because the plants to be assayed were selected as per their availability and not at the same time. These plants were growing under experimental growth conditions since the growth chambers were being standardized for growing healthier *Arabidopsis* plants at that time.

<table>
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<tr>
<th>Average Specific activity nmol NADPH/mg protein/min</th>
<th>Wt (line1)</th>
<th>Line1</th>
<th>Wt (line2)</th>
<th>Line2</th>
<th>Wt (line5)</th>
<th>Line5</th>
<th>Wt (line6)</th>
<th>Line6</th>
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<tr>
<td>Plant 1</td>
<td>1.24</td>
<td>1.59</td>
<td>10.30</td>
<td>12.27</td>
<td>9.03</td>
<td>14.82</td>
<td>6.19</td>
<td>6.71</td>
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<tr>
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<td>3.83</td>
<td>12.81</td>
<td>2.32</td>
<td>3.33</td>
<td>15.15</td>
<td>19.47</td>
<td>19.85</td>
<td>20.45</td>
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<tr>
<td>Plant 3</td>
<td>4.23</td>
<td>4.38</td>
<td>5.46</td>
<td>5.48</td>
<td>14.50</td>
<td>13.22</td>
<td>8.06</td>
<td>12.88</td>
</tr>
<tr>
<td>Plant 4</td>
<td>3.77</td>
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<td></td>
<td>6.37</td>
<td>8.78</td>
<td></td>
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<tr>
<td>Mean Spec. Act.</td>
<td>3.27</td>
<td>5.79</td>
<td>6.03</td>
<td>7.03</td>
<td>11.26</td>
<td>14.07</td>
<td>11.37</td>
<td>13.35</td>
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</tbody>
</table>

**Table 1: Specific activity of transgenic and wild-type plants**

The above table shows the specific activity of each assay performed on different plants of each line and their respective wild-types. It further shows the mean specific activity, standard deviation, standard error of the mean and the P-values.
Figure 20: Graphical representation of enzyme activity in transgenic and wild-type plants
The bar graph represents the mean specific activity for each transgenic line and its corresponding wild-type plant (Line1 P=0.17, Line2 P=0.39, Line5 P=0.19, Line6 P=0.37). The error bars are the standard error of the mean of the plants.

SDS-PAGE and Western blotting

Once specific activity of the protein was determined, the next step was to detect the presence of the maize malic enzyme protein in the crude extract. SDS-PAGE was carried out to separate proteins based on their charge and molecular weight. This experiment was performed only on line1 plants. Crude extract from two separate line1 plants and the wild-type plants were used. The purified malic enzyme with the C-terminal his-tag and the N-terminal his-trx tag was used as a positive control.

Western blot analysis was performed thereafter on the separated protein gel and treated with anti-his antibodies to check for the presence of the his-tagged ME protein. The Western blot detected the control his-tagged and his-trx-tagged protein at the right size of 72kadal and did not show the protein in the wild-type as expected.
However it failed to show the presence of the protein in the transgenic plants (Figure 21).

![Western Blot Image]

**Figure 21: Western Blot**

The above western blot was performed on the crude protein extract of two different line1 plants and two wild-type plants of same age and same growth stage. The control showed the presence of the 72kd his-tagged and his-trx-tagged maize malic enzyme protein. The wild-type and the transgenic lines did not show the presence of the his-tagged protein. Lane1 showed the Benchmark his-tagged protein standard (Invitrogen), Lane 8 showed the Benchmark protein ladder (Invitrogen)

**Western blotting on guard cell protoplast.**

Guard cell protoplast was isolated from transgenic line1 and line2 plants and also from wild-type plants of same age and growth stage. The proteins were separated by using SDS-PAGE followed by western blot to detect the presence of the protein. The purified malic enzyme with the C-terminal his-tag and the N-terminal his-trx tag was used as a positive control. Neither line1 nor line2 guard cell protoplast shows the presence of the protein; the positive control was detected at 72kd (Figure 22).
Figure 22: Western blot of guard cell protoplast

The above western blot was performed on the guard cell protoplast extract of line1 and line2 plants and wild-type plants. The control showed the presence of the band at 72kd. The wild-type and the transgenic lines did not show the presence of the his-tagged protein. Lane1 showed the prestained protein ladder (Invitrogen). Lane 8 shows the Benchmark his-tagged protein standard (Invitrogen).
DISCUSSIONS

The present study was designed by taking into consideration the decrease in stomatal conductance of the transgenic tobacco plant due to the over-expression of the maize $C_4$ form of NADP-ME using a constitutive promoter (Laporte et al., 2002). Another basis for the study was the fact that malate is lost from guard cells, through their respective ion channels, upon stomatal closure where malate can also be metabolized or converted back to starch (Assman, 1999). Making the guard cells a direct target for $C_4$ maize ME expression could detect the effect of the increased malic enzyme activity on the guard cell malate leading to stomatal closure.

The presence of the guard cell biased promoter KAT2 and the maize ME gene in the PCR study shows that the maize ME gene was successfully transformed into the *Arabidopsis* plant with the pMP535 vector construct. Multiple homozygous transgenic lines were obtained, after herbicide screening, by performing independent transformation events. These lines were also confirmed to have the malic enzyme gene and its transcript by PCR and RT-PCR. In the above study, the repeated enzyme assays on several plants of all the transgenic lines show an increase in the production of NADPH when the absorbance was measured at 340 nm. The most important observation made in the study was that the increased activity of malic enzyme was seen in the transgenic plants, with relative difference in the level of activity within two lines of transformants, more so than the wild-type plants. However, since the statistical data did not support any significant rise in the specific activity more enzyme assays need to be performed. The reason for the variation in the specific activity, in individual assay of the wild-type plants and transgenic plants is likely that the plants were growing under standardizing conditions of the growth chambers. It is important to select all transgenic line plants and wild-type plants growing under same
growth conditions and of the same age to make a true comparison of the specific activity.

The above study included the chloroplast form of maize malic enzyme. This form is a more active form and is synthesized in the cytoplasm with a chloroplast transit peptide that leads it into the bundle sheath chloroplasts (Rothermel and Nelson 1989). This C₄ maize malic enzyme with an optimum pH 8 is found to be active in the Arabidopsis guard cell pH 7.5. Thus it could be concluded that there was no suppression of the foreign gene by the host plant, Arabidopsis. However, use of the guard cell biased promoter KAT2 and the elimination of the chloroplast transit peptide of the maize ME should ideally lead the protein into the guard cell cytoplasm. This can be further determined by performing RT-PCR on the guard cell protoplast and detecting the expression of this gene. Detecting the protein only in the guard cell protoplast and not in any other plant cell could further support the hypothesis.

Transgenic Arabidopsis plants, which would express maize ME throughout the plant using the constitutive promoter, could be used as controls for the above and for the rest of the suggested studies.

The failure to detect the presence of the protein in the crude extract of the transgenic plant in western blot could be due to the minuscule amount of the his-tagged malic enzyme protein in the crude extract. The enzyme is targeted only to the guard cell cytoplasm, which makes a very small volume of the complete plant enzyme. To resolve this, the guard cell protoplast was isolated from the transgenic plants, and protein was extracted from this protoplast. However, the western blot performed on the protoplast protein failed to work. Since the his-tag is present both on the N- as well as the C- terminal of the control, in comparison to his-tag only on the C- terminal end of the protein, it is not certain as to which end his-tag of the control
protein is being detected. These results were from a preliminary study and thus it is difficult to draw any conclusion. Another approach to the protein detection study could be by purifying malic enzyme using the histidine-binding nickel bead resin chromatography column from the crude extract or protoplast followed by a western blot. Immunohistochemistry studies could also be performed where the leaf can be fixed and sectioned and then treated with anti-his antibodies followed by fluorescent secondary antibody to detect the fluorescence under fluorescent microscope.

The direct effect of this study is supposed to be on the malate ion pool within the guard cell, so it is important to check the amount of malate in the guard cell protoplast of these transformants. Performing enzyme linked spectrophotometric analysis by providing malic enzyme to the assay and measuring the amount of malate formed in both, the crude extracts and isolated protoplast could detect the changes in the malate ion pool.

It is important to study the stomatal phenotype of the homozygous transgenic plants that were generated in the above study. The water conservation efficiency of the engineered plants can be verified by performing dry down experiments or hydroponic experiments on these homozygotes (Laporte et al., 2002). Under dry down conditions, the plant pots can be saturated with water and then covered with plastic wrap and aluminum foil to prevent evaporation of water, and the percentage soil moisture of the wild-type and transgenic plant can be calculated. Plants can be grown under hydroponic conditions and the fresh weight gain of the plants over the water-used ratio could be studied in a course of time. Another important study in progress is the measurement of the stomatal conductance or the measurement of gas exchange of the various transgenic lines in comparison with the wild-type plants by using LiCor 6400 Photosynthesis System. It is the measure of stomatal aperture size based on the
rate of water loss from the leaf. The larger the pore size, the greater the stomatal conductance-to-water-vapor, and the more water is lost from the plant. Comparing the change in the phenotype of the stomata with respect to the malate ion pool in the guard cell would support the hypothesis that it is the malate in the guard cell that is responsible to an extent for the stomata movement. It will also show the direct effect of the increase in the enzyme on the stomatal aperture and amount of water loss from the open stomata.

A key component of the study was to develop transgenic *Arabidopsis* plants that over-express maize NADP ME, which has been achieved. The above study has created groundwork by producing several lines of homozygous transformants that have been confirmed by both herbicide screening, PCR and RT-PCR, which exhibit increased malic enzyme expression. The suggested future study should now provide data on the effect of this expression on the malate ion pool of the guard cell and on the stomatal conductance of these homozygote transformants.

The manifestation of such a project is mainly on cultivating crops in countries where there is stress of the growing population on the environment, leading to decreased water resources for agriculture and at the same time increasing the demand of crop production. Altering stomatal guard-cell malate metabolism could overcome the need for water in areas with low water availability and would still fulfill the need for high crop yield.
REFERENCES


APPENDIX

Primers
Primers used for amplification of the KAT2 promoter and Malic enzyme gene.

KAT2 NdeI Right 5’CGATCATATGAAGGTTAGTTATAAAATATAGTGA 3’
KAT2 XmaI Left 5’CGATCCCGGGAAAAACGCAAAGTGAATCCTCTCT 3’
ME-NcoI-His-Right 5’
CGATCCATGGCTACACCACCACCACCACCACCACCACCGTAGTTGCGGTAGACGG 3’
ME NdeI Left 5’CGATCATATGGCGATGGTCTCCAACCGGGAGA 3’

Sequencing primers
M13 Forward 5' TGTTAAACGACGCGCCAGT 3'
M13 Reverse 5' CAGGAAACAGCTATGACC 3'

Custom sequencing primers for the KAT2 promoter and Malic enzyme gene.

KAT2 838 Right 5’ TTTTCTCTAATTCATAATGCTTTTTC 3’
KAT2 812 Left 5’ TGAAAAACGATTATGAATTAAGGAAA 3’
KAT2 1601 Right 5’ TTCCACTATTCGTTAGTAGATCCAA 3’
KAT2 1576 Left 5’ TTTCATCTACTTAGAATAGTTGGA 3’
Maize ME 362 Left 5'AGGAGCTGCCCAGTCATGCCCTGGG 3'
Maize ME 1027 Right 5' CATCATACTCCTCGCCAGTCG 3'
Maize ME 1495 Left 5'ATCGGGACATCTGGAGTTG 3'
Maize ME 1603 Right 5'AATGCGAGGTTGCTTGG 3
pMP535 minibinary vector

Sequence of pMP535 minibinary vector and the coordinates of various regions.

1-630 RK2 oriV
631-1636 nptIII
1637-3129 plasmid RK2 TrfA region
1935-3083 TrfA
3130-3299 TDNA RB
3944-4048 E9 term
3945-4048 MCS
4326-4049 nos term
4878-4327 BAR
5199-4879 nos promoter
5239..5405 TDNA LB

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<thead>
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<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
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</table>
1  CGCTCACCGG GCTGGTTGCC CTCGCCGCTG GGCTGGCGGC CGTCTATGGC
101  CGCGCCGGGT TGTTGATACC TCACGGAAAA CTTGGCCCTC ACTGACAGAT
201  ACAGATGAGG GCCAGGCTCG ATTTCGGCCG GCGACGTGGA GCTGGCCACG
301  TGTGGACAAG CCTGGGGATA AGTGCCCTGC GGTATTGACA CTTGAGGGGC
401  TGCTGACAGA TGAGGGGCGC ACCTATTGAC ATTTGAGGGG CGTGCACACG
501  TACCTGTCTT TTTAACCGC TTTTAAACCA ATATTTATAA ACCTTGTTTT
601  CCAACCACCTG TCGACACCTCG CGGCCCGCTG CTGAGGCTTG ACACATCACCC
701  CTCTTGTCTA CACATCTGGGT TTAAGAATTG CAGGCGGCGA CTGCTATGGC
801  CGCGCGCGCGG GTGGCGTGGG ATCTGCTCAT GAGTGAGGCC GATGGCGTCC
901  TATCTGCTCAT GAGTGAGGCC GATGGCGTCC TTTGCTCGGA AGAGTATGAA
1001  ACCTATGATG TGGAACGGGA AAAGGACATG ATGCTATGGC TGGAAGGAAA

56
GCTGTATGAT TTTTAAAGA CGGAAAAGCC CGAAGAGGAA CTTGTCTTTT 1400
1401 CTCAGCGGCA AGATCGGGGA TACCAAGTAC GAGAAGGACG 1800
1501 CCCACGGCGA CCTGGGAGAC AGCAACATCT TTGTGAAAGA TGGCAAAGTA
1801 GCCAGACTGC CGCCGCCGAAC CCGAGTCTCT CCGCATCAAG 1700
1901 GCCAGTGGT GCACGAGGAG TACCAAGTAC GAGAAGGACG 2000
2001 CATGCGGAAA CCATGCGGAC CGAGAGGAG TACCAAGTAC GAGAAGGACG 2400
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4301 GCCAGACTGC CGCCGCCGAAC CCGAGTCTCT CCGCATCAAG 100
5001 GCCAGACTGC CGCCGCCGAAC CCGAGTCTCT CCGCATCAAG 000
NCBI BLAST

NCBI BLAST of the insert (KAT2 and Maize ME) sequences in the pCRII-TOPO vector

> gi|12666979|emb|AJ288900.1|ATH288900 Arabidopsis thaliana kat2 gene for inward rectifying K+ channel, exons 1-11
Length=5369

Score = 4478 bits (2259), Expect = 0.0
Identities = 2259/2259 (100%), Gaps = 0/2259 (0%)
Strand=Plus/Plus
There is nucleotide difference seen at 963-965 and 1919bp (highlighted in the figure above) in the core sequence and that of the NCBI sequence (ZmChlMe1). However these base pairs are identical to the base pairs of an isoform (ZmChlMe2) of NADP-ME, which codes for the 62kd protein (Tausta et al., 2002).
Sequencing

Sequencing performed on the complete insert using Sequencher (Gene Codes)

The above figure shows the complete contig that is obtained using sequences of the complete insert DNA from the sequencing core. The whole contig runs around 4kb, which is the expected size of the complete insert, KAT2 and ME.

The above figure highlights the Xmal site that is at the N-terminal of the KAT2 promoter. The sequence was obtained from the sequencing core using the M13 reverse primer.
The above figure highlights the NdeI restriction site that is present on the C-terminal end of the KAT2 and N-terminal end of ME gene. The NdeI site also acts as a start codon of the maize malic enzyme gene.

The above Figure highlights the NcoI restriction site on the C-terminal of the Maize malic enzyme. It also shows the presence of the Histidine tag before the restriction site, which will be expressed, and a stop codon after the restriction site.

The above figure highlights the NcoI restriction site on the C-terminal of the Maize malic enzyme. It also shows the presence of the Histidine tag before the restriction site, which will be expressed, and a stop codon after the restriction site.