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# Observing *CSLA* Expression Patterns in *Arabidopsis thaliana* Using Promoter-Gus Fusion Analysis

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# Observing CSLA Expression Patterns in *Arabidopsis thaliana* Using Promoter-Gus Fusion Analysis

## **Abstract**

Plant cell walls are the world's most abundant source of renewable biomass. Consisting mainly of carbohydrates, including mannans, plant cell walls are vital to humanity as a source of food, health products, and biofuels. Previous research suggests diverse functions of mannans and implicates several members of the *CELLULOSE SYNTHASE-LIKE A (CSLA)* gene family as mannan synthases, proteins involved in mannan backbone synthesis. Prior research using *Arabidopsis thaliana* (*A. thaliana*) *cs/a* single mutants showed no obvious phenotypic abnormalities, with the exception of *cs/a7*, which was embryo lethal; however, ectopic expression of *CSLA9* complemented the *cs/a7* mutant phenotype, suggesting *cs/a7* and *cs/a9* enzymes make similar carbohydrate products. Additionally, an -81% reduction in inflorescence stem glucomannan content, reduced quantity and growth rate of lateral roots, as well as a reduced susceptibility to *Agrobacterium tumefaciens*-mediated transformations have been observed in *cs/a9* knockout mutants. Questions regarding the specific biological functions of CSLA proteins await further investigation. Thus, it was hypothesized in *A. thaliana*, *AtCSLA1* and *AtCSLA9* have unique expression patterns in various tissues during specific stages of development. The expression patterns of the *AtCSLA7* and *AtCSLA9* genes during plant development were studied in *A. thaliana* using promoter-GUS fusion analysis. The results demonstrated unique expression patterns of the *AtCSLA7* and *AtCSLA9* genes, with numerous examples of overlapping expression at specific developmental stages, supporting the hypothesis. Understanding *AtCSLA7* and *AtCSLA9* gene expression patterns and functions will also improve understanding of the roles of mannan carbohydrates in plants.

## **Degree Type**

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## **Department**

Biology

## **First Advisor**

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**OBSERVING CSLA EXPRESSION PATTERNS IN *ARABIDOPSIS THALIANA* USING  
PROMOTER-GUS FUSION ANALYSIS**

By

Evan R. VandenBosch

A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

in Partial Fulfillment of the Requirements for Graduation

with Honors in Biology

Approved at Ypsilanti, Michigan, on this date April 25, 2012

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## Abstract

Plant cell walls are the world's most abundant source of renewable biomass. Consisting mainly of carbohydrates, including mannans, plant cell walls are vital to humanity as a source of food, health products, and biofuels. Previous research suggests diverse functions of mannans and implicates several members of the *CELLULOSE SYNTHASE-LIKE A (CSLA)* gene family as mannan synthases, proteins involved in mannan backbone synthesis. Prior research using *Arabidopsis thaliana* (*A. thaliana*) *csla* single mutants showed no obvious phenotypic abnormalities, with the exception of *csla7*, which was embryo lethal; however, ectopic expression of *CSLA9* complemented the *csla7* mutant phenotype, suggesting *csla7* and *csla9* enzymes make similar carbohydrate products. Additionally, an ~81% reduction in inflorescence stem glucomannan content, reduced quantity and growth rate of lateral roots, as well as a reduced susceptibility to *Agrobacterium tumefaciens*-mediated transformations have been observed in *csla9* knockout mutants. Questions regarding the specific biological functions of CSLA proteins await further investigation. Thus, it was hypothesized **in *A. thaliana*, *AtCSLA7* and *AtCSLA9* have unique expression patterns in various tissues during specific stages of development.** The expression patterns of the *AtCSLA7* and *AtCSLA9* genes during plant development were studied in *A. thaliana* using promoter-*GUS* fusion analysis. The results demonstrated unique expression patterns of the *AtCSLA7* and *AtCSLA9* genes, with numerous examples of overlapping expression at specific developmental stages, supporting the hypothesis. Understanding *AtCSLA7* and *AtCSLA9* gene expression patterns and functions will also improve understanding of the roles of mannan carbohydrates in plants.

## Background and Introduction

### Plant Cell Walls

Plant cell walls are the world's most abundant source of renewable biomass and are vital to humanity because they provide a source of food, health products, and biofuels (Pauly and Keegstra, 2008; Liepman et al., 2010). Plant cell walls consist mainly of carbohydrates including cellulose, hemicelluloses, and pectins. Hemicelluloses are a group of various polysaccharides with (1,4)- $\beta$ -linked backbones of glucose, mannose, or xylose that interact with cellulose (Scheller and Ulvskov, 2010). In plant cell walls, hemicelluloses link together cellulose microfibrils, providing strength and support to the cell wall (Cosgrove, 2005).

### Cellulose Synthase-Like (CSL) Genes

Although many genes involved in plant cell wall biogenesis have been elucidated, they represent a small fraction of over 2,000 estimated genes encoding proteins involved in a diversity of processes (e.g. secretion, synthesis and interconversion of nucleotide sugars substrates, substrate transporters, etc.) essential to cell wall biogenesis (Carpita et al., 2001). The *CELLULOSE SYNTHASE-LIKE (CSL)* genes are a family of genes suggested to encode proteins that synthesize hemicelluloses (Richmond and Summerville, 2000; Hazen et al., 2002). *CSL* genes have been divided into subclasses *A* through *J* based on amino acid sequence similarity, with distinct distributions of these genes present in different plants (Fincher, 2009).

Prior studies have confirmed the hypothesis that CSL proteins are involved in the synthesis of hemicelluloses. Several CSLA proteins have been characterized and shown to be (1,4)- $\beta$ -mannan synthases involved in mannan synthesis (Dhugga et al., 2004; Liepman et al., 2005; Suzuki et al., 2006; Liepman et al., 2007; Gille et al., 2011). Several CSLF proteins (Burton et al., 2006; Taketa et al., 2012; Vega-Sanchez et al., 2012) and a CSLH protein (Doblin et al., 2009) are (1,3;1,4)- $\beta$ -D-glucan synthases involved in  $\beta$ -glucan synthesis. Two members of the CSLC protein family are (1,4)- $\beta$  glucan synthases involved in xyloglucan synthesis (Cocuron et al., 2007), however, it has been suggested other CLSC proteins have other functions (Dwivany et al., 2009). The function(s) of CSLD proteins are not yet clear. CSLD proteins appear to play a role in arabinoxylan synthesis (Li et al., 2009), cellulose content (Li et al., 2009; Luan et al., 2011), xylose levels (Luan et al., 2011), and homogalacturonan content (Li et al., 2009) in *Oryza sativa* (rice), mannan synthesis in *Arabidopsis thaliana* (*A. thaliana*; Verhertbruggen et al., 2011) and *in vitro* (Yin et al., 2011), as well as (1,4)- $\beta$ -glucan synthesis in root-hair cells (Park et al., 2011).

### **Cellulose Synthase-Like A (CSLA) Genes**

The *CSLA* gene family in *Arabidopsis thaliana* (*A. thaliana*), a model organism in plant biology, is composed of nine members (Table 1) (Richmond and Somerville, 2000). With its nine members, the *CSLA* gene family is the largest *CSL* gene family in *A. thaliana*. Previous research shows that *CSLA* genes from numerous plants, including *A. thaliana*, each encode proteins exhibiting mannan synthase activity (Dhugga et al., 2004; Liepman et al., 2005; Suzuki et al., 2006; Liepman et al., 2007; Goubet et al.,

2009; Gille et al., 2011). As previously indicated, mannan synthases catalyze the polymerization of the backbone of mannans, a type of hemicellulosic polysaccharide found in plant cell walls (Liepman et al., 2005). Mannans serve as structural elements and carbohydrate storage reserves during plant development, and may serve additional potential functions (Liepman et al., 2007). Despite the *CSLA* gene family being the largest CSL gene family and the known roles of mannans during plant development, mannans are less abundant than some other hemicelluloses in *A. thaliana* (Zabackis et al., 1995), raising questions regarding why so many *CSLA* genes are present in *A. thaliana*.

**Table 1 – The *CSLA* genes of *Arabidopsis thaliana*, along with their unique genomic identifiers (AGI IDs) and enzymatic functions (where known). My thesis focuses upon AtCSLA7 and AtCSLA9.**

<b>Gene</b>	<b>AGI ID</b>	<b>Enzymatic Function</b>	<b>Reference</b>
<i>AtCSLA1</i>	At4g16590	Mannan Synthase	Liepman et al., 2007
<i>AtCSLA2</i>	At5g22740	Mannan Synthase	Liepman et al., 2005
<i>AtCSLA3</i>	At1g23480	Mannan Synthase	Goubet et al., 2009
<b><i>AtCSLA7</i></b>	<b>At2g35650</b>	<b>Mannan Synthase</b>	<b>Liepman et al., 2005</b>
<b><i>AtCSLA9</i></b>	<b>At5g03760</b>	<b>Mannan Synthase</b>	<b>Liepman et al., 2005</b>
<i>AtCSLA10</i>	At1g24070	Unknown	-
<i>AtCSLA11</i>	At5g16190	Mannan Synthase	Arnold and Liepman, unpublished
<i>AtCSLA14</i>	At3g56000	Mannan Synthase	Arnold and Liepman, unpublished
<i>AtCSLA15</i>	At4g13410	Unknown	-

### **Studies of *A. thaliana* *csla* Mutants**

The *Arabidopsis* research community has generated a large collection of mutant plants (Alonso et al., 2003). These mutants are useful in identifying the consequences of the loss of function of a gene. However, in some cases, it may be difficult to discern



a phenotype in mutant plants because of factors including genetic redundancies and minor novel phenotypes. *A. thaliana* knockout mutant studies suggest overlapping expression of various *CSLA* genes, such as *CSLA2*, *CSLA3*, and *CSLA9* in inflorescence stems. Unique and important roles of *CSLA* genes were documented as well, such as *CSLA9* in root development and branching and *CSLA7* in embryogenesis (Goubet et al, 2003; Zhu et al., 2003; Goubet et al., 2009). Analysis using *A. thaliana* *csla* single mutants, for all nine *AtCSLA* genes, showed no obvious phenotypic abnormalities, with the exception of *csla7*, which was embryo lethal (Goubet et al., 2003). It was also demonstrated ectopic expression of *CSLA9* complemented the *csla7* mutant phenotype, suggesting *CSLA7* and *CSLA9* enzymes make similar carbohydrate products, yet there was asynchrony of embryogenesis (Goubet et al., 2009). Microscopy and carbohydrate compositional analysis demonstrated an ~81% reduction in glucomannan content of the inflorescence stem of a *csla9* knockout mutant (Goubet et al., 2009). When a *csla2*, *csla3*, and *csla9* (*csla2/3/9*) triple knockout mutant was analyzed, glucomannan was nearly undetectable in the inflorescence stems (Goubet et al., 2009). Despite lacking detectable glucomannan, the stem strength and development of the *csla2/3/9* triple knockout mutant was not observably altered (Goubet et al., 2009). Another study demonstrated *CSLA9* promoter activity in the zone of elongation of *A. thaliana* roots (Zhu et al., 2003). In a *csla9* knockout mutant, known as *rat4*, there was a reduced quantity and growth rate of lateral roots, as well as a reduced susceptibility to *Agrobacterium tumefaciens*-mediated root transformation (Zhu et al., 2003), indicating *CSLA9* is involved in root development and branching in *A. thaliana*.

## **Microarray Expression Analysis of CSLA Genes**

Microarray expression analysis is a useful technique for analyzing gene expression in homogenized tissue samples; however, spatial information about cells prior to tissue disruption is lost when tissues are homogenized. Previous microarray expression analysis has shown distinct expression patterns among the *AtCSLA* genes (Hamann et al., 2004). Further investigations using microarray expression analysis have demonstrated similar tissue-specific expression patterns of *AtCSLA* genes in various vegetative and floral tissues; more specifically, *AtCSLA7* and *AtCSLA9* demonstrated expression in the hypocotyl, root, leaf, and stem tissues, with a noticeably high expression level of *AtCSLA9* in stem tissue (Liepman et al., 2007). In floral tissues, *AtCSLA7* demonstrated low expression levels in sepal, petal, stamen, carpal, and pollen tissues, while *AtCSLA9* showed low expression levels in petal and carpal tissues, moderate expression in stamen tissue, and very high expression levels in pollen tissue (Liepman et al., 2007). Additionally, although mannans are a relatively minor constituent in some *A. thaliana* tissues, including leaves, there are nine *AtCSLA* genes and multiple *CSLA* transcripts were present simultaneously in *A. thaliana* leaf tissues (Liepman et al., 2007).

## **Hypothesis and Introduction to Current Study**

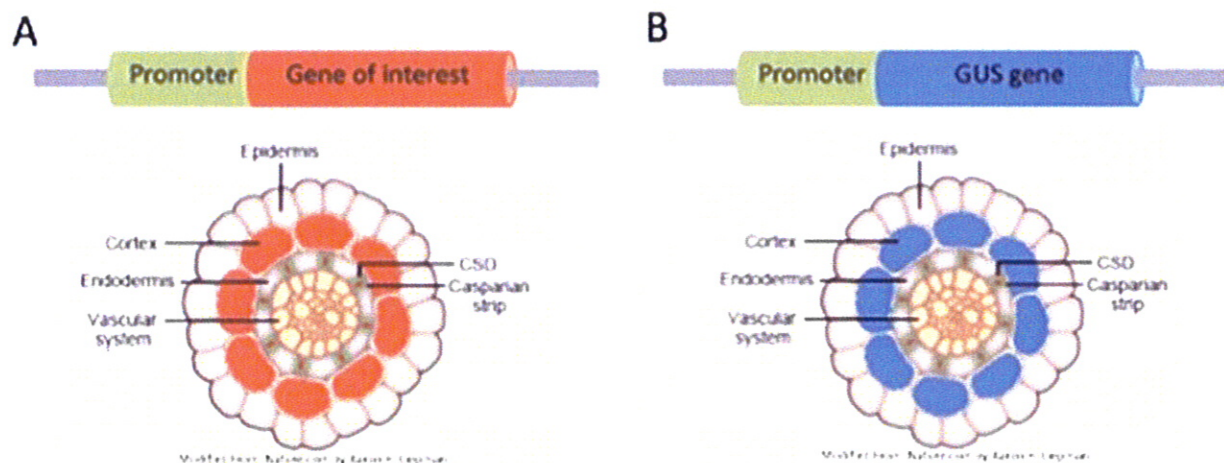
The disconnect between the number of *AtCSLA* genes and mannan abundance in some *A. thaliana* tissues (Liepman et al., 2007), combined with the results from the knockout mutant analysis (Goubet et al., 2003; Zhu et al., 2003; Goubet et al., 2009) and microarray expression analysis (Hamann et al., 2004; Liepman et al., 2007), raises

further questions about the expression patterns of *AtCSLA* genes in *A. thaliana*, specifically the expression patterns of *AtCSLA7* and *AtCSLA9*. While *AtCSLA* gene expression at the tissue level has been previously documented, very little is known about the spatiotemporal expression patterns of *AtCSLA* genes at the cellular level within these tissues, warranting further investigation using a different biological research tool capable of detecting spatiotemporal gene expression patterns at the cellular level. Thus, it was hypothesized **in *A. thaliana*, *AtCSLA7* and *AtCSLA9* have unique expression patterns in various tissues during specific stages of development.** The hypothesis was investigated using promoter-*GUS* fusion analysis (Jefferson et al., 1987). Promoter-*GUS* fusion analysis preserves spatial information lost using techniques where tissues are homogenized, allowing the mapping of gene expression at a cellular level. Promoter-*GUS* fusion analysis is a sensitive technique used to visualize expression patterns of genes by generating an indigo precipitate or “stain” where and when a gene is expressed (Jefferson et al., 2001). The location of this stain can be visualized using microscopy.

### **Promoter-*GUS* Fusion Analysis Theory**

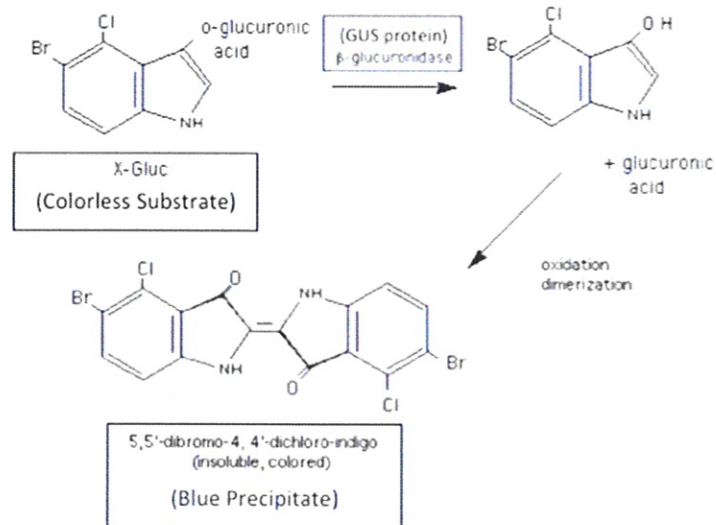
The promoter of a gene specifies the expression pattern of that gene. In a wild type (WT), or “normal,” organism the promoter of a gene specifies the expression pattern of that gene. If the promoter of a gene is fused to a different gene, ideally that promoter will then specify the same expression pattern, but for the new fused gene. For example, the promoter of a gene may specify a gene be expressed in specific plant tissue(s) (Figure 1A); however, if the promoter of that gene is fused to another gene

such as the *Escherichia coli*  $\beta$ -glucuronidase (*GUS*) reporter gene, then the *GUS* reporter gene will be expressed in the same tissue(s) as the original gene (Figure 1B). It is important to note, although ideally the expression pattern of the promoter will be retained when fused to the *GUS* reporter gene, elements within a gene also can contribute to its regulatory pattern.



**Figure 1 – Theory of promoter-*GUS* fusion analysis. (A) A promoter upstream of its corresponding gene specifies the expression pattern of the wild-type gene of interest. (B) Fusion of the same promoter to the *GUS* reporter gene, to direct expression of the *GUS* gene in the same pattern as the gene of interest. (Images modified from Nature.com by Aaron H. Liepman)**

The *GUS* staining assay requires transgenic plants be incubated in 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronic acid (X-Gluc), a colorless substrate which the *GUS* protein cleaves, allowing dimerization and the formation of a blue precipitate, as shown in Figure 2, which can be observed at the cellular level using microscopy.



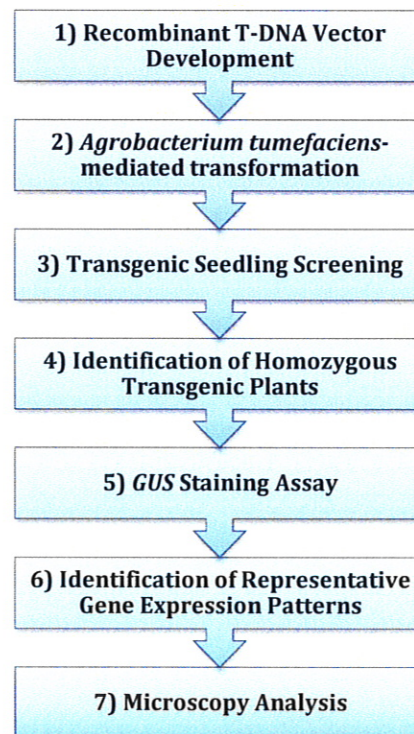
Modified from: [www.bio.purdue.edu/people/faculty/karcher/blue2000/fig1.gif](http://www.bio.purdue.edu/people/faculty/karcher/blue2000/fig1.gif)

**Figure 2 – The enzymatic activity of the *Escherichia coli* β-glucuronidase (GUS) protein when exposed to the colorless substrate X-Gluc. The GUS protein cleaves the glucuronic acid substituent off of X-Gluc, allowing the resulting molecules to undergo oxidative dimerization, resulting in an insoluble blue precipitate, which may be observed using microscopy.**

## Materials and Methods

### Promoter-*GUS* Fusion Analysis Overview

A conceptual flow diagram of the general steps used during this experiment is displayed in Figure 3. As shown in Figure 3, recombinant T-DNA vectors were developed and transformed into *Agrobacterium tumefaciens* (*A. tumefaciens*), which was used to transform wild-type, flowering *A. thaliana* plants. Transgenic plants were selected on the basis of their hygromycin resistance and homozygous plants were



**Figure 3 – Conceptual diagram of the general steps used in this experiment.**

generated and identified. The *GUS* staining assay was used to identify representative seeds for each gene that were used for detailed analysis using microscopy (Figure 3).

### **Recombinant T-DNA Vector Development**

Recombinant DNA constructs containing the *GUS* gene fused to either the *AtCSLA7* promoter (1435 bp) or the *AtCSLA9* promoter (2421 bp) were generated by former members of the Liepman lab. Adnan K. Syed developed the *AtCSLA9-GUS* construct and Alexis I. Stein developed the *AtCSLA7-GUS* construct using standard recombinant DNA techniques. The sequence of each promoter fragment was verified to be error-free by using DNA sequencing. The *AtCSLA-GUS* constructs (pCAMBIA-*AtCSLA7* and pCAMBIA-*AtCSLA9*) were created by amplifying the particular *CSLA* promoters, cloning them in a cloning vector, sequencing them to verify absence of mutations, and using restriction enzymes and ligation to fuse the promoters adjacent to the *GUS* gene within the T-DNA region of the pCAMBIA 1305.1 vector. Additional notable components of the T-DNA region of the pCAMBIA 1305.1 vector include a hygromycin (herbicide) resistance marker, to facilitate selection of the transformed seedlings, and a kanamycin (antimicrobial) resistance marker, to select transformed bacterial cells. The genetic maps of the pCAMBIA-*AtCSLA7* and pCAMBIA-*AtCSLA9* constructs are displayed in Figures 4 and 5, respectively.

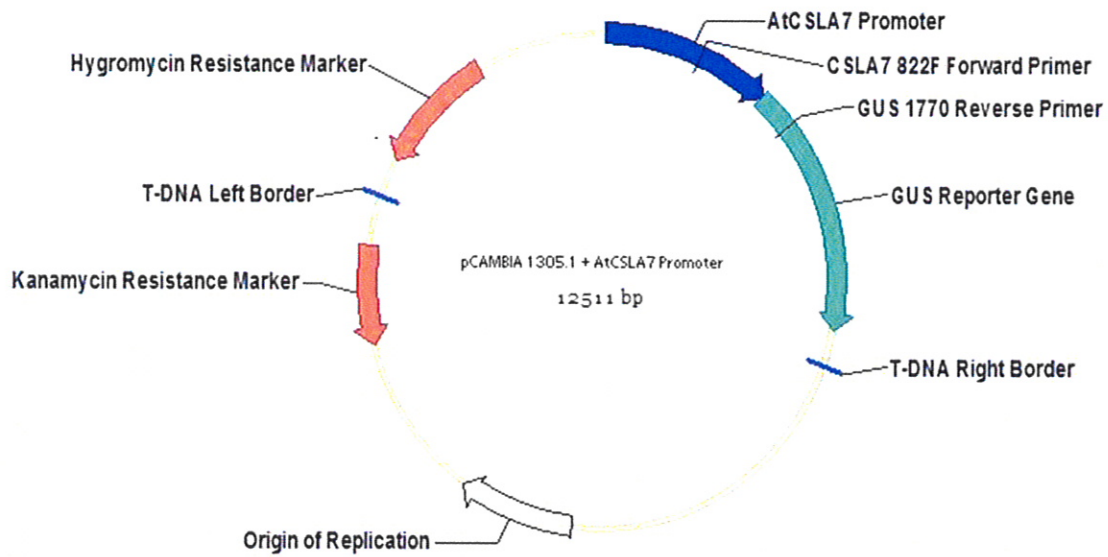


Figure 4 – pCAMBIA 1305.1 vector containing the *AtCSLA7* promoter-*GUS* reporter gene fusion (pCAMBIA-*AtCSLA7*)

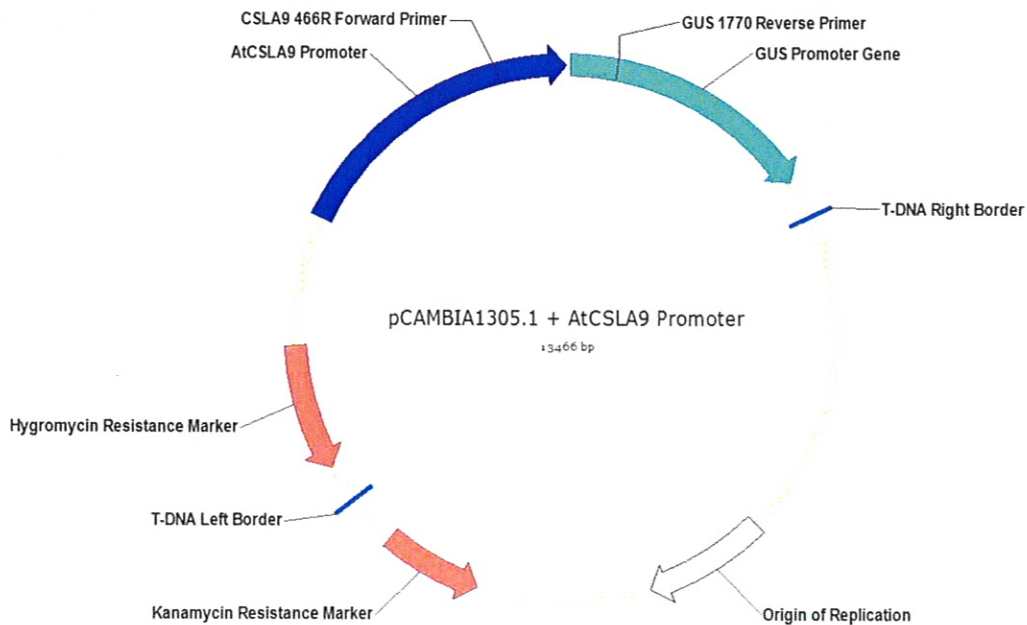


Figure 5 – pCAMBIA 1305.1 vector containing the *AtCSLA9* promoter-*GUS* reporter gene fusion (pCAMBIA-*AtCSLA9*)

### **Transformation of *A. tumefaciens* with *GUS* constructs**

A 0.5µg sample of the recombinant T-DNA vector (pCAMBIA-*AtCSLA7* or pCAMBIA-*AtCSLA9*) was concentrated to a final volume of 5µl and combined with 100µl of EHA10S competent *A. tumefaciens* cells, which had been stored at -80°C. The solution containing the vector sample and competent cells was thawed for five minutes at 37°C, refrozen in liquid nitrogen, and thawed again for five minutes at 37°C. 900µl of liquid half salt LB media (10 mg/ml Tryptone Broth, 5 mg/ml Yeast Extract, 5 mg/ml NaCl, recipe in appendix A) was added to the competent cell and vector mixture. The solution was incubated at 28°C for 3 hours with shaking at 200 RPM. The bacterial cells in the solution were sedimented using centrifugation at 6,000 x g for 10 minutes at room temperature. The resulting pellet was resuspended in 100µl of supernatant and spread on half salt LB plates, pH 7.5, containing 30 mg/L rifampicin and 50 mg/L kanamycin. The plates were incubated for two days at 28°C to allow for colony development.

### ***Agrobacterium tumefaciens*-mediated transformation**

10 ml broth cultures of liquid half salt LB media containing 30 µg/ml rifampicin and 50 µg/ml kanamycin were individually inoculated using separate colonies from the incubated plates. The broth cultures were incubated at 28°C for 3 days with shaking at 200 RPM. After 3 days, 300 ml of liquid half salt LB media containing 30 µg/ml rifampicin and 50 µg/ml kanamycin was placed in a 500 ml Erlenmeyer flask and inoculated with 2 ml of broth culture. The inoculated broth



**Figure 6 – *A. thaliana* plant early in flowering stage.**



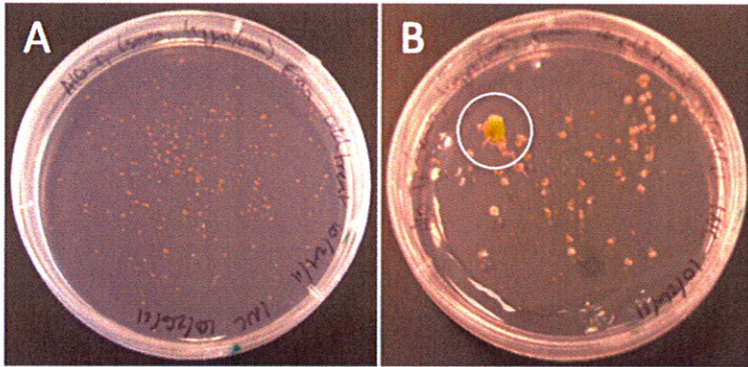
culture was incubated at 28°C for approximately 16 hours with shaking at 200 RPM. The cell densities of the broth cultures were monitored spectrophotometrically by measuring the optical density of the liquid at 600 nm ( $OD_{600}$ ) until a value between 0.8 and 1.0 was reached. The cells from the culture were sedimented at 11,000 x g for 10 minutes at room temperature, the supernatant was discarded, and the pellet was resuspended in 300 ml of resuspension solution containing 5% (w/v) sucrose and 45mM  $MgCl_2$ . The resuspended cells were poured into a small plastic tray and Silwet was mixed into the solution to a final concentration of 0.5% (v/v). Wild-type *A. thaliana* plants of the Columbia ecotype (approximately 3 to 4 weeks old, early in the flowering stage; Figure 6), were dipped in the solution and a dropper was used to ensure solution penetrated developing flowers. The transformed plants were covered in thin plastic wrap (Saran Wrap) for 24 hours and grown under long day growth conditions (16 hours of light) at 22°C for the remainder of their life cycle. The resulting  $T_0$  seeds were collected and archived.

### **Seed Sterilization**

Seeds were sterilized in a 20% (v/v) bleach solution for 30 minutes, rinsed in sterile deionized water three times, then plated as indicated in each protocol. All seed sterilization and plating procedures were conducted in a laminar flow hood, to minimize contamination.

### **Transgenic Seedling Screening**

$T_0$  seeds were sterilized as previously indicated and plated on Murashige &



**Figure 7 – Identification of T<sub>0</sub> transgenic seeds using hygromycin selection (A) Sterilized seeds placed on MS hygromycin media for selection (B) Two-week-old seedlings grown on MS hygromycin for selection of rare surviving, transgenic, seedlings.**

Skoog (MS) solid media containing hygromycin (50 mg/L), recipe in appendix A (MS+H media), as shown in Figure 7A, and grown with constant light for two weeks. Candidate transgenic seedlings were identified on the basis of their ability to survive on hygromycin

(non-candidate seedlings would be expected to die on hygromycin-containing media), as shown in Figure 7B. The T<sub>0</sub> seedlings that survived selection were transferred to soil and grown under long day growth conditions (16 hours of light) to set seeds, producing the next generation (T<sub>1</sub> generation).

### **PCR Amplification and Gel-Electrophoresis Genotyping**

Leaves of developing T<sub>0</sub> plants at approximately 4 weeks of age were placed between wax paper and Whatman<sup>®</sup> FTA<sup>®</sup> cards and crushed with a pestle, generating archived DNA samples on the Whatman<sup>®</sup> FTA<sup>®</sup> cards using a procedure detailed at ([http://microscopy.tamu.edu/lab-protocols/protocols-FTA\\_paper\\_processing.pdf/view](http://microscopy.tamu.edu/lab-protocols/protocols-FTA_paper_processing.pdf/view)).

Small punches of the archived DNA cards were washed twice for 5 minutes in FTA<sup>®</sup> Purification Reagent (Whatman<sup>®</sup>) then twice in TE<sup>-1</sup> buffer (10 mM Tris pH 8, 0.1mM EDTA). The washed punches containing DNA samples were dried at room temperature for at least one day.

Punches containing DNA samples of potential transgenic lines (or wild-type

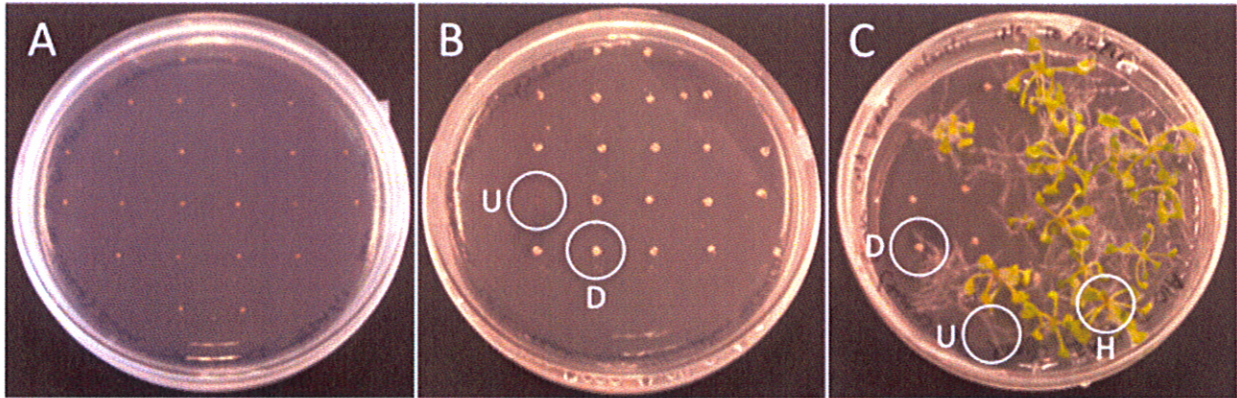
control), were placed in thin-walled PCR tubes containing 20  $\mu$ l of 1X GoTaq, 0.2 $\mu$ M forward primer (CSLA7-822F or CSLA9 466R, Appendix B), and 0.2 $\mu$ M reverse primer (GUS 1770R, Appendix B) reaction solution. A no DNA (negative control) PCR reaction and a miniprep (1:100,000 dilution, positive control) PCR reaction using the corresponding pCAMBIA-*AtCSLA7* or pCAMBIA-*AtCSLA9* vector were also conducted. The PCR amplification conditions used were: (1 cycle: 95°C for 2 minutes; 30 cycles: 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 1 minute and 15 seconds; 1 cycle: 72°C for 5 minutes; Appendix B).

PCR products were electrophoresed on 0.7% agarose (w/v) gels containing, 0.5X TBE buffer (45mM Tris base, 0.275% (w/v) boric acid, 0.001M EDTA, pH 8.0) and 1X gel red. For electrophoresis, gels were submerged in 0.5X TBE buffer in a gel rig (Owl Separation Systems, Model: B2). The gel was run at ~100 volts for approximately 45 minutes and visualized with UV transillumination using a gel documentation system (Bio-Rad).

### **Identification of Homozygous Transgenic Plants**

A sample of the collected, transgenic T<sub>1</sub> seeds along with a sample of wild-type seeds as a control, were sterilized as previously indicated. 48 of the sterilized transgenic T<sub>1</sub> seeds were evenly divided with 24 seeds placed in a grid pattern on each of two MS+H media plates, as shown in Figure 8A. After 2 weeks of growth under constant illumination, seeds were classified as germinated and healthy (H), germinated and dead (D), or ungerminated (U), as shown in Figures 8B and 8C. As a control for the herbicide, it was expected all of the germinated wild-type seeds would die (D = 100%),

as shown in Figure 8B, since the wild-type seedlings were not hygromycin resistant. Transgenic seedlings survived as germinated and healthy (H) seedlings on the MS+H media, while non-transgenic seedlings germinated but died (D), as shown in Figure 8C. Any ungerminated seeds (U) were assumed to be non-viable.



**Figure 8 – Identification of homozygous transgenic seeds using hygromycin selection (A) Seeds placed in grid on MS hygromycin media for homozygous seed selection (B) Dead two-week-old wild-type seedlings in grid on MS hygromycin media as a control for hygromycin efficacy (C) Some surviving, transgenic, two-week-old seedlings on MS hygromycin.**

The segregation ratio ( $S$ ; the number of germinated and healthy seedlings ( $H$ ), divided by the total number of germinated seedlings ( $H+D$ ), and expressed as a percent; or  $[H/(H+D)] \cdot 100\% = S$ ) was used to infer whether the parental plant was heterozygous or homozygous for the *CSLA-GUS* T-DNA insert. If 95% or more of the seedlings were healthy ( $S \geq 95\%$ ) the parent plant was considered homozygous, however, if roughly 75% of the seedlings were healthy ( $S \approx 75\%$ ), the parent plant was considered heterozygous. Six surviving, transgenic  $T_1$  seedlings from each parent were planted on soil, grown under long day growth conditions (16 hours of light), and PCR genotyped. If the seedlings from a single parent plant demonstrated a homozygous segregation ratio and PCR genotyping verified the seedlings were transgenic, the batch

of seeds from the homozygous parent plant was ready for *GUS* staining and microscopy analysis. If the seedlings demonstrated a heterozygous segregation ratio, the T<sub>1</sub> seedlings that passed PCR genotyping were grown for T<sub>2</sub> seed collection. The collected T<sub>2</sub> seeds were archived. A sample of T<sub>2</sub> seeds was sterilized and gridded on MS+H media to observe the segregation ratios of the seedlings. This process of selection was continued until seeds from a homozygous parental plant from at least five different transgenic integration events for each gene were collected.

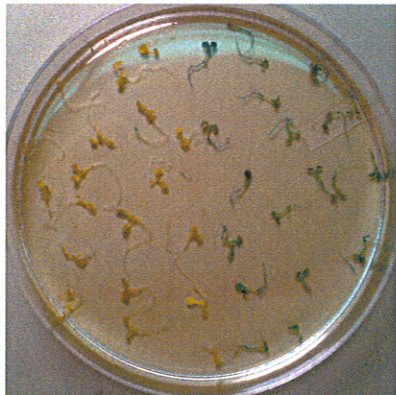
### ***GUS* Staining Assay**

The *GUS* staining assay was modified from the histochemical assay described by Jefferson et al. (2001). The seedlings and plants were harvested and placed in ice cold 90% acetone and allowed to incubate for 20 minutes at room temperature. The 90% acetone was removed and equilibration buffer solution (0.2% (v/v) Triton-X100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, sodium phosphate buffer 100 mM pH 8.0, recipe in Appendix A) was added to the plant samples in tubes on ice. The equilibration buffer solution was removed and staining buffer solution (0.2% (v/v) Triton-X100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, sodium phosphate buffer 100 mM pH 8.0, 2.4 mM X-gluc in dimethylformamide, recipe in Appendix A) was added. The plant samples were vacuum infiltrated (Welch, Model: 2522B-01) for 30 minutes. Plant samples were incubated at 37°C for 30 minutes to 2 hours. Staining solution was removed from samples and 20% (v/v) ethanol was added to samples. Samples incubated in 20% (v/v) ethanol for 30 minutes. The 20% (v/v) ethanol solution was removed and 35% (v/v) ethanol was added. Samples incubated in

35% (v/v) ethanol for 30 minutes, followed by 50% (v/v) ethanol for 30 minutes. The 50% ethanol (v/v) was replaced with FAA solution (50% (v/v) ethanol, 5% (v/v) formaldehyde, 10% (v/v) acetic acid) and samples incubated in FAA for 30 minutes. The FAA solution was removed and samples were stored in 70% (v/v) ethanol.

### **GUS Phenotyping Assay**

As an alternative to PCR genotyping, a protocol based on the *GUS* staining assay was established to determine whether a parental transgenic plant was homozygous or heterozygous. Potentially transgenic seeds and wild-type seeds were sterilized and plated on MS media, incubated in the dark at 4°C for 2 days to synchronize germination, and grown under constant light for 2 weeks.



**Figure 9 – 50% staining in diffusion control tube of *GUS* phenotyping assay.**

The seedlings were separated into three tubes: a “sample tube” containing 40 potentially transgenic seedlings, an “assay control tube” containing 40 wild-type seedlings, and a “diffusion control tube” containing 20 wild-type seedlings and 20 potentially transgenic seedlings (Table 2). The *GUS* staining protocol was performed on all three tubes under the same conditions.

The number of stained plants in each tube was counted. If none of the 40 seedlings in the assay control tube stained, it suggested the assay was executed correctly and the results were valid (Table 2). If 50% of the seedlings in the diffusion control tube were stained, it suggested the precipitate had not diffused into non-transgenic seedlings, as shown in Figure 9, if more than 50% of the seedlings were

stained the results were considered erroneous, most likely due to diffusion of the blue precipitate from transgenic plants to non-transgenic plants (Table 2). If 100% of the seedlings in the sample tube were stained, it suggested the parental plant was homozygous, however, if roughly 75% of the seedlings in the sample tube were stained, it suggested the parental plant was heterozygous (Table 2).

**Table 2 – Treatments, rationales, expectations, and conclusions for each of the three tubes (the sample tube, the assay control tube, and the diffusion control tube) used during the *GUS* phenotyping assay.**

<b>Tube</b>	<b>Treatment</b>	<b>Rationale</b>	<b>Expectations</b>	<b>Conclusions</b>
<b>Sample Tube</b>	40 potentially transgenic seedlings	To identify if the seeds came from a heterozygous or homozygous parent	Between 75% and 100% of the seedlings stained blue	If roughly 75% of seedlings stained blue, parent was heterozygous. If 100% of seedlings stained blue, parent was homozygous.
<b>Assay Control Tube</b>	40 wild-type seedlings	To ensure the <i>GUS</i> phenotyping assay was conducted properly	0% (none) of the seedlings stained blue	If 0% (none) of the seedlings stained blue, assay was conducted properly. If any seedlings stained blue, assay not conducted properly.
<b>Diffusion Control Tube</b>	20 potentially transgenic seedlings and 20 wild-type seedlings	To ensure the blue precipitate didn't diffuse from transgenic seedlings to non-transgenic seedlings	50% (or less) of the seedlings stained blue	If 50% (or less) of seedlings stained blue, no diffusion occurred, suggesting accurate results. If more than 50% of seedlings stained blue, diffusion occurred, suggesting inaccurate results.

### **Growth Conditions of Seedlings and Plants for *GUS* Staining Analysis**

Homozygous transgenic seeds were sterilized as previously described, plated on MS media, and incubated in the dark at 4°C for 2 d to synchronize germination.

Seedlings were grown for 1 to 7 d at 25°C under constant light (Table 3). 1 to 5-day-old etiolated seedlings were grown under the same conditions as the 1 to 7-day-old constant light grown seedlings, however, the media plates containing the etiolated seedlings were wrapped in foil to exclude light (Table 3). The seeds for 2 week, 4 week, and 7 week analysis were similarly sterilized, rinsed, planted, and incubated in the dark at 4°C for 2 d to synchronize germination, but were grown on plates under short day growth conditions (8 hr of light) for 2 weeks (Table 3). After 2 weeks, the 2-week-old plants were collected for analysis and the plants for 4-week-old and 7-week-old analysis were transferred to soil. The plants for 4-week-old were collected after another 2 weeks, for a total growth period of 4 weeks (Table 3). After the 4-week-old plants were collected the conditions were changed to long day conditions (16 hr of light) for the final 3 weeks of growth before the 7-week-old plants were collected for analysis (Table 3).

**Table 3 – Table of growth conditions used for growing 1 to 5-day-old (etiolated), 1 to 7-day-old (light-grown), 2-week-old, 4-week-old, and 7-week-old plants for *GUS* analysis of *AtCSLA7-GUS* and *AtCSLA9-GUS* expression in *A. thaliana*.**

Age	Growth Conditions
1 to 5-Day-Old (Etiolated)	No Light: 24 hr dark (25°C) for specified time of growth (1-5 days)
1 to 7-Day-Old (Light Grown)	Constant Light: 24 hr light (25°C) for specified time of growth (1-7 days)
2-Week-Old	Short Day Growth: 8 hr light (22°C) and 16 hr dark (18°C), for 2 weeks
4-Week-Old	Short Day Growth: 8 hr light (22°C) and 16 hr dark (18°C), for 4 weeks
7-Week-Old	Short Day Growth: 8 hr light (22°C) and 16 hr dark (18°C), for 4 weeks then, Long Day Growth: 16 hr light (22°C) and 8 hr dark (18°C), for 3 weeks
Note - All seeds were grown in the dark at 4°C for 2 days to synchronize germination before being grown under the specified conditions above	



## Identification of Representative Gene Expression Patterns

Because the T-DNA integrates semi-randomly into the *A. thaliana* genome, and the location where integration occurs can affect expression of the transgene, it is necessary to examine multiple transgenic lines to identify a representative pattern. To control for multiple integrations, or integrations into heterochromatin, seeds from a minimum of five different integration events for each gene were observed to identify a representative expression pattern. The seeds were sterilized as previously described and plated on MS media, incubated in the dark at 4°C for 2 d to synchronize germination, and grown under constant light at 25°C for 2 weeks. The seedlings were *GUS* stained and observed for similar staining patterns in seven different areas: the hypocotyl, cotyledons, first true leaves, true leaves, trichomes, primary roots, and lateral roots. Similar staining patterns were identified for each gene. If staining patterns from at least three of the five different integration events were similar, one of the integration events was chosen, using seedling viability, to be the representative expression pattern for that gene. Seeds subjected to representative expression pattern analysis for *AtCSLA7* were Line 5, Line 7, Line 12, Line 17, and Line 18, and for *AtCSLA9* were Line 1, Line 2, Line 3, Line 4, and Line 5. Seeds with expression patterns deemed representative were *AtCSLA7* Line 17 and *AtCSLA9* Line 2. Seeds with representative patterns were subjected to detailed analysis using microscopy.

## Microscopy Analysis

Using microscopy, the indigo precipitate resulting from the action of the *GUS*

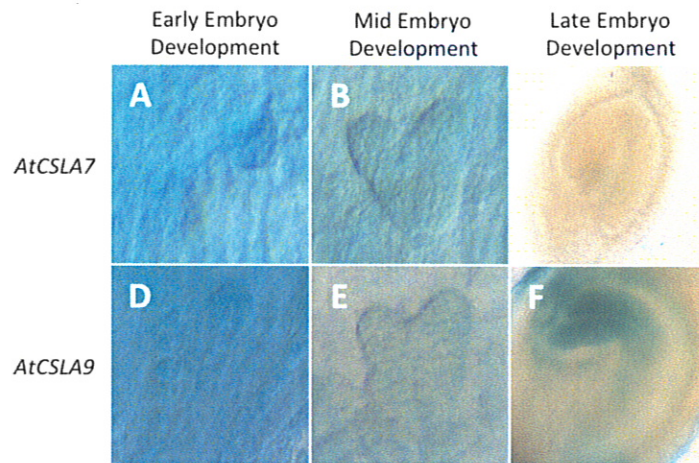
reporter protein upon X-gluc was visually observed; the pattern of precipitate observed suggested the location of the corresponding active *CSLA* gene promoter. Gene expression patterns were observed and photographed by Mary E. Skinner using a Canon EOS Rebel XSi digital camera mounted to an Olympus dissecting microscope, or an Olympus compound microscope. The computer and software used were the DSLR Remote Pro software on an Apple iMac7,1 computer.

## Results

### ***AtCSLA7-GUS* and *AtCSLA9-GUS* Expression Patterns:**

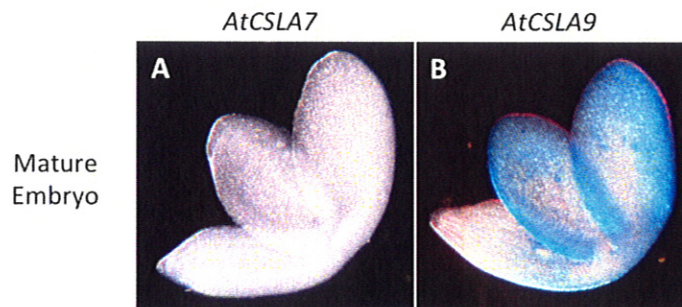
#### **Embryonic Stages**

*AtCSLA7-GUS* and *AtCSLA9-GUS* expression was analyzed at three different stages of embryo development: early, mid, and late embryo development. In the early and mid stages of embryo development *AtCSLA7-GUS* is expressed, however, in the late stages of embryo development there was no expression observed (Figures 10A-10C). *AtCSLA9-GUS* expression was observed throughout embryo development (Figures 10D-10F).



**Figure 10 – *AtCSLA7-GUS* and *AtCSLA9-GUS* expression patterns during embryo development. *AtCSLA7-GUS* expression in early (A), mid (B), and late (C) embryo development. *AtCSLA9-GUS* expression in early (D), mid (E), and late (F) embryo development.**

*AtCSLA7-GUS* gene expression was not observed in mature embryos, however *AtCSLA9-GUS* expression was observed around the periphery of the cotyledons (Figures 11A and 11B).



**Figure 11 – *AtCSLA7-GUS* and *AtCSLA9-GUS* expression in mature embryos. *AtCSLA7-GUS* (A) and *AtCSLA9-GUS* (B) expression in mature embryos.**

### ***AtCSLA7-GUS* and *AtCSLA9-GUS* Expression Patterns:**

#### **1 to 5-Day-Old Etiolated Seedlings**

In 1-day-old etiolated seedlings there was no observable expression of *AtCSLA7-GUS*, however, *AtCSLA9-GUS* expression was observed in the radicle (Figures 12A and 12F). In 2-day-old etiolated seedlings *AtCSLA7-GUS* expression was observed in the shoot and root apical meristems, tips of cotyledons, hypocotyl, and radicle (Figure

12B). *AtCSLA9-GUS* expression was observed in the shoot and root apical meristems, tips of cotyledons, hypocotyl, and radicle of 2-day-old etiolated seedlings (Figure 12G). In 3-day-old etiolated seedlings *AtCSLA7-GUS* expression was observed in the root apical meristem, tips of cotyledons, hypocotyl, and radicle (Figure 12C). *AtCSLA9-GUS* expression was observed in the shoot and root apical meristems, tips of cotyledons, hypocotyl, and radicle of 3-day-old etiolated seedlings (Figure 12H). In 4-day-old etiolated seedlings *AtCSLA7-GUS* expression was observed in the root apical meristem, hypocotyl, and radicle (Figure 12D). *AtCSLA9-GUS* expression was observed in the shoot and root apical meristems, tips of cotyledons, hypocotyl, and radicle of 4-day-old etiolated seedlings (Figure 12I). In 5-day-old etiolated seedlings *AtCSLA7-GUS* expression was observed in the root apical meristem, hypocotyl, and radicle (Figure 12E). *AtCSLA9-GUS* expression was observed in the root apical meristem, hypocotyl, and radicle of 5-day-old etiolated seedlings (Figure 12J).

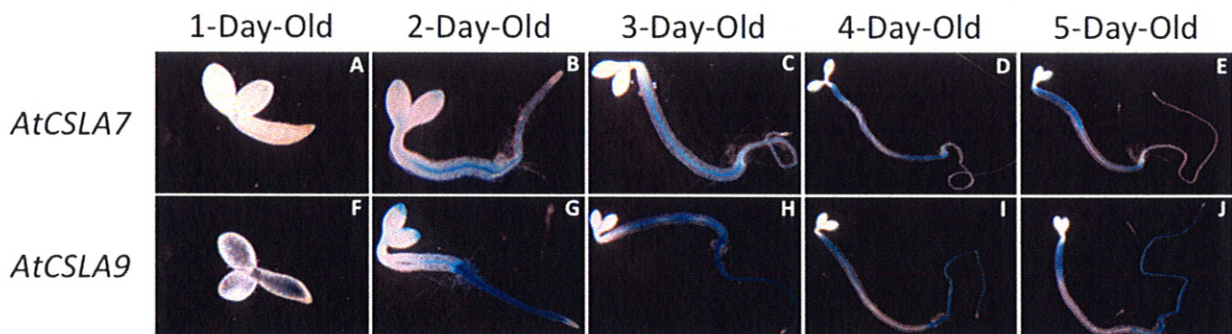


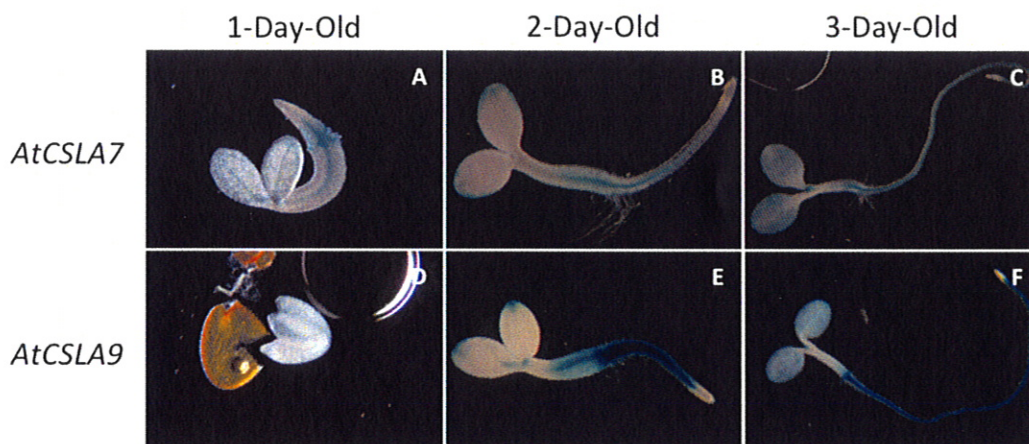
Figure 12 – *AtCSLA7-GUS* and *AtCSLA9-GUS* expression in 1 to 5-day-old etiolated seedlings. *AtCSLA7-GUS* expression (A-E), *AtCSLA9-GUS* expression (F-J).

### ***AtCSLA7-GUS* and *AtCSLA9-GUS* Expression Patterns:**

#### **1 to 7-Day-Old Light-Grown Seedlings**

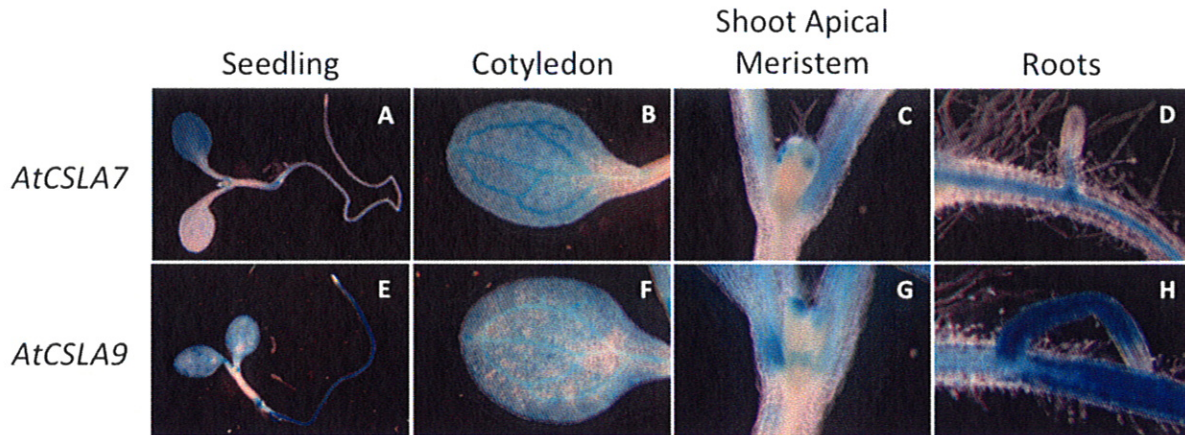
In 1-day-old light-grown seedlings, *AtCSLA7-GUS* expression was observed in the radicle, hypocotyl, and tips of cotyledons (Figure 13A), while there was no

observable *AtCSLA9-GUS* expression (Figure 13D). In 2-day-old light-grown seedlings, *AtCSLA7-GUS* expression was observed in the radicle, hypocotyl, and tips of the cotyledons (Figure 13B), with observed *AtCSLA9-GUS* expression in the radicle, hypocotyl, shoot apical meristem, and tips of the cotyledons (Figure 13E). In 3-day-old seedlings grown under continuous light, both *AtCSLA7-GUS* and *AtCSLA9-GUS* expression were observed in the radicle, hypocotyl, and cotyledons (Figure 13C and Figure 13F), with additional expression of *AtCSLA7-GUS* in the shoot apical meristem (Figure 13C).



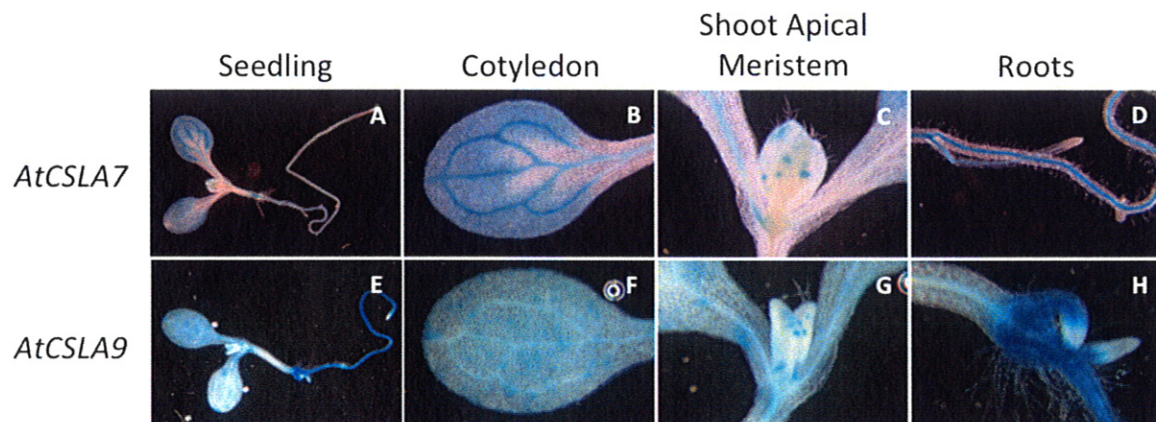
**Figure 13 – *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression in 1 to 3-day-old seedlings grown under constant light. *AtCSLA7-GUS* expression (A-C), *AtCSLA9-GUS* expression (D-F).**

In 4-day-old seedlings, *AtCSLA7-GUS* expression was observed in the primary root (Figure 14D), developing axial roots (Figure 14D), hypocotyl (Figure 14A), shoot apical meristem (Figure 14C), cotyledons (Figure 14B), and first true leaves (Figure 14C). *AtCSLA9-GUS* expression was observed in the primary root (Figure 14H), developing axial roots (Figure 14H), hypocotyl (Figure 14E), shoot apical meristem (Figure 14G), cotyledons (Figure 14F), and first true leaves (Figure 14G) of the 4-day-old light-grown seedlings.



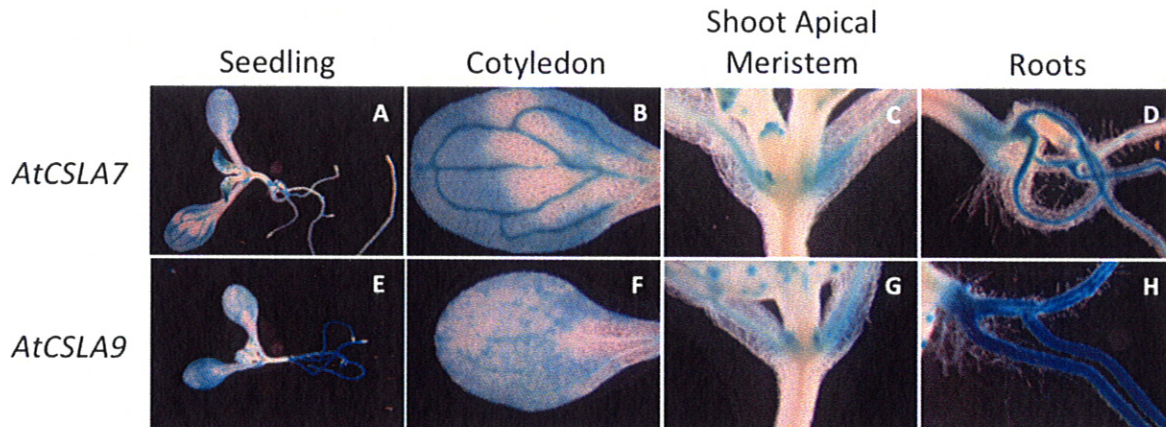
**Figure 14 – *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression in 4-day-old seedlings grown under constant light. *AtCSLA7-GUS* expression in 4-day-old light-grown seedlings (A), cotyledons (B), shoot apical meristem (C), and roots (D). *AtCSLA9-GUS* expression in 4-day-old light-grown seedlings (E), cotyledons (F), shoot apical meristem (G), and roots (H).**

In 5-day-old light-grown seedlings, *AtCSLA7-GUS* expression was observed in the primary root (Figure 15D), developing axial roots (Figure 15D), hypocotyl (Figure 15A), shoot apical meristem (Figure 15C), cotyledons (Figure 15B), first true leaves (Figure 15C), and developing trichomes (Figure 15C). *AtCSLA9-GUS* expression was observed in the primary root (Figure 15H), developing axial roots (Figure 15H), hypocotyl (Figure 15E), shoot apical meristem (Figure 15G), cotyledons (Figure 15F), and first true leaves (Figure 15G) of the 5-day-old light-grown seedlings.



**Figure 15 – *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression in 5-day-old seedlings grown under constant light. *AtCSLA7-GUS* expression in 5-day-old light-grown seedlings (A), cotyledons (B), shoot apical meristem (C), and roots (D). *AtCSLA9-GUS* expression in 5-day-old light-grown seedlings (E), cotyledons (F), shoot apical meristem (G), and roots (H).**

In 6-day-old seedlings grown under constant light, *AtCSLA7-GUS* expression was observed in the primary root (Figure 16D), axial roots (Figure 16D), hypocotyl (Figure 16A), shoot apical meristem (Figure 16C), cotyledons (Figure 16B), first true leaves (Figure 16C), and developing trichomes (Figure 16C). *AtCSLA9-GUS* expression was observed in the primary root (Figure 16H), developing axial roots (Figure 16H), hypocotyl (Figure 16E), shoot apical meristem (Figure 16G), cotyledons (Figure 16F), first true leaves (Figure 16G), and developing trichomes (Figure 16G) of the 6-day-old light-grown seedlings.



**Figure 16 – *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression in 6-day-old seedlings grown under constant light. *AtCSLA7-GUS* expression in 6-day-old light-grown seedlings (A), cotyledons (B), shoot apical meristem (C), and roots (D). *AtCSLA9-GUS* expression in 6-day-old light-grown seedlings (E), cotyledons (F), shoot apical meristem (G), and roots (H).**

In 7-day-old seedlings grown under constant light, *AtCSLA7-GUS* expression was observed in the primary root (Figure 17D), axial roots (Figure 17D), hypocotyl (Figure 17A), cotyledons (Figure 17B), and first true leaves (Figure 17C). *AtCSLA9-GUS* expression was observed in the primary root (Figure 17H), developing axial roots (Figure 17H), hypocotyl (Figure 17E), shoot apical meristem (Figure 17G), cotyledons (Figure 17F), first true leaves (Figure 17G), and developing trichomes (Figure 17G) of the 7-day-old light-grown seedlings.

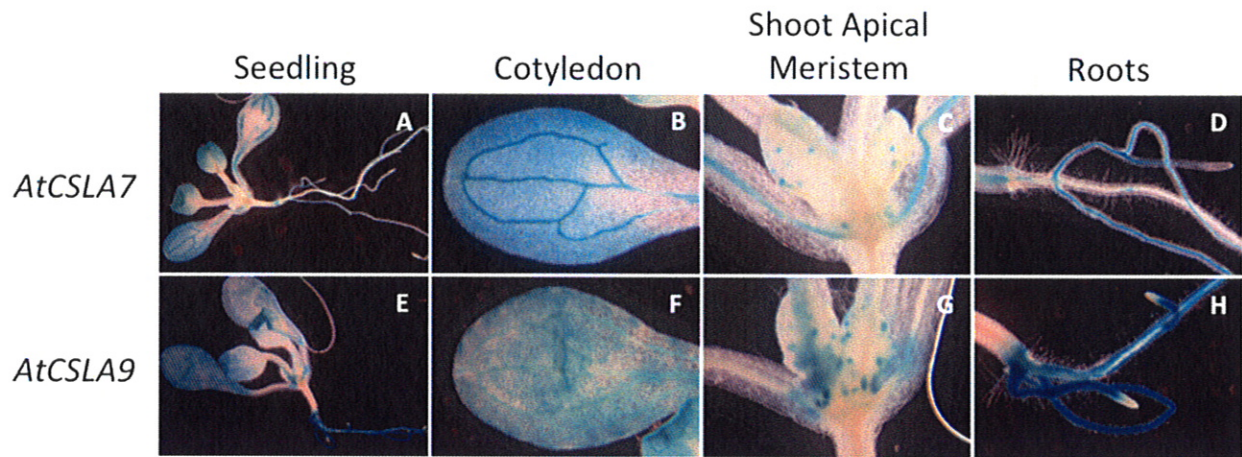


Figure 17 – *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression in 7-day-old seedlings grown under constant light. *AtCSLA7-GUS* expression in 7-day-old light-grown seedlings (A), cotyledons (B), shoot apical meristem (C), and roots (D). *AtCSLA9-GUS* expression in 7-day-old light-grown seedlings (E), cotyledons (F), shoot apical meristem (G), and roots (H).

### ***AtCSLA7-GUS* and *AtCSLA9-GUS* Expression Patterns:**

#### **2-Week-Old Plants**

In 2-week-old plants *AtCSLA7-GUS* expression was observed in the axial roots (Figure 18A and Figure 18C), shoot apical meristem (Figure 18B), and hypocotyl (Figure 18C). *AtCSLA9-GUS* expression was observed in the axial roots (Figure 18F), shoot apical meristem (Figure 18E), and hypocotyl (Figure 18D and Figure 18F).

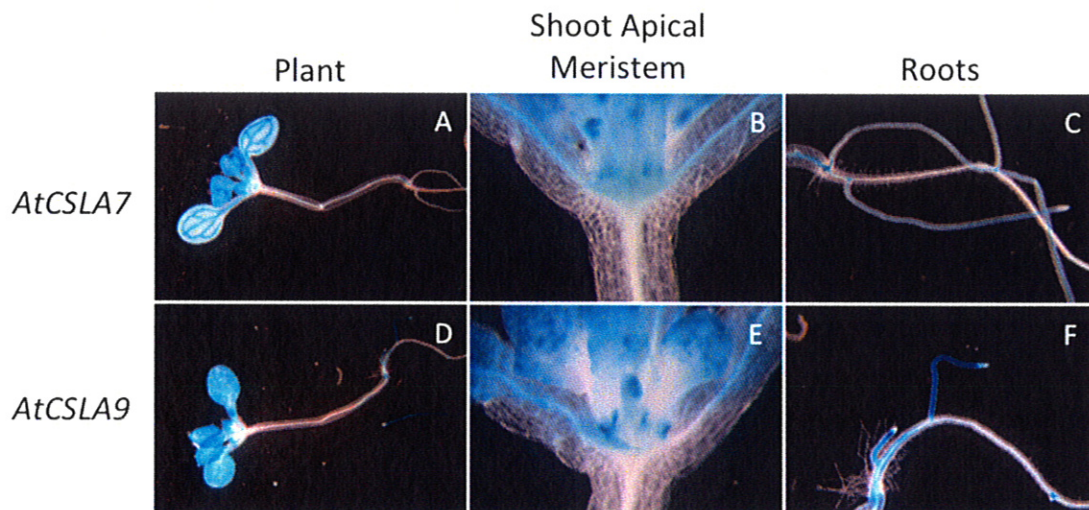
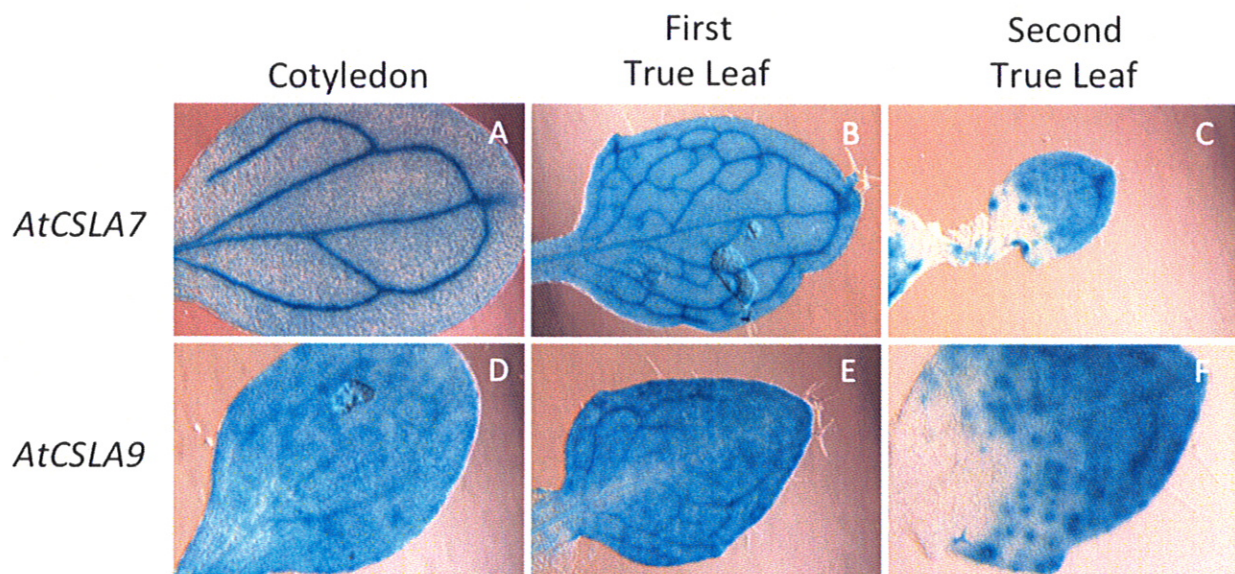


Figure 18 – *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression in 2-week-old plants. *AtCSLA7-GUS* expression in 2-week-old plants (A), shoot apical meristem (B), and roots (C). *AtCSLA9-GUS* expression in 2-week-old plants (D), shoot apical meristem (E), and roots (F).



*AtCSLA7-GUS* was also observed in the vasculature of the cotyledons (Figure 19A), first true leaves (Figure 19B), second true leaves (Figure 19C), and developing trichomes on second true leaves (Figure 19C) in 2-week-old plants. *AtCSLA9-GUS* expression in the cotyledons (Figure 19D), first true leaves (Figure 19E), second true leaves (Figure 19F), and developing trichomes on second true leaves (Figure 19F) of the 2-week-old plants was also observed.



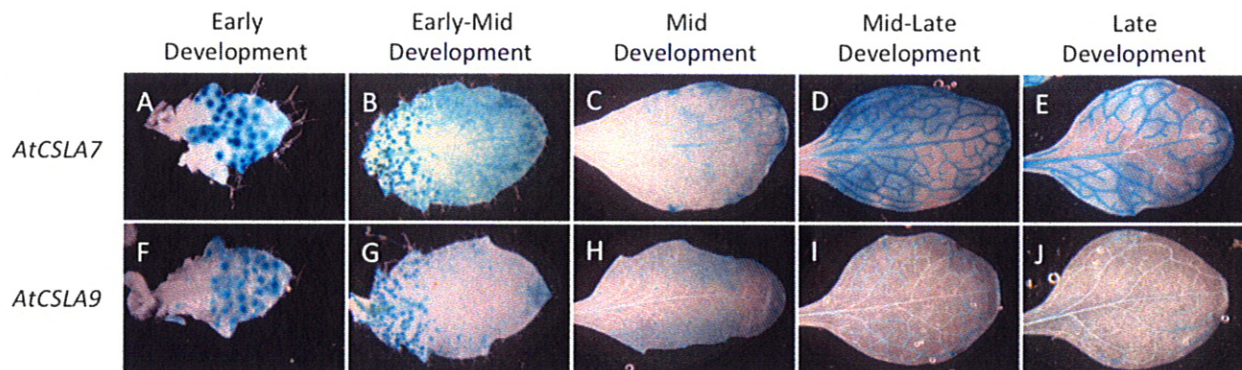
**Figure 19 – *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression in 2-week-old seedling cotyledons and leaves. *AtCSLA7-GUS* expression in 2-week-old cotyledons (A), first true leaves (B), and second true leaves (C). *AtCSLA9-GUS* expression in 2-week-old cotyledons (D), first true leaves (E), and second true leaves (F).**

### ***AtCSLA7-GUS* and *AtCSLA9-GUS* Expression Patterns:**

#### **4-Week-Old Plants**

In 4-week-old plants *AtCSLA7-GUS* expression was observed in developing trichomes during early leaf development (Figure 20A), in developing trichomes, some guard cells, hydathodes, and in most leaf tissues except for the central region during early-mid leaf development (Figure 20B), in hydathodes and vascular tissues during mid leaf development (Figure 20C), in vascular tissues and most leaf tissues except for the

central region during mid-late leaf development (Figure 20D), and in vascular tissues during late leaf development (Figure 20E). *AtCSLA9-GUS* expression was observed in developing trichomes during early leaf development (Figure 20F), in developing trichomes, some guard cells, and in the tip of the leaf during early-mid leaf development (Figure 20G), slightly in vascular tissues around the margins of the leaf during mid leaf development (Figure 20H), slightly in vascular tissues during mid-late leaf development (Figure 20I), and slightly in vascular tissues during late leaf development (Figure 20J).



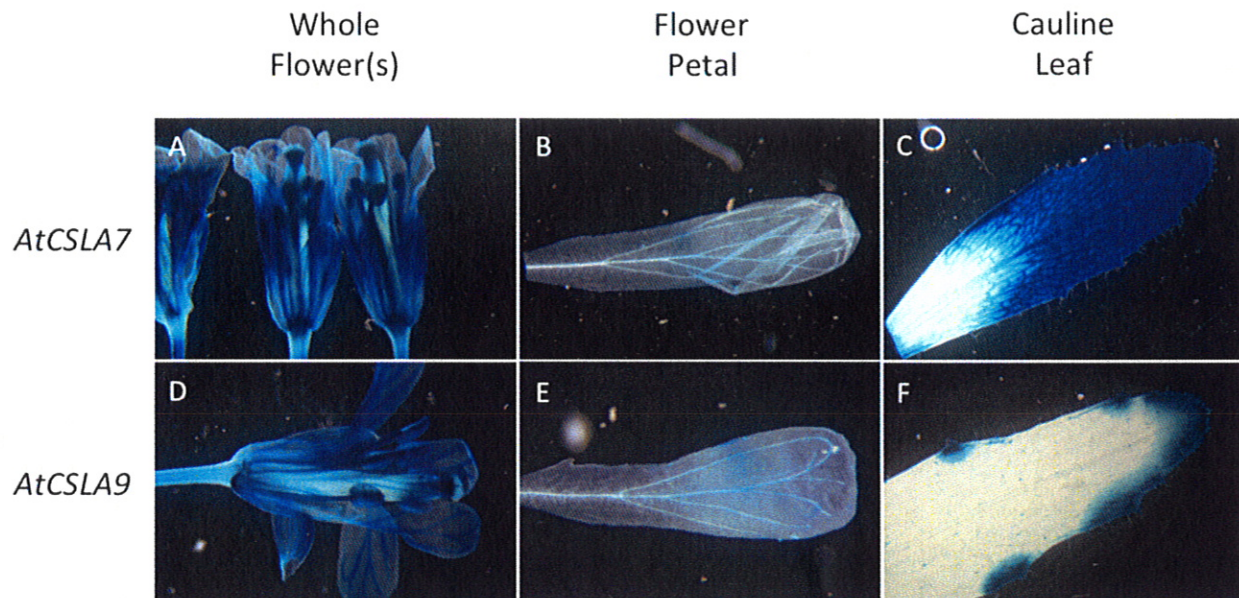
**Figure 20 – *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression in 4-week-old leaves. *AtCSLA7-GUS* expression in 4-week-old leaves in early (A), early-mid (B), mid (C), mid-late (D), and late (E) development. *AtCSLA9-GUS* expression in 4-week-old leaves in early (F), early-mid (G), mid (H), mid-late (I), and late (J) development.**

### ***AtCSLA7-GUS* and *AtCSLA9-GUS* Expression Patterns:**

#### **7-Week Old Plants**

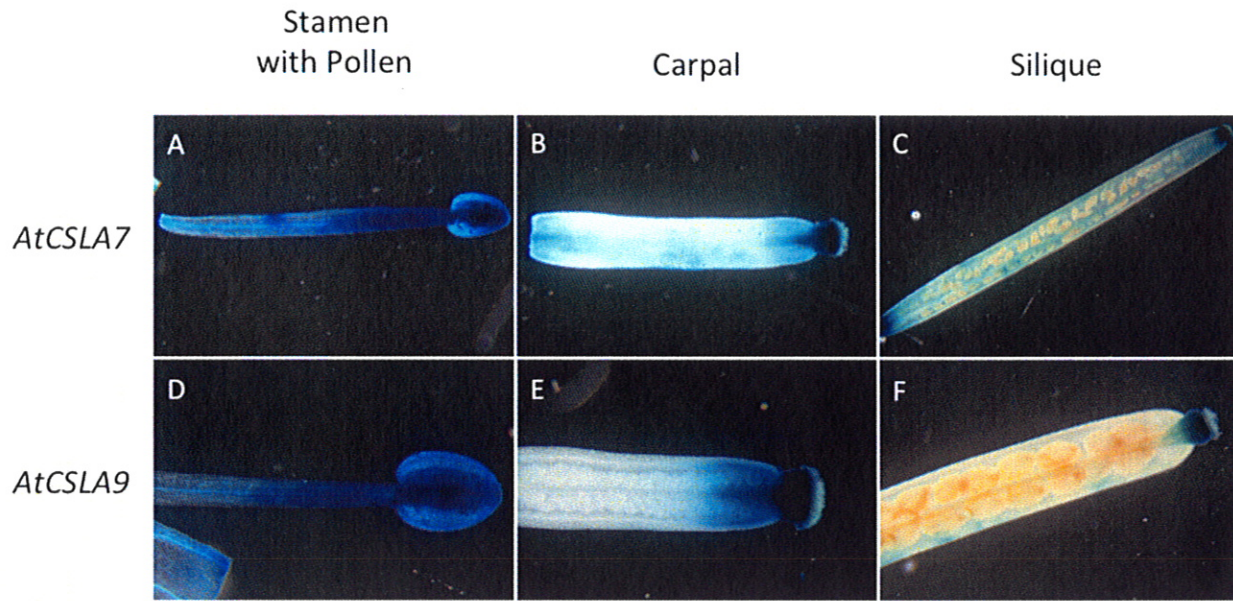
In 7-week-old plants *AtCSLA7-GUS* expression was observed in many floral tissues (Figure 21A). *AtCSLA7-GUS* expression was observed in trichomes, vascular tissue, and most of the leaf tissue aside from the base in the cauline leaves (Figure 21C). Additionally, *AtCSLA7-GUS* expression was observed in flower petals (Figure 21B), although staining was inconsistent. *AtCSLA9-GUS* expression was observed in many floral tissues as well (Figure 21D). *AtCSLA9-GUS* expression was observed in trichomes and hydathodes of cauline leaves (Figure 21F). Additionally, similarly to

*AtCSLA7-GUS*, *AtCSLA9-GUS* expression was observed in flower petals (Figure 21E), although it was not consistent.



**Figure 21 – *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression in 7-week-old flowers, flower petals, and cauline leaves. *AtCSLA7-GUS* expression in 7-week-old flowers (A), flower petals (B), and cauline leaves (C). *AtCSLA9-GUS* expression in 7-week-old flowers (D), flower petals (E), and cauline leaves (F).**

In other reproductive tissues (i.e. stamens, pollen, carpals, and siliques) there was a significant degree of overlap in the expression of *AtCSLA7-GUS* and *AtCSLA9-GUS*. *AtCSLA7-GUS* expression was also observed in the filament, anther, and pollen grains in the stamen (Figure 22A), in the style and stigma of the carpel (Figure 22B), and in the style of siliques (22C) in 7-week-old plants. *AtCSLA9-GUS* expression in the filament, anther, and pollen grains in the stamen (Figure 22A), in the style and stigma of the carpel (Figure 22B), and in the style of siliques (22C) in 7-week-old plants was also observed.



**Figure 22 – *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression in 7-week-old stamens, carpals, and siliques. *AtCSLA7-GUS* expression in 7-week-old stamens (A), carpals (B), and siliques (C). *AtCSLA9-GUS* expression in 7-week-old stamens (D), carpals (E), and siliques (F).**

## Discussion

It was hypothesized in *A. thaliana*, *AtCSLA7* and *AtCSLA9* have unique expression patterns in various tissues during specific stages of development. The expression patterns of the *AtCSLA7* and *AtCSLA9* genes during plant development were studied in *A. thaliana* using promoter-*GUS* fusion analysis. *AtCSLA7-GUS* and *AtCSLA9-GUS* expression pattern analysis in *A. thaliana* throughout development revealed examples of overlapping and non-overlapping expression, supporting the hypothesis of unique expression patterns.

During embryo development, overlapping and non-overlapping expression patterns were observed. In the early and mid stages of embryo development *AtCSLA7-GUS* is expressed, however, in the late stages of embryo development there was no

expression observed (Figures 10A-10C). *AtCSLA9-GUS* expression was observed throughout embryo development (Figures 10D-10F). The observed expression pattern of *AtCSLA7-GUS* during early embryo development is consistent with a role of CSLA7 at this stage during embryogenesis, which has been previously highlighted in *csla7* mutants (Goubet et al., 2003; Goubet et al., 2009). Additionally, since both *AtCSLA7-GUS* and *AtCSLA9-GUS* expression was detected in embryos during early and mid embryo development (Figures 10A, 10B, 10D, and 10E), another explanation may be required for the embryo lethal phenotype of *csla7* knockout mutants (Goubet et al., 2003; Goubet et al., 2009). One possibility is that *AtCSLA7* and *AtCSLA9* might be expressed in different cells during these stages and the absence of *AtCSLA7* in these cells results in the lethal phenotype.

*AtCSLA7-GUS* gene expression was not observed in mature embryos (Figure 11A), however *AtCSLA9-GUS* expression was observed in the cotyledons of the mature embryos (Figure 11B). This illustrates the distinct expression patterns of *AtCSLA7-GUS* and *AtCSLA9-GUS* in *A. thaliana*, despite frequently overlapping gene expression throughout development.

In 7-day-old seedlings, *AtCSLA7-GUS* and *AtCSLA9-GUS* expression was observed in hypocotyl, root, leaf, and stem tissues (Figures 17A-17H). These gene expression patterns are consistent with previous microarray expression analysis (Liepman et al., 2007). High expression levels of *AtCSLA9-GUS* were observed in developing roots at 7-days-old (Figure 17H), this is supported by previous mutant studies (Zhu et al., 2003)

In 4-week-old *A. thaliana* plants, both *AtCSLA7-GUS* and *AtCSLA9-GUS* gene

expression was observed in younger leaves and tended to decrease as the leaves aged (Figures 20A-20J). This generalized decrease in *AtCSLA7-GUS* and *AtCSLA9-GUS* expression coincides with observations described in previous microarray expression analysis (Hamann et al., 2004).

In floral tissues of 7-week-old *A. thaliana* plants, both *AtCSLA7-GUS* and *AtCSLA9-GUS* gene expression were observed in whole flowers, flower petals, stamens, pollen, and carpals (Figures 21A-21F and Figures 22A-22F), which is consistent with previous microarray expression analysis of floral tissues (Liepman et al., 2007).

## Future Directions

The expression patterns of *AtCSLA7-GUS* and *AtCSLA9-GUS* could be further investigated by sectioning various tissues and observing the cell-specific expression in those sectioned tissues using microscopy. The expression patterns of *AtCSLA7-GUS*, *AtCSLA9-GUS*, and the other seven *AtCSLA-GUS* fusions throughout *A. thaliana* development will be compiled into a comprehensive “expression atlas.” This expression atlas may be used in future studies to enhance understanding of *AtCSLA* gene functions. Future studies may aim to further understand the roles of each *AtCSLA* gene and the physiological functions of mannans in *A. thaliana*, as well as other plants. The *AtCSLA-GUS* expression atlas would be instrumental in further research by providing a map of locations at specific developmental periods to anticipate possible phenotypic variations in single and multiple gene knockout mutants, narrowing a potentially

exhaustive search to identify morphological defects in these mutants. This study provides foundational knowledge of *AtCSLA* gene expression patterns critical for further explorations into the functions of *CSLA* genes and mannans in *A. thaliana*, which could be applied to enhance future crop health, medical treatments, and biofuel production (Pauly and Keegstra, 2008; Liepman et al., 2010).

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## **Appendix A - Media Recipes**

### **Liquid Half Salt LB Media**

To make 100 ml of liquid half salt LB media place roughly 70 ml of deionized water in a beaker. Add a magnetic stir bar to the water and begin stirring gently. Weigh and add 1.0 g of Tryptone Broth, 0.5 g of Yeast Extract, 0.5 g of solid sodium chloride (NaCl) to the stirring deionized water in the beaker. Using 3 M NaOH, bring the solution to a pH of 7.5. Bring the media solution to a final volume of 100 ml in a graduated cylinder and place in autoclave safe bottle. To sterilize the media, autoclave the liquid media solution at 121°C for 25 minutes.

### **MS Media and MS Hygromycin Media**

To make 1 L of MS media or MS hygromycin media place roughly 800 ml of deionized water in a 1 L beaker. Add one packet of MS Basal Salts (4.33 g) and 30 g of sucrose to the water and dissolve to solids using a magnetic stir bar. Using 3 M NaOH bring the solution to a pH of 5.8. Bring the solution to volume in a graduated cylinder, pour the solution into a 2 L Erlenmeyer flask, add 5.0 g of phyto agar, and a magnetic stir bar. To sterilize the media and melt the agar, autoclave the solution at 121°C for 25 minutes. Place the media with the molten agar on a stir plate in a sterile hood, to reduce contamination, and add sterile Murashige and Skoog vitamin mixture (1X) from PhytoTechnology Laboratories to the media using aseptic technique. To make MS hygromycin media add hygromycin (50 mg/L) to the molten media as well as the vitamins. Stir the solution of media to mix in the molten agar, which tends to settle at the

bottom of the flask, and pour the molten media into sterile petri dishes in the sterile hood. Label and return the plates to the sterile sleeves and store in a 4°C cooler.

### **Staining Buffer Solution and Staining Solution**

To make 10 ml of staining buffer solution add 1 ml of 0.1 M sodium phosphate buffer (pH 7), 200 µl of 10% (v/v) Triton X-100, 200 µl of 100 mM potassium ferrocyanide ( $K_4[Fe(CN)_6] \cdot 3H_2O$ , FW: 422.41 g/mol), and 200 µl of 100 mM potassium ferricyanide ( $K_3[Fe(CN)_6]$ , FW: 329.26 g/mol) to a beaker on ice. Bring to volume with molecular biology grade water (MBG H<sub>2</sub>O). To make 10 ml of staining solution add the same reagents to the beaker on ice, as well as 240 µl of 100 mM X-Gluc in dimethylformamide (DMF).

Note: To make 0.1 M sodium phosphate buffer (pH 7), first make a 0.1 M sodium phosphate dibasic ( $Na_2HPO_4$ , FW: ) solution and a 0.1 M sodium phosphate monobasic ( $NaH_2PO_4$ , FW: ) solution. Add 0.1 M sodium phosphate monobasic solution to 0.1 M sodium phosphate dibasic solution until pH 7 obtained. Filter sterilize the final buffer solution in a sterile hood.

## Appendix B - Polymerase Chain Reaction (PCR) Amplification

### PCR Thermal Cycler

BIO-RAD, MyCycler™ Thermal Cycler

### PCR Primers

CSLA7-822F (forward primer for CSLA7-GUS)

5'-GCATGGTGTAATCTTACTATATATACTATGTTT-3'

CSLA9 466R (forward primer for CSLA9-GUS)

5'-CACACACACAACACTGTGTC-3'

pCAMBIA1305 GUS 1770 Rev (reverse primer for all CSLA-GUS)

5'-GGTCAGCTTGCTTTCGTACCAC-3'

### PCR Program

Initial Denaturation: (1 cycle)

95°C for 2 minutes

Denaturation, Annealing, and Elongation: (30 cycles)

95°C for 30 seconds

52°C for 30 seconds

72°C for 1 minute and 15 seconds

Final Extension: (1 cycle)

72°C for 5 minutes