UNIVERSITY OF CALIFORNIA SAN DIEGO

Exploring the *Arabidopsis thaliana* Clock Function in Specific Tissues

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

in

Biology

by

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The Thesis of Vivian Li Lin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Professor Jose L. Pruneda-Paz, Chair
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The circadian clock is an internal time-keeping mechanism that allows an organism to anticipate external changes by synchronizing its biological processes with the environment. In plants, previous evidence showed that the circadian clock function, although intrinsic to each cell, is compartmentalized in different tissues and that these tissue-specific clocks are organized

in a hierarchical fashion. However, the function of cell-intrinsic clock components in each tissue is not well known. In this thesis, we developed a tissue-specific estradiol-inducible system for *Arabidopsis thaliana* aiming to better understand the role of clock proteins in a tissue-specific manner. We identified gene promoters that are active only at the shoot apex, root, or leaf tissues. We also experimentally confirmed the tissue-specific activities of these promoters and created conditional overexpression constructs for core clock genes. Additionally, we discussed possible phenotypes that could result from clock genes overexpression in each tissue. The strategy developed in this study provides a useful tool that could be universally implemented to study tissue-specific functions of any gene of interest.

Introduction

Organisms on earth adapted their physiology and behavior to the periodic environmental cycles that occur due to the earth's rotation. For instance, plants adjust to the differences in the availability of light sources during day and night cycles through leaf movements (McClung 2006). Interestingly, most organisms from bacteria to humans can anticipate these periodic environmental changes and adapt before the events occur. Such anticipatory response is driven by an internal time-keeping mechanism known as the circadian clock, which organizes internal biological processes throughout the day (Michael et al. 2003). For example, sunflowers can orient their leaves towards the east by the end of the night where the sun is expected to rise the next day (Atamian et al. 2016). This occurs because the sunflower's endogenous clock-controlled rhythms are synchronized with the daily oscillations in environmental conditions. This synchronization enhances plant growth by, among others, increasing the efficiency of photosynthesis reactions (Dodd et al. 2005). Likewise, the circadian clock function has been shown to maximize organismal fitness in different species through controlling a wide range of biological processes (Ouyang et al. 1998; Green et al. 2002; Dodd et al. 2005).

The circadian clock drives endogenous rhythms that have periods of about 24 hours (McClung 2006). At the molecular level, the circadian clocks in all species studied thus far are built by proteins that are organized in regulatory feedback loops. For example, in the plant model organism *Arabidopsis thaliana*, these clock proteins include two morning-expressed MYB transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LONG ELONGATED HYPOCOTYL (LHY), as well as an evening-expressed pseudo-response regulator, TIMING OF CAB EXPRESSION1 (TOC1) (Alabadí et al., 2001). CCA1 and LHY protein levels peak at dawn and repress the transcription of *TOC1* (Alabadí et al. 2001). As the

day passes, CCA1 and LHY protein levels are gradually reduced to relieve the repression of *TOC1* gene expression. Consequently, TOC1 protein levels increase and peak at dusk (Más et al. 2003). The TOC1 proteins repress *CCA1* and *LHY* transcriptions at the beginning of the night, but as the night passes TOC1 proteins are degraded to relieve the expression of *CCA1* and *LHY* the following day (Gendron et al. 2012; Más et al. 2003). In *Arabidopsis*, this basic feedback circuitry is believed to be present in all tissues and cell types.

Misregulation of the circadian clock function leads to a reduction in plant fitness, as the plant would be unable to synchronize its endogenous physiological responses with environmental changes (Dodd et al. 2005). For example, *CCA1* overexpression in *Arabidopsis* causes a short circadian period that results in abnormal growth and developmental phenotypes such as hypocotyl hyper-elongation (Más et al. 2003; Strayer et al. 2000; Wang and Tobin 1998). These phenotypes are a result of plants not being able to sense and regulate light responses properly, and as many biological processes in plants rely on light signaling, this is harmful for plant survival (Wang and Tobin 1998). In addition, *CCA1* overexpression in *Arabidopsis* also leads to root growth phenotypes, where mainly lateral root length and to a lesser extent primary root length were reduced compared to wild-type plants (Ruts et al. 2012). Furthermore, the direction of the lateral root growth was more horizontal in the *CCA1* overexpression plants, implying there were defects in their gravity sensing ability (Ruts et al. 2012). All of these irregularities are disadvantageous for the plants to absorb nutrients properly and stabilize in soil. Moreover, *CCA1* overexpression in *Arabidopsis* has longer petiole and smaller lamina phenotypes as well (Ruts et al. 2012). Having a smaller leaf surface area can bring down many vital processes such as light-harvesting activities, which decreases fitness.

In multicellular organisms, such as plants and animals, circadian clocks are intrinsic to each cell. However, the circadian clock in each cell of a tissue requires coupling to perform essential cell-to-cell communications as tissue-specific clocks (Endo et al. 2014). Each tissue-specific coupled clock system is further organized in a hierarchical fashion where the clock function in some tissues can influence the clock function in different tissues, meanwhile the clocks in other tissues may not have such influence (Endo et al. 2014; Takahashi et al. 2015). The architecture of such organismal clock systems has been studied previously. In mammals, it was shown that there is a central pacemaker in the suprachiasmatic nucleus of the hypothalamus that controls peripheral clocks in other tissues, such as liver, lung, and cornea (Aton and Herzog 2005; Yoo et al. 2004). In *Arabidopsis*, there is evidence showing a similar coupled system, where the shoot apex clock influences circadian rhythms in the roots through intercellular communications (Takahashi et al. 2015). However, how changes in a tissue-specific clock affect the physiology of that tissue or other tissues is largely unknown.

To add onto current findings, we are interested in knowing how plants respond to the dysregulation of core clock components in a certain tissue. Specifically, we wanted to know how changes in a tissue-specific clock affect the physiology of the same tissue or other tissues. To answer these questions, we focused on developing an experimental system that could be used to disrupt the clock function in a tissue-specific manner. With this system, we aimed to investigate how *CCA1* overexpression in the shoot apex, leaves, or roots affect tissue-specific phenotypes, such as hypocotyl growth, primary and secondary root development, or petiole elongation and leaf lamina changes. The approach developed here could be applied universally to understand the role of local (tissue-specific) changes in the expression of any given gene.

Result

Identification of tissue-specific and constitutively expressed genes

To create a conditional tissue-specific expression system, we first identified genes that are only expressed in the shoot apex, leaves, or root according to publicly available dataset repository (Austin et al. 2016). From the list of tissue-specific genes, we then selected those that display a constitutive expression throughout the day according to publicly available datasets (Mockler et al. 2007). The latter is important to avoid variations of the expression system depending on the time of the day the experiment is conducted. Using this two-step selection criteria, we identified three shoot apex-specific genes (AT3G59270, AT3G50170, AT5G59330), two root-specific genes (AT3G01190, AT5G47450) and one leaf-specific gene (AT3G16670) (Figure 1A). The levels of expression between the selected genes vary, but overall are specific to each tissue in different developmental stages (Figure 1A). However, it should be noted that some of the shoot apex-specific genes (AT3G59270, AT3G50170) are expressed in the inflorescence phase so they would only be suitable for experiments using plants in the vegetative phase of development (Mockler et al. 2007). Regarding their daily expression patterns, four genes (AT3G59270, AT3G50170, AT5G59330, and AT3G01190), displayed constitutive transcript levels in all four diurnal conditions (Figure 1B) and all four constant conditions (Figure 1C). It should be noted that one gene (AT5G59330) exhibited large peaks in the LDHH_ST diurnal condition (Figure 1B) and in the LL_LLHC and LL23_LDHH constant conditions (Figure 1C). However, apparent daily expression changes cannot be considered a true daily rhythm as the circadian wave correlation values for the expression pattern of AT5G59330 in each of the conditions mentioned above were found to be below the standard cutoff value of 0.8 (Mockler et al. 2007). Two of the selected genes (AT5G47450 and AT3G16670) were found to exhibit

circadian rhythms in one condition, LLHC (AT5G47450) with a circadian wave correlation value of 0.87 and LL_LLHC (AT3G16670) with a circadian wave correlation value of 0.8 (Figures 1B, 1C). The expressions of these two genes were constitutive in all other conditions (Figures 1B, 1C). Overall, the selected genes fulfilled the two criteria that we considered important for developing a conditional tissue-specific overexpression system. However, having transcripts synthesized in one tissue can be due to various mechanisms, such as transcripts in all other tissues being degraded. Thus, both the tissue-specificity and constitutive expression pattern of the promoters from the selected genes had to be confirmed.

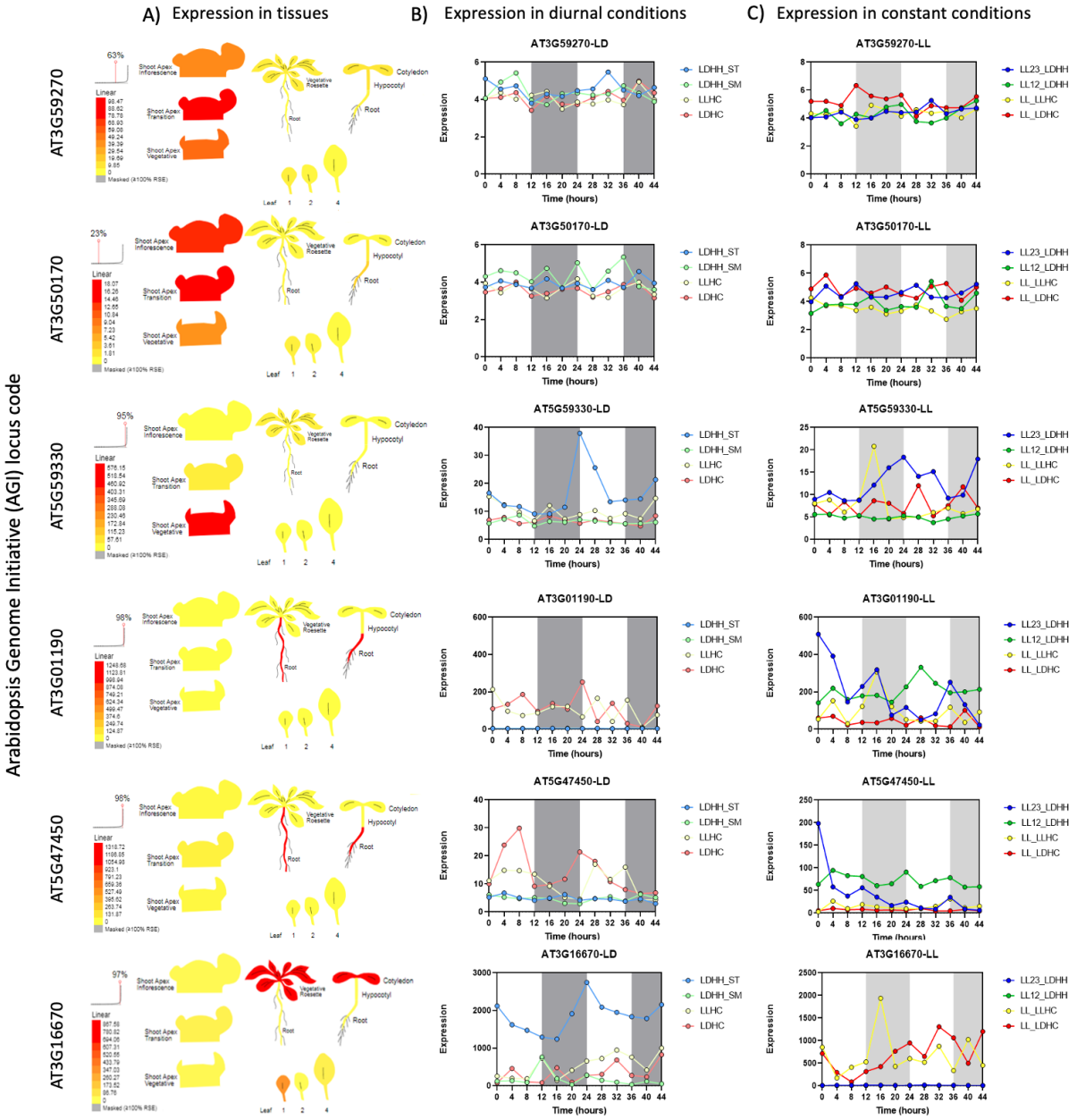

Figure 1. Selection of constitutive tissue-specific promoters for *Arabidopsis thaliana.* A) RNA expression of each tissue-specific gene (AGI locus code: AT3G59270, AT3G50170, AT5G59330, AT3G01190, AT5G47450, AT3G16670) in apex, rosette, root, cotyledon, hypocotyl, and leaf. **B)** Expression pattern of each tissue-specific gene in plants grown under diurnal cycles over 44 hours (LDHH_ST: 12h light/12h dark at 22°C, LDHH_SM: 12h light/12h dark at 20°C, LLHC: continuous light at 12h 22°C/12h 12°C, LDHC: 12h light/12h dark at 12h 22°C/12h 12°C). **C)** Expression pattern of each tissue-specific gene in free-running (constant) conditions over 44 hours (LL23_LDHH and LL12_LDHH: entrained on 12h light/12h dark at 22°C then subjected to continuous light at 22°C, LL_LLHC: entrained on continuous light at 12h 22°C/12h 12°C then subjected to continuous light at 22°C, LL_LDHC: entrained on 12h light/12h dark at 12h 22°C/12h 12°C then subjected to continuous light at 22°C).

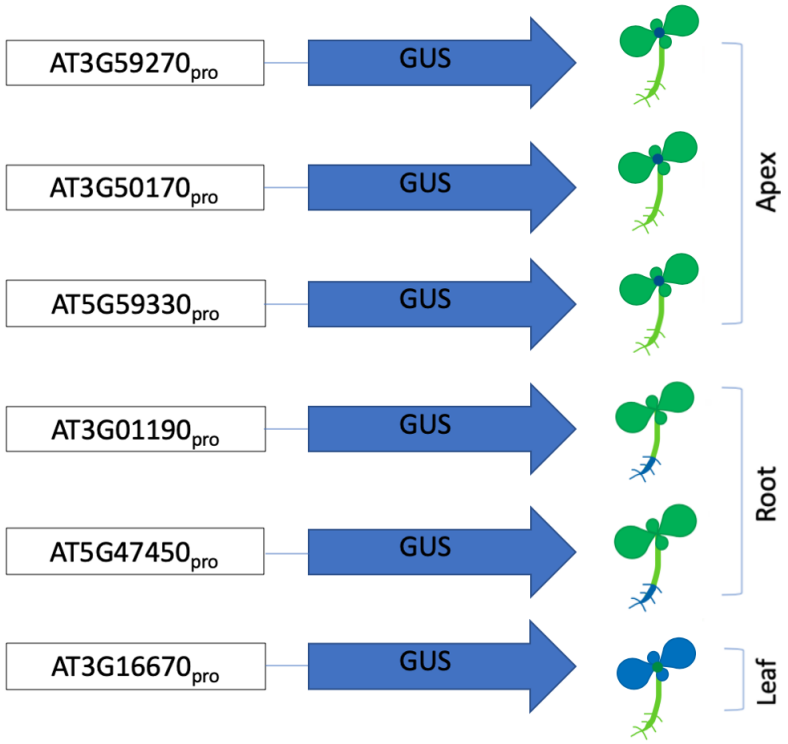

Figure 2. Expected β-glucuronidase (GUS) assay results to confirm tissue-specific activity for the promoters of selected genes. Depicted hypothesis of β-glucuronidase (GUS) assay outcomes for transgenic *Arabidopsis* lines carrying constructs with the selected tissue-specific gene promoters driving the *GUS* gene constitutively. It was expected that each promoter would drive GUS protein expression in the specific tissue and that the level of expression would not change when the experiment is conducted at different times of the day.

β-glucuronidase (GUS) assay confirmed the tissue-specific activity of the selected gene promoters

To confirm that the promoters of the genes selected using online database could drive tissue-specific gene expression, each promoter of the selected genes was cloned into the pBI101.1 vector, which carries the coding region of the *β-glucuronidase (GUS)* reporter gene (Figure 2). By using the *GUS* reporter gene, it is possible to visualize the location and the level of expression driven by each promoter. Eight to ten homozygous *Arabidopsis* transgenic lines carrying each promoter::GUS construct were generated. The GUS assay was optimized for each one of the lines carrying the reporter constructs in order to avoid non-specific GUS staining. Finally, two representative transgenic lines for each reporter construct were used to analyze the GUS expression pattern at different developmental stages (4, 8, and 16 days post-germination).

For plants carrying the AT3G59270 gene promoter, the GUS proteins are only expressed specifically at the shoot apex in all developmental stages tested (Figure 3). In plants that carry the AT3G50170 gene promoter, no expression of GUS was observed at 4 days old (Figure 3). However, in 8 days old plants, very low levels of GUS activity were detected at the shoot apex, and in 16 days old plants, GUS proteins were found in spots at the shoot apex periphery (Figure 3). It is worth noting that no other tissues showed GUS expression for this construct at all developmental stages (Figure 3). Plants carrying the AT5G59330 promoter showed no GUS expression in 4 days old plant, and low GUS expression was seen only at the shoot apex in 8 days old plants, while GUS proteins were observed in spots at the shoot apex periphery in 16 days old plants (Figure 3). In plants carrying the AT3G01190 gene promoter, GUS expression was detected at the entire root, except the root tips, at all times tested (Figure 3). However, this line displayed GUS activity at two small localized spots near the shoot apex after 16 days of

growth (Figure 3). For plants carrying the AT3G16670 gene promoter, we observed that only the leaf tips showed GUS expression at all the developmental stages tested (Figure 3). It is important to note that no GUS expression was detected in any tissue at all times tested in non-transgenic wild-type control plants (Figure 3).

These results showed that the promoters of most genes selected previously from public datasets exhibited a tissue-specific activity. However, some of them showed activity in other tissues at least in the 16 days old plants, in particular, at the apex for the root-specific AT3G01190 gene. In addition, the shoot apex-specific genes AT3G50170 and AT5G59330 seemed less consistent than AT3G59270 as different developmental stages had different expression patterns. In the root-specific gene AT3G01190, there were clear cut-offs at the end of the roots where the tips were not stained at all, showing that the gene is not expressed at the root apex. Lastly, the leaf-specific gene AT3G16670 did not show expression all over the leaves but only at the tip where the mesophyll cell expansion initiates (Pyke, Marrison, and Leech 1991).

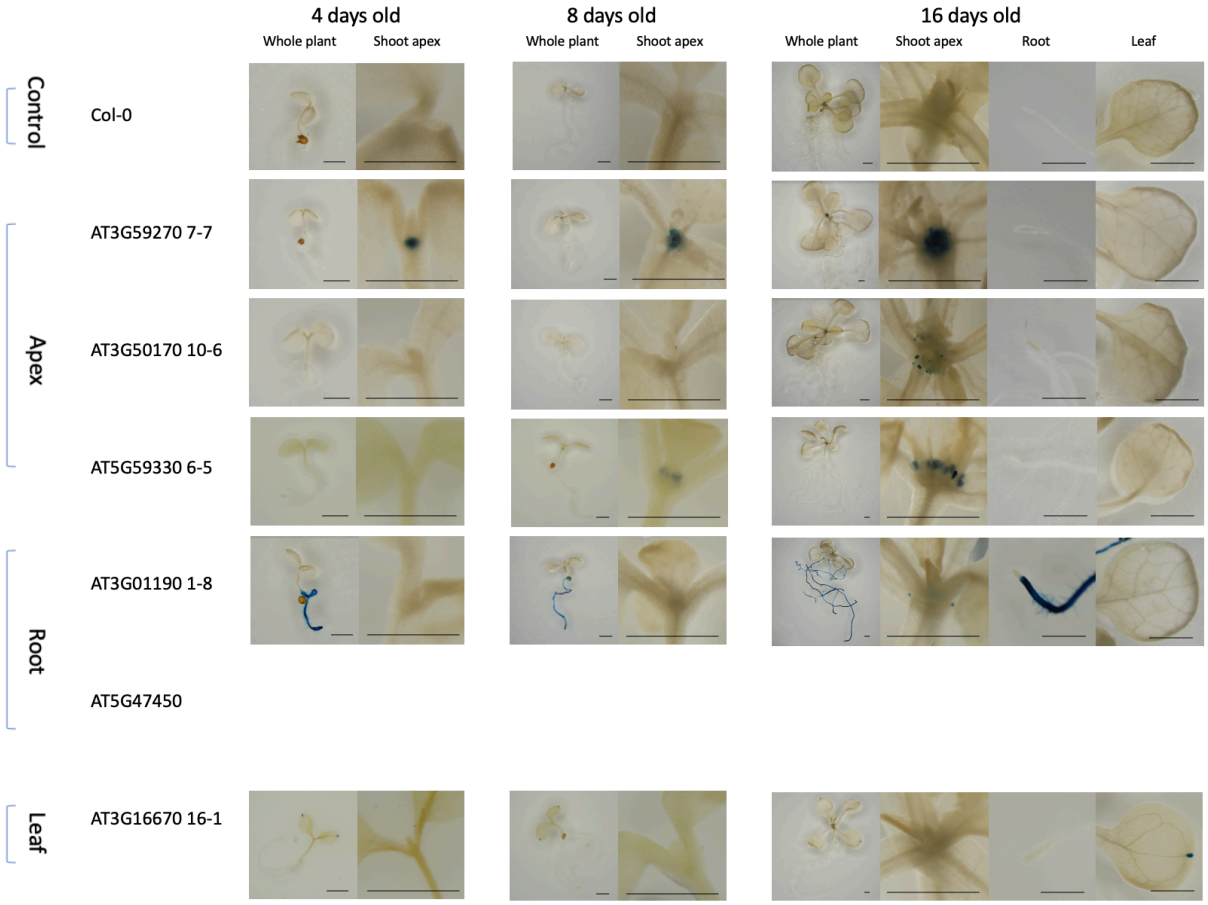

Figure 3. *ß-glucuronidase* (GUS) assay results of *Arabidopsis* transgenic lines carrying the selected tissue-specific gene promoters driving the *GUS* reporter gene expression. *Arabidopsis* carrying the *β-glucuronidase* gene driven by shoot apex-specific gene promoters (AT3G59270, AT3G50170, AT5G59330), root-specific gene promoters (AT3G01190, AT5G47450) and leaf-specific gene promoter (AT3G16670) were stained at different developmental stages. Images focusing on different tissues are displayed and the bars at the bottom of each image represent 1mm in length.

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

To achieve tissue-specific overexpression of a gene for a short duration of time, an estrogen receptor-based vector was utilized. With this, the application of estradiol directly to the whole plant can induce local gene expression. This system includes the synthetic chimeric transcription factor XVE, which consists of a DNA-binding domain from the bacterial repressor LexA (X), the activation domain of Virus protein 16 (V) and the ligand binding domain of the human estrogen receptor (E) (Zuo, Niu, and Chua 2000). The XVE receptor synthesis is constitutive, and the protein remains in the cytosol until the external addition of estradiol, which moves the receptor into the nucleus and binds onto the LexA promoter to drive the synthesis of a target gene (Figure 4A). To make the system tissue-specific, we cloned the promoters of each tissue-specific gene selected previously upstream of the XVE coding region so that the XVE receptor will only be present at the tissue where the promoter is active (Figure 4A). Because estradiol is not naturally produced in plants, the gene of interest would be activated only by the externally applied estradiol.

First, to test this conditional tissue-specific gene overexpression system, the coding region of the *GUS* gene was cloned downstream of the *LexA* promoter for each tissue-specific construct. Then, eight to ten homozygous *Arabidopsis* transgenic lines carrying each construct were generated. We plan to optimize the GUS assay for each one of the lines carrying the reporter constructs in order to avoid non-specific GUS staining and to select two representative transgenic lines for each reporter construct to analyze the GUS expression pattern at different developmental stages (4, 8 and 16 days post-germination).

Since the XVE receptor will only be expressed in a single tissue, the GUS proteins are also only expected to be synthesized in the same tissue upon the estradiol treatment for all

constructs (Figure 4B). However, it is possible that the XVE protein may be able to travel within the plant, which would lead to GUS protein expression in other tissues. For instance, after applying estradiol and performing GUS assay in these plants, the blue coloration should only be seen at the shoot apex when driven by the shoot apex-specific promoter (Figure 4C). If the *GUS* reporter gene was seen in both the shoot apex and the root, then the XVE receptors might have traveled, which discards the tissue-specificity of this system (Figure 4C). In the control plant carrying the inducible system without a tissue-specific promoter, the entire plant should express the GUS proteins (Figure 4C). Generally, if the GUS proteins are observed in a different tissue other than the one targeted, or in more than one tissue, then the system would be considered leaky and unusable. However, since the XVE transcription factor has been used to induce gene expression in a tissue-specific manner, and many published works support the notion that this system is not leaky, we expected to see *GUS* activity only in the tissues targeted.

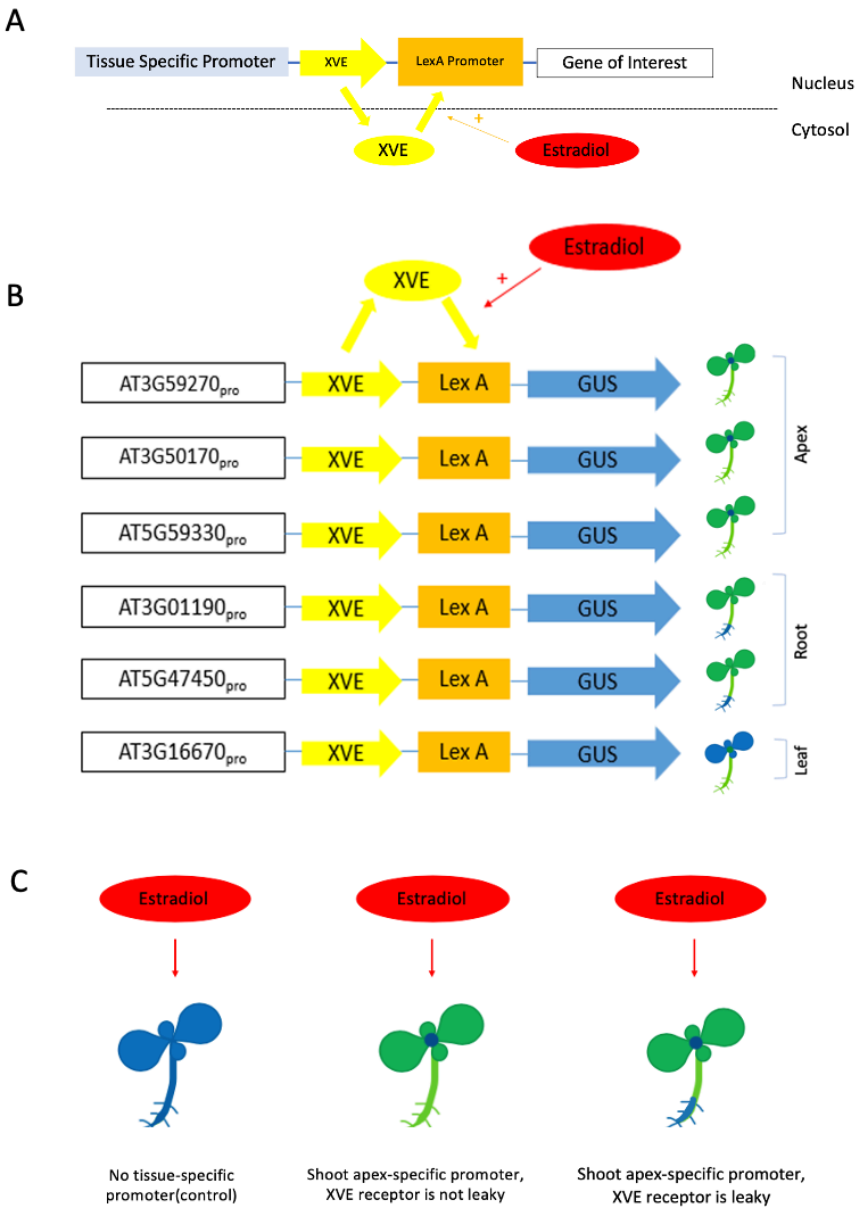

Figure 4. Construction of an *Arabidopsis* conditional tissue-specific overexpression system. A) Schematics of the estradiol-inducible system used in this study. B) Expected results of the β-glucuronidase assay for *Arabidopsis* containing the estradiol-inducible overexpression system driving the expression of the *GUS* reporter gene. C) Possible outcomes of the β-glucuronidase assay results to assess the tissue-specificity of the inducible system driven by the selected promoters.

Tissue-specific *CCA1* overexpression leads to local and distal alterations in plant growth and development

Once a conditional tissue-specific overexpression system is established, we can use it to find out how the physiology of *Arabidopsis* changes when the clock protein CCA1 is overexpressed tissue-specifically. First, we cloned the coding region of the *CCA1* gene downstream of the LexA promoter for each tissue-specific construct. Then eight to ten homozygous *Arabidopsis* transgenic lines carrying each construct were generated (Figure 5A). By overexpressing the CCA1 proteins only at one tissue at a time, we can analyze the clocks in which tissue play a bigger role in controlling different phenotypes that are seen in plants with CCA1 proteins constitutively overexpressed. It is known that overexpression of CCA1 proteins in the whole plant causes hyper-elongated hypocotyl and reduced root growth, as well as petiole lengthening and reduced lamina size (Wang and Tobin, 1998; Ruts et al., 2012). Our hypothesis is that disruption of clock function at the shoot apex may lead to all CCA1-sensitive phenotypes mentioned above because in previous reports it was shown that clock arrhythmicity in the shoot apex can lead to arrhythmicity in other tissues such as the root (Figure 5B). Meanwhile, overexpression of CCA1 at the root may only lead to root phenotypes because previous evidence showed that clock arrhythmicity in roots does not lead to arrhythmicity in the shoot apex (Figure 5B). Lastly, CCA1 overexpression at the leaf may only lead to leaf phenotypes because the leaf clock has not been shown to influence clocks in other tissues (Figure 5B). The control plant which contains the inducible system, pMDC7 CCA1, is expected to have all mentioned phenotypes because the CCA1 protein would be induced without a tissue-specific promoter (Figure 5B). It is important to note that we have only proposed a few possibilities from a variety of potential phenotypes that can be seen in these plants.

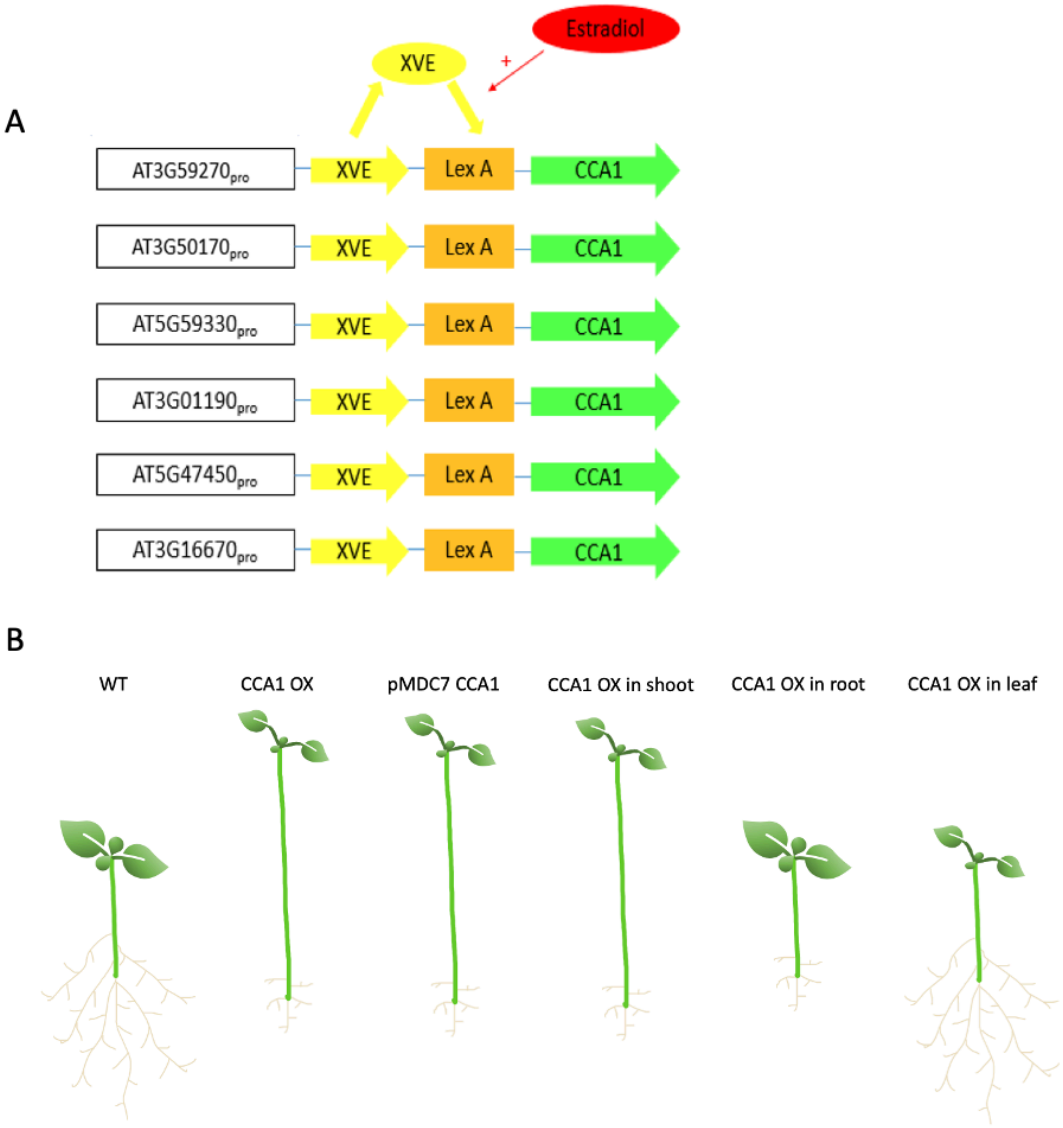

Figure 5. Constructions of estrogen-based inducible CCA1 overexpression system. A) Schematics of the estradiol-inducible overexpression system with the tissue-specific promoters driving the expression of *CCA1* gene. B) Possible phenotypes of *Arabidopsis* with CCA1 proteins overexpressed at different tissues.

Discussion

Overexpression of genes has been widely used as a way to analyze gene function. Through the development of the *β*-estradiol-inducible system, scientists are now able to overexpress genes temporally and spatially. The substitution of promoters and coding regions of target genes is simple in this system, making it efficient to use. The level of overexpression induced can also be adjusted, thus this system is very flexible. This method has been utilized to induce gene expression in specific cell types or tissues of various organisms, and it is dependable as long as the promoters selected are truly specific to the target cell type or tissue. There were other techniques used before to study the clock function tissue-specifically, but with the strategy presented in our study, we can achieve the same analysis in non-invasive and time-independent manners. Here, we have proposed the use of an estrogen-based inducible system as a potential way to study circadian clock gene function and their phenotypes tissue-specifically in *Arabidopsis thaliana.*

The circadian clock plays an important role in regulating the endogenous rhythms of organisms to match the changes in the environment. When the clock function is disrupted, the fitness of organisms is reduced (Dodd et al. 2005). For instance, it was previously discovered that the overexpression of the core clock protein CCA1 in *Arabidopsis* causes hyper-elongated hypocotyl, delayed flowering, and lowered root growth as well as lengthened lamina phenotypes which are disadvantageous to plants (Wang and Tobin 1998; Ruts et al. 2012). When looking at the circadian clock cellularly in *Arabidopsis*, it was found that clock components are coupled within a tissue that give rise to different clock functions between tissues (Endo et al. 2014). Interestingly, the clocks in different tissues are organized in a hierarchy where some tissues can influence the circadian rhythms of other tissues while some cannot (Takahashi et al. 2015).

Knowing these, we were interested in understanding how the core clock protein CCA1 functions in different tissues. To answer our question, we utilized online databases and found three shoot apex-specific (AGI locus code AT3G59270, AT3G50170, AT5G59330), two roots-specific (AT3G01190, AT5G47450), and one leaf-specific (AT3G16670) promoters. Next, we incorporated the promoters of these genes into the estrogen-based, tissue-specific overexpression system. We then predicted the possible phenotypes of *Arabidopsis* which the overexpression of *CCA1* in each selected tissue can cause.

We hypothesized that plants will have hyper-elongated hypocotyl from *CCA1* overexpression in the shoot apex because the shoot apex has a major role in the development of the stem, which arose from hypocotyl. Since it has been shown that the clock in the shoot apex can govern the clocks in other tissues, we also anticipate root and leaf CCA1-sensitive phenotypes to be present in these plants. Similarly, we expect having a non-functional clock in the roots would lead to a decreased root growth phenotype because the root-specific clock might have a role in the development and lengthening of the roots. Likewise, the long petiole and small lamina are anticipated in plants with *CCA1* overexpressed only in the leaf because the clocks in the leaf might have important functions in controlling these phenotypes. If these hypotheses are true, it can be confirmed that tissue-specific clocks regulate critical proteins in their respective cell types, and these clocks can affect the overall physiology of *Arabidopsis*.

The use of the β-estradiol-inducible system can be helpful in studying the circadian clock function of *Arabidopsis*. The easy substitution of promoters and genes of interest may be applied to a wide variety of organisms, making its applications broad. However, it is possible that the XVE receptors in this system can move to tissues that are not where the selected promoters are active. If this is the case, that means the location where the inducible construct is inserted in the

Arabidopsis genome matters. To solve this problem, a split system that separates the inducible constructs into two parts would be needed. The first construct would include the tissue-specific promoter with the coding region of the XVE transcription factor. The second construct would contain the LexA promoter and the coding region of the target gene. Each of these constructs should be transformed into *Arabidopsis* and separately then the plants should be crossed together. The initial crossing requires the use of *GUS* as the gene of interest to see where the expression is. Once a line of plant is confirmed to be expressing the GUS protein at the desired tissue, the gene expressed in the second construct can be replaced with a clock gene or any other genes of interest. This way, it can be confirmed that the inducible system is not leaky.

The next step of this project will be to test the functions of other clock proteins, such as LHY and TOC1, tissue-specifically through the methods provided in this study. These will help us better understand the role of core clock proteins. Additionally, we can induce overexpression of a clock component in a single tissue with the addition of a clock reporter, then observe the clocks in other tissues to further analyze the effect of overexpression on clock function.

Materials and Methods

Tissue-specific promoter selection

To find genes that are only expressed in specific tissues, an online *Arabidopsis* gene expression dataset was used (Austin et al. 2016). The Expression Angler displays the expression pattern for each gene in different developmental stages. Those genes with expression only in the shoot apex, roots, or leaves were further analyzed using another online database, Diurnal to see whether the gene expression oscillates in different external inputs throughout the day (Mockler et al. 2007). Only tissue-specific genes that do not oscillate in expression for all conditions are chosen.

Building constructs

To generate promoter::GUS constructs, the promoter of each selected gene (Table 1) was cloned into the pBI101.1 binary vector. For that, each promoter was PCR amplified (using primers indicated in Table 2) and cloned into *Sma*I digested pBI101.1 using DNA assembly (New England Biolabs). Each construct (pBI101.1-AT3G59270, pBI101.1-AT3G50170, pBI101.1-AT5G59330, pBI101.1-AT3G01190, pBI101.1-AT5G47450, pBI101.1-AT3G16670) was transformed into Col-0 plants through Agrobacterium-mediated transformation as described below.

To generate pMDC7 constructs carrying tissue-specific promoters upstream of the synthetic XVE transcription factor coding region, the G10-90 constitutive promoter in pMDC7 was replaced by the selected promoter regions. For that, we first built the pMDC7ΔG10-90pro vector, which lacks the G10-90 promoter region and contains an EcoRV restriction site instead. To generate this vector the regions flanking the G10-90 promoter (and carrying the EcoRV restriction site) were PCR amplified using the following primer pair:

5’AAACACTGATAGTTTGATATCATGAAAGCGTTAACGGCCAGGC 3’ and 5’AACGCTGCGCGATTTCCGCA3’. The PCR product was cloned into *Pme*I/*Mlu*I digested pMDC7 using DNA assembly (New England Biolabs). To generate pMDC7-AT3G59270, pMDC7-AT3G50170, pMDC7-AT5G59330, pMDC7-AT3G01190, pMDC7-AT5G47450, and pMDC7-AT3G16670, the promoter of each selected gene (Table 1) was cloned into the pMDC7ΔG10-90pro binary vector. For that, each promoter was PCR amplified (using primers indicated in Table 3) and cloned into *Eco*RV digested pMDC7ΔG10-90pro using DNA assembly (New England Biolabs). Each construct (pMDC7-AT3G59270, pMDC7-AT3G50170, pMDC7-AT5G59330, pMDC7-AT3G01190, pMDC7-AT5G47450, and pMDC7-AT3G16670) was transformed into Col-0 plants through Agrobacterium-mediated transformation as described below.

Construction of pENTR-CCA1 was described previously (Pruneda-Paz et al. 2009). To generate pENTR-GUS, the coding sequence for the *GUS* gene was PCR amplified from pBI101.1 using the following primers: 5’ CACCATGTTACGTCCTGTAGAAAC 3’ and 5’ TCATTGTTTGCCTCCCTGCT 3’. The PCR product was cloned into the pENTR/D-TOPO vector following the manufacturer’s protocol (Life Technologies).

Tissue-specific pMDC7 vectors carrying the *GUS* or *CCA1* coding sequences were generated by transferring, the constructs previously created were used as vectors. pENTR-GUS and pENTR-CCA1 were each cloned into the tissue-specific pMDC7 vectors by Gateway LR recombination following the manufacturer’s protocol (Invitrogen). GUS constructs transformed into Col-0 plants and CCA1 constructs were transformed into CCA1::LUC+ plants.

Creating transgenic plants

Agrobacterium-mediated transformation of *Arabidopsis thaliana* was performed as described before with some modifications (Zhang et al. 2006). Briefly, the constructs built previously were transformed into AGL0 Agrobacterium through electroporation. The Agrobacterium was incubated in LB broth for one hour at room temperature. Then it was transferred onto selection plates and further incubated at room temperature for 2 days. The selected colony was inoculated in 400ml of LB with appropriate antibiotics until Agrobacterium reaches a stationary phase in growth (OD of about 1.5-2.0). The culture is then centrifuged to collect cells, and the cells were resuspended in a solution containing 500ml of 5%sucrose and 0.02% Silwet. Pots each with about 30 *Arabidopsis* plants grown on soil for 3-4 weeks where inflorescence peaks were used for this transformation. The pots were inverted to dip the aerial part of the plants into Agrobacterium suspension for 30 seconds, then the pots were removed from suspension to allow draining. The dipped plants were covered with plastic bags and placed on their sides to create a high humidity environment for one day. Then, the plastic bags were removed to allow plants to continue growing until the seeds were ready to be collected. The collected T1 seeds were sterilized and grown on 1% sucrose MS selection plates to identify successfully transformed plants. Single insertion lines were identified in the T2 generation and homozygous lines were selected in the T3 generation.

GUS staining assay

The β-glucuronidase (GUS) assay was performed as described previously with modifications (Kim et al. 2006). In short, sterilized T3 seeds were grown on MS medium with 1% sucrose for 14 days. Samples were harvested and placed into 90% acetone on ice, and after all samples were harvested, the tissues were incubated at room temperature for 20 minutes. After incubation, the acetone was removed and replaced with GUS staining wash buffer (50mM sodium phosphate buffer-pH 7.0, 0.2% Triton X-100, 2mM potassium ferrocyanide, 2mM potassium ferricyanide). The samples were washed with the GUS wash buffer solution 3 times, then the buffer was replaced with GUS staining solution with (50mM sodium phosphate buffer-pH 7.0, 0.2% Triton X-100, 2mM potassium ferrocyanide, 2mM potassium ferricyanide, and 1mM X-Gluc). The samples were vacuumed in 5 minutes intervals for a total of 20 minutes, then incubated at 37°c for 6 hours in the dark. After incubation, the GUS staining solution was replaced with 70% ethanol to de-stain the tissues overnight. Then the 70% ethanol was replaced with 90% ethanol every day until the tissues have been completely de-stained.

Each line was stained in 4 different concentrations of potassium ferrocyanide and potassium ferricyanide, including 1mM, 2mM, 5mM, and 10mM. This was done to see which concentration is optimal for avoiding non-specific staining. The most representative lines were selected to be stained at different developmental ages, including 4, 8, and 16 days old. This was done to see if the expression changes at different developmental stages.

Table 1 - List of tissue-specific, non-oscillating genes selected through online databases.

Arabidopsis Genome Initiative (AGI) locus code	Description	Promoter region cloned (bp upstream of ATG)
AT3G59270	FBD-like domain family	1228
AT3G50170	Transmembrane protein, putative (DUF247)	1202
AT5G59330	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	1597
AT3G01190	Peroxidase superfamily protein	1323
AT5G47450	Tonoplast intrinsic protein 2;3	680
AT3G16670	Pollen Ole e 1 allergen and extensin family protein	2465

Table 2 - pBI101 GUS constructs primers.

Arabidopsis Genome Initiative (AGI) locus code	Forward Primer	Reverse Primer
AT3G59270	TAGAGGATCCCCCAGTGTCA AAGAACCTCTGA	GACTGACCACCCCTTTTTCA AATGCAAATCACCAC
AT3G50170	CTAGAGGATCCCCATTCTGTT GTTGTTTCCTTTC	GGACTGACCACCCTGGTTTT GCTGGTTGAAGC
AT5G59330	TAGAGGATCCCCGAAGACAT GCTCAATGATTTTTC	GACTGACCACCCTGTGTTTG CTCTATTTTGTTTTGG
AT3G01190	TAGAGGATCCCCATGAAACA CCAGTGTCGATA	GACTGACCACCCTTTCTTAA AAAATCTTTAGTTTGTTGC
AT5G47450	CTAGAGGATCCCCTGAGCTG GCTGAATGGTAAT	GGACTGACCACCCTTTTTGC TTTGGTTTTGTTACTTC
AT3G16670	TAGAGGATCCCCAAGAATTA ATTTGGATACATTTTAAACCC	GGACTGACCACCCCTTTTTT GGATTACTTGTATAT

Table 3 - pMDC7 GUS constructs primers.

Arabidopsis Genome Initiative (AGI) locus code	Forward Primer	Reverse Primer
AT3G59270	TGATAGTTTGATCAGTGTCAA AGAACCTCTGA	CGCTTTCATGATCTTTTTC AAATGCAAATCACCAC
AT3G50170	TGATAGTTTGATATCTTCTGTT GTTGTTTCCT	CGCTTTCATGATATCTGGTT TTGCTGGTTGAAGC
AT5G59330	TGATAGTTTGATGAAGACATG CTCAATGATTTTTC	CGCTTTCATGATTGTGTTT GCTCTATTTTGTTTTGG
AT3G01190	CACTGATAGTTTGATATGAAA CACCAGTGTCGATA	AACGCTTTCATGATTTTCT TAAAAAATCTTTAGTTTG
AT5G47450	TGATAGTTTGATTGAGCTGGC TGAATGGTAATG	CGCTTTCATGATTTTTTGCT TTGGTTTTGTTACTTC
AT3G16670	CACTGATAGTTTGATAAGAAT TAATTTGGATACATTT	CGCTTTCATGATCTTTTTTG GATTACTTGTATAT

Reference

Alabadí, D., T. Oyama, M. J. Yanovsky, F. G. Harmon, P. Más, and S. A. Kay. 2001. "Reciprocal Regulation between TOC1 and LHY/CCA1 within the Arabidopsis Circadian Clock." *Science* 293 (5531): 880–83.

Atamian, Hagop S., Nicky M. Creux, Evan A. Brown, Austin G. Garner, Benjamin K. Blackman, and Stacey L. Harmer. 2016. "Circadian Regulation of Sunflower Heliotropism, Floral Orientation, and Pollinator Visits." *Science* 353 (6299): 587–90.

Aton, Sara J., and Erik D. Herzog. 2005. "Come Together, Right… Now: Synchronization of Rhythms in a Mammalian Circadian Clock." *Neuron* 48 (4): 531–34.

Austin, Ryan S., Shu Hiu, Jamie Waese, Matthew Ierullo, Asher Pasha, Ting Ting Wang, Jim Fan, Curtis Foong, Robert Breit, Darrell Desveaux, Alan Moses, Nicholas J Provart. 2016. "New BAR Tools for Mining Expression Data and Exploring Cis-Elements in Arabidopsis Thaliana." *The Plant Journal: For Cell and Molecular Biology* 88 (3): 490–504.

Dodd, Antony N., Neeraj Salathia, Anthony Hall, Eva Kévei, Réka Tóth, Ferenc Nagy, Julian M. Hibberd, Andrew J. Millar, and Alex A. R. Webb. 2005. "Plant Circadian Clocks Increase Photosynthesis, Growth, Survival, and Competitive Advantage." *Science* 309 (5734): 630–33.

Endo, Motomu, Hanako Shimizu, Maria A. Nohales, Takashi Araki, and Steve A. Kay. 2014. "Tissue-Specific Clocks in Arabidopsis Show Asymmetric Coupling." *Nature* 515 (7527): 419–22.

Gendron, Joshua M., José L. Pruneda-Paz, Colleen J. Doherty, Andrew M. Gross, S. Earl Kang, and Steve A. Kay. 2012. "Arabidopsis Circadian Clock Protein, TOC1, Is a DNA-Binding Transcription Factor." *Proceedings of the National Academy of Sciences of the United States of America* 109 (8): 3167–72.

Green, Rachel M., Sonia Tingay, Zhi-Yong Wang, and Elaine M. Tobin. 2002. "Circadian Rhythms Confer a Higher Level of Fitness to Arabidopsis Plants." *Plant Physiology* 129 (2): 576–84.

Kim, Kye-Won, Vincent R. Franceschi, Laurence B. Davin, and Norman G. Lewis. 2006. "Beta-Glucuronidase as Reporter Gene: Advantages and Limitations." *Methods in Molecular Biology* 323: 263–73.

Más, Paloma, David Alabadí, Marcelo J. Yanovsky, Tokitaka Oyama, and Steve A. Kay. 2003. "Dual Role of TOC1 in the Control of Circadian and Photomorphogenic Responses in Arabidopsis." *The Plant Cell* 15 (1): 223–36.

McClung, C. Robertson. 2006. "Plant Circadian Rhythms." *The Plant Cell* 18 (4): 792–803.

Michael, Todd P., Patrice A. Salomé, Hannah J. Yu, Taylor R. Spencer, Emily L. Sharp, Mark A. McPeek, Jose M. Alonso, Joseph R. Ecker, and C. Robertson McClung. 2003. "Enhanced Fitness Conferred by Naturally Occurring Variation in the Circadian Clock." *Science* 302 (5647): 1049–53.

Mockler, T. C., T. P. Michael, H. D. Priest, R. Shen, C. M. Sullivan, S. A. Givan, C. McEntee, S. A. Kay, and J. Chory. 2007. "The DIURNAL Project: DIURNAL and Circadian Expression Profiling, Model-Based Pattern Matching, and Promoter Analysis." *Cold Spring Harbor Symposia on Quantitative Biology* 72: 353–63.

Ouyang, Y., C. R. Andersson, T. Kondo, S. S. Golden, and C. H. Johnson. 1998. "Resonating Circadian Clocks Enhance Fitness in Cyanobacteria." *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.95.15.8660.

Pruneda-Paz, Jose L., Ghislain Breton, Alessia Para, and Steve A. Kay. 2009. "A Functional Genomics Approach Reveals CHE as a Component of the Arabidopsis Circadian Clock." *Science* 323 (5920): 1481–85.

Pyke, K. A., J. L. Marrison, and A. M. Leech. 1991. "Temporal and Spatial Development of the Cells of the Expanding First Leaf of Arabidopsis Thaliana (L.) Heynh." *Journal of Experimental Botany* 42 (11): 1407–16.

Ruts, Tom, Shizue Matsubara, Anika Wiese-Klinkenberg, and Achim Walter. 2012. "Aberrant Temporal Growth Pattern and Morphology of Root and Shoot Caused by a Defective Circadian Clock in Arabidopsis Thaliana." *The Plant Journal: For Cell and Molecular Biology* 72 (1): 154–61.

Strayer, C., T. Oyama, T. F. Schultz, R. Raman, D. E. Somers, P. Más, S. Panda, J. A. Kreps, and S. A. Kay. 2000. "Cloning of the Arabidopsis Clock Gene TOC1, an Autoregulatory Response Regulator Homolog." *Science* 289 (5480): 768–71.

Takahashi, Nozomu, Yoshito Hirata, Kazuyuki Aihara, and Paloma Mas. 2015. "A Hierarchical Multi-Oscillator Network Orchestrates the Arabidopsis Circadian System." *Cell* 163 (1): 148–59.

Wang, Z. Y., and E. M. Tobin. 1998. "Constitutive Expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) Gene Disrupts Circadian Rhythms and Suppresses Its Own Expression." *Cell* 93 (7): 1207–17.

Yoo, Seung-Hee, Shin Yamazaki, Phillip L. Lowrey, Kazuhiro Shimomura, Caroline H. Ko, Ethan D. Buhr, Sandra M. Siepka, Hee-Kyung Hong, Won Jun Oh, Ook Joon Yoo, Michael Menaker, and Joseph S. Takahashi. 2004. "PERIOD2::LUCIFERASE Real-Time Reporting of Circadian Dynamics Reveals Persistent Circadian Oscillations in Mouse Peripheral Tissues." *Proceedings of the National Academy of Sciences of the United States of America* 101 (15): 5339–46.

Zhang, Xiuren, Rossana Henriques, Shih-Shun Lin, Qi-Wen Niu, and Nam-Hai Chua. 2006. "Agrobacterium-Mediated Transformation of Arabidopsis Thaliana Using the Floral Dip Method." *Nature Protocols* 1 (2): 641–46.

Zuo, Jianru, Qi-Wen Niu, and Nam-Hai Chua. 2000. "An Estrogen Receptor-Based Transactivator XVE Mediates Highly Inducible Gene Expression in Transgenic Plants." *The Plant Journal: For Cell and Molecular Biology* 24 (2): 265–73.