Expression Properties of Two Promoters During Callus and Shoot Development in Transgenic Poplar

by
Anna Brousseau

A THESIS

submitted to

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Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

Presented May 23, 2022
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AN ABSTRACT OF THE THESIS OF

Anna Brousseau for the degree of Honors Baccalaureate of Science in Bioengineering presented on May 23, 2022. Title: Examination of Promoter Activity for Timed Excision of CRISPR-Cas9 Machinery in Poplar Trees.

Abstract approved:_____

Steven Strauss

The *Arabidopsis thaliana* developmental promoters of *WUSCHEL* (*WUS*) and *COLD SHOCK DOMAIN PROTEIN 3* (*CSP3*) were tested for their suitability as potential control elements for the Cre-Lox recombination system in poplar cells. To remove the integrated transgene after CRISPR machinery has modified the genome, timely control of Cre is required. Both promoters are expressed in *A. thaliana* meristematic tissue, a crucial location of cell division, and we tested if the same would hold true when in transgenic poplar tissue. Both promoters were cloned upstream of a green fluorescent protein (GFP) encoding gene to track promoter activity. Transformed poplar plantlets were grown *in vitro*, then imaged and scored weekly to record GFP brightness level and tissue types. *CSP3* was found to be the more reliable and consistent promoter due to higher activity during early shoot stages, and more consistency between genetic insertion events. However, neither *WUS* or *CSP3* showed meristem-dominant expression as desired; both showed strong expression in callus tissue well before meristem differentiation, and also showed high variation in expression patterns and intensity between gene insertion events. Thus, additional layers of expression control, or other means of controlling Cre activity, are needed to meet system design goals.

Keywords: CRISPR, promoter, recombinase, gene editing, *WUSCHEL, COLD SHOCK DOMAIN PROTEIN 3,* Cre-Lox, transgene, poplar, biotechnology

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APPROVED:

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Anna Brousseau, Author

Table of Contents

Literature Review

Biotechnology in Clonally Propagated Plants

Clonally propagated crops encompass a diverse range of the plants grown today. Among the most common crops are strawberry, apple, grape, mint, potato, hardwoods, and sugar cane ("Clonal Propagated Crops : USDA ARS," 2020). Clonally propagated plants belong to 34 different families and serve to provide fruit, nuts, herbs, lumber, and shrubbery (McKey et al., 2010). Biotechnology methods such as micropropagation continue to expand the amount of clonally propagated plants used in research and crop production. As discussed in more depth below, other biotechnology methods include grafting, rooting, genomic analysis, genetic engineering, and gene transfer. In general, clonal and woody crops are more difficult to genetically modify than are seed propagated crops, though there is great variation among species and genotypes in both types.

A challenge associated with clonal crops is that they often have reduced maximum rates of biomass growth in comparison to seed crops. Additionally, due to their propagation, clonal crops often have a root system containing mostly lateral roots, compared to seeds that produce a true tap root. Tap roots enhance stability and nutrient uptake from deep soil layers (Albrecht et al., 2017). Many forest trees, including the poplars that I studied in my research, are clonally propagated during production or breeding.

Poplars

The genus *Populus* is widespread in the Northern Hemisphere and comprises 35 species including *Populus tremuloides* (aspens), *Populus aigeiros* (cottonwoods), and *Populus alba* (white poplars) ("Populus - New World Encyclopedia," n.d.). Poplars are dioecious plants and can reproduce asexually through cultivation of stem or root cuttings. Due to the lightweight and fast-growing wood, poplars are desirable to humans for their various resources. Poplars are most used to produce pulp, paper, wood veneer, lumber, and energy (Bradshaw et al., 2000). Poplars can also be planted to limit erosion and sequester excess soil carbon (Hansen, 1993). Additionally, *Populus* is the most commonly used forest tree genus in genetic modification studies and is often considered the model tree for tree biotechnology due to its ease of

transformation and regeneration, rapid propagation by *in vitro* culture, and abundant genomic resources including a high quality reference genome (Marchadier and Sigaud, 2004).

Biotechnology

Biotechnology is defined in a wide variety of ways by scientists and practitioners. For the purposes of this thesis, I define biotechnology as the diverse set of practices that modify organism physiology or genetics for human benefit ("Agricultural Biotechnology Glossary," n.d.). Transformation and genetic engineering (GE) are examples of common biotechnology. Common desired traits that are altered by GE in trees, and specifically in poplars, consist of wood quality, flowering onset and sterility, herbicide resistance, and pest resistance. Altering wood characteristics in poplar trees requires targeting the process of lignin or carbohydrate biosynthesis. Modification of lignin concentration could help paper pulping properties and forage digestibility by reducing the energy or chemicals needed to remove lignin from cellulose used for paper and biofuel production (Tilston et al., 2004). The ability to genetically regulate flowering time can reduce the extensive juvenile stage of poplars (Yuceer et al., 2011), delay flowering to limit the spread of transgenes (Goralogia et al., 2021a), or cause the production of non-functional flowers (Elorriaga et al., 2021). Weeds are also a major threat to crops, but with the modification of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, plants have been able to resist commonly used broad-spectrum herbicides such as glyphosate (Zhao et al., 2015). Other traits that have been modified include drought, pest, salinity, cold, and disease resistance (Strauss et al., 2001).

Along with genetic modification comes concerns regarding the risks involved, whether it be by conventional or recombinant DNA methods. Reservations about recombinant methods include the quasi-random nature of gene insertion, to be discussed in detail below. Additionally, there has been apprehension over the effects of modifying species on ecosystem function and services, such as biodiversity. In altering the traits of as critical a component of an ecosystem as for a dominant forest tree species, other species that rely on that component could suffer or be required to adapt (Halpin et al., 2007). However, current regulations are slowly shifting to be less focused on the process of genetic modification, and more on the final products (Strauss and Sax, 2016). This should enable public sector scientists and small companies more room for

innovation, particularly with efficient methods for modifying native genes like CRISPR.

The CRISPR Gene Editing System

Gene editing, where specific changes can be induced in genomes at high frequency, is considered one of the major scientific breakthroughs of the last decade, and CRISPR is the most popular and efficient form (Phelan, 2015). CRISPR is based on a natural bacterial defense mechanism. It is now routinely used to modify genomes of nearly all organisms; over 200 species have been modified by CRISPR including fungi, insects, plants, and animals (Reardon, 2019). CRISPR is an acronym for Clustered Regularly Interspaced Short Palindromic Repeats. The system uses a type II Cas protein, naturally found in bacteria, that employs only three Cas components (Mir et al., 2018). In combination with the Cas9 protein, the embedded RNA molecule (guide RNA) can cut DNA sequences at specifically targeted locations. Through creating cuts in the DNA and letting natural repair systems take over, CRISPR-Cas9 can create genetic mutations and alter DNA sequences in desirable ways (Zhang et al., 2021).

Agrobacterium, a natural plant pathogen, is the most common method for delivering the genes that encode the CRISPR machinery through the cell wall and membrane. T-DNAs carry the CRISPR sequences. They are encoded by *Agrobacterium* as single stranded molecules that get more-or-less randomly integrated into the plant genome (Gelvin, 2000). The T-DNA is engineered by scientists to contain all the necessary CRISPR editing components including the: Cas9 nuclease, guide RNA/s, antibiotic resistance marker, and optionally a fluorescent protein marker to aid in identification of transgenic cells.

Once a mutation is stably introduced into the genome by CRISPR-Cas9, it is no longer needed. The elimination of CRISPR-Cas9 machinery from crops would render it non-GMO, this potentially qualifying for an exemption or reduced regulatory scrutiny when intended for commercial use ("USDA APHIS | About the SECURE Rule," 2021).

Transgenes

Transgenic plants are those that have foreign pieces of DNA integrated into their genomes. In the case of *Agrobacterium* transformation, the transgene is what is encoded on the T-DNA. The process of T-DNA integration is not well known, and it is hard to control where and how many times the transgene will be inserted into the genome (Gelvin, 2003). Therefore,

transgenic plants often express variability and instability. However, transgenic plants are often selected for stable transformation and proper plant development and propagated to only contain single and fully integrated transgenes (Strauss et al., 2001).

Transgenes can still be an undesirable result of the genetic modification process due to their regulatory scope. Previously, the use of *Agrobacterium* for DNA delivery caused transgeneitc plants to be classified as a potential plant pest. Under the 2020 SECURE Rule, DNA source is not a trigger, but recombinant methods are (Entine et al., 2021). In unique cases, transgenes can even spread their edited traits to offspring, bypassing the natural Mendelian inheritance, and possibly create unintentional gene drives (Gantz and Bier, 2016). Having the ability to perform transgene-free, or "clean" edits that do not contain the integrated transgene and its components would help avoid these concerns.

Transgene Excision Methods

Because transgenes in crops of commercial interest are usually single-copy and heterozygous, they can be eliminated by self-segregation during sexual reproduction. This is the most common means to remove CRISPR machinery from crops after edits are made. However, because this is not available (or extremely slow and inefficient) in long-lived and heterozygous vegetatively propagated plants such as poplars, other methods must be explored. In order to create "clean" edit varieties that lack the CRISPR machinery, site-specific recombinases are often employed. Site-specific recombinases are commonly used enzymatic tools in molecular biology that catalyze strand-switching reactions between two recombinase binding sites on a DNA sequence. Depending on the associated recombinase binding sites, the enzymes can excise, integrate, or invert specific genetic sequences (Cody et al., 2020).

Cre is the most widely used recombinase capable of removing large pieces of DNA, and thus potentially up to several undesired transgenes. Cre is a tyrosine integrase (as opposed to a serine recombinase), derived from the P1 bacteriophage. Its recombinase activity is directed at Locus of Crossover in P1 (LoxP) recognition sites at flanking regions of the transgene. Through a looping mechanism Cre is able to attach to both LoxP sites to break and rejoin single strands to form a Holliday junction intermediate (Grindley et al., 2006). This double stranded break of the DNA at both locations allows for the removal of the transgene when the LoxP sites are in direct orientation, and inversion when they are in opposite orientations (Goralogia et al., 2021b). When

using the system of Cre recombinase and LoxP sites for the removal of CRISPR after gene editing occurs, the timing of transgene removal is crucial.

Methods of Gene Expression Control

Three common controls of protein expression include regulatory protein induction, mRNA stability, and mRNA recruitment. Gene expression is primarily controlled at the transcriptional level which can be induced or repressed depending on the regulatory proteins being expressed. Additionally, the rate of mRNA turnover is appropriately adjusted to determine the rate of mRNA synthesis and thus the level of gene expression (Wada and Becskei, 2017). Protein expression can be controlled by translational efficiency which governs the rate of mRNAs recruitment into the ribosome and adjusts the expression of genes that respond to internal and external signals. Changing the level of rate-limiting protein factors that participate in the process of translation allows for control over the process of translation (Hershey et al., 2012). For biotechnology applications, transcriptional regulation such as through the use of well-characterized promoters is commonly used.

Promoters as Tools for Directing Transgene Expression

The term promoter is defined as both the region typically found two kilobase pairs upstream of the transcription start site, and the specific sequence region that directly initiates transcription (Schmitz et al., 2022). For the purpose of this thesis, the former definition of promoters as sequences of DNA, commonly placed upstream of genetic transcription sites will be used. Promoters have the ability to control gene expression, including which cells display expression and when expression occurs (Margulies, n.d.). Many *cis* elements present within promoters and contribute to controlling gene expression within the plant genome. *Cis* regulatory elements are nucleotide fragments of non-coding DNA that are commonly located upstream of the gene coding sequences, and include elements such as: enhancers, silencers, and insulators. Enhancers and silencers are often located further away from the transcription start site (Biłas et al., 2016). Two main promoter types are inducible promoters and developmental promoters. Inducible promoters are expressed only when exposed to specific chemicals, or stress, such as cold shock or heat exposure. Developmental promoters are active during specific growth stages, and in specific cells. Any of these types of promoters can also be transcriptionally fused to

control the Cre recombinase, thus triggering Cre activity and transgene removal during the ideal growth period.

Recombinase Control

Tight control over the timing of recombinase excision is necessary to ensure that the transgenes are present in the plant cells for the correct amount of time. The transgenes need enough time to be able to make proper genomic edits in all of the transformed cells yet need to be eliminated efficiently as possible afterwards to minimize chimerism of the resulting plants. In order to limit premature excision, but still allow for transgene removal, developmental promoters can be used to express Cre recombinase activity (Goralogia et al., 2021b). By placing Cre under control of developmental promoters such as those derived from the *WOX*, *CLV3*, or *WUS* genes, Cre expression should be directly associated with distinct developmental stages and cell types (Van Ex et al., 2009), at least in the majority of transgenic plants. However, because transgene insertion is quasi-random, promoter behavior may be affected by nearly enhancer elements, as well as epigenetic modification during gene insertion. We expect a useful number, but not all gene insertions, to show the desired Cre expression patterns.

Poplar Regeneration and Growth Stages

The process of regenerating a plant from single cells to a whole plant varies based on the organism. In poplar, and many other dicot species, an indirect somatic organogenesis system is often used (Han et al., 1996). This involves the dedifferentiation of cells into an early-stage mass of unorganized plant cells, otherwise known as a callus. Following this stage, the cells redifferentiate into shoots and other more complex tissue types. Plant regeneration in its various stages is primarily controlled by changes in the ratio of auxin to cytokinin in the culture medium (Raemakers et al., 2006).

Meristem-Active Promoters and Reporter System

Meristems are plant tissues that contain undifferentiated stem cells responsible for plant growth. Through cell division, the meristematic cells produce daughter cells that differentiate into specialized cells and create distinct tissues. Continuous growth of a plant creates a flow of cells out of the meristem (Hay and Tsiantis, 2005). Control of meristematic cell function,

including *in vitro*, is primarily guided by auxin and cytokinin hormonal signaling pathways (Stahl and Simon, 2010). Apical meristems are the main site of plant growth; and can be found at the tips of shoots and roots. Apical meristems are responsible for novel organogenesis like creating new leaves and flowers. The organizing center is a small population of stem cells located in the center of the apical meristem that replenishes and arranges the undifferentiated cells (Bao et al., 2009). Lateral meristems can give rise to branches in roots and shoots, and a specialized type called the cambium can give rise to secondary growth and formation of woody tissues (Ikeuchi et al., 2019). In theory, meristematic cells can develop into all other types of cells and are thus known as "totipotent". Thus, promoters active in the meristems should allow for the creation of edited cells and the elimination of transgenes that can be regenerated into plants with the same changes in all of its derived cell types and organs.

WUSCHEL

The gene *WUSCHEL* (*WUS*) (AT2G17950) was isolated from the model plant *Arabidopsis thaliana*. *Arabidopsis* is widely used as a model research plant in the field of biology due to its rapid life cycle, fully sequenced genome, and efficient transformation methods utilizing *Agrobacterium* ("TAIR - About Arabidopsis," n.d.). Since poplars share the majority of their meristem regulatory framework with *Arabidopsis*, research conducted on regulatory genes in *Arabidopsis* can be applied to poplars. Expression of *WUS* is required in order to keep the stem cells in their undifferentiated state. *WUS* is primarily expressed in the organizing center of *Arabidopsis* apical meristems, thus we chose its 5' upstream regulatory region in the hope of abstaining similar expression when fused to Cre or a reported gene like GFP.

In an analysis of the expression of β-glucuronidase (GUS) driven by *WUS* promoter fragments in *Arabidopsis*, strong expression was observed in young floral meristems while weaker expression was observed in the organizing center of inflorescence meristems. Additionally, it was found that the *WUS* promoter contains regulatory regions responsible for the control of both tissue specificity and levels of transcription (Bäurle and Laux, 2005). In another study, one performed in my host labortary over a decade ago, the *WUS* promoter was studied in poplar throughout callus and shoot growth They found that 50-60% of the transgenic events displayed expression in the apical and axillary meristem tissue. However, it was also found that expression was common in other areas such as leaf tissue and vascular pores (Bao et al., 2009).

Based on promoter behavior in *Arabidopsis* and recent studies in poplar, we chose the *WUS* promoter as a potential candidate for limiting Cre expression to newly formed meristems.

Cold Shock Domain Protein 3

The *Arabidopsis* gene *COLD SHOCK DOMAIN PROTEIN 3* (*CSP3*) (AT2G17870) is involved in the acquisition of freezing tolerance in plants and displays tissue dominant expression. The gene is highly expressed in tissues with active growth and cell division, such as the shoot and root apical meristem regions of *Arabidopsis* (Kim et al., 2009). Although *CSP3* is widely expressed in various tissues, elevated expression has been found primarily in meristematic and nearby tissues (Yang and Karlson, 2012). The promoter of the *CSP3* gene was evaluated with an *CSP3* promoter::GUS fusion. In early stages of development (2-10 days) GUS activity was detected in primary roots tips and shoot apices (Kim et al., 2009). The promoter for the gene *CSP3* was selected for testing in the excision system due to its expression throughout the entire meristematic region.

Green Fluorescent Protein

Tracking expression patterns of specific promoters in various plants and tissues can be achieved using visual markers. The most commonly used visual fluorescent marker is green fluorescent protein (GFP). In 1991 the gene for GFP was first cloned from the green, bioluminescent jellyfish *Aequorea victoria*. GFP is a unique light-emitting protein in that it does not require any additional cofactors or substrates to generate its green light (Misteli and Spector, 1997). One main use of GFP is to visualize protein expression, which entails locating the monitored promoter in front of the GFP coding sequence. This transcriptional GFP fusion allows for visualization under a microscope with suitable excitation lights and filters (Lee et al., 2002). With the use of fluorescence microscopy, levels of expression can be monitored much more easily than with methods requiring biochemical detection.

GFP has a tight barrel structure that protects the integrity of the protein, ensuring that its fluorescent properties will not degrade in the presence of larger proteins. With the expression of GFP from a specific promoter, the visualization of activity occurring within the cells can be non-invasively tracked over time. Repeated monitoring of the same living tissue can thus be conducted without protein degradation of GFP (Misteli and Spector, 1997). These features of

GFP allow for an accessible characterization of promoter expression in early stages of plant development; however, once the tissues become more complexly layered, GFP visibility is harder to detect. My research therefore focused on early stages of callus and shoot primordium development.

Overview of Experimental Goal

We tested the *Arabidopsis thaliana* developmental genes of *WUSCHEL* (*AtWUS*) and *COLD SHOCK DOMAIN PROTEIN 3* (*AtCSP3*), specifically examining if these promters would be likely to impart desired expression patterns for inducing the Cre-Lox recombination system transgenic poplar cells shortly after meristem formation. If so, they could be key parts of a system to remove gene editing components such as CRISPR after editing is complet. *AtWUS* has been well studied in stem cells and is restricted to the organizing center (Bao et al., 2009; Bäurle and Laux, 2005), while *AtCSP3* is less well studied but found to be active throughout the meristem, covering a larger population of cells (Kim et al., 2009; Yang and Karlson, 2012). We measured during the process of transgenic callus and shoot differentiation, using GFP to allow promote expression and location to be easily tracked. Our intened outcome was to identify the best promoter that would induce expression strongly during early meristem formation, with little or no expression in callus or mature shoot tissue.

Methods

Construct Generation and Plant Transformation

The DNA sequences for *Arabidopsis WUS* (AT2G17950) and *Arabidopsis CSP3* (AT2G17870) were obtained from TAIR (https://www.arabidopsis.org/) as a source to accurately amplify the promoters. The forward and reverse primers used in the polymerase chain reaction (PCR) for the construction of *WUS::GFP* were 5'-agatcccctgtttctcactgcatgc-3' and 5'-

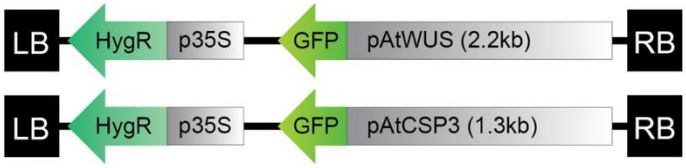

Figure 1: *Arabidopsis WUS* promoter (top) and *Arabidopsis CSP3* promoter (bottom) placed before the coding sequence for the GFP gene, 35S promoter placed before the coding sequence for the hygromycin resistance gene. Constructs not drawn to scale.

gtgtgtttgattcgacttttgttcacaaagtt-3', respectively. The forward and reverse primers used for the construction of *CSP3::GFP* were 5'-tacaatcgcatatcgtgaattccgatgt-3' and 5'-tagatcagagaaaaaggttaacaaaaactcgaa-3', respectively. Both promoters were cloned upstream of the GFP encoded gene (Figure 1) using a T/A cloning binary vector as described by Jiang and colleagues (Jiang et al., 2014).

Hybrid white poplar clone 717-1B4 (female, *Populus tremula* × *P. alba*) from INRA, France was used for all transformations (Han et al., 2000). All plants were transformed using *Agrobacterium tumefaciens* strain LBA4404.

Explant Propagation

Forty- to fifty-day-old *in vitro* grown poplar plantlets served as material for explants to be used in regeneration assays. Ten events of each *AtWUS* and *AtCSP3* were originally chosen as source material for the observed explants. Two events of each promoter were lost during the propagation phases, resulting in eight final events per promoter. From each event, three plates containing 12 explants were propagated for a total of 36 explants per event. Microcuttings (4 mm) of internodal stem and petiole sections were initially cultured on hormonal callus-induction medium (CIM). In addition to gelling agents, the CIM contained 5 μM 2-isopentenyladenine (2iP), 10 μM 1-naphthaleneacetic acid (NAA), half-strength Murashige and Skoog basal medium (MS), and L-glutamine (0.8 g/L). All explants were then moved to shoot-induction medium (SIM). In addition to gelling agents, the SIM contained 1 μM thidiazuron (TDZ), half-strength Murashige and Skoog basal medium (MS), and L-glutamine (0.8 g/L).

Growth Timeline

All explants were cultured on CIM at 25℃ in darkness for three weeks to produce callus tissue. Following this period, explants were grown on SIM at 25℃ in chambers under 40 µmol fluorescent light for three weeks to form shoots. As displayed in Figure 2, a one-week staggered system was utilized. In four stages, four events of explants were placed on CIM per week to allow for timely imaging.

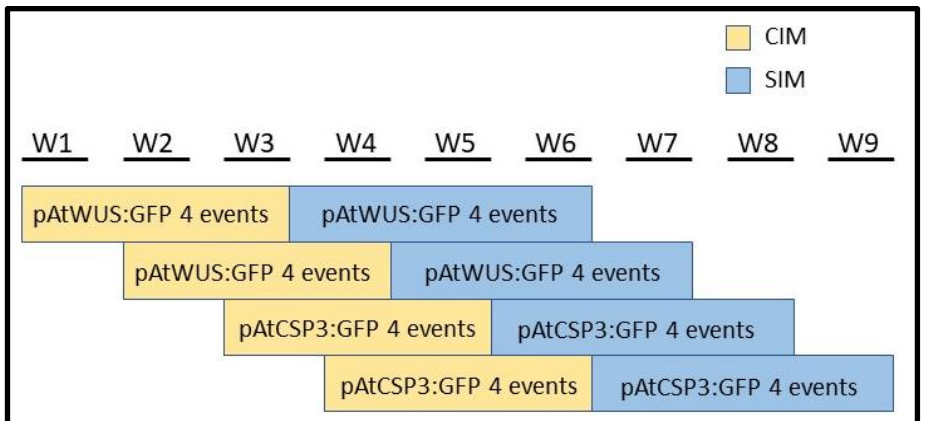

Figure 2: Weekly staggered schedule of eight p*AtWUS*:GFP and eight p*AtCSP3*:GFP events on CIM (three weeks) and SIM (three weeks).

Imaging and Scoring System

Weekly scoring of explants began after two weeks (growth week 3) on CIM. The number of calli, number of shoots, shoot stage, and brightness of the callus/shoot were recorded for each explant when applicable. We considered a single callus one that is not physically connected to any others (Figure 3). A single shoot was also considered one that is not in physical contact with any others.

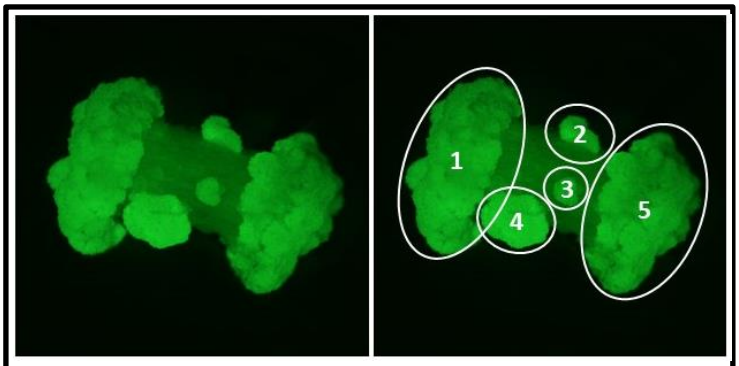

Figure 3: *AtWUS*:GFP event 6 containing five individual calli circled and numbered in the right-hand image. Each callus was scored individually.

As seen in Figure 4a, five different shoot stages were determined. Stage I shoots had initial leaf primordia visible. Stage II represented shoots that contained leaf primordia and the initial meristem bulge. Stage III displayed elongated leaf primordia. Stage IV contained circularly organized leaf primordia around the meristem. Finally, Stage V shoots displayed fuller leaves and more matured meristem centers.

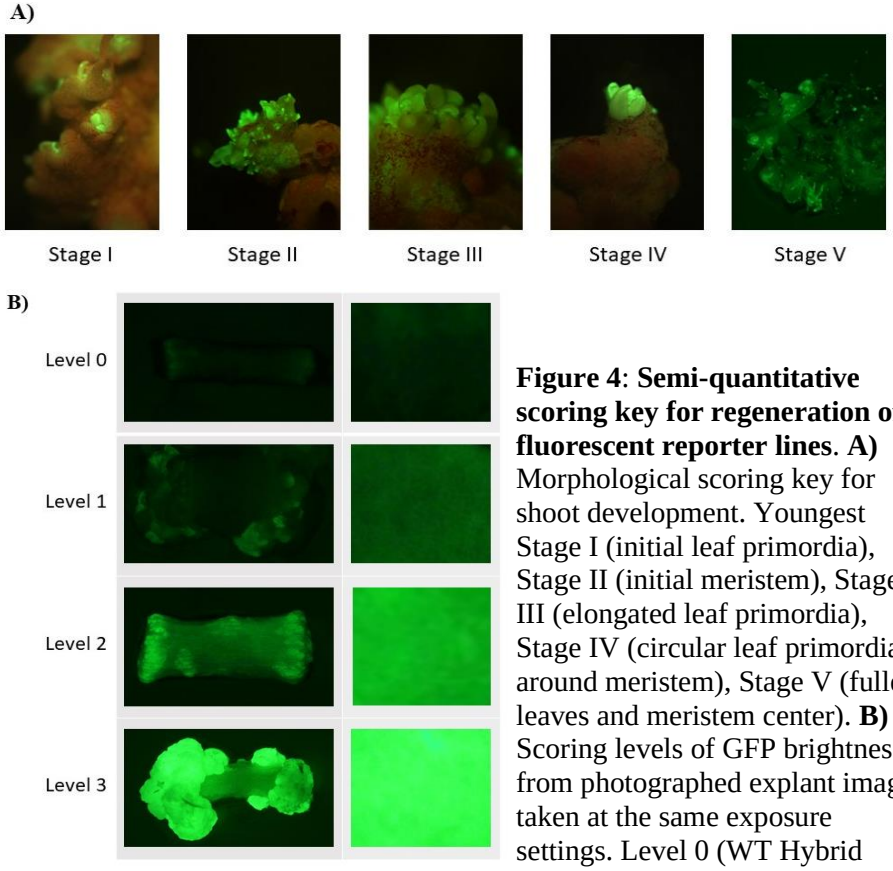

Figure 4: **Semi-quantitative scoring key for regeneration of fluorescent reporter lines**. **A)** Morphological scoring key for shoot development. Youngest Stage I (initial leaf primordia), Stage II (initial meristem), Stage III (elongated leaf primordia), Stage IV (circular leaf primordia around meristem), Stage V (fuller leaves and meristem center). **B)** Scoring levels of GFP brightness from photographed explant images taken at the same exposure settings. Level 0 (WT Hybrid Aspen clone 717-1B4), Level 3 (Ubiquitin10 controlled GFP).

The scale for fluorescent brightness was the same for both callus and shoot. As seen in Figure 4b, the scale ranges from zero (no visible fluorescent light) to three (brightest). In order to compare the level of natural autofluorescence displayed, a wild-type (WT) Hybrid Aspen clone 717-1B4 was observed under the fluorescent microscope. This level of brightness corresponded to a score of zero. The positive control was a single event of 717-1B4 with Ubiquitin10 controlled GFP expression (Čermák et al., 2017). This level of fluorescent brightness corresponded to a score of three.

In addition to scoring each explant, brightfield and fluorescent images were taken. All images were captured using a Nikon Epifluorescence Stereomicroscope SMZ25 with a DS-Ri2 color digital camera. All comparative images were taken with the same exposure, gain, and aperture settings.

Statistical Analysis

Following completion of explant scoring, the data was verified for normality by visiaul inspection of Q-Q plots and linear regressions. Homogeneity of the data was also verified by visual inspection of residuals. In order to average the three plants of each event into an event mean, Main Effects ANOVAs were performed to analyze the difference between plate brightness's of the same event. Both promoter and event trends were graphed over time and observed to initially determine any visual differences. The difference between promoter activity at the tissue type level was then analyzed using a two-tailed T-test. Finally, variation between the two promoters and event variation within the promoters were tested using a two-tailed F-test and Main Effects ANOVA, respectively. All statistical analysis was conducted using Microsoft Excel and/or Statistica.

Results and Discussion

Meristem Reporters During Early Regeneration

In order to assess successful control of the Cre-Lox recombination system in poplar cells, green fluorescent protein was used to track and locate *WUS* and *CSP3* promoter activity. Hybrid Aspen 717-1B4 clones were transformed and grown *in vitro* for forty to fifty days. Micro cuttings were taken and cultured on CIM for three weeks then transferred to grow on SIM for an additional three weeks (Figure 5a; Figure 5b). Starting on growth week three, each explant was imaged (Figure 5c; Figure 5d) and scored weekly according to the brightness

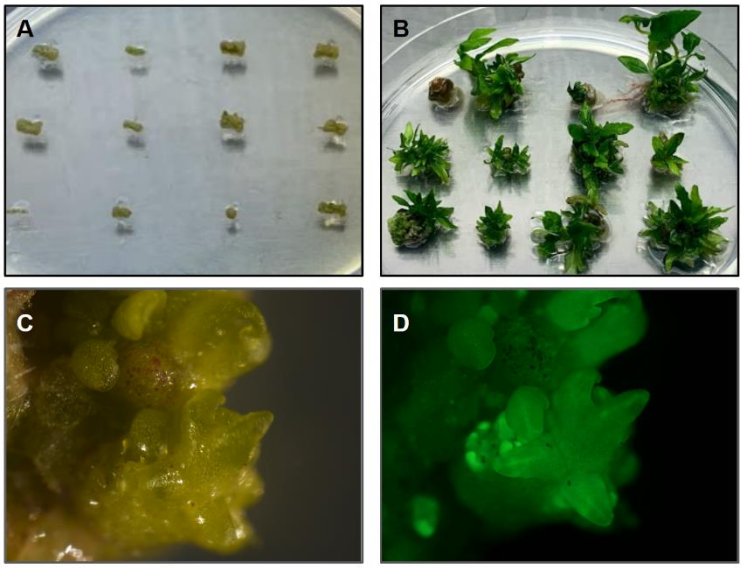

Figure 5: Explant tissue development and imaging over six weeks. A) 12 explants after growth on callus induction medium (CIM) for three weeks. **B)** 12 explants after growth on CIM for two weeks, followed by growth on shoot induction medium (SIM) for three weeks. **C)** Brightfield image of explant with shoot tissue. **D)** Fluorescent image of explant with shoot tissue and promoter activity displayed by GFP.

and growth development scales. The scored data was then statistically analyzed following the outline displayed in Table 1.

Table 1: Statistical analysis outline. Testing topic and the related statistical analysis in order of presentation.

Testing Topic	Statistical Analysis
Normality	Q-Q Plot
Homogeneity	Explant Residual Brightness Change
Difference Between Plates of Same Event	Main Effects ANOVA
Promoter Trends Over Time	Plate Brightness vs Time
Event Trends Over Time	Event Brightness vs Time
Difference Between Promoter Tissue Brightness	Two-tailed T-test
Promoter Activity Correlations	Callus vs Early & Late Shoot Brightness
Promoter Variation	Two-tailed F-test
Event Variation	Main Effects ANOVA

Validation of Data Set

Prior to data analysis, explant brightness scores were compiled and tested for normality. Scores from both promoters fit a normal distribution allowing for continuation in the data validation process (Figure S1; Figure S2). Next, in order to determine homogeneity of the data, the residual change in individual explant brightness was tracked over time for each promoter construct. It was determined that the data is homogenous with the exception of a few individual explants that had drastic decreases by three levels of brightness throughout their growth. However, less than 2% of explants displayed this behavior and thus would not substantially affect statistical analysis (Figure S3; Figure S4).

In order to create event means and ensure that there was not a significant difference in brightness between plates of the same event, a Main Effects ANOVA with Tukey HSD post-hoc was performed. With the exception of *WUS* stage Shoot IV ($p=0.049$), no significant difference in plate brightness was found at any tissue stage (Table S1-S9). This allowed for the three plates of each event to be averaged into event means. The *WUS* Shoot IV significant difference was most likely due to the small sample size of stage Shoot IV tissue compared to other tissue types.

Similar Promoter Activity Trends by Developmental Stage

The two promoters gave very similar patterns over developmental time (Figure 6). A two-tailed t-test at each developmental timepoint (Table S10), showed that there were no significant differences in brightness between the promoters at any developmental stage. Ideally promoter activity in the callus phase would appear much lower, as it was hoped that gene editing is highly active at this time and then ends with excision during meristem formation. High expression in callus is likely non-optimal for the recombinase excision system because activity in early callus growth is exactly when other transgenic components are needed to make proper genomic edits, reduce chimerism through the selectable marker gene. Losing cells to excision during this critical stage may increase chimerism and reduce gene editing efficiency.

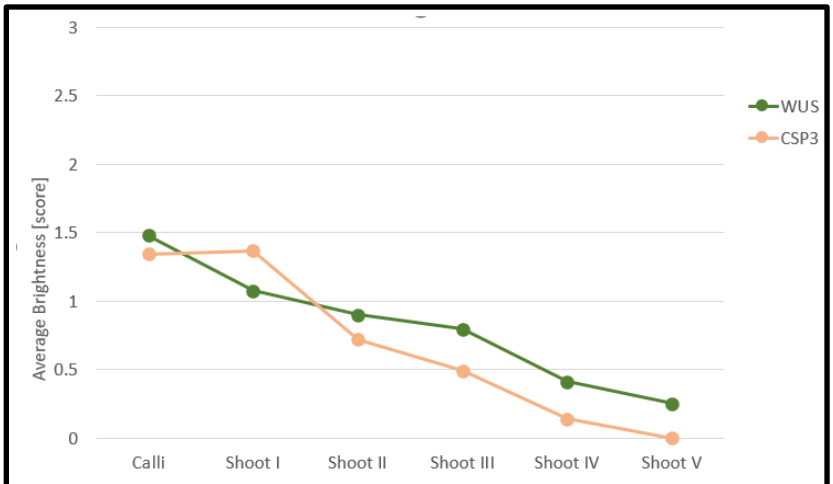

Figure 6: Average tissue type brightness. *WUS* and *CSP3* average brightness across all weeks (y-axis) by tissue type (x-axis).

To further investigate the trends of promoter activity across developmental stages, brightness correlations between different tissue stages were created. For both promoters, event average calli brightness was compared to average Shoot I brightness and fit to a linear model. *WUS* (Figure 7) and *CSP3* (Figure 8) displayed poor fits to the linear model, negating a consistent correlation between promoter activity in calli and early-stage shoots. Event averages at two different time points were included on the graph and often displayed similar locations signifying that events displayed consistent brightness.

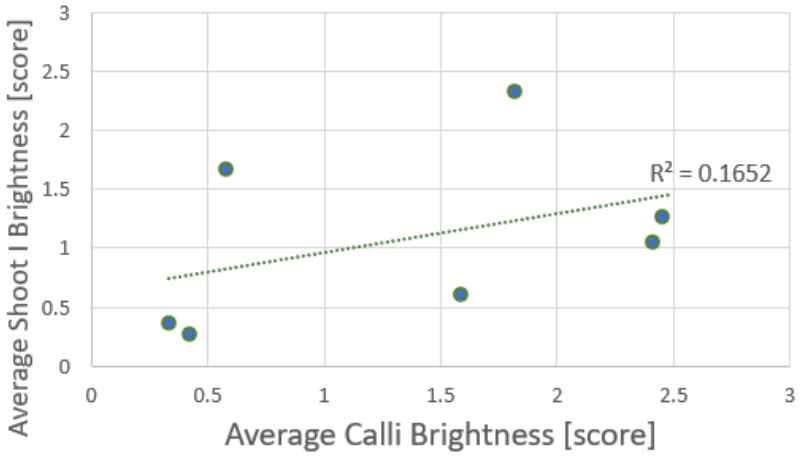

Figure 7: *WUS* early shoot versus calli brightness correlation. Event average shoot I brightness (y-axis) compared to event average calli brightness (x-axis).

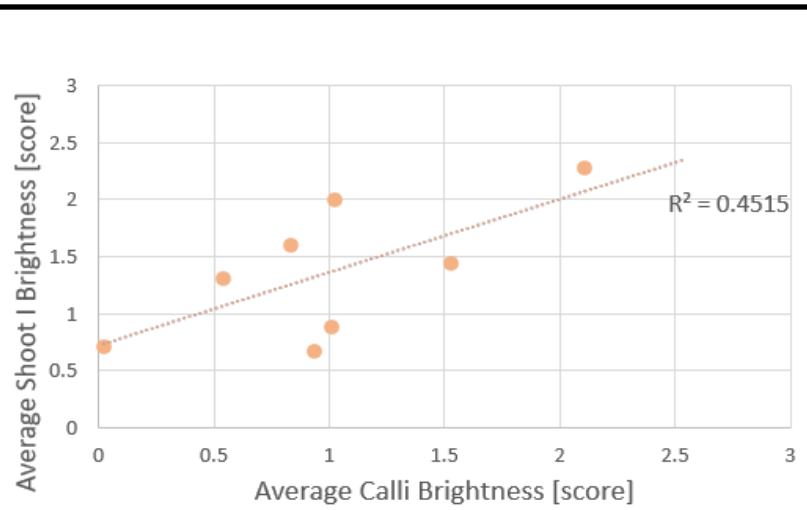

Figure 8: *CSP3* early shoot versus calli brightness correlation. Event average shoot I brightness (y-axis) compared to event average calli brightness (x-axis).

Similarly, in comparing average calli brightness to average Shoot III brightness there was no correlation for *WUS* (Figure 9) nor *CSP3* (Figure 10). This lack of correlation demonstrates that initial promoter activity in callus tissue does not determine the level of promoter activity in early or late stage shoots. Thus promoter expression in the callus stage is not a strong indicator of how the promoter will express in shoots. However, further exploration of the relationships between shoot stage I and II (Figure S6), and shoot stage II and

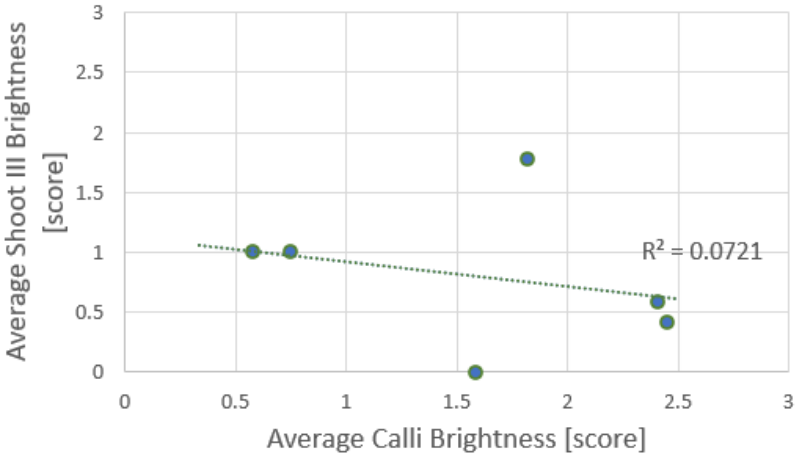

Figure 9: *WUS* late shoot versus calli brightness correlation. Event average shoot III brightness (y-axis) compared to event average calli brightness (x-axis).

III (Figure S7), displayed a strong correlation between average brightness for *WUS*. The *WUS* correlations display the trend of similar promoter expression between shoot stages. Despite having a strong correlation, the promoter activity variation between events needed to be explored.

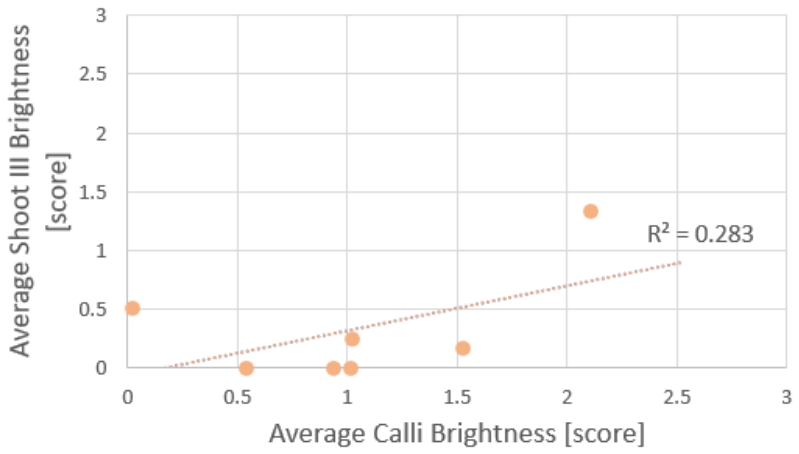

Figure 10: *CSP3* late shoot versus calli brightness correlation. Event average shoot III brightness (y-axis) compared to event average calli brightness (x-axis).

High Levels of Inter-Event Variation

While the overall trend gives a general look at the activity of the promoter, it is important to break down and investigate the influence of specific gene insertion events. In an ideal case promoter activity would behave the same and have limited variation in order to reliably control Cre recombinase. Individual event averages were plotted over time (Figure 11; Figure 12). In observing the varied event trends, the dispersion of *WUS* and *CSP3* averages visually displayed the high standard deviations previously found in the overall trends. The decreased standard

deviation of *CSP3* is visible with the convergence of event average brightness at week six. Ideally there would be less deviation at each time point, signifying that the promoters are displaying similar levels of activity regardless of their placement in the gene. Evaluation of promoter activity over time is useful; however, each tissue type has unique biology and requires further exploration of promoter activity by developmental stage.

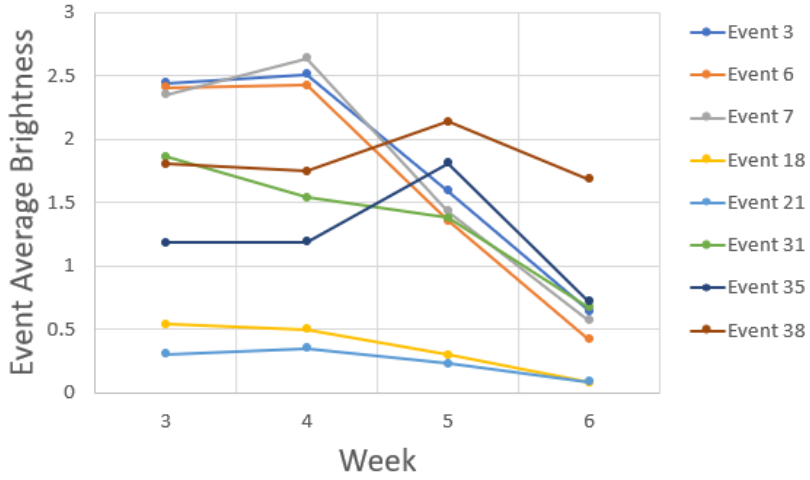

Figure 11: Variation among expression profiles for *WUS* events. Event average brightness of all tissue types (y-axis) as a function of time since explant propagation (x-axis).

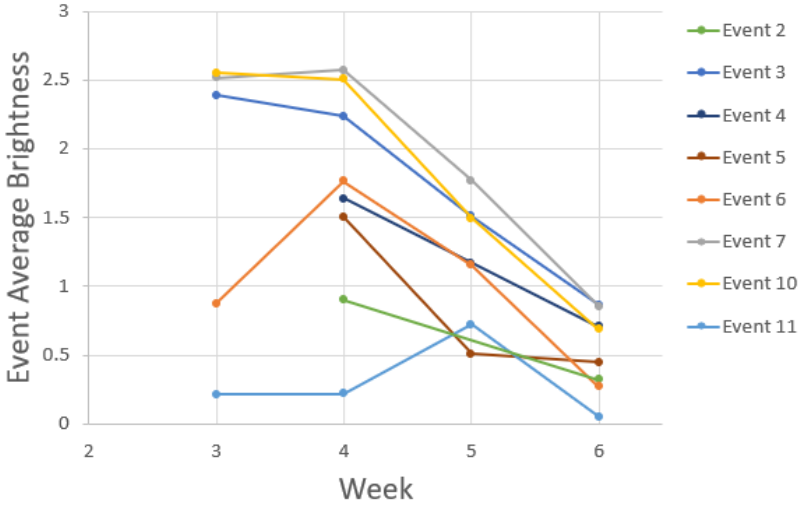

Figure 12: Variation among expression profiles for *CSP3* events. Event average brightness of all tissue types (y-axis) as a function of time since explant propagation (x-axis).

To assess the difference in promoter activity variation, a two-tailed F-test was performed at each tissue stage. In comparing the standard deviations of event weekly average brightnesses, it was found that shoot stage IV was the only stage with significantly different variance between

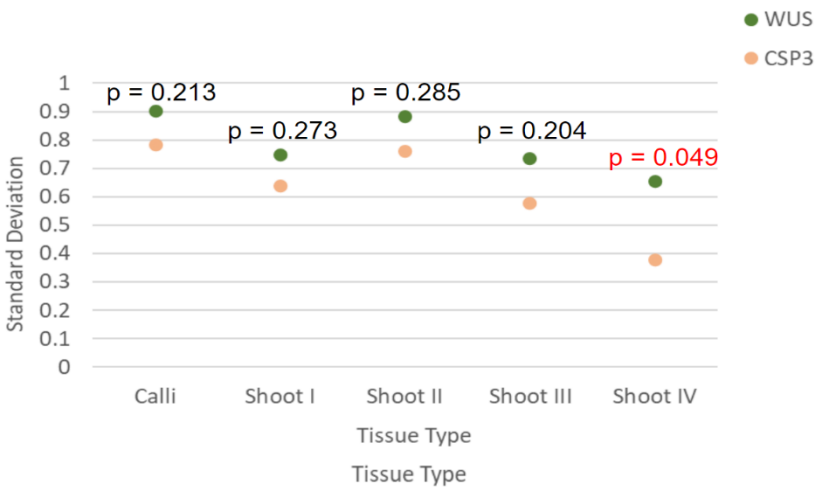

Figure 13: *WUS* and *CSP3* variation comparison at each tissue type. Standard deviation of event weekly average brightness (y-axis) for each tissue type (x-axis). Displayed p-values from two-tailed F-test with $\alpha = 0.05$. Red values represent a significant amount of variation between promoters.

promoters (Figure 13). It was also found that neither promoter displayed significantly more overall variance regardless of tissue type (Table S11).

An ideal promoter for controlling the recombinase system would also have consistent outcomes regardless of gene insertion placement. Thus, in order to assess variation between events within the individual promoters, a Main Effects ANOVA with Tukey HSD post-hoc test was performed. The fraction of significantly different *WUS* (Figure 14a) and *CSP3* (Figure 14b) events were observed for each tissue type.

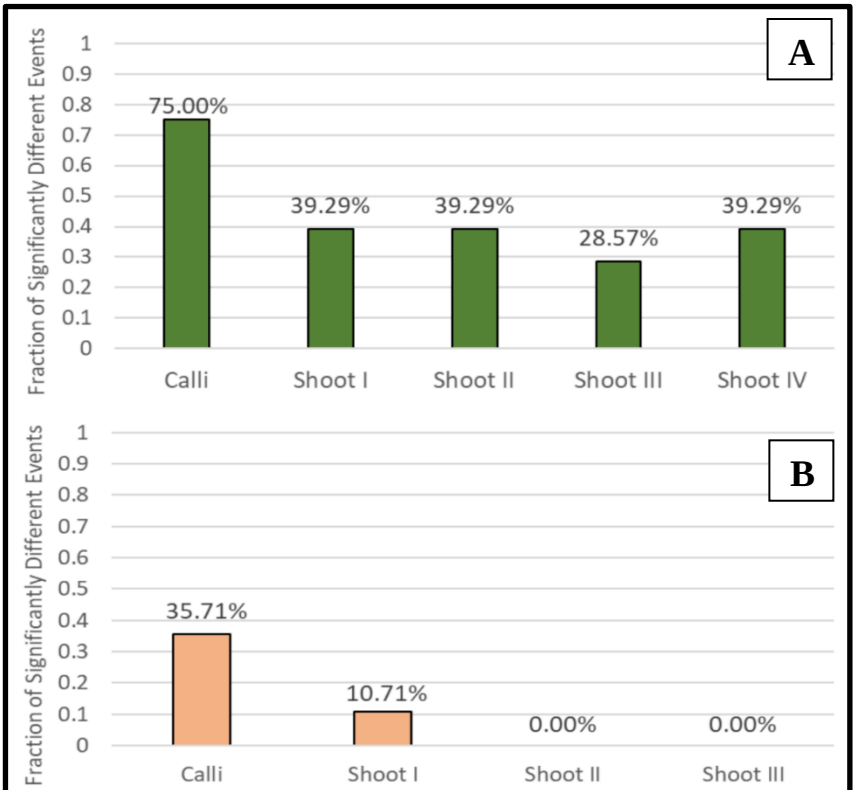

Figure 14: A) *WUS* and B) *CSP3* variation between events. Fraction of significantly different events out of 28 event comparisons (y-axis) per tissue type (x-axis). Significantly different events determined using Main Effects ANOVA with Tukey HSD post-hoc test, $\alpha = 0.05$.

Graphing individual event trends over developmental stages for *WUS* (Figure 15) and *CSP3* (Figure 16) also allows for a visual interpretation of variation differences. In comparing the promoters, it was found that *WUS* displayed higher variability between events than *CSP3*. Therefore, *CSP3* can be deemed a more consistent promoter, as gene insertion event has less of an effect on promoter activity.

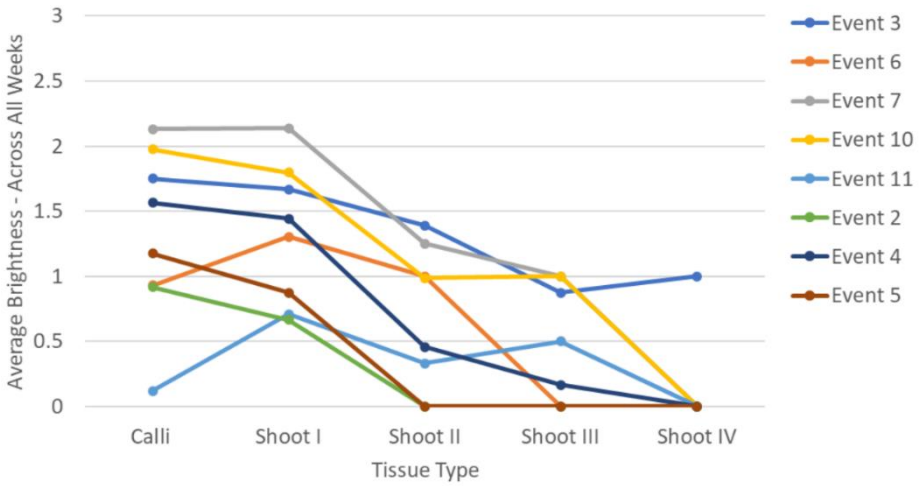

Figure 16: *CSP3* tissue type average brightness. Event average brightness across all weeks (y-axis) by tissue type (x-axis).

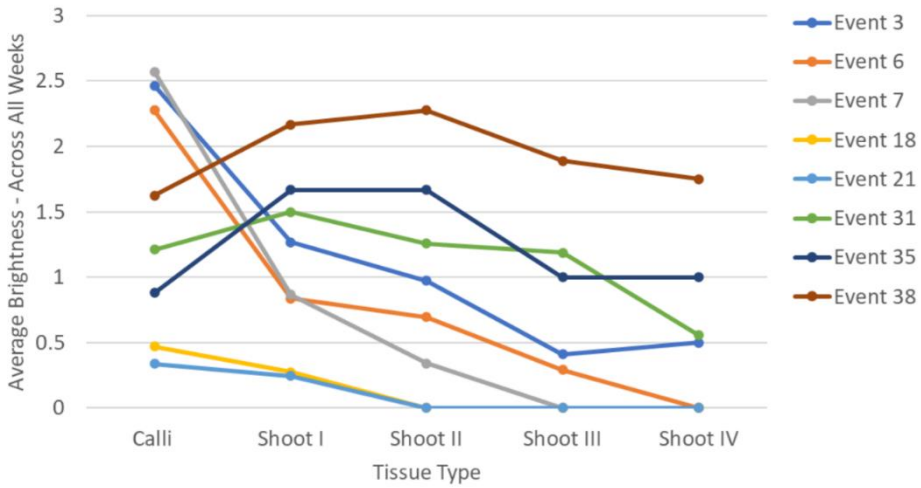

Figure 15: *WUS* tissue type average brightness. Event average brightness across all weeks (y-axis) by tissue type (x-axis).

Conclusion

Both *CSP3/WUS* are Non-Ideal Promoters for the Cre-Lox System

In order to determine the more ideal promoter for control of the Cre-Lox recombination system in poplar cells, I conducted an in-depth comparison of *WUS* and *CSP3* promoter expression. Promoter construct behavior was evaluated using GFP which allowed for the activity to be fluorescently tracked through early regeneration stages.

After scoring and statistically analyzing the promoter construct activity, neither promoter is well suited for control of the Cre-Lox recombination system. Both promoters displayed high levels of activity in callus tissue which is undesired because the transgenes would not have enough time to make proper edits before being removed from the genome. Additionally, both promoters were inconsistent and presented significant variation between gene insertion events – though this is not uncommon with transgenic methods. This suggests that a greater scan of usable promoters that express highly in early shoot regeneration but very low in callus tissue is warranted if this approach is to be used.

If one promoter had to be chosen amongst the two investigated, *CSP3* has some advantages over *WUS*. *CSP3* displayed high promoter construct activity in callus tissue, but still showed increased activity during the callus-shoot I transition that *WUS* did not, and it is most important that the promoter is active during this period. *CSP3* also had much less variation between events than *WUS* did, showing that *CSP3* is the more reliable and consistent promoter. However, further systems and tools need to be explored, and perhaps leaky callus expression is something that cannot be adequately through promoter selection.

Future Tools Needed for Further Advancement of Promoter Controlled Systems

With the rapid growth of gene editing in clonally propagated plants, the ability to remove genetic editing machinery has proven to be difficult. The exploration of additional promoters for this recombinase system is required in order to find an ideal promoter. Promoter selection for plant systems is often low throughput (taking months to years), due to the required transformation and propagation. Automated promoter selection based on gene expression profiles would speed up the initial selection process while allowing for a wider variety of promoters to be considered (Fleur et al., 2021). In addition, alternative solutions for recombinase

control could include temperature shock and chemical induction that could be used in combination with a developmental promoter to create a system with tighter control.

As previously mentioned, gene insertion position greatly affects the resulting level of promoter construct activity. DNA delivery into plant cells via *Agrobacterium* results in varying copies of the DNA inserted at random locations. Having access to genetic engineering tools that allow for site-specific insertion into precise location might increase the reliability of the excision system. This would allow for reduced transgene positional affect and promoter construct activity to be less dependent on placement in the genome (Dong and Ronald, 2021). However, techniques for targeting transgenes into a specific integration site in the genome are limited and further exploration needs to be taken. CRISPR is one promising solution on the horizon, as it can direct transgenes to specific genome location, but the efficiency is low at present.

Another method of reducing variability between gene insertion events is the incorporation of genetic insulators. Insulators are a class of DNA that possess the ability to protect expressing genes, or promoters, from surrounding environmental signals. Insulators are able to prevent enhancers from activating gene expression, and block chromatin that may silence expression (West et al., 2002). While insulators have been well characterized in animals, use in genetic engineering of plants is not common practice. Having access to this genetic tool would potentially allow for maximized promoter expression and minimized variability. Promoter activity would be less dependent on genetic placement as it would be protected from surrounding signals. This would reduce variability between gene insertion events and allow for a more comprehensive understanding of true promoter activity (Pérez-González and Caro, 2019). Further efforts to investigate genetic insulators in plants are needed for enhanced promoter control and the future of plant biotechnology.

Continuing Need for Timely Transgene Removal System

Genetic engineering of clonal crops, and specifically *Populus*, is a growing field due to the potential of enhancing desired traits such as wood quality, pest resistance, and herbicide resistance. Alteration of such traits comes with concerns regarding the potential risk of modifying species, the process of T-DNA integration, and variable expression of transgenes. Having the ability to create transgene-free edits that do not contain the integrated transgenes would help to mitigate these concerns. The Cre-Lox recombination system is a possible solution

on the condition that a proper control mechanism is found. Due to the observed non-ideal behavior of both *WUS* and *CSP3* promoters, additional mechanisms need to be researched for the timely control of the Cre-Lox system.

Supplemental Figures and Tables

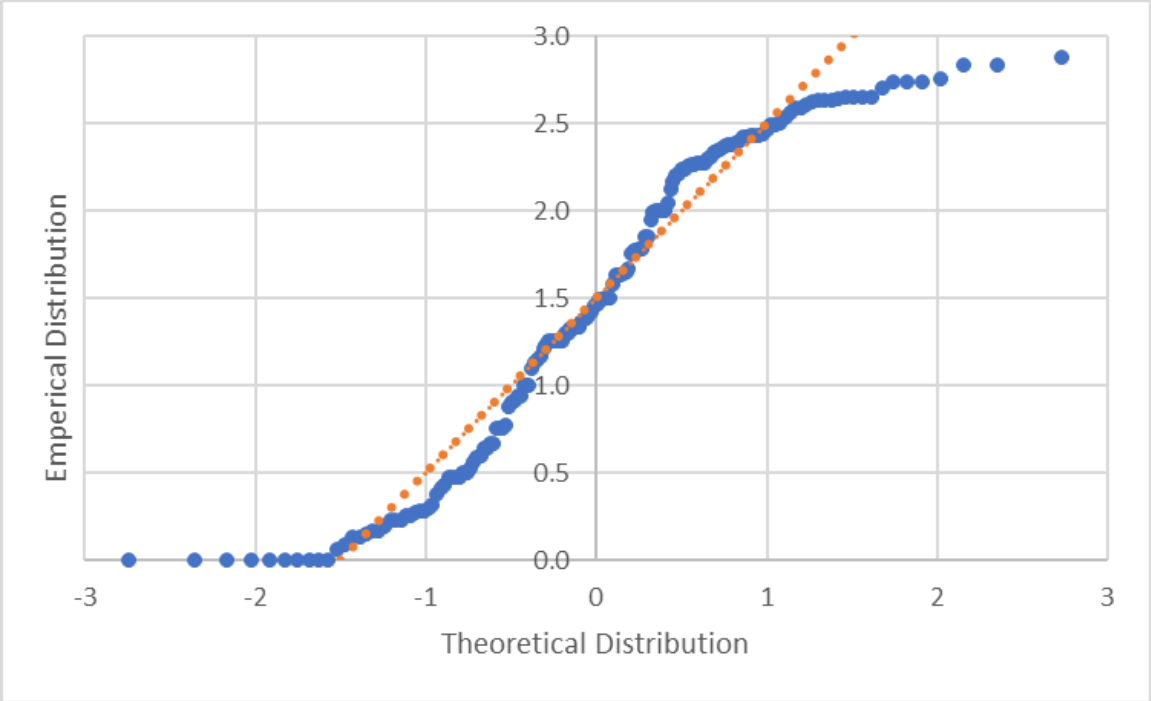

Figure S1: Quantile-Quantile plot of average plate brightness of all calli. Average plate brightness of calli (y-axis) as a function of quartile z-scores expected from a normal distribution with the same mean and variance as the empirical distribution (x-axis). Orange line displays normally distributed data. Note sigmoidal shape due to asymptotes at y = 0 and y = 3.

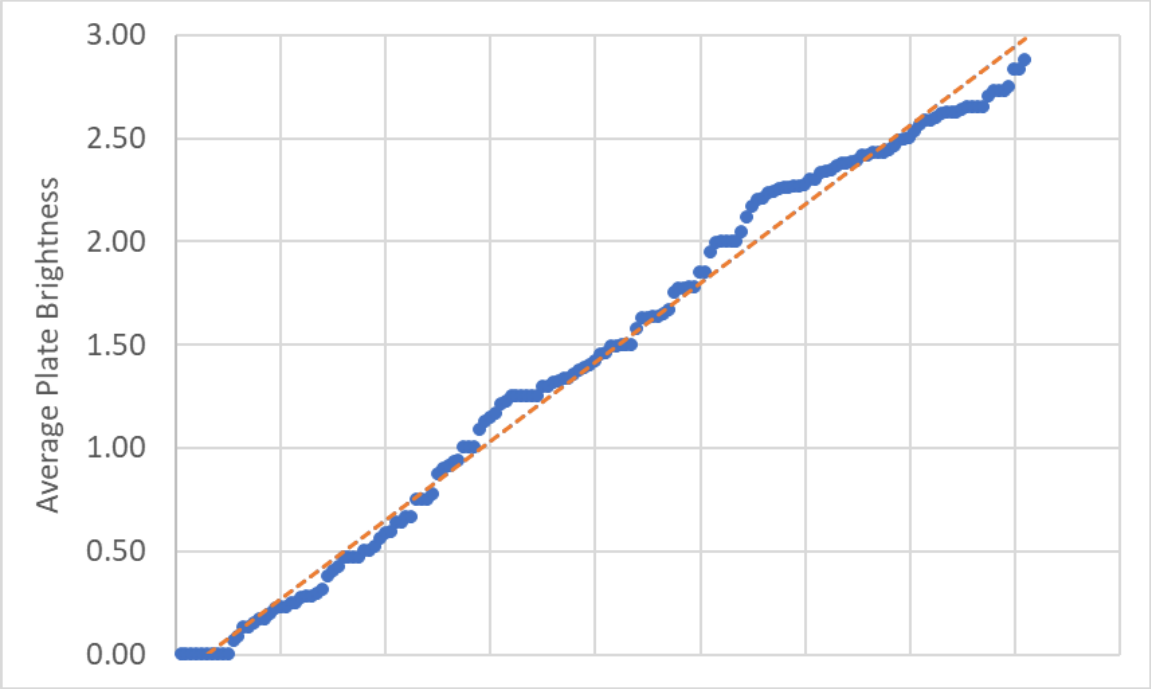

Figure S2: Average plate brightness poled over all tissue types. Average plate brightness of all tissue types (y-axis) as a function of ascending order (x-axis). Orange line represents expectation for normally distributed data.

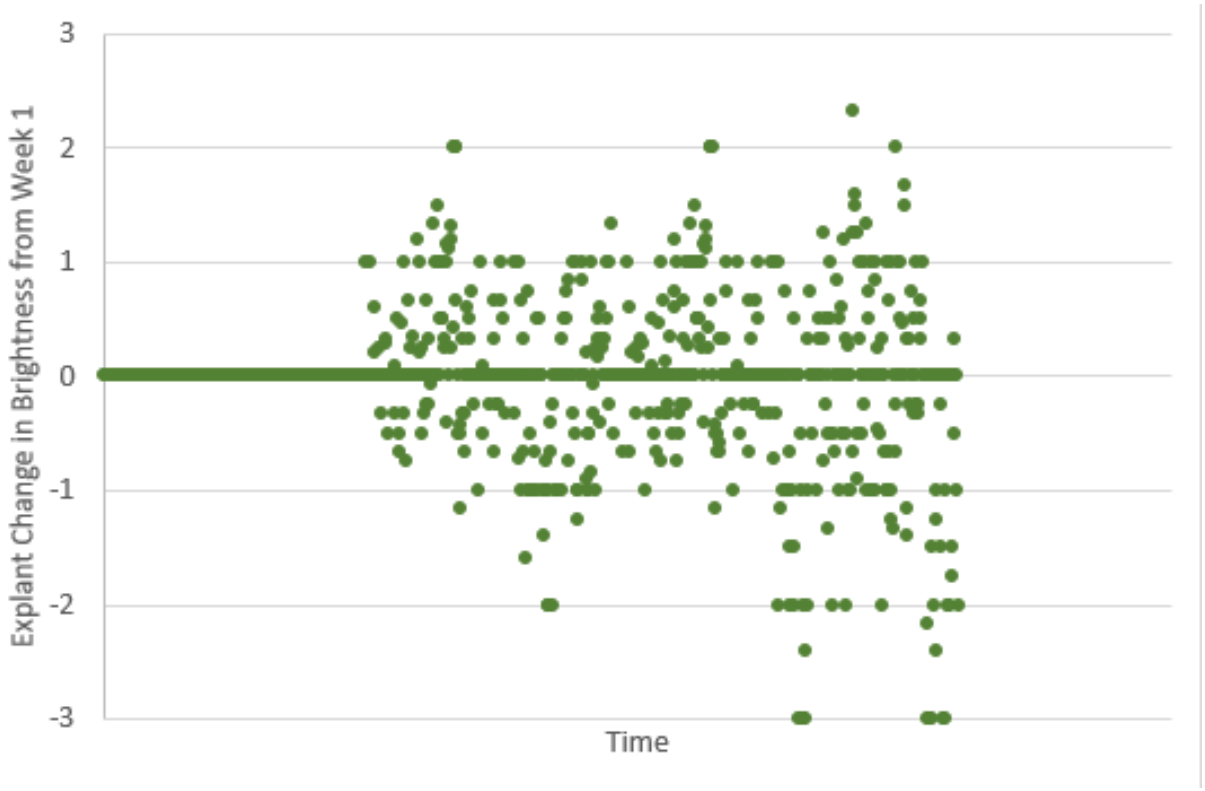

Figure S3: *WUS* residual explant brightness change. Change in individual explant brightness from growth-week 3 (y-axis) as a function of time since explant propagation (x-axis). Brightness change of each explant calculated by subtracting explant brightness at Week 3 from current week explant brightness.

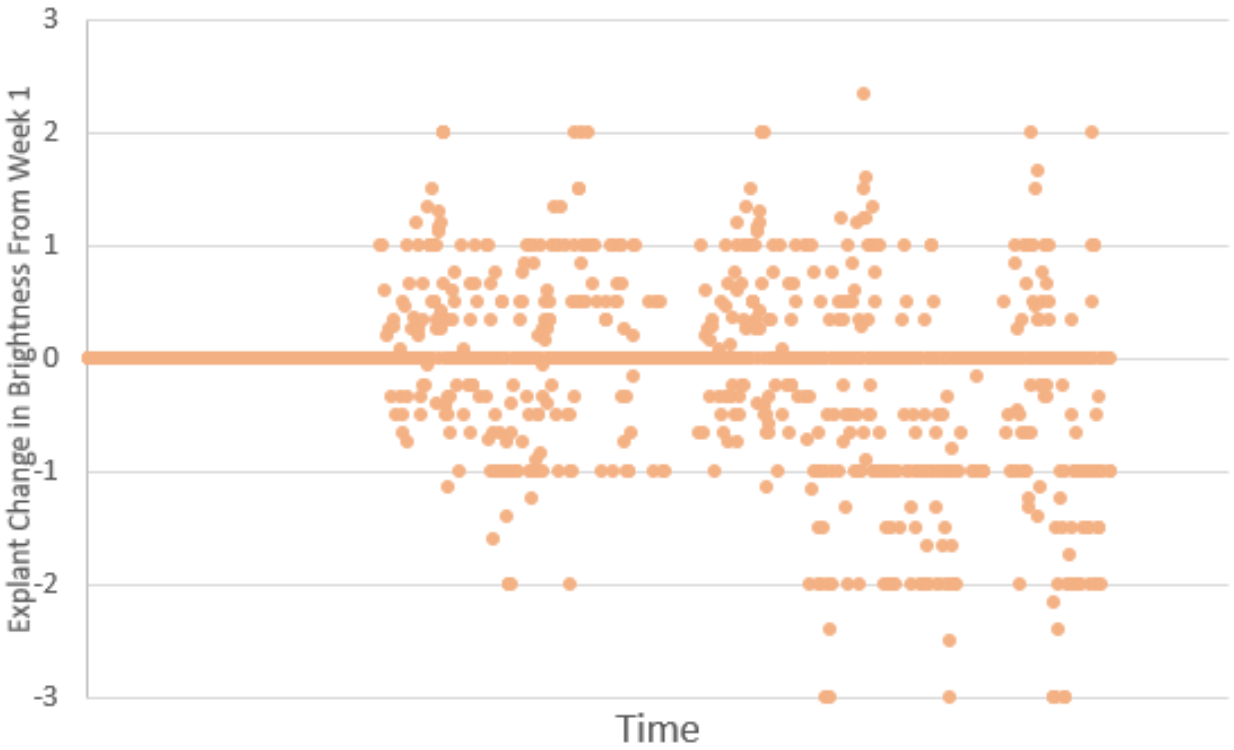

Figure S4: *CSP3* residual explant brightness change. Change in individual explant brightness from growth-week 3 (y-axis) as a function of time since explant propagation (x-axis). Brightness change of each explant calculated by subtracting explant brightness at Week 3 from current week explant brightness.

Table S1: *WUS* average plate calli brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test using $\alpha = 0.05$. Red values represent a significant difference in brightness between plates.

WUS Average Callus Brightness			
	Plate 1	Plate 2	Plate 3
Plate 1		0.955	0.811
Plate 2	0.955		0.638
Plate 3	0.811	0.638	

Table S2: *WUS* average plate shoot I brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test using $\alpha = 0.05$. Red values represent a significant difference in brightness between plates.

WUS Average Shoot I Brightness			
	Plate 1	Plate 2	Plate 3
Plate 1		0.562	0.183
Plate 2	0.562		0.718
Plate 3	0.183	0.718	

Table S3: *WUS* average plate shoot II brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test using $\alpha = 0.05$. Red values represent a significant difference in brightness between plates.

WUS Average Shoot II Brightness			
	Plate 1	Plate 2	Plate 3
Plate 1		0.737	0.974
Plate 2	0.737		0.860
Plate 3	0.974	0.860	

Table S4: *WUS* average plate shoot III brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test using $\alpha = 0.05$. Red values represent a significant difference in brightness between plates.

WUS Average Shoot III Brightness			
	Plate 1	Plate 2	Plate 3
Plate 1		0.620	0.931
Plate 2	0.620		0.428
Plate 3	0.931	0.428	

Table S5: *WUS* average plate shoot IV brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test using $\alpha = 0.05$. Red values represent a significant difference in brightness between plates.

WUS Average Shoot IV Brightness			
	Plate 1	Plate 2	Plate 3
Plate 1		0.013	0.013
Plate 2	0.013		0.975
Plate 3	0.013	0.975	

Table S6: *CSP3* average plate calli brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test using $\alpha = 0.05$. Red values represent a significant difference in brightness between plates.

CSP3 Average Calli Brightness			
	Plate 1	Plate 2	Plate 3
Plate 1		0.804	0.116
Plate 2	0.804		0.342
Plate 3	0.116	0.3422	

Table S7: *CSP3* average plate shoot I brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test using $\alpha = 0.05$. Red values represent a significant difference in brightness between plates.

CSP3 Average Shoot I Brightness			
	Plate 1	Plate 2	Plate 3
Plate 1		0.990	0.798
Plate 2	0.990		0.738
Plate 3	0.798	0.738	

Table S8: *CSP3* average plate shoot II brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test using $\alpha = 0.05$. Red values represent a significant difference in brightness between plates.

CSP3 Average Shoot II Brightness			
	Plate 1	Plate 2	Plate 3
Plate 1		0.505	0.249
Plate 2	0.505		0.781
Plate 3	0.249	0.781	

Table S9: *CSP3* average plate shoot III brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test using $\alpha = 0.05$. Red values represent a significant difference in brightness between plates.

CSP3 Average Shoot III Brightness			
	Plate 1	Plate 2	Plate 3
Plate 1		0.917	0.785
Plate 2	0.917		0.942
Plate 3	0.785	0.942	

Table S10: Comparison of *WUS* and *CSP3* average tissue type brightness across all weeks. P-values from two-tailed T-test assuming equal variances and $\alpha = 0.05$. Black values represent no significant brightness difference between promoters.

		P-Value two-tail
WUS vs CSP3	Calli	0.544
	Shoot I	0.259
	Shoot II	0.508
	Shoot III	0.272
	Shoot IV	0.355
	Shoot V	0.410

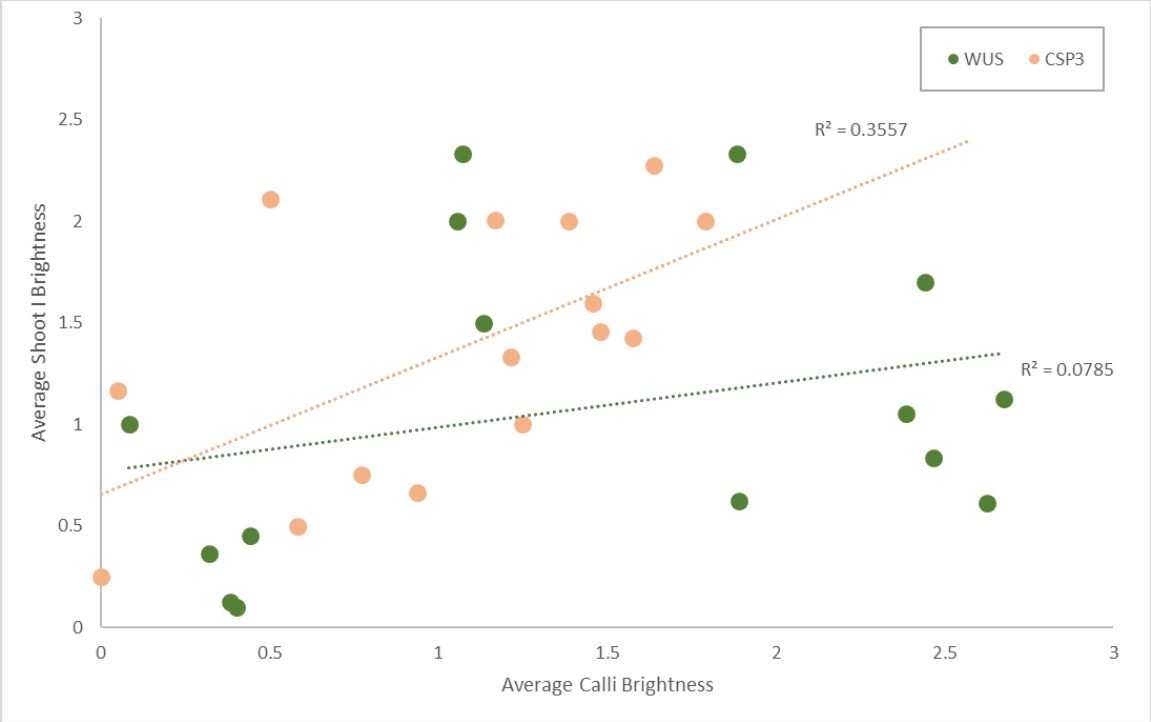

Figure S5: *WUS* and *CSP3* shoot I versus calli brightness correlation. Event average shoot I brightness (y-axis) compared to event average calli brightness (x-axis).

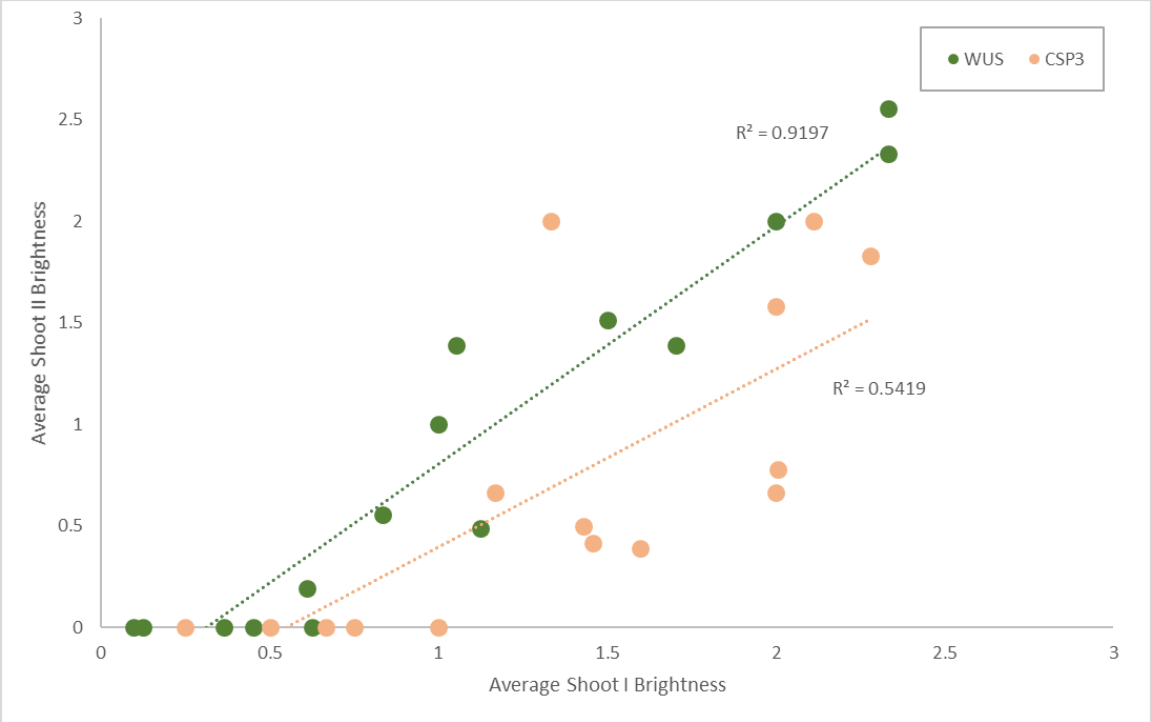

Figure S6: *WUS* and *CSP3* shoot II versus shoot I brightness correlation. Event average shoot II brightness (y-axis) compared to event average shoot I brightness (x-axis).

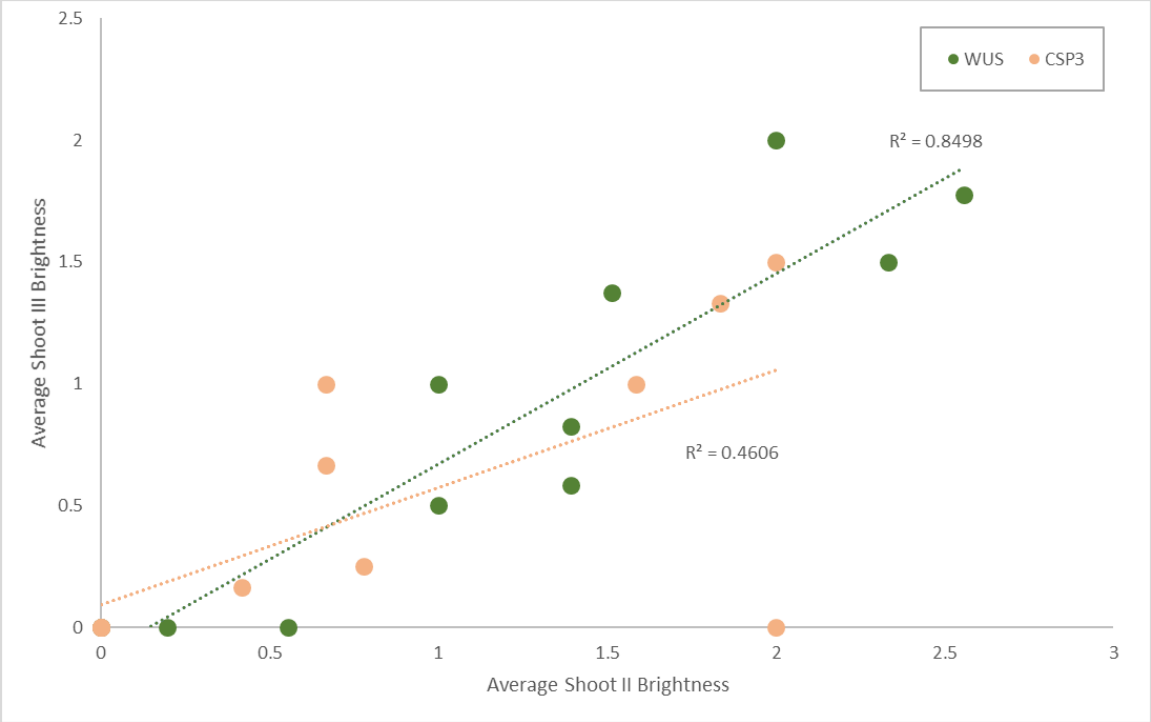

Figure S7: *WUS* and *CSP3* shoot III versus shoot II brightness correlation. Event average shoot III brightness (y-axis) compared to event average shoot II brightness (x-axis).

Table S11: Two-Tailed F-Test comparing variance of *WUS* and *CSP3* for each tissue type. Standard deviation is of event weekly average brightness, degrees of freedom (DOF) are the count of event weekly average brightnesses, F-critical determined using DOF and $\alpha = 0.05$.

	Calli	Shoot I	Shoot II	Shoot III	Shoot IV	Overall
WUS Standard Deviation	0.902	0.748	0.884	0.736	0.655	0.105
CSP3 Standard Deviation	0.782	0.638	0.761	0.576	0.378	0.163
DOF	32	15	15	12	7	4
F-statistic	1.329	1.375	1.348	1.633	3.001	2.429
F-critical	2.457	2.602	2.602	2.681	2.998	3.747
P-value	0.213	0.273	0.285	0.204	0.049	0.206
Null Hypothesis Rejected?	NO	NO	NO	NO	YES	NO

Table S12: *WUS* event average calli brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test, $\alpha = 0.05$. Red values represent a significant difference in average brightness between events.

WUS AVG Calli Brightness									
	Tukey HSD test; variable AVG Brightness Calli								
Cell No.	Event	3	6	7	18	21	31	35	38
1	3		0.9517	0.9984	0.0001	0.0001	0.0001	0.0001	0.0002
2	6	0.9517		0.6581	0.0001	0.0001	0.0001	0.0001	0.0052
3	7	0.9984	0.6581		0.0001	0.0001	0.0001	0.0001	0.0001
4	18	0.0001	0.0001	0.0001		0.9936	0.0010	0.2439	0.0001
5	21	0.0001	0.0001	0.0001	0.9936		0.0002	0.0382	0.0001
6	31	0.0001	0.0001	0.0001	0.0010	0.0002		0.5253	0.2337
7	35	0.0001	0.0001	0.0001	0.2439	0.0382	0.5253		0.0009
8	38	0.0002	0.0052	0.0001	0.0001	0.0001	0.2337	0.0009	

Table S13: *WUS* event average shoot I brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test, $\alpha = 0.05$. Red values represent a significant difference in average brightness between events.

WUS AVG Shoot I Brightness									
	Tukey HSD test; variable Avg Brightness Shoot I								
Cell No.	Event	3	6	7	18	21	31	35	38
1	3		0.6029	0.6830	0.0046	0.0033	0.9915	0.9255	0.0161
2	6	0.6029		1.0000	0.2763	0.2224	0.3254	0.2361	0.0003
3	7	0.6830	1.0000		0.2216	0.1759	0.3812	0.2756	0.0004
4	18	0.0046	0.2763	0.2216		1.0000	0.0043	0.0051	0.0001
5	21	0.0033	0.2224	0.1759	1.0000		0.0033	0.0041	0.0001
6	31	0.9915	0.3254	0.3812	0.0043	0.0033		0.9998	0.2713
7	35	0.9255	0.2361	0.2756	0.0051	0.0041	0.9998		0.7129
8	38	0.0161	0.0003	0.0004	0.0001	0.0001	0.2713	0.7129	

Table S14: *WUS* event average shoot II brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test, $\alpha = 0.05$. Red values represent a significant difference in average brightness between events.

WUS AVG Shoot II Brightness									
	Tukey HSD test; variable Avg Brightness Shoot II								
Cell No.	Event	3	6	7	18	21	31	35	38
1	3		0.9837	0.4637	0.0585	0.0585	0.9644	0.0870	0.0043
2	6	0.9837		0.9402	0.3437	0.3437	0.5593	0.0124	0.0005
3	7	0.4637	0.9402		0.9497	0.9497	0.0851	0.0008	0.0002
4	18	0.0585	0.3437	0.9497		1.0000	0.0063	0.0002	0.0001
5	21	0.0585	0.3437	0.9497	1.0000		0.0063	0.0002	0.0001
6	31	0.9644	0.5593	0.0851	0.0063	0.0063		0.5371	0.0674
7	35	0.0870	0.0124	0.0008	0.0002	0.0002	0.5371		0.9508
8	38	0.0043	0.0005	0.0002	0.0001	0.0001	0.0674	0.9508	

Table S15: *WUS* event average shoot III brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test, $\alpha = 0.05$. Red values represent a significant difference in average brightness between events.

WUS AVG Shoot III Brightness									
	Tukey HSD test; variable Avg Brightness Shoot III								
Cell No.	Event	3	6	7	18	21	31	35	38
1	3		0.9972	0.9044	0.9044	0.9044	0.1313	0.7719	0.0034
2	6	0.9972		0.9994	0.9994	0.9994	0.0976	0.5684	0.0043
3	7	0.9044	0.9994		1.0000	1.0000	0.0338	0.3178	0.0015
4	18	0.9044	0.9994	1.0000		1.0000	0.0338	0.3178	0.0015
5	21	0.9044	0.9994	1.0000	1.0000		0.0338	0.3178	0.0015
6	31	0.1313	0.0976	0.0338	0.0338	0.0338		0.9960	0.6992
7	35	0.7719	0.5684	0.3178	0.3178	0.3178	0.9960		0.4673
8	38	0.0034	0.0043	0.0015	0.0015	0.0015	0.6992	0.4673	

Table S16: *CSP3* event average calli brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test, $\alpha = 0.05$. Red values represent a significant difference in average brightness between events.

CSP3 AVG Calli Brightness									
	Tukey HSD test; variable AVG Brightness Calli								
Cell No.	Event	2	3	4	5	6	7	10	11
1	2		0.3702	0.7776	0.9992	1.0000	0.0444	0.1188	0.4225
2	3	0.3702		0.9962	0.6226	0.0138	0.5768	0.9521	0.0001
3	4	0.7776	0.9962		0.9606	0.3496	0.3973	0.7676	0.0002
4	5	0.9992	0.6226	0.9606		0.9959	0.0756	0.2161	0.0344
5	6	1.0000	0.0138	0.3496	0.9959		0.0002	0.0007	0.0155
6	7	0.0444	0.5768	0.3973	0.0756	0.0002		0.9945	0.0001
7	10	0.1188	0.9521	0.7676	0.2161	0.0007	0.9945		0.0001
8	11	0.4225	0.0001	0.0002	0.0344	0.0155	0.0001	0.0001	

Table S17: *CSP3* event average shoot I brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test, $\alpha = 0.05$. Red values represent a significant difference in average brightness between events.

CSP3 AVG Shoot I Brightness									
	Tukey HSD test; variable Avg Brightness Shoot I								
Cell No.	Event	2	3	4	5	6	7	10	11
1	2		0.5212	0.8970	1.0000	0.9544	0.2233	0.5785	1.0000
2	3	0.5212		0.9753	0.4504	0.8392	0.9844	1.0000	0.0443
3	4	0.8970	0.9753		0.9272	1.0000	0.6016	0.9912	0.4202
4	5	1.0000	0.4504	0.9272		0.9782	0.1209	0.5109	0.9988
5	6	0.9544	0.8392	1.0000	0.9782		0.2779	0.8984	0.5436
6	7	0.2233	0.9844	0.6016	0.1209	0.2779		0.9270	0.0027
7	10	0.5785	1.0000	0.9912	0.5109	0.8984	0.9270		0.0468
8	11	1.0000	0.0443	0.4202	0.9988	0.5436	0.0027	0.0468	

Table S18: *CSP3* event average shoot II brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test, $\alpha = 0.05$. Red values represent a significant difference in average brightness between events.

CSP3 AVG Shoot II Brightness									
	Tukey HSD test; variable Avg Brightness Shoot II								
Cell No.	Event	2	3	4	5	6	7	10	11
1	2		0.8087	0.9996	1.0000	0.9946	0.8791	0.9320	0.9999
2	3	0.8087		0.8278	0.5415	0.9600	1.0000	0.9986	0.5202
3	4	0.9996	0.8278		0.9981	1.0000	0.9206	0.9724	1.0000
4	5	1.0000	0.5415	0.9981		0.9787	0.6663	0.7745	0.9994
5	6	0.9946	0.9600	1.0000	0.9787		0.9900	0.9988	0.9985
6	7	0.8791	1.0000	0.9206	0.6663	0.9900		1.0000	0.6919
7	10	0.9320	0.9986	0.9724	0.7745	0.9988	1.0000		0.8249
8	11	0.9999	0.5202	1.0000	0.9994	0.9985	0.6919	0.8249	

Table S19: *CSP3* event average shoot III brightness comparisons. P-values from Main Effects ANOVA with Tukey HSD post-hoc test, $\alpha = 0.05$. Red values represent a significant difference in average brightness between events.

CSP3 AVG Shoot III Brightness									
	Tukey HSD test; variable Avg Brightness Shoot III								
Cell No.	Event	2	3	4	5	6	7	10	11
1	2		0.9715	1.0000	1.0000	1.0000	0.8364	0.9241	0.9999
2	3	0.9715		0.9783	0.9715	0.9125	0.9979	0.9995	0.9788
3	4	1.0000	0.9783		1.0000	1.0000	0.7874	0.9363	1.0000
4	5	1.0000	0.9715	1.0000		1.0000	0.8364	0.9241	0.9999
5	6	1.0000	0.9125	1.0000	1.0000		0.6155	0.8611	0.9995
6	7	0.8364	0.9979	0.7874	0.8364	0.6155		1.0000	0.7013
7	10	0.9241	0.9995	0.9363	0.9241	0.8611	1.0000		0.9404
8	11	0.9999	0.9788	1.0000	0.9999	0.9995	0.7013	0.9404	

Appendix

As part of my thesis research, I attempted to analyze cellular level expression patterns from the different promoters. This work did not yield clear results, so is not included in the body of my thesis. The background and method below summarize that work.

Background

Internal Expression of Prepared Fluorescent Tissue Analysis

In the later stages of plant growth, plant tissues become difficult to image due to more complex tissue structures and photosensitivity. An outdated solution is the use of histochemical GUS staining. This involved incubating entire plants in X-Gluc solution, followed by a bleaching stage in order to stain and clear the tissues, respectively. This allowed for the detection of promoter expression, including both location and intensity (Bao et al., 2009). However, this method destroys the plant and does not allow for *in vivo* visualization of the plant.

In order to overcome the challenges of tracking gene expression in later stages of plant growth, the newer chemical technique of ClearSee was created at Nagoya University. Through the process of screening a chemical library, the chemical clearing recipe was formulated (N-sarkosyl, Urea, Xylitol). The goal of chemically fixing plant tissues with ClearSee is to diminish the natural chlorophyll autofluorescence while preserving fluorescent proteins such as GFP. The method of clearing can also be combined with chemical staining (i.e. fluorescent dyes) to help differentiate between different plant organs or cell types (Kurihara et al., 2015). The simultaneous use of clearing and dying creates fixed, mature plant tissues that are more accessible for imaging and observation under fluorescent microscopy.

Methods

Whole Plant Observation

In order to determine the most effective plant fixative, Experiment #1 compared Formaldehyde Alcohol Acetic Acid (FAA), Paraformaldehyde (PFA), and water. Each fixative was tested on root, stem, and leaf sections. Each sample was initially placed in the appropriate fixative and vacuumed inside the vacuum chamber for 20 minutes total (releasing and resealing vacuum every five minutes) to incorporate the fixative into the plant tissue. The fixative was drained, and plant tissues were placed in the ClearSee solution (N-sarkosyl, Urea, Xylitol). All samples were placed in the dark on a shaker table.

Experiment #2 tested the ClearSee solution with the addition of Citric Acid and Ferric Citrate to prevent browning of the tissue. Plant samples were fixed in PFA following the same vacuuming schedule, then placed in the enhanced ClearSee solution. All samples were placed in the dark on the shaker table.

Experiment #3 incorporated soaking root, stem, and leaf samples in 20% Acetone on ice for 10 minutes before being vacuumed in PFA. The plant samples were then placed in the enhanced ClearSee solution and left in the dark on the shaker table.

Following the ClearSee experiments, fresh cut shoot tips (forty- to fifty-day-old *in vitro* grown) and roots from *AtWUS*, *AtCSP3*, WT, and Ubiquitin10 driven GFP expression were hand-sliced vertically with a razor blade to expose internal tissues. Brightfield and fluorescent images were then taken of plant samples, specifically focused on apical and axillary meristematic regions.

Results and Discussion

To evaluate promoter activity in later growth stages, meristem tissue from small whole plants was exposed and observed for GFP brightness (Figure A1). To ensure that natural autofluorescence was not perceived as GFP signal, wild type (WT) meristems were used as a negative control.

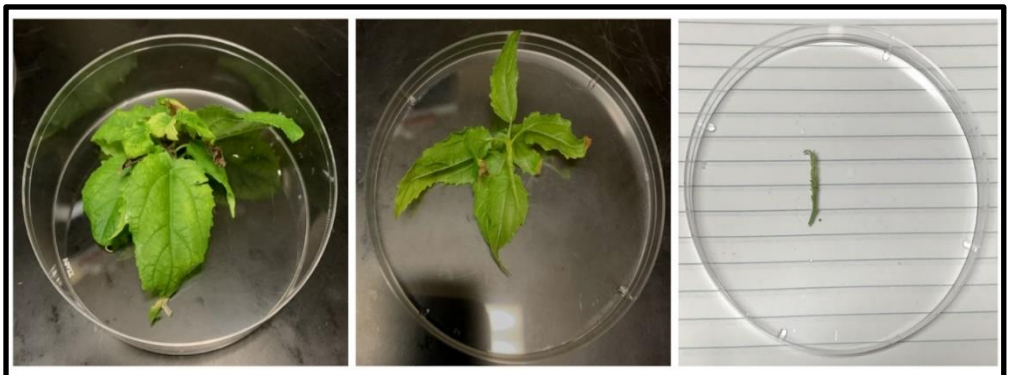

Figure A1: Process of cutting leaves off small whole plant. Tip from small whole plant before and after leaf trimming.

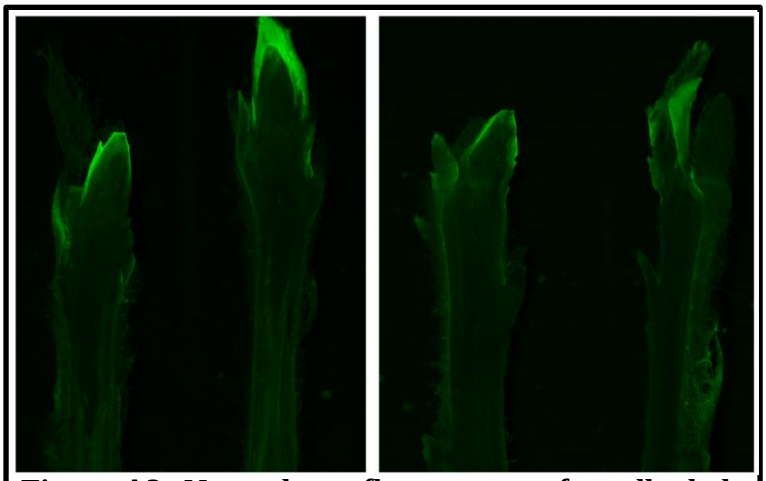

Figure A2: Natural autofluorescence of small whole plant tip. 717 fresh tissue negative control.

It was observed that WT meristems displayed autofluorescence in leaf tips but did not show any autofluorescence in the controlling center region (Figure A2). Therefore, transgenic leaf tips displaying similar levels of fluorescent activity do not correlate to promoter activity.

Small whole plants from *WUS* events 6 and 38 were observed because they were previously observed to have the greatest overall promoter activity, regardless of their lack of fit to the ideal trend. Three meristem tips from each event were observed. Event 6 displayed slight promoter activity in one of the three observed tips (Figure A3). However, the other two tips did not show any promoter activity at this stage (Figure A4). Despite being the brightest event in callus and

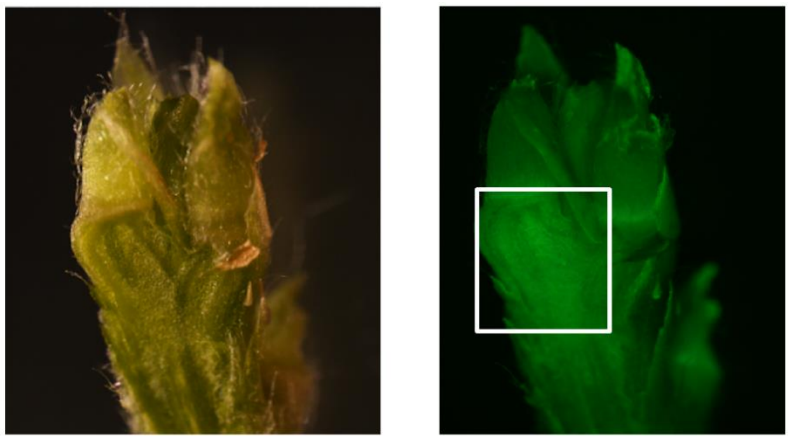

Figure A3: GFP fluorescence from *WUS* promoter. Shown is event 6. Meristematic tissue imaged in brightfield (left) and fluorescent (right).

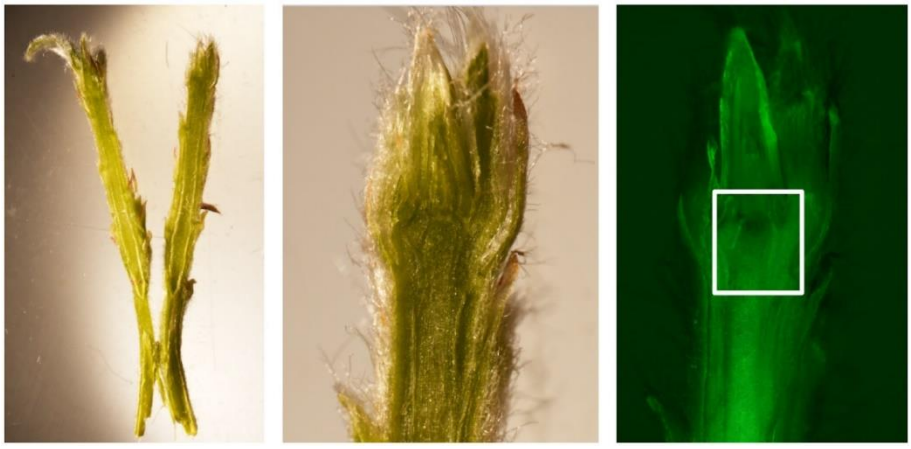

Figure A4: *WUS* Event 6. Entire shoot tip after vertical cut (left), brightfield closeup (middle), and fluorescent closeup (right).

shoots, event 38 did not display any GFP signal in meristematic tissue (Figure A5). The lack of

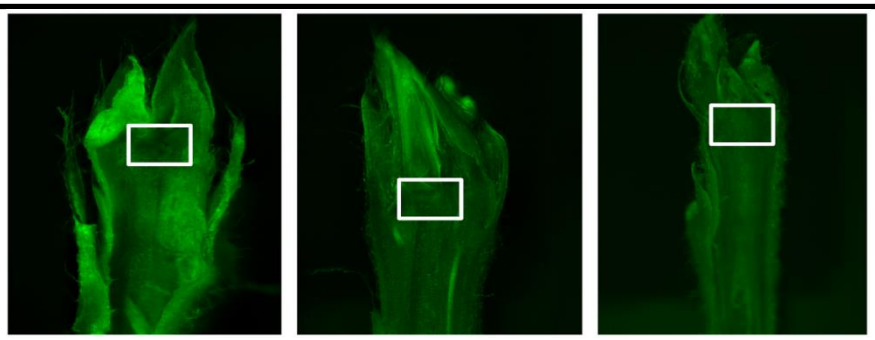

Figure A5: *WUS* Event 38. Three different shoot tip samples fluorescently imaged.

GFP signal in both *WUS* event 6 and 38 signifies that in later growth stages the *WUS* promoter construct does not have strong levels of activity in the meristem.

CSP3 events 6 and 11 were observed due to their high promoter construct activity in callus and shoot tissue. Shoot tips from event 6 and event 11 displayed promoter construct activity in all six of the examined meristems. Notably, individual cells in the organizing center could be seen due to the concentrated promoter activity in this region (Figure A6). *CSP3* consistently displayed strong promoter construct activity in the organizing center, even in small whole plants. The drastic difference between *WUS* and *CSP3* activity in later growth stages further supports the previous claim that *CSP3* is a more ideal promoter for controlling the Cre-Lox recombinase system.

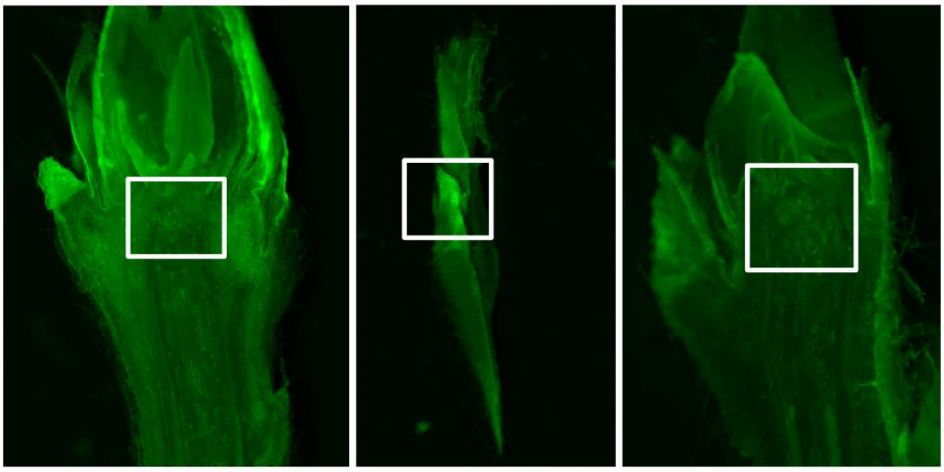

Figure A6: Most notable *CSP3* meristematic expression. Three different shoot tips fluorescently imaged. Event 6 (left), event 11 (middle, right).

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