ABSTRACT

AMY JOY BURRELL
Analysis of the Transcriptional Expression of *Arabidopsis GAUT1* and *GAUT7*: Members of a Pectin Biosynthetic α-1,4-Galacturonosyltransferase Complex
(Under the Direction of DR. DEBRA MOHNEN)

The plant cell wall provides the structural basis upon which the morphogenesis of plant cells, tissues, and organs rests. Pectin, a major wall component, is a family of polysaccharides that provides many of the biochemical properties that define cell wall form and function. Regulation of enzymes that synthesize wall components could potentially be altered to affect wall formation and therefore modify the growth and development of the plant. A specific family of enzymes involved in pectin biosynthesis known as Galacturonosyltransferases (GalATs) catalyze the transfer of galactosyluronic acid (GalA) residues from uridine diphosphate-GalA (UDP-GalA) to the growing pectic polysaccharide chain. The first gene that encodes a pectin GalAT in *Arabidopsis thaliana*, known as GAlactUronosylTransferase1 (*GAUT1*), was previously identified and BLAST analyses indicated a gene superfamily with high-sequence similarity. To understand the biological significance of *GAUT1* and *GAUT7*, transcriptional expression was analyzed using a β-Glucuronidase (GUS) reporter gene system. The *GAUT* promoters were amplified, fused to the GUS gene, and transformed into *Arabidopsis* plants. The activities of the promoter:GUS constructs were assayed to reveal spatial and temporal expression patterns. This data shows that *GAUT1* and *GAUT7* are expressed heavily in meristematic regions, vascular tissues, and pollen, suggesting a role in primary and secondary wall growth. Extensive overlap of expression provides evidence that these proteins work in a complex. This study provides new insight to guide the design of future studies on *GAUT* function in pectin synthesis and can be used with other evidence to further elucidate the physiological relevance of these genes.

INDEX WORDS: Cell wall, Pectin, Galacturonosyltransferase, β-Glucuronidase, GUS, Transcriptional expression

ANALYSIS OF THE TRANSCRIPTIONAL EXPRESSION OF *ARABIDOPSIS GAUT1* AND *GAUT7*: MEMBERS OF A PECTIN BIOSYNTHETIC α-1,4-GALACTURONOSYLTRANSFERASE COMPLEX

by

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CHAPTER ONE
INTRODUCTION

All plant cells are surrounded by at least one of two different types of cell wall, which are both composed of polysaccharides and proteins (see Fig. 1). Primary cell wall forms around growing cells while secondary wall forms in differentiated cells in between the plasma membrane and the primary cell wall (which is pushed outward) (Mohnen, 1999). Pectin is the most structurally complex polysaccharide located primarily in the primary cell wall of all plants and possibly in algae, liverworts, mosses, and ferns (Ridley *et al*., 2001; Mohnen, 2002). Pectin accounts for many of the biochemical properties of the growing plant cell wall and thus has an important role in the growth, morphology, and development of the entire plant (Sterling *et al*., 2006). Pectin is also located in the middle lamella (the area between two adjacent cells) and in small amounts in the secondary wall (Mohnen, 1999). Pectins also contribute to cell wall structural integrity, plant defense responses, cell-cell adhesion, signaling, cell expansion, ion-

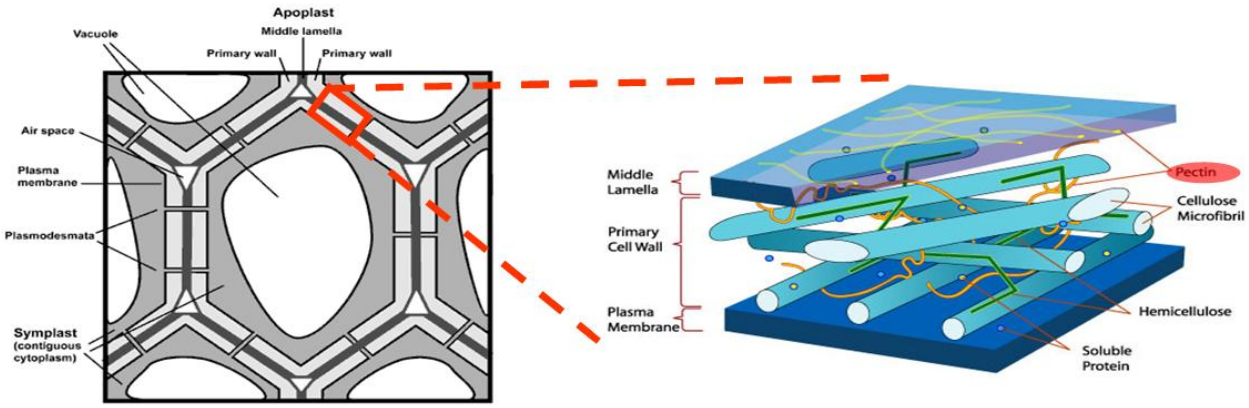

Figure 1. Plant Cell Wall Model. Figure shows location of primary cell wall and its components. **Figures modified from www.ccrc.uga.edu and wikipedia.com**

binding, wall porosity, seed hydration, leaf abscission, and fruit development (Ridley et al., 2001; Willats et al., 2001; Mohnen, 2008).

Pectin has been shown to have nutritional, biomedical, pharmaceutical and industrial properties. In the food industry, pectin is commonly used as a gelling agent, thickener, emulsifier, and fat/sugar replacer (Thakur *et al.*, 1997). Pectin has also been shown to lower blood cholesterol and serum glucose levels in humans and to induce apoptosis in human prostate cancer cells *in vitro* (Behall and Reiser, 1986; Thakur et al., 1997; Jackson et al., 2007). Understanding pectin structure and synthesis is also crucial to facilitate cell wall modification for

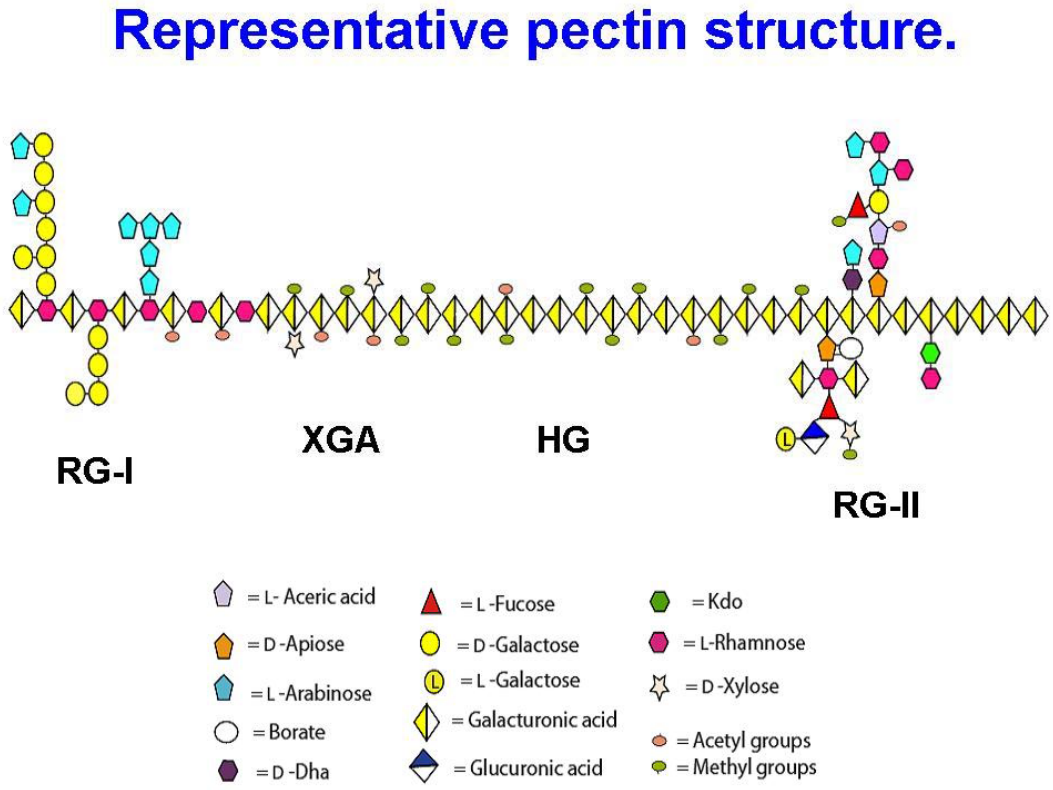

Figure 2. Model of Pectin Structure. To approximate the amounts of pectins in walls, increase HG 12.5-fold and RG-I 2.5-fold. Figure from Mohnen, *Curr. Opin. Plant Biol.* 2008.

the reduction of plant recalcitrance and degradation of biomass for biofuel production. The diversity of epitopes in pectin's complex structure allows for the wide variety of uses and functions (Mohnen, 2008).

Pectin is a family of pectic polysaccharides, of which there are three major classes: homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and the substituted galacturonans which include rhamnogalacturonan-II (RG-II) and xylogalacutronan (XGA). Each of the pectic polysaccharides contains 1,4-linked α-D-galactosyluronic acid (GalA) residues as shown in Figure 2 (Ridley et al., 2001; Mohnen, 2008). HG, a linear chain of 1,4-linked α-D-GalA, is the most abundant pectic polysaccharide. The others are structurally more complex with side branches of different sugars with different linkages. Some of the pectic polysaccharides, such as RG-I, are structurally variable in different cell types (Mohnen, 2008). Based on mutant phenotype studies and co-elution of pectin with other polysaccharides, pectic polysaccharides are believed to be linked to other cell wall polymers, such as cell wall hemicellulose, xyloglucan, and xylan (Mohnen, 2008) although the evidence is not yet definitive.

Because of the complexity of pectin, it requires a large number of enzymes (at least 67) for its synthesis, all of which are transferases (glycosyl-, methyl-, and acetyl-) located in the Golgi (Mohnen, 2002). The Mohnen lab is interested in a specific family of enzymes involved in pectin biosynthesis known as galacturonosyltransferases (GalATs). GalATs catalyze the transfer of GalA residues from uridine diphosphate-GalA (UDP-GalA) to the pectic polysaccharide chain (Ridley *et al.*, 2001). The Mohnen lab has previously identified a gene that encodes a pectin GalAT in *Arabidopsis thaliana* known as galacturonosyltransferase1 (*GAUT1*) through a proteomic approach using partially-purified, detergent-solubilized membrane protein preparations (Sterling *et al.*, 2006). BLAST analyses indicate the existence of a superfamily of

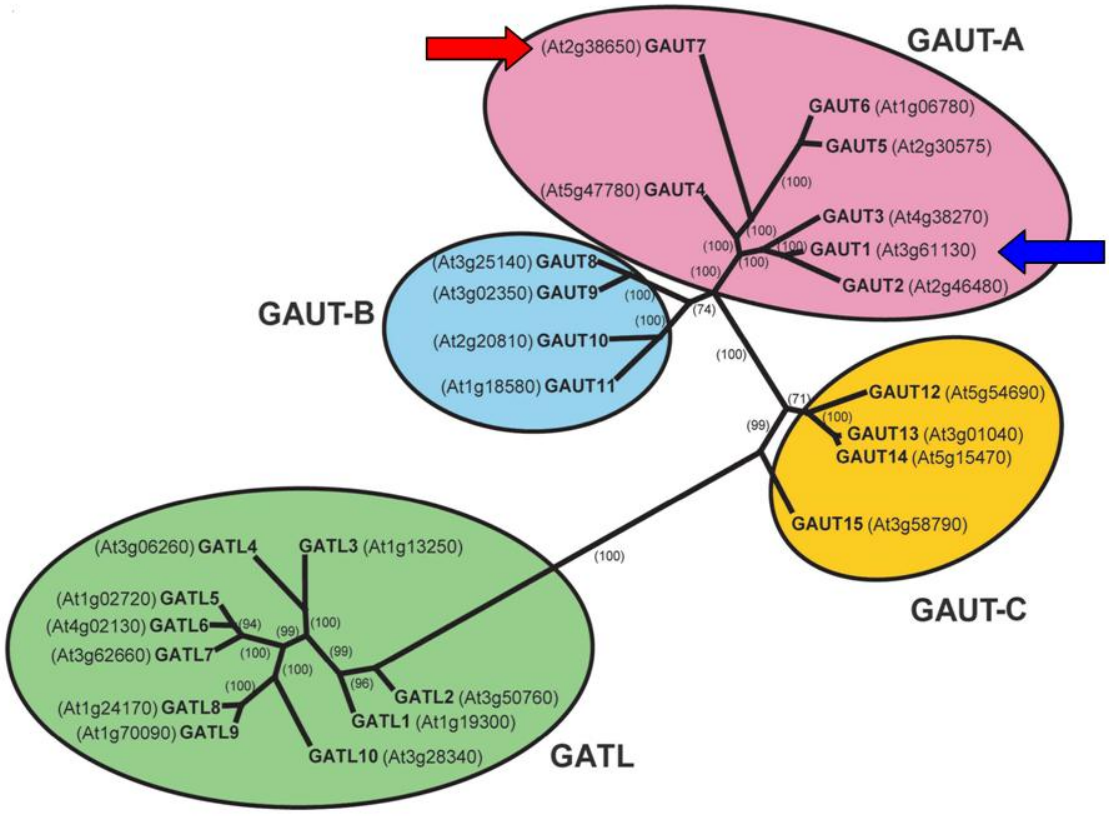

Figure 3. ***Arabidopsis GAUT1*-related gene superfamily.** Red arrow indicates *GAUT7* (At2g38650) and blue arrow indicates *GAUT1* (At3g61130). From Sterling et al. *PNAS*. 2006 Mar 28;103(13):5236-41

genes shown in Figure 3 with high-sequence similarity to *GAUT1* (Sterling *et al.*, 2006). Many of these genes are also hypothesized to play a role in cell wall synthesis. One of these genes produces *GAUT7*, which was co-identified with *GAUT1* and has been hypothesized to operate in a complex with *GAUT1* (Sterling *et al.*, 2006).

Growing evidence that pectin structure differs in different species, cell types and developmental states suggests that pectin biosynthetic enzymes also differ in distinct plant, tissue, cell type, and developmental states (Mohnen, 2008). Understanding the biological function of these enzymes along with the biochemical function is a key to understanding pectin structure and function. To understand the biological significance of the GAUT genes, it is

important to understand where within the plant their expression occurs (Ridley *et al.*, 2001). The goal of my research project was to qualitatively analyze where the *GAUT1* and *GAUT7* genes are expressed temporally and spatially in the plant through the utilization of a reporter gene system. A reporter gene system allows for analysis of the expression of each GAUT gene in plants at specific environmental conditions and at specific developmental stages. The reporter gene system also provides detailed cellular resolution, which is an advantage when compared to other expression analyses (e.g. microarray studies), which generally reflect expression in tissues containing multiple cell types. A comparison of the tissue and cell-type specific expression of each *GAUT* gene will provide information about potential *GAUT* gene redundancy. Such information is useful in the interpretation and design of *GAUT* mutant studies. Furthermore, analysis of the expression will provide information about the relationship between *GAUT1* and *GAUT7*. If the expressions of the *GAUT1* and *GAUT7* genes are similar or overlapping, it would provide supporting evidence that the proteins may operate in a complex.

A hypothesis about how the genes may be expressed can be formed from RT-PCR studies and microarray databases, which give information about where *GAUT1* and *GAUT7* transcripts are expressed at the organ level. Microarray experiments measure the relative amounts of mRNA in the entire plant at a specific stage in specific organs. RT-PCR is a variation of basic PCR in which the starting nucleic acid that is amplified is RNA or mRNA. The enzyme, reverse transcriptase (RT), is utilized in order to synthesize a DNA complementary strand, producing a cDNA molecule, which can serve as a template for further PCR or for combinatorial libraries (Garrett and Grisham, 2005). Analysis of the microarray and RT-PCR results showed that *GAUT1* and *GAUT7* genes are expressed in the roots, stems, and leaves of the plants (K. Caffall, unpublished data). Microarray studies have produced similar but more comprehensive data than

the RT-PCR studies, but the data are still only at the organ level. Microarray data from Genevestigator (www.genevestigator.ethz.ch), which contains the results of thousands of microarray experiments, are shown in Figures 4 and 5. *GAUT1* and *GAUT7* are expressed in all developmental stages (Fig. 4) and in all adult tissues (Fig. 5) but at different levels. *GAUT1* and *GAUT7* are expressed at highest levels in bolting and flowering tissues. *GAUT7* is also expressed highly in the hypocotyls, inflorescences nodes, and root elongation zone while *GAUT1* is expressed highly in petals and stamen. Therefore, my hypothesis is that *GAUT1* and *GAUT7* expression occurs throughout the plant, but at different levels in different cell types during all developmental stages.

The promoter-β-glucuronidase (GUS) reporter gene system was selected to provide comprehensive information about the expression of the *GAUT* genes (Karcher, 2002). A reporter gene system is particularly advantageous to analyze the activity of the GAUT genes because the expression of the *GAUT* genes is not easily identified due to their complex and unknown regulation that varies according to developmental stage, environmental influences, and possibly a wide range of other factors (Karcher, 2002). The GUS gene encodes an enzyme that has an easily assayable activity. The GUS gene is placed in a vector in which the promoter region of the GUS reporter gene has been removed and the promoter of the gene of interest is inserted in its place (Karcher, 2002). The new chimeric gene construct containing the particular gene's promoter attached to the GUS gene is then transformed back into the wild-type *Arabidopsis* plant (Karcher, 2002). The *Agrobacterium*-mediated transformation produces transgenic seeds containing the GUS construct (Karcher, 2002). To prepare for assessment of the GUS expression, the seeds are grown on antibiotic-supplemented media, planted, and allowed to grow into mature plants (Karcher, 2002).

The assay for GUS expression involves histochemical staining (Karcher, 2002) (Figure 6). The GUS gene encodes the enzyme β-glucuronidase (GUS) which cleaves the color-generating substrate X-gluc (5-bromo-4-chloro-3-indolyl-β-d-glucoronic acid) producing a compound that is insoluble and bright blue (Karcher, 2002). Since GUS activity is not present naturally within plant cells, the blue color indicates that the promoter that is driving the transcription of the chimeric gene is active (Karcher, 2002). Furthermore, it is an indication that the *GAUT* gene is actively expressed in such tissues because the *GAUT* promoter is used to express GUS. The blue-coloring of the transgenic plants can be closely studied and the complex transcript expression patterns of the genes can be analyzed in detail to provide valuable information about the transcription of the *GAUT* genes. For the fall semester of 2006, I worked to obtain the correct sequence of the promoter regions within a cloning vector, pGEMT-Easy (pGEMTE). For the spring semester, I obtained the correct sequence of the promoter regions within the expression vector, pBI101. For the summer and fall semesters of 2007, I worked to produce transgenic *Arabidopsis thaliana* plants that contained the reporter gene constructs and analyzed the expression of the *GAUT1* and *GAUT7* genes.

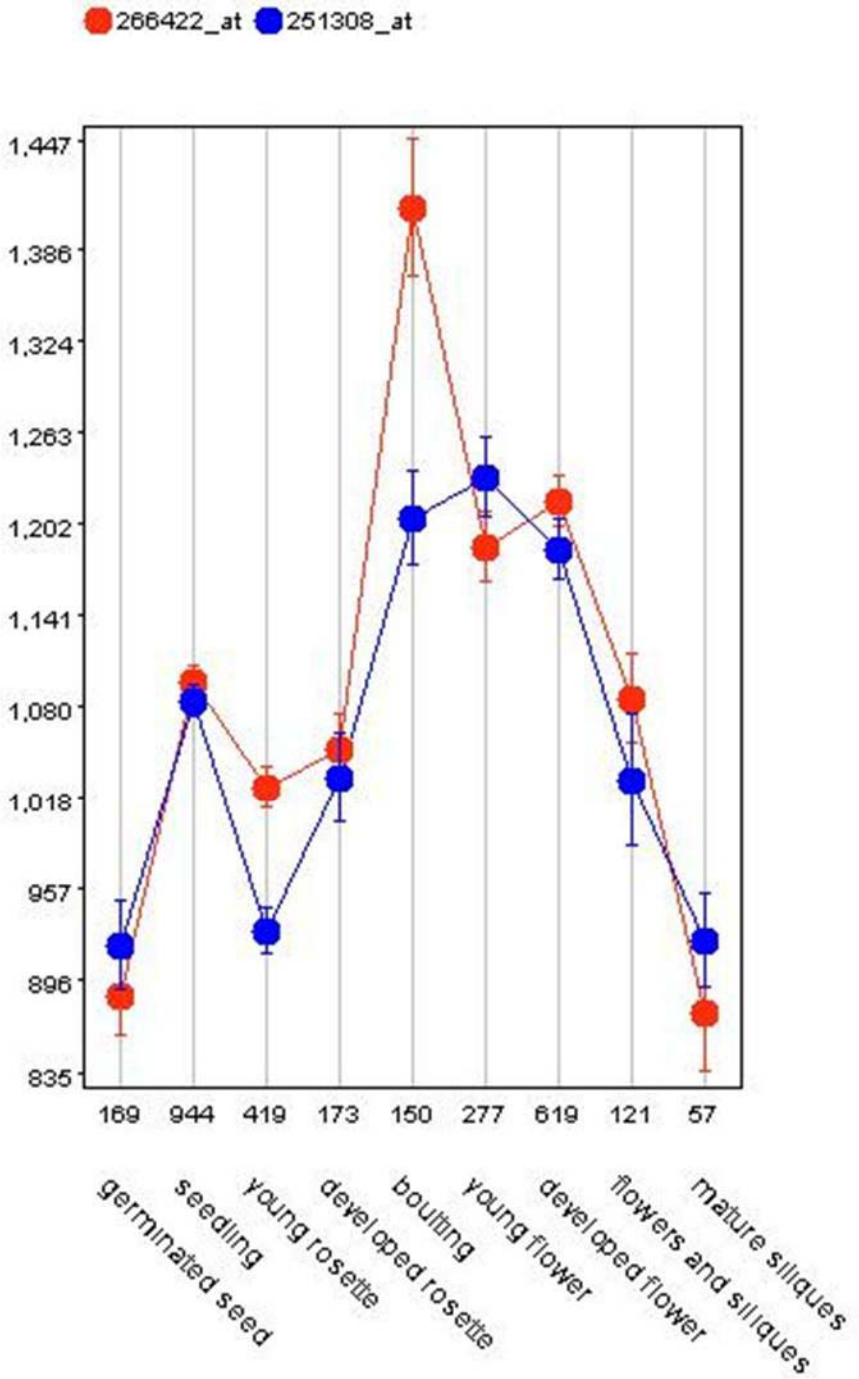

Figure 4. Genevestigator microarray data showing relative expression levels of *GAUT1* and *GAUT7* at specific developmental stages. *GAUT1* (251308_at) is shown in blue and *GAUT7* (266422_at) is shown in red. Obtained from https://www.genevestigator.ethz.ch

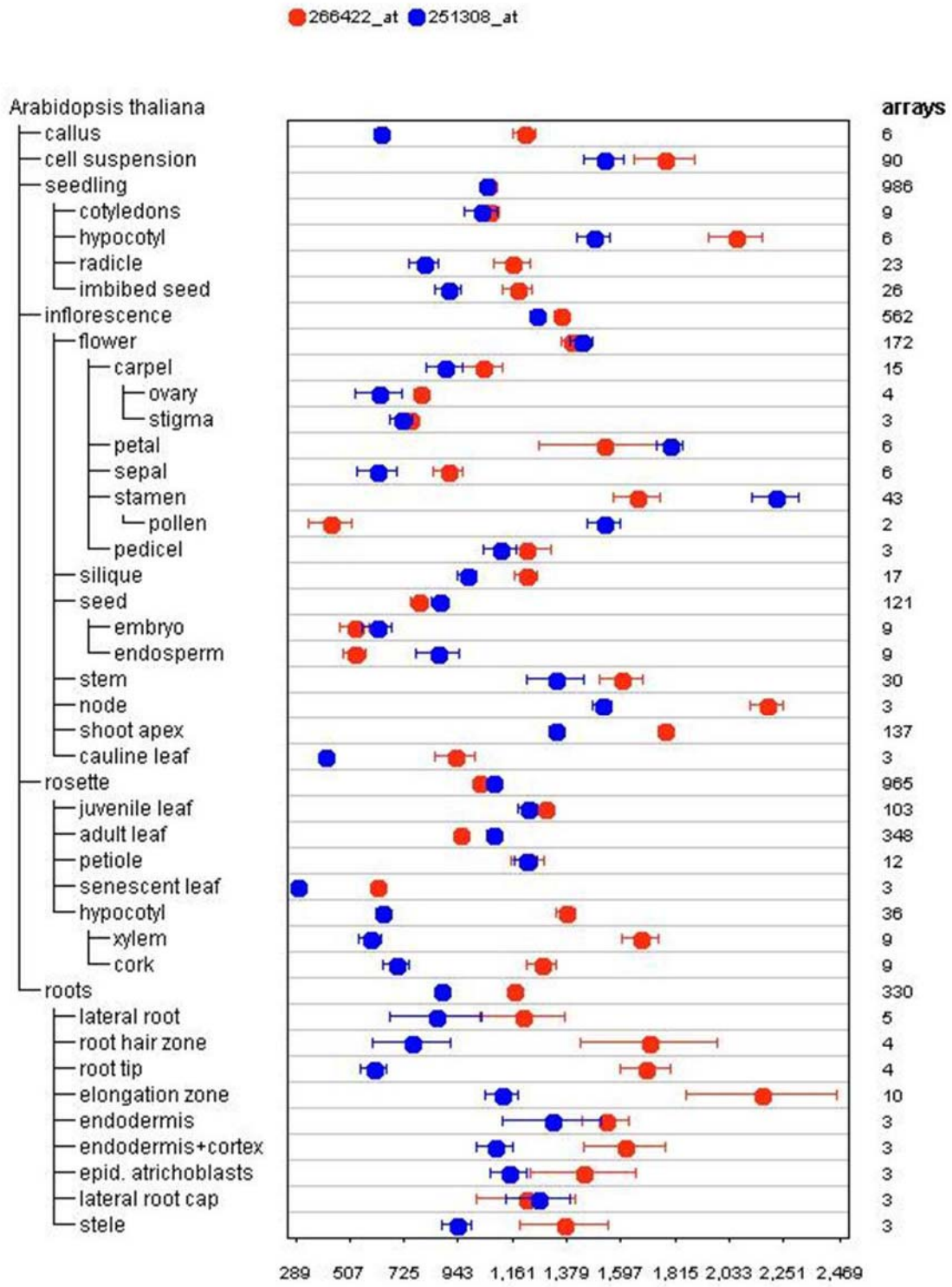

Figure 5. Genevestigator microarray data showing relative expression levels of *GAUT1* and *GAUT7* in specific tissues. *GAUT1* (251308_at) is shown in blue and *GAUT7* (266422_at) is shown in red. Obtained from https://www.genevestigator.ethz.ch

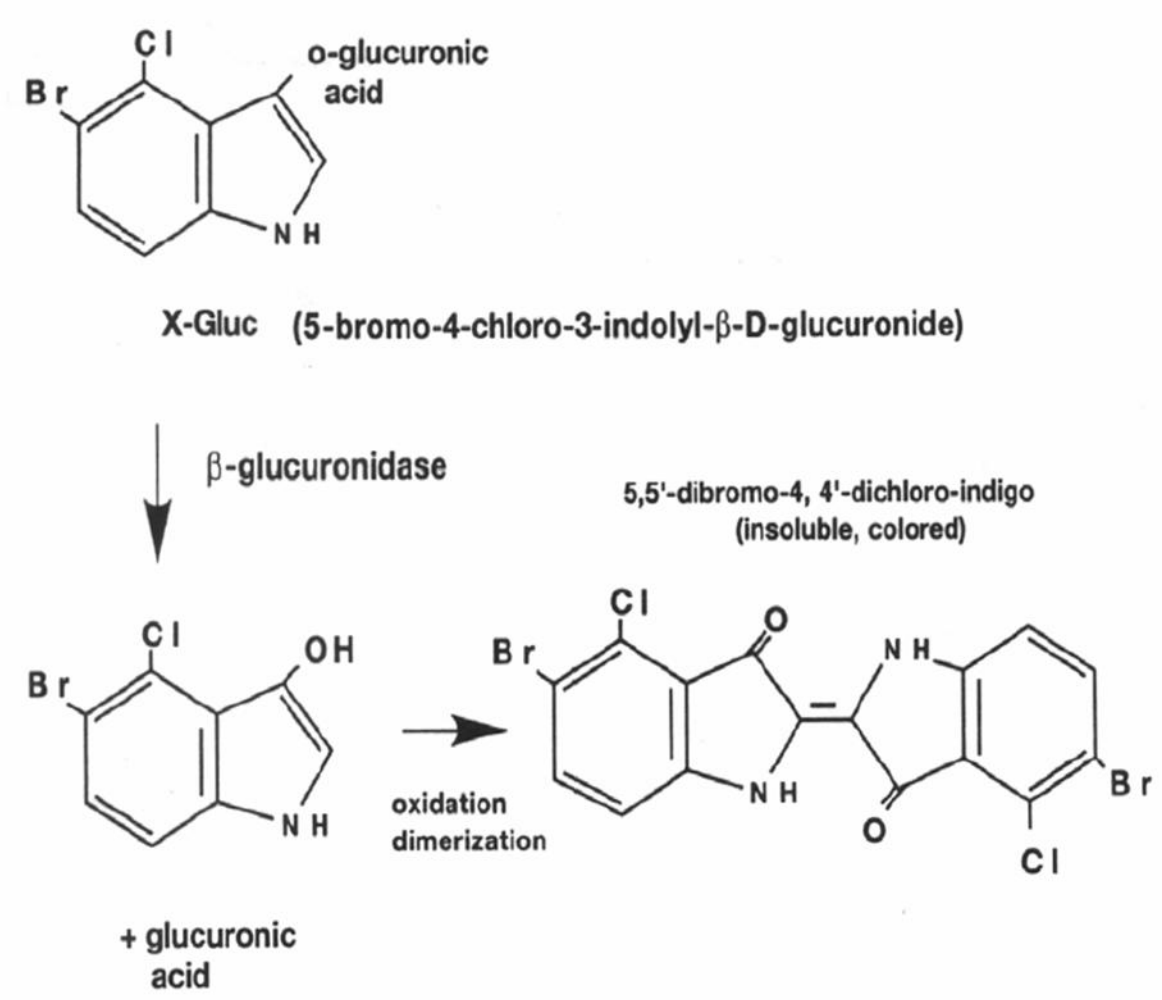

Figure 6. Cleavage of the substrate X-gluc (5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid) by β-glucuronidase produces chloro-bromoindigo, which when oxidized produces an insoluble blue precipitate. Figure from Karcher (2002).

CHAPTER TWO
MATERIALS AND METHODS

Designing *GAUT1* and *GAUT7* Promoters

The sequences for *GAUT1* and *GAUT7* promoters were obtained from The *Arabidopsis* Information Resource (TAIR) website (http://www.*Arabidopsis*.org) using gene codes At3g61130 (*GAUT1*) and At2g38650 (*GAUT7*). According to the advice of Dr. Maor Bar-Peled, the promoter region was determined to be contained within the intergenomic region, i.e. the sequence between the stop codon of the previous gene and the start codon of the respective *GAUT* gene. Since the intergenomic sequence was less than 900 basepairs for *GAUT7*, an additional construct was made containing the intergenomic region plus approximately 1200 nucleotides upstream into the previous gene, and was named "*GAUT7* long version."

Once the promoter regions were determined, primers for promoter and gene amplification and DNA sequencing were designed. Primers were designed to optimize sequence fidelity. The criteria for primers, which were formulated based on the advice and experience of colleagues within the Mohnen lab and CCRC, were that the primers should be 19-40 basepairs that would anneal to the 5'-end of the promoter region and that the primer's NCBI BLAST analysis would indicate that it did not match with high fidelity to another sequence within the *Arabidopsis* genome in order to prevent amplification of other unwanted regions. Additional criteria were that 50% of the primer bases be guanine and cytosine (50% GC-content) and that the 3'-end of the primer (where DNA polymerase extends from) would have its last two bases be guanines

and/or cytosines (known as a "GC clamp"), because guanine and cytosine form three hydrogen bonds (instead of two) when paired, thereby providing extra stability.

The primers for promoter amplification must contain restriction sites at the 5'-ends so that the amplified promoter regions can be easily removed and inserted from and into vectors. Using JustBio.com (http://www.justbio.com) hosted tools, the restriction enzymes that do and do not cut the promoter regions were determined. The restriction enzymes that cut in the multiple cloning site of the pBI101 vector, but also did not cut within the promoter region were used to insert the region into the pBI101 vector. These restriction enzymes also were crosschecked to make sure that they did not cut within the pGEMTE vector, which was used as the cloning vector. The restriction enzymes that cut in the multiple cloning site of each vector are shown in Figures 7 and 8 (Jefferson et al., 1987; Promega, 2005)

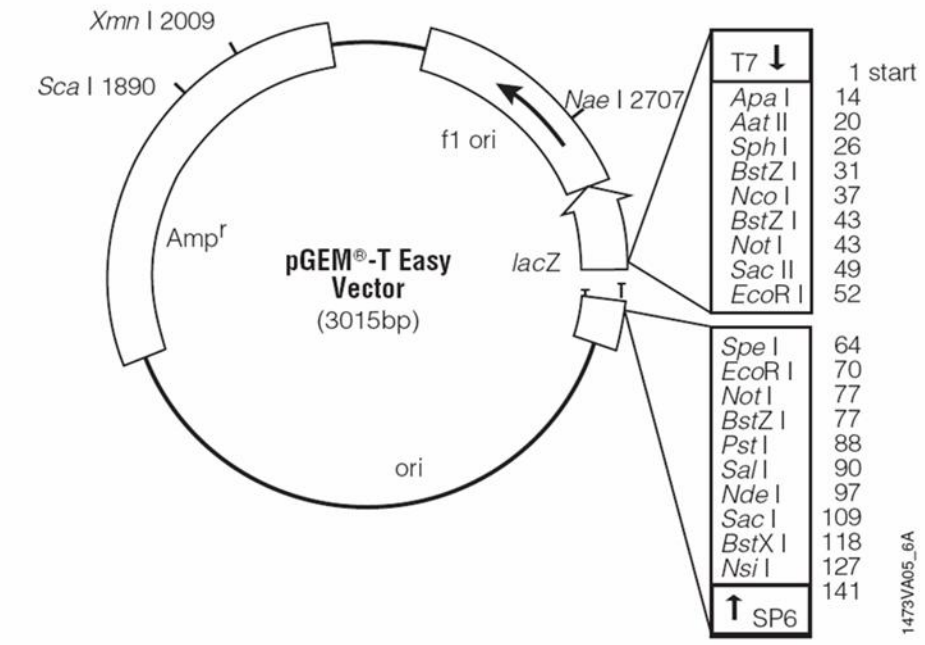

Figure 7. Map of pGEM-T Easy (pGEMTE) vector showing location of the lac-Z gene, ampicillin-resistance gene and the multiple cloning site. (Taken from Promega (2005)).

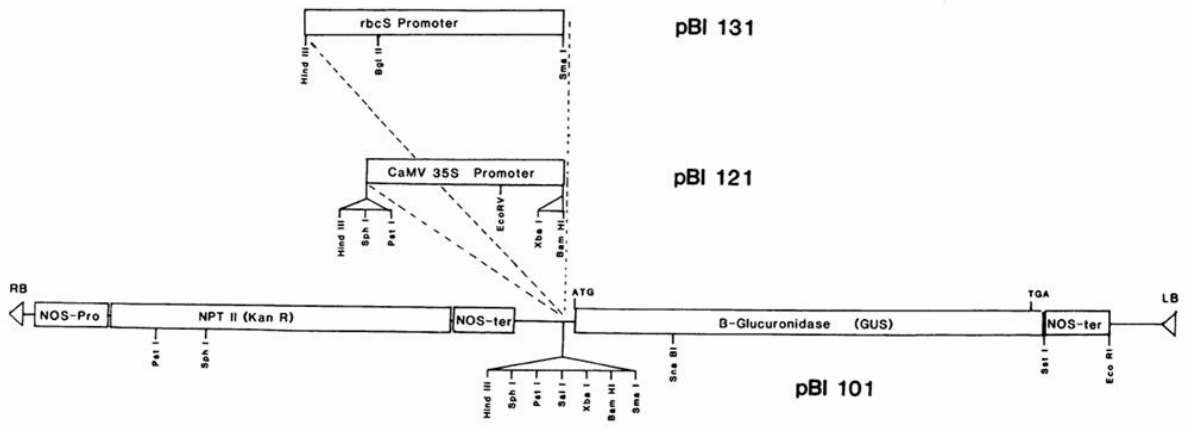

Figure 8. Map of the T-DNA region of pBI101 containing kanamycin resistance gene, GUS gene, and multiple cloning site. (Taken from Jefferson et al (1987).) The multiple cloning site contains restriction sites for the enzymes HindIII, SphI, PstI, SalI, XbaI, BamHI, and SmaI.

Insertion of *GAUT1* and *GAUT7* Promoters into pGEMT-Easy Cloning Vector for Sequencing and Large Scale Production

Isolation of Genomic DNA

In order to amplify the promoter region for insertion into the cloning vector, the genomic DNA needed to be obtained. The genomic DNA was isolated using an E.Z.N.A. Plant Miniprep Kit purchased from Omega Bio-Tek, Inc. (Doraville, GA). The directions from the kit were closely followed. Two preparations of 200 mg of 7-day old suspension culture cells were obtained. One preparation utilized liquid nitrogen and the other utilized a homogenizer for grinding of the 200 mg sample. Each sample was applied to a HiBind DNA spin-column from the kit, followed by two wash steps, which removed contaminants. The pure DNA was eluted using 100 µl of Elution Buffer to produce the first sample. A second elution of another 100 µl of Elution Buffer produced the second sample, which contained a lower concentration of the DNA. The genomic DNA was then analyzed by separation using gel electrophoresis (1% agarose gel in 1x TAE (40 mM Tris-acetate, 2 mM Na_2EDTA, pH of 8.5), run at 100 volts) and analyzed by

spectrophotometry (A_{260} and A_{280}) in order to confirm that the DNA was intact and at a concentration high enough for use (i.e. a concentration of 1-10 μg/ml). The expected size of the DNA was determined to be that which corresponded to the size of the smallest chromosome. According to The *Arabidopsis* Information Resource (TAIR) website (http://www.*Arabidopsis*.org) the smallest chromosome in the plant is chromosome 4, which is approximately 20 Mbp.

Promoter Amplification, PCR amplification

The polymerase chain reaction parameters, polymerase, and reaction mixture were experimented with several times in order to find the optimal parameters to produce the highest-fidelity promoter region. The final parameters that produced the optimal PCR product are indicated in Table 1 and the reaction mixture is indicated in Table 2.

The PCR was carried out using Platinum Taq High Fidelity Polymerase (Invitrogen, Carlsbad, CA) because it adds an extra adenosine base to the 3'ends of the PCR product, (known as an "A-overhang") which allows for ligation to the pGEMTE vector. The entire PCR product was then resolved on a 1% agarose gel from which a quick UV snapshot was taken. The visible bands were then excised from the gel using a scalpel. The PCR products were purified from the excised bands using a Qiagen Gel Extraction Kit following the manufacturer's protocol.

Table 1. Parameters for polymerase chain reaction. The denaturing, annealing, and extension steps are repeated a total of 30 times after the initial denaturation step and before the final extension step.

Number of Cycles: 30		
Step	Temperature (°C)	Time
Initial denaturation	95	2 minutes
Denaturing	95	20 seconds
Annealing	55	20 seconds
Extension	72	2.5 minutes
Final Extension	72	5 minutes
---	4	indefinite

Table 2. Reaction mixture for polymerase chain reaction. These amounts of the specified concentration of components were combined into a PCR tube.

Component	1 Reaction (µl)
10X High Fidelity PCR Buffer	5
50 mM $MgSO_4$	2
10 mM dNTP Mix	1
2 µM sense primer	5
2 µM antisense primer	5
Platinum Taq High Fidelity	0.2
cDNA	2
DEPC-treated water	29.8
Final Volume	50

Cloning the Promoter Regions into the pGEMTE Vector

In order to verify the sequence of the PCR product, which should have the same sequence as the promoter, it needed to first be inserted into a cloning vector. Use of a cloning vector is advantageous because it will contain a single PCR product segment from which more copies can be made through transformation of the plasmid into *E. coli.* After the bacteria are allowed to grow into colonies, each bacterium in the colony will contain a copy of the same insert. The plasmids can then be harvested from the bacteria in the colony, producing a solution of plasmids that contain the same PCR insert.

pGEMTE was chosen because its antibiotic resistance gene (ampicillin-resistance) is different than that of the pBI101 vector (kanamycin resistance) and because it has thymine on the 5'-ends of the linearized vector (5'-T overhangs) used for annealing to the 3'-A overhangs generated by Invitrogen's Taq polymerase during PCR. pGEMTE also allows for blue/white colony selection because it contains the lac-Z gene which produces the enzyme β-galactosidase,

which creates a blue color upon digestion of substrate X-Gal (Promega, 2005). The PCR product is inserted in the middle of this gene, therefore if the plasmid contains the insert, it will be indicated visibly by an absence of blue coloring due to the absence of the enzyme (Promega, 2005).

Ligation

T4 DNA Ligase is an enzyme that catalyzes the joining of two strands of DNA in either a blunt-ended or cohesive-ended configuration. In the pGEMTE-PCR product reaction used, T4 DNA Ligase joined the 5'T overhangs of pGEMTE to the 3'A overhangs of the PCR product. Three µl of approximately 20-25 ng/µl PCR product was ligated into 1 µl of 50 ng/µl linear pGEMTE. Two µl of the positive control unknown DNA was used as according to the Technical Manual which was followed closely (Promega, 2005). One µl of T4 DNA ligase (3 Weiss units/µl) was added and the reaction was incubated for 1 hour at room temperature.

Transformation

Two µl of the ligation reaction was added to 50 µl of JM109 High Efficiency Competent *E. coli* cells for transformation. The transformation occurred by the heat shock method, which begins by placing the mixture on ice for 20 minutes and then quickly placing it in a water bath at 42°C for 45-50 seconds. The cells are then returned back to ice for 2 minutes, followed by the addition of 950 µl SOC medium (autoclaved 10 mM NaCl, 2.5 mM KCl, 2 g/100ml Bacto-tryptone and 0.5 g/100ml Bacto-yeast extract plus filter-sterilized 20 mM Mg^{2+} stock and 20 mM glucose, at pH of 7.0.) The cells were then incubated for 1.5 hours at 37°C with shaking at 150 rpm.

Growth of E. coli Colonies and Selection

The transformation culture (100 µl) was plated onto plates of LB medium (10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, 5 g/L NaCl, pH of 7.0) with 15 g/L agar (and 0.5 mM IPTG, 80 µg/ml X-Gal, and 100 µg/ml ampicillin) and incubated for 14 hours at 37°C. The transformed bacteria were allowed to grow into colonies. Five white round colonies were selected from each pGEMTE:GAUT promoter plate and 2 white round colonies were selected from the positive control. A portion of the selected colonies was transferred to patch plates (to serve as a back-up culture) and the rest was used to inoculate 5 mL of liquid LB media, giving the cells more medium in which to grow. The liquid LB media culture was incubated overnight at 37°C with shaking (225-250 rpm.) A long-term stock was also made by mixing 600 µl of the liquid culture and 150 µl of sterile 50% glycerol, which were subsequently stored at -80°C.

The plasmid was harvested out of 5 mL of the liquid *E. coli* culture using a Qiagen Miniprep kit to prepare the plasmid for verification of its sequence. The kit's manual, which was closely followed, utilized different solutions to lyse the cells and purify the DNA. The DNA was eluted with 75 µl sterile deionized water.

Before the plasmid was sent for sequencing, a restriction digest was performed on the plasmid to see if expected fragments would be produced. EcoRI and HindIII restriction enzymes were used to perform two separate digests. Eight µl of the plasmid solution was added to 1 µl of enzyme (10 U/µl HindIII, 12 U/µl EcoRI) and 1 µl of buffer and incubated at 37°C for 2 hours. The anticipated sizes of the fragments were compiled using ApE software (http://www.biology.utah.edu/jorgensen/wayned/ape/) (see Table 3 and 4). The plasmid clones (plasmids harvested from a single colony) that produced fragments of the expected size were sent to the Integrated Biotechnology Laboratory (IBL) for sequencing.

Table 3. **Expected fragment size from digestion of plasmids with EcoRI is shown underneath the Size (bp) column**. Cut Site refers to the different locations of the EcoRI restriction sites on each plasmid.

pGEMT-E: GAUT7			pGEMT-E: GAUT1			pGEMT-E: GAUT7L		
Size (bp)	Cut Site 1	Cut Site 2	Size (bp)	Cut Site 1	Cut Site 2	Size (bp)	Cut Site 1	Cut Site 2
2997	971	71	2997	2035	71	2997	2103	71
319	71	390	1673	362	2035	866	656	1522
262	559	821	291	71	362	585	71	656
169	390	559				262	1691	1953
130	821	951				169	1522	1691
20	951	971				130	1953	2083
						20	2083	2103

Table 4. **Expected fragment size from digestion of plasmids with HindIII is shown underneath the Size (bp) column**. Cut Site refers to the different locations of the HindIII restriction sites on each plasmid. For the pGEMT-E:*GAUT7* and pGEMT-E:*GAUT1* plasmids, there is only one restriction site.

pGEMT-E: GAUT7			pGEMT-E: GAUT1			pGEMT-E: GAUT7L		
Size (bp)	Cut Site 1	Cut Site 2	Size (bp)	Cut Site 1	Cut Site 2	Size (bp)	Cut Site 1	Cut Site 2
3897	334	334	4961	1867	1867	4333	1466	770
						424	770	1194
						272	1194	1466

Sequencing of Colonies

A high concentration of the harvested plasmid, which was measured using the spectrophotometer, was obtained by speed-vacuum centrifugation. Upon obtaining a high concentration of plasmid, the cloning vector (pGEMTE) containing the *GAUT* promoter sequence (pGEMTE:PG1 and pGEMTE:PG7) and the primers needed to sequence the vector were sent to the Integrated Biotechnology Laboratory (IBL, UGA campus) for sequencing. Since the polymerase used for sequencing makes many mistakes in the first 100 basepairs sequenced, primers were designed to anneal approximately 100 basepairs upstream from the desired promoter sequence. This facilitated obtaining high quality sequence information in the

DNA region of interest. Additionally, the polymerase could only sequence 500-800 basepairs from the start of the primer. Therefore, multiple primers were designed in order to obtain the entire sequence of the promoter region.

The sequencing resulted in multiple overlapping sequences that were compared using a software program called ApE (http://www.biology.utah.edu/jorgensen/wayned/ape/). These sequences were compiled and compared to the intergenomic sequence (directly upstream of the *GAUT* gene) obtained from The *Arabidopsis* Information Resource (TAIR) website. If the sequence contained a point mutation, the original chromatogram from IBL was referred to in order to ensure that there was indeed a mutation.

Construction of GAUT1 Promoter Sequence within the Cloning Vector

pGEMTE:PG1 harvested from five different *E. coli* colonies did not contain the correct *GAUT1* promoter sequence. Two of the plasmids harvested from colonies (designated clone 2 and clone 3) only contained one point mutation. Therefore, clone 2 and clone 3 were used to produce the correct sequence by digesting and ligating the sequences that do not contain the point mutations. Figure 9 shows a map of two clones with the location of their mutations and restriction sites labeled. Digestion with the restriction enzymes BseRI and SacI produced a fragment that was 497 bp (designated as the insert) and another fragment that was approximately 4500 bp (designated as the vector). The 497 bp region of clone 2 did not contain a point mutation and the 4500 basepair region of clone 3 did not contain a point mutation. These two fragments were ligated to produce pGEMTE:PG1 with the correct sequence.

Plasmids of each of the above-described clones were harvested using Qiagen Miniprep kit. The DNA concentration was measured and calculations were made for the reaction mixture, as shown in Table 5 and Table 6. The digestion mixture was incubated for 2 hours at 37°C. The

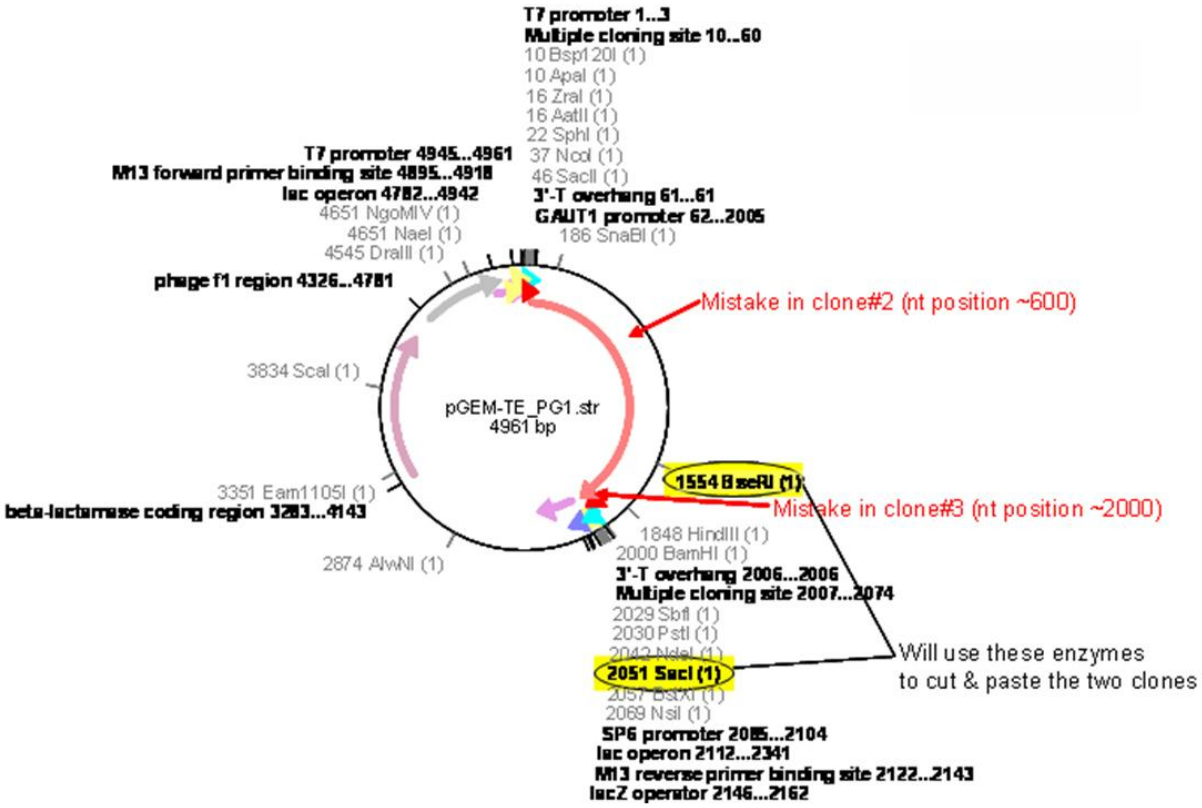

Figure 9. Map comparing two different pGEMTE:PG1 clones (plasmids harvested from two different colonies.) The map shows the location of point mutations in the two plasmids and the position of restriction enzymes that could be used to digest the two plasmids.

digestion of clone 3, which produced the vector (larger ~4500 bp fragment), was treated with phosphatase as described previously for the digestion of pBI101. The digestion mixtures were resolved on a 1% agarose gel and the correct bands were extracted and purified using the Qiagen Gel Extraction kit. The fragments were combined in a microcentrifuge tube according to the ligation mixture in Table 7 and incubated for 2 hours at 25°C, followed by 16 hours at 16°C in the thermocycler. Ten µl of the ligated vector were transformed into 100 µl of *E. coli* cells using the heat shock method as previously described.

The transformed cells were plated on LB plates supplemented with 50 µg/ml ampicillin and incubated overnight at 37°C. Twelve colonies were selected for PCR amplification of the *GAUT1* promoter region. The PCR reaction mixture is shown in Table 8. Reaction parameters

were the same as those used in Table 1. The colonies in which the *GAUT1* promoter region was successfully amplified were sent for sequencing and designated as pGEMTE:PG1C.

Table 5. Digestion of pGEMTE:PG1.C2 to obtain the 497 bp "insert."

10x Buffer B1	5 µl
NEB Acetylated BSA (10 mg/ml, 100x)	0.5 µl (100 µg/ml)
DNA (~1 µg/µl)	3 ul (3 µg)
Sterile water	38.5 µl
Mix by pipetting, then add:	
BseRI (4 U/µl)	l.5 ul (6 U)
SacI (20 U/µl)	1.5 µl (30 U)
Final Volume	**50 µl**

Table 6. Digestion of pGEMTE:PG1.C3 to obtain the 4464 bp "vector."

10x Buffer B1	5 µl
NEB Acetylated BSA (10 mg/ml, 100x)	0.5 µl (100 µg/ml)
DNA (~0.8 µg/µl)	5.6 µl (4.5 µg)
Sterile water	34.4 ul
Mix by pipetting, then add:	
BseRI (4 U/µl)	2.25 ul (9 U)
SacI (20 U/µl)	2.25 µl (45 U)
Final Volume	**50 µl**

Table 7. Ligation of digested pGEMTE:PG1.C3 (vector) to digested pGEMTE:PG1.C2 (insert).

Vector (120 ng/µl)	1.5 µl (180 ng)
Insert (10 ng/µl)	7.17µl (72 ng)
Ligase buffer 10x	1 µl
Ligase (3 U/ 1 µl)	0.33 µl (1.0 U)
Final volume	10 µl

Table 8. Reaction mixture for polymerase chain reaction of *E. coli* transformed with pGEMTE:PG1.

5x PCR buffer	4 µl
dNTP (10 µM)	0.4 µl (0.2 µM)
Forward Primer (PG1F) (2 µM)	2 µl (0.2 µM)
Reverse Primer (PG1R) (2 µM)	2 µl (0.2 µM)
GoTaq Flexi polymerase	0.5 µl (2.5 µM)
Distilled water	11.1 µl
Final volume	20 µl

Insertion of *GAUT1* and *GAUT7* Promoters into GUS-Reporter Gene Expression Vector, pBI101 to Produce pBI101:PG1 and pBI101:PG7

Digestion of pGEMTE:PG7, pGEMTE:PG1 and Expression Vector pBI101

pGEMTE:PG7 was digested according to the mixture listed in Table 9 using SalI and XbaI from New England Biolabs (NEB). The expression vector pBI101 was also digested according to the reaction mixture in Table 10 using the same restriction enzymes. After placing the mixture in a tube, the tube was centrifuged for several seconds and then placed in thermocycler for incubation at 37°C for 2.5 hours.

After incubation, the mixture containing pBI101 was treated with 0.15 µl (1.5 units) of the enzyme, phosphatase (NEB) and incubated at 37°C for 60 minutes. This phosphatase treatment was performed in order to prevent the reannealing of the expression vector to itself during the next step, ligation. After the phosphatase treatment of the expression vector, the digestion products were immediately analyzed by using gel electrophoresis (1% agarose gel in 1x TAE (40 mM Tris-acetate, 2 mM Na_2EDTA, pH of 8.5), electrophoresis at 150 volts). The entire purified product was resolved on the agarose gel and the desired bands were extracted using a clean scalpel blade. The excised DNA was purified using Qiagen's Gel Extraction Kit.

Double digestion of pGEMTE:PG1C and pBI101 with SalI and BamHI followed by ligation and transformation was not successful even though positive ligation and transformation controls produced transformed colonies. Therefore, the digestion protocol was modified. A single digestion of pGEMTE:PG1C and pBI101:PG7 was performed and resolved on an agarose gel along with another sample of uncut plasmid for comparison. pBI101:PG7 was chosen instead of pBI101 because the restriction sites were compatible and its digestion to obtain the

linearized vector produced a large product (the *GAUT7* promoter region) that was visible on the gel, allowing us to confirm that the vector had been completely digested.

The vectors were excised and purified using Qiagen's Gel Extraction Kit and the concentration was determined. The linearized vectors were then digested with the second restriction enzyme (followed by phosphatase-treatment of the pBI101 vector) and resolved on an agarose gel, extracted and purified. Each digestion reaction, shown in the Tables below, was performed for 2-3 hours at 37°C. Several digestion reactions were performed and DNA was combined in order to obtain a high product concentration.

Table 9. Reaction mixture for digestion of cloning vector containing *GAUT7* promoter sequence using SalI and XbaI.

10x Buffer B3	5 µl
NEB Acetylated BSA (10 mg/ml, 100x)	0.5 µl (100 µg/ml)
DNA (~0.3 µg/µl)	15 µl (4.5 µg)
Sterile water	24.5 µl
Mix by pipetting, then add:	
SalI (20 U/µl)	4 µl (80 U)
XbaI (20 U /µl)	1 µl (20 U)
Final Volume	50 µl

Table 10. Reaction mixture for digestion of pBI101 expression vector using SalI and XbaI.

10x Buffer B3	5 µl
NEB Acetylated BSA (10 mg/ml, 100x)	0.5 µl (100 µg/ml)
DNA (~0.5 µg/µl)	6 µl (~3 µg)
Sterile water	33.5 µl
Mix by pipetting, then add:	
SalI (20 U/µl)	4 µl (80 U)
XbaI (20 U/µl)	1 µl (20 U)
Final Volume	50 µl

Table 11. First digestion of pBI101:PG7 and pGEMTE:PG1C.

	pBI101:PG7	pGEMTE:PG1C
Water	24.5 ul	33 ul
10x buffer	5 ul	5 ul
Acetylated BSA 10 ug/ul (100x)	0.5 ul	0.5 ul
DNA (~0.125 ug/ul pBI101:PG7 and ~0.35 ug/ul pGEMTE:PG1C)	16 ul (2 ug)	9 ul (3 ug)
Restriction Enzyme (20 U/ul)	4 ul (80 U SalI)	3 ul (60 U BamHI)
TOTAL	50 ul	50 ul

Table 12. Second digestion of linearized pBI101:PG7 and pGEMTE:PG1C to obtain vector and insert for ligation.

	pBI101:PG7	pGEMTE:PG1C
Water	24 ul	36.75 ul
10x buffer	5 ul	5 ul
Acetylated BSA 10 ug/ul (100x)	0.5 ul	0.5 ul
DNA (~0.1 ug/ul pBI101:PG7 and ~0.8 ug/ul pGEMTE:PG1C)	17.5 ul (1.75 ug)	3.75 ul (3 ug)
Restriction Enzyme (20 U/ul)	3 ul (60 U)	4 ul (80 U)
TOTAL	50 ul	50 ul

Ligation of Promoter Region to the Expression Vector

Since the promoter region and the expression vector were digested with the same restriction enzymes in the previous step, they contained the same complementary cohesive ends needed for ligation. Using the spectrophotometer the concentration of the vector was determined to be approximately 20 ng/µl and the insert (promoter region) was determined to be approximately 15 ng/µl. In order to fully saturate the vector, the vector:insert ratio must be 1:3. The amount needed to obtain the ratio was calculated. The following equation, which takes the size of the vector into account, was used for calculations:

$$\frac{\text{ng vector} \times \text{kb insert}}{\text{kb vector}} \times \frac{3}{1} = \text{ng of insert needed for 3:1 ratio}$$

A background reaction mixture containing no insert (only vector) was also performed and served as the positive control for transformation. The resulting reaction mixtures are shown in Table 13 and 14. The ligation mixture was incubated for 2 hours at 25°C, followed by 16 hours at 16°C in the thermocycler.

Table 13. Standard ligation reaction mixture. The vector:insert ratio is 1:3.

Vector (20 ng)	2.5 µl (50 ng)
Insert (15 ng)	6.2 µl (93 ng)
10x ligase buffer	1 µl
Ligase (3 u/ 1 µl)	0.33 µl (1.0 u)
Final volume	10 µl

Table 14. Background ligation reaction mixture. No insert added.

Vector (20 ng)	2.5 µl (50 ng)
Insert (15 ng)	-----
10x ligase buffer	1 µl
Ligase (3 u/ 1 µl)	0.33 µl (1.0 u)
Final volume	10 µl

Transformation of pBI101:PG1 and pBI101:PG7 into E. coli

pBI101:PG7 was transformed into JM109 *E. coli* (Promega) while pBI101:PG1 was transformed into 10-beta *E. coli* (New England Biolabs (NEB)) because the later strain is more efficiently transformed with larger plasmids like pBI101:PG1 (New England Biolabs (2007).) Ten µl of the pBI101:PG7 standard ligation reaction was added to 100 µl of JM109 High Efficiency Competent *E. coli* cells for transformation. For the positive control, 10 µl of the background ligation reaction was added to 100 µl of JM109 *E. coli* cells. For the negative control, 0.1 µl of digested pBI101 (linearized) was mixed with 9.9 µl of water and added to 100 µl of JM109 *E. coli* cells. The three mixtures were added to three sterile polypropylene tubes and transformed using the same heat shock method as previously described.

The heat shock method used to transform the 10-Beta *E. coli* cells was slightly different as suggested by the New England Biolabs (NEB) protocol, which was closely followed. Ten µl of the pBI101:PG1 standard ligation reaction was added to 50 µl of 10-beta Competent *E. coli* cells (NEB) in an eppendorf tube and gently flicked 4-5 times to mix the cells. Positive control

and negative controls (as previously described) were also transformed into 50 µl of 10-beta Competent *E. coli* cells. The mixtures were placed on ice for 30 minutes without mixing and then moved to a water bath at 42°C for 30 seconds. The cells were then returned back to ice for 5 minutes, followed by the addition of 950 µl SOC medium and incubated for 1 hour at 37°C with shaking at 250 rpm.

The transformation culture (1000 µl) was centrifuged and the top layer was decanted. The remaining cells were resuspended in 100 µl of LB medium and plated onto agar plates of LB medium (50 µg/ml kanamycin) and incubated for 14 hours at 37°C. Twelve colonies were picked for PCR amplification of the promoter region using a combination of primers that anneal to the promoter region and the vector region. Each colony was picked with a pipette tip, which was used to transfer the cells to 11.1 µl of distilled water, which was used for PCR. The PCR reaction mixture is shown in Table 15. The PCR reaction parameters are shown in Table 1.

Table 15. **Reaction mixture for polymerase chain reaction to confirm presence of plasmid in transformed colonies.** These amounts of the specified concentration of components were combined into a PCR tube.

5x PCR Buffer	4 µl
dNTP (10 µM)	0.4 µl (0.2 µM)
Forward Primer (2 µM)	2 µl (0.2 µM)
Reverse Primer (2 µM)	2 µl (0.2 µM)
GoTaq Flexi polymerase	0.5 µl (2.5 µM)
Distilled water	11.1 µl
Final Volume	20 µl

Patch plates of each colony were created (to serve as a back-up) and 5 mL of liquid LB media was inoculated to allow the cells to grow. The liquid LB media culture was incubated overnight at 37°C with shaking (225-250 rpm). A long-term stock of the culture was also made

by mixing 600 µl of the liquid culture and 150 µl of sterile 50% glycerol, which was subsequently stored at -80°C.

Electroporation of pBI101:PG1 and pBI101:PG7 into *Agrobacterium*

Plasmid from transformed *E. coli* colonies (confirmed by the presence of the promoter region using PCR) was harvested and prepared for transformation into *Agrobacterium tumefaciens,* a bacterium that, in the wild-type state, is pathogenic to the plant, but in the disarmed state (used here) facilitates the transfer of desired DNA into the plant cells. Once transformed into the *Agrobacterium* (strain GV3101::pMP90), the pBI101:PG7 and pBI101:PG1 vector will serve as *Agrobacterium*'s Tumor-Inducing (Ti) plasmid. The T-DNA region of pBI101, which contains a gene for kanamycin resistance and the *GAUT1* and *GAUT7* promoters fused to the GUS gene, will be integrated into the plant's genome by the *Agrobacterium*.

Five separate controls and treatments were transformed into the *Agrobacterium*: positive transformation control using PB277 DNA, a negative transformation control using water, a negative control for staining using unlinearized pBI101, and the two treatment DNAs pBI101:PG1 and pBI101:PG7 DNA. PB277, donated by Dr. Mary Tierney (University of Vermont), is a pBI101 vector containing the promoter region for a gene that encodes for an *Arabidopsis* cell wall protein, AtPRP3 (Bernhardt and Tierney, 2000). Two µl of each plasmid were mixed with 80 µl of *Agrobacterium* cells and transferred to an electroporation cuvette. The electroporation machine was set to a 100 ohms (resistance), 250 µFD (capacitance), 2.5 V (voltage), and 25 µFd (capacitance) according the machine protocol. The cuvette was placed in the electroporation machine and an electrical current was applied for 8-10 seconds. One ml of LB medium was added to the cuvette to transfer the cells to a microcentrifuge tube. The cells

were then incubated in the dark room for 2 hours with shaking at 200 rpm. After 2 hours, the 150 µl of the culture was plated on LB plates containing 50 µg/ml rifampicin, 50 µg/ml gentamycin, and 50 µg/ml kanamycin and incubated for three days at 28°C.

In order to verify that the cells were transformed, 10 *Agrobacterium* colonies transformed with pBI101:PG1, 10 colonies transformed with pBI101:PG7, 10 colonies transformed with PB277, and 3 colonies transformed with pBI101 were picked for PCR amplification. The PCR reaction mixture and parameters were followed according to those listed in Table 1 and Table 14. The PCR products were then analyzed using gel electrophoresis. Colonies that were PCR-positive were used to inoculate 5 mL of LB medium (supplemented with 50 µg/ml rifampicin, 50 µg/ml gentamycin, and 50 µg/ml kanamycin), incubated in the dark room for 2 hours with shaking at 200 rpm, and then used to make long-term stock.

Transformation of *Arabidopsis* Plants with *Agrobacterium* harboring the *GAUT1* and *GAUT7* GUS-Reporter Gene Constructs, the Floral Dip Method

Four weeks prior to transformation, over 36 wild-type *Arabidopsis thaliana* seeds were planted. Two days prior to plant transformation, 5 ml of LB medium (with 50 µg/ml rifampicin, 50 µg/ml gentamycin, and 50 µg/ml kanamycin) were inoculated with transformed *Agrobacterium* and incubated in the dark room overnight at room temperature with shaking at 250 rpm. The evening before transformation, the flowering *Arabidopsis* plants were watered and a 2800 mL flask containing 300 ml of YEP medium (10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl, adjusted to pH 7.0) supplemented with appropriate antibiotics was inoculated with 5 mL of the previous culture and incubated in the dark room overnight at room temperature with shaking at 250 rpm.

The *Agrobacterium* was then pelleted by centrifugation at 6000 rpm for 15 minutes at room temperature. The top layer was decanted and the cells were resuspended in 150 ml of infiltration media (1/2x Murashige and Skoog salts, 1x Gamborg's B5 vitamins, 5% (w/v) sucrose, 0.044 μM benzylamino purine). The solution was transferred to a 150 mL beaker and the solution was stirred for 5 minutes using a magnetic stirbar with Silwet L-77 (final concentration 0.3%). The inflorescence shoots of 10-12 plants for each solution (Agro:PB277, Agro:pBI101, Agro:pBI101:PG1 and Agro:pBI101:PG7) were "dipped" or inverted and fully submerged for approximately 30 seconds, moving the shoots up and down 3-5 times in the solution. After dipping, the plants were laid on their sides in a plastic container and covered for 24 hours. The plants were then returned to their normal growing conditions and watered after 3 days in order to prevent the *Agrobacterium* from being washed away with the water before infection had occurred.

Growth of *Arabidopsis* Plants

Arabidopsis plants were grown by planting seed with forceps on dampened soil in plastic trays, covering with a plastic dome, and placing the trays in an *Arabidopsis* growth chamber (19°C day, 15°C night, photoperiod of 14/10). After 5-10 days, when cotyledons became visible, the plastic dome was removed. The soil surrounding the seedling was watered lightly each day until shoots began to develop. Upon emergence of shoots, plants were watered every 3-5 days and treated with fertilizer (1 tsp/gal Peters Professional All Purpose Plant Food) and biopesticide (5 ml/L Gnatrol) once a week.

Harvesting *Arabidopsis* Seed

When seedpods of mature plants began to brown, the plants were placed in ArabiSifter floral sleeves (a plastic covering that protects the seed from dispersal) and left unwatered for 2-3 weeks until the entire plant was completely dry and brown. The plastic covering was then removed and the plants were cut at the stem over a sheet of clean newspaper. The plant was then crumbled in between clean hands and collected into the center of the paper where large debris was separated from the seed with fingers and placed into a biohazard bag for autoclaving. The remaining plant material was then transferred to a sheet of paper (8 ½ x 11 inches) for easier handling. The plant material was slowly transferred to another sheet of paper, allowing the seeds to fall, and other debris and dirt to stay behind. Seeds were transferred from one paper to the other until only a few pieces of dirt remained, which were removed with forceps. The seeds were left exposed to air for 2-3 days and then stored with Drierite in sealed 1.5 mL eppendorf tubes at 4°C.

Germinating *Arabidopsis* Seed

In order to grow seedlings on agar plates (1/2 x Murashige and Skoog (MS) salts, pH to 5.8, 0.7% plant agar), the seed was placed in a sterile 1.5 mL eppendorf tube and sterilized by soaking in 70% ethanol for 1 minute followed by 50% v/v commercial bleach with 0.05% v/v Triton-X-100 for 5 minutes and washing with 5 changes of sterile distilled water. Seeds were then suspended in 1 mL of sterile 0.1% agarose, transferred to, and spread onto plates using a sterile 1 mL pipette tip. Clumps of seeds were separated 1-3 cm apart using sterile forceps. The plates were left open in laminar flow hood for 10-15 minutes to allow agarose to harden and then

sealed with 3 M microporous tape. Plates were placed at 4°C for two to three days to break dormancy and then moved to growth chamber (16/8 photoperiod at a constant 24°C).

Selection and Identification of Transformed Plants

The first wild-type plants grown for floral dipping were designated T0 (generation zero). After dipping, T0 plants (approximately 4 weeks old at time of dip) were grown for 3 more weeks and then allowed to dry out (not watered) for 2 weeks before T1 seed (first generation post-dip) was harvested from the plants. T1 seed was picked from the seed pods and separated from other plant material and air-dried for several days. T1 seeds were surface sterilized and plated on agar plates (0.7% agar, ½X MS) supplemented with 50 μg/ml kanamycin for selection of transformed seed. Kanamycin-resistant (T1) seedlings that continued to grow for 10 days on the media supplemented with kanamycin (50 μg/ml) were gently removed from the agar with roots preserved and placed in fresh soil. These T1 seedlings were carefully watered for 5 weeks until plants were flowering and producing seed pods. These T1 plants were confirmed to contain the promoter:GUS construct by PCR using primers that annealed to the promoter region and to the GUS gene. Sigma-Aldrich's "Extract-N-Amp" kit was used to isolate genomic DNA and perform PCR. PCR parameters were same as those shown in Table 1.

For each promoter:GUS construct, 5-10 T1 lines were confirmed to be transgenic by analyzing the PCR product resolved on an agarose gel. Once the seeds pods of these PCR-confirmed T1 plants turned brown, the plants were no longer watered. After 3 weeks of no watering, T2 seed was harvested from these T1 plants. Some of the seed was plated in order to stain seedlings and some of the seed was planted in soil for staining of mature plants. This procedure is summarized by Figure 10.

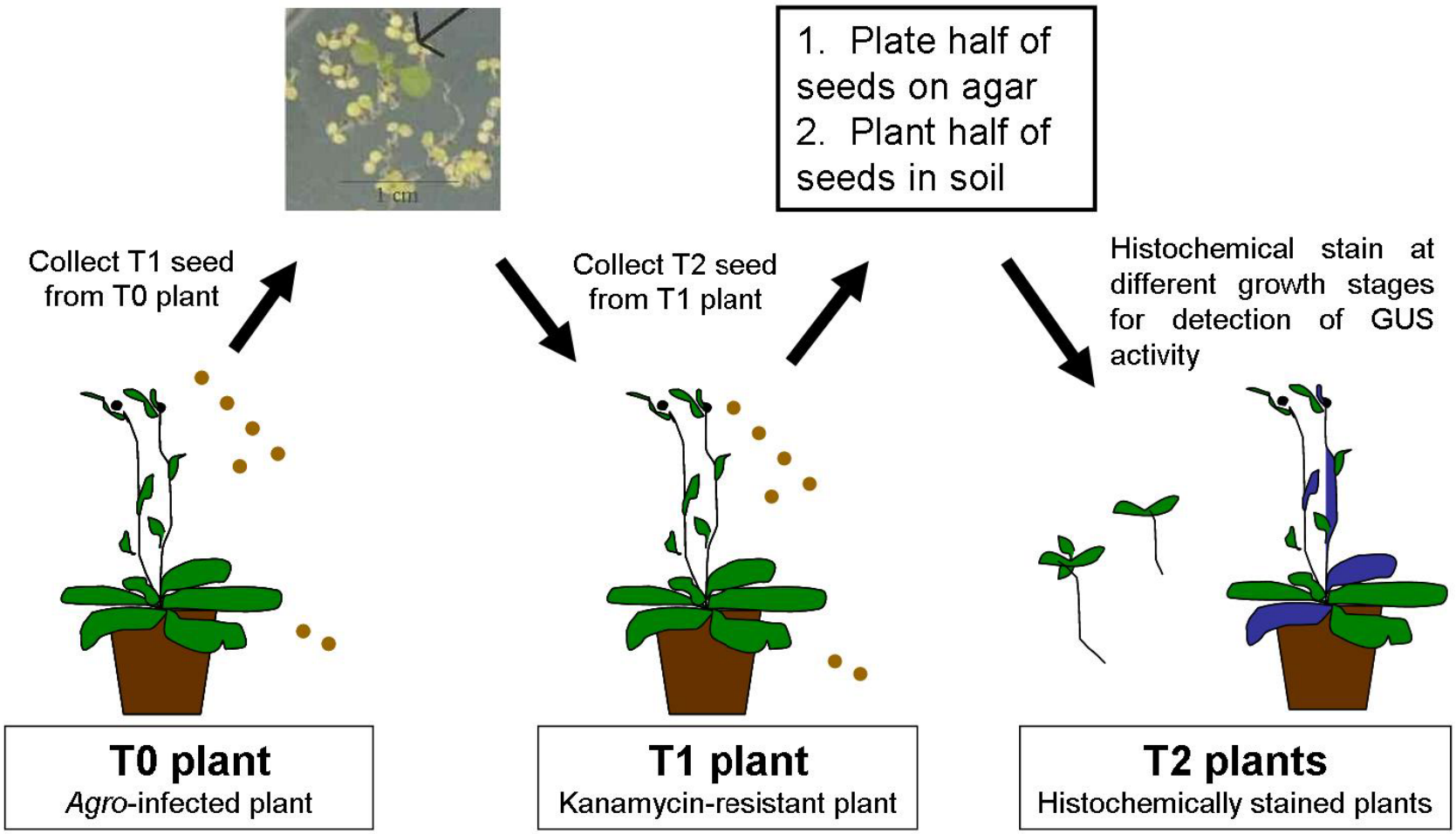

Figure 10. Obtaining T2 plants for histochemical staining. Photo from http://www.bioinformatics.vg/Methods/arabitransformationf.htm

Histochemical Staining of Second Generation GUS Construct-Transformed *Arabidopsis* Plants

Plant materials harvested from T2 generation were submerged in X-gluc staining solution (50 mM sodium phosphate (pH 7.0), 15% (v/v) methanol, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.05% (v/v) Tween-20, and 2 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid) in 2 mL eppendorf tubes on ice. Many different experiments were previously performed and published in order to determine the effect of each component of the staining solution on GUS expression and intensity (Jefferson et al., 1987; Craig, 1992; Stomp, 1992; Kim et al., 2005). Table 16 shows the function of each component of the solution. After submerging tissues in the X-gluc solution, the tissues were placed under a 600 mm Hg vacuum for 10 minutes to remove air bubbles. The tissues were placed in an incubator at 37°C for 0.5 to 72 hours. In order to remove chlorophyll, tissues were then washed every 30 minutes with several changes of 70% ethanol until no traces of chlorophyll remained.

Table 16. Components of the X-gluc staining solution and its function in the staining process (Jefferson et al., 1987; Craig, 1992; Stomp, 1992; Kim et al., 2005).

Component of solution	**Function**
50 mM sodium phosphate (pH 7.0)	Buffer, reduces background GUS activity
15% (v/v) methanol	Reduces background GUS activity
0.5 mM potassium ferricyanide	Catalyst, accelerates oxidative cleavage of intermediate to final indigo product
0.5 mM potassium ferrocyanide	Catalyst, accelerates oxidative cleavage of intermediate to final indigo product
0.05% (v/v) Tween-20	Penetration of X-gluc into plant tissues
2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc)	Substrate for β-glucuronidase
pH between 7 and 8	Reduces background GUS activity

Incubation times vary greatly depending on many different factors such as promoter strength and copy number (number of promoter:GUS insertions in genome). Staining intensity also varies between different T1 lines because promoter:GUS constructs are randomly inserted

into the genome in different places. Most GUS protocols advised incubation until visible blue coloring of tissue appears while avoiding overly long incubation (>24 hours) in order to prevent tissue deterioration (Stomp, 1992). Tissues were first stained at half-hour time intervals until tissues were fully saturated with substrate. Tissues were then evaluated under a microscope in order to determine the incubation time that produced the most saturated staining pattern without leakage of the substrate.

We required consistent staining of multiple T2 seedlings and adult tissues from a minimum of six independent transgenic T1 lines in order to make definite conclusions about the staining patterns. Wild-type and negative control tissues were also stained in order to look for the presence of endogenous or background GUS activity. Seedlings were stained at several intervals: 3-day, 5-day, 7-day, 10-day, and 15-day. Different organs of mature T2 plants were stained at four weeks: flowers, leaves, taproot cross-section, and lower, middle, and upper stem-cross-sections (hand-cut).

Analyses of Staining Patterns

Tissues were submerged under a thin film of water for analysis of expression using a dissecting scope. Expression patterns and intensities at different developmental stages from 5-10 T1-lines for each construct were studied and recorded extensively. The photographs most indicative of the observed staining pattern were produced when using bright field microscopy with additional lighting sources from the top. The compound light microscope was used for higher magnification of some tissues, but it was, however, harder to obtain photos with good resolution using high magnification due to the number of cell layers required to obtain visible GUS staining. For leaf and cotyledon tissues, epidermal peels (delicate separation of top

epidermal layer from rest of tissue using extremely sharp forceps) were performed in order to obtain clearer resolution of cells. Types of tissue and cell types were identified using multiple books, papers, and advice from Glenn Freshour, Kimberly Hunt, and other CCRC colleagues (Esau, 1953, 1977; Bowman, 1994; Turner and Somerville, 1997; Fowler et al., 1999; Ye et al., 2002; Kim et al., 2005; Ko et al., 2007).

CHAPTER THREE
RESULTS

Designing *GAUT1* and *GAUT7* Promoter Sequences

The promoter region for *GAUT1* was determined to be the 1932 nucleotides upstream of the start codon of the gene and the promoter region of *GAUT7* was determined to be the 868 nucleotides upstream of the start codon of *GAUT7*. The version of the *GAUT7* promoter known as "*GAUT7* long" consists of the 2000 nucleotides upstream from the start codon.

Analysis of restriction sites within the promoter regions and within the pGEMTE and pBI101 vectors determined which restriction sites should be added to 5'-ends of the promoter. For both versions of the *GAUT7* promoter, the restriction sites of SalI and XbaI fit the criteria for use. For the *GAUT1* promoter, the restriction sites used were SalI and BamHI. The primers that were designed for PCR contained these restriction sites on the 5' end. The forward primer consisted of the 5'-> 3' restriction site plus the 5'-> 3' beginning of the promoter region. The reverse primer consisted of the end of the promoter region oriented 3'-> 5' plus the 3' -> 5' orientation of the restriction site. Primers designed are shown in Table 17.

Table 17. Primers designed for the GAUT genes. The underlined nucleotides are the restriction site. *GAUT7* and *GAUT7* long share the same reverse primer.

Primer	**Sequence**
GAUT1 Forward	5'-**GTCGAC**CTCTCACTCGCTCTCTCTCTTCTTT**CTACG**-3'
GAUT1 Reverse	3'-CGAACTAAATCAAAAAAAAAACTTAAACTATCCC**CCTAGG**-5'
GAUT7 Forward	5'-**GTCGAC**ACTCAAAACTAAAAGAACAGTCAC-3'
GAUT7 Reverse	3'-GTAAGTTAAGATTTAGCCCTTA**AGATCT**-5'
GAUT7 Long Forward	5'- **GTCGAC**TGACCACAGCATTTTTTCCTGC-3'

Insertion of *GAUT1* and *GAUT7* Promoters into pGEMTE for Sequencing and Large Scale Production

The preparation of genomic DNA that produced the clearest band without any fragmentation was the sample that was prepared using liquid nitrogen. In Figure 11, this sample can be seen in lanes 1 and 2. Lanes 5 and 6 are overloaded. The total size of the band is around 12,000 basepairs which is significantly smaller than the expected size which was 20 megabasepairs. This discrepancy suggests that either the DNA is fragmented or the E.Z.N.A. Plant MiniPrep Kit only selects for small sized DNA. Even though the genomic DNA was possibly fragmented, the decision was made to continue with PCR because the promoter regions of *GAUT1* and *GAUT7* could remain intact within such fragment sizes.

The first preparation had a lower concentration of genomic DNA but had a higher purity (Table 18). Higher purity means that there is less contamination and the DNA is more suitable for amplification. Therefore, the decision to use the first preparation containing the best quality DNA for PCR amplification was made. After the promoter region was PCR-amplified, resolved on an agarose gel, extracted and purified from the gel, the promoter was then ligated to the pGEMTE cloning vector and transformed into JM109 *E. coli*. Figure 12 shows the gel from which the PCR product was extracted. The colonies

Table 18. Concentration of isolated *Arabidopsis* genomic DNA samples were determined using A260 on a spectrophotometer where 1 O.D. unit contained 50 µg/ml. The quality or presence of impurities in the DNA was based on the ratio of the A260/A280 where ideal purity is 2.

Sample	Concentration (mg/µl)	Quality (ratio)
Preparation 1 1st elution	0.02255	1.479
Preparation 2 2nd elution	0.0246	1.103
Preparation 2 1st elution	0.086	1.599
Prepration 2 2nd elution	0.02265	1.015

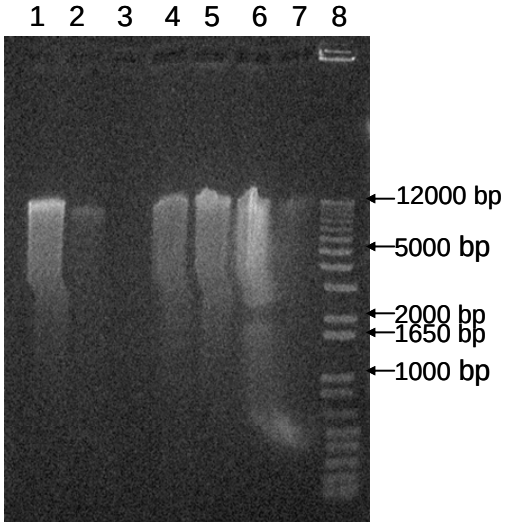

Figure 11. Agarose gel (1%) stained with Ethidium Bromide containing *Arabidopsis* genomic DNA. Lanes 1, 2, and 5 are from preparation 1, while lanes 4, 6, and 7 are from preparation 2. Lanes 5 and 6 contain 10 µl while lanes 1, 2, 4, and 7 contain 3 µl. Lanes 2 and 7 are second elutions of each preparation, which contain an even lower concentration of DNA than the first elution. Lane 8 is a DNA marker.

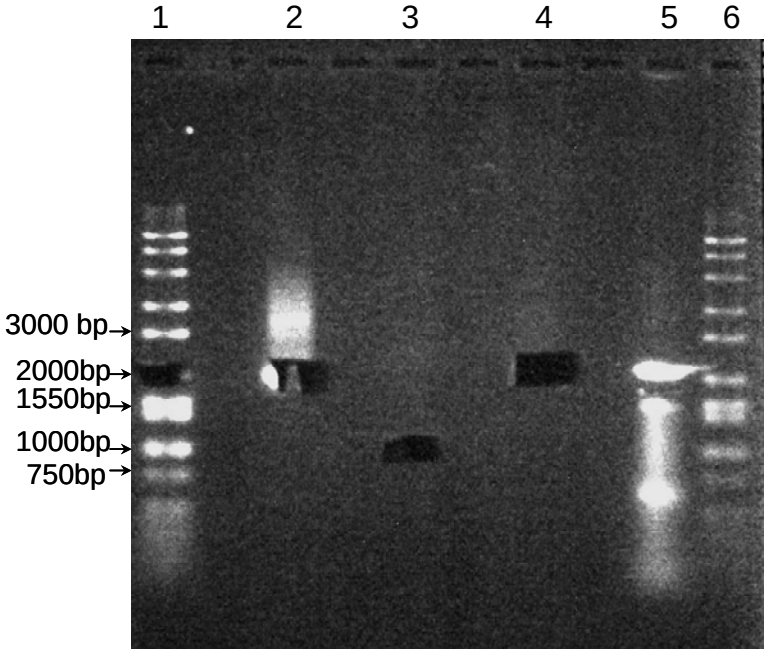

Figure 12. PCR products of the promoter region of *GAUT1* (lane 2), *GAUT7* (lane 3), and *GAUT7* long version (lane 4) are resolved on 1% agarose gel and stained with Ethidium Bromide. The bands used have been excised and purified from the gel. Excision marks can be seen in positions where the bands previously existed, which correspond to the expected size of the promoter regions. Lanes 1,5, and 6: DNA markers. PCR product (40 µl) was loaded into each well along with 8 µl of 6X loading buffer.

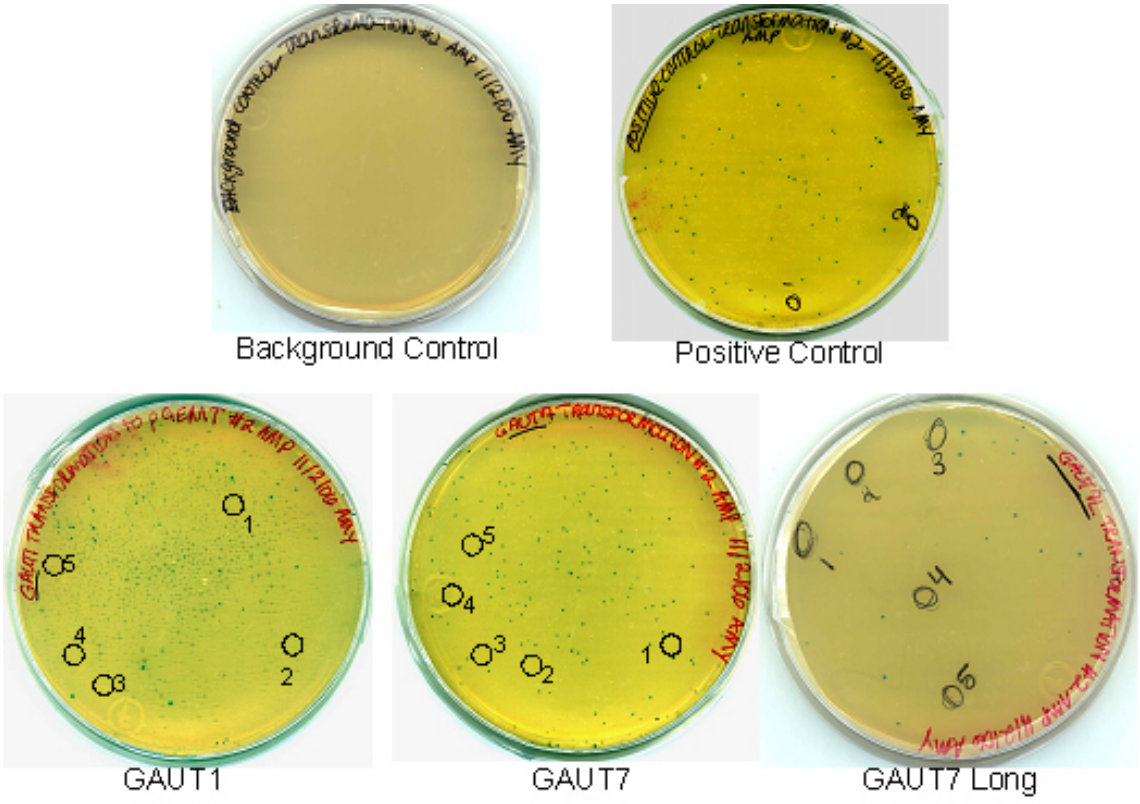

Figure 13. **Results of 14-hour incubation of *E. coli* harboring pGEMTE constructs or ampicillin-supplemented agar plates.** The positions of the positive (white) colonies that were selcted to be transferred to liquid media are labeled 1-5 for each plate.

produced from the 14-hour incubation of transformed *E. coli* cells grew into nice round colonies (Figure 13.) The positive control contained many white colonies and the background control contained no colonies, which is exactly as expected.

The bands resulting from the digestion of pGEMTE:*GAUT1* and pGEMTE:*GAUT7* long did not correspond to the anticipated results (Figure 14). The digestion of pGEMTE:*GAUT7*, did however, produce the anticipated fragments. It is possible that *GAUT1* and *GAUT7* long were not amplified with the highest fidelity. This hypothesis is formed based on the fact that *GAUT1* and *GAUT7* long are both approximately 2000 nucleotides while *GAUT7* is only 868 nucleotides.

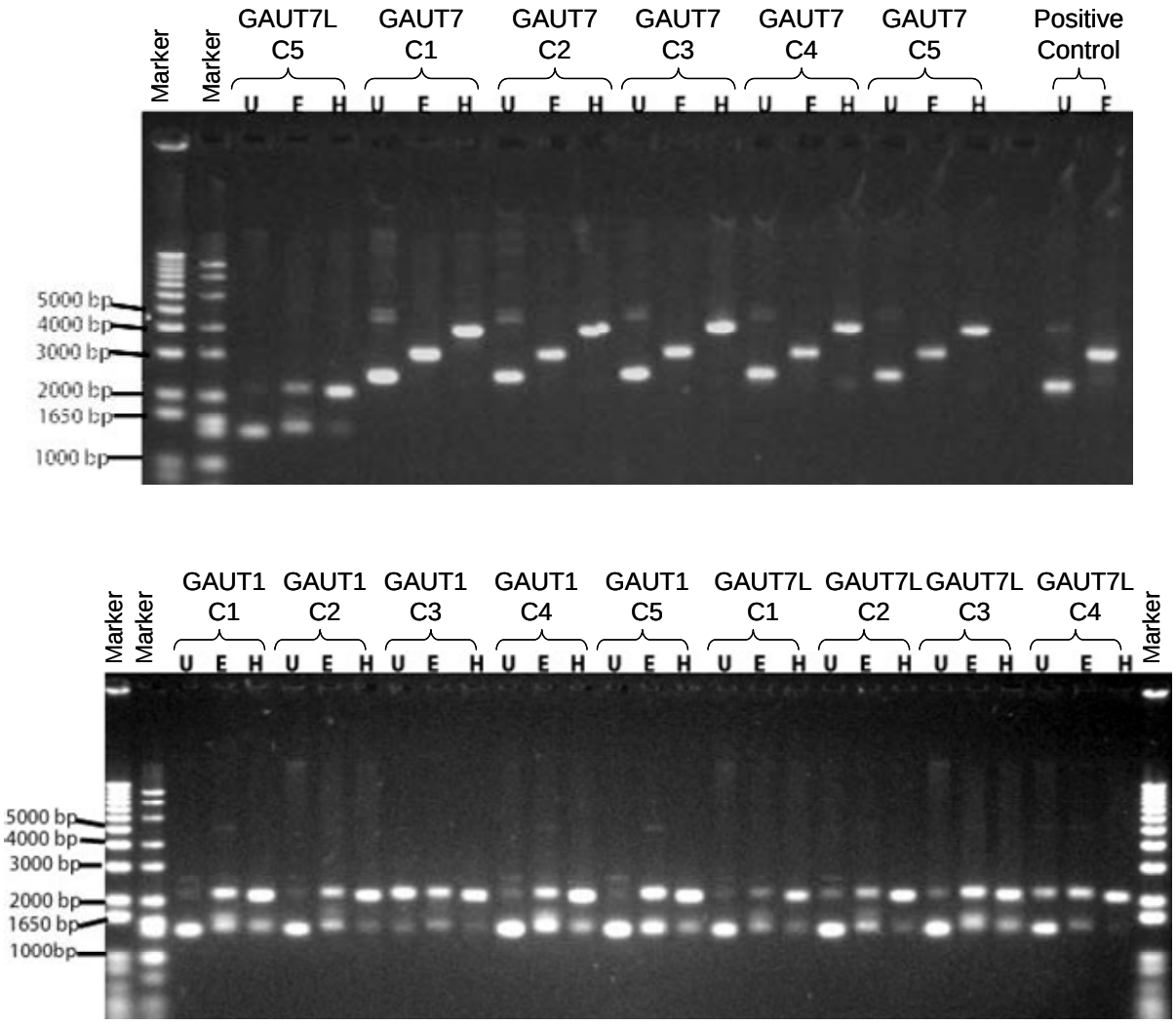

Figure 14. Each pGEMTE construct was digested with EcoRI and HindIII. The resulting fragments were resolved on a 1% agarose gel, which was subsequently stained with Ethidium Bromide. Uncut constructs were also included as a positive control. U- uncut plasmid. E- plasmid digested with EcoRI. H- plasmid digested with HindIII. C- Clone.

Since *GAUT1* and *GAUT7* long have a longer nucleotide sequence, there is a higher possibility that the DNA polymerase made mistakes during PCR amplification.

It was also possible that *GAUT1* and *GAUT7* long do contain the specific GAUT promoter region even though the size does not correspond to the size of the expected fragments on the gel. Since the pGEMTE:*GAUT1* or pGEMTE:*GAUT7* long contain so many basepairs

(>5000 basepairs), it was possible that the sequence was twisted into a configuration that hindered its movement. Therefore, in order to determine if the plasmids contained the correct inserts, plasmids from several colonies were harvested and sent for sequencing at the Integrated Biotechnology Laboratory (IBL).

The plasmid harvested from the second *E. coli* colony was verified to obtain the correct sequence of the *GAUT7* promoter region. Plasmid harvested from three different *E. coli* colonies did not contain the correct *GAUT1* and *GAUT7* long version promoter sequence. Since the correct *GAUT7* long version sequence was not successfully amplified and cloned, we decided not to repeat amplification due to lack of time. As described in the Materials and Methods section, the plasmids from two different colonies were digested and ligated in order to obtain the correct *GAUT1* sequence within the promoter region. The plasmid constructed from ligation of fragments from two different plasmids was denoted as pGEMTE:PG1C and was verified by PCR amplification using PG1F and PG1R primers. Ten µl of PCR product was resolved on a 1% agarose gel. The gel shown in Figure 15 confirms the presence of the *GAUT1* promoter (approximately 2000 bp). The integrity of the sequence was confirmed by sequencing at IBL.

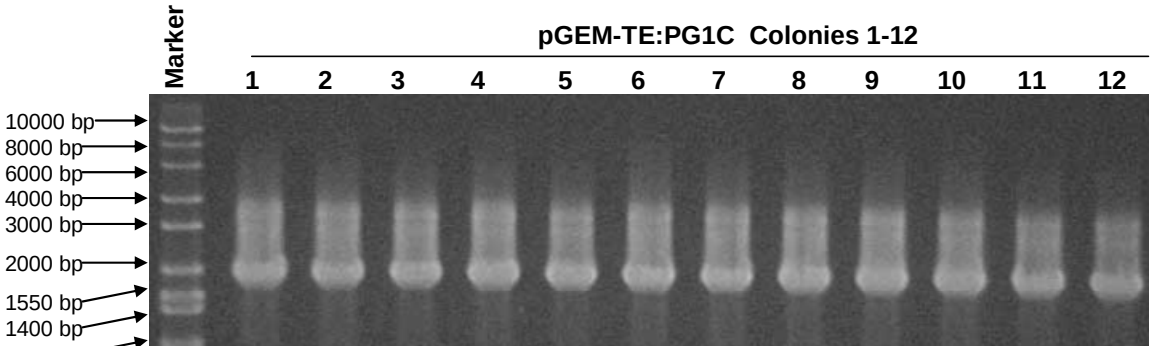

Figure 15. PCR on *E. coli* containing pGEMTE:PG1 using primers that anneal to the *GAUT1* promoter region. The expected size of the PCR product is 1932 bp.

Insertion of *GAUT1* and *GAUT7* Promoters into GUS-Reporter Gene Expression Vector, pBI101

After purification of the digested expression vector and promoter insert, 2 µl of the purified DNA was resolved on a 1% agarose gel in order to confirm that the DNA was intact and at a high enough concentration for ligation. After ligation and subsequent transformation of the ligation product, the kanamycin-resistant *E. coli* colonies were verified to contain the plasmid by PCR. PCR amplification of the *GAUT1* and *GAUT7* promoter regions in 12 transformed *E. coli* colonies are shown in Figure 16. Each colony except for *GAUT7* colony #10 was successfully transformed with the pBI101:PG vectors.

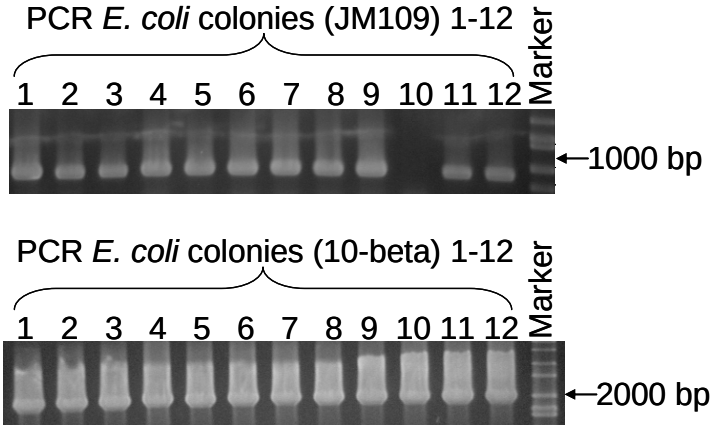

Figure 16. PCR amplification of the promoter regions in transformed colonies. Top gel shows PCR of the *GAUT7* promoter region in JM109 *E. coli* colonies 1-12 transformed with pBI101:PG7. Twenty µl of PCR product is resolved on a 1% agarose gel (top). PCR of the *GAUT1* promoter region in 10-beta *E. coli* colonies 1-12 transformed with pBI101:PG1C. Ten µl of PCR product is resolved on a 1% agarose gel.

Transformation of *GAUT1* and *GAUT7* GUS-reporter expression vectors (pBI101) into *Agrobacterium tumefaciens*

Primers that anneal either to the vector and/or to the insert were used in different combinations to confirm that the integrity of pBI101:PG7 that had been maintained during the transformation. One primer that annealed to the GUS gene and another that annealed to the *GAUT1* promoter region were used to amplify *Agrobacterium* transformed with pBI101:PG1C.

Figures 17 and 18 show the products of PCR on *E. coli* colonies containing pBI101:PG7 and pBI101:PG1C, respectively, resolved on 1% agarose gels. Figure 19 shows the product of PCR on *E. coli* transformed with pBI101, which was expected to be 283 bp. Figure 20 shows the product of PCR on *E. coli* transformed with PB277, expected to be approximately 1700 bp.

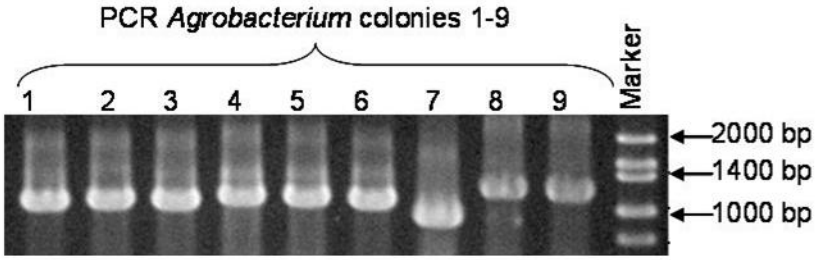

Figure 17. PCR amplification of transformed *Agrobacterium* colonies transformed with pBI101:PG7 using a combination of primers listed in Table 4. Expected size is around 1100 bp.

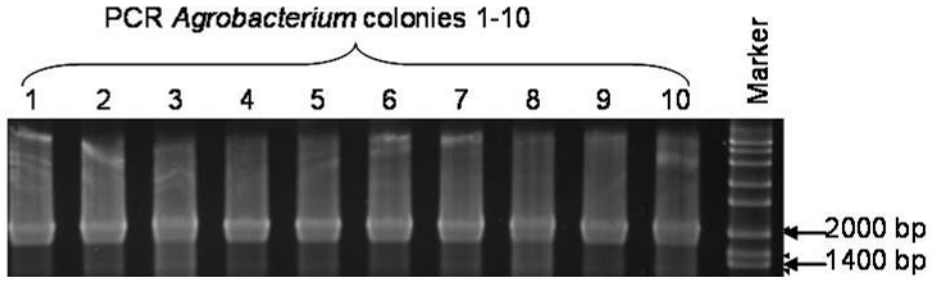

Figure 18. PCR amplification of transformed *Agrobacterium* colonies transformed with pBI101:PG1C using a primer that annealed to the GAUT1 promoter sequence. Expected fragment size is 2059 bp.

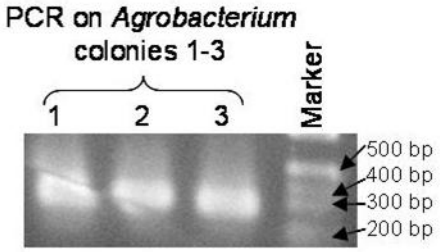

Figure 19. PCR of Agrobacterium cells transformed with pBI101 plasmid. Expected fragment size is 283 bp.

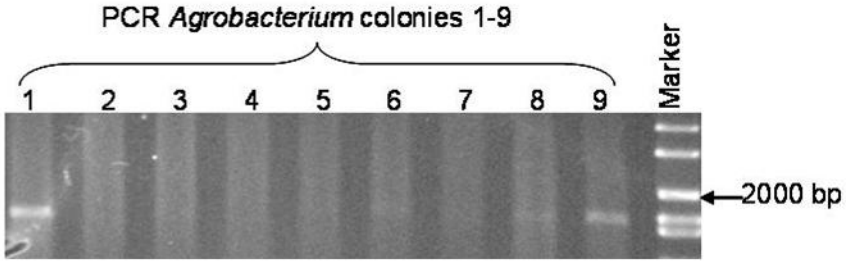

Figure 20. PCR of *Agrobacterium* cells transformed with PB277 plasmid. Expected fragment size is 1783 bp.

Transformation of *Arabidopsis* plants with *Agrobacterium* harboring *GAUT1* and *GAUT7* GUS-Reporter Gene Constructs, the Floral Dip Method

Figures 21 and 22 show the plants during the floral dip procedure and during the 24 hour period after the floral dip.

Figure 21. *Arabidopsis* plants are dipped into *Agrobacterium* transformed with PB277.

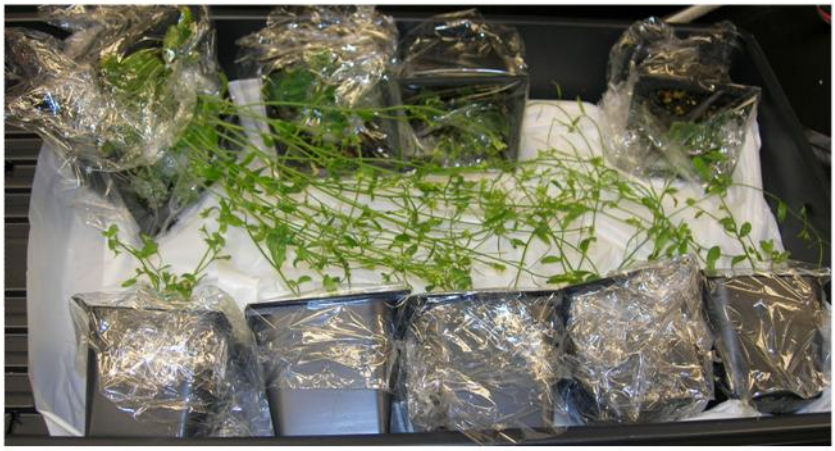

Figure 22. *Arabidopsis* during 24-hour period after floral dip.

Recovery of *GAUT1* and *GAUT7* GUS-Reporter Vector Transformed *Arabidopsis*

T1 seeds plated on kanamycin plates began to grow after 7-10 days. Seeds that were not transformed grew yellow and brown plantlets. Transformed seeds yielded plantlets that grew quickly and appeared bright green (see Figure 23). Seeds were germinated on the 0.8% agar plates (1/2 X Murashige and Skoogs (MS) salt and 50 µg/ml kanamycin). The transformed seedlings were labeled and transferred from the media to soil for growth. After 6-8 weeks, the plants began to flower and produce seed, which was harvested and stored according to the T1 lines from which it was collected.

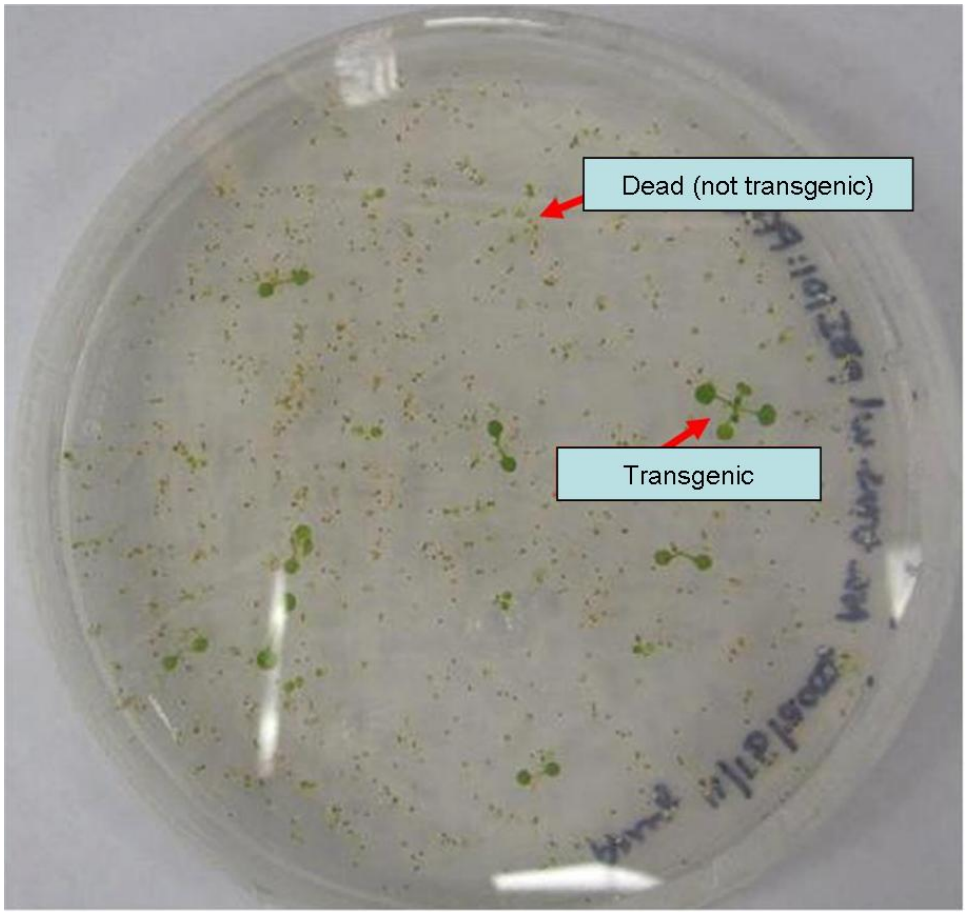

Figure 23. Selection of transformed *Arabidopsis* seedlings. Arrow labeled "transgenic" points to seedlings grown from transformed seeds. Arrow labeled "dead (not transgenic)" points to brown seedlings, which represent seedlings grown from untransformed seeds.

GUS Staining Pattern of *GAUT1* and *GAUT7* Promoter Construct Transformed Plants

Expression Analysis

While expression intensities varied amongst different T1 lines, expression patterns did not vary. *GAUT7* tissues produced much weaker staining than *GAUT1* tissues, often not even visible at 24 hour incubation times. Thus *GAUT7* tissues were therefore incubated for 24 to 72 hours. For *GAUT1* and *GAUT7* promoter construct transformed tissues, longer incubation times showed expression in every tissue and cell type. Negative and background controls were performed at each time interval and analyzed. The controls yielded no endogenous GUS expression.

Expression Pattern in Seedlings

GAUT1 is strongly expressed, while *GAUT7* is weakly expressed, in the vascular region of the cotyledon and hypocotyl with greater intensity at the hypocotyl base. *GAUT1* and *GAUT7* are expressed strongly in leaf primordia, leaf outer edge, leaf vasculature, and in some stomata of the cotyledon and true leaves as summarized in Table 19. As leaves mature, the expression pattern in *GAUT1* and *GAUT7* leaves fades and becomes less uniform. As cotyledons mature, the expression becomes most concentrated in the highly lignified area at the cotyledon tip. *GAUT1* is expressed strongly in root tips and root vasculature but is absent in the vasculature of root elongation zones. *GAUT7* is expressed more weakly in root and is particularly weak in the root tip and root cap. Detailed expression in a 7-day old seedling is shown in Figure 25 and 26, while the expression over a 15-day timespan is shown in Figure 24.

Table 19. Summary of expression in *GAUT1*:GUS and *GAUT7*:GUS seedlings.

	GAUT1	***GAUT7***
Cotyledon	Strong in vascular regions and tip.	Strong in vascular regions and tip.
Hypocotyl	Strong in vascular region with greater intensity at base.	Very weak in vascular region with greater intensity at base.
Leaves	Strong in leaf primordial and outer edge. Less uniform expression as leaf ages.	Strong in leaf primordial and outer edge. Less uniform expression as leaf ages.
Root	Strong in root tip and vascular region. Absent in elongation zones.	More weakly expressed than *GAUT1*, especially in root tip.

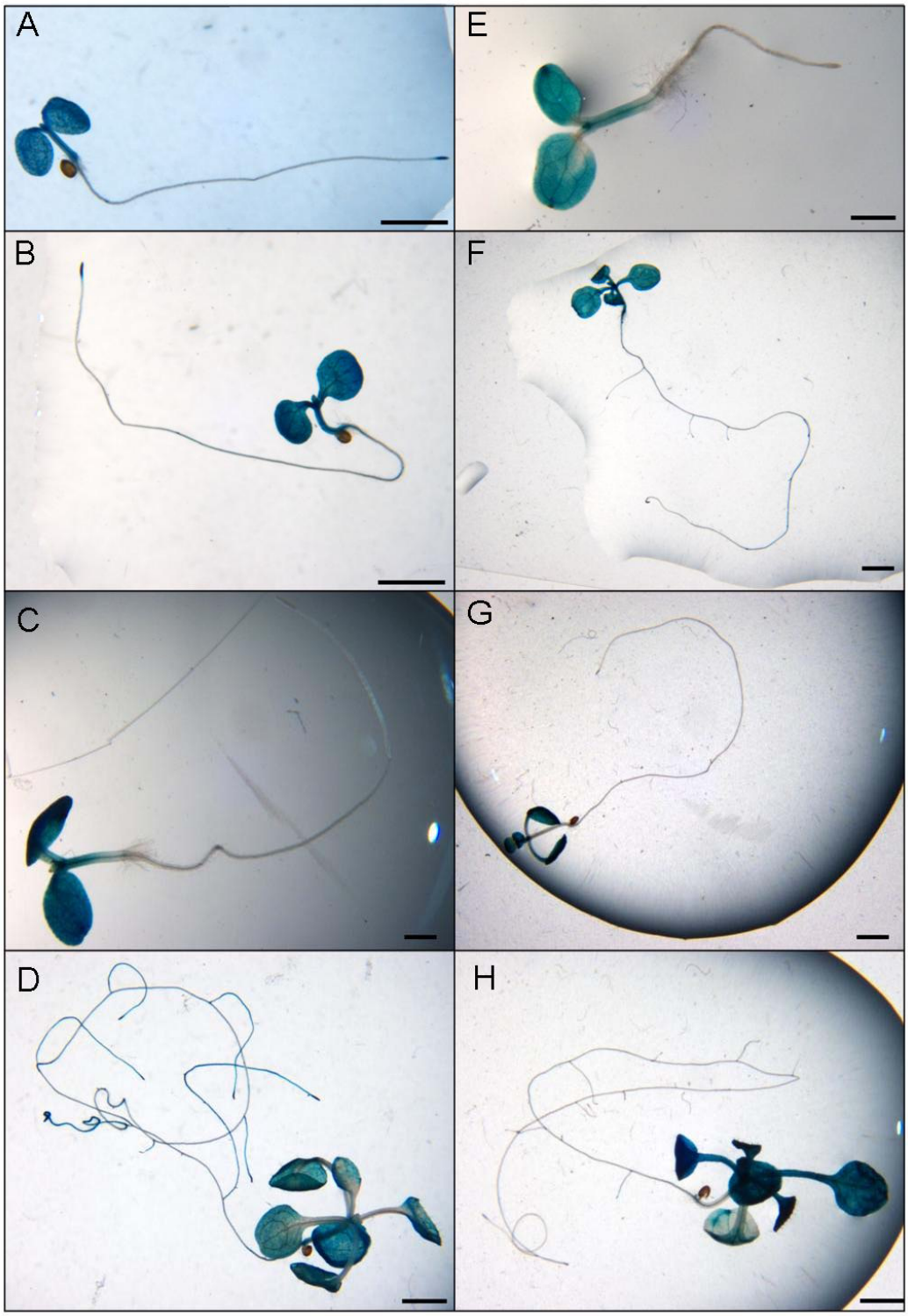

Figure 24. Staining patterns of *GAUT1* and *GAUT7* promoter constructs in seedlings at specific intervals over a span of 15 days. A-D show expression of *GAUT1* at 3-day, 5-day, 10-day, and 15-days (after placement of plates in growth chamber). E-H show expression of *GAUT7* at 3-day, 5-day, 10-day, and 15-days. Scale bar is 2mm.

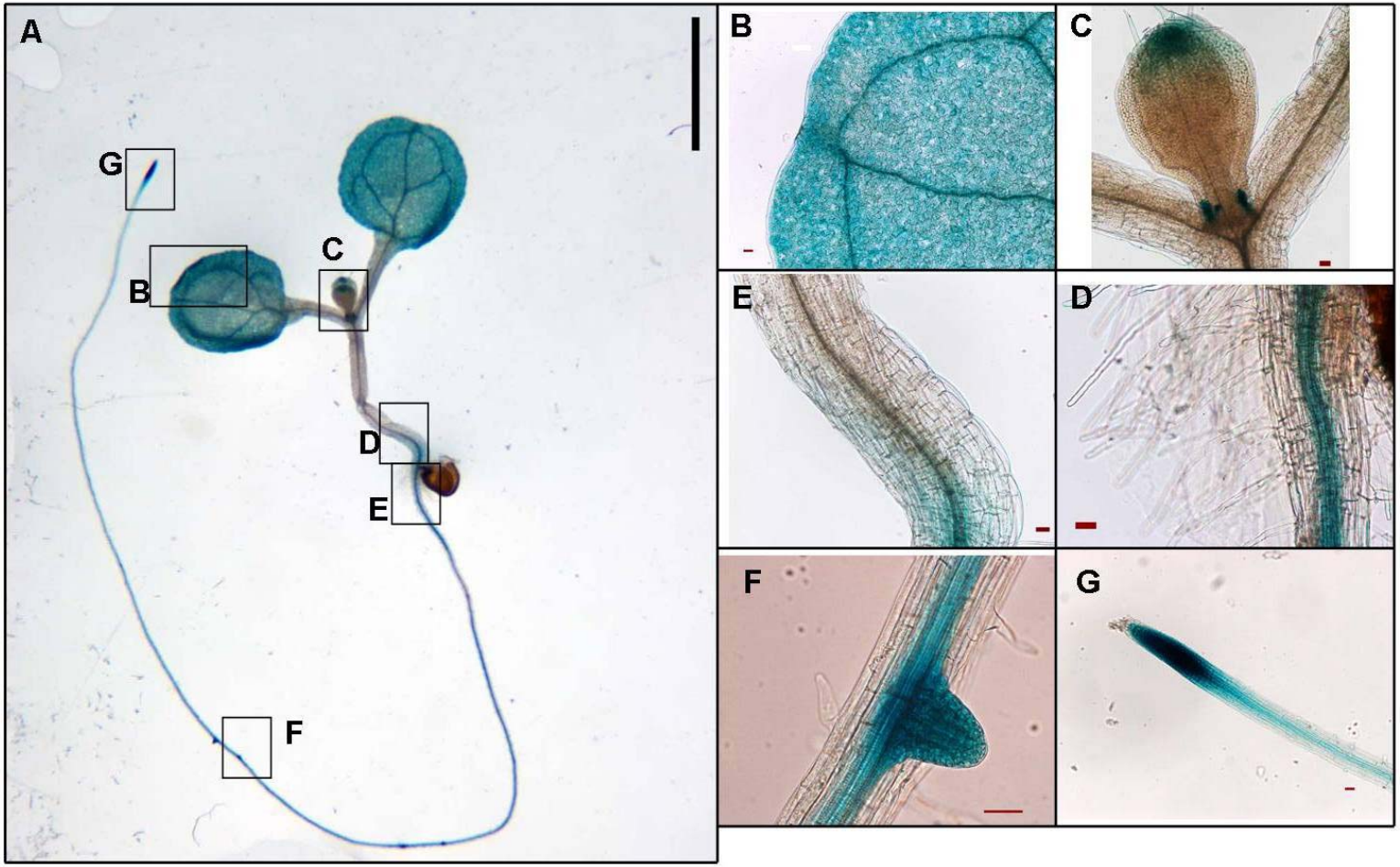

Figure 25. Detailed expression of a *GAUT1*:GUS 7-day old seedling. Scale bar in A is 2 mm. Scale bars in B-G is 100 μm.

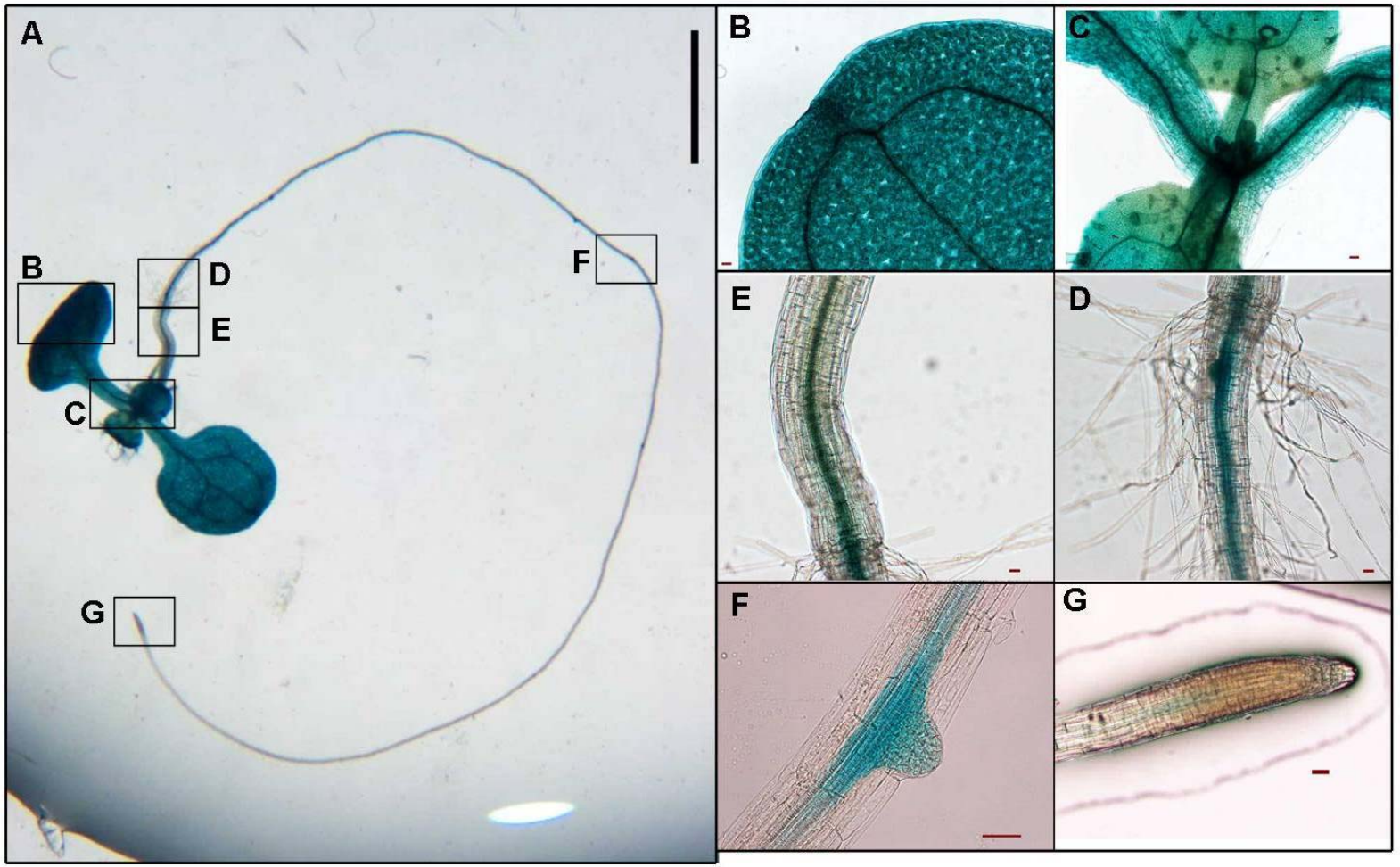

Figure 26. Detailed expression of a *GAUT7*:GUS 7-day old seedling. Scale bar in A is 2 mm. Scale bars in B-G is 100 μm.

Expression Pattern in Mature Tissues

In 6-8 week old (mature) plants, *GAUT1* and *GAUT7* are most strongly expressed in pollen grains, vascular cambium and phloem. The expression pattern in flowers is shown in Figure 27 and summarized in Table 20. *GAUT1* is expressed towards the end of the stigma, in the papillae, anthers, sepals, and pollen grains. *GAUT7* is expressed in these same tissues with the exception of the papillae. In both the stem and root, *GAUT1* and *GAUT7* are very highly expressed in the vascular cambium and phloem. In stem, the expression of both genes in xylem, pith, and epidermis is weaker when compared to the vascular cambium and phloem (see Figure 28). In root, GUS expression in xylem tissues is more ambiguous due to the natural pigment of the tissue, which masks the GUS staining pattern (see Figure 29).

Table 20. Summary of expression in *GAUT1*:GUS and *GAUT7*:GUS 6-8 week old plants.

	GAUT1	***GAUT7***
Flower	Distal end of stigma, papillae, anthers, pollen grains, sepals.	Distal end of stigma, anthers, pollen grains, sepals.
Stem	Strong in cambium and phloem. Also present in epidermis, cortex, metaxylem and protoxylem.	Strong in cambium and phloem. Also present in epidermis, cortex, metaxylem and protoxylem.
Root	Strongest in vascular cambium and phloem. Weaker in xylem, cortex and epidermis when compared to other tissues.	Strongest in vascular cambium and phloem. Weaker in xylem, cortex and epidermis when compared to other tissues.

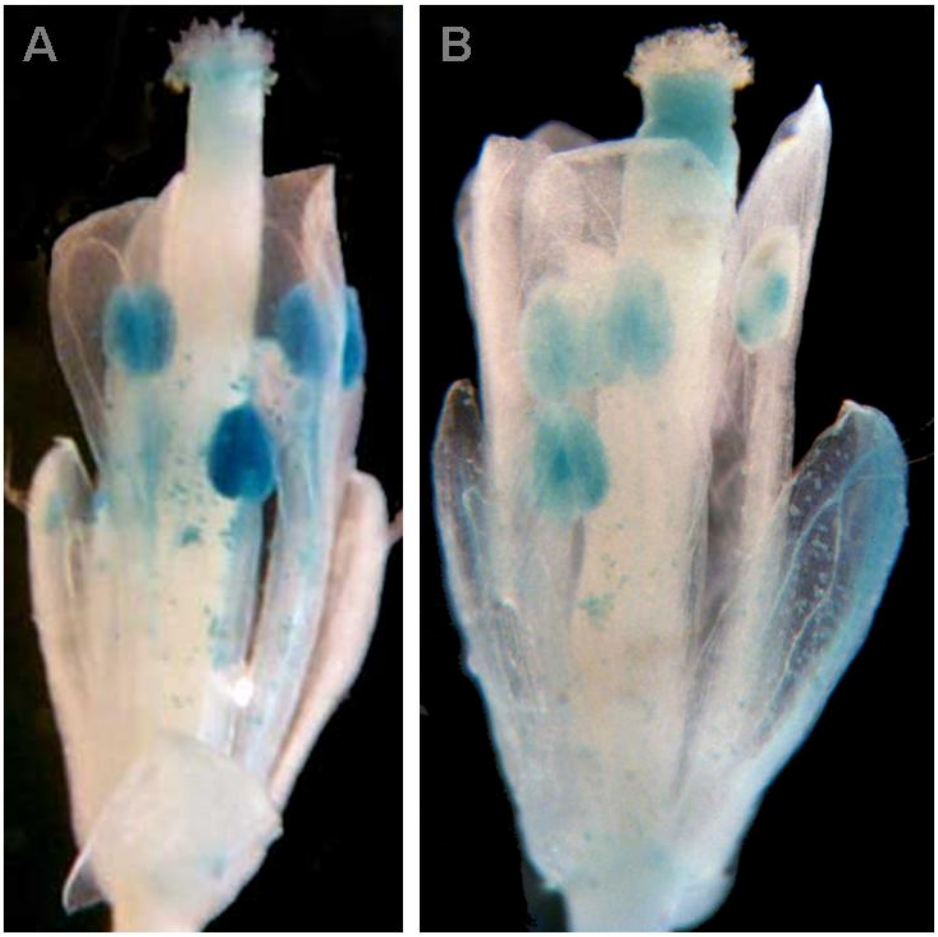

Figure 27. Expression of *GAUT1* and *GAUT7* promoter constructs in flowers. A shows the staining pattern of *GAUT1*. B shows the staining pattern of *GAUT7*.

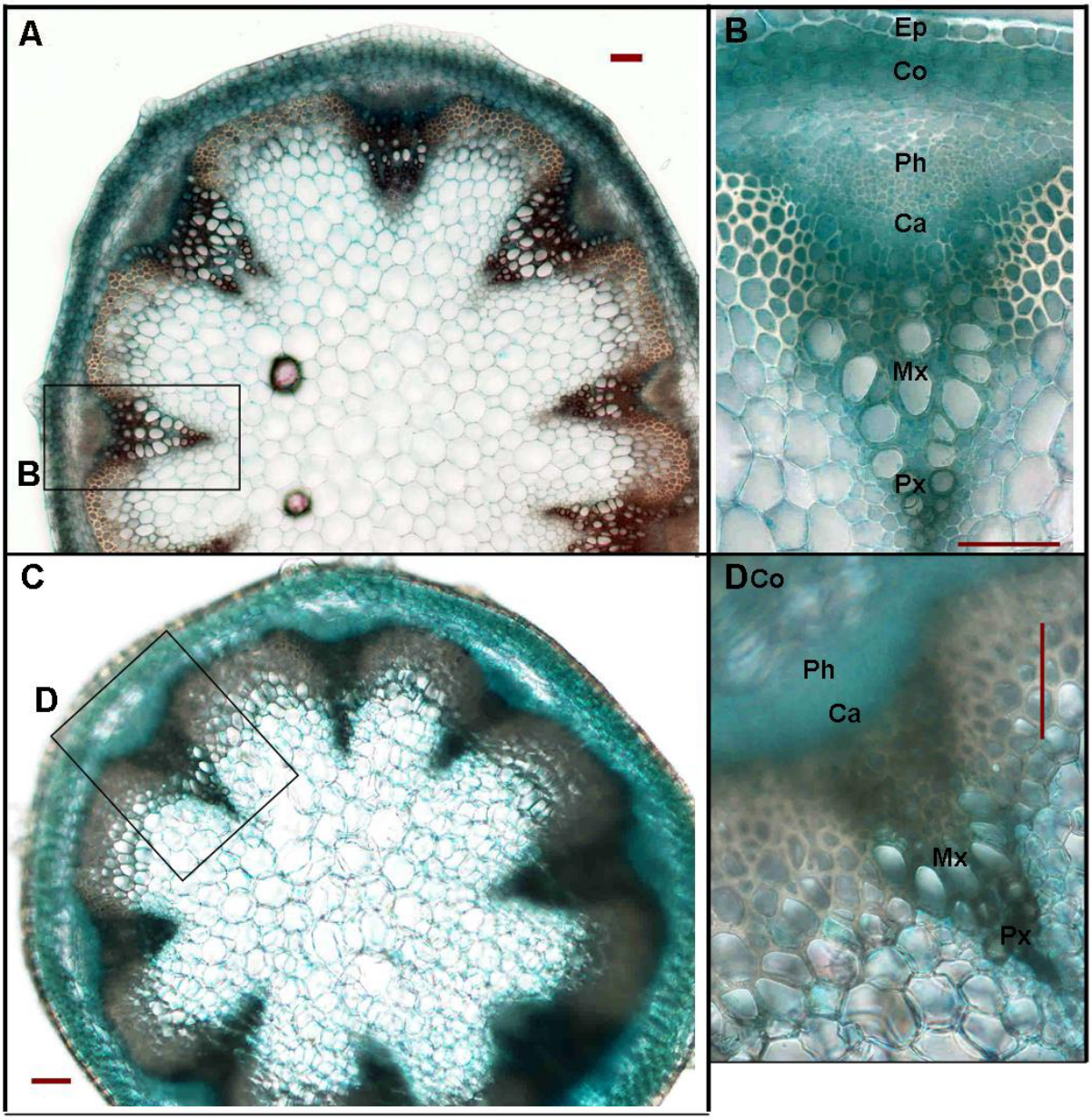

Figure 28. Expression of *GAUT1* and *GAUT7* promoter constructs in stems. A and B show *GAUT1*:GUS expression in stem. C and D show *GAUT7*:GUS expression in root. Scale bar is 200 μm. Px, protoxylem; Mx, metaxylem; Ca, cambium; Ph, phloem; Co cortex; Ep, epidermis.

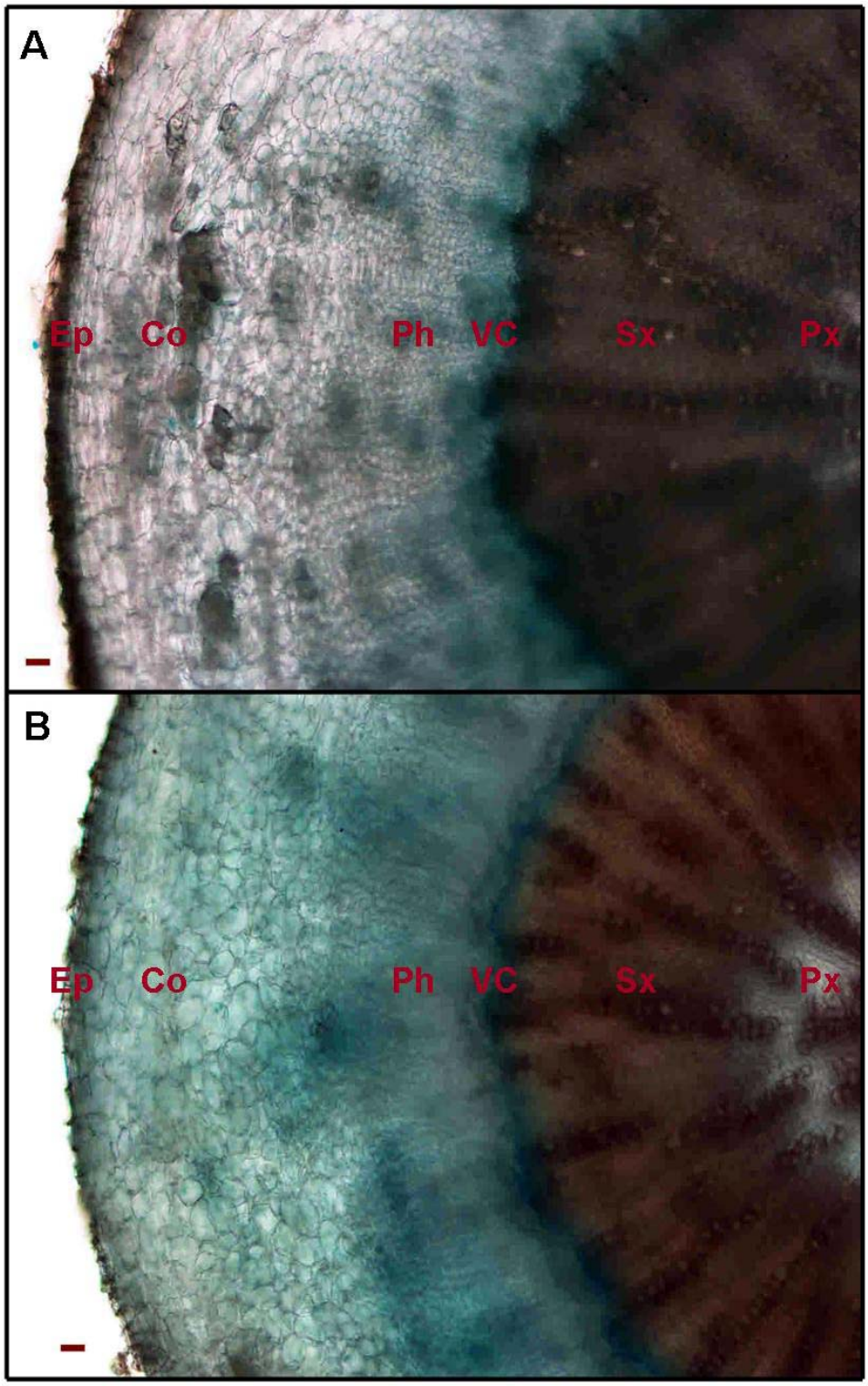

Figure 29. Expression of *GAUT1* and *GAUT7* promoter constructs in roots. A shows *GAUT1*:GUS expression in root. B shows *GAUT7*:GUS expression in root. Scale bar is 200 µm. Px, primary xylem; Sx, secondary xylem; VC, vascular cambium; Ph, phloem; Co, cortex; Ep, epidermis.

CHAPTER FOUR
DISCUSSION

Interpreting the Results

GUS reporter gene constructs have given new insight into the potential physiological relevance of *GAUT1* and *GAUT7*. While previous RT-PCR studies only revealed expression data in major tissues, the GUS reporter system has been used to reveal expression patterns with more detail. Different methods of detecting gene expression each have their own limitations and qualifications. As mentioned, RT-PCR studies are limited to whole organs, while microarray data is limited because it is a compilation of data from many experiments performed in different laboratories and therefore under different conditions. It is important to verify gene expression using multiple approaches and interpret the results with caution.

One of the disadvantages of the GUS reporter gene system is its dependency on the characteristics of the reporter substrate (the substrate penetrability) and reporter product (the product stability and diffusibility). The relationship between incubation times, staining intensity and expression levels is often ambiguous because GUS staining is dependent on several variable factors. These factors include the promoter strength, the insertion site and copy number of the construct within the genome, and the possibility of additional transcriptional regulatory elements that were not included in the construct. Therefore, GUS expression data cannot be used to make definite conclusions about gene expression levels based on relative intensity differences between two different constructs. Although a significantly longer incubation time is required for the *GAUT7*:GUS construct when compared to the *GAUT1*:GUS construct, it cannot be concluded

that *GAUT7* is expressed at a lower level than *GAUT1* because expression intensities can only be compared within a single construct. However, GUS activity can be used to compare levels of expression within one construct. For example, the *GAUT7*:GUS expression is more intense in pollen grains than in the root tips, so one can conclude that it is more strongly expressed in pollen grains.

The conclusions of this study are based on comparison of expression patterns and levels of multiple T1 lines from the same construct at different incubation times. Because long incubation times produced coloring at some level, however faint, in all *GAUT1*:GUS and *GAUT7*:GUS tissues, while wild-type and negative controls showed no GUS expression, it seems that expression of both genes occurs throughout the plant in the tissues observed.

GUS Expression Compared to RT-PCR and Microarray Data

GAUT1:GUS and *GAUT7*:GUS expression is consistent with previous RT-PCR results (K. Caffall, unpublished data). *GAUT1* and *GAUT7* expression was found in the roots, stems, and leaves even if expression was very low. However, GUS expression allowed for identification of specific tissue and cell type expression within the roots, stems, and leaves. For example, a higher intensity of expression was observed in root vascular regions than in other surrounding tissues.

GUS expression varied slightly from the microarray data obtained from Genevestigator. In agreement with the Genevestigator data, *GAUT1*:GUS and *GAUT7*:GUS expression did occur in almost all developmental stages (Fig. 4) and adult tissues (Fig. 5). However, the expression intensity within each construct occured at levels that differed from those reported by Genevestigator. For example, the data from Genevesitgator in Figure 5 shows *GAUT7* to be expressed highly in the root tip in comparison to other tissues, but *GAUT7*:GUS expression

showed faint root staining patterns in the root tip in comparison to other tissues. One possible explanation for this discrepancy may be found in the limitations of microarray studies as previously discussed. Another explanation for the discrepancy could be due to the possibility that the *GAUT7*:GUS construct does not have all of the necessary regulatory elements that serve to enhance expression. Regulatory elements not included within the construct could be sequences that lie within introns in the gene, within the 3' untranslated region, or within areas upstream of the intergenomic region (the intergenomic region for *GAUT7* was 868 basepairs while the intergenomic region for *GAUT1* was 1932 basepairs).

GUS Expression in Regards to Potential *GAUT1* and *GAUT7* Protein Complexes

The spatial and temporal expression patterns of *GAUT1*:GUS and *GAUT7*:GUS are similar in most tissues. Strong expression for both constructs was observed in pollen grains, vascular cambium, phloem, and in vascular regions of the cotyledon, leaves, root, and base of hypocotyls. Medium expression was observed in both constructs in root elongation zones, distal end of stigma, xylem, cortex, pith, and epidermis. Both constructs showed a weak level of expression overall. *GAUT1* and *GAUT7* expression differed notably in two specific tissues: the root tip of the seedling and in the papillae of the stigma. *GAUT1* is highly expressed in the root tip and root cap, while *GAUT7* is expressed much weaker and more prominent in the vascular region of the root tip. *GAUT1* is highly expressed in papillae while there was no clear GUS activity observed in the papillae of *GAUT7*:GUS transformants.

These results suggest that *GAUT1* and *GAUT7* may not always work together in a complex, but may exist in a complex in most tissues. However, the floral differences may be accounted for in that the flowers observed appear to be in slightly different developmental stages (the stigma in the *GAUT1* flower is slightly longer than the stigma in the *GAUT7* flower in Fig.

27). Further work in understanding floral developmental stages might reveal subtle changes in expression as the flower develops into the seed pod. This work was not performed due to time limitations, but future research in this area might provide more insight into these apparent differences. Additionally, the root expression differences may be explained by the possibility that the *GAUT7* promoter region in the construct was not sufficient enough to drive expression in all tissues.

Implications and Future Research

High levels of *GAUT1*:GUS and *GAUT7*:GUS expression in young tissues, areas of new growth, and in meristematic regions demonstrate the importance of these genes in plant growth and development. Combined with Genevestigator data showing that these genes are most highly expressed during bolting (Fig. 4), this study provides supporting evidence that *GAUT1* and *GAUT7* play an important role in deposition of the primary cell wall. Since homogalacturonan is most abundant in the primary cell wall, it was expected that *GAUT1* would be involved predominantly in primary cell wall synthesis, not in secondary wall synthesis. However, high levels of *GAUT1*:GUS and *GAUT7*:GUS expression in the vasculature and in tissues not thought to be synthesizing primary cell wall suggests that these genes also play a role in the secondary growth of the plant. A recent study has shown the existence of pectin-like fibrillar cell wall deposits in poplar xylem fibers (Arend et al., 2008) and another study has shown that lateral water flow between adjacent xylem vessels is controlled by the shrinking and swelling of the pectin matrix in pit membranes in response to ions (Zwieniecki et al., 2001). *GAUT1* and *GAUT7*, which show GUS expression in stem xylem, may contribute to the synthesis of the pectin that regulates water flow through xylem, which is most likely not homogalacturonan (Nardini et al., 2007).

The expression of these genes in pollen, stigma, and papillae (only *GAUT1* is expressed in papillae) also suggests a possible role in pollen viability, fertilization, adhesion of the pollen grain to the papillae, or hydration of the pollen grain by the stigma. The Mohnen lab has been unsuccessful in obtaining homozygous *GAUT1* mutants, which also suggests that this gene is necessary for reproduction. Further research targeted at understanding the role of these genes in plant reproduction would certainly aid in understanding these genes.

Research comparing *GAUT1* and *GAUT7* expression patterns to other GAUT family and cell-wall related genes might reveal more information about the biological activities of these genes. For example, *GAUT1* and *GAUT7* show similar expression patterns to a cell wall-localized lipid transfer protein, LTP1. These three genes show GUS co-expression at the hypocotyl base, leaf vasculature, lignified areas of the cotyledon tip, shoot meristems, stipules, guard cells, lateral roots, pollen grains, and stigma (Thoma et al., 1994). Gene co-expression can be used to make inferences about their relationships and activities.

This study has given several clues to the biological function of *GAUT1* and *GAUT7* by suggesting roles in primary and secondary cell wall synthesis and in plant reproduction. These data will be useful in further GAUT expression studies and designing GAUT mutant studies. The similar expression patterns also suggest that *GAUT1* and *GAUT7* may work combinatorially, at least some of the time, or in complex with one another and/or with other proteins. This study provides direction and guidance in designing future studies and its results can be used in combination with other evidence to determine the biological function of these genes in cell wall synthesis.

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