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Tissue specificity of *gus* gene activity in *Agrobacterium*-mediated transformed tobacco plants

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Abstract. The β-glucuronidase (*gus*) gene was isolated from the bacterium *Escherichia coli*. Due to the blue pigment it produces, it has been widely used as a reporter gene in genetically modified organisms serving to study gene expression and tissue specificity. The focus of this study is to use the *gus* gene to monitor the tissue specificity of the promoter CaMV 35S in *Agrobacterium*-mediated genetically modified plants. *Agrobacterium* is a pathogenic bacterium that has the ability to integrate foreign DNA into a plant's DNA. Promoters are DNA sequences that drive gene expression and determine where a gene is expressed. If a promoter of unknown specificity is used to express *gus* in an organism, then β-glucuronidase will only be present in the tissues the promoter specifies. In 1988 Benfey et al. used the *gus* gene to elucidate on the specificity of the CaMV 35S promoter in *Agrobacterium*-meditated transformed tobacco plants[1]. CaMV 35S originates from the cauliflower mosaic virus and is widely used in the laboratory to drive strong expression of any gene of interest. Benfey et al. was successful in demonstrating the specificity of CaMV 35S at 6, 10, and 15 days of development and at 7 weeks. The results of their study showed that CaMV 35S activates *gus* expression in all cells during 6, 10 and 15 days in development[1]. In 7 week old plants CaMV 35S activates *gus* in all cells of the leaves and roots, but only in the vascular tissue of the stem[1]. The goal of this study was to use the *gus* gene to elucidate on the tissue specificity of CaMV 35S in *Agrobacterium*-meditated transformed tobacco plants at 4 weeks in development. It is expected that the 4 week tobacco plants will show no specificity as they are still categorized as seedlings and are closer in physical traits to 15 day old plants.

Introduction

The Biotechnological industry plays a big role in agriculture. It is a small fraction of farmers that own naturally fertile lands and many that have to deal with pests invading their crops. The biotechnological industry has been able to make the inconveniences of working on the land easier to deal with by genetically modifying plants to be hardier in difficult climates and more resistant to pesticides. These modifications are possible by using naturally occurring genes in bacteria and viruses that serve as an advantage under such conditions.

The β-glucuronidase (*gus*) gene was isolated in 1986 from *Escherichia coli*[5] and since then has been widely used as a reporter gene in genetically modified plants, serving to study gene expression and tissue specificity of different promoter sequences. *Gus* codes for β-glucuronidase (GUS), an enzyme that catalyzes the hydrolysis of β-D-glucuronides[3].When GUS reacts with 5-bromo-4-chloro-3-indolyl-β-glucuronide (x-gluc reagent) it cleaves the β-glucuronide and produces 5-bromo-4-chloro-3-indolyl which then oxidizes into a blue insoluble compound. Any tissue (plants, animals or microbes) expressing *gus*, and exposed to x-gluc, will appear blue to the naked eye.

Promoters are DNA sequences that drive gene expression and determine where a gene is expressed. If a promoter of unknown specificity is used to express *gus* in an organism, then β-glucuronidase will only be present in the tissues the promoter specifies. In 2007, Inaba et al. used *gus* to study the specificity of the ASA2 promoter in soybeans[4]. In 1988 Benfey et al. used the *gus* gene to elucidate on the specificity of the CaMV

35S promoter in *Agrobacterium*-meditated transformed tobacco plants[1]. CaMV 35S originates from the cauliflower mosaic virus and is widely used in the laboratory to drive strong expression of any genes of interest. Benfey et al. was successful in demonstrating the specificity of CaMV 35S at 6, 10, and 15 days of development and at 7 weeks. The goal of this study is to use the *gus* gene to elucidate on the tissue specificity of CaMV 35S in *Agrobacterium*-meditated transformed tobacco plants at 4 weeks in development.

Literature Review

It is common in the biotechnological industry to use *Agrobacterium* as a vector for genetic modification of plants as it is a reliable and cost effective method. Most pesticide resistant plants have been modified using this bacterium. *Agrobacterium* is a soil pathogenic bacterium that has the ability to integrate a part of its plasmid genome into a plant's nuclear genome and cause the plant to produce crown galls (tumors) to nurture the bacteria. In the wild it can only attack dicotyledonous plants[11]. In the lab the modified *Agrobacterium* strains are commonly used for the genetic modification of plants by substituting the oncogenic genes for any genes of interest.

The substitution takes place between a 25 bp long direct repeat in the tumor-inducing (Ti)-plasmid, designated the right and left boarders. This region of the plasmid contains the only genes that are transferred into the plant and is therefore named the Transfer (T)-DNA (Fig.1). When the T-DNA is replicated and ready for transport, it is referred to as the Transfer (T)-Strand. The Ti-plasmid also holds the virulence genes *vir*A, B, C, D, E, G, and H, which code for proteins involved in the copying and transfer of T-DNA[9, 10] (Fig. 1). The chromosome holds the genes chvA and B, pscA, and att, which code for proteins involved in cell recognition and attachment [11].

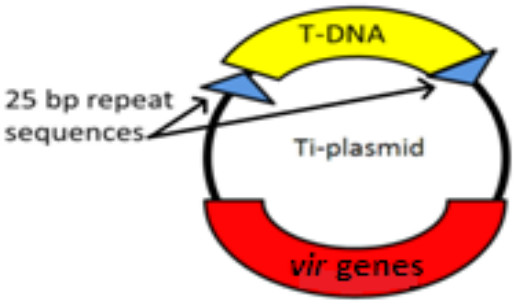


Figure 1 Ti-plasmid. A simplified diagram depicting the general organization of the Ti-plasmid.

An injured plant cell secretes phenolic compounds which attract the bacteria, induce the production of cellulose filaments, and initiate the *vir* signaling pathway. The cellulose filaments anchor the bacterial cell to the plant cell wall and the virulence proteins initiate the transcription of the T-strand. The T-strand is then transported to the cell nucleus and integrated into the plant genome (Fig. 2).

The integration is non-specific and the T-Strand is replicated by the host cell DNA polymerase. Any tissue specificity of *gus* can be attributed to the promoter, CaMV 35S, and not the process of integration itself. The results of the study done by Benfey et al. showed that CaMV 35S activates *gus* expression in all cells during 6, 10 and 15 days in development[1]. In 7 week old plants CaMV 35S activates *gus* in all cells of the leaves and roots but only in the vascular tissue of the stem[1]. It is expected that the 4 week tobacco plants will show no specificity as they are still categorized as seedlings and are closer in physical traits to 15 day old plants.

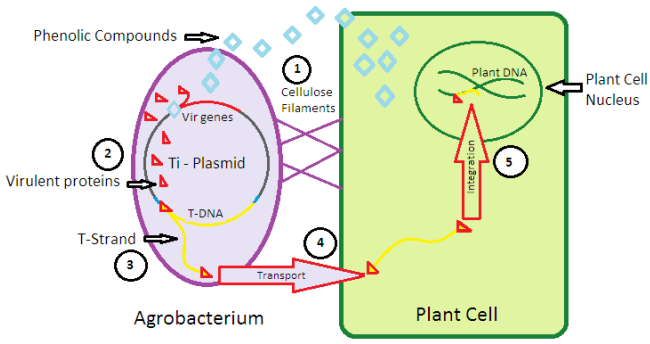


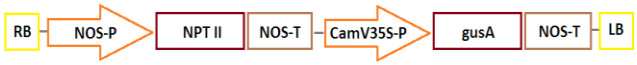
Figure 2 DNA transfer from *Agrobacterium* to plant cell nucleus. (1) *Agrobacterium* reacts to plant phenolics by attaching to the plant cell wall with cellulose filaments and initiating the *vir* signaling pathway. (2) Transcription of the *vir* proteins occurs. (3) *vir* proteins transcribe the T-DNA. (4) *vir* proteins transport the T-strand into the plant cell. (5) *vir* proteins integrate the T-strand into the plant genome.

Methods and Materials

The bacterium chosen for the study carried the pBI121 plasmid. Transgenic plants were created by cutting pieces of leaves from a wild type tobacco plant and incubating them in a broth containing the bacterium. After transformation they are placed in regeneration media for cloning. The seeds of the cloned transgenic tobacco plants are then harvested and germinated. Some of these seeds will be composed entirely of modified cells and are termed homozygous modified. Only plants germinated from these seeds were used for the study of CaMV 35S specificity.

Agrobacterium Species and Plasmid Material

The *Agrobacterium* species used was the *A. tumefaciens.* The Ti-plasmid present in this line of *A. tumefaciens* is the pBI121. We chose this plasmid because NPT II and *gus* substitute the oncogenic genes in the T-DNA region. NPT II is the kanamycin resistance gene that will aid in isolating transformed plants. *Gus* is the β-



glucuronidase gene that will demonstrate the specificity of CaMV 35S (Fig. 3).

Figure 3 Structure of the transformation plasmid pBI121. From left to right: T-DNA right border, NOS promoter, NPTII gene coding region confers kanamycin resistance for plant cell selection, NOS terminator, CaMV 35S promoter drives the gene expression for gusA, *gus* gene coding region, NOS terminator, T-DNA left border.

Transformation of Tobacco Leaf Cells

Six sterile 50mL tubes were filled with 50mL of the pBI121-containing *A. tumefaciens* culture and centrifuged at 3,000 rpm for 15 minutes at room temperature. The supernatant was poured off and the pellet of cells was re-suspended with 10mL of co-cultivation solution containing 200-400μM of acetosyringone (a phenolic compound that aids in *vir* gene activation) and 0.05% Pluronic F68 (a surfactant that prevents cell breakage and foaming).

A leaf from six wild type tobacco plants was cut into 5 × 5mm disks and placed into a tube, each for 40 minutes. The tubes were inverted at 5 minute intervals to ensure the disks came into contact with the bacteria. The leaf disks were then removed and blotted dry on sterile napkins. Once dry, they were placed on filter paper abaxial up in a petri dish (small plastic container) containing co-cultivation media, acetosyringone, and Pluronic F68 for three days in the dark at 26°C[12]. The co-cultivation media allows for the bacteria to stay nourished so they can complete the infection of the leaf disks. During this time *A. tumefaciens* is producing T-DNA, transferring it into the host cell, and integrating it into the plant genome (Fig. 2). Each transformed tobacco leaf represented a different plant line and it was named after the person who performed the transformation.

Regeneration of Wild Type and Modified Tobacco Leaves

After three days transformed leaf disks were transferred into regeneration media which is composed of glucose, salts, minerals, and growth hormones, along with kanamycin and timentin. Hormones in the media induce cell division leading to the growth of new plants. The kanamycin antibiotic allows for isolation of transformed plants containing the NPTII gene which confers kanamycin resistance. Timentin is another antibiotic that kills any remaining *Agrobacterium* but will not harm the tobacco plant itself. They were left in this media for three weeks, at 26°C, 16hrs in light, and 8hrs in dark [12].

One leaf from a wild type tobacco plant was cut into approximately 5 × 5mm and placed into the same regeneration media as above, except for the antibiotic kanamycin. These leaf disks were left in this media for three weeks, at 26°C, 16hrs in light, and 8hrs in dark[12] and served as a control for the experiment. After three weeks shoots with visible meristems from both the experimental and control group were cut and placed in new media for further development (Fig. 4a).

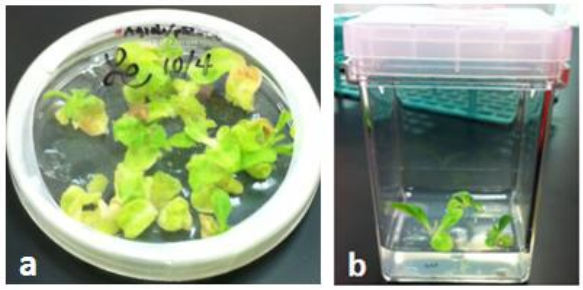


Figure 4 Regeneration of tobacco plants from leaf disks. (a) Tobacco plant shoots in regeneration media after three weeks. (b) Transgenic tobacco plants transferred into rooting media after 5-6 weeks of *Agrobacterium* transformation.

Identification of Transformed Tobacco Plants

Once the shoots developed leaves they were placed in rooting media. This media was the same as the regeneration media minus the hormones. Rooting media allows for further isolation of transformed plants as those without the NPTII gene will not develop roots. Plantlets that developed roots were transferred into a Magenta culture box and labeled. A culture box is a container that allows plants more space to grow while keeping the same conditions as the petri dish (Fig. 4b).

DNA Analysis

The DNeasy Plant Mini Kit (Qiagen) was used to isolate the DNA from the *transgenic* plants and the control plant. For the manual disruption of plant tissue, half a leaf from each transgenic plant was cut and ground manually in a centrifuge tube with the extraction buffer following the kit protocol. The same was done for the wild type plant. The wild type DNA would serve as a negative control.

Once the DNA was isolated, the *gus* gene sequence was amplified by polymerase chain reaction (PCR) (Table 1). PCR exponentially duplicates a specified DNA sequence if present in your DNA extraction. This allows for the presence of *gus* to be confirmed by gel electrophoresis. 1µl of DNA from each sample was used. 19µl of PCR master mix was added to each tube (Table 2). The DNA of a previously confirmed transgenic plant served as the positive control.

A gel electrophoresis was done on the PCR product of each sample. This method uses the electrical charge of DNA to separate DNA strands according to their size. The size of *gus* is known and can be identified on the gel. 1.5µl of loading dye was used to stain each sample. 10µl of each sample was then loaded into separate wells of a 1.5% agarose gel containing ethidium bromide (ETBr). The ETBr allows us to see the DNA bands under UV light. The gel was run at 70 volts for 30 minutes.

Acclimatization of Transgenic Plants

Once all tests were done, the confirmed transgenic plants were removed from their food source and transferred to potted soil. From this point on the plants will have to undergo photosynthesis to obtain energy. A moist environment, similar to the magenta box in the growing chamber, must be provided for the plants to become acclimated to growth[8].

Table 1 PCR Conditions

Temperature	**Time**
95˚C	2 minutes
95˚C	20 seconds
52˚C	20 seconds
72˚C	40 seconds
Cycles	30

Six 6-inch diameter pots were filled with top soil and generously watered with tap water. A hole was dug into the dirt with forceps about three inches deep. The plants were then gently removed from the rooting media, making sure that the roots were not injured. This was done by using forceps to loosen the agar from the roots and then pulling the plants out. After removal, the agar was carefully washed off the roots with tap water.

The plants were then transferred to the pot and watered generously once more (Fig. 5). The pots were placed in a plastic re-sealable bag filled with about two cups of tap water. The bags were closed and placed in a growing chamber at 26˚C with 16 hrs of light daily for four days. The bags were then opened and left under the same conditions for another four days. After the eight days of acclimatization, the pots were removed from the bags.

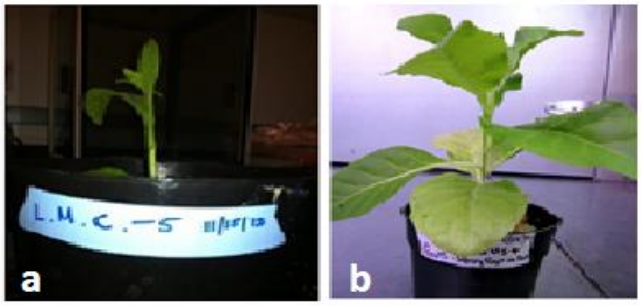


Figure 5 Two representative transgenic plants grown in soil. (a) An example of a six week old plant. (b) An example of a three month old plant

Table 2 Master Reaction Mix Concentrations

Components	**Volume**	**Final Concentration**
dH_2O	246 µL	-
10X Buffer	32 µL	-
dNTPs	6.4 µL	0.2 µL
gus Primer 1	6.4 µL	0.2 µL
gus Primer 2	6.4 µL	0.2 µL
Thermal Stable DNA Polymerase	7.5 µL	0.5 µL
DNA from wild type or transgenic plants	1 µL	36 ~ 50 ng DNA

Seed Collection and Germination

Seeds collected from the flowering plants were surface-sterilized by wrapping them in a pre-labeled cheese cloth and sealing them in with a paper clip. Each bundle of seeds was dropped into a 10% bleach solution and stirred for 20 minutes. The seeds were then removed and washed with sterile water four times. Then the seeds were unwrapped and planted into a petri dish containing rooting media with kanamycin.

The seeds were germinated for four weeks in the culture chamber at 26ºC with 16 hrs of light. The new seedlings are the next generation of the modified plant lines (T1), some of which are homozygous transformed plants. Once the seedlings sprouted they were scored for ratio of transformation by comparing the number of seedlings sensitive to kanamycin versus the total number.

When plants are initially modified with pBI121, only one of the two homologous chromosomes (one from mother and the other from father) has the T-DNA insertion. When the self-fertilizing parent produces the T1 seeds, the T-DNA will be segregated according to Mendel's laws of inheritance. This is why the seedlings (T1) are scored for kanamycin sensitivity instead of the parent generation. Furthermore, since NPTII is tightly linked to *gus* (Fig. 3), these two genes are known to segregate together.

Most nuclear genes follow classical Mendelian genetics. The promoter of the NPTII gene in pBI121 is man-made and is known to have strong expression in every tissue; therefore, NPTII is determined to have dominant expression. Scoring the seedlings for kanamycin resistance is a way to determine how our genes of interest have segregated during reproduction.

GUS Assay

The GUS assay was used to determine the specificity of the CaMV 35S promoter. As described earlier, this promoter is what drives the expression of *gus* in the T-DNA inserted into the plants genome. The GUS enzyme will be present wherever the gene is expressed. Plants incubated in the x-gluc solution turn blue wherever *gus* is being expressed due to enzymatic activity. Being able to see the expression of *gus* with the naked eye provides a convenient and accurate way of detecting any CaMV 35S specificity.

After four weeks one resistant seedling from each plant line was tested for the specificity of CaMV35S using the GUS assay. 150µL of x-gluc solution was added to six tubes and each seedling was incubated overnight at 37°C[2, 5, 6]. The samples were then rinsed with 70% ethanol several times to remove the chlorophyll in order to facilitate the observation of GUS stained cells. Leaf, stem, and root tissue was then observed under a microscope to identify any staining present in any cell. Navy blue stained areas represent the expression of GUS (Fig. 6).

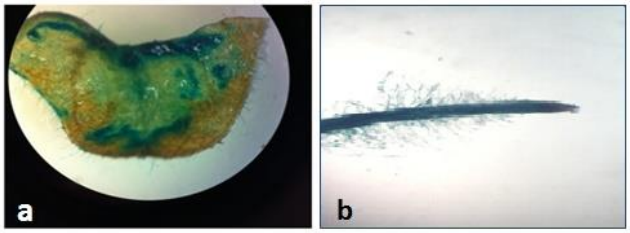


Figure 6 GUS assay. (a) GUS positive leaf sample that stained blue in x-gluc solution. (b) GUS positive root sample that stained blue in x-gluc solution. The leaf sample was ethanol bleached for better visibility of stain.

Results

Insertion of pBI121in Regenerated Tobacco Plants

After four weeks in rooting media eight modified plants formed roots. Only six were confirmed to contain the pBI121 T-DNA by PCR amplification of *gus*. The PCR fragment of *gus* gene is 525 base pairs (bp) long. The gel clearly showed that the negative control (WT) did not show any band for the *gus* gene, whereas the positive control (AP) showed a bright band near the 500 bp mark (Fig. 7). The results of the negative and positive controls allow the certainty that the procedure was carried out correctly.

Plant lines KEG, LMC-5, JMR, LC-1, HD-1, and HD all contained the *gus* gene while plant lines JC and AGZ did not. Only plant lines that were confirmed to be *gus* positive by the gel were chosen for study of CaMV 35S specificity.

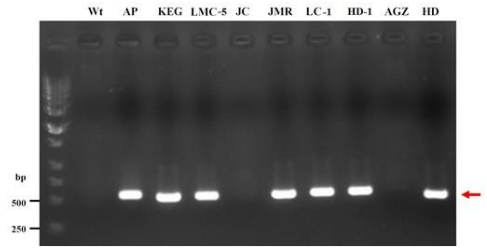


Figure 7 The gel electrophoresis done on the TAIL-PCR amplification of *gus*. Gus band is visible near the 500bp. mark. The amplified *gus* DNA is 525bp long. Six out of eight plant lines were confirmed to carry the *gus* gene.

Scoring of T1 Generation

The average phenotypic ratio of the plant lines for kanamycin sensitivity in this study was calculated to be 0.32±0.11 (Table 3), which is close to the classic Mendelian ratio of 1/3 or 0.33. Genetic inheritance may vary from the standard inheritance pattern based on the type of gene. This result allowed us to analyze the expression of *gus* based on classic Mendelian laws of inheritance, which is the simplest type of inheritance to work with.

GUS Assay

Plant lines LC-1, LMC-5, and KEG-2 were the only seedlings to show GUS activity. Plant lines HD-1, HD, and JMR did not show any GUS activity (Table 4). Those plant lines that showed GUS activity only had it present in the leaves and roots (Fig. 6). No GUS activity was seen in the stems of any of the plants. These results imply that the CaMV 35S promoter might have some level of specificity in four week old seedlings.

Table 3 Scoring of T1 Generation

Plant Line	Sensitive Seedlings	Resistant seedlings	Ratio
LC-1	6.0	33	0.18
LMC-5	1.0	34	0.03
KEG-2	0.0	39	0.00
HD-1	16	38	0.42
HD	8.0	15	0.53
JMR	40	60	0.67
Average	12	37	0.32

Table 4 Levels of GUS Expression

Plant Line	**Roots**	**Stems**	**Leaves**
LC-1	**E**	**N**	**E**
LMC-5	**E**	**N**	**E**
KEG-2	**N**	**N**	**E**
HD-1	**N**	**N**	**N**
HD	**N**	**N**	**N**
JMR	**N**	**N**	**N**

E=Expression, N=No Expression

Discussion

Based on the antibiotic isolation technique, eight plants did undergo insertion of the pBI121 T-DNA by *A. tumefaciens*. Even though plant lines JC and AGZ exhibited significant root elongation, PCR results did not confirm the presence of *gus* in their genome. This may be attributed to incomplete T-DNA integration[11]. If only the antibiotic resistance gene was incorporated into the genome, then plants would be able to survive in the selection media without carrying *gus*. However, further study is needed to confirm this hypothesis

Plant lines HD-1, HD, and JMR, although DNA analysis confirmed the presence of *gus* in their genome, did not express the gene product. It is important to understand that because the insertion site of the T-DNA is random, its location of insertion is unknown[10, 11]. There is a chance that *gus* gene activity might be inhibited simply because of where it integrated in the plant nuclear genome[1]. The lack of expression in plant lines HD-1, HD, and JMR may be the result of a type of gene silencing due to its location in the genome. Identification of the insertion sites of the pBI121 T-DNA should be studied[7]. Only three out of the six confirmed *gus* modified plant lines (LC-1, LMC-5, KEG-2) expressed the gene product. They showed enzyme activity in the epidermal cells of leaves and roots only.

Even though there was some variance between plant lines, our end results indicated that the specificity of *gus* in four week old plants is closer in comparison to the seven week old plants than to the 15 day old seedlings. We did not observe any expression of GUS in the epidermal cells of the stem. The lack of GUS in the epidermal cells of the stems in 4 week old plants suggests that 4 week old plants are closer in development to the 7 week old plants than the 15 day old plants. The vascular tissue of the stem was not observed and further study should be done to confirm the presence or lack of GUS in the vascular tissue of the stem. This study has served to elucidate on the growth stages of tobacco plants as well as CaMV 35S activity in those stages.

Further studies on different stages should also be performed on these plant lines in order to confirm the current data. Confirming any specificity to CaMV 35S and identification of insertion location will contribute to the understanding of CaMV 35S and *Agrobacterium* virulent mechanisms. The application of this knowledge can

serve to enhance the efficiency and accuracy in modifying plants for the agricultural industry.

Acknowledgements

This paper describes the major results of the experiments carried out by undergraduate students who took Dr. Xing-Hai Zhang's "Plant Biotechnology" course (BOT 4734C) in the fall semester of 2013. Although not all of the classmates were able to co-author this paper, we thank them for their participation and discussion. Laura Cruz further did a directed independent study in Dr. Zhang's lab to finish up this project in spring 2014, and is grateful for the support of the FAU's undergraduate research award.

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