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Isolation and Analysis of the KATI Promoter from Arabidopsis Thaliana

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Abstract
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Expression of NADP-ME in Arabidopsis thaliana required the isolation of a strong, guard-cell specific promoter. A promoter of a potassium channel gene, KAT1, is strongly expressed in guard cells. Analysis of the Arabidopsis genome was done to locate KAT1. The KAT1 promoter was amplified and isolated through Polymerase Chain Reaction (PCR). Sequence analysis confirmed isolation of KAT1 with minor mutations. Visualization of KAT1 expression was confirmed through the analysis of transgenic plants with the KAT1 promoter fused to the GUS reporter gene. Significant expression of KAT1 was detected exclusively in guard cells of 9-day-old seedlings. The KAT1 promoter will be inserted into a binary vector and then Agrobacterium to transform Arabidopsis thaliana and create transgenic plants. Expression of KAT1 with NADP-ME in Arabidopsis thaliana should result in lower concentrations of malate in guard cells, decreased aperture size of stomata, and a decrease in transpiration rate during gas exchange.

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Isolation and Analysis of the KAT1 Promoter from *Arabidopsis thaliana*

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TABLE OF CONTENTS

ABSTRACT

Plants that reduce water loss by transpiration present less agricultural stress to the environment. Transpiration is limited by the size of pores, or stomata, on the surfaces of leaves. Stomatal aperture is related to the ion concentration in surrounding guard cells, which varies in response to fluctuating concentrations of potassium ions, the principle counter-ion chloride, and the organic counter-ion malate. Levels of malate in guard cells may be altered by increased activity of NADP-Malic Enzyme (NADP-ME) expression. The goal of our research is to create transgenic *Arabidopsis thaliana* plants with increased NADP-ME expression in the guard cells, limiting transpiration through decreased stomatal aperture size. Such plants are expected to have less open stomata, be sufficient in yield, and retain the ability to respond to changing environmental conditions.

Expression of NADP-ME in *Arabidopsis thaliana* required the isolation of a strong, guard-cell specific promoter. A promoter of a potassium channel gene, KAT1, is strongly expressed in guard cells. Analysis of the *Arabidopsis* genome was done to locate KAT1. The KAT1 promoter was amplified and isolated through Polymerase Chain Reaction (PCR). Sequence analysis confirmed isolation of KAT1 with minor mutations. Visualization of KAT1 expression was confirmed through the analysis of transgenic plants with the KAT1 promoter fused to the GUS reporter gene. Significant expression of KAT1 was detected exclusively in guard cells of 9-day-old seedlings. The KAT1 promoter will be inserted into a binary vector and then *Agrobacterium* to transform *Arabidopsis thaliana* and create transgenic plants. Expression of KAT1 with NADP-ME in *Arabidopsis thaliana* should result in lower concentrations of malate in guard cells, decreased aperture size of stomata, and a decrease in transpiration rate during gas exchange.

INTRODUCTION

In 2007, the Intergovernmental Panel on Climate Change (IPCC) released a summary of scientific research confirming the effects of global warming on the Earth's climate. Increasing global temperatures correlate with considerable changes in weather patterns, precipitation levels, and increased occurrences of drought and flooding. The IPCC claims a projected decrease of water availability by 10-30% over some dry regions at mid-latitudes and in the dry tropics by mid-century (IPCC, 2007). Locally, the impact of rising temperatures is most evident in the decrease of water levels in the Great Lakes. Since 1998, the water levels of Lakes Michigan and Huron have dropped at the fastest pace ever recorded, primarily due to decreases in precipitation (GLERL, 2001).

The impacts of decreased precipitation and increased prevalence of drought conditions will be most noticeable in the world's agriculture. Approximately 70% of currently available freshwater is used for agriculture, and 40% of the world's food is grown on irrigated soils (Somerville and Briscoe, 2001). The additional use of fertilizers, chemicals, and widespread development of irrigation systems, present massive ecological burdens and will become difficult to sustain in future dry climates. Specifically, irrigation systems contribute largely to inefficient agriculture and can be improved by how well crop plants use water and conduct photosynthesis.

A significant amount of scientific research is dedicated to creating more water-efficient agriculture. Historically, crop improvement was limited to strengthening or reducing already present characteristics, through selective breeding practices. With the expansion of the molecular understanding of plant function and growth, a large amount of biotechnology is devoted to creating novel phenotypes through genetic engineering

(Vinocur and Altman, 2005; Umezawa et. al., 2006). Such engineering would ideally create phenotypes that require less water, fertilizers, pesticides and herbicides, and still produce sufficient, sustainable crop yields (Chaves and Oliveira, 2004).

Research dedicated to creating water-efficient plants focuses on reducing the amount of water lost through transpiration during growth and in response to drought conditions. The onset of drought signals a number complex plant responses designed to aid in water conservation. Transpiration is limited collectively through alterations in gene expression, accumulation of metabolites or other compounds involved in osmoregulation such as abscisic acid (ABA), and activation of proteins that function to scavenge reactive oxygen species (Umezawa et. al., 2006; Chaves and Oliveira, 2004). Initially, research was focused on engineering drought tolerant plants, which conserve water in response to arid conditions and increased drought signals. A significant area of research goes beyond this by engineering drought avoidant plants, which conserve water during growth in addition to responding to environmental changes. Traditional irrigation systems have allowed plants to grow in constant water surplus, and slowed the natural development of drought avoidant phenotypes (Laporte et. al., 2002).

The engineering of drought avoidant plants focuses on preventing extensive water loss through transpiration. Transpiration is regulated by the size of pores, or stomata, mainly found on leaf surfaces of plants. Stomata open to allow for gas exchange necessary for photosynthesis, but in the process, lose water to the surrounding dry air. Therefore, the fundamental role of stomata is to balance the trade-off between photosynthetic activity and transpiration according to varying environmental conditions. The balance between gas exchange and transpiration is regulated by varying stomatal

aperture size, which in turn, is regulated by the amount of turgor pressure in the surrounding guard cells.

Guard cells respond to varying environmental factors such as soil moisture, light conditions, humidity, CO_2, varying ion concentrations, and hormonal signaling (Rob et. al., 2005; MacRobbie, 1998). Turgor pressure in the guard cells is primarily the result of the influx of potassium ions into the cell via inwardly-rectifying potassium ion channels. The influx of positive ions creates osmotic potential, driving the movement of water into guard cells. There is also evidence that salt-enriched soils allow for the accumulation of sodium and result in similar changes in osmotic movements in guard cells, however, are often irreversible (Rob et. al., 2005; Willmer and Mansfield, 1969). The influx of potassium ions and water into guard cells causes the opening of stomata (Raschke et. al., 1988).

The significant role of potassium ion channels in osmoregulation has made them a topic of intense research. There are three known families of membrane spanning potassium channels in plants; the *Shaker* super-family first identified in *Drosophila* fruit flies, Tandem Pore K+ channels, and Kir-Like channels, which share similarities in structure and function (Lebaudy et. al., 2007; Gambale and Uozumi, 2006). *Arabidopsis thaliana* has been shown to contain at least one channel in each potassium channel family (Lebaudy et. al., 2007). The two principle inwardly-rectifying voltage-gated potassium channels present in guard cells are KAT1 and KAT2 (Pilot et. al., 2001; MacRobbie, 1998; Nakamura et. al., 1995).

The first guard cell-specific ion channel to be cloned and intensely studied was KAT1. KAT1 has been shown to localize to the plasma membrane during stomatal

opening by the use of electrophysiological studies and confocal microscopy (Lebaudy et. al., 2007; Hurst et. al., 2004). Specifically, expression of the KAT1 promoter fused to Green Fluorescent Protein (GFP) has been identified *in vivo* in guard cells (Chytilova et. al., 1999). Activation of KAT1 is brought about by light, which stimulates a H+-ATP-ase causing membrane hyperpolarization, thereby opening KAT1 (Nakamura et. al., 1995). Recent research indicates that KAT1 is also phosphorylated, most likely by a calcium-dependent kinase, in response to ABA (Gambale and Uozumi, 2006; Li et. al., 1998). KAT1 activity may also be influenced by auxin (indole-3-acetic acid) however, is limited to cells involved in hypocotyl elongation and likely insensitive in guard cells (Phillipar et. al., 2004).

KAT2 is likewise involved in potassium transport, but expressed at much lower levels than KAT1 (Rob et. al., 2005; Szyroki et. al., 2001). Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) indicates that KAT1 transcripts are approximately three times higher than KAT2 transcripts (Lebaudy et. al., 2007; Szyroki et. al., 2001). KAT2 is primarily expressed the phloem of minor veins but also in guard cells (Pilot et. al., 2001). Experimental data also shows that KAT1 and KAT2 may functionally interact with each other (Lebaudy et. al., 2007; Pilot et. al., 2001).

In *Vicia faba* guard cells, potassium ion uptake is counterbalanced by accumulations of chloride ions and the organic anion, malate (Raschke and Schnabl, 1978). Malate, a dicarboxylic acid, is synthesized in the cytosol of guard cells from the degradation of starches stored in chloroplasts by phospho*enol*pyruvate carboxylase (Outlaw et. al., 1981). During stomatal opening, malate accumulations increase 6-fold in guard cells, creating the osmotic potential necessary for the subsequent water influx

(Outlaw and Lowry, 1977). This balance of potassium ions and counter-ions determines the aperture size of stomata.

Events leading to the closure of stomata are still somewhat unclear (Outlaw et. al., 1981; Laporte et. al., 2002). Very few guard cell-specific membrane channels have been identified that correlate with stomatal closure (Negi et. al., 2008; Vahisalu et. al., 2008). A guard cell-specific, outward-rectifying potassium channel, GORK, shows involvement in potassium efflux from the cell (Ache et. al., 2000; Gambale and Uozumi, 2006). Recently, a guard cell-specific anion channel, SLAC1 has been shown to be predominately expressed in guard cells and localizes to the plasma membrane. SLAC1 anion channels are permeable to malate and chloride, and is likely involved in guard cell osmoregulation (Negi et. al., 2008; Vahisalu et. al., 2008). The efflux of potassium and malate may sufficiently cause subsequent water efflux, causing stomatal closure (Negi et. al., 2008; Vahisalu et. al., 2008; Rob et. al., 2005; Hedrich et. al., 1994; Schmidt and Schroeder, 1994).

It is hypothesized that altering levels of malate may decrease stomatal aperture during gas exchange, preventing excessive water loss through transpiration and still demonstrate normal growth patterns (Laporte et. al., 2002). NADP-Malic Enzymes (NADP-MEs) catalyze the oxidative decarboxylation of L-malate yielding pyruvate, CO_2, and NADPH. NADP-MEs are present in a large number of plant species, grouped into four related phylogenetic groups (Drincovich et. al., 2001; Wheeler et. al., 2005). NADP-MEs are responsible for a variety of metabolic reactions dependent on the method of photosynthesis and location in the cell. Additional research of NADP-MEs shows involvement in plant defense, fatty acid biosynthesis, and control of cytosolic pH (Liu et.

al., 2007). Studies in rice, *Oryza sativa*, also indicate NADP-MEs may aid in plant growth, including increased salt tolerance in alkaline soils (Cheng and Long, 2007; Liu et. al., 2007).

There is a large variety in NADP-MEs with certain isoforms exhibiting higher levels of activity. The maize NADP-ME1 isoform is one of the principle enzymes involved in C_4 photosynthesis, an alternate method of photosynthesis that reduces transpiration. The NADP-ME1 isoform in maize is thought to be sufficiently active that if expressed in other plants, may potentially affect stomatal aperture, thus limiting transpiration through enzymatic activity. Recent research has confirmed this notion, as maize NADP-ME1 was isolated and expressed in tobacco resulting in decreased stomatal conductance (Laporte et. al., 2002). The observed decreases in stomatal aperture size is either due to (1) a decrease in the concentration of the counter-ion malate within guard cells, creating lower osmotic potential and thus preventing the full opening of stomata, or (2) an increase in the intercellular CO_2 concentration, a product of NADP-ME1 activity, in mesophyll cells which is thought to contribute to stomatal closure (Laporte et. al., 2002).

In order to better understand the effects of maize NADP-ME1 expression in other plants, it has been expressed in *Arabidopsis thaliana*. Experimentation is ideal in *Arabidopsis thaliana* because it serves as a model organism for typical C_3 plants, including future agricultural crops. The recent completion and availability of the entire sequenced *Arabidopsis* genome also encourages its use. Measurements of stomatal conductance have been attempted by over-expressing a gene encoding maize NADP-ME1 in the genome of a model plant type *Arabidopsis thaliana* (Laporte et. al.,

unpublished data). The goal of our research is to successfully express NADP-ME1 driven by a strong, guard cell-specific promoter. Analysis of the resulting phenotype will shed light on the mechanisms of stomatal closure and the role of NADP-ME1 in producing a drought avoidant phenotype in *Arabidopsis thaliana*.

Initial experimentation involved the use of the constitutive Mannopine synthase promoter to drive NADP-ME1 expression throughout the entire plant. The resulting transgenic plants however, developed necrotic spots and were difficult to analyze. Whole plant expression of maize NADP-ME in *Arabidopsis thaliana* has been shown to cause accelerated senescence in dark conditions (Fahnenstich et. al., 2007). This suggested that NADP-ME1 expression could be improved by localizing it in guard cells. Further experimentation involved the promoter of KAT2 gene, responsible for guard cell-specific potassium ion channels. However, as previously mentioned KAT2 has relatively weak expression patterns and also localizes to other areas of the plant, such as phloem veins. Consequently, it is difficult to detect NADP-ME1 expression driven by the KAT2 promoter and large variations in stomatal conductance have not been consistently observed (Laporte et. al., unpublished data).

Expression of maize NADP-ME1 in *Arabidopsis thaliana* requires a promoter that will both strongly express the gene as well as localize it to guard cells. It is hypothesized that such expression patterns may be successful though the use of the promoter of a guard cell-specific potassium ion channel gene, KAT1. The high expression of the KAT1 gene in guard cells provides an ideal promoter to similarly express maize NADP-ME1 in *Arabidopsis thaliana.*

Our goal was to isolate the KAT1 promoter in order to create transgenic lines expressing NADP-ME1 in *Arabidopsis thaliana* for analysis of stomatal conductance. Isolation of the KAT1 promoter was similar to the procedure published by Nakamura et. al., (1995). The KAT1 promoter will be inserted in a binary vector along with maize NADP-ME1 and used to transform *Agrobacterium*, then *Arabidopsis thaliana*.

Another goal was to confirm the localization of KAT1 to guard cells. This has been confirmed by growing and analyzing KAT1::GUS transgenic plants. In these plants, the KAT1 promoter is fused to β-Glucuronidase (GUS), a reporter gene. GUS expression is indicative of KAT1 expression, and is readily confirmed through staining procedures. GUS expression was present exclusively in the guard cells of 9 day-old seedlings. GUS expression in older leaves was not found, and requires further experimentation.

MATERIALS AND METHODS

Analysis of *Arabidopsis thaliana* Genome: Location of KAT1 Promoter

Location of the KAT1 promoter and corresponding gene was identified using the databases of the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov and The Arabidopsis Information Resource (TAIR) http://www.arabidopsis.org. Sequence comparison between the genomic mRNA coding strand (ch. 5, KAT1 gene; NM_123993) and complete chromosome 5 (NC_003076) was done by using BLAST 2 Sequences, http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi, available through NCBI. Sequence was compared to published data according to Nakamura et. al (1995) and their clone (GenBank accession No. U25088).

DNA Extraction

DNA was extracted from wild-type *Arabidopsis thaliana* plants, Columbia ecotype, using MasterPure™ Plant Leaf DNA Purification Kit by Epicentre® (Madison, Wisconsin, USA) protocol and reagents. Quantification of DNA was done using a Beckman Coulter DU-800 spectrophotometer to measure absorbance at 260nm and 280nm to determine the concentration.

Custom Primers

Custom primers with similar melting points were designed to amplify and sequence the KAT1 promoter using Primer 3 program, http://biotools.umassmed.edu/bioapps/primer3_www.cgi. Delta G values for homodimer,

heterodimer, and hairpin loops were determined using OligoAnalyzer 3.0, http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/, by Integrated DNA Technologies. Unlike Nakamura et. al. (1995), custom primers were designed to amplify a slightly longer segment of the promoter, 3.7 kilobase pairs, for future adjustments necessary for insertion into the binary vector pMP 535. Mutagenic PCR primers were also designed to add an NdeI site to the 3' end of the KAT1 promoter for future insertion in the binary vector pMP 535 containing the NADP-ME1 gene.

PCR Primers	**Left**: CTTTTTGATGATCTCTAAAGACAAAGAAA **Left with added NdeI site**: CATATGCTTTTTGATGATCTCTAAAG	**Right**: CAAATGGTACATTGCGAAAACA
Custom Sequencing Primers	**Forward**	**Reverse**
	F550: GCATTTGAAGGTGGGAGTGA	R313bp: TTACGCCTTTTCTTCACCATTG
	F1158bp: CTGGCTCGCAAGCAATATGT	R847bp: TGATTGGTTTTGGGGACTAGAAA
	F1731bp: TGACATGGGAAAACAAAAGAAAAG	R1415bp.: TCTAGCACACTAGAGAAATGAAAATGAAAACATACA
	F2001bp: TTTGTTTCTGCGTCGATCTGA	R1969bp: GGCGGAAAGGTTTGAAGAATT
	F2264bp: AAATGAATTATTTCGGCTTTCAAATT	R2449bp: ACAACCCCACTTGCACTAGGA
	F2827bp: ACGACCAGATTGAACCATGCT	R2574bp: AGACCAACCGATTCCAAGACTT
	F3391bp: GTTTGTGTAATGTCGGCATGATT	R3085bp: CAATAAAAGTCTCAACTGCCTTTACA
	F3453bp: GCTTGATATATGGGTCCTTCCA	

Table 1: Sequence of PCR primers and custom designed sequencing primers (5' → 3'). PCR using Left primer with NdeI site was used to amplify KAT1 promoter with the added NdeI site to allow correct insertion into binary vector pMP 535.

Polymerase Chain Reaction (PCR) Amplification of KAT1 Promoter

PCR reagents and protocol were obtained from Invitrogen® (Carlsbad, California, USA) and used at listed concentrations in 25μL reactions. A magnesium curve from 2mM to 4mM was performed to identify optimal reaction conditions for High Fidelity Taq Polymerase. 1μL of 575ng/μL genomic template DNA was used in each reaction. Separate reactions used different genomic template DNAs from two different wild-type plants to ensure usefulness of both. A negative control without template DNA was performed in order to identify any potential errors. PCR was performed under varying conditions until successful. PCR amplification of the KAT1 promoter was accomplished using the following program:

1. Denaturing Temperature at 94^{o}C for 2 minutes
2. Denaturing Temperature at 94^{o}C for 30 seconds
3. Annealing Temperature at 49.4^{o}C for 30 seconds
4. Extension Temperature at 68^{o}C for 4 minutes
5. Go To step 2, 30 cycles
6. Final Extension Temperature at 68^{o}C for 10 minutes
7. Hold at 4^{o}C

Gel Electrophoresis

Gel electrophoresis was used to visually confirm the presence of PCR products or results of restriction enzyme digests. 1% agarose gel was cast using 0.5X TBE buffer and Gel Red® by Biotium® (Hayward, California, USA) 1.5mL/45mL gel was added to stain the gel. 6X loading dye was added to each sample at appropriate dilutions. Banding

lengths were determined by comparison to the 1Kb Plus Ladder by Invitrogen® (Carlsbad, California, USA). Gels were typically run at 110V, until banding patterns were evident.

Luria-Bertani (LB) Media Plates

Nutrient rich plates of LB media were created to culture bacteria for blue-white colony selection. The solution for 1L consisted of 10g of Tryptone, 2.5g of Yeast Extract, 5g of NaCl. After bringing the solution to a pH of 7 by adding 0.5M NaOH, 7.5g of agar was added to the solution and autoclaved. 1mL of 50mg/L kanamycin was added, and media was poured in sterile hood.

TOPO®TA Cloning

Once PCR amplification of the desired KAT1 promoter fragment was successful, it was cloned into *E.coli* cells. TOPO®TA One Shot Cloning into TOP 10 F' competent cells was done according to the Invitrogen® (Carlsbad, California, USA) protocol and reagents. 4μL of PCR product was used in the TOPO® Cloning reaction, 2μL of which was added to TOP 10 F' cells. After heat shocking cells at 42 ^{o}C, and 2 minute incubation on ice, cells were centrifuged at 14,000 x g. The supernatant was discarded, and the pellet was resuspended in 700μL of liquid LB media. LB plates were warmed at 37 ^{o}C, and 40μL of X-gal and IPTG was spread and incubated for 30 minutes. Various amounts of bacteria cells and S.O.C nutrient broth were plated to yield adequate numbers of colonies. White colonies resistant to kanamycin were expected to contain the 3.7Kb insert of the KAT1 promoter, and were selected for future culture.

Liquid Culture

Four healthy, white colonies per PCR product were selected for liquid culture. Selection of white colonies was done using a pipette tip. Samples were incubated in a 1000:1 volume ratio of LB liquid broth and 50mg/L kanamycin solution. Samples were shaken at 37^{o}C overnight at 200rpm until cloudy.

Plasmid Purification

Plasmid purification was done using both the centrifugation and vacuum protocol of the SV Mini-Preps: DNA Purification Kit by Promega® (Madison, Wisconsin, USA). Samples were quantified using Beckman Coulter DU-800 spectrophotometer to measure absorbance at 260nm and 280nm to determine the concentration.

Restriction Enzyme Digests

Protocols varied depending upon the brand of restriction enzyme used and corresponding optimum conditions. EcoRI (5'-GAATTC-3') digests excised 3.7 kilobase pair insert of the KAT1 promoter from PCRII vector, were used to identify successful amplification of PCR reactions. SmaI (5'-CCCGGG-3') and NdeI (5'-CATATG-3') restriction enzyme digests of purified plasmid DNA were used to isolate the desired final 3.2 kilobase pair insert of the KAT1 promoter. SmaI and NdeI digests were also used to confirm the presence of the added site on the KAT1 promoter necessary for insertion into the binary vector pMP 535 for future transformation. To prevent transformation of the template plasmid, EcoRI/PstI double digests were used prior to PCR. Restriction

enzymes were deactivated according to the protocol used. Enzyme digests of DNA were confirmed through gel electrophoresis.

Preparation of Binary Vector pMP 535

The pMP 535 binary vector originates from a related vector pCB302 (Prigge et. al., 2005; Xiang et. al., 1999). pMP 535 is a derivative of the pCB302 vector that has been improved with additional multiple cloning sites and a reduced size, which enable more cloning options for recombination. The pMP 535 binary vector was previously created with the KAT2 promoter and NADP-ME1 (P. Thakur, 2006). To use this vector, KAT2 was removed from the vector by performing sequential SmaI and NdeI digests. Reactions were deactivated and run on a 1% gel as stated above. Bands were excised from the gel, and DNA was purified using the SV Gel and PCR Clean-Up Kit by Promega® (Madison, Wisconsin, USA). Directional cloning of the KAT1 promoter preceding the NADP-ME1 gene into pMP535 requires a 5' SmaI and 3' NdeI restriction enzyme site. The SmaI site is naturally present; however, the 3' NdeI site will be added through PCR with mutagenic primers.

Sequence Analysis of KAT1 Promoter

Isolated DNA from plasmid purification was sequenced at The University of Michigan DNA Sequencing Core using M13 Forward and Reverse primers, as well as designed custom sequencing primers. Sequencing data spanning the entire region of the KAT1 promoter was assembled using either Sequencher 4.7, or Vector NTI Advance 10 software programs. The four PCR products that successfully amplified were sent for full

sequencing. The PCR product with the fewest number of mutations furthest away from the start codon was selected for further experimentation. This PCR product was then used as a template for PCR with mutagenic primers to add the NdeI site at the 3' end of the KAT1 promoter.

Murashige-Skoog (MS) Media: Growth of KAT1::β-glucuronidase (GUS) Seedlings and Plants

MS Media (Murashige and Skoog, 1962) was used to grow healthy seedlings for β-glucuronidase (GUS) staining. KAT1::GUS transgenic seeds were obtained from the Arabidopsis Biological Research Center at Ohio State University http://www.arabidopsis.org/servlets/TairObject?type=germplasm&id=1005211800 (#CS3763; Nakamura et. al., 1995). Seeds were sterilized in 20% bleach solution for 30 minutes and rinsed three times with autoclaved water. Seeds were dried on filter paper under a fume hood and subsequently transferred uniformly onto MS Media plates. Plates were incubated for 9 days in 12 hour white light and dark cycles prior to staining. Seedlings were transplanted to soil, Metro Mix 360 from SunGro®, and grown in Conviron E-15 growth chambers. Growth chambers provided a constant environment of 8 hours of white light at 500$\mu Em^{-2}s^{-1}$ at 20°C, and 16 hours of darkness at 15°C. The relative humidity remained constant at 60%. GUS staining was also attempted on leaves from 51 and 85-day-old plants.

Whole Mount β-Glucuronidase (GUS) Staining Protocol

Leaves selected for staining were from plants of similar age and size. The section of the leaf used for staining was from mid-section to leaf tip, and was done on similarly sized leaves. Seedlings used for staining were 9-days-old, and the entire seedling was used. The procedure was modified from The Arabidopsis Manual (Weigel and Glazebrook, 2002) and Nakamura et. al., (1995). Because of the difficulties in visualizing expression of KAT1::GUS in leaves, the two protocols were compared in order to find optimum staining conditions for both seedlings and leaves.

The protocol in The Arabidopsis Manual (Weigel and Glazenbrook, 2002) was intended for leaves, whereas the protocol in the Nakamura et. al., (1995) paper was designed for seedlings. GUS staining solutions were prepared according to both protocols and were performed as cited.

According to The Arabidopsis Manual mentioned above, seedlings or leaf tips were collected and placed into 90% acetone on ice. Samples were then incubated at room temperature for 20 minutes while staining buffer was prepared. The staining buffer composed of: 0.5M Na_2HPO_4/NaH_2PO_4 buffer (pH 7.2), 10% Triton X-100, 2mM of both $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ diluted in autoclaved water to a constant volume of 1mL. Samples with increased concentration of 5mM $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ were also prepared to determine the effects on substrate metabolism by GUS. Final concentration of the chromogenic substrate X-Gluc (5-bromo-4-chloro-3-indolyl- β-D-glucuronide) was consistently 2mM. Samples were vacuum infiltrated for 30 minutes, and incubated overnight at room temperature. The Arabidopsis Manual then follows with a series of

washes in 20%, 35%, 50%, 70% ethanol for 30 minutes each, and finally fixed in 100% ethanol.

According to Nakamura et. al., (1995) paper mentioned above, seedlings or leaf tips were collected and placed into 90% acetone on ice. Samples were then incubated at room temperature for 20 minutes while staining buffer was prepared. The staining buffer composed of: 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0), 2% Triton X-100, 10mM of Na_2EDTA, and 2mM of both $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ diluted in autoclaved water to a constant volume of 1mL. Samples with increased concentration of 5mM $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ were again prepared to determine the effects on substrate metabolism by GUS. Final concentration of the chromogenic substrate X-Gluc (5-bromo-4-chloro-3-indolyl- β-D-glucuronide) was consistently 2mM. Samples were vacuum infiltrated for 30 minutes, and incubated overnight at room temperature. The protocol cited by Nakamura et. al., (1995) then follows with a series of washes in 20%, 35%, 50% ethanol, then FAA (50% ethanol, 5% formaldehyde, 10% glacial acetic acid) for 30 minutes each. These samples were then fixed in 70% ethanol.

Samples were observed using a light microscope and Leica DFC320 camera full frame at different magnifications. The expression of GUS, the reporter gene, is driven by the activity of the KAT1 promoter. Detection of GUS is observed through its metabolism of the chromogenic substrate X-Gluc, shown by the presence of blue pigment. GUS staining was attempted on whole seedlings as well as mature leaves.

RESULTS

Analysis of *Arabidopsis thaliana* Genome: Location of KAT1 Promoter

Analysis of KAT1 promoter using NCBI and TAIR located the KAT1 gene on chromosome 5 of the *Arabidopsis thaliana* genome. Previous research by Nakamura et. al., (1995) identified the region 3.4 kilobase pairs upstream of the start codon to the KAT1 gene to function in showing guard cell-specific expression. Comparison of the mRNA coding sequence and chromosome 5 using BLAST 2 sequences indicated the start codon was located at 18,763,488 bp. We amplified the region of DNA from 18,763,776-18,767,488bp, an extended region than identified by Nakamura et. al., (1995). According to the direction of the sequence, the KAT1 promoter and gene was found on the complementary strand.

DNA Extraction

Extracted DNA from wild type *Arabidopsis* leaves was quantified at 575ng/μL and confirmed by the presence of a band through gel electrophoresis (Figure 1).

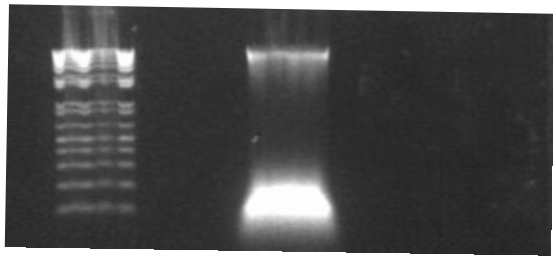

Figure 1: Genomic DNA extraction from wild type *Arabidopsis thaliana* ecotype Columbia. Well 1 contains 1 Kb Plus ladder by Invitrogen® (Carlsbad, California, USA) to estimate band sizes. Well 3 shows successful extraction of genomic DNA. The concentration of the DNA was 575ng/μL.

Polymerase Chain Reaction (PCR) Amplification of KAT1 Promoter

PCR was performed using custom designed primers to amplify the DNA sequence of the KAT1 promoter (Table 1). Comparison of two samples of template genomic DNA, referred to as LP template and CN template, and a curve of $MgSO_4$ concentrations were used due to previous difficulties in amplification. The LP template showed a DNA band at 2mM and 3mM, whereas the range was slightly higher in CN template at 3mm and 4mM $MgSO_4$ concentrations (Figure 2).

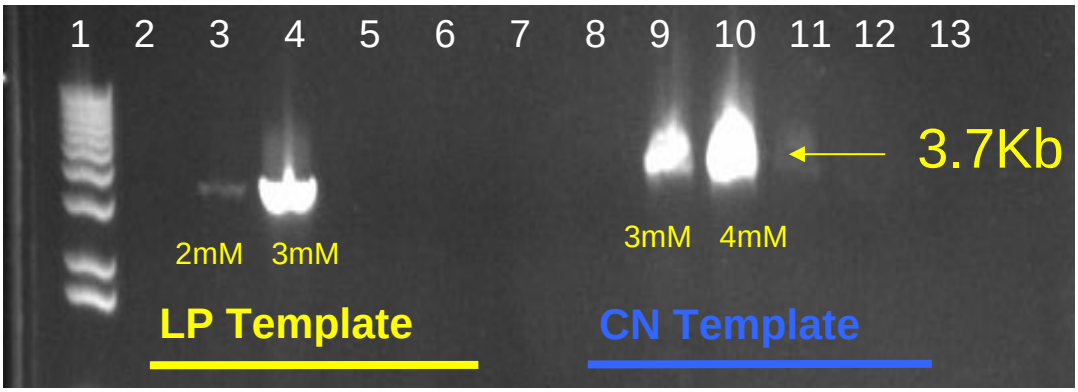

Figure 2: PCR results of $MgSO_4$ curve. Well 1 contains 1 Kb Plus ladder by Invitrogen® (Carlsbad, California, USA) to estimate band sizes. Wells 2-7, and 8-13 represent two different samples of wild type genomic DNA template, LP template and CN template. Four different concentrations from 1mM to 4mM of $MgSO_4$ were tested per template, wells 2-5, 8-11 respectively. A sample without DNA served as a negative control for each template, wells 7 and 13. Amplification occurred in the LP template at 2mM and 3mM, and 3mM and 4mM in the CN template.

TOPO®TA Cloning and Confirmation through Restriction Enzyme Analysis

Each of the PCR products that showed amplification was cloned into *E.coli* competent cells using the TOPO® TA Cloning Protocol and spread on LB Media plates. Four white, kanamycin resistant, colonies were selected per PCR sample to purify DNA from. Each DNA sample was digested with EcoRI restriction enzyme to further confirm

the presence of inserted DNA (Figure 3). All of the digested samples showed the expected banding patterns: undigested vector of approximately 7.7 kilobase pairs, PCR II vector of approximately 4 kilobase pairs, and the KAT1 promoter of 3.7 kilobase pairs. Three DNA bands were visible in each lane, indicating that the PCR amplification of DNA was successful and digestion was incomplete

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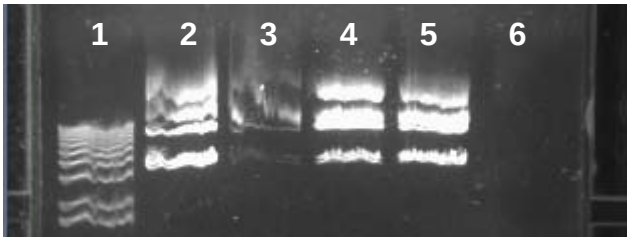


Figure 3: EcoRI digest of four PCR products amplified in varying $MgSO_4$ concentrations. Well 1 is the 1 Kb Plus ladder by Invitrogen® (Carlsbad, California, USA) used to estimate band sizes. Wells 2-5 represent each of the PCR products that had a DNA band visible after gel electrophoresis, indicating successful amplification. Digestion of these PCR products indicates the insertion of the PCR product into PCRII. The top band corresponds to undigested vector of approximately 7.7 kilobase pairs, the middle band is the PCR II vector of approximately 4 kilobase pairs, and the KAT1 promoter of 3.7 kilobase pairs.

Sequence Analysis of KAT1 Promoter

All samples were sent for sequencing with M13 Forward and Reverse primers and custom designed sequencing primers. Sequence analysis revealed minor mutations in all samples. Each sequence was analyzed individually using Sequencher® 4.7, or Vector NTI Advance 10® software (Figure 4). The PCR product showing the fewest number of mutations that were also furthest away from the start codon was chosen for further experimentation (LP template DNA at 3mM $MgSO_4$). Deletions occurred from base pairs 884-886, missing a triplet of thymines, and had an insertion of an adenine at 2313.1 base pairs from the start codon (Figure 5).

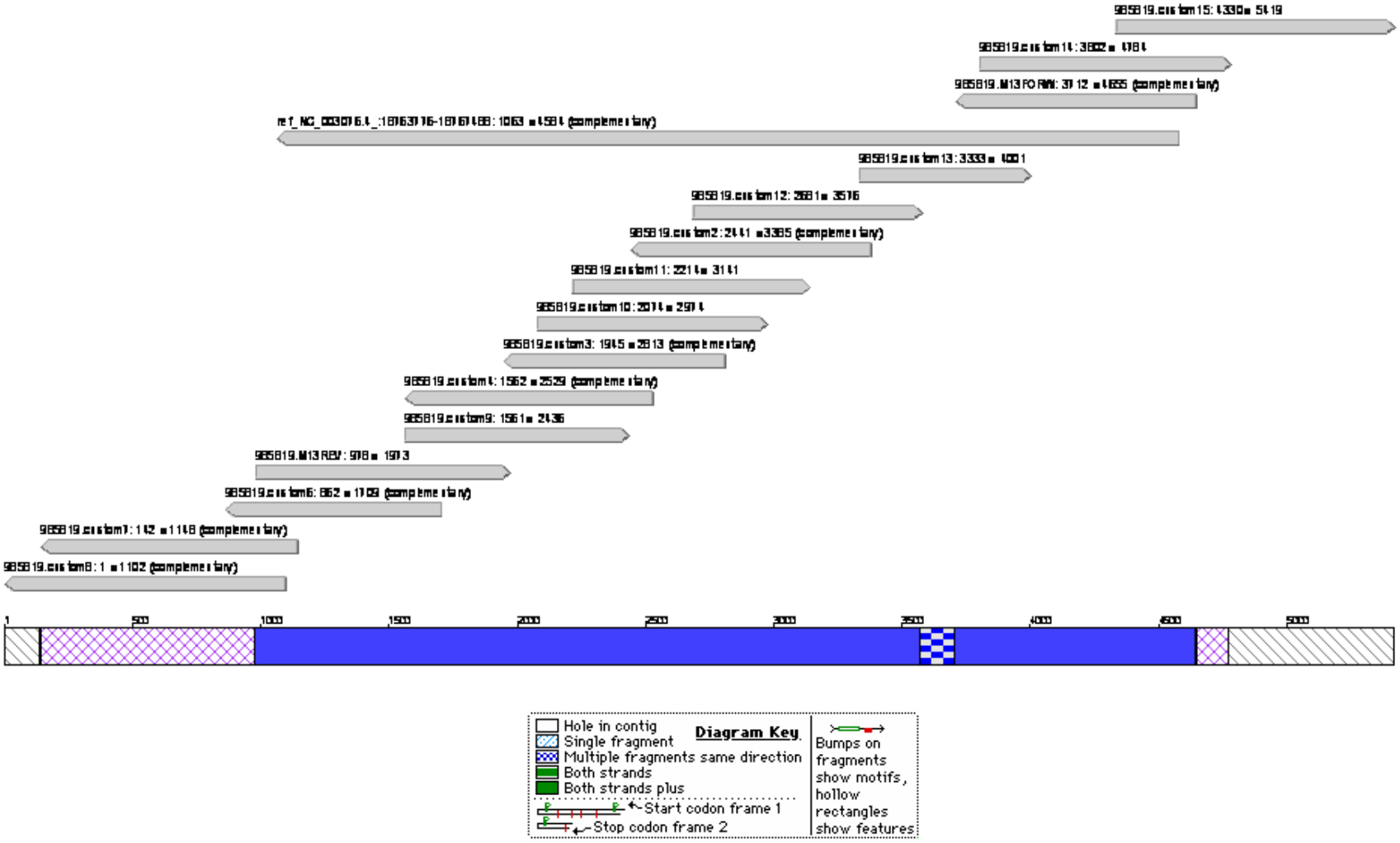
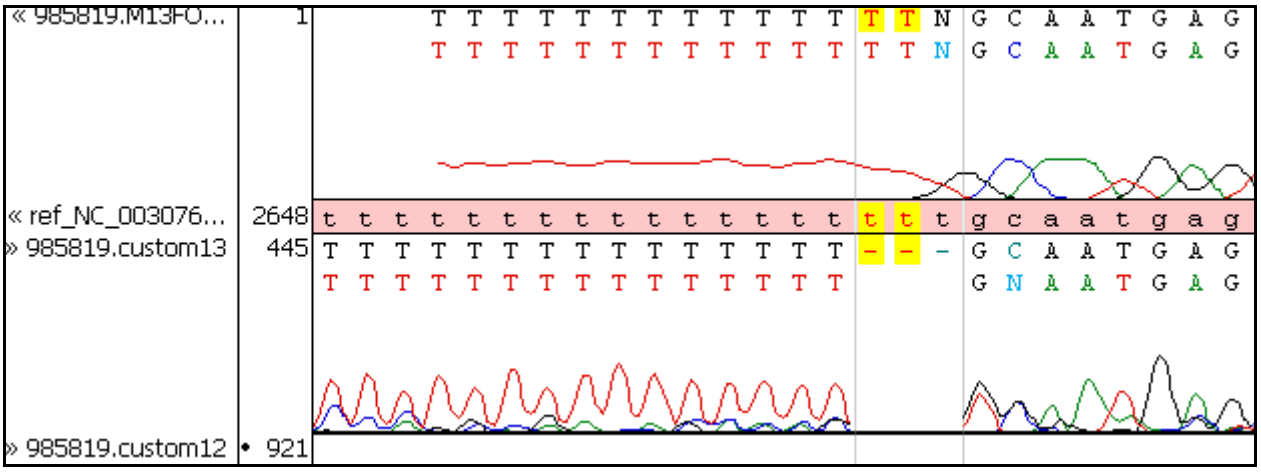


Figure 4: Contig assembly of primers spanning entire length of KAT1 promoter. An analysis of sequence data was supported by the presence of multiple fragments in comparison to published reference sequence of KAT1 from the *Arabidopsis* genome.

A



B

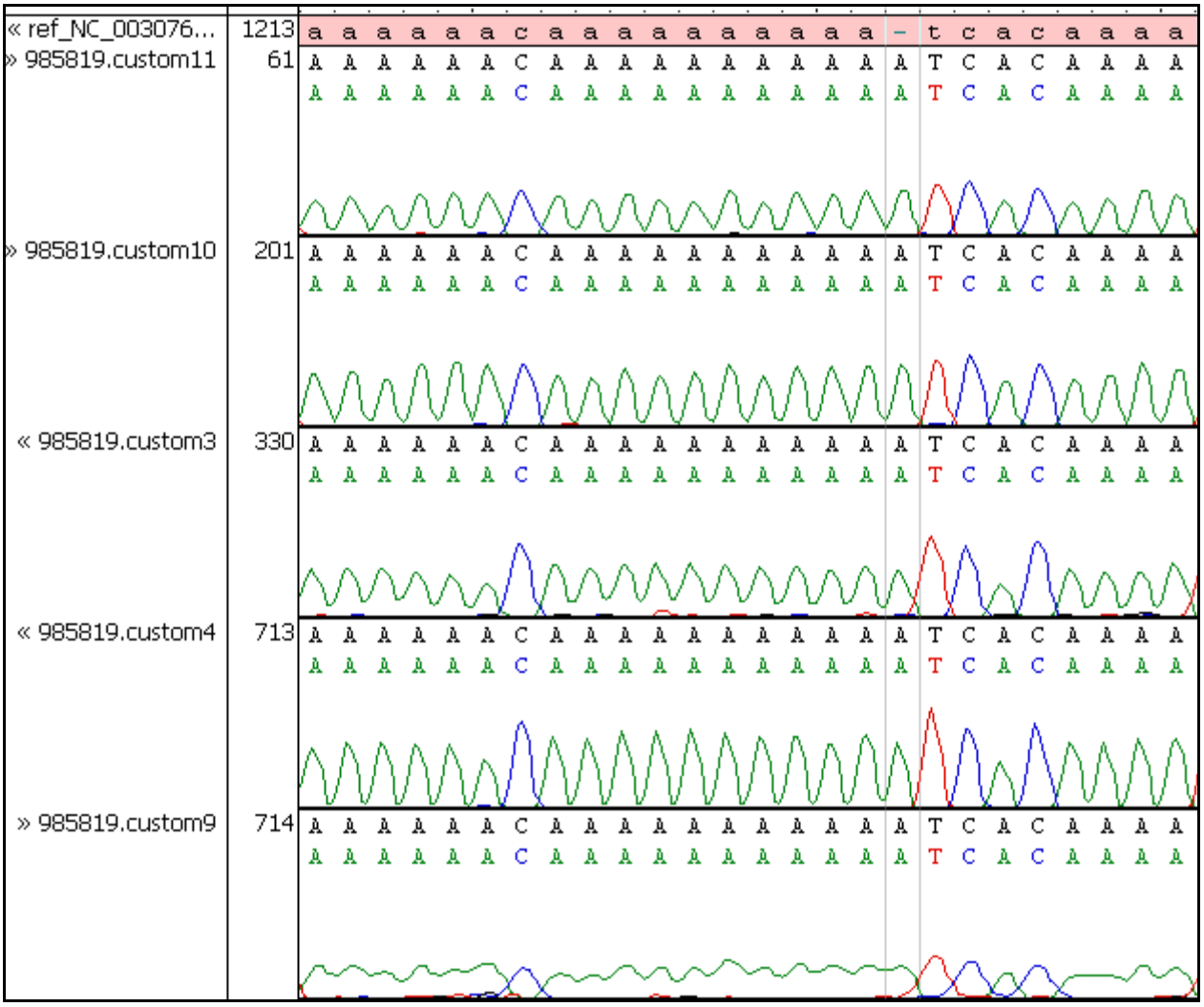


Figure 5: Chromatograms showing minor mutations occurring in the KAT1 promoter emphasized by gray lines, and confirmed by sequence from multiple custom primers. [A] Possible triplet deletion from bases 884-886bp from the start codon. [B] Insertion of an Adenine at position 2313.1 not present in the reference sequence.

Preparation of Binary Vector pMP535

Preparation of pMP 535 binary vector was done through a series of restriction enzyme digests with SmaI and NdeI. The pMP535 vector had been previously modified to incorporate the KAT2 promoter driving the NADP-ME1 gene provided by P. Thakur, 2006. The sequential SmaI and NdeI digests effectively removed KAT2, leaving a promoter-less pMP535 construct with an intact NADP-ME1 gene. This was confirmed through gel electrophoresis (Figure 6). The band corresponding to the promoter-less vector DNA was excised from the gel. DNA was gel purified and quantified at 9ng/μL.

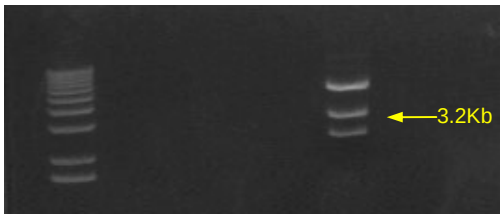


Figure 6: Excision of the KAT2 promoter from binary vector pMP 535 using sequential SmaI and NdeI restriction enzyme digests. Well 1 is the 1 Kb Plus ladder by Invitrogen® (Carlsbad, California, USA) used to estimate band sizes. Well 6 is a digested sample of pMP 535, separated by empty wells to allow for convenience in gel excision. The top band corresponds to the pMP535 vector with the KAT2 promoter of 5405 bp, the middle band corresponds to the pMP535 vector without KAT2 of 3147 bp, and the bottom band corresponds to the KAT2 promoter of 2258 bp.

Mutagenic Addition of NdeI site to the 3' End of KAT1 Promoter

Directional cloning of the KAT1 promoter into the binary vector pMP 535 required the addition of an NdeI site to the 5' end of the previous left primer (Table 1). PCR was performed using the newly designed left primer with the added NdeI site (5'-CATATGCTTTTTGATGATCTCTAAAG-3'), using purified plasmid DNA rather than

extracted genomic DNA. The PCR product was run on a 1% gel, which confirmed successful amplification (Figure 7).

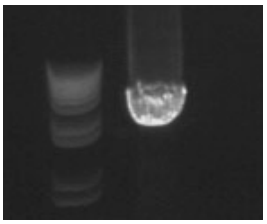


Figure 7: PCR using custom designed mutagenic primer with NdeI site. Well 1 is the 1 Kb Plus ladder by Invitrogen® (Carlsbad, California, USA) used to estimate band sizes. Well 2 is a PCR sample using purified plasmid DNA as template and 2mM $MgSO_4$. Presence of a DNA band indicates the PCR was successful using the mutagenic primer.

The PCR product was subsequently TOPO®-cloned into *E.* coli competent cells followed by plasmid purification. To ensure the KAT1 promoter was in fact inserted into the PCRII vector, the purified plasmid DNA was subjected to an EcoRI digest to confirm insertion. Two EcoRI sites are present in the PCRII vector, and upon digestion, excise the insertion of the KAT1 promoter and indicated successful digestion (Figure 8).

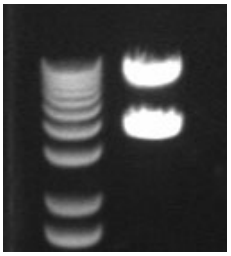


Figure 8: EcoRI digest showing successful excision of KAT1 promoter from PCR II vector. Well 1 is the 1 Kb Plus ladder by Invitrogen® (Carlsbad, California, USA) used to estimate band sizes. Well 2 is an EcoRI digest of purified plasmid DNA containing the KAT1 promoter amplified with mutagenic PCR primers with the added NdeI site. The top band corresponds to the PCRII vector of approximately 4Kb pairs, the second band corresponds to the KAT1 promoter of 3.2Kb pairs.

DNA purified from these plasmids was subject to restriction enzyme analysis with SmaI and NdeI (Figure 9). The PCRII vector was not digested by the restriction enzymes SmaI and NdeI. This result suggests that the plasmid DNA used as a template was transformed into the bacteria rather than the TOPO®-cloned plasmid.

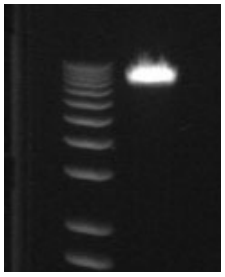


Figure 9: Sequential restriction enzyme digests using SmaI and NdeI. Well 1 is the 1 Kb Plus ladder by Invitrogen® (Carlsbad, California, USA) used to estimate band sizes. Well 2 is purified plasmid DNA from the bacteria transformed with TOPO®-cloning reaction. SmaI and NdeI restriction enzymes did not digest the vector.

This sample was sent for complete sequencing with all primers. Sequencing results confirmed that the NdeI site was not present (Figure 10).

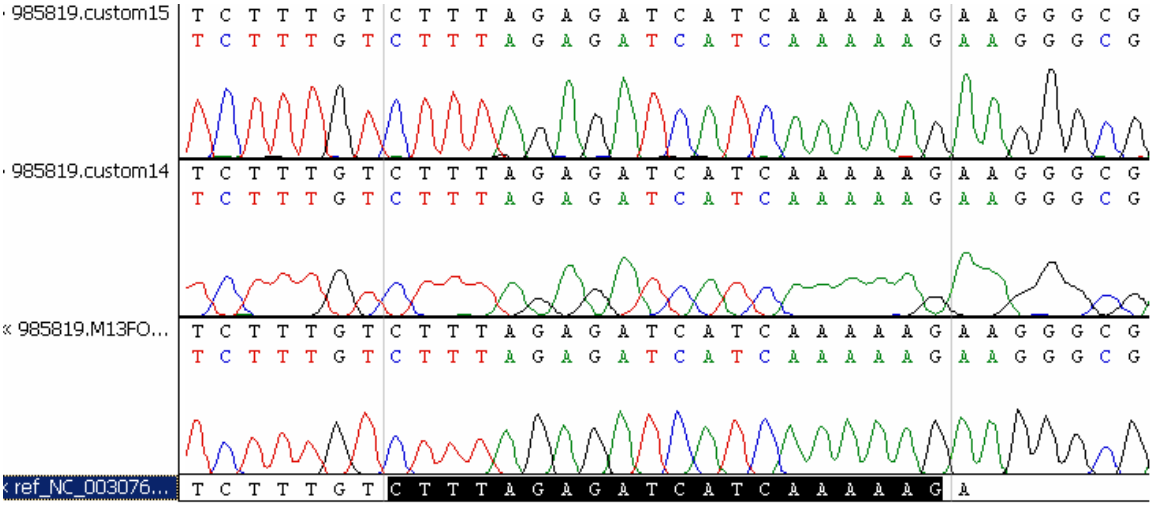


Figure 10: Sequencing (reversed and complement) results of PCR performed with mutagenic custom designed left primer with the additional NdeI site. The primer sequence that matches the reference sequence has been highlighted in black. The six bases to the right of the highlighted sequence were added to include the NdeI site (5'-CATATG-3'), but were not present in the plasmid.

Other *E. coli* colonies were analyzed to ensure none contained the KAT1 promoter with the correct addition of the NdeI site. DNA was plasmid purified from these additional colonies, and subject to sequential SmaI and NdeI restriction enzyme digests. None of the colonies were digested, indicating the template plasmid DNA had been transformed in each case rather than the TOPO®-cloned plasmid (Figure 11).

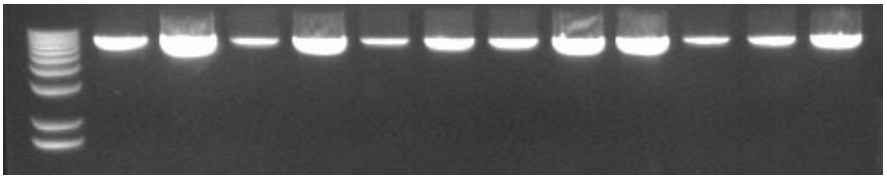


Figure 11: Sequential SmaI and NdeI restriction enzyme digests of purified plasmid DNA containing the KAT1 promoter amplified with mutagenic PCR primers. Well 1 is the 1 Kb Plus ladder by Invitrogen® (Carlsbad, California, USA) used to estimate band sizes. Wells 2-13 are different *E. coli* colonies that do not contain plasmids with the NdeI site.

A second attempt was made to add a 3' NdeI site to the KAT1 promoter. To prevent the transformation of competent *E. coli* cells with the template plasmid DNA, it was digested prior to PCR. Two restriction enzymes, EcoRI and PstI, were used to cut the PCRII vector and not the desired section of the KAT1 promoter. The digest was then subjected to PCR using the right primer, and mutagenic left primer with added NdeI site. This PCR product was immediately cloned into *E.coli* cells, and plasmid purified. Upon digestion with SmaI and NdeI however, five of the six colonies appeared to be undigested (Figure 12). A single colony showed the excision of a fragment of DNA approximately 3Kb which could correspond to the KAT1 promoter.

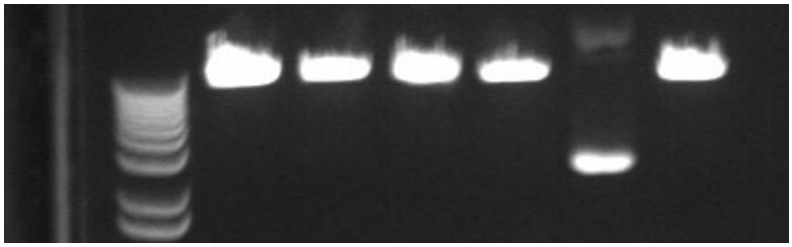


Figure 12: SmaI and NdeI digest of plasmid DNA showing no plasmids with the addition of the KAT1 promoter and functional NdeI site. Well 1 is the 1 Kb Plus ladder by Invitrogen® (Carlsbad, California, USA) used to estimate band sizes. Wells 2-7 are SmaI and NdeI digests of purified plasmid DNA from cells transformed using PCR products following digestion with EcoRI and PstI. Well 6 has two DNA bands present. The top band may correspond to undigested vector, and the more clear, bottom DNA band at approximately 3Kb, could correspond to the KAT1 promoter.

The single sample of DNA that showed digestion by SmaI and NdeI was sent for M13 Forward and Reverse sequencing. The results of sequencing returned with no data to analyze.

Whole Mount β-Glucuronidase (GUS) Staining Protocol

KAT1::GUS seedlings and plants at different growth points were used for β-Glucuronidase (GUS) staining. Two different staining protocols were used to identify optimal visualization of blue pigment and therefore KAT1::GUS expression. Both protocols were successful on the 9-day-old seedlings but not in 51 and 85 day-old-plants. Blue pigment corresponding to KAT1 expression was observed clearly in the guard cells surrounding stomata. There was also a small amount of blue pigment in the roots (Figure 13). Expression in the roots is consistent with Nakamura et. al., (1995) but not with more recent research (Lebaudy et. al., 2007; Gambale and Uozumi, 2006; Phillipar et. al., 2004; Chytilova et. al., 1999).

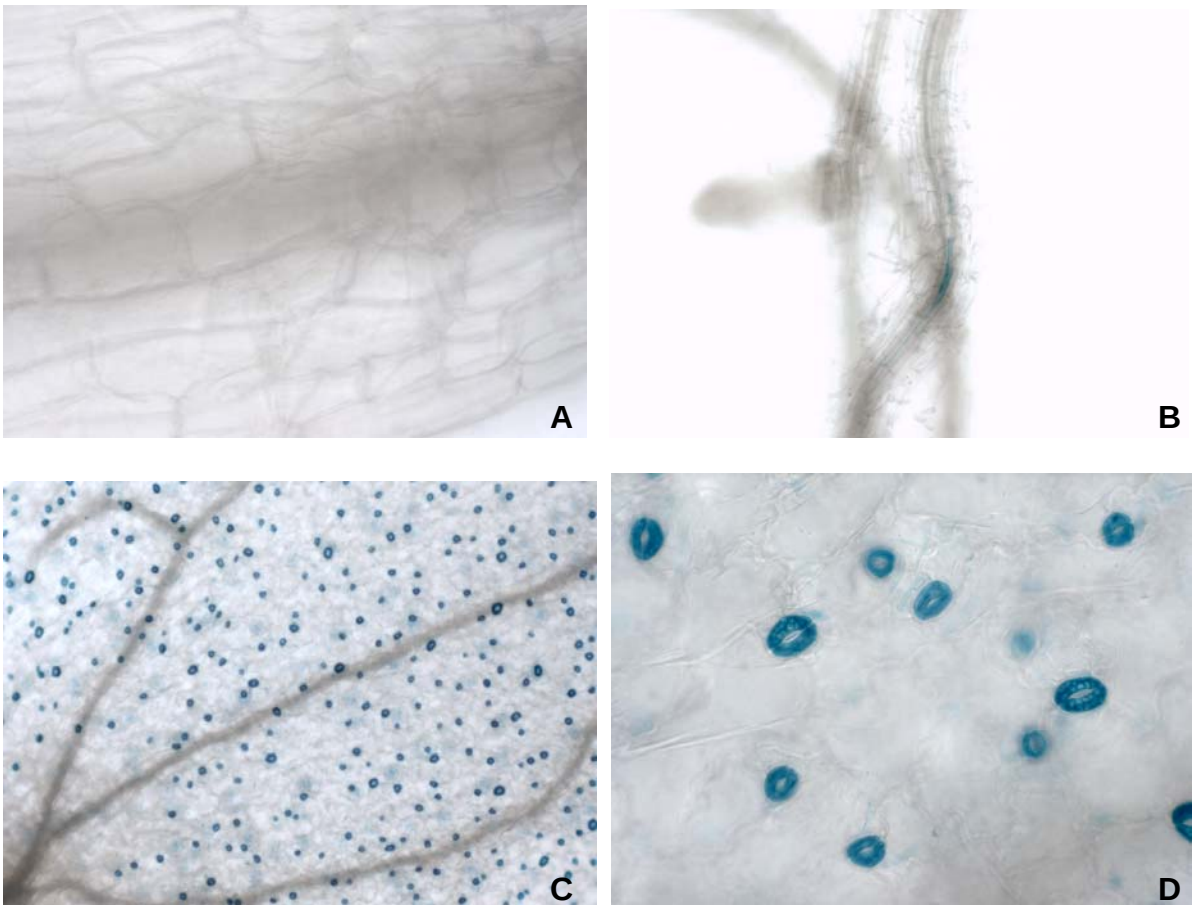


Figure 13: KAT1::GUS staining of 9-day-old seedlings grown on MS Media. [A] Root cells at 400X, indicating no presence of blue pigment or GUS expression. [B] Presence of a small amount of blue pigment in roots at 100X, indicating possible expression. [C] Localized KAT1::GUS expression in guard cells of leaf tip at 50X. [D] Localized KAT1::GUS expression in guard cells of leaf tip at 400X.

DISCUSSION

Isolation of the KAT1 promoter is a necessary and important process in engineering plants with reduced transpiration. Transpiration can be limited by reducing stomatal aperture, involving the alteration of ion concentrations in the surrounding guard cells. Guard cell osmoregulation is dependent upon concentrations of potassium, chloride, and organically synthesized malate (Raschke and Schnabl, 1978). Levels of malate may be altered by increased expression of NADP-Malic Enzyme (NADP-ME) from maize (Laporte et. al., 2002).

Expression of NADP-ME1 in *Arabidopsis thaliana* required the isolation of a strong, guard-cell specific promoter. The promoter of a potassium channel gene, KAT1, was selected because of strong expression patterns and localization specifically to guard cells. Such characteristics render the KAT1 promoter useful in driving the expression of NADP-ME1 in *Arabidopsis thaliana*. It is hypothesized that transgenic *Arabidopsis thaliana* plants with KAT1::NADP-ME1 expression will have less open stomata, reducing the amount of transpiration yet retain the ability to respond to changing environmental conditions (Laporte et. al., 2002).

Cloning of KAT1 Promoter

The entire 3.7 kilobase pair sequence of the KAT1 promoter identified by Nakamura et. al. (1995) was isolated with minor mutations and analyzed. We designed custom primers to amplify and sequence the entire 3.7 kilobase pair region of the KAT1 promoter. We isolated an extended length of the promoter, from 3.4 kilobase pairs to 3.7 kilobase pairs, than Nakamura et. al., (1995) had previously as a precautionary measure.

The additional length allowed for flexibility in the selection of restriction enzymes sites required to insert into the binary vector pMP535. In PCR reactions, custom primers amplified the correct region of the promoter at the optimal concentration of 3mM $MgSO_4$.

Mutations were identified as variants from the established reference sequence of the mRNA coding sequence provided by NCBI. Mutations occurred due to random errors of High Fidelity Taq polymerase. Mutations such as the missing triplet of thymines at 884-886bp were confirmed in more than one sequencing reaction, providing support that these were in fact mutations and not sequencing anomalies. This triplet deletion specifically happened in a region containing 16 consecutive thymines according to the database. The fact that multiple PCR products lack this triplet provides support that it may be an error within the database. According to our results, we contend the correct number of thymines in this location may actually be 13 rather than 16 as the reference sequence indicates. Because these mutations were fairly distant from the start codon of KAT1 promoter, their effects will be minor and most likely will not impede proper function.

Preparation of pMP 535 Binary Vector

Preparation of pMP 535 binary vector was done through a series of restriction enzyme digests with SmaI and NdeI, followed by gel purification. This effectively removed the KAT2 promoter, creating a promoter-less pMP535 construct with an intact NADP-ME1 gene. Following the mutagenic addition of a 3' NdeI site, future experimentation will require the KAT1 promoter to be ligated into the pMP 535 vector.

This KAT1::NADP-ME1 construct will then be used to transform a bacterial culture of *Agrobacterium*, which will then be used to transform *Arabidopsis thaliana* plants.

Mutant *Arabidopsis thaliana* plants will have to be identified, and a few homozygous lines will be chosen to further analyze. These mutants are expected to show an increased activity of NADP-ME1 expression in guard cells. As a result, levels of the organic counter-ion malate will be depleted, allowing for an increased turgor pressure in guard cells and therefore decreased stomatal aperture. Stomatal conductance and biochemical assays confirming the presence of NADP-ME1 will have to be done to verify any changes in transpiration in comparison to wild-type controls.

Mutagenic Addition of NdeI Aite to 3' End of the KAT1 Promoter

Directional cloning of the KAT1 promoter into the pMP535 vector containing the NADP-ME1 gene requires 5' SmaI and 3' NdeI restriction enzyme sites. The KAT1 promoter has an intrinsic 5' SmaI site, and the addition of a 3' NdeI site will allow for proper insertion. Analysis of the KAT1 promoter revealed the SmaI site to be sufficiently distant from the start codon by 3255 base pairs. The KAT1 promoter did not contain an NdeI site and needed to be added by the use of mutagenic PCR primers.

The addition of the NdeI site was attempted using a number of different methods, but has not been successful. The initial attempt to add the NdeI site involved the design of a new custom-made left primer. This site was added to the 5' end of the previous primer, in the complementary direction in which the KAT1 promoter runs, placing the start codon in the correct reading frame. Using this primer, the PCR successfully amplified the promoter region. After transforming *E.coli* cells with this PCR product

however, none of the transformants indicated that they contained plasmids with the addition of the NdeI site. This was confirmed by the samples not being digested with NdeI. Sequencing results from these colonies indicate that the plasmid DNA used as a template transformed the bacteria rather than the TOPO®-cloned plasmid.

To prevent the transformation of the template DNA, it was digested prior to PCR with two restriction enzymes, EcoRI and PstI, to eliminate vector sequence, and retain the KAT1 promoter. These digests should have linearized template DNA molecules, resulting in the KAT1 promoter in the PCRII vector to be the only form of circular DNA. Since *E. coli* cells cannot be transformed with linear DNA, this technique prevents them from being transformed with template. Using this method, only one sample showed possible digestion, and was submitted for sequencing. However, sequence analysis returned with no data.

Whole Mount β-Glucuronidase (GUS) Staining Protocol

Two different β-Glucuronidase (GUS) staining protocols provided by The Arabidopsis Manual (Weigel and Glazenbrook, 2002) and by Nakamura et. al. (1995) were used to identify optimal visualization of blue pigment and therefore expression. Our results suggest that KAT1 may be developmentally controlled in later growth of leaves, although requiring further analysis. Using the 9-day-old seedlings, both protocols showed an abundance of blue pigment in the guard cells, indicating KAT1::GUS expression. The protocol provided by The Arabidopsis Manual was also attempted on leaves from 51 and 85-day-old plants, but showed no evidence of any blue pigment.

The lack of blue pigment indicates the lack of GUS expression and therefore the lack of KAT1 promoter activity. Since both protocols were effective in producing blue pigment in 9-day-old seedlings, the lack of blue pigment detection in older leaves is likely not due to variable responses to differences in protocols. Further experimentation of KAT1::GUS expression throughout the growth of the plant will be necessary to support our claim of the developmental control of KAT1 expression. KAT1::GUS seeds and plants grew at somewhat variable rates, and were less hearty than expected. The KAT1::GUS seeds came directly from the Arabidopsis Biological Research Center at Ohio State University and were assumed to be homozygous. Seed homozygosity was not confirmed through experimentation, however.

Further analysis can easily be accomplished by comparing pigment intensities, and thus expression levels, of plants throughout their growth. Analysis of KAT1 expression patterns have been published (Lebaudy et. al., 2007; Szyroki et. al., 2001) but significant quantitative data regarding KAT1 expression throughout growth has yet to be firmly established.

The protocols provided by The Arabidopsis Manual and by Nakamura et. al. (1995) were both successful in GUS staining using 9-day-old seedlings. This data supports previous research that KAT1 promoter expression is specific to guard cells (Lebaudy et. al., 2007; Hurst et. al., 2004; Pilot et. al., 2001; Chytilova et. al., 1999; MacRobbie, 1998; Nakamura et. al., 1995). The expression of KAT1 in root cells was first identified by Nakamura et. al., (1995) however, was not consistent with more current research using the same 3.4Kb segment of the KAT1 promoter (Lebaudy et. al., 2007; Gambale and Uozumi, 2006; Phillipar et. al., 2004; Chytilova et. al., 1999). Our study

found a single sample with a small amount of blue pigment in the roots, indicating GUS expression. Interpretation of such results is limited due to the small sample size, and may be due to natural variation, or non-homozygous seed. Further experimentation on numerous samples will confirm whether or not KAT1 is expressed in the root system.

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