UNIVERSITY OF CALIFORNIA SAN DIEGO

Investigating the Tissue-specific Role of the Circadian Clock in *Arabidopsis thaliana*

A Thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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2021

The Thesis of Ji Sun Chin is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Investigating the Tissue-specific Role of the Circadian Clock in *Arabidopsis thaliana*

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Ji Sun Chin

Master of Science in Biology

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Professor Jose L. Pruneda-Paz, Chair
Professor Martin F. Yanofsky, Co-chair

Plants, like many organisms, possess the ability to synchronize their biological processes with the diurnal conditions of the surrounding environment. This ability is largely controlled by an internal time-keeping mechanism known as the circadian clock. Clock function is intrinsic to each cell in the plant organism and at the same time is compartmentalized in the form of tissue-

specific clocks. In addition, previous studies have shown that these tissue-specific clocks are further organized in a hierarchical structure. Still, the tissue-specific role of core clock components is largely unknown and is the focus of ongoing research. In this thesis, we tested a previously constructed tissue-specific estradiol-inducible system in *Arabidopsis thaliana*, and we discovered results that suggested a need for modifying this system to yield more effective conditional tissue-specific gene expression. In addition, phenotyping experiments were conducted to test this systems capacity to overexpress core clock elements and it was observed that for certain clock genes, the timing of induction is an important factor that should be taken into consideration for future experiments utilizing similar conditional systems. Altogether, the approach outlined in this study poses a useful tool for not only elucidating the tissue-specific role of clock genes in plants, but also for investigating tissue-specific functions of any gene of interest.

Introduction

Plants, like many organisms on earth, have evolved to anticipate periodic environmental changes that occur due to Earth's rotation about its axis. Moreover, due to the sessile nature of plants, their survival and evolutionary success are heavily dependent on their ability to adapt to their surroundings as they are unable to avoid unfavorable environmental conditions. Therefore, it stands to reason that plants' reliance on their ability to synchronize their biological processes with the diurnal conditions of their environment is relatively greater compared to that of their animal counterparts (Nohales et al. 2016). Many higher plants, such as *Arabidopsis thaliana*, exhibit phototrophic behaviors, facilitated by differential growth patterns in response to photoreceptor activity in aerial tissues, to maximize their exposure to sunlight for photosynthesis (Pedmale et al. 2010). Plants also modulate stomatal apertures throughout the day to differentially control transpiration rates in efforts to minimize water loss and to maximize metabolic efficiency (Chavez et al. 2016). Given the status of climate change and the increasing prevalence of droughts across the globe, understanding the mechanisms underlying the control of transpiration will serve to be a critical factor in facilitating drought escape in the coming years of limited freshwater supply (Chavez et al. 2016, Xu et al. 2019). Likewise, these adaptations along with a multitude of other evolutionary traits exhibiting significant periodicity are subjects of great interest during these pressing times in which scientists are searching for new ways to adapt crops to changing environmental conditions. Further knowledge regarding such traits may be acquired by investigating the internal time-keeping mechanism known as the Circadian Clock: the system facilitating endogenous periodicity in plants and other living organisms (McClung 2006).

The Circadian Clock is composed of an intricate molecular oscillatory system that normally functions on a 24-hour cycle (McClung 2006). The molecular components that

comprise the clock are present in each cell in a complex network of regulatory feedback loops; understanding the specific interactions within these regulatory pathways remains a topic of major inquiry in the field. In the plant model *Arabidopsis thaliana,* there are several key components of the Circadian Clock that have been elucidated, but the main drivers of the clock have been identified as the morning genes (*CCA1, LHY*) and evening gene (*TOC1*). *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) both encode MYB transcription factors that peak at dawn and repress the expression of *TIMING OF CAB EXPRESSION 1* (*TOC1*), which is a pseudo-response regulator that peaks at dusk and works to subsequently inhibit the expression of *CCA1* and *LHY* in the evening (Caluwe et al. 2016). This fundamental transcriptional feedback loop along with other related feedback mechanisms allow plants to synchronize environmental inputs such as light and temperature with their intricate biological processes that have been fine-tuned for their survival (Caluwe et al. 2016).

Mis-regulating the Circadian Clock in plants hinders their ability to optimally coordinate their basic survival functions with their constantly changing environment and as a result can be a large detriment to overall plant fitness. For instance, overexpression of *CCA1* in Arabidopsis is characterized by a variety of unique phenotypes, including elongated hypocotyls, elongated petioles, reduced root growth, and decreased lamina size (Más et al. 2003; Strayer et al. 2000; Wang and Tobin 1998). *CCA1* overexpression (CCA1-OX) is known to hinder plants' ability to sense and respond to light and as a result, it leads to hyper-elongated hypocotyls as the growing seedling continues to proliferate hypocotyl tissue in efforts to search for more light exposure (Wang and Tobin 1998). Similarly, decreased lamina size also decreases the amount of exposure to sunlight which reduces photosynthetic productivity (Ruts et al. 2012)). As for the CCA1-OX root phenotype, the reduction in both primary and secondary root growth suggests that the

plants' ability to sense gravity is hindered by the mis-regulation of CCA1 and as a result prevents the roots from developing normally (Ruts et al. 2012).

The circadian clock exists in all plant cells; however, in complex organisms, these internal time-keeping mechanisms need to function across different cells and tissues to coordinate essential functions throughout the plant (Endo et al. 2014). Evidence has been shown that tissue-specific clocks are coupled together to maintain rhythmicity throughout the plant organism (Endo et al. 2014). Furthermore, recent studies have shown that tissue-specific clocks are also organized in a hierarchical fashion, in which clock function in certain tissues such as the shoot apex may have great influence on the clocks of other tissues like the roots, whereas the inverse may not hold true (Takahashi et al. 2015). Nonetheless, it is still largely unknown how alterations in certain tissue-specific clocks influence the physiology of other plant tissues.

To investigate tissue-specific mis-regulation of circadian clock genes in *Arabidopsis*, an experimental estrogen receptor-based conditional system was previously constructed to selectively overexpress core clock genes in specific organs of transgenic *Arabidopsis* lines. Non-oscillating organ-specific *Arabidopsis* promoters for the shoot apical meristem, root, and leaf tissues have previously been identified using expression data from a publicly available gene database, and those promoters were utilized to drive the tissue specificity of the conditional expression system employed in this study (Austin et al. 2016, Mockler et al. 2007). Plants carrying this conditional system were treated with an estradiol inducer at different developmental stages and screened for OX phenotypes. Previous studies have demonstrated that clock function in Arabidopsis plants can be detected as early as 2 days after seed imbibition (Salomé et al. 2008). Therefore, part of our investigation will involve testing how overexpressing one of the

core clock genes, *CCA1*, during various stages in early development will influence the emergence of hypocotyl elongation, a well-known CCA1 OX phenotype.

Previous studies have reported that this estradiol-inducible system is very robust and there are not many reported instances of non-specific or leaky expression patterns (Brand et al. 2006, Zuo, Niu, and Chua 2000). Nonetheless, the same reports have stated that aberrant expression patterns, although rare, can result from the estradiol-inducible system. Therefore, one of the primary objectives of this study will be to elucidate any adverse gene expression patterns that arise from the previously constructed tissue-specific estradiol-inducible system.

A single vector configuration for most inducible systems in plants have displayed various complications that may pose significant difficulties in producing viable expression lines. First, positional effects following Agrobacterium Transformation may lead to variable expression for both driver and effector components (Borghi 2010). Also, it has been shown that high expression levels of the driver component can lead to post-transcriptional gene silencing and nonspecific binding which may result in unpredictable downstream effects for the system as well as the overall development of the plant (Borghi 2010). Furthermore, GFP Reporter studies utilizing a single vector estradiol-inducible system in *Arabidopsis* have shown that some transgenic lines display a nonuniform pattern of expression as well as no expression at all in rare cases (Zuo, Niu, and Chua 2000). These issues may be circumvented by adopting a binary system approach in which the driver and effector components are encoded by two separate vectors that are independently introduced into wild type lines. Therefore, following transformation, these driver and responder lines can be individually examined for optimal activity, thus minimizing aberrant expression patterns in plants carrying both elements of the inducible system (Borghi 2010).

In this study, we first demonstrated the capacity of the single vector estradiol-inducible approach to facilitate tightly controlled tissue-specific gene overexpression in *Arabidopsis*. We have accomplished this by conducting a series of β-glucuronidase (GUS) reporter assays that screened inducible plants for aberrant expression phenotypes. The results of these experiments further validated the motivations for pursuing a binary variation of the estradiol-inducible system. Additionally, we also demonstrated the importance of the treating plants early on in development to generate maximal CCA1 OX phenotypes in whole plant inducible lines. Altogether, the findings of this project should help set the foundation for how future investigations utilizing the split estradiol-inducible system should be carried out to study tissue-specific expression of *Arabidopsis* genes.

Results

β-glucuronidase (GUS) assay confirmed tissue specificity of a root-specific promoter.

This experiment was performed as part of a larger experiment aimed toward confirming the tissue specificity of previously identified promoters that were reported on an online database to display tissue-specific expression patterns that remain constant throughout the day. In order to confirm the tissue-specificity of one of these promoters, previously identified as a root-specific promoter (AT5G47450pro), the AT5G47450 gene promoter was cloned into a binary vector carrying the *β-glucuronidase (GUS)* gene known as pBI101.1 (Figure 1A). The GUS reporter gene allows for visualization of the intensity as well as localization of the AT5G47450 promoter activity. GUS staining protocol was previously optimized for transgenic Arabidopsis lines carrying the AT5G47450pro::GUS construct in order to minimize nonspecific GUS staining. Eight homozygous independent transgenic lines were generated and stained for GUS activity at 8 days old, and two representative lines were grown to various ages (4, 8, and 16 days post-germination) and stained to analyze the GUS expression pattern across different developmental stages. The images shown were taken from one of these representative lines (Figure 1B).

Plants carrying the AT5G47450pro::GUS construct displayed localized GUS activity in the roots starting at 4 days old. Throughout the three different developmental stages, GUS reporter activity is present in all root tissues, except the region surrounding the root meristem (Figure 1B). In addition, it was observed in the 4 days old plant that there seemed to be weak GUS signal in the leaf, shoot apex, and hypocotyl regions. However, the same nonspecific signal was not observed in either the 8 days old or 16 days old plants (Figure 1B). It should also be noted that out the eight transgenic lines that were stained for this root-specific construct, seven lines showed localized GUS signals in the roots to varying degrees, while one of the lines

showed no signal throughout the entire plant. Lastly, no GUS signal was observed for any of the wild type Col-0 control plants that were subject to GUS staining treatment alongside the transgenic lines (Figure 1B).

These results effectively completed previous work done to investigate the expression patterns of non-oscillatory tissue-specific promoters in *Arabidopsis*, and it was confirmed that all six identified promoters, which consist of the shoot apex gene promoters (AT3G59270, AT3G50170, AT5G59330), root gene promoters (AT3G01190, AT5G47450), and leaf gene promoter (AT3G16670), displayed localized activity in their respective tissue types in 8 days old Arabidopsis. This information set the precedent for future GUS reporter assays for the conditional tissue specific gene overexpression system.

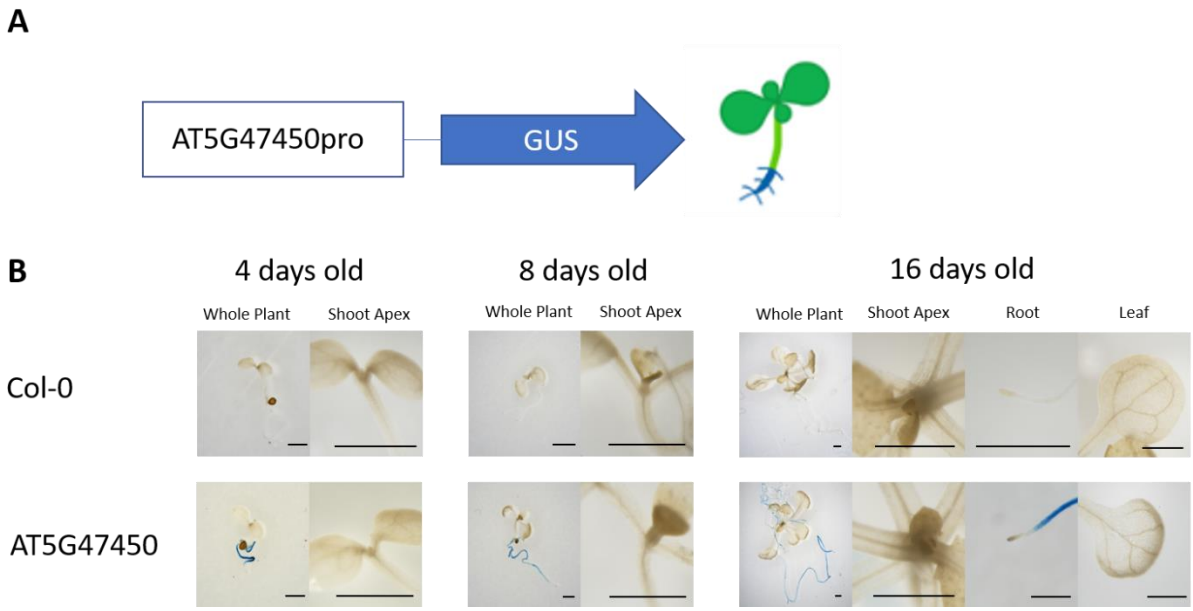


Figure 1. β-glucuronidase (GUS) assay result for root specific promoter driving the expression of the GUS reporter Gene. A) Graphic illustrating the assembly of the implemented reporter construct as well as the hypothesized outcome of the subsequent GUS assay. B) Arabidopsis plants carrying the *β-glucuronidase* gene driven by a root specific promoter AT5G47450 were stained at 3 different developmental stages (4, 8, 16 days post-germination). Images of whole plants as well as those focused on specific tissues are displayed and each scale bar is equivalent to 1mm.

Aberrant β-glucuronidase (GUS) expression was observed using a single vector conditional tissue-specific overexpression system in *Arabidopsis*.

To induce the overexpression of genes in a spatially and temporally controlled manner, an estrogen receptor-based vector (pMDC7) was utilized. Transgenic *Arabidopsis* plants carrying this vector can be treated directly with estradiol to induce gene expression. This system encodes a chimeric transcription activator known as XVE, which is composed of the DNA-binding domain of a bacterial repressor LexA (X), the activation domain of VP16 (V), and the ligand-binding domain of the human estrogen receptor (E) (Zuo, Niu, and Chua 2000). The expression of XVE is controlled by a constitutive promoter (G10-90) and XVE remains in the cytosol until it binds to estradiol and localizes to the nucleus. Once in the nucleus, XVE activates the inducible LexA promoter and induces the expression of a target gene of interest (Figure 2A). This inducible system was modified by cloning non-oscillating, tissue-specific promoters upstream of the region encoding XVE, such that XVE would only be present in tissues where the promoter is active. Therefore, following external estradiol treatment, plants carrying the tissue-specific pMDC7 constructs should only express the gene of interest in their respective tissues.

To investigate the efficacy of this conditional system, transgenic *Arabidopsis* lines carrying tissue-specific pMDC7 constructs driving the expression of the β-glucuronidase (*GUS*) reporter gene were screened for any abnormal GUS phenotypes. Homozygous lines for two shoot apex-specific (pMDC7-59330-GUS, pMDC7-50170-GUS) and two root-specific (pMDC7-01190-GUS, pMDC7-47450-GUS) estradiol-inducible constructs were tested and they displayed a variety of phenotypes, most of which deviated from the expected conditional and tissue-specific outcome. A few lines with the expected GUS signal were identified for one of the shoot apex-specific constructs (pMDC7-59330-GUS) and both root-specific constructs (Figure 2B). In the shoot apex-specific lines that worked well, blue coloration indicative of GUS activity is

localized in the shoot apex only when plants were treated with estradiol (Figure 3A). Similarly, for both root-specific constructs, GUS signal is evident only in the roots, excluding the root meristem, when the plants are treated with estradiol (Figures 5A, 6A).

On the other hand, all tissue-specific estradiol-inducible constructs displayed a significant degree of aberrant expression patterns. Some root-specific lines displayed a strictly leaky phenotype, for which a strong GUS signal can be observed in only the roots for the estradiol treatment condition, but a similar root-localized signal, although significantly weaker, is seen to leak through in the absence of estradiol treatment (Figures 2B, 5B). In addition, a significant proportion of one of the shoot-apex specific constructs (pMDC7-AT3G50170-GUS) as well as one root-specific construct (pMDC7-47450-GUS) displayed strictly nonspecific expression patterns (Figure 2B). For cases exhibiting this strictly nonspecific phenotype, there was no GUS signal throughout the plant in the absence of estradiol; however, in the treatment condition, GUS signal was detected in non-target tissues (Figures 4B, 6B).

Furthermore, for all four tissue-specific estradiol-inducible constructs that were tested, there seemed to be a common nonspecific and leaky dual phenotype, characterized by nonspecific expression patterns that occur with and without estradiol treatment (Figures 2B, 3B-C, 4C, 5C, 6C). It should also be noted that within this nonspecific and leaky phenotypic category there exists additional variation in expression patterns. For some lines, the same GUS signal pattern is seen in both treatment and no treatment conditions (Figures 4C, 6C). On the contrary, a couple lines from one of the shoot apex-specific (pMDC7-59330-GUS) and root-specific (pMDC7-01190-GUS) constructs show nearly no leaky signal in their target tissues whereas the nonspecific signal tends to leak in the no treatment condition (Figures 3C, 5C). Also, there are even other lines, such as the one observed for the shoot apex-specific construct

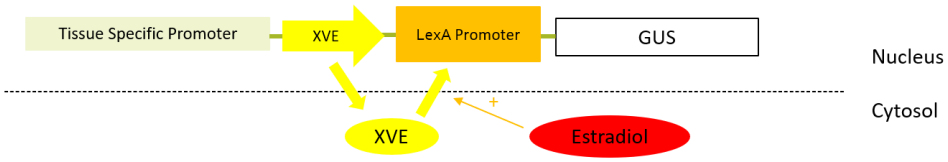
pMDC7-59330-GUS, in which the nonspecific signal does not leak but the target signal does (Figure 3B).

It should also be noted that for both shoot apex-specific constructs, several lines displayed no visible GUS activity even after estradiol treatment (Figures 2B, 4A). This result does not necessarily suggest that *GUS* induction failed for these lines as it may be possible that the induced GUS signal may have simply been too weak to be macroscopically visible.

To further contribute to these aberrant results, whole plant estradiol-inducible-GUS (pMDC7-GUS) lines were also screened for any unexpected phenotypes. Some pMDC7-GUS lines showed the expected phenotype, displaying strong GUS signal throughout all tissues only in the estradiol treatment condition (Figure 7A). However, several other lines showed aberrant phenotypes comparable to those observed in the tissue-specific estradiol-inducible lines. A few lines possessed a leaky phenotype in the absence of estradiol treatment (Figure 7B). Other lines exhibited incomplete GUS signal in which not all tissues were stained in the estradiol treatment group which can be likened to the strictly nonspecific phenotype that was described previously (Figure 7C).

Altogether, these GUS-reporter results suggest that the single vector pMDC7 conditional system as previously constructed is innately flawed as it produces a variety of unexpected phenotypes in *Arabidopsis* plants in both the whole plant and tissue-specific variations. It is clear certain modifications must be made to this system to facilitate tightly controlled tissue-specific gene overexpression that can be later applied to overexpress core clock genes in *Arabidopsis.*

A



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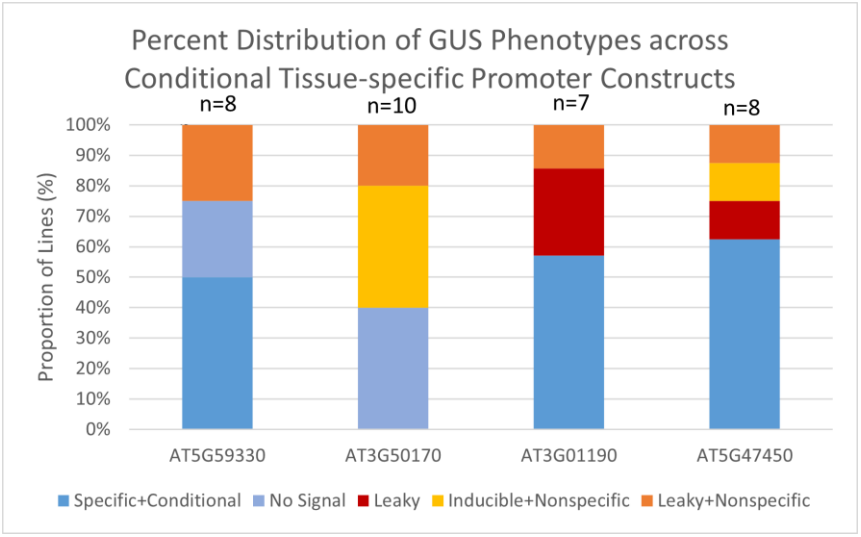

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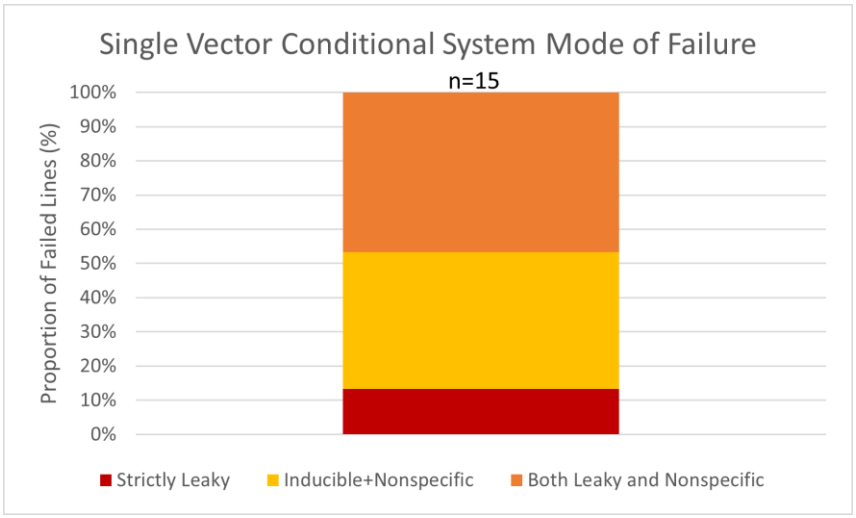


Figure 2: Construction and validation of conditional tissue-specific gene overexpression system. A) Schematic of the estradiol-inducible pMDC7 construct displaying the mechanism regulating the induction of gene expression. B) GUS reporter assay results showing distribution of phenotypes observed in homozygous transgenic lines across all estradiol-inducible pMDC7 constructs C) Graph summarizing the distribution of the aberrant phenotypes observed across transgenic *Arabidopsis* lines carrying the tissue specific pMDC7 GUS constructs.

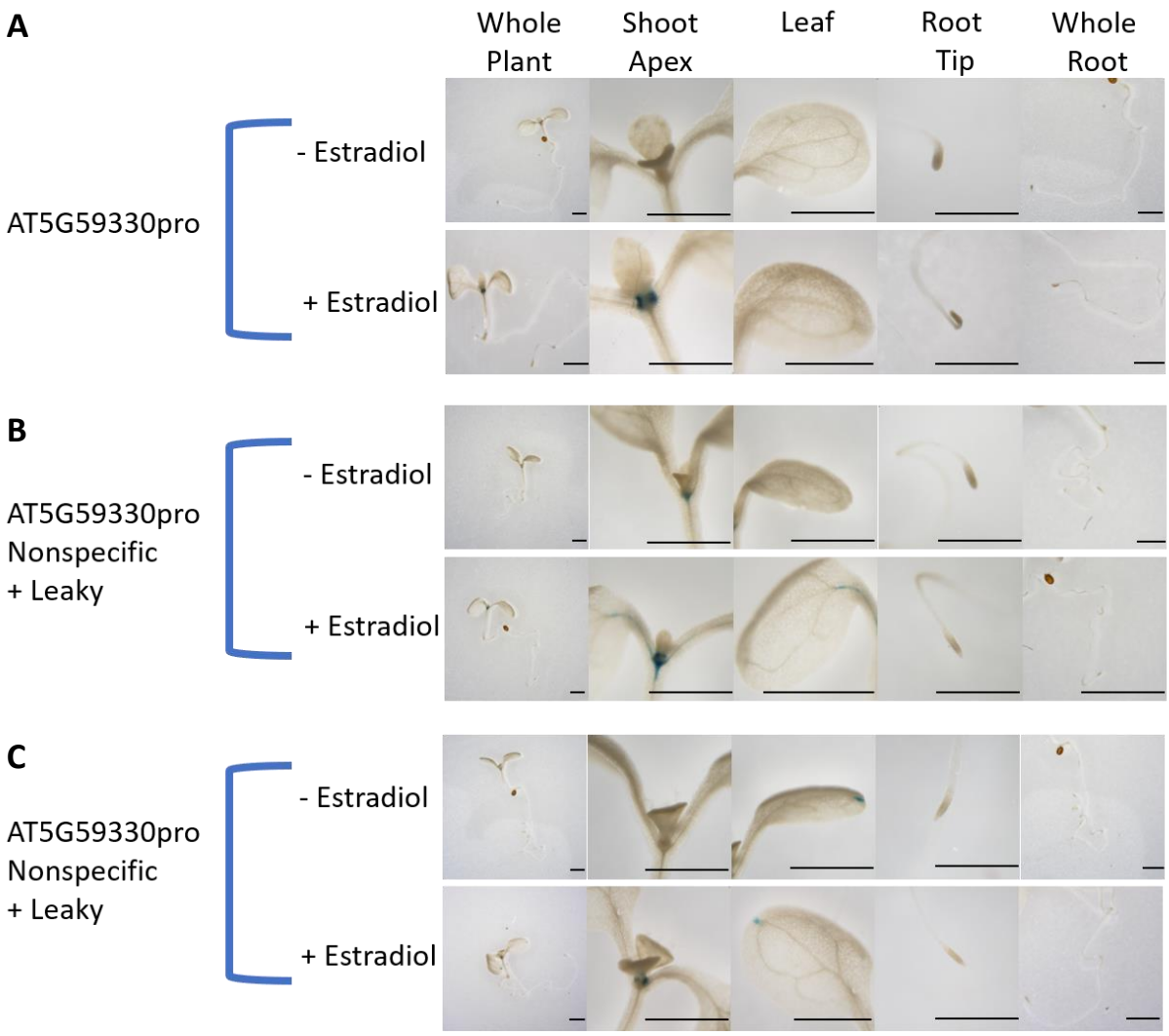


Figure 3. β-glucuronidase (GUS) assay results of *Arabidopsis* lines carrying the conditional shoot apex-specific pMDC7-AT5G59330-GUS construct. Transgenic *Arabidopsis* lines carrying the pMDC7-AT5G59330-GUS construct were treated with Estradiol-containing medium and harvested for GUS staining. Images of whole seedlings and specific tissues were taken for three representative lines: A) one line displaying the expected phenotype B-C) two lines displaying both nonspecific and leaky aberrant phenotypes. Each scale bar represents 1mm in length.

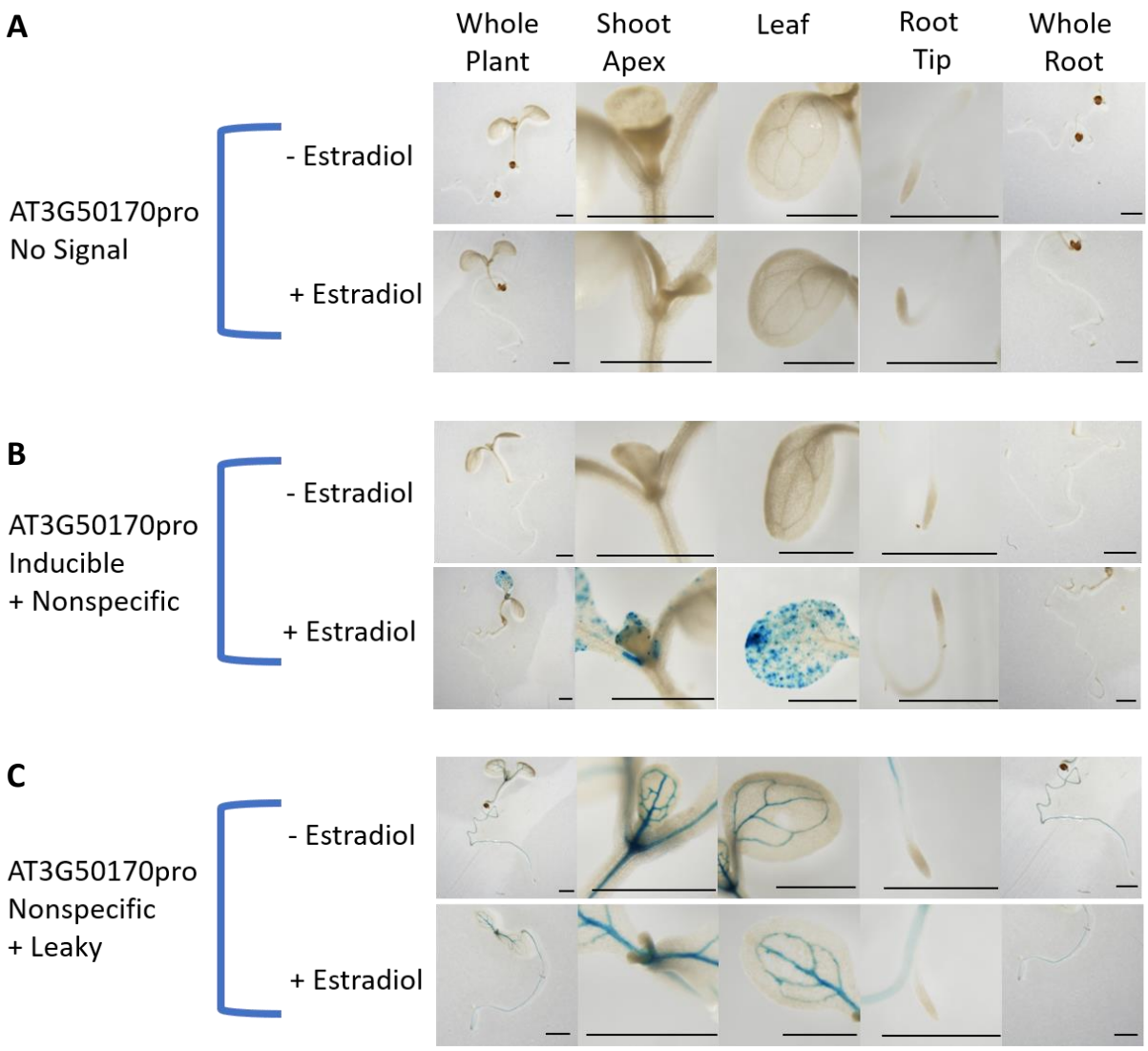


Figure 4. β-glucuronidase (GUS) assay results of *Arabidopsis* lines carrying the conditional shoot apex-specific pMDC7-AT3G50170-GUS construct. Transgenic *Arabidopsis* lines carrying the pMDC7-AT5G50170-GUS construct were treated with estradiol-containing medium and harvested for GUS staining. Images of whole seedlings and specific tissues were taken for three representative lines, all of which display various aberrant phenotypes: A) no signal B) leaky signal C) nonspecific signal. Each scale bar represents 1mm in length.

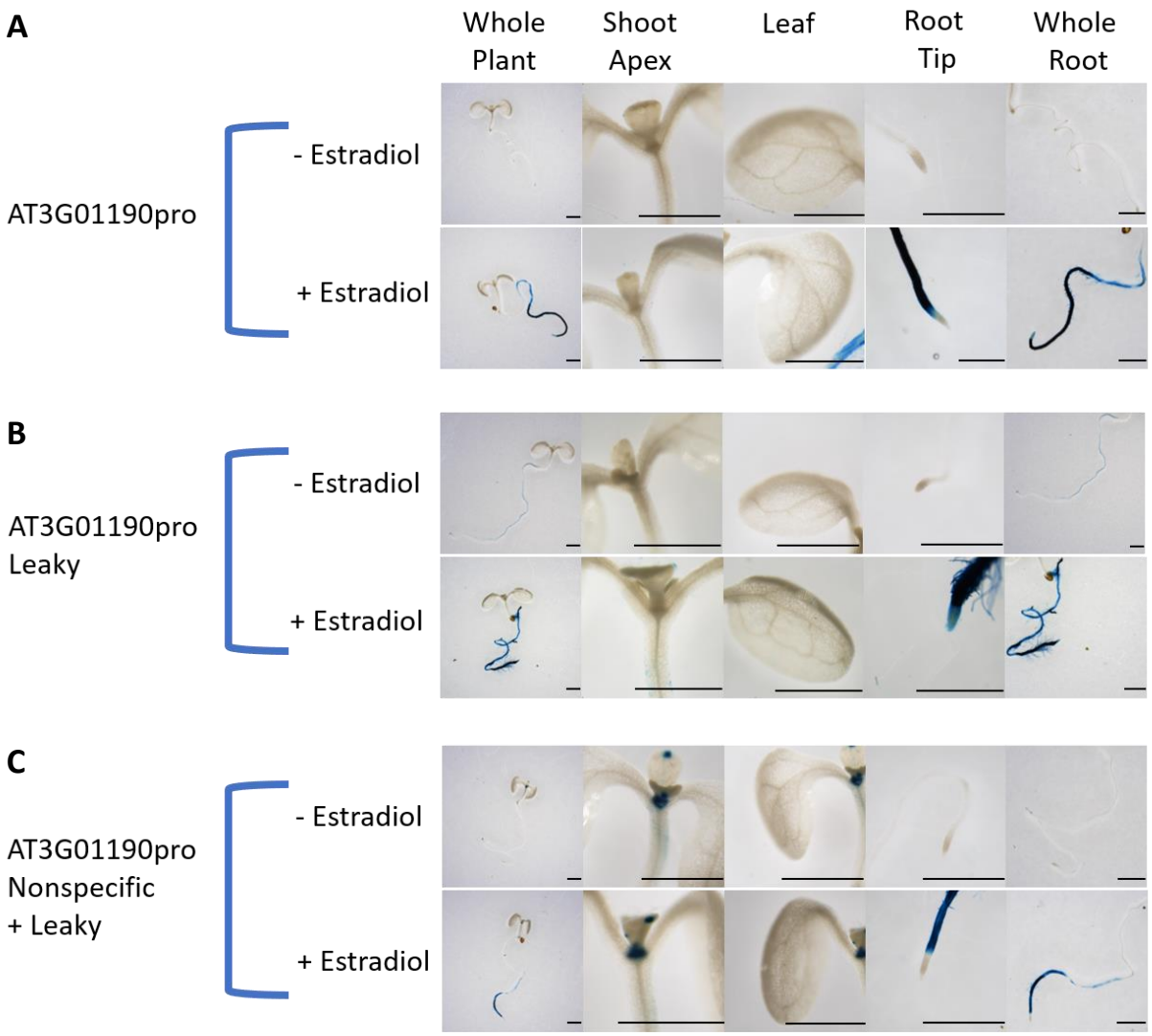


Figure 5. β-glucuronidase (GUS) assay results of *Arabidopsis* lines carrying the conditional root-specific pMDC7-AT3G01190-GUS construct. Transgenic *Arabidopsis* lines carrying the pMDC7-AT3G01190-GUS construct were treated with Estradiol-containing medium and harvested for GUS staining. Images of whole seedlings and specific tissues were taken for three representative lines: A) one line displaying the expected phenotype B) one line with a leaky phenotype C) one line with a both nonspecific and leaky phenotype. Each scale bar represents 1mm in length.

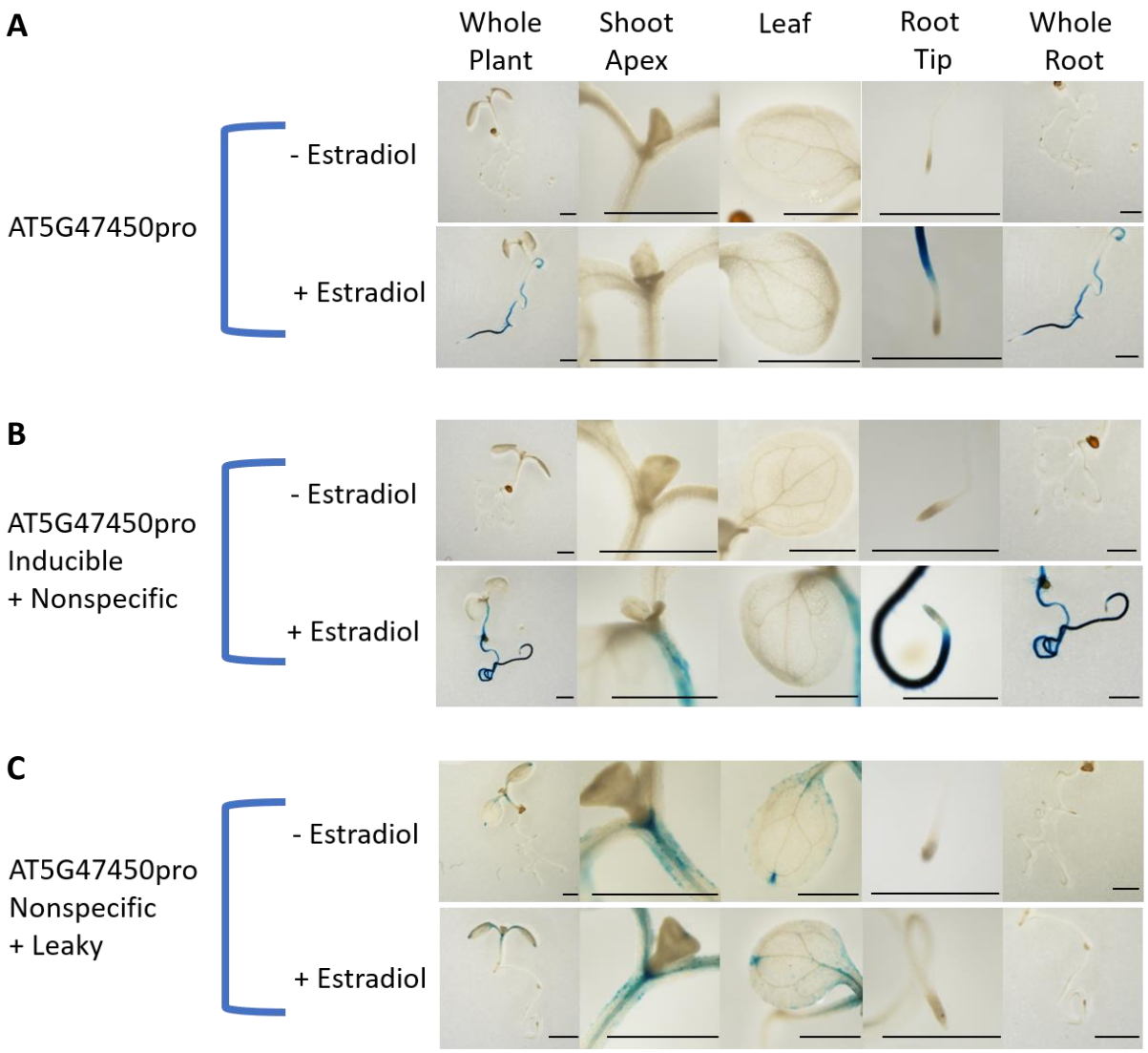


Figure 6. β-glucuronidase (GUS) assay results of *Arabidopsis* lines carrying the conditional root-specific pMDC7-AT5G47450-GUS construct. Transgenic *Arabidopsis* lines carrying the pMDC7-AT5G47450-GUS construct were treated with Estradiol-containing medium and harvested for GUS staining. Images of whole seedlings and specific tissues were taken for three representative lines: A) one line displaying the expected phenotype B) one line with a nonspecific phenotype C) one line with a both nonspecific and leaky phenotype. Each scale bar represents 1mm in length.

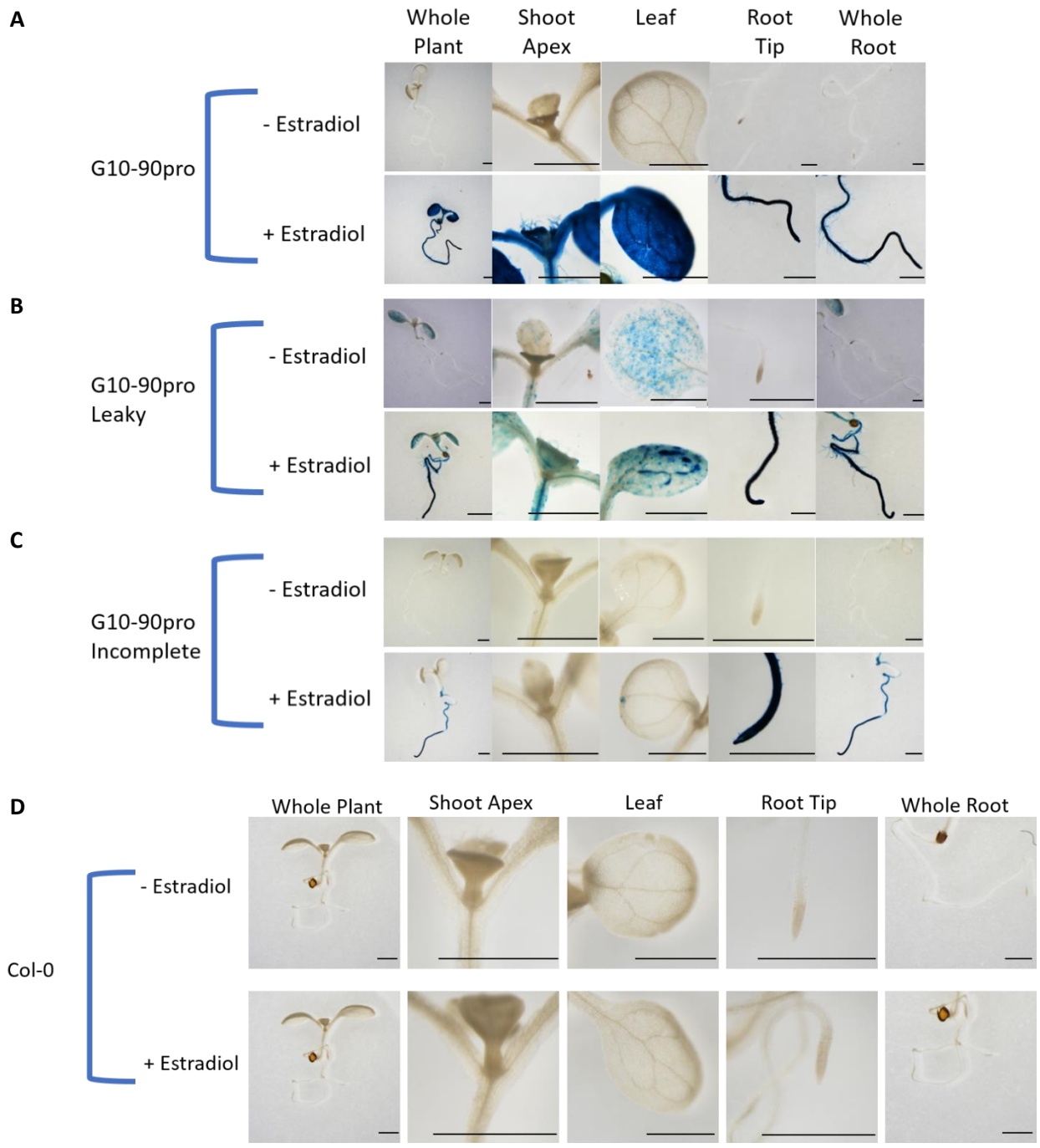


Figure 7. β-glucuronidase (GUS) assay results for whole plant Estradiol-Inducible-GUS lines and Col-0 control A) Transgenic homozygous *Arabidopsis* lines carrying the pMDC7 GUS construct were stained for GUS activity following estradiol treatment. Images of whole seedlings and specific tissues were taken for three representative lines: A) one of displaying the expected phenotype B) one displaying an aberrant leaky phenotype C) one displaying an aberrant incomplete phenotype. D) Wildtype Col-0 plants were treated with Estradiol and stained alongside all estradiol-inducible transgenic lines. Each scale bar represents 1mm in length.

Optimization of Estradiol Treatment Protocol

In the initial GUS assay results of the previously described conditional system, several shoot apex-specific lines displayed no visible signal and some whole plant estradiol-inducible lines also showed patchy expression patterns throughout the aerial tissues (Figures 4A, 7A). We suspected that the estradiol medium treatment method may not be providing optimal penetration in the aerial tissues, so we hypothesized that spraying the plants directly with estradiol would allow greater infiltration of estradiol in the regions that previously displayed weak signal. In addition, we also hypothesized that increasing the incubation time for estradiol treatment would result in stronger GUS signals given that previous studies have described that reporter gene activity peaks between 24-48 hours of incubation with estradiol (Brand et al. 2006). We performed an experiment with a whole plant estradiol-inducible line that previously displayed good results (Figure 7A) and we subjected plants from the same lineage to four different estradiol treatment combinations prior to GUS staining to test the differential *GUS* expression patterns from spray vs medium treatment and 24 vs 48 hr incubation time.

Overall, the GUS assay results showed that spraying the plants with estradiol led to stronger GUS signal on average than the medium treatment, and that incubating the plants for an additional 24 hours leads to stronger GUS signals for both spray and medium treatment methods (Figure 8). Although the differences observed across the treatment conditions aren't extreme, they may very well contribute to the weak GUS expression patterns that are prevalent throughout the estradiol-inducible lines and we propose that plants should be treated by the spray method and incubated for 48 hours prior to phenotypic screening to optimize reporter activity in the aerial tissues.

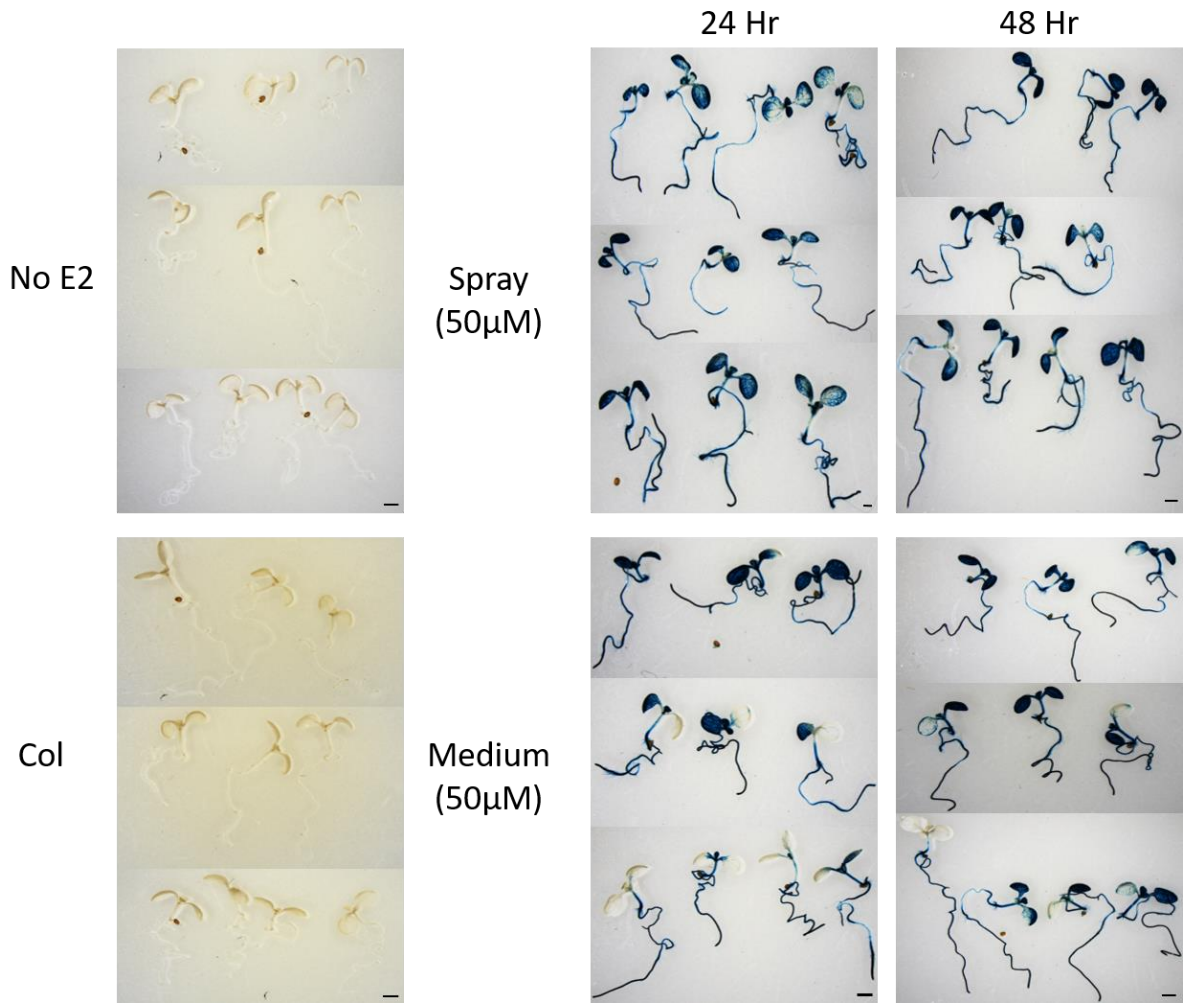


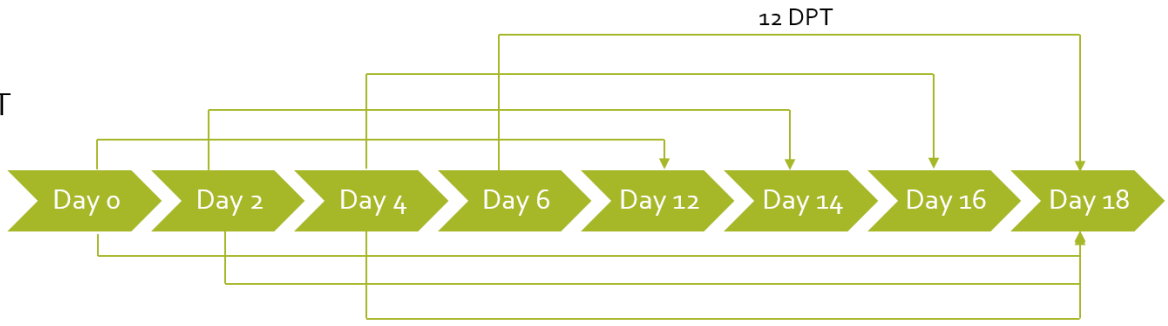
Figure 8. β-glucuronidase (GUS) assay results of homozygous *Arabidopsis* transgenic plants carrying pMDC7 GUS construct following Incubation with Estradiol for 24 and 48 hours using both spray and medium treatment methods.
A) Images displaying β-glucuronidase (GUS) assay results for four sets of Arabidopsis plants carrying the conditional pMDC7 GUS construct that were treated under different estradiol incubation times (24hrs and 48hrs) as well as different delivery methods (spray and medium treatment). Plants were treated with 50µM estradiol in all treatment conditions and all plants were harvested at 8 days old for GUS staining. Each scale bar represents 1mm in length.

Conditional Overexpression of CCA1 in *Arabidopsis* resulted in varying degrees of hypocotyl elongation depending on timing of CCA1 induction.

To investigate this estradiol-inducible system's capacity to overexpress circadian clock genes, whole plant estradiol-inducible-CCA1 (pMDC7-CCA1) *Arabidopsis* lines were treated with estradiol at various developmental stages (0, 2, 4, 6 days post-germination) and the resulting plants were screened for the elongated hypocotyl phenotype commonly observed in CCA1 OX lines. Two sets of plants were grown for each treatment condition as well as for the no treatment, CCA1 OX, and wildtype Col-0 controls. One set was harvested for hypocotyl measurement at 12 days post-treatment and 12 days post-germination for the control plants. The other set was harvested at 18 days post-germination to account for differences in hypocotyl length determined by differences in developmental stage (Figure 9A).

Based on the following results, there seems to be a significant difference in the degree of hypocotyl elongation depending on the timing of estradiol treatment. The day 0 treatment group (18 DO) displayed significantly longer hypocotyls than the day 6 treatment group (18 DO), and hypocotyl lengths for all treatment groups were significantly higher than that of the no treatment control (Figure 9B). It should also be noted that marginal differences were observed between the plants in different developmental stages once the plants reached 12 days, so even though the different treatment groups were observed at various developmental stages (12, 14, 16, 18 days post-germination), it does not likely invalidate the overall trend in hypocotyl length across the different treatment times (Figure 9B). Also, no treatment group produced longer hypocotyls than the CCA1 OX control, which suggests that the estradiol treatment regimen requires further optimization to reach high levels of gene overexpression (Figure 9B).

A



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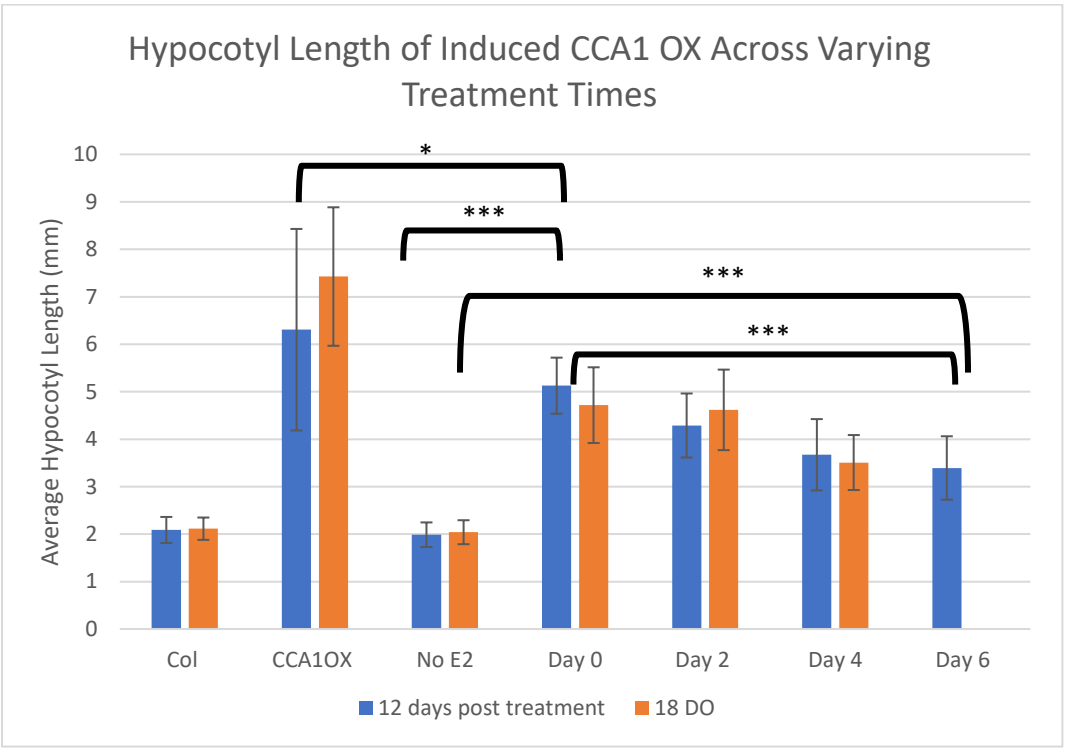

Figure 9. Elongated hypocotyl phenotype analysis for whole plant estradiol-inducible-CCA1 *Arabidopsis* line subject to estradiol treatment at various developmental stages. A) Schematic describing when plants were harvested for hypocotyl measurement. *Arabidopsis* plants from a homozygous whole plant estradiol-inducible-CCA1 line were treated with estradiol at different developmental stages (0, 2, 4, 6 days post-germination). Two replicates were grown for each treatment group: one was harvested at a fixed time after treatment (FTAT) at 12 days post-treatment and the other was harvested at a fixed developmental stage (FDS= 18 days post-germination). Col-0 and CCA1 OX lines were grown in parallel as controls. B) Mean bar graph showing the average measured hypocotyl length of each whole plant estradiol-inducible-CCA1 replicate (n=20). Statistical significance was determined by a 2-tailed T-Test (*p<=0.05, ***p<=0.001).

Development of split conditional tissue-specific gene overexpression system for *Arabidopsis.*

By splitting the previously utilized pMDC7 conditional system into independent driver and effector constructs, we aim to establish a more effective method of generating transgenic *Arabidopsis* lines that display tight regulation of conditional gene overexpression in specific tissues. The effector construct, which will be referred to as pMDC3.5, contains the same elements, including the Hygromycin resistance marker, of the original pMDC7 construct but encodes only the effector component (LexA-Gene of Interest). On the other hand, the driver construct, referred to as pG229-XVE, which encodes the BASTA selection marker, contains only the driver component (promoter-XVE). Similar to the previous system, pMDC3.5 contains gateway cloning sites so any gene of interest can easily be cloned into this effector construct. For the purpose of future studies investigating the tissue-specific role of core circadian clock genes, pMDC3.5 effector constructs for *CCA1*, *TOC1*, and *LHY* were generated in addition to the pMDC3.5-GUS reporter construct. As for the driver constructs, promoters from previously characterized tissue-specific genes (AT3G59270, AT3G16670, AT3G01190) as well as the oscillatory CCA1 promoter and constitutive G10-90 promoter were cloned into the pG229-XVE vector.

Both sets of Driver and Effector constructs will be independently transformed into wildtype *Arabidopsis* plants. pMDC3.5 GUS reporter lines can be stained without estradiol treatment to screen for leaky GUS signal in the homozygous T3 generation. We could then cross those pMDC3.5 GUS lines displaying no leaky phenotype to the various homozygous T3 driver lines. The progeny of those crosses will then be treated with estradiol and stained for GUS activity, and F1 lines displaying no aberrant phenotypes would be traced back to their parent driver lines to identify optimal driver lines for future crosses with different effectors (Figure 10).

In the previous pMDC7 conditional system, we observed three categories of aberrant expression patterns for which we have arrived at multiple hypotheses that provide potential explanations for these results. First, when we observe strictly leaky phenotypes, we suspect that either the LexA promoter in the effector component may display leaky activity due to positional effects, or it could be the case that similar positional effects may be causing the driver component to exhibit high levels of activity, leading to oversaturation of XVE in the cytosol and subsequently causing XVE to migrate into the nucleus in the absence of estradiol. Previous GUS assay results for Arabidopsis carrying the pMDC3.5 GUS effector construct reveal that leaky GUS signal is relatively uncommon and is only seen in 20% of the investigated T1 lines, and in addition, the intensity of the GUS signal in those leaky lines is also relatively low (Figure 11). This result suggests that positional effects on the effector component is likely not a large contributor to the overall leaky phenotype that was previously observed in the pMDC7 system. Therefore, we expect that the driver component is more susceptible to influence from positional effects and we maintain support for splitting the system to isolate optimal driver lines for generating non-leaky expression patterns.

Secondly, we also observed strictly nonspecific phenotypes in the tissue-specific pMDC7-GUS lines, and this aberrant phenotype is most likely due to positional effects on the driver component of the pMDC7 system. It is possible that in some cases the expression of XVE may be enhanced due to nearby cCREs, and this increase in XVE expression may oversaturate the cells with XVE and may allow XVE to travel within the plant to off-target tissues. It could also be the case that in some instances the selected tissue-specific promoters may not behave in a tissue-specific manner due to positional effects, but since nonspecific phenotypes were not observed in the previous GUS reporter studies investigating the same tissue-specific promoters,

the nonspecific signal that was observed in the estradiol-inducible pMDC7 lines are most likely not due to faulty tissue-specific promoter activity (Figure 1).

Lastly, we consistently observe lines displaying both leaky and nonspecific phenotypes for all tissue-specific pMDC7 constructs. This could be due to a combination of positional effects on both driver and effector components as previously described, or it could also be primarily due to leaky LexA promoter activity, which would theoretically result in both leaky nonspecific expression patterns as portrayed in previous GUS assay results with T1 pMDC3.5 GUS lines (Figure 11). However, as previously mentioned, leaky activity of the LexA promoter was rarely observed in the T1 generation and so it seems unlikely that the LexA promoter is largely responsible for the nonspecific and leaky dual phenotype. In conclusion, regardless of which hypotheses hold true, the proposed split system method allows one to filter out driver and effector lines that produce any of the three aforementioned aberrant phenotypes and increase the chances of generating transgenic *Arabidopsis* plants that display tightly controlled induced overexpression of genes in specific tissues.

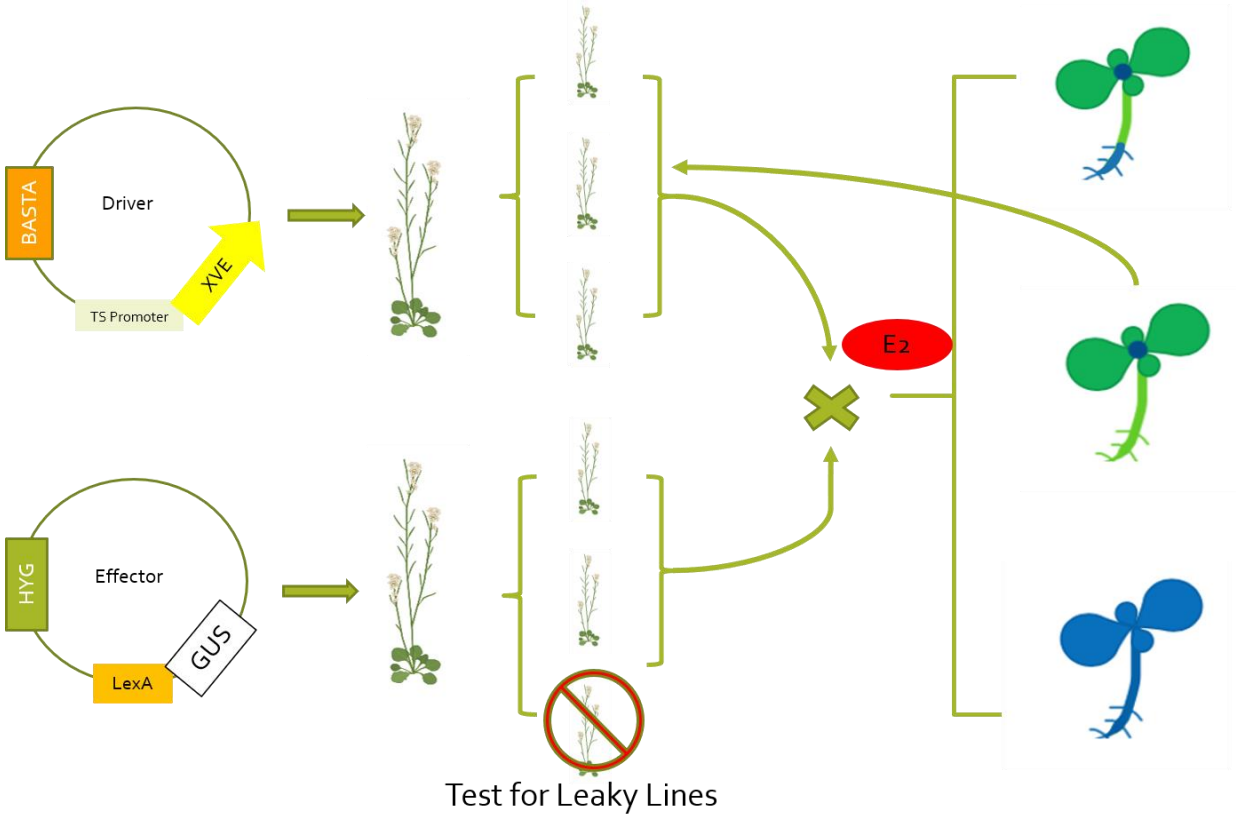

Figure 10. Split conditional gene overexpression system schematic. Graphic describing the proposed plan for splitting the previously investigated pMDC7 estradiol-inducible system.

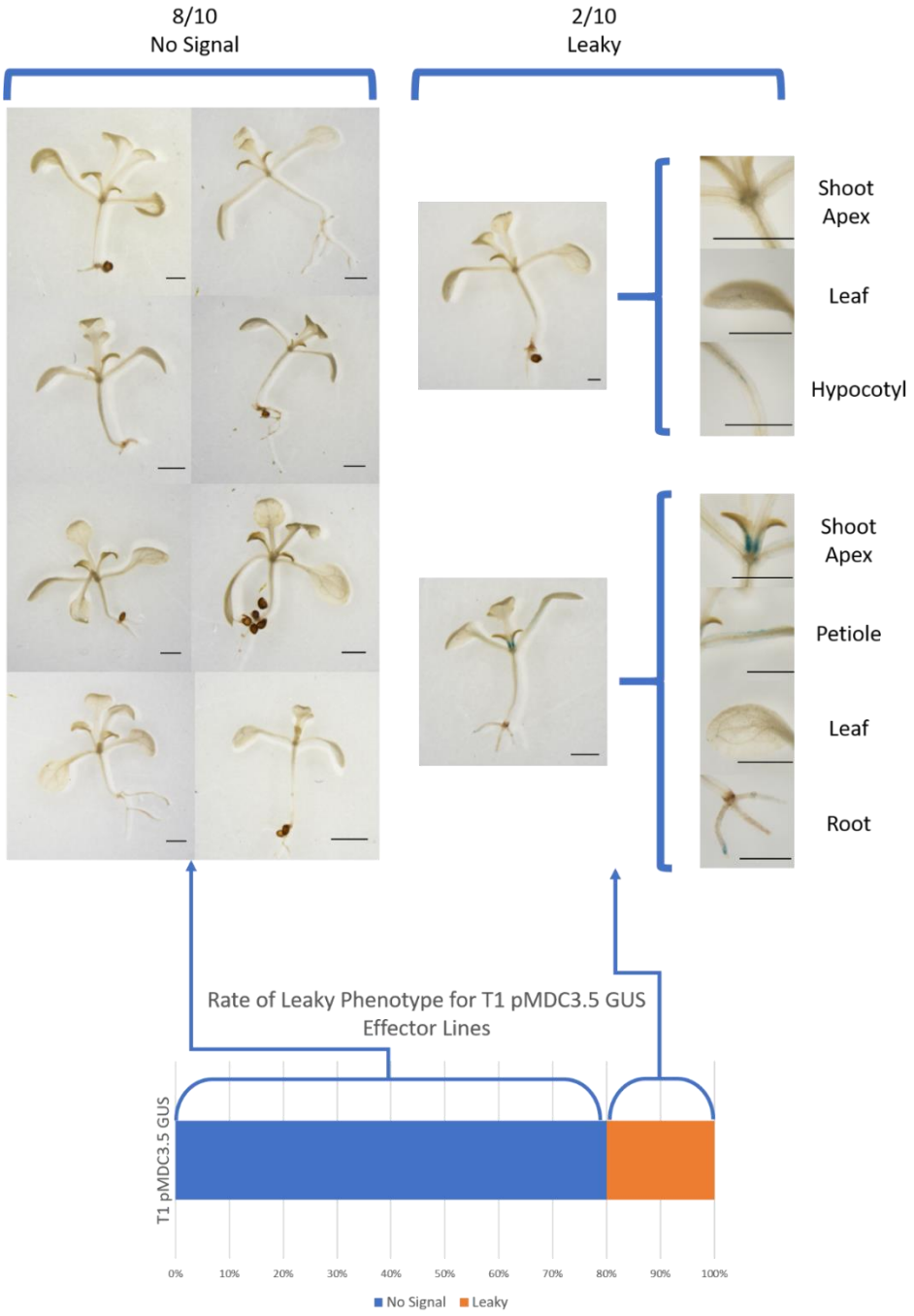

Figure 11. β-glucuronidase (GUS) assay results of T1 *Arabidopsis* lines carrying the pMDC3.5 GUS effector construct. Images displaying GUS assay results for transgenic lines in the T1 generation carrying the inducible LexA-GUS effector construct. 10 lines were tested, and images are split into two categories based on GUS phenotype. For lines displaying a leaky phenotype, additional images focusing on specific tissues are shown. Each scale bar represents 1mm in length.

Discussion

Systems designed to effectively overexpress genes in a spatiotemporally controlled manner are highly sought after in the field of biology for their capacity to investigate gene function in the context of complex organisms, whose biological processes vary throughout development. The β-estradiol-inducible system is one of several inducible systems that have been utilized in *Arabidopsis* that has demonstrated the ability to induce gene overexpression in specific loci given that appropriate tissue-specific promoters are used. This system is also simple to modify with different tissue specific promoters and various genes of interest which further increases its utility in studying gene function.

In this study, we corroborated favorable findings from previous research that utilized estradiol-inducible systems to induce gene expression in specific tissues of *Arabidopsis* plants. However, we also identified significant aberrant expression patterns produced by the single vector approach. For all estradiol-inducible constructs that were screened for GUS reporter activity in this study, they displayed abnormal phenotypes including leaky and nonspecific expression patterns. Some lines even exhibited no apparent GUS signal at all which could suggest that in those cases the inducible cassette was inserted in a non-transcribed region of the genome, but the prevalence of no visible expression patterns exceeded what has been reported in previous literature (Brand et al. 2006, Zuo, Niu, and Chua 2000). Therefore, we suspect that our method of analyzing GUS signal may not be sensitive enough, and we propose that for future reporter assays, cross sections of tissues should be taken to better detect weaker GUS signals that may not be visible microscopically.

Nevertheless, the aberrant phenotypes presented by the single vector variation of the estradiol-inducible system suggests that certain modifications must be made to the currently

constructed system to minimize aberrant expression patterns for future experiments. Therefore, we designed a new split system approach that would better accommodate the aberrant expression phenotypes that were observed in the single vector scenario. In *Arabidopsis* as well as other plant organisms, the position of T-DNA integration is known to significantly affect the expression of transgenes (Gelvin et al. 2017). As a result, we hypothesized that the aberrant expression patterns observed utilizing the single vector approach is largely due to inherent positional effects that alter the activity of the driver and/or effector components. Therefore, to eventually succeed in establishing a tightly controlled system for inducing genes in specific tissues, we designed a new split system approach that would allow for independent identification of optimal driver and effector lines for minimizing aberrant expression patterns.

Although this new split configuration is expected to increase the efficiency of the estradiol-inducible system, one major drawback of the presently constructed split system methodology is the labor-intensive nature of testing non-reporter effector constructs. As currently devised, non-reporter effector lines would need to be analyzed using procedures such as RT-qPCR or FISH to confirm leaky effector activity. To resolve this issue, we propose creating a new effector construct that encodes both the GOI as well as the *Luciferase* reporter gene. Luciferase is a great reporter candidate due to its high sensitivity and non-lethal screening procedure (Southern et al. 2006). Utilizing this new effector construct, T1 effector lines could be screened for luminescence, which would be indicative of leaky expression of the GOI, and directly moved to subsequent generations for efficient identification of optimal effector lines. With this new effector modification in place in conjunction with the previously devised split system methodology, we should be able to more efficiently identify tissue-specific, inducible plant lines for any gene of interest.

In the field of circadian biology, it is becoming more evident that understanding the role of core clock genes in specific tissues represents one of the next steps in elucidating the complex inner workings of the circadian clock and how it regulates biological processes in response to rhythmic environmental conditions. Mis-regulation of core clock genes, such as overexpressing *CCA1,* in *Arabidopsis* has been discovered to produce significant changes in plant phenotypes, including hypocotyl elongation, reduced root growth, and decreased lamina size (Más et al. 2003; Strayer et al. 2000; Wang and Tobin 1998). It is also known that tissue-specific clocks are coupled in a hierarchical fashion such that the clocks of certain tissues possess differential degrees of influence on the clocks of other tissues (Endo et al. 2014, Takahashi et al. 2015). However, it is not clear what physiological effects arise when core clock genes are mis-regulated in specific tissues throughout the plant. Unlike the previous single vector approach, the split conditional system may allow for sufficient tissue-specific regulation of gene expression such that we can begin to unveil the tissue-specific role of circadian clock genes.

Through this initial study, we confirmed the hypocotyl elongation phenotype in whole plant estradiol-inducible plants. In addition, our findings displayed a correlation between the hypocotyl elongation phenotype and the timing of *CCA1* induction, which is consistent with previous reports identifying clock function in Arabidopsis plants as early as 2 days post-germination (Salomé et al. 2006). However, we also found that regardless of how early the plants were treated with estradiol, the hypocotyls never elongated to the extent of the CCA1 OX control. One of the many advantages of the estradiol-inducible system is the ability to modulate the strength of gene induction with the amount of inducer that is used in plant treatment (Zuo et al. 2002). It is possible that the concentration of estradiol used was not high enough to achieve the same levels of *CCA1* expression as the control. On the other hand, it might be the case that

the method and duration of treatment may pose an additional factor in determining the strength of gene induction. The estradiol treatment optimization experiment performed in this study revealed that higher levels of GUS reporter activity could be generated in aerial tissues by both spraying plants rather than growing them on estradiol medium and incubating the plants longer with estradiol. Therefore, it seems that future treatment optimization experiments need to be conducted to determine whether modifying the estradiol treatment protocol generates hypocotyl phenotypes more closely resembling the CCA1 OX control.

Altogether, the next steps for this project will be to finish generating transgenic lines carrying the modified split conditional system, and to conduct GUS reporter experiments to confirm lower rates of aberrant expression patterns in the new inducible lines. Then we can start to perform phenotyping experiments similar to the previously conducted *CCA1* overexpression experiment using tissue-specific inducible CCA1 lines. Once we successfully demonstrate the ability to conditionally overexpress *CCA1* in specific tissues, we could then apply the same system to other clock elements such as *LHY* and *TOC1* and determine whether this system is in fact a versatile tool for investigating the tissue-specific role of circadian clock genes.

Materials and Methods

Building Constructs

Promoter::GUS constructs were previously generated by cloning PCR amplified tissue specific promoters into the pBI101.1 binary vector. The GUS assay results for one such promoter::GUS construct, pBI-47450-GUS, were previously shown (Fig. 1).

To create the pMDC7 constructs containing the tissue specific promoters driving the expression of XVE, PCR amplified promoters were cloned into the previously constructed pMDC7ΔG10-90pro vector (Lin 2020). The resulting tissue-specific pMDC7 vectors were used to clone in the coding sequences for CCA1 and GUS using LR Gateway Recombination (Invitrogen). Gus constructs were transformed into Col-0 plants and CCA1 constructs were transformed into *CCA1::LUC+* plants.

More information regarding the construction of both the *promoter*::GUS and tissue-specific pMDC7 constructs can be found in a thesis written by a former Master's student who had performed these procedures (Lin 2020).

To generate the tissue-specific driver constructs controlling XVE, the XVE coding sequence was PCR amplified from the pMDC7-PL2 construct using the following primer pair: 5'CCGGGCCCCCCCTCGAGATGAAAGCGTTAACGGCCAG 3' and 5'CACCGCGGTGGCGGCCGCGTTTGGGATGTTTTACTCCT 3'. The amplified XVE was then cloned into *XhoI+NotI* digested pGREEN229 binary vector through Gibson assembly (New England Biolabs). Next, promoters from tissue-specific *Arabidopsis* genes (AT3G59270, AT3G16670, AT3G01190) were PCR amplified (using primers indicated in Table 1) using the previously generated tissue-specific pMDC7 constructs (pMDC7-AT3G59270, pMDC7-AT3G16670, pMDC7-AT3G01190) as templates and were cloned into *XhoI* digested pGREEN229-XVE through Gibson assembly (New England Biolabs). The same DNA assembly

was performed using PCR amplified *G10-90* and *CCA1* promoters (primers indicated in Table 1) to create pG229-G10-XVE and pG229-CCA1-XVE constructs. All driver constructs were then transformed into Col-0 plants by Agrobacterium floral dipping (Zhang et al. 2006).

To generate effector constructs for the proposed two-component system, the previously generated pMDC7-AT3G50170 construct was digested with *HindIII* and *EcoRV* in order to remove the AT3G50170pro-XVE portion of the construct, and a fill-in reaction was performed on the resulting *HindIII* overhang to generate a blunt end using Klenow DNA polymerase (New England Biolabs). Following subsequent ligation, the PMDC3.5 construct containing the *LexA::Gateway* cassette fusion was generated. Finally a series of LR Gateway recombination reactions were performed between the PMDC3.5 construct and the following pENTR constructs carrying the GUS reporter as well as circadian clock genes of interest: pENTR-GUS, pENTR-CCA1, pENTR-TOC1, pENTR-LHY (Lin 2020, Pruneda-Paz et al. 2009). The resultant pMDC3.5-GUS, pMDC3.5-CCA1, pMDC3.5-TOC1, and pMDC3.5-LHY constructs were then transformed into Col-0 plants by Agrobacterium-mediated transformation.

Generating Transgenic Plant Lines

Arabidopsis lines containing the split conditional system were generated by Agrobacterium- mediated transformation for which the protocol has been previously described (Zhang et al. 2006). Briefly, the previously obtained constructs cloned into AGL0 agrobacterium through electroporation. The transformed Agrobacterium were then incubated in LB broth for an hour at 28°C and plated on selection plates and left to grow at 28°C for 2 additional days. Colonies were selected and inoculated in 400 mL of selective LB broth and incubated until the Agrobacterium reached a stationary growth phase (OD 1.5-2.0). The culture was spun down in a centrifuge and the cells were resuspended in 500 mL 5% sucrose and 0.02% Silwet solution. Pots containing ~30 Arabidopsis plants grown for 3-4 weeks were inverted and dipped into the Agrobacterium sucrose solution for 30 seconds. The pots were drained, covered with plastic bags and left on their side for 1 day, away from direct light exposure, to incubate in a high humidity environment. Plastic bags were removed and the plants were allowed to grow in LD conditions until seeds were ready for harvesting. Finally, T1 seeds were sterilized and grown on selective (Hygromycin for effector lines and Basta for driver lines) 1% sucrose MS plates to isolate successfully transformed plants.

GUS Enzymatic Assay

The β-glucuronidase (GUS) assay was conducted as previously detailed with a few slight modifications to optimize staining for the conditional system (Kim et al. 2006). First, T4 seeds were grown on top of filter paper on 1% sucrose MS plates for 8 days. ~12 seedlings per line were harvested and placed in cold 90% acetone and they were left to incubate for 15-20 min. The seedlings were then removed from ice, the acetone was removed and replaced with GUS wash buffer (25 mM sodium phosphate buffer-pH 7.0, 0.1% Triton X-100, 2mM potassium ferrocyanide, 2mM potassium ferricyanide). The samples were then washed 2 more times with GUS wash buffer and the final wash was replaced with GUS staining solution (25 mM sodium phosphate buffer-pH 7.0, 0.1% Triton X-100, 2mM potassium ferrocyanide, 2mM potassium ferricyanide, 1mM X-Gluc). The samples were then placed under vacuum for a total of 20 minutes for 5 minute intervals, gently shaking the samples in between, and then incubated at 37°C for 6 hours. After incubation, samples were de-stained with a series of ethanol washes (1 per day). Samples were first stored in 70% ethanol at 4°C then washed with 90% ethanol for each consecutive day thereafter until plants were ready for visualization.

Hypocotyl Length Measurements

To measure the hypocotyl phenotype of the pMDC7-CCA1 lines, seedlings were grown on non-selective 1% sucrose MS plates on top of filter paper. One set of seedlings were sown directly onto 50uM estradiol-containing plates prior to stratification (2 days) and several additional sets of plates were grown on 1% sucrose MS plates and transferred to 50uM estradiol plates at different stages of development (2, 4, and 6 days post-germination). Multiple sets of CCA1-OX and Col-0 plants were also grown in parallel on filter paper in 1% sucrose MS plates. Seedlings were harvested at designated times and placed in plastic transparent sleeves such that their hypocotyls were clearly visible. Sleeves were then scanned, and the hypocotyl images were analyzed on ImageJ. In ImageJ, the segmented line tool was used to trace the hypocotyls and the calibrated analyze>measure tool was used to generate the lengths of all the hypocotyls.

Table 1. pG229-prom-XVE constructs primers

Arabidopsis Genome Initiative (AGI) locus code / Promoter Name	Forward Primer	Reverse Primer
AT3G59270	GGGCCCCCCCTCGACAGTG TCAAAGAACCTCTGA	TTAACGCTTTCATCCTTTTTC AAATGCAAATCAC
AT3G01190	GGGCCCCCCCTCGAATGAA ACACCAGTGTCGATA	TTAACGCTTTCATCTTTCTTA AAAAATCTTTAGT
AT3G16670	GGGCCCCCCCTCGAAAGAA TTAATTTGGATACAT	TTAACGCTTTCATCCTTTTTT GGATTACTTGTAT
G10-90	GGGCCCCCCCTCGATAGTT TAAACTGAAGGCGGG	TTAACGCTTTCATCGGGATCC CAGCGTGTCCTCT
CCA1 promoter	GGGCCCCCCCTCGATGTCT CTGGTCTTTTTTAGC	TTAACGCTTTCATCCACTAAG CTCCTCTACAC

Table 2. Seed Repository for pBI101, pMDC7, pMDC3.5, pG229-prom-XVE constructs

Construct	Homozygous Lines
T3-T4 pBI-LHY-GUS	4-1, 8-1-4, 1-6, 9-2, 15-3, 16-7, 4-2, 6-3, 3-6
T3-T4 pBI-CCA1-GUS	5-1, 3-4, 14-3, 17-8
T3-T4 pBI-59270-GUS	27-5, 3-4, 28-6, 20-1, 16-4, 15-6, 6-5, 7-7, 9-5
T3-T4 pBI-50170-GUS	18-1, 23-2 21-3, 25-4, 26-2, 10-6, 1-2, 9-3, 14-4
T3-T4 pBI-59330-GUS	2-1, 6-5, 17-1, 3-6, 5-5, 12-6, 10-2
T3-T4 pBI-16670-GUS	29-6, 7-5, 14-8, 9-2, 5-8, 20-2, 27-3, 16-1, 24-4
T3-T4 pBI-01190-GUS	6-2, 4-5, 10-4, 11-3, 1-8, 13-2, 12-4, 2-3
T3-T4 pBI-47450-GUS	4-8, 19-5, 18-2, 27-2, 26-2, 2-7, 20-3, 9-3
T3-T4 pMDC7-GUS	25-7, 27-4, 31-2, 19-8, 15-6, 22-5, 32-5, 20-5, 10-2
T3-T4 pMDC7-59270-GUS	12-4, 7-4, 21-3, 26-2, 25-2, 10-4, 22-2, 4-5, 13-3
T3-T4 pMDC7-50170-GUS	2-5, 30-7, 15-2, 6-3, 29-1, 28-1, 22-1, 9-2, 24-7, 4-3
T3-T4 pMDC7-59330-GUS	1-1, 26-4, 25-3, 21-3, 7-5, 19-6, 17-5, 23-7
T3-T4 pMDC7-16670-GUS	10-6, 17-2, 5-5, 2-7, 13-4, 4-2, 20-3, 9-3
T3-T4 pMDC7-01190-GUS	6-2, 14-5, 1-4, 10-3, 2-3, 9-4, 13-1
T3-T4 pMDC7-47450-GUS	17-7, 20-2, 21-3, 28-7, 19-5, 8-3, 15-4, 23-5
T3-T4 pMDC7-CCA1	4-6, 11-2, 8-7, 14-4, 13-3, 20-1, 2-6, 15-4
T3-T4 pMDC7-59270-CCA1	20-4, 5-4, 2-3, 41-4, 10-7, 18-2, 7-5, 32-4, 30-1
T3-T4 pMDC7-50170-CCA1	1-6, 14-6, 12-3, 28-6, 17-3, 30-2, 32-2, 27-3, 22-6
T3-T4 pMDC7-59330-CCA1	10-1, 20-8, 2-5, 15-7, 21-2, 23-7, 16-3
T3-T4 pMDC7-16670-CCA1	2-1, 42-5, 1-1, 37-3, 17-1, 36-7, 33-7, 15-6, 28-4, 41-8
T3-T4 pMDC7-01190-CCA1	6-5, 20-4, 9-5,
T3-T4 pMDC7-47450-CCA1	14-8, 12-5, 11-7, 28-5, 27-8, 18-7, 20-3
T1 pMDC3.5-GUS	None yet identified
T1 pMDC3.5-CCA1	None yet identified
T1 pMDC3.5-LHY	None yet identified
T1 pMDC3.5-TOC1	None yet identified
T1 pG229-G10-XVE	None yet identified
T1 pG229-CCA1pro-XVE	None yet identified
T1 pG229-59270-XVE	None yet identified
T1 pG229-16670-XVE	None yet identified
T1 pG229-01190-XVE	None yet identified

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